Joseph A. Impellizeri *Editor*

Electroporation in Veterinary Oncology Practice

Electrochemotherapy and Gene Electrotransfer for Immunotherapy

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For my kids, Samantha, Joseph, and Ryan. You bring more joy than you can ever imagine into my life. Soar and never stop climbing to reach your goals and dreams. Love, Dad

Foreword

It was almost the end of the 1990s when I came to learn, from a colleague returning from an international congress, of a new technique called "in vivo electroporation." At that time, I was working at a research center in Italy called IRBM P. Angeletti, part of the Merck and Co. Research division (Merck Research Laboratories) near Rome where I coordinated an ambitious program aimed at developing somatic gene therapy for various diseases. My immediate reaction was of pure skepticism. As molecular biologist, I had been used to applying high-voltage electroporation only to cells in vitro to transfect DNA for gene expression purposes. I could not imagine how it could have been possible to apply what looked to me to have been such harsh treatment to a living organism without causing any adverse reactions and lack of tolerability. However, after having done some background reading behind the theoretical basis of in vivo electroporation in seminal papers by Lluis Mir and, most importantly, after having obtained impressive results when we first applied in vivo electroporation to mice using a very simple in-house built device, I realized that this type of technology could have greatly helped us to boost our program and be a true game changer.

What was the simple experiment we carried out and what was its impressive results? We injected a small amount (micrograms) of a plasmid encoding beta-galactosidase into the muscle tibialis of a group of anesthetized mice, and a few minutes after DNA injection mice were subjected to an escalating number of high-frequency low-voltage electrical bipolar pulses. The procedure was well tolerated; mice recovered from anesthesia returned to their normal activity. After one week, mice were sacrificed, and the tibialis muscles were stained for beta-galactosidase activity. As expected, we observed that in non-electroporated muscles staining was minimal with a very small number of fibers turning blue. In contrast, and to our great surprise, we noticed that electroporated muscles had acquired an intense blue staining and that this staining was proportional to the number of electrical pulses used. In fact, the muscles exposed to the highest number of pulses had turned entirely blue! Which meant that we had been able to transfect together with DNA the majority of the fibers of that muscle in vivo, which under normal conditions would be an impossible result to achieve because normal cells are relatively impermeable to naked DNA. This was truly impressive! For our gene therapy program, this result would mean a big jump-start ahead!

Since then, more than two decades have passed and in vivo electroporation has evolved into a robust and diversified technology with a vast array of applications both in the human and in the veterinary fields. Therefore, the publication of this book is indeed very timely; it recapitulates the enormous progress made over the past few years and constitutes a much-needed compendium of the current clinical applications of in vivo electroporation, in particular electrochemotherapy which has become clinical practice in several European countries, as well as representing an outline of several promising technological discoveries such as irreversible or calcium electroporation. The most influential investigators in the field have contributed to various chapters, which taken together nicely provide a reference document for those who are willing to learn about the past, the present, and the future of this approach.

One comment about safety and tolerability: my initial concern about this aspect has been lifted by the empirical observations that this technology is safe and well tolerated. Not only when we consider electrochemotherapy in anesthetized cancer patients, but also when we talk about therapeutic and prophylactic vaccines, where thousands of patients have been enrolled into clinical trials of gene-electro-transfer for vaccination purposes without adverse events. Hence, if gene-electro-transfer does not yet have a marketed product, it is not for these reasons but probably because there has not been sufficient investments made in this technology to obtain robust Phase III data in support of product registration.

I would like to close this short preface by mentioning three people who have been an inspiration for the entire field of in vivo electroporation, namely Luigi (Gigi) Aurisicchio, Ruggero Cadossi, and Joe Impellizeri. Gigi Aurisicchio has been instrumental in continuing the IRBM P. Angeletti tradition in gene-electro-transfer in Takis Biotech that has flourished under his guidance. Gigi is pursuing with commendable perseverance ambitious goals regarding the use of this technology for cancer vaccines and beyond and has been the first one to demonstrate the clinical efficacy of a cancer vaccine for lymphoma delivered by gene-electro-transfer in dogs. Ruggero Cadossi with the company Igea, and thanks to the design and the development of Cliniporator, and his capability to establish a network of outstanding clinicians all over Europe, has been able to ride the successful implementation of electrochemotherapy. Finally, Joe Impellizeri has been instrumental in initially introducing electrochemotherapy in the veterinary field in the USA.

I offer my best wishes and great success to this initiative and my sincere thanks to all the contributors involved.

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Acknowledgements

First, I would like to thank all my contributors. This inaugural textbook has been long overdue and I am honored to work with so many distinguished colleagues. This textbook will expand the concepts of electroporation as an invaluable tool for research and therapeutic intervention and I am beyond proud and humbled to be part of it.

To the students reading this textbook, never stop pursuing something better for your patients—that is how the world changes and we advance our profession.

Finally, deep gratitude to my clients and patients for allowing me to be part of their healthcare team and to Drs. Luigi (Gigi) Aurisicchio and Gennaro Ciliberto for introducing me to the topic of Electroporation over a decade ago, allowing me to help so many patients here in the United States. For that, I will be forever grateful.

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Part I

Technology and Science of Electroporation: Fundamentals

Electroporation: Technology and Science

Marie-Pierre Rols, Muriel Golzio, and Justin Teissié

Abstract

Enormous progress has been made in pulsed electric field-based therapies since the first reports describing the occurrence of electric field-induced transient pores in phospholipid bilayer vesicles. The term electroporation took some time to anchor within the vocabulary of the scientific community. Cell and tissue electroporation, visualized at the single-cell level, can be described as a succession of different steps. By a good selection of the pulses parameters, it is possible to induce a transient or irreversible permeabilization of the cell membrane, and to transfer non-permeant or poorly permeant molecules into cells and tissues. The development and current use of electroporation in oncology practice indeed highly benefitted from the understanding of the mechanisms and underlying biological processes of this method, from the optimization of pulses generators and electrodes, and from the standardization of the operating procedures. Classical electrochemotherapy and irreversible electroporation, already proved their efficacy to treat cancer both in human and veterinary clinics. Gene electrotransfer for immunotherapy and the combination of calcium ions with high-intensity electric field pulses, on another hand, have been developed in the last years, and are emerging as two additional clinical applications of electroporation.

Marie-Pierre Rols and Muriel Golzio dedicate this chapter to Dr. Justin Teissié, who lived for and by Science. Dr. Teissié was a pioneer in a number of applications of electroporation and in the elucidation of its mechanisms. He was the PhD supervisor of Marie-Pierre Rols and, together, they performed one of the first clinical trials using ECT in veterinary medicine for sarcoids treatment on horses. Dr. Teissié passed away in September 2020, but his electric field-mediated battle against cancer will indeed go on.

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Keywords

Electroporation · Electrochemotherapy · Veterinary oncology · Membrane permeabilization · Molecules delivery · Cytotoxic drugs · Companion animals · Cancer treatment · Technology · Mechanisms

1 Introduction

Cell membranes act as a selective barrier that tightly regulates the exchanges of molecules between the cell and the external medium. The free access of non-permeant or poorly permeant molecules into the cytoplasm is therefore hindered, preventing cytotoxic drugs (such as cisplatin and bleomycin) or nucleic acids (siRNA, DNA) to reach their intracellular targets, namely the cytoplasm or the nuclei. As the delivery of such molecules is mandatory in clinics to treat cancer or to treat genetic disease, this led, during the recent decades, to the continuous development and improvement of methods to efficiently and safely deliver drugs into cells and tissues. Today, there is still no gold standard method.

The treatment of cancer has indeed undergone continuous changes for centuries mainly due to a better understanding of the disease and of its underlying biological processes (Falzone et al. 2018). Surgery, the oldest method has already been used in ancient Egypt. Hormone therapy and physical methods such as radiation therapy have started to be developed in the nineteenth century. Other treatment modalities such as chemotherapy, immunotherapy, and gene therapy (a treatment option for a number of diseases as inherited disorders and cancer) appeared during the twentieth century. All these treatments have been developed and optimized to increase, in addition to effectiveness and lifespan, the quality of life of the patients. Nowadays, the percentage of people with cancer alive at 5 years after diagnosis has increased up to 60% worldwide, but we are still far from the goal to heal all patients (Allemani et al. 2018). Clinicians are currently still lacking efficient, safe, simple, reproducible, and versatile methods. These methods are required not only in Human clinics but also in veterinary clinics to treat companion animals.

A very promising and, nowadays, efficient method, the electroporation, has been developed during the last 50 years. This method, together with a panoply of derived approaches, also known as electroporation-based applications, emerged in medicine and in food industry, leading to the creation of the ISEBTT (International Society for Electroporation-Based Technologies and Treatments) in 2016 (Yarmush et al. 2014). Electroporation phenomena are based on cell membranes permeabilization by local application of electric field pulses. The first reports on the use of electric fields in medicine can be traced back to the eighteenth century when Pierre Jean Claude Mauduyt de la Varenne, used electricity to treat rheumatisms, paralysis (Zelbstein 1984), l'abbé Nollet observed red spots on human skin and Benjamin Franklin reported that electricity could be fatal to small animals (Franklin 1956). At that

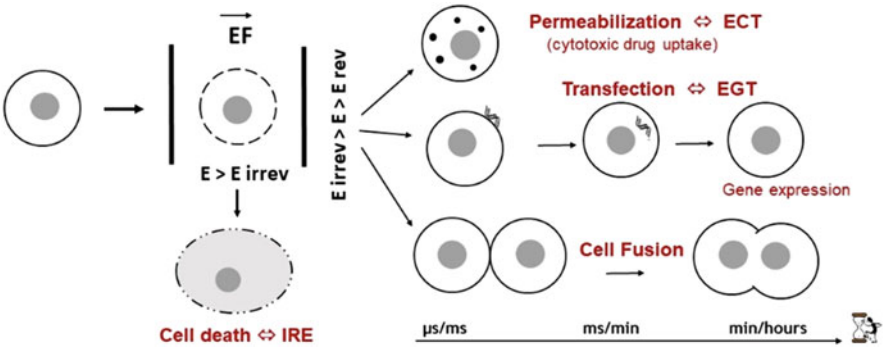

Fig. 1 Different applications of electroporation. According to the electric field intensity value, membrane electropermeabilization can be reversible (Erev < E < Eirrev) or irreversible (E > Eirrev), leading to different potential medical applications such as the delivery of cytotoxic drug (ECT), of nucleic acid (EGT), cell fusion, or when E exceeds a threshold, cell death (IRE).

time, the causes of such effects were not understood at all. Later on, in the twentieth century, once the basic description of the process and the control of the electric field parameters were achieved, electroporation began to be translated from the laboratories to the industry as a method to eradicate bacteria and sterilize water. Medical applications followed, when electric field parameters and pulses generators were adapted to transiently permeabilize the cell membranes leading to different applications. These applications comprise electrofusion of cells in contact (Teissie et al. 1982; Teissie and Rols 1986), the delivery of cytotoxic drugs or nucleic acids into the cells, respectively named electrochemotherapy (ECT) (Mir et al. 1998), and electrogenetherapy (EGT) (Rols et al. 1998) (Fig. 1). All these applications were made possible thanks to the transient destabilization of the cell membrane, i.e., reversible electroporation. As will be described later in this chapter, irreversible electroporation (IRE) is another option to induce cell death. The technology is also used in food industry to extract molecules of high added value from plants or microalgae or to sterilize liquids (Parniakov et al. 2016; Puertolas et al. 2009; Aguilar-Machado et al. 2020; Monfort et al. 2012), and is known as PEF (Pulsed Electric Field) technology. As IRE does not induce any thermal damage, the concept has also been developed in medicine to safely treat unresectable tumors.

In the 1970s, Neumann et al. demonstrated that the application of calibrated external electric field pulses can transiently permeabilize the vesicular cell membrane (Neumann and Rosenheck 1972). In the 1980s, it was shown that this reversible electroporation of membranes allows non-permeant or poorly permeant cytotoxic drugs to enter cells in large quantities, potentiating their activity. The proof of concept of electrochemotherapy was conducted with bleomycin, where the cytotoxicity of this drug was enhanced by 700-fold after electroporation, compared to the drug alone (Orlowski et al. 1988). In addition to be efficient, the method also appeared to be safe. The side effects of the drug were highly limited thanks to the direct and local delivery of the drug by electric pulses, which were applied directly to

the cancerous tissue, placed between the electrodes. ECT has become an alternative approach to classical chemotherapy. Electrochemotherapy has been successfully applied for a wide variety of tumors in human clinical trials (Mir et al. 1998; Cemazar et al. 1999). This method has been used for more than 10 years and in more than 150 centers and clinics throughout Europe. Moreover, its use is increasingly expanding over the globe. ECT has also demonstrated its effectiveness in naturally occurring tumors in domestic animals, including horses (Rols et al. 2002), cats, and dogs (Cemazar et al. 2008) not only in Europe but also in South America (Maglietti et al. 2020). The first clinical study, in veterinary medicine, was also conducted in 1997 by Mir and colleagues for the treatment of cats (Mir et al. 1997). The first studies pioneering ECT in equids started at the beginning of the millennium (Rols et al. 2002; Tamzali et al. 2001).

In addition to ECT, calcium electroporation was proposed as a potential novel anticancer treatment (Frandsen et al. 2012). High concentrations of calcium ions are introduced into the cells thanks to electroporation, leading to cell death. This new treatment modality, introduced by Gehl and colleagues 10 years ago, is efficient and easy to implement. Its perspectives for cancer treatment are highly promising in humans (Agoston et al. 2020) and veterinary clinics (Frandsen et al. 2020).

Beside reversible electroporation, irreversible electroporation has also been developed. The approach is of high interest for tissue ablation (Davalos et al. 2005) allowing cancer treatment of deep-seated tumors as hepatic and pancreatic ones (Niessen et al. 2012) and long-term survival of patients after percutaneous irreversible electroporation of inoperable colorectal liver metastases (Schicho et al. 2019).

Electroporation is also a well-known technique of cell transfection used in laboratories. Despite the fact that the pioneering work on plasmid DNA electrotransfer in cells was initiated 40 years ago by Neumann and colleagues (Neumann et al. 1982), and shown to be an efficient method for gene transfection, the mechanisms underlying membrane electropermeabilization and DNA electrotransfer remained elusive for a long time. Nowadays, vaccination and oncology gene therapy are major fields of application of DNA electrotransfer in clinics. Translation of EGT preclinical studies into clinical trials started more than 10 years ago. The first clinical trial of plasmid electroporation carried out by Daud, Heller, and collaborators in patients with metastatic melanoma, has shown promising results (Daud et al. 2008). The method has also been successfully used for the treatment of companion animals (Impellizeri et al. 2016) and shows great potential in immunotherapy strategy for cancer in veterinary medicine (Maglietti et al. 2020). Even if in vitro electrotransfer is efficient in almost all cell lines, in vivo gene delivery and expression in tumors were not as efficient as in viral vectorization. In order to increase gene transfer and expression, while preserving safety, it became mandatory to understand the exact phenomenon underlying the mechanisms and responses of membrane permeabilization, as well as cells and tissues responses to electric pulses.

This chapter, therefore, aims to describe both the science and the technology of electroporation. We thus provide insight into basic research of the mechanisms following membrane electropermeabilization, and describe gene delivery in cells.

In addition, we describe the optimization of devices, which led to the establishment of standard operating procedures that are mandatory to efficiently and safely use electroporation in clinics.

2 Science: Cells and Tissue Electroporation

2.1 Electroporation at the Single-Cell Level

According to Maxwell equations and electrodynamics of dielectrics, plasma membrane can be considered as a dielectric separating two conductive media, the cytoplasm and the external medium. When submitted to an external electric field, the cell behaves as a closed capacitor. As a result, the field induces a size and position-dependent membrane voltage modulation leading to membrane permeabilization or poration, when the field-induced transmembrane voltage reaches a critical value (Teissie and Rols 1993). Cells have a resting transmembrane potential of a constant value (close to −60 mV for mammalian cells) all along their plasma membrane. Exposure to short and intense electric pulses induces position-dependent changes of this potential. Being dependent on the angle between the electric field direction and the normal to the membrane, the electric field effects are not the same along the membrane. Maximum effects are present at the poles of the cells facing the electrodes when the resulting transmembrane potential reaches a threshold value (close to 200 mV) (Rols 2006), above which permeabilization of the cell membrane occurs (Fig. 2).

At the single-cell level, electropermeabilization, also called electroporation (due to the simplest description of the process based on the formation of pores), can be described as a succession of different steps (Teissie et al. 2005): (1) "Induction step" (time scale microsecond or less) where the field-induced membrane potential difference reaches the critical threshold value; (2) "Expansion step" (micro to millisecond) where membrane defects expend as long as the field is present; (3) "Stabilization step" (millisecond) which brings the membrane to a permeabilized state; (4) "Resealing step" occurring on a scale of minutes, during which the membrane repairs, which is mandatory to preserve cell viability; and (5) "Memory effect" present on a longer time scale (hours) allowing transport of molecules through other pathways such as micropinocytosis (Rols et al. 1995). For drug delivery and ECT, the main fact is that the cell membrane stays permeable for seconds to minutes after the pulse, during the so-called resealing step. A short-lived electrophoretic loading is present during the pulse, but a passive loading occurs during the slow resealing, resulting in a high increase of anticancer drug intracellular concentration, which is multiplied up to 100-fold, explaining why ECT is so efficient (Escoffre et al. 2011). This is of major importance for drug delivery. High cytotoxicity of polar toxic drugs (bleomycin or cisplatin) can be obtained even if their external concentration is low, therefore limiting their adverse effects.

The use of video microscopy allowed visualization of the permeabilization phenomenon at the single-cell level (Escoffre et al. 2011; Golzio et al. 2002).

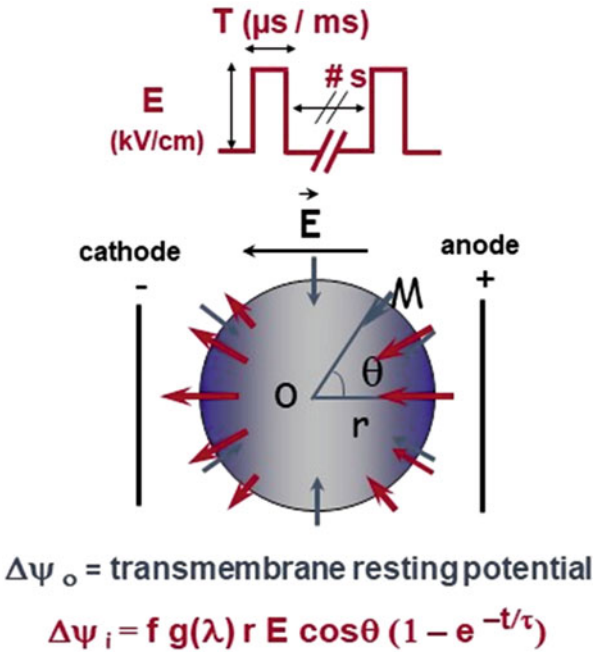

Fig. 2 Membrane potential difference modulation along electropermeabilization process. Schematic representation of the effect of the external electric field applied on a cell. The external electric field induces a change in the resting transmembrane potential (which is uniform along the membrane, blue arrows represent its gradient). The value of the induced change depends on the shape f of the cell and the conductivity of the media $g(\lambda)$. r is the radius of the cell, E is the electric field strength, and $\theta(M)$ is the angle between the direction of the field and the normal of the cell's surface at the point M (red arrows have different directions, and lengths mimicking their value)

Fluorescent indicators of membrane permeabilization, such as propidium iodide (PI), are indeed very convenient to detect the electrotransfer of molecules into the cytoplasm and to image to real-time processes of the electrotransfer of molecules (Golzio et al. 2002). Whatever the molecules used to detect permeabilization (if they are small enough and charged), a direct transfer into the cell cytoplasm is observed. When added after electropulsation, molecules can still penetrate into cells but less efficiently, because electric field acts on both the permeabilization of the membrane and the electrophoretic drag of the charged molecules into the cytoplasm. The electrotransfer mechanism involved is indeed specific and correlates with the physicochemical properties of the molecule (Golzio et al. 2002; Paganin-Gioanni et al. 2011). In addition to membrane permeabilization, DNA electrotransfer is dependent on DNA electrophoresis. The oligonucleotide must indeed be present during the pulse to be later on transferred in the cytoplasm. Short pulses with high field strength can be used, but are less effective than long pulses with lower field strength. Therefore, pulses parameters have to be determined to permeabilize the membrane, while preserving as much as possible cell viability (keep it above 30–50%). Reporter

Table 1 Models used in evaluations of electropermeabilization and gene delivery processes

Model	Membrane permeabilization	DNA electrotransfer
GUV	Direct visualization of membrane permeabilization and its consequences (deformation, lipid loss)	Fail to address DNA/membrane interaction (DNA is directly transferred inside the vesicle)
2D Cell cultures	Kinetics of permeabilization and its consequences (lateral and transverse mobility of lipids and proteins)	Visualization of DNA/membrane complex formation and access to DNA traffic into the cells
3D Cell cultures	Molecules diffusion and transfer that mimics complex in vivo situations (contacts between cells, junctions, extracellular matrix)	Allow to address DNA delivery in 3D and mimic the in vivo (decrease in gene expression from the periphery to the core)

Table 2 Kinetics of the different steps involved in gene delivery

Time scale	Steps involved in DNA electro-mediated delivery	References
µs	Plasma membrane facing the electrodes is permeabilized	Golzio et al. (2002)
ms	Electrophoretic migration of DNA toward the membrane	Escoffre et al. (2011), Golzio et al. (2002)
s	DNA/membrane complex formation	Faurie et al. (2010)
min	Conversion of the metastable form of the DNA/membrane complex to a stable one	Escoffre et al. (2011)
hour	DNA translocation/diffusion across the membrane	Rosazza et al. (2011, 2013)
day	DNA transport toward the nucleus along the cytoskeleton	Rosazza et al. (2016)

genes are useful to optimize the protocol. As for electropermeabilization, single-cell microscopy and fluorescent plasmids can be used to visualize and determine the different steps of electrotransfection.

In order to understand the whole process of membrane permeabilization and DNA electrotransfer in tissues, it has been necessary to develop and use different models, from simple lipid vesicles to tumor multicellular tumor spheroids, which more accurately represent the in vivo situation. Each of these models has advantages and limits. However, combined together, they helped elucidate the basic mechanisms (Table 1).

The electropermeabilization of the plasma membrane is a prerequisite for gene electrotransfer, since nucleic acids are highly charged and large molecules that cannot enter cells. The entry of plasmid DNA occurs through a multistep mechanism involving the electrophoretically driven interaction of DNA molecules with the destabilized membrane during the pulse, a stabilization step during the second following the pulse, and the passage of DNA across the membrane (Golzio et al. 2002; Paganin-Gioanni et al. 2011; Escoffre et al. 2009) (Table 2). Therefore, successful DNA electrotransfer into cells depends not only on cell permeabilization but also on the way plasmid DNA interacts with the plasma membrane.

The first electroporation-mediated gene transfer experiment was published more than 30 years ago, and since then, the translation to clinics has benefited from increased knowledge of the mechanisms involved in the electrotransfer of nucleic acids. As for electropermeabilization, single-cell studies aided in describing the process of DNA electrotransfer. In tumors (i.e., for clinical developments), the first evidence for gene electrotransfer has been reported on mice by Rols and collaborators in 1988 (Rols et al. 1998). A critical problem to the low efficiency of in vivo GET tightly correlates with plasmid diffusion, which is hindered by the extracellular matrix (Henshaw and Yuan 2008).

2.2 Electrodelivery in Tissues

Classical theories of electropermeabilization present some limits to give a full description of the transport of molecules through membranes. While some effects involving electric field parameters and the consequent membrane permeabilization, as well as the associated transport of molecules, are well established, an important fraction of phenomena occurring at the molecular level remains speculative. Molecular models of lipid bilayers and electropore formation are giving interesting new insight into the process. Electroinduced destabilization of the membrane includes both lateral and transverse redistribution of lipids and proteins, leading to mechanical and electrical modifications, which are not yet fully understood (Escoffre et al. 2014a, b). One may suggest that such modifications, which may vary according to the microenvironment, can be involved in the subsequent transport of molecules such as the DNA. Experimental verification of the basic mechanisms leading to the electropermeabilization and other changes in the membrane, cells, and tissues, remain a priority given the importance of these phenomena for processes in cell biology and in medical applications. In vivo gene electrotransfer will face other challenges such as the necessity to control electric field distribution and gene expression, both in space (targeted DNA delivery to the cells) and in time. Guidelines for successful DNA delivery are still necessary, and fundamental studies will indeed help to optimize standard operating procedures, which will yield efficient treatments.

The description and knowledge of the mechanisms of electropermeabilization at the single-cell level could indeed be adapted to be valid on tissues. A fair agreement could be found between mathematical simulation and experimental observations. The local field at the cell level is not the "macro-field" present externally on the assembly (tissue). The free diffusion of the drug can be hindered by the close contact of the cells and by the external matrix (Teissie and Rols 1993; Rols 2006).

Permeabilization (and associated local delivery) occurs as long as it is possible to locally provide a field strong enough to be larger than the critical value reported in Step 1. This is obtained by a proper design of the electrodes taking into account their geometry and the specific dielectric properties of a tissue. The efficiency in delivery is then controlled by the pulse cumulated duration. This parameter acts on the extent of permeabilization (step 2) and on the kinetics of the slow resealing (Step 4). Drug

delivery is obtained and appears to be further enhanced by a tissue response, the so-called "vascular lock." The application of electric pulses to the tumors induces instant but transient tumor blood flow reduction. This is followed by a vascular disrupting action by an indirect action on the endothelial cells of the blood vessels (Teissie et al. 2005).

This means that the drug does not need to be locally injected (IT) but its IV injection will be effective on the local tissue (tumor) where the electric pulse is applied. Again, a massive inflow occurs allowing the injection of a drastically lower doses of the drug.

As written by Stewart et al. "The complex mechanisms of established methods and their often unpredictable impact on cell behaviour have dramatically limited the scope of biological experiments and reduced efficacy of potentially promising cell therapy concepts. The biomedical research community would benefit greatly from a more mechanistic and transparent understanding of intracellular delivery, both to further the development of more robust techniques and to realize key medical and industrial applications" (Teissie and Rols 1993). In this context, the electroporation technology is probably the most promising and versatile one. The development of the electroporation-based technologies has benefited for years from the continuous increase of the knowledge of the mechanisms underlying the phenomena and the development of the technology.

3 Technology

3.1 Pulses Generators/Pulse Parameters

The capacitor discharge is the oldest concept of pulse generator. It has been primarily used in vitro (Neumann and Rosenheck 1972) but also in vivo (Okino and Mohri 1987). The generator operates in two phases, charge and discharge, and generates exponentially decaying pulses. The time constant of the discharge τ is the product between the capacitance and the impedance of the biological sample. As the impedance varies during the pulse, the time constant changes during the pulse. Therefore, it is highly difficult to find optimum conditions for pulses delivery leading to the electropermeabilization of all the cells while preserving their viability. Such pulses generators can, however, be used on cells, protected by a cell wall, such as bacteria or yeasts, and became a standard method in the laboratories for electrotransformation of bacteria.

For a better control of the electric field parameters, and repeatability of the experiments, square wave pulse generators have been introduced. The concept is different from the one of the capacitor discharge, due to the fact that the voltage power supply constantly charges the capacitor. The amplitude of the pulse is defined by the amplitude of variable power supply and remains constant during all the pulse duration, whatever the conductivity of the biological sample. In addition to pulse strength, pulse duration, pulse number, and repetition frequency can also be defined independently from the cells' responses to electric pulses.

Further developments came from reversing the polarity of the electric pulses or by changing their orientation. Both led to transfection level increases due to an increase in the cell membrane area where DNA interacts with to form transient DNA/membrane complexes. These studies, performed by our group, demonstrated the relationship between DNA/membrane surface interaction and gene transfer efficiency, and indicated how to define experimental conditions to optimize the yield of transfection of mammalian cells (Faurie et al. 2004, 2010). Based on that, and with the aim to increase the efficacy of electroporation-based technologies for gene electrotransfer, pulse generators and electrodes have been developed by Miklavcic and collaborators to automatically change the electric field direction between the electrical pulses. Finite elements method was used to calculate and evaluate the electric field homogeneity between these electrodes, improving cell transfection without affecting cell survival (Rebersek et al. 2007). This is the direct confirmation indicating that a better understanding of the process of molecule delivery by electric pulses helped to the development of new generators and electrodes.

In addition to μs and ms pulse duration, used to deliver cytotoxic drugs or plasmid DNA into cells, nanosecond electroporation of cell organelles is being studied since more than a decade. A new high-voltage diode opening switch (DOS)-nanosecond pulse generators for laboratory use for in vitro experiments in electroporation cuvettes have been developed (Pirc et al. 2019). These generators are not yet used for veterinary purposes.

If there are a number of laboratory electroporators, only a few are available for treating patients, by contrast to the different types of electrodes available for a variety of applications. From a research point of view, it is useful to have a wide range and control over pulse parameters (pulse strength, pulse duration and number, frequency). The choice of an electroporator is therefore driven by the requirements for electric pulse parameters for a given purpose (for instance drug delivery, gene electrotransfer, cell eradication). The electrode design is quite simple and sometimes hand-made (for instance, for a direct visualization under the microscope). On the opposite, for clinical purposes, electrodes are designed to produce homogeneous electric fields, and to be easily sterilized. The pulse parameters have to be well optimized before, in respect to the electrodes and following standardized operating procedures.

3.2 Standard Operating Procedures

From the technology (design of pulses generators and electrodes), it is possible to specifically target certain tissues within the body, regardless of the molecules (Hojman 2010).

The most impressive success of electroporation in drug delivery is the clinical development of electrochemotherapy and electrogenetherapy. From the efforts of several teams with supports of the European Union (FP5 Cliniporator, FP6 Esope), the treatment was standardized in the framework of the European Standard

Operating Procedure on Electrochemotherapy (ESOPE) multicenter trial, first released in 2006 (Gehl et al. 2006) and recently updated (Gehl et al. 2018). The objective of ESOPE was to validate the clinical applications of electroporation of cells in tissues (electrochemotherapy and electrogenetherapy) and to establish standard operating procedures (SOP) for their rapid dissemination in Europe.

The protocol consists of associating cytotoxic drug injection with the application of calibrated electric field pulses delivered locally at the tumor site. The cytotoxic drugs (Bleomycin or cisplatin) are injected either IV or IT. A series of 8 pulses of 100μs (frequency up to 5 kHz) are applied at a 1300 V to electrode width (cm) with plate or needle electrodes. ECT is now proved to be an effective treatment in the palliative management of unresectable recurrent disease in solid cutaneous and subcutaneous tumors with overall objective response rates of approximately 80–90%. ECT is also now a loco-regional therapy for disseminated cutaneous and subcutaneous tumor lesions, and is known to improve patient's quality of life.

Currently, new electrochemotherapy strategies are under development. European Standard Operating Procedure on Electrochemotherapy-equivalent protocols are available for the delivery of nontoxic drugs such as calcium. Calcium electroporation has been proposed as a simple tool for anticancer therapy. Calcium can help overcome the drawbacks of standard drugs (side effects, cost, difficult handling, storage, etc.). In vitro studies permitted the optimization of pulsing protocols that can be used in clinics (Romeo et al. 2018).

Last but not least, tumor eradication can also be obtained by inducing irreversible permeabilization (so-called IRE) by using a higher number of more intense pulses. No drug injection is required and the associated mechanisms are clearly different. IRE has shown promise for the therapeutic treatment of focal disease. A consensus study of panelists has been recently performed to provide a uniform protocol addressing (contra) indications, procedural parameters, perioperative care, and follow-up of irreversible electroporation (IRE) for the treatment of hepatic malignancies (Ruarus et al. 2020). The method can be used for a number of cancers including human prostate tissue (Scheltema et al. 2019) and brain tumors in canine patients (Garcia et al. 2017) with MRI-based treatment planning. The treatment planning method consists of building patient-specific finite element models and using them to compute electric fields used in the IRE treatment, using medical imaging analysis and reconstruction, numerical modeling, and real-time electrode placement guidance.

4 New Development/Perspectives

Electrochemotherapy (ECT) is a very potent method for the treatment of tumors in veterinary oncology. Based on successful results of clinical studies in veterinary oncology, ECT became a standard treatment method for the specific type and location of tumors (cutaneous, subcutaneous, oral tumors) in dogs, cats, equids, and other pets (Impellizeri et al. 2016; Tozon et al. 2016). ECT in veterinary oncology uses two chemotherapeutic drugs: cisplatin and bleomycin. For the

treatment of dogs and horses, cisplatin is mainly used in combination with electric pulses, while for the use in cats, since cisplatin is not indicated; bleomycin is the drug of choice. This combined approach can be used as a single therapy or as an adjuvant therapy to surgery. Moreover, ECT can be used for treatment of inoperable tumors. It can be effective as a one-time treatment only, or it can be repeated several times with equal or improved effectiveness in case of failure or partial tumor response. The advantages of ECT are its simplicity, short duration of treatment sessions, low chemotherapeutic doses, and no side effects, as determined by the Veterinary Cooperative Oncology Group toxicity scale (Veterinary Co-operative Oncology 2004) in follow-up examinations. The results of published clinical trials suggest a safe and effective use of ECT (Impellizeri et al. 2016; Tozon et al. 2016; Tamzali et al. 2012; Spugnini and Baldi 2019).

Partial necrosis of the tumors, more frequent after bleomycin treatment, does not require additional wound healing approaches and causes minimal inflammation reactions of surrounding tissue, which seem to be painless. Ulceration with mild inflammatory reaction in surrounding tissue is expected in the first week, followed by a superficial scab formation, which subsides within 5 weeks. Furthermore, cosmetic and functional effects after treatment are excellent. For equid sarcoids, the treatment is favorably combined with surgery, which makes it effective in most cases and provides satisfactory functional and esthetic results (Tamzali et al. 2012).

As the use of cisplatin is currently limited due to very restrictive regulations on anticancer drugs in veterinary medicine in many countries, electroporation with calcium (Electroporation Calcium Therapy or ECaT) emerges as a favorable alternative. A study evaluating the effects of ECaT in equine sarcoids (spontaneous skin tumors) on an animal cohort demonstrated that ECaT appears to be an effective alternative to ECT. Indeed, 13 of the 16 treated tumors showed necrosis. Among them, 9/13 were necrotic to >50% after a single treatment of ECaT. One adverse effect was a local inflammatory reaction. Surrounding tissues were not affected (Galant et al. 2019) Therefore, treatment with ECaT offers advantages in comparison to other treatments of sarcoids, and is beneficial for the horse, the owner, and the veterinarian, as this treatment is not associated with any constraints of biosecurity linked to the use of anticancer drugs. Calcium ions-mediated approach could also significantly reduce the cost of the electroporation treatment. This advantage could thus allow a larger spreading of the electroporation technique in veterinary facilities refractory to the implementation of restrictive biosecurity rules.

4.1 Immunotherapy Strategy to Stimulate Immune System

The type of cell death induced by the ablation modality, is a critical aspect of therapeutic success, and can affect the efficacy of the treatment as well as impact systemic antitumor immune system responses. In an attempt to increase systemic antitumor effectiveness of ECT, gene electrotransfer (GET) with immunomodulatory effect could be used as adjuvant treatment (Maglietti et al. 2020). The route of

administration for gene transfer of IL-12 can be intratumoral, peritumoral, or intramuscular (Kishida et al. 2003; Cemazar et al. 2017; Calvet and Mir 2016).

Several reports showed, both in human clinical trials and in veterinary trials, that intratumoral administration of different vectors (plasmid, viral vectors, etc.) carrying the interleukin-12 (IL-12) genes generates a strong systemic therapeutic effect in several models of metastatic digestive tumors, venereal tumors or in cutaneous tumors such as melanoma. IL-12 enhances CD4+ T cell differentiation into Th1 cells, stimulates cytotoxic functions of CD8+ T cells, NK cells, and NKT cells by increasing IFNγ secretion, and shows antiangiogenic properties (Kishida et al. 2003; Heinzerling et al. 2001).

The combination of Bleomycin-ECT, which is potent at eradicating primary superficial tumors, and IL-12 EGT, which can induce antitumor immunity, have been successful in eradicating and preventing recurrence of melanoma and mammary cancer in murine models (Kishida et al. 2003; Torrero et al. 2006). In addition, the combination of partial IRE and GET IL-12 can also induce antitumor immunity, resulting in tumor eradication and prevention of recurrence of melanoma in murine models (Pasquet et al. 2019).

In dogs, the combination of cytoreductive surgery, ECT, and IL-12 GET, repeated up to five times, depending on the clinical response to the treatment, may be beneficial for the treatment of canine oral malignant melanoma (Milevoj et al. 2019). Moreover, combined ECT and GET IL-12 induced a cellular response against neoplastic cells characterized mainly by the recruitment of T-lymphocytes and macrophages and a fibrotic proliferation with reduction of microvessels in mast cell tumors (MCT) (Salvadori et al. 2017) (Fig. 3).

Since electrochemotherapy (ECT) can induce immunogenic cell death of the tumor cells, adjuvant gene electrotransfer of Il-12 (GET IL-12) on the peritumoral tissue could lead to locoregional effect such as release of antigens and activation of the dendritic cells (DC). Activated DC can migrate to the draining lymph node (LN) and stimulate the production of specific T lymphocytes (CD4+ and CD8+). These specific lymphocytes can then act on the treated area but also produce an abscopal effect on distant untreated metastases. Therefore, the combination of electrochemotherapy with peritumoral IL-12 electrotransfer acts as an in situ vaccination.

5 Conclusions

Electric pulses-mediated drug delivery is now at a mature state. As electroporation-based treatments have a high response rate in biomedical applications, these approaches have been increasingly used as therapeutic procedures in both: human and veterinary oncology. While such treatments are rather simple in all cases, their success depends on several factors. First, the type of electrodes used and their configuration, as well as electrical parameters. The amplitude, duration, and the number of electric pulses have to be adequate to the type of electroporation-based treatment. Second, it is primordial to correctly place the electrodes to cover the tumor

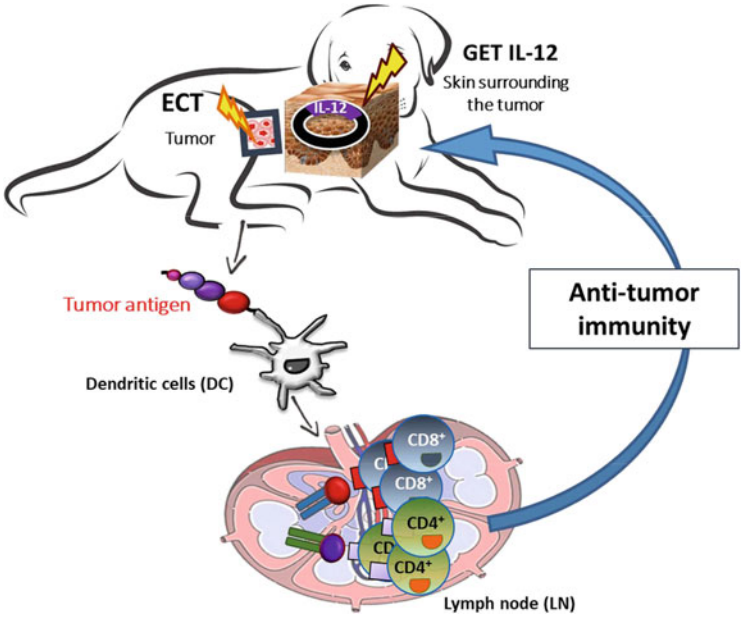

Fig. 3 Electro-chemo-gene-immuno-therapy as a strategy to stimulate the immune system

with an adequate electric field. Third, in electrochemotherapy it is important to provide a sufficient concentration of the drug at the site of treatment, while in tumor ablation by irreversible electroporation it is important to ablate the entire malignant zone. Finally yet importantly, in an attempt to increase the systemic antitumor effectiveness the electrotransfer of genes with immunomodulatory effect (immunogene electrotransfer) could be used as adjuvant treatment. Since electrochemotherapy can induce immunogenic cell death, adjuvant immunogene electrotransfer to peritumoral tissue could lead to locoregional effects, as well as abscopal effects on distal (untreated) metastases.

Electrochemotherapy is now used in routine oncology clinical practice after almost 20 years of preclinical research. In addition, electroporation might be useful in novel applications, and will indeed continue to benefit from an increasing knowledge on the level of the molecular mechanism, where the process remains poorly understood.

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References

Agoston D et al (2020) Evaluation of calcium electroporation for the treatment of cutaneous metastases: a double blinded randomised controlled phase II trial. Cancers 12(1):179

Aguilar-Machado D et al (2020) Enzymatic processes triggered by PEF for astaxanthin extraction from xanthophyllomyces dendrorhous. Front Bioeng Biotechnol 8:857

Allemani C et al (2018) Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. Lancet 391(10125):1023–1075

Calvet CY, Mir LM (2016) The promising alliance of anti-cancer electrochemotherapy with immunotherapy. Cancer Metastasis Rev 35(2):165–177

Cemazar M et al (1999) Increased platinum accumulation in SA-1 tumour cells after in vivo electrochemotherapy with cisplatin. Br J Cancer 79(9-10):1386–1391

Cemazar M et al (2008) Electrochemotherapy in veterinary oncology. J Vet Intern Med 22 (4):826–831

Cemazar M et al (2017) Efficacy and safety of electrochemotherapy combined with peritumoral IL-12 gene electrotransfer of canine mast cell tumours. Vet Comp Oncol 15(2):641–654

Daud AI et al (2008) Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. J Clin Oncol Off J Am Soc Clin Oncol 26(36):5896–5903

Davalos RV, Mir IL, Rubinsky B (2005) Tissue ablation with irreversible electroporation. Ann Biomed Eng 33(2):223–231

Escoffre JM et al (2009) What is (still not) known of the mechanism by which electroporation mediates gene transfer and expression in cells and tissues. Mol Biotechnol 41(3):286–295

Escoffre JM et al (2011) Electromediated formation of DNA complexes with cell membranes and its consequences for gene delivery. BBA-Biomembranes 1808(6):1538–1543

Escoffre JM et al (2014a) Membrane disorder and phospholipid scrambling in electropermeabilized and viable cells. BBA-Biomembranes 1838(7):1701–1709

Escoffre JM et al (2014b) Evidence for electro-induced membrane defects assessed by lateral mobility measurement of a GPi anchored protein. Eur Biophys J Biophys Lett 43(6-7):277–286

Falzone L, Salomone S, Libra M (2018) Evolution of cancer pharmacological treatments at the turn of the third millennium. Front Pharmacol 9:1300

Faurie C et al (2004) Effect of electric field vectoriality on electrically mediated gene delivery in mammalian cells. BBA-Biomembranes 1665(1–2):92–100

Faurie C et al (2010) Electro-mediated gene transfer and expression are controlled by the life-time of DNA/membrane complex formation. J Gene Med 12(1):117–125

Frandsen SK et al (2012) Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis. Cancer Res 72(6):1336–1341

Frandsen SK et al (2020) Calcium electroporation of equine sarcoids. Animals 10(3):517

Franklin B (1956) Farther experiments and observations in electricity. Science 123(3185):47–50

Galant L et al (2019) Calcium electroporation: the bioelectrochemical treatment of spontaneous equine skin tumors results in a local necrosis. Bioelectrochemistry 129:251–258

Garcia PA et al (2017) Predictive therapeutic planning for irreversible electroporation treatment of spontaneous malignant glioma. Med Phys 44(9):4968–4980

Gehl J et al (2006) Results of the ESOPE (European Standard Operating Procedures on Electrochemotherapy) study: efficient, highly tolerable and simple palliative treatment of cutaneous and subcutaneous metastases from cancers of any histology. J Clin Oncol 24 (18):464s

Gehl J et al (2018) Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol 57(7):874–882

Golzio M, Teissie J, Rols MP (2002) Direct visualization at the single-cell level of electrically mediated gene delivery. Proc Natl Acad Sci U S A 99(3):1292–1297

Heinzerling LM et al (2001) Tumor regression induced by intratumoral injection of DNA coding for human interleukin 12 into melanoma metastases in gray horses. J Mol Med 78(12):692–702

Henshaw JW, Yuan F (2008) Field distribution and DNA transport in solid tumors during electric field-mediated gene delivery. J Pharm Sci 97(2):691–711
Hojman P (2010) Basic principles and clinical advancements of muscle electrotransfer. Curr Gene Ther 10(2):128–138
Impellizeri J et al (2016) Electroporation in veterinary oncology. Vet J 217:18–25
Kishida T et al (2003) Electrochemo-gene therapy of cancer: intratumoral delivery of interleukin-12 gene and bleomycin synergistically induced therapeutic immunity and suppressed subcutaneous and metastatic melanomas in mice. Mol Ther 8(5):738–745
Maglietti F et al (2020) Electroporation as the immunotherapy strategy for cancer in veterinary medicine: state of the art in Latin America. Vaccine 8(3):537
Milevoj N et al (2019) A combination of electrochemotherapy, gene electrotransfer of plasmid encoding canine IL-12 and cytoreductive surgery in the treatment of canine oral malignant melanoma. Res Vet Sci 122:40–49
Mir LM et al (1997) First clinical trial of cat soft-tissue sarcomas treatment by electrochemotherapy. Br J Cancer 76(12):1617–1622
Mir LM et al (1998) Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy. Br J Cancer 77(12):2336–2342
Monfort S et al (2012) Inactivation of Salmonella spp. in liquid whole egg using pulsed electric fields, heat, and additives. Food Microbiol 30(2):393–399
Neumann E, Rosenheck K (1972) Permeability changes induced by electric impulses in vesicular membranes. J Membr Biol 10(3):279–290
Neumann E et al (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J 1(7):841–845
Niessen C et al (2012) Ablation of a liver metastasis with irreversible electroporation (IRE) in liver segment II adjoining the area nuda. RoFo 184(10):937–938
Okino M, Mohri H (1987) Effects of a high-voltage electrical impulse and an anticancer drug on in vivo growing tumors. Jpn J Cancer Res Gann 78(12):1319–1321
Orlowski S et al (1988) Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. Biochem Pharmacol 37(24):4727–4733
Paganin-Gioanni A et al (2011) Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells. Proc Natl Acad Sci U S A 108(26):10443–10447
Parniakov O et al (2016) Extraction assisted by pulsed electric energy as a potential tool for green and sustainable recovery of nutritionally valuable compounds from mango peels. Food Chem 192:842–848
Pasquet L et al (2019) Pre-clinical investigation of the synergy effect of interleukin-12 gene-electrotransfer during partially irreversible electropermeabilization against melanoma. J Immunother Cancer 7:161
Pirc E, Miklavcic D, Rebersek M (2019) Nanosecond pulse electroporator with silicon carbide mosfets: development and evaluation. IEEE Trans Biomed Eng 66(12):3526–3533
Puertolas E et al (2009) Pulsed electric fields inactivation of wine spoilage yeast and bacteria. Int J Food Microbiol 130(1):49–55
Rebersek M et al (2007) Electroporator with automatic change of electric field direction improves gene electrotransfer in-vitro. Biomed Eng Online 6:25
Rols MP (2006) Electropermeabilization, a physical method for the delivery of therapeutic molecules into cells. BBA-Biomembranes 1758(3):423–428
Rols MP, Femenia P, Teissie J (1995) Long-lived macropinocytosis takes place in electropermeabilized mammalian-cells. Biochem Biophys Res Commun 208(1):26–35
Rols MP et al (1998) In vivo electrically mediated protein and gene transfer in murine melanoma. Nat Biotechnol 16(2):168–171
Rols MP, Tamzali Y, Teissie J (2002) Electrochemotherapy of horses. A preliminary clinical report. Bioelectrochemistry 55(1-2):101–105
Romeo S et al (2018) ESOPE-equivalent pulsing protocols for calcium electroporation: an in vitro optimization study on 2 cancer cell models. Technol Cancer Res Treat 17:1533033818788072

Rosazza C et al (2011) The actin cytoskeleton has an active role in the electrotransfer of plasmid DNA in mammalian cells. Mol Ther 19(5):913–921
Rosazza C et al (2013) Intracellular tracking of single-plasmid DNA particles after delivery by electroporation. Mol Ther 21(12):2217–2226
Rosazza C et al (2016) Endocytosis and endosomal trafficking of DNA after gene electrotransfer in vitro. Mol Ther Nucleic Acids 5:1–11
Ruarus AH et al (2020) Irreversible electroporation for hepatic tumors: protocol standardization using the modified delphi technique. J Vasc Interv Radiol 31(11):1765–1771 e15
Salvadori C et al (2017) Effects of electrochemotherapy with cisplatin and peritumoral IL-12 gene electrotransfer on canine mast cell tumors: a histopathologic and immunohistochemical study. Radiol Oncol 51(3):286–294
Scheltema MJ et al (2019) Numerical simulation modeling of the irreversible electroporation treatment zone for focal therapy of prostate cancer, correlation with whole-mount pathology and T2-weighted MRI sequences. Ther Adv Urol 11:1756287219852305
Schicho A et al (2019) Long-term survival after percutaneous irreversible electroporation of inoperable colorectal liver metastases. Cancer Manag Res 11:317–322
Spugnini EP, Baldi A (2019) Electrochemotherapy in veterinary oncology: state-of-the-art and perspectives. Vet Clin North Am Small Anim Pract 49(5):967–979
Tamzali Y, Teissie J, Rols MP (2001) Cutaneous tumor treatment by electrochemotherapy: preliminary clinical results in horse sarcoids. Rev Med Vet 152(8–9):605
Tamzali Y et al (2012) Successful treatment of equine sarcoids with cisplatin electrochemotherapy: a retrospective study of 48 cases. Equine Vet J 44(2):214–220
Teissie J, Rols MP (1986) Fusion of mammalian-cells in culture is obtained by creating the contact between cells after their electropermeabilization. Biochem Biophys Res Commun 140 (1):258–266
Teissie J, Rols MP (1993) An experimental evaluation of the critical potential difference inducing cell-membrane electropermeabilization. Biophys J 65(1):409–413
Teissie J et al (1982) Electric pulse-induced fusion of 3t3 cells in monolayer-culture. Science 216 (4545):537–538
Teissie J, Golzio M, Rols MP (2005) Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of ?) knowledge. BBA-Gen Subjects 1724(3):270–280
Torrero MN, Henk WG, Li S (2006) Regression of high-grade malignancy in mice by bleomycin and interleukin-12 electrochemogenetherapy. Clin Cancer Res 12(1):257–263
Tozon N et al (2016) Operating procedures of the electrochemotherapy for treatment of tumor in dogs and cats. J Vis Exp 116:54760
Yarmush ML et al (2014) Electroporation-based technologies for medicine: principles, applications, and challenges. Annu Rev Biomed Eng 16:295–320
Zelbstein MU (1984) Not available. Hist Sci Med 18(3):295–306

Electrodes and Electric Field Distribution in Clinical Practice

Helena Cindrič, Bor Kos, and Damijan Miklavčič

Abstract

The success of electroporation-based treatments (i.e., reversible electroporation for electrochemotherapy and gene electrotransfer and irreversible electroporation as a tissue ablation method) depends on complete coverage of the clinical target volume with a sufficiently strong electric field (exceeding the reversible or irreversible electroporation threshold, depending on the type of treatment). Electric field distribution in biological tissue is a complex phenomenon that depends on several parameters, such as the electrode geometry, parameters of delivered pulses and tissue composition and electrical properties. Numerical modelling has proven to be an indispensable tool in investigating and designing electroporation-based treatments and preparing patient-specific treatment plans. In this chapter, the basic principles regarding electric field distribution in biological tissue are explained, with the aim to enable effective electroporation-based treatments. Biological effects observed in electroporation on tissue level are described, common electrode designs used in clinical practice are shown and equipped also with simple models of electric field distribution. The basic principles of numerical modeling and treatment planning are also explained.

Keywords

Electric field distribution · Electrodes for electroporation · Numerical models · Treatment planning · Clinical practice · Tissue electroporation

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1 Introduction

Electroporation is a phenomenon in which short high-voltage electric pulses are used to change the structural integrity of the cell membrane and consequently increase the membrane permeability. With an appropriate selection of pulse parameters—namely the amplitude, duration, and number of applied pulses—the phenomenon can be either reversible or irreversible. In reversible electroporation, the cell membranes quickly return to their original state and the cells' ability to divide and function is not affected in the long term, while in irreversible electroporation the cells lose their functionality due to high exposure and die (Neumann and Rosenheck 1972; Kotnik et al. 2011, 2019; Rems and Miklavčič 2016). Electroporation has sparked interest for use in medicine, biotechnology, and food processing (Yarmush et al. 2014; Mahnič-Kalamiza et al. 2014; Kotnik et al. 2015; Geboers et al. 2020). In medical applications reversible electroporation is used for facilitating the transport of various molecules into cells, the most promising treatments being electrochemotherapy (ECT) and gene electrotransfer (GET) (Mir et al. 1991; Serša and Miklavčič 2008; Rosazza et al. 2016; Stepišnik et al. 2016), while irreversible electroporation (IRE) is used for ablation of tumor and cardiac tissue (Davalos et al. 2005; Edd et al. 2006; Rubinsky 2007; Meijerink et al. 2018; Jiang et al. 2015; Scheffer et al. 2014; Reddy et al. 2018; Wittkampf et al. 2018; Stewart et al. 2019).

It is generally accepted that reversible and irreversible electroporation occurs in tissue at a specific electric field strength, i.e., the reversible and irreversible electroporation threshold (Fig. 1). A complete coverage of the target tissue volume with electric field above either the reversible or the irreversible threshold is required to achieve a therapeutic effect in electroporation-based treatments (Miklavčič et al.

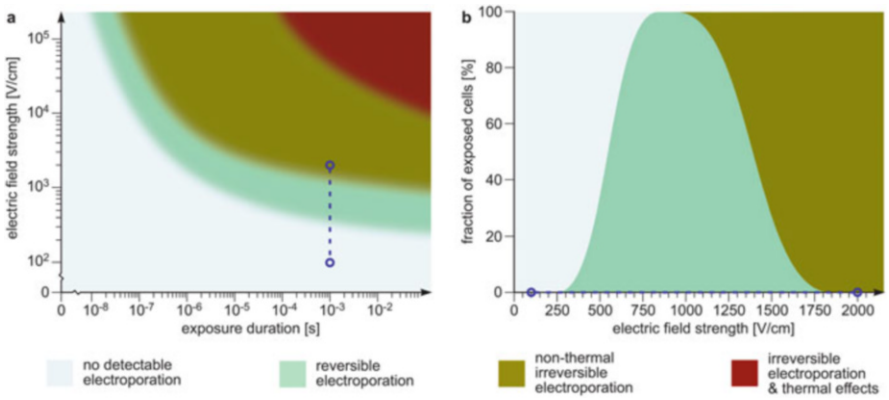

Fig. 1 (**a**) Reversible and irreversible electroporation and thermal effects are functions of both the electric field strength and the duration of tissue exposure (mainly the number and duration of applied pulses). Electroporation may occur at lower electric field threshold if exposure duration is increased. (**b**) For a fixed exposure duration of 1 ms (dashed line in panel **a**), the fraction of electroporated cells is a function of the electric field strength. The image is reproduced from Yarmush et al. (2014)

2006). Electric field distribution in tissue depends on the electrode geometry, parameters of delivered pulses, and tissue electrical properties. Several electrode designs and configurations have been developed through the years to support different therapeutic needs—from plate electrodes mainly used for the treatment of superficial tumors (e.g., skin metastases), fixed needle arrays, individual needle electrodes for treatment of deep-seated tumors, to electrodes tailored to specific treatments such as finger electrodes (Miklavčič et al. 2014; Geboers et al. 2020). The geometry of the electrodes largely determines the distribution of electric fields in tissue.

A wide range of different pulses and pulse protocols is used in practice—from $8 \times 100\mu s$ pulses used in ECT to $90 \times 100\mu s$ pulses used in IRE, and millisecond pulses used in GET. The most straightforward approach to controlling the electric field strength is by adjusting the applied voltage amplitude according to the distance between paired electrodes. The manufacturers of pulse generators usually suggest a fixed voltage-to-distance ratio as the guideline for applied voltage amplitude. However, voltage-to-distance ratio should not be confused with the electroporation thresholds (despite sharing the same unit of measure—V/cm), as it is generally higher than the actual threshold. Electroporation thresholds also depend on the duration of exposure of target tissue to the electric field, i.e., the number and duration of applied pulses (Pucihar et al. 2011). In other words, electroporation may occur at a lower threshold if more pulses or longer pulses are applied, as demonstrated in Fig. 1.

Another important factor affecting the electric field distribution is tissue properties. Target tissue is often not a homogenous structure and may include different tissue types (e.g., skin, fat, and muscle in case of treatment of cutaneous and subcutaneous targets), which have different electrical and thermal properties. The local electric field strength depends on the electrical conductivity of the medium (i.e., tissue), therefore, any inhomogeneity in tissue electrical conductivity affects the electric field distribution, and needs to be accounted for when planning the treatment (Dermol-Černe et al. 2020). Furthermore, tissue electrical conductivity is not constant during electroporation but increases due to electroporation, which depends on the local electric field strength in the tissue, and due to increased temperature. This rise in electrical conductivity in turn affects the local electric field (Šel et al. 2005; Ivorra et al. 2009; Corovic et al. 2013).

Electric field distribution in biological tissue is a complex phenomenon with multiple factors to be considered and is, therefore, often not intuitive or easy to determine. This chapter provides an overview of basic principles regarding electric field distribution in biological tissue, with the aim to enable effective electroporation-based treatments (i.e., reversible electroporation for electrochemotherapy and gene electrotransfer and irreversible electroporation as a tissue ablation method). Biological effects of electroporation on tissue level are explained and common electrode designs for clinical practice are shown and equipped with results based on contemporary numerical models. The basics of numerical modelling and patient-specific treatment planning are also provided.

2 Gross Biological Effects Observed in Electroporation

2.1 Reversible Electroporation

When a biological cell is exposed to an external electric field of sufficient strength structural and chemical changes occur primarily in the cell membrane but also in other subcellular structures, such as the cytoskeleton. This process is known as electroporation or electropermeabilization. So far, three main mechanisms affecting the cell membrane have been identified (Kotnik et al. 2019): (a) the formation of hydrophilic pores in the lipid bilayer, (b) chemical changes in membrane lipids (e.g., peroxidation), and (c) electrically induced modulation of membrane protein function (e.g., voltage-gated channels). These mechanisms contribute to a transiently increased permeability of the cell membrane to water, ions, and molecules of various sizes, for which the membrane is otherwise impermeable or poorly permeable. The membrane gradually recovers its physiological level of impermeability (resealing) within a few minutes after the termination of pulse delivery and, unless the damage inflicted is leading to cell death, the membrane returns to its natural state within a few hours. Electroporation causes the formation of additional conductive pathways in the otherwise poorly conducting cell membrane thus also resulting in an increased bulk electrical conductivity of affected tissue. Figure 2 shows a diagram of various gross biological effects encountered in electroporation treatments. The zone of reversible electroporation is indicated in Fig. 2 with a yellow contour.

Facilitation of cellular uptake by reversible electroporation is the basis of electrochemotherapy (ECT) and gene electrotransfer (GET), in which cytotoxic agents and genetic material, respectively, are introduced into cells. The membrane resealing mechanism traps the introduced material within the cell thus increasing its effectiveness. ECT has already achieved a firm place in clinical and veterinary oncology (Cemazar et al. 2008; Kulbacka et al. 2017; Campana et al. 2019) with the introduction of the European Standard Operating Procedures of ECT (ESOPE) in 2006 (Mir et al. 2006; Gehl et al. 2018). GET is not yet in widespread use in humans, but ongoing studies show promising results. Currently, the most advanced applications of GET are DNA vaccination and cytokine therapy (Heller and Heller 2015; Lambricht et al. 2016; Geboers et al. 2020).

2.2 Irreversible Electroporation

If the applied electric field exceeds a critical threshold, the cell membrane is damaged to the extent that it can no longer repair itself, resulting in loss of cell homeostasis. The damaged cells eventually die in a mechanism that is similar to the programmed cell death known as apoptosis (Yarmush et al. 2014; Rems and Miklavčič 2016). This process is known as irreversible electroporation and has attracted interest as an alternative method for ablation of various soft tissues due to its essentially nonthermal cell kill mechanism (Davalos et al. 2005; Scheffer et al. 2014; Aycock and Davalos 2019).

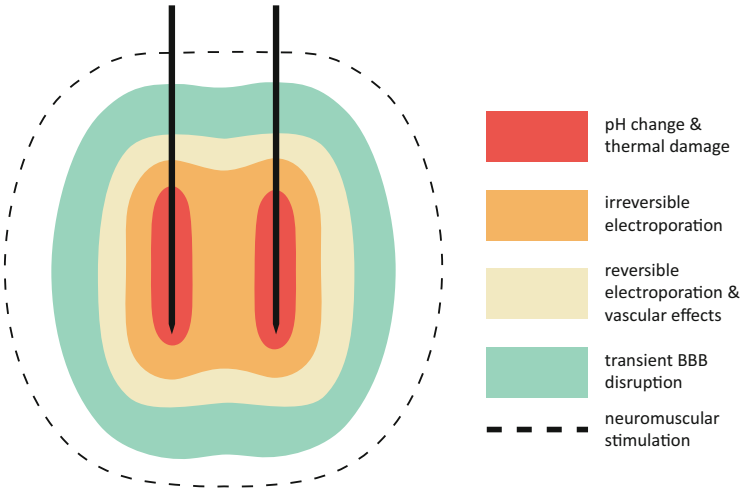

Fig. 2 Regions of gross biological effects observed in electroporation. Mechanical damage occurs along the path of needle electrode insertion. Thermal damage and possible pH changes occur in close proximity to the electrode surface (red), followed by a zone of irreversible electroporation (orange), a zone of reversible electroporation and vascular effects (yellow), and a zone of transient blood–brain barrier (BBB) disruption (green). The dashed contour indicates the zone where excitable tissues, such as nerves and muscles, are stimulated. It should be noted that this diagram is for illustrative purposes only. The size of specific regions varies with many parameters, including electrode spacing and length of exposed tip, duration and number of applied pulses, and tissue type. Moreover, the specific effects do not necessarily overlap with reversible/irreversible electroporation, for instance vascular effects may manifest already at lower electric fields than reversible electroporation

In the therapeutic setting, several cell death mechanisms are likely to act in addition to apoptosis, including necrosis caused by the inevitable thermal component (Ben-David et al. 2012; Garcia et al. 2014), necroptosis, and the contribution of the systemic immune response targeting the tumor and potential metastatic sites (Brock et al. 2020). Irreversible electroporation affects mainly the cells, leaving other structures and proteins in the extracellular matrix intact, thus maintaining the structural integrity of the tissue. This promotes tissue regeneration after treatment and reduces tissue scarring, which is a significant improvement compared to existing treatment modalities (Vogel et al. 2016). The zone of irreversible electroporation is indicated in Fig. 2 with an orange contour.

Even though lower pulse amplitudes and significantly fewer pulses are used in electrochemotherapy, an area of irreversibly electroporated tissue is also present. However, this area is limited to a few millimeters from the electrode surface and has little clinical significance for ECT (Zmuc et al. 2019). Similarly, a zone of IRE is also to be expected in GET, as longer pulses are used. In GET reversible electroporation and cell survival are imperative for successful transfection, therefore, irreversible electroporation is undesirable (Lacković et al. 2009). Moreover, the associated

heating and pH changers may also negatively affect the plasmid DNA (Maglietti et al. 2013).

2.3 Thermal Effects

Electroporation, and especially IRE ablation, is commonly referred to as a nonthermal modality, which can lead to a wrongful assumption that the thermal component is completely absent from the treatment. No clinically relevant heating has been observed in treatments with reversible electroporation. However, in IRE ablation, several studies have shown that current clinical protocols may lead to elevated temperatures sufficient to cause thermal damage to varying degrees (Ben-David et al. 2012; Faroja et al. 2013; Dunki-Jacobs et al. 2014; Wagstaff et al. 2015). When an electric current flows through a conducting medium, thermal energy is generated in a process called Joule heating. High voltages (up to 3000 V) are used in IRE ablation and several hundred pulses are sequentially delivered in a single session, especially when treating large volumes of tissue with multiple electrode pairs. While the predominant cell death mechanism in IRE is still nonthermal, these factors do contribute to a distinct thermal component that results in an area of necrotic tissue consistent with thermal injury. The area of thermal damage is mostly confined to the vicinity of the electrodes and may extend further into the surrounding tissue as the number and duration of pulses increases (Garcia et al. 2011; O'Brien et al. 2018). The zone where thermal damage is expected is indicated in Fig. 2 with a red contour.

The currently most common application of IRE ablation is in treatment of tissue volumes where the use of conventional treatments, such as surgery, radiofrequency ablation, and microwave ablation, are contraindicated for various reasons. Due to its predominantly non-thermal mechanism of cell kill, IRE ablation has shown promise for use in cases where other ablation modalities present an unacceptable risk to nearby critical anatomical structures such as vessels, bile ducts, and nerve bundles that are highly susceptible to thermal damage (Maor et al. 2010; Schoellnast et al. 2013; Cannon et al. 2013). However, in such cases, the thermal component of IRE ablation cannot be neglected. Special care must be taken to place the electrodes at a sufficient distance from the critical anatomical structures and pulsing protocols used during treatment must be carefully considered. Recent studies have demonstrated the potential of internally cooled electrodes (O'Brien et al. 2018) and algorithmically controlled pulsing protocols (Sano et al. 2020) for regulating the temperature during ablation. In addition, IRE is unaffected by the heat sink effect and can, therefore, be successfully used for ablation in the vicinity of large blood vessels, where the efficacy of thermal ablation modalities is reduced (Ahmed et al. 2011).

2.4 Blood Flow Modifying Effects of Pulsed Electric Fields

It is well known that electroporation induces a decrease in local blood flow, which may be roughly divided into two phases. Immediately after the initiation of pulse delivery the local blood flow reduces nearly to zero within a few seconds. This rapid decrease is mainly attributed to the constriction of smooth muscles in the vessel walls (vasoconstriction). This phase is relatively short, as blood flow recovers to about 40% of pre-treatment levels in up to 15 min after pulse application (Jarm et al. 2010). After the initial recovery, a stall in blood flow recovery is observed in the second phase, mainly due to structural changes in the endothelium. Electroporation causes disruption of the cytoskeletal filaments and intercellular junctions resulting in increased permeability of the endothelial monolayer (Kanthou et al. 2006; Čemažar 2017). Loss of barrier integrity leads to fluid leakage, resulting in increased interstitial fluid pressure and decreased intravascular pressure (Jarm et al. 2010; Graybill and Davalos 2020). These factors together manifest in decreased local perfusion, which may persist for several hours after treatment. The decrease in perfusion is more pronounced in tumor tissue than in healthy tissue because of the already abnormal vasculature of tumors, and may persist for up to 24 h after treatment. The zone of vascular effects corresponds roughly with the zone of reversible electroporation and is indicated in Fig. 2 with a yellow contour.

The vascular effects of electroporation have further relevance in electroporation-based treatments. While the application of pulses alone causes a temporary decrease in blood flow, the addition of the chemotherapeutic agent in ECT has been shown to cause permanent disruption of local vasculature (Čemažar 2017). The vascular disrupting effect of ECT is already effectively used in the treatment of bleeding metastases (Jarm et al. 2010) and is also beneficial in the treatment of highly vascularized organs, such as the liver. Moreover, it prevents washout of cytostatic drugs in ECT and plasmids in GET. On the other hand, a reduction in tissue perfusion also leads to a decreased cooling ability of the affected tissue. In IRE ablation, a large number of pulses is applied to the target tissue; therefore, Joule heating by the electrodes is more pronounced and results in a greater thermal component. A decrease in tissue blood perfusion may contribute even further to the undesirable thermal component by reducing the amount of heat that the blood is able to extract from tissue.

2.5 Nerve and Muscle Stimulation

Excitable tissues, such as muscles, nerves, and myocardium, respond to external electrical stimuli, and the electric field threshold for triggering the action potential in excitable cells is much lower than that of electroporation. Therefore, the application of electroporation pulses causes stimulation of nerves and muscles in the vicinity of the treatment zone, resulting in muscle contractions and acute pain (Golberg and Rubinsky 2012; Mercadal et al. 2017). The zone of neuromuscular stimulation is indicated in Fig. 2 with a dashed contour.

Several advances have been made to reduce the neuromuscular stimulation during electroporation-based treatments. Local or general anesthesia (depending on treatment type) is used to reduce pain during pulse application, while muscle relaxants are used to reduce muscle contractions; however, contractions are still observed near the electrodes (Golberg and Rubinsky 2012). Aside from unpleasant sensations, muscle contractions can displace the electrodes during treatment. This is particularly important during IRE ablation of deep-seated tissues, where long needle electrodes are carefully placed around the target volume. Displacement of the electrodes during pulses alters the electric field distribution and increases needle trauma, which can lead to undertreatment and harmful effects on nearby vital structures (Martin et al. 2015). Therefore, in current clinical practice, all IRE procedures are performed under general anesthesia with complete neuromuscular blockade. Furthermore, to eliminate the risk of triggering cardiac arrhythmias during procedures in the thoracic region, synchronization of pulse delivery with the patient's ECG cycle is required; pulses are timed to be delivered during the absolute myocardial refractory period (Cannon et al. 2013; Mali et al. 2015).

Several studies have shown that altering pulse dynamics, for example, increasing the pulse repetition rate of monopolar pulses from 1/s to 5000/s in ECT (Miklavčič et al. 2005; Zupanic et al. 2007), can significantly reduce muscle stimulation and associated adverse effects without compromising treatment efficacy. A new pulse protocol for IRE, referred to as high-frequency IRE or H-FIRE has been introduced in recent studies (Arena et al. 2011; Yao et al. 2017; Ringel-Scaia et al. 2019), in which conventional monopolar pulses with a repetition rate of 1–5000/s are replaced by bursts of bipolar pulses with a repetition rate of 50,000–125,000/s.

2.6 Blood–Brain Barrier Disruption

The blood–brain barrier (BBB) is a multicellular endothelial structure that separates the central nervous system from the peripheral vasculature and restricts the passage of pathogens and various molecules from the blood into the cerebrospinal fluid (Obermeier et al. 2013). Due to its high selectivity, BBB is a major obstacle to the effective penetration of systemically administered therapeutics, thus limiting the efficacy of treatment for tumors and various neurodegenerative diseases. Recently, it was discovered that electroporation (EP) applied in the brain can cause a transient disruption of the BBB lasting for 24–48 h, which may facilitate the uptake of therapeutic agents (Sharabi and Mardor 2016). The exact mechanism of EP-induced BBB disruption is still unclear, but it has been reported to occur at lower thresholds than reversible electroporation of endothelial cells (Sharabi et al. 2019). As with electroporation, BBB disruption is also dependent on electric field strength as well as exposure duration, meaning that the threshold will decrease with increasing exposure duration. The zone of transient BBB disruption is represented in Fig. 2 with a green contour.

EP-induced BBB can be used alone (reversible electroporation only) or in combination with IRE ablation. The latter may be particularly useful in treatment

of brain tumors, where IRE is used to ablate most of the tumor mass, while the area of reversible electroporation and BBB disruption surrounding the ablated tissue is treated with therapeutic drugs to eliminate infiltrating tumor cells while sparing healthy brain tissue (Hjouj et al. 2012; Sharabi et al. 2016).

2.7 pH Changes

In recent years new studies have emerged, examining a process previously overlooked in EP-based treatments—the role of pH changes in cell death mechanisms (Turjanski et al. 2011; Maglietti et al. 2013; Olaiz et al. 2014). The theory is based on previous experience in electrochemical treatment of tumors (EChT) where it was shown that two opposing pH fronts emanate from the two electrodes, acidic from the anode and basic from the cathode (Miklavčič et al. 1993), which are related to the extent of the necrotic area (Turjanski et al. 2009). It is suggested that a similar effect may be present in EP-based treatments. While the presence of tissue necrosis is not particularly bothersome in IRE ablation, it is significantly more undesirable in GET. Significant pH changes in the medium can be detrimental to the plasmids used in GET, as DNA denaturation is influenced by pH (Maglietti et al. 2013).

The pH change is attributed to the ion transport, which follows the free diffusion dynamics and is more pronounced at the site of the anode than the cathode (Turjanski et al. 2009). The transport of ions results in strong anodic acidification and cathodic alkalinization. A recent in vitro study on cell survival and GET efficacy showed, that overall cell survival was better in slightly acidic extracellular conditions, however, the efficacy of GET was decreased (Potočnik et al. 2019).

3 Tissue Properties and Computation of Electric Field

Different biological tissues have very different dielectric and thermal properties. The dielectric character of tissues is described by electrical conductivity (the ability to transfer electrical charge) and relative permittivity (the ability to store charge and rotate molecular dipoles). Both properties are frequency dependent. Electrical conductivity increases significantly at frequencies above 100 kHz, while permittivity decreases with frequency. However, most EP-based treatments are performed in the lower frequency range, where electrical conductivity can be considered frequency independent, and displacement currents, which are affected by the permittivity, can be neglected. Thermal properties of tissue are mainly characterized by thermal conductivity, specific heat capacity, and tissue perfusion rate. This chapter mainly focuses on the electrical conductivity, since it directly affects the electric field distribution in the target tissue. Nevertheless, some relevant thermal properties are also discussed. At the end of the chapter, the basics of numerical modeling commonly used to study and visualize electric field distribution in tissue are explained as well.

3.1 Physiological State of Tissue

There is a large discrepancy in measured values of electrical conductivity reported in literature. Several factors contribute to this variability, for example, tissue inhomogeneity and anisotropy, biological variability, and method of measurement. The physiological state of measured tissue, for instance, pathological changes and whether the measurements were performed in vivo or ex vivo, also introduce significant variability within the same tissue type.

In vivo measurements of dielectric properties are quite challenging; therefore, measurements are often performed ex vivo in excised tissue samples. However, the dielectric properties of tissues change rapidly and significantly after death, especially in the lower frequency range (below 1 MHz). For most tissues, the electrical conductivity and permittivity decrease immediately after excision; for example, in liver tissue, the dielectric properties measured in vivo can be 16–43% higher than ex vivo in the GHz range (O'Rourke et al. 2007).

Pathological changes to tissue, such as the presence of fibrosis in cirrhotic liver and increased fat content in liver steatosis, can have a significant effect on the dielectric properties, due to inherently different cellular architecture (O'Rourke et al. 2007; Peyman et al. 2015). For example, cirrhotic liver has a slightly higher conductivity than normal liver, while liver with steatosis has a lower conductivity due to the low conductivity of fat.

Malignant tissues have significantly different properties from healthy tissue. It is generally accepted, that tumors have higher electrical conductivity than normal tissue (Haemmerich et al. 2009; Laufer et al. 2010; Peyman et al. 2015). This is mainly attributed to altered cell membrane composition and abnormal microvasculature, which results in a presumably higher ion and water content in tumor tissue (Peyman et al. 2015). Laufer et al. (2010) and Haemmerich et al. (2009) measured the conductivity of excised hepatic tumors and normal liver parenchyma. In both studies, the reported conductivity of tumors was approximately 5 times the value of normal liver tissue. The dielectric properties of tumors also vary with the tumor type. For instance, Peyman et al. showed that liver metastases exhibit even higher dielectric properties compared to primary hepatic tumors (HCC), since they originate from a different tissue.

Contrary to dielectric properties, thermal properties are not significantly affected by the pathological changes. Furthermore, changes following death (tissue excision) occur much later than with electrical properties, and are attributed mainly to tissue drying (Duck 2012).

3.2 Tissue Anisotropy

Some biological tissues, such as the skeletal muscles, nerves, and bones, exhibit a distinct directional organization of cells and extracellular structures. This allows an easier flow of electric current in a specific direction, meaning that the electrical conductivity of tissue is not equal in all directions. For example in skeletal muscles,

the electrical conductivity parallel to muscle fibers can be up to 5 times higher than the conductivity perpendicular to fiber orientation (Gabriel et al. 2009). Anisotropy may affect the distribution of electric fields in tissue as the electric current preferably flows along the direction with less resistance. At higher frequencies (~1 MHz) the electrical properties become essentially isotropic.

3.3 Tissue Perfusion

Tissue perfusion can roughly be divided into microvascular perfusion, which occurs at the capillary level, and macrovascular perfusion, which refers to blood vessels with a diameter greater than 3 mm. Both types of perfusion affect the convective heat transfer; while the well-known heat sink effect is mainly associated with the macrovasculature, microvascular perfusion plays an important role in heat deposition and tissue self-cooling ability (Schutt and Haemmerich 2008). In EP-based treatments, the thermal effects are usually not detrimental, however, in IRE ablation the reduced cooling ability due to vascular effects may impair the nonthermal character of treatment.

The characteristics of tumor vasculature and blood flow are very different from that of normal tissues. Solid tumors are often poorly perfused and oxygenated in comparison with the surrounding tissue, which is generally homogeneously vascularized. Variability in the density of capillaries and oxygenation levels within the tumor volume is often present, abnormal blood vessel walls result in an inferior blood flow regulation and leaky capillaries. These changes contribute to the development of hypoxic regions in the tumor volume and to acidic tissue environment (Jarm et al. 2010). Abnormal tumor blood flow can represent an obstacle for antitumor treatments, as it can impede the interstitial delivery of chemotherapeutic agents. On the other hand, these abnormalities can make the tumor vulnerable due to vascular disrupting effects, present for example in ECT (Cemazar et al. 2001).

3.4 Nonhomogenous Tissue

The treated area often consists of more than one tissue type, resulting in a distinct inhomogeneity of tissue properties, especially the electrical conductivity. Examples of tissues with the lowest baseline electrical conductivity are bones, fat tissue, and the outermost layer of the skin (the stratum corneum), while fluids, such as blood and bile, and tissues with a high ionic content, such as the prostate and kidney, have the highest electrical conductivity in the human body (Gabriel et al. 1996, 2009; Duck 2012).

Electric field strength depends on electrical conductivity of the medium (i.e., tissue); therefore, any inhomogeneity in tissue composition needs to be accounted for when calculating the electric field distribution. This is especially important when treating target volumes that contain tissues with significantly different conductivities, as the majority of voltage drop, and consequently electric field

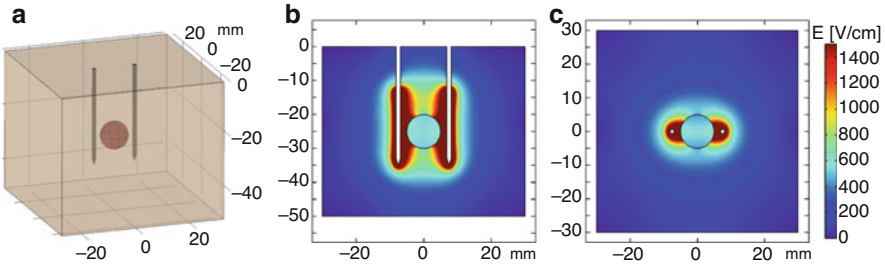

Fig. 3 A simple numerical model of a deep-seated spherical tumor surrounded by fat (**a**). Computed electric field distribution in tissue shown in side view (**b**) and axial view at the largest diameter of the tumor (**c**). Tumor volume is outlined in black. The baseline electrical conductivity of the tumor is 10 times higher than the surrounding fat tissue. The model parameters are taken from Miklavčič et al. (2010)

strength, occurs in tissues with low conductivity. Examples commonly encountered in clinical practice are deep-seated tumors surrounded by fat tissue (Miklavčič et al. 2010; Denzi et al. 2015) and treatment of subcutaneous tumors through skin (Pavšelj et al. 2005). The stratum corneum presents a major obstacle in transdermal treatment, such as in the case of plate electrodes. Due to its low conductivity, the majority of voltage drop occurs in the skin, and therefore limits the depth of field penetration.

Figure 3 shows a simple model of a deep-seated tumor surrounded by fat tissue. Two needle electrodes are inserted into the tissue 1.5 cm apart and 1500 V is applied to the left electrode. Tumor has a much higher conductivity than fat; therefore, it significantly alters the electric field distribution. We can see on panels B and C, that the electric field strength is lower in the tumor volume than in the surrounding fat. This is due to a higher resistance and the resulting voltage drop in the fat tissue.

3.5 Dynamic Tissue Conductivity

Under normal conditions, the cell membrane acts as an electric insulator. During electroporation, the structural changes in the membrane result in the opening of newly formed current pathways, thus increasing the bulk electrical conductivity of affected tissue (Ivorra et al. 2009). The increase in bulk conductivity is generally characterized by a nonlinear dependence on the local electric field. If the electric field strength exceeds the threshold for reversible electroporation, tissue conductivity increases from its baseline value as a function of field strength. If the electric field exceeds a certain threshold, the conductivity increases to its maximum value and does not increase further with the applied electric field (Pavšelj et al. 2005). Various mathematical functions can be used to describe the increase in conductivity, however, the most commonly used are functions of a sigmoid shape (Corovic et al. 2013):

Table 1 Parameters of tissues composing a simplified model of a subcutaneous tumor

Tissue type	σ_0 (S/m)	A (−)	E_1 (V/cm)	E_2 (V/cm)	References
Skin	0.008	100	400	1200	Corovic et al. (2013)
Muscle	0.135	3	200	800	Kos et al. (2010) and Corovic et al. (2013)
Tumor	0.3	3	400	800	Kos et al. (2010) and Corovic et al. (2013)
Fat	0.02	3.5	100	800	Kos et al. (2010)

$$\sigma(E) = \sigma_0 \cdot (1 + \text{sigmoid}(E, E_1, E_2, \sigma_0, A)), \tag{1}$$

where σ_0 is the baseline electrical conductivity of target tissue and E is the local electric field. The shape of the sigmoid function in Eq. (1) is defined by E_1, which is the electric field threshold at which the conductivity starts increasing, E_2, which is the electric field at which the conductivity stops increasing, and A, which is the factor of maximum conductivity increase at electric fields above E_2.

The dynamics of conductivity increase are not the same for all tissue types. In addition to different baseline conductivity values, the factor of conductivity increase and the respective electric field thresholds are all tissue dependent. Table 1 shows an example of tissue parameters (σ_0, A, E_1, and E_2) used in a simplified model of a subcutaneous tumor. We can see that the fat and muscle conductivities start to increase at a lower electric field than tumor and skin. Furthermore, the factor of increase is much higher for skin than for muscle or tumor, as the outermost skin layer, the stratum corneum, is extremely nonconductive in its unperturbed state. Figure 4 shows the dynamic conductivity as a function of electric field strength [Eq. (1)] for four different tissues shown in Table 1. The thresholds where the conductivity starts to increase (E_1) and saturates (E_2) are indicated with dashed vertical lines.

Conductivity of biological tissue is also affected by temperature. This is particularly important in IRE where significant heat is produced close to the electrodes. The relationship between electrical conductivity and temperature increase is commonly characterized with a linear equation that assumes a constant temperature coefficient (Rossmanna and Haemmerich 2014):

$$\sigma(T) = \sigma_0 \cdot (1 + \alpha_T \Delta T), \tag{2}$$

where α_T is the temperature coefficient (rate of increase), ΔT is the temperature difference with respect to the initial tissue temperature and σ_0 is the baseline electrical conductivity before the increase. Elevated temperature can increase the electrical conductivity even beyond its plateau value caused by the local electric field strength. The dynamic electrical conductivity of tissue is a function of the both electric field and temperature. One example of modelling this co-dependency is by combining Eqs. (1) and (2):

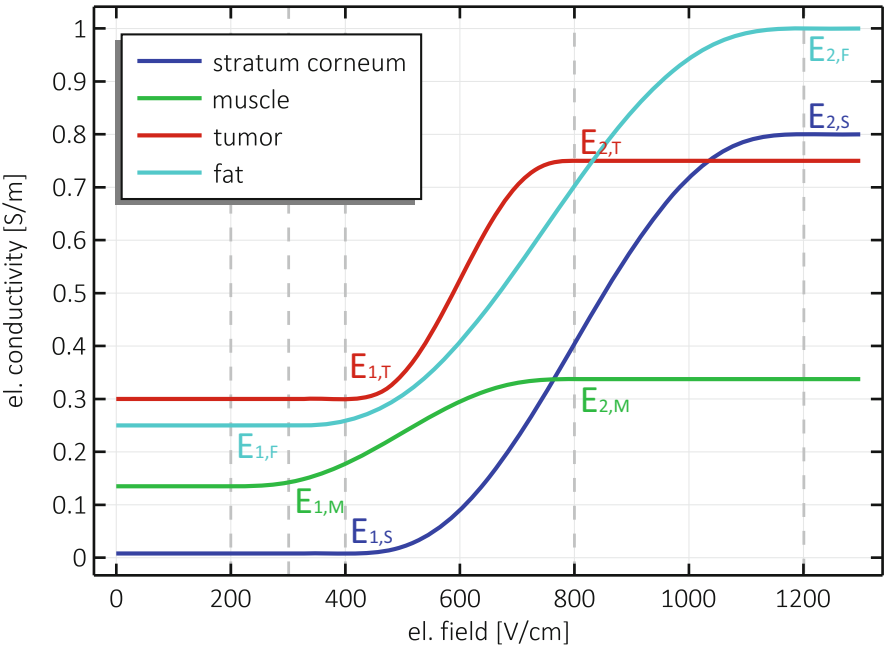

Fig. 4 The effect of electroporation on the electrical conductivity of various tissues can be approximated with sigmoid functions. The baseline conductivity increases when the applied electric field exceeds the threshold for reversible electroporation. When the irreversible threshold is exceeded, the conductivity reaches its maximum value and does not increase further with increasing field strength. The reversible (E_1) and irreversible (E_2) thresholds are indicated with dashed vertical lines (S—stratum corneum, M—muscle, T—tumor, F—fat). The baseline conductivities, factors of conductivity increase, and respective thresholds are taken from Kos et al. (2010 and Corovic et al. (2013) and are summarized in Table 1

$$\sigma(E,T) = \sigma(E) \cdot (1 + \alpha_T \Delta T), \tag{3}$$

where $\sigma(E)$ represents the nonlinear increase due to electroporation effect. Other approaches to modelling the combined thermal and electroporation effects are reported in literature, since it is difficult to decouple the two mechanisms (Garcia et al. 2011; Zhao et al. 2020).

3.6 Numerical Computation of Electric Field Distribution in Tissue

To achieve a therapeutic effect, complete coverage of target tissue volume with sufficiently high electric field is required in all EP-based treatments. Due to tissue-specific properties and various electrode geometries and pulse parameters used in treatments, the distribution of electric field is often not easy to determine. Numerical modelling is an effective way of predicting the shape and strength of the applied

electric field for a selected set of parameters, electrodes, and target tissues. It is based on constructing a simplified anatomical model of the target tissue and solving a set of algebraic equations using the finite element method. The most common numerical models of electroporation are based on solving the partial differential equation for electric potential in stationary conditions (Šel et al. 2007; Pavšelj and Miklavčič 2008; Županič et al. 2012):

$$\nabla \cdot (\sigma \nabla V) = 0, \tag{4}$$

$$E = -\nabla V, \tag{5}$$

where V is the electric potential, σ is the tissue conductivity and electric field E is defined as the gradient (∇) of electric potential. Most EP-based treatments use 50–100μs long pulses, which means all transient phenomena of electroporation will have settled long before the end of the pulse, and steady-state conditions can be used for computation. The electrical conductivity change during electroporation is implemented in the model in the form of a nonlinear function of the local electric field strength [Eq. (1)].

The initial step in numerical modelling is constructing the model geometry that reflects the patients' conditions as closely as possible. For treatment planning complex geometries are constructed based on patients' medical images (Grošelj et al. 2015; Kos et al. 2015), however, for investigative purposes (such as in this chapter) simplified geometries are used, where tumors are usually represented by spheres or spheroids surrounded by blocks of healthy tissue of sufficiently larger dimensions. The appropriate electrode geometry is built on the model in the form of voltage terminals; for deep-seated tumors needle electrodes are generally used, while for superficial tumors plate or various fixed array electrodes are used. When the geometry is defined, voltage is applied to the electrodes and electric potential and field are computed in the whole model domain according to Eqs. (4) and (5). The model complexity can be further increased by including additional anatomical objects, such as nearby vessels and tissue layers, and by including tissue anisotropy and inhomogeneity (Kos et al. 2010, 2015).

If thermal effects need to be investigated, for example, in IRE ablation, the computation needs to be transformed from stationary conditions to time domain. Thermal effects are most commonly modelled by the modified Pennes' bioheat equation (Pennes 1948; Agnass et al. 2020):

$$\rho C \frac{\partial T}{\partial t} = \nabla (k \nabla T) - Q_{\text{perf}} + Q_{\text{met}} + \sigma |E|^2, \tag{6}$$

where ρ, C and k are density, thermal capacity, and thermal conductivity of tissue, respectively, T is tissue temperature, t is time, Q_{perf} is the blood perfusion term, Q_{met} is the metabolic heat generation term and $\sigma|E|^2$ is the Joule heating term. In this case the nonlinear conductivity function is a function of electric field and temperature [Eq. (3)].

4 Electrode Designs and Configurations

Different therapeutic needs necessitate the development of different electrode types and models; from noninvasive electrodes intended for transdermal treatment to various needle electrodes with fixed or variable geometry for treatment of superficial or deep-seated tumors. Finger and small cavity electrodes have been developed for treatment in hard-to-reach locations, for example, in the oral cavity. Special electrode designs are continuously being developed for treatment of specific organs; for instance, thicker needle electrodes (1.8 mm instead of standard 1.2 mm diameter) with a trocar tip intended to penetrate the rigid bone tissue (Miklavčič et al. 2012), or electrodes with internal cooling to reduce heating during IRE ablation (O'Brien et al. 2018). In this section, a few examples of commercially available electrodes are shown, although new electrode models are being studied and developed for clinical use. Some examples of most commonly used electrode types in current clinical practice are also illustrated with simple numerical models to show the expected electric field distribution in tissue.

4.1 Noninvasive Electrodes

Noninvasive electrodes mainly consist of various types of plate and L-shaped electrodes and are intended for the treatment of skin and small superficial tumor nodules, e.g., various skin metastases. These electrodes cannot be inserted into the tissue, so the skin must be considered when evaluating the treatment response. Conductive gels are often used to provide better electrical contact between the electrodes and skin surface.

The plate electrode (Fig. 5a) was most commonly used in early studies of electrochemotherapy (Miklavčič et al. 2014). Due to a simple geometry consisting of two parallel plates, the electric field was most often estimated as the ratio between the applied voltage and the distance between the inner surfaces of the plates. However, this simplification is only valid if the distance between the electrodes is much smaller than their surface area and if the target tissue is homogenous with electrical conductivity independent of the applied electric field, which is not to be expected in a real clinical application. The electric field strength depends on the thickness and conductivity of the tissue in question, but generally, the tissue with lower conductivity (e.g., skin) experiences a higher electric field. Figure 6 shows the electric field distribution in a simple model of a skin fold treated with plate electrodes with 8 mm spacing. With this type of electrodes $8 \times 100\mu s$ pulses are delivered with a pulse repetition rate of 5000/s with the suggested pulse amplitude of 960 V, resulting in a voltage-to-distance ratio of 1200 V/cm. Due to an inhomogeneous tissue and dynamic electrical conductivity, the actual electric field experienced by the tissue is less than 1200 V/cm as can be seen in Fig. 6. The strongest electric field is found in the outer layer of skin at the surface of the electrodes and decreases with distance from the electrodes. For this reason, the distance between the electrodes must be small compared to their surface area. Panels c and d in Fig. 6 show the

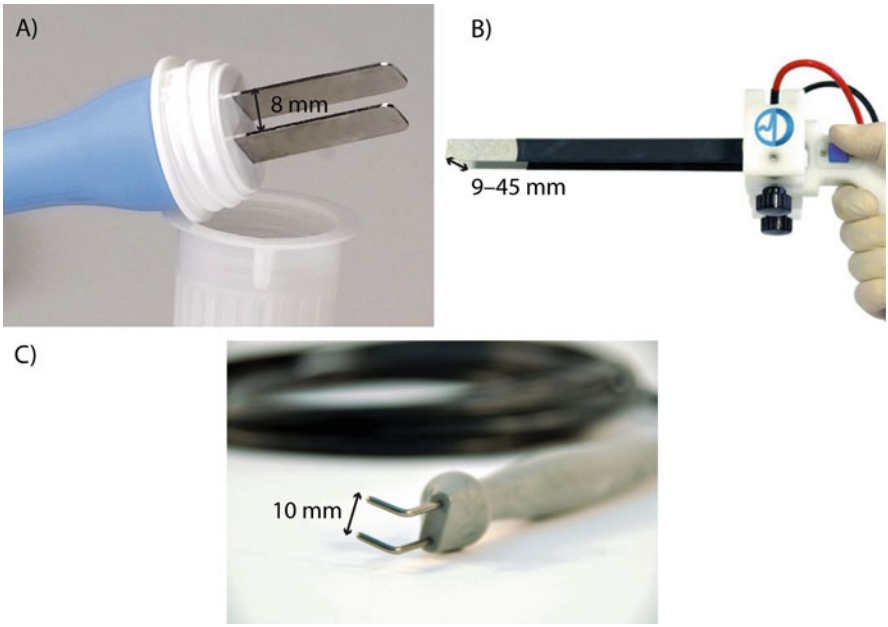

Fig. 5 Examples of noninvasive electrodes. (**a**) A plate electrode with a fixed spacing for clinical use (P-30-8B, EPS Series, IGEA S.p.A., Italy). (**b**) Clamp plate electrode with adjustable spacing for veterinary use (M1 Clamp, OnkoDisruptor, Biopulse Biotech, Italy). (**c**) L-shaped electrode for veterinary use (ELECTROvet, Leroy Biotech, France)

skinfold model with added small tumor nodule (4 mm diameter). The electric field distribution is affected by the higher conductivity of the tumor compared to surrounding fatty tissue.

The main advantages of plate electrodes are their noninvasiveness and a relatively easy visualization of the area treated by electric field. However, the applicability of plate electrodes is limited to small superficial areas, such as earlobes, nose, or superficial exophytic tumor nodules that can be compressed between the plates. In addition to plate electrodes with fixed spacing, clamp plate electrodes with adjustable spacing (Fig. 5b) have also been developed for veterinary electrochemotherapy of tumors of various sizes. L-shaped electrodes are also used for treatment of skin and small superficial tumors (Fig. 5c). Multiple applications are generally advised with the rotation of the electrode for 90 degrees in between, in order to treat the target tissue from all directions (Serša et al. 1996). With the L-shaped electrode shown on Fig. 5c, 4 × 100μ pulses of 1300 V amplitude are applied in each position. In all noninvasive electrode models, the depth of effective electric field penetration is very limited—usually to only a few millimeters from the surface.

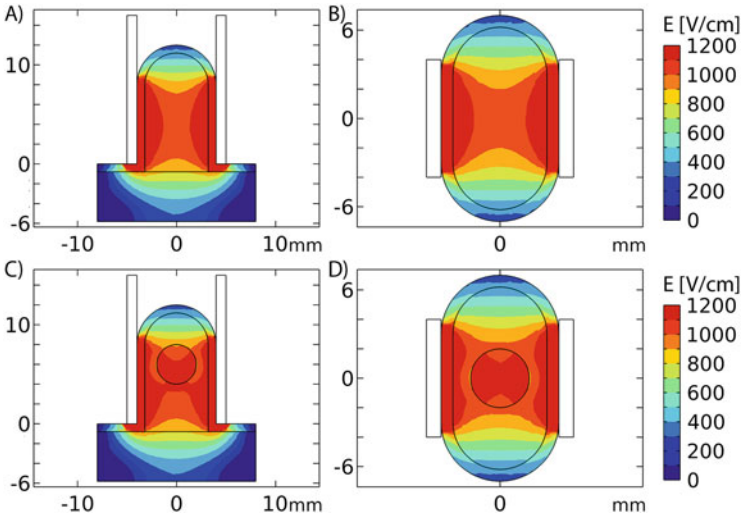

Fig. 6 Electric field distribution with plate electrodes in side view (**a**, **c**) and axial view (**b**, **d**). Panels (**a**) and (**b**) show a simple skinfold model, consisting of skin and fat. Panels (**c**) and (**d**) show the same model with the addition of a tumor with a 4 mm diameter. The distance between the plates is 8 mm and applied voltage is 960 V, resulting in a voltage to distance ratio of 1200 V/cm. Due to an inhomogeneous tissue and a dynamic electrical conductivity, the actual electric field experienced by the majority of tissue volume is lower than 1200 V/cm (dark red contour). The electric field is strongest in the skin layer due to its low conductivity and immediate proximity to the electrode surface. The addition of tumor volume (**c**, **d**) alters the field distribution even further. The color scale is adjusted to the range of up to 1200 V/cm for better visibility

4.2 Needle Electrode Arrays (Fixed Geometry)

Needle electrodes with a fixed geometry are the most commonly used electrode type in ECT. Unlike non-invasive electrodes, needle electrodes must be inserted into the tissue. Figure 7, panels a–d show some of the commercially available needle electrodes with fixed geometry. Although there are many possibilities, the most common models have either a linear (Fig. 7a, b) or a hexagonal (Fig. 7c) needle configuration and are used for treatment of superficial tumors. Figure 7d shows a different design of a needle electrode, intended for endoscopic and laparoscopic uses. The electrode has a flexible shaft with a modular needle exposure and is designed for minimally invasive treatment of lesions localized in the abdominal parenchyma.

The most common model of linear needle electrodes consists of two rows of four needles, separated by 4 mm (Fig. 7a). The needles of each row are connected together, therefore, the electrical connection of the electrode is effectively bipolar (Bertacchini 2017). Fixed linear electrodes are used for treatment of smaller tumor nodules. Typically 8 × 100μs pulses are delivered with a pulse repetition rate of 5000/s and pulse amplitude of 400 V. Figure 8 shows an example of the electric field

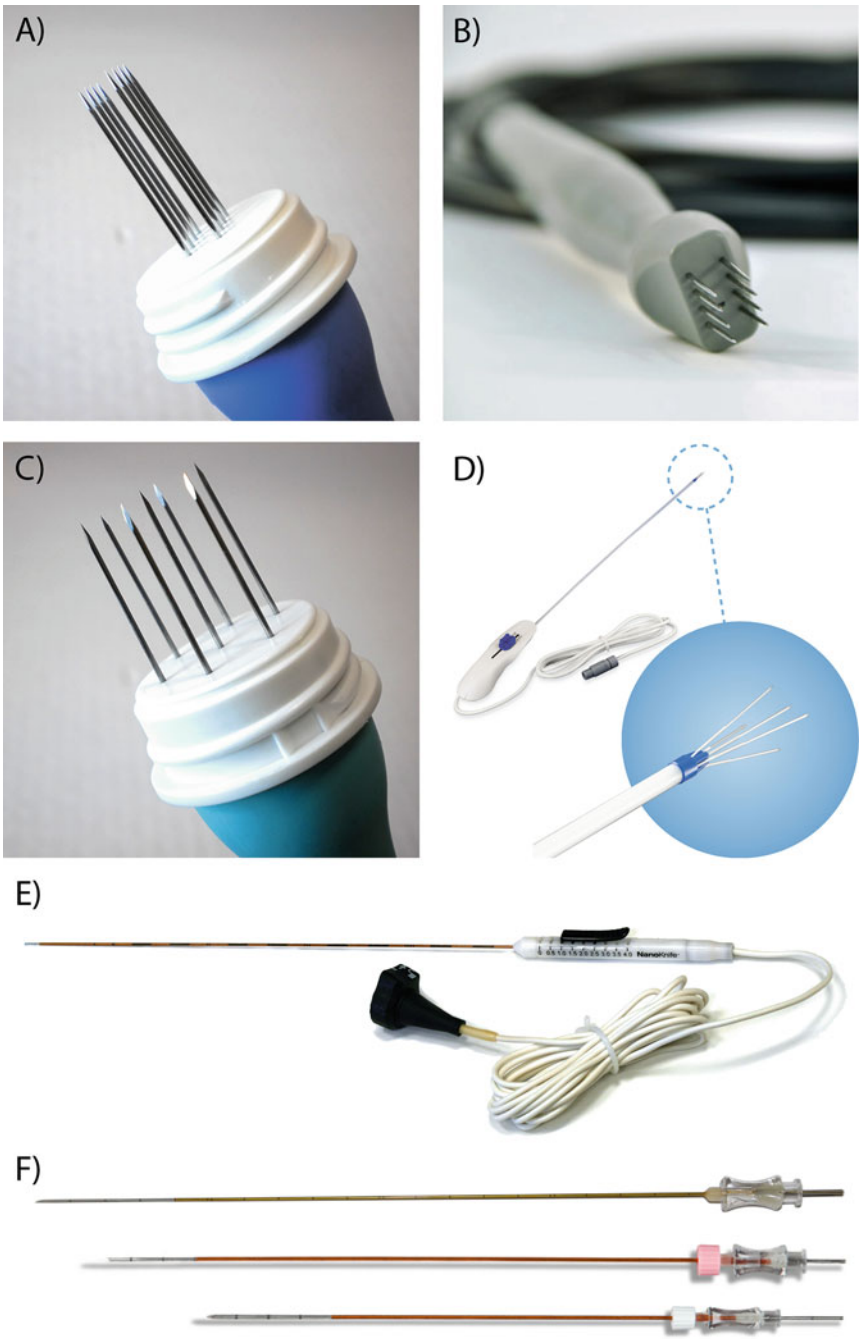

Fig. 7 Examples of needle electrodes. (**a**) Electrode array with a fixed linear geometry (4 mm spacing) for clinical use (N-xx-4B, EPS Series, IGEA S.p.A., Italy). (**b**) Electrode array with a fixed linear geometry (5.9 mm spacing) for veterinary use (ELECTROvet, Leroy Biotech, France). (**c**) Electrode array with a fixed hexagonal geometry for clinical use (N-xx-HG, EPS Series, IGEA S.p.

distribution using a linear electrode in a homogenous tissue model (a, b) and nonhomogeneous tissue model (c, d)—a subcutaneous tumor of 6 mm diameter.

Hexagonal electrodes consist of six needles distributed in a hexagonal configuration around the central (seventh) needle. In the electrode model shown in Fig. 7c, the distance between the needles is 7.3 mm, resulting in cylindrically shaped treatment area with an approximately 15 mm diameter. This geometry enables the treatment of larger tumor nodules compared to linear electrodes. Typically 8 × 100μs pulses (4 + 4 with reversed electrode polarity) are delivered with the pulse repetition rate of 5000/s and 730 V amplitude to each needle pair individually. The seven needles form together 12 unique electrode pairs, resulting in 96 total pulses delivered in a single application. Figure 9 shows an example of the electric field distribution using a hexagonal electrode in a homogenous tissue model (a, b) and nonhomogeneous tissue model (c, d)—a subcutaneous tumor of 12 mm diameter.

For both electrode models, the needle length can be adjusted from 10 to 40 mm. The depth of electrode insertion dictates the depth of penetration of electric field. However, with longer electrodes (longer electrode exposure), higher electric currents are to be expected during treatment, i.e., the current amplitude depends on the depth of electrode insertion. With fixed needle electrodes the electric field distribution is more complex than with plate electrodes, since pulses are applied to multiple electrode pairs and in different directions. Nevertheless, the geometries and necessary parameters have been extensively studied, therefore, the user needs only to ensure the treated tumor is contained within the needle boundaries (Bertacchini 2017). For larger tumors, multiple applications with repositioning of the electrodes are often performed (for both linear and hexagonal configurations).

4.3 Single Needle Electrodes (Variable Geometry)

For minimally invasive treatment of deep-seated tumors, single monopolar needle electrodes are used in pairs to deliver high-voltage electric pulses to target tissue. Single needle electrodes are the most versatile electrode type, however, their placement is challenging and requires an experienced interventional radiologist (Rossmeisl et al. 2015; Garcia et al. 2017). Moreover, given the limitations of available pulse generators in terms of maximum voltage and electric current delivery, the treatment typically requires the use of more than one pair of electrodes, further complicating the determination of the electric field distribution in the tissue. Nevertheless, even a single electrode pair allows effective coverage of larger tissue volumes than other electrode types. The number and placement of electrodes depend on the size of the tumor, but typically 3–6 electrodes are used in a polygonal

Fig. 7 (continued) A., Italy). (**d**) A flexible electrode with modular needle exposure for endoscopic and laparoscopic use (Stinger, EGPS Series, IGEA S.p.A., Italy). (**e**) Single needle electrode for IRE ablation of deep-seated tumors (NanoKnife, Angiodynamics Inc., USA). (**f**) Single needle electrodes for ECT of deep-seated tumors (VGD Series, IGEA S.p.A., Italy)

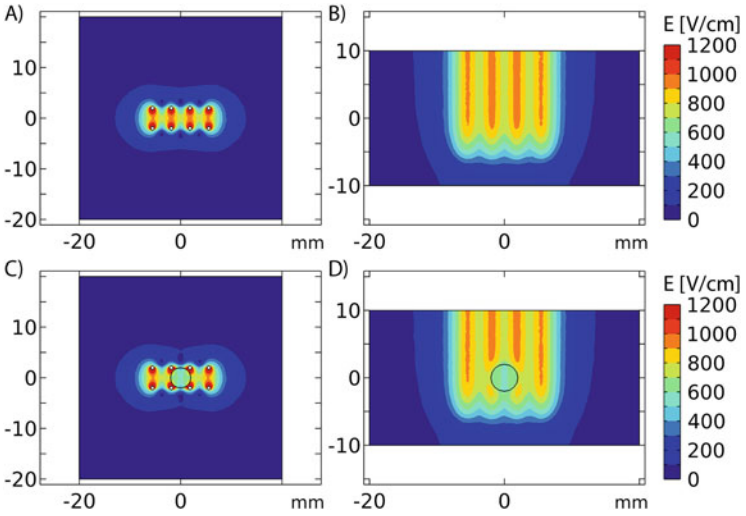

Fig. 8 Electric field distribution in homogenous (**a**, **b**) and nonhomogeneous (**c**, **d**) tissue model using the fixed linear needle electrodes. In the nonhomogeneous model a spherical tumor with 4 mm diameter is positioned in the center of electrode geometry (black outline). The distance between the rows is 4 mm, insertion depth is 15 mm and the applied voltage is 400 V, as is recommended for ECT with this electrode type, resulting in a voltage to distance ratio of 1000 V/cm. Dynamic electrical conductivity is used in both models. In the nonhomogeneous model, the tumor conductivity is approximately 3-times the value of surrounding tissue conductivity. The color scale is adjusted to the range of up to 1200 V/cm for better visibility. Panels (**a**) and (**c**) show the axial view at the depth of the largest tumor diameter. Panels (**b**) and (**d**) show the side view in the middle between the needle rows

configuration in close proximity or even inside the tumor mass. The electrodes should be positioned parallel to each other and at the same depth to achieve a predictable electric field shape. In practice, this is often difficult to achieve due to anatomical constraints; moreover, the electrodes are long and thin and tend to bend. The electrodes also have an adjustable active length (1–4 cm), but in practice 2 cm are usually used, because longer electrodes result in a (too) high electric current, which may cause automatic termination of pulse delivery by the pulse generator.

The electric field is the strongest in immediate vicinity of the electrode and decreases rapidly with distance from the electrode. The proximity of the counter electrode in the pair is crucial in establishing the necessary field strength in the whole target volume. Figure 10 illustrates the effect of inter-electrode distance on the electric field distribution in a simple model with two needle electrodes in a homogeneous tissue. A fixed voltage-to-distance ratio of 1500 V/cm is used to determine the applied voltage, as this value is most commonly recommended for IRE treatment of deep-seated tumors. The distance between the electrodes is set to either 1 cm, 1.5 cm or 2 cm. At 1 cm distance, the electric field appears almost homogenous in the middle between the electrodes with a strength of approximately 1200 V/cm. When

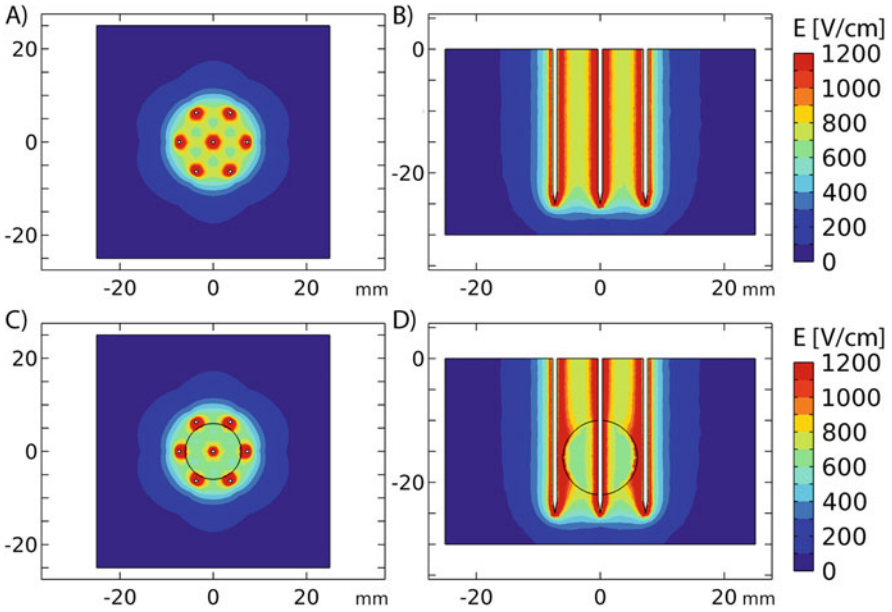

Fig. 9 Electric field distribution in homogenous (**a**, **b**) and nonhomogeneous (**c**, **d**) tissue model using the fixed hexagonal needle electrodes. In the nonhomogeneous model, a tumor with 12 mm diameter is positioned in the center of electrode geometry (black outline). The distance between the needles is 7.3 mm, insertion depth is 25 mm and the applied voltage is 730 V, as is recommended for ECT with this electrode type, resulting in a voltage to distance ratio of 1000 V/cm. Dynamic electrical conductivity is used in both models. In the nonhomogeneous model, the tumor conductivity is approximately 3-times the value of surrounding tissue conductivity. The color scale is adjusted to the range of up to 1200 V/cm for better visibility. Panels **a** and **c** show the axial view at the depth of the largest tumor diameter. Panels **b** and **d** show the side view at the central electrode aligned with the *x*-axis

the electrodes are positioned further apart, the electric field strength in the middle decreases and becomes more inhomogeneous. To compensate for this decrease, a higher voltage needs to be applied to the electrodes; however, the maximum voltage that can be supplied by the currently approved medical pulse generators is limited to 3000 V, therefore limiting the distance between the electrodes. For example, the general guideline for IRE ablation is that the electrodes should not be further than 2 cm apart to ensure the recommended voltage-to-distance ratio of 1500 V/cm.

A fixed voltage-to-distance ratio is usually suggested by the manufacturer of the pulse generator, as a guideline for determining the voltage applied to the electrodes. However, the applied voltage can then be adjusted by the user. In literature, the voltage-to-distance ratio is often confused with the electric field threshold, required for electroporation, as both have the same unit of measure—V/cm. The recommended voltage-to-distance ratio is much higher than the necessary threshold for electroporation, to compensate for the decrease in field strength with distance.

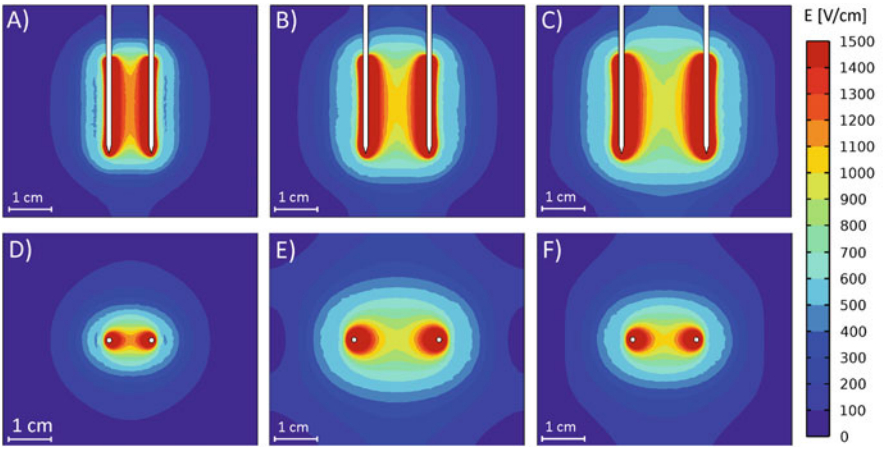

Fig. 10 An example of electric field distribution in homogeneous tissue at different distances between needle electrodes—1 cm (**a**, **d**), 1.5 cm (**b**, **e**), and 2 cm (**c**, **f**). A fixed voltage-to-distance ratio of 1500 V/cm was used to determine the applied voltage. The active length of the electrodes was 2 cm. The electric field is strongest in the immediate vicinity of the electrodes, but decreases rapidly with the distance from the electrode surface. For better visibility, the color scale is adjusted to a range of up to 1500 V/cm, however, very high electric fields (up to 10,000 V/cm) can be found in tissue directly at the electrode surface

For instance, in Fig. 10 the voltage-to-distance ratio was set to 1500 V/cm. However, we can see that the 1500 V/cm field strength (dark red contour) only occurs in a few millimeters from the electrodes, while in most of the tissue between the electrodes the field strength is considerably lower.

According to manufacturer instructions, the electrodes should be placed parallel to each other and at the same depth, since it is easier to predict the distribution and homogeneity of the resulting electric field. However, in biological tissues this placement is almost impossible to achieve, due to electrode bending and anatomical constraints. Any imperfections in the electrode positions result in a much more complex distribution of electric field. Figure 11 shows the effect of electrode angulation and skewness on the shape of the electric field in a homogeneous tissue. In a clinical setting some angulation and skewness, as shown in panel d of Fig. 11, is to be expected. Examples shown in Figs. 10 and 11 are computed in a homogenous tissue with dynamic conductivity. In reality, biological tissue is inhomogeneous considering its conductivity, and any inhomogeneity in target tissue properties alters the electric field distribution even further.

4.4 Finger/Cavity Electrodes

In some anatomical locations, for instance some orifices and cavities, standard electrodes could not be used. Therefore, electrodes have been designed shaped specifically for treatment in these challenging locations. Figure 12 shows two

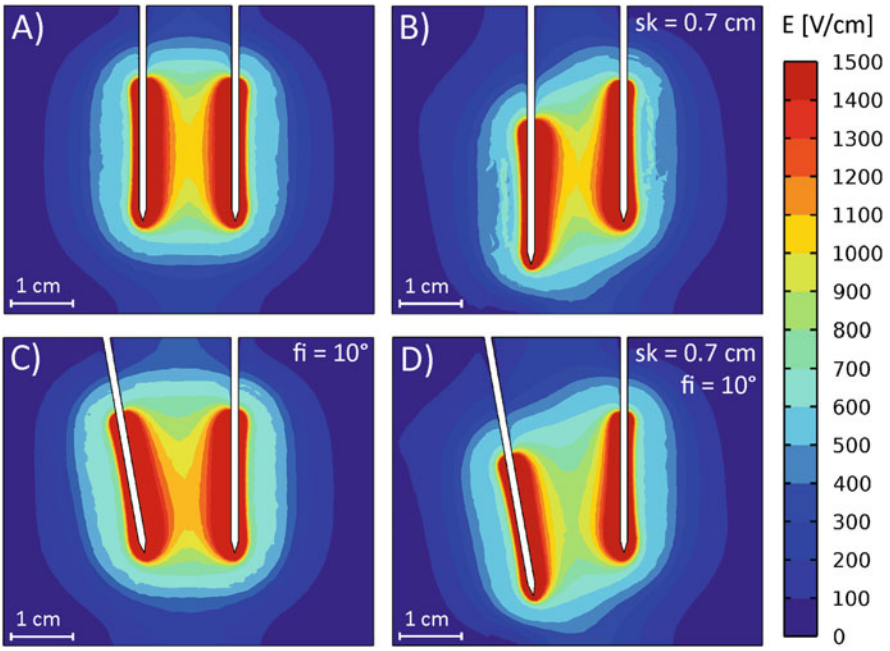

Fig. 11 The effect of electrode skewness and angulation on electric field distribution in homogeneous tissue. A fixed voltage-to-distance ratio of 1500 V/cm was used to determine the applied voltage. The active length of the electrodes was 2 cm. (**a**) Ideal placement—parallel electrodes, same depth of insertion. Distance between the needles is 1.5 cm. (**b**) Parallel electrodes, left electrode is placed 0.7 cm deeper. (**c**) Same depth of insertion, left electrode is inserted at a 10° angle with respect to the vertical axis of insertion. (**d**) Left electrode is inserted at an angle and deeper than the counter electrode. This scenario is most commonly encountered in clinical practice

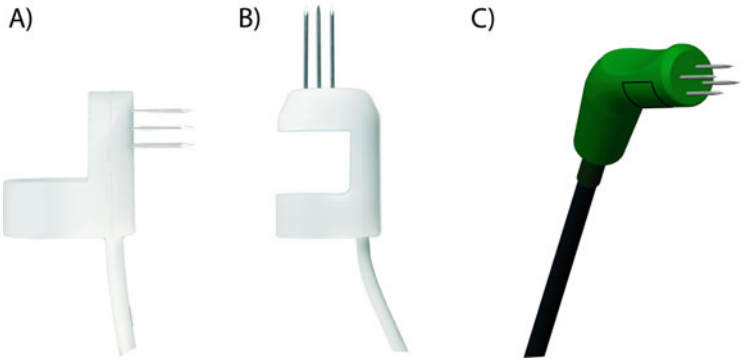

Fig. 12 Examples of electrodes for treatment in locations with difficult access. (**a**) Orthogonal model (F-15-NO, NFD Series, IGEA S.p.A., Italy) and (**b**) longitudinal model (F-xx-NL, NFD Series, IGEA S.p.A., Italy) of a finger electrode with linear needle configuration for treatment in the human orifices. (**c**) Cavity electrode, designed specifically for treatment in the oral cavity in veterinary oncology (ELECTROvet, Leroy Biotech, France)

models of finger electrodes, designed for ECT treatment in the human orifices. As the name suggests the electrode is to be worn on the finger of the user and has the linear needle configuration. The distance between the rows is 4 mm while the active length can be either 5 or 10 mm. The needles can be positioned either perpendicular to the finger (orthogonal model) or at the tip of the finger (longitudinal model) to allow treatment of difficult-to-reach sites (Bertacchini 2017; Campana et al. 2019). Panel c of Fig. 12 shows a cavity electrode, designed for ECT of tumors in the oral cavity of animal patients, which consists of four 10 mm long needles.

5 Pulse Parameters (Treatment Protocols)

Electroporation-based treatments have different needs regarding the desired therapeutic effect and therefore require the use of pulse parameters and delivery protocols, tailored to each type of treatment. The classical electroporation protocol of 8 × 100μs pulses, developed in the first studies of electrochemotherapy, is still the most widely used protocol in clinical practice, however, several new protocols have also been developed.

5.1 Electrochemotherapy

Electrochemotherapy (ECT) uses reversible electroporation to facilitate transmembrane transport of chemotherapeutic agents with intercellular action, e.g., bleomycin and cisplatin. The most widely known ECT protocol consists of 8 pulses of 100μs duration and a pulse repetition rate of 1/s (Mir et al. 1991). In some commercially available devices today, such as the Cliniporator (IGEA S.p.A., Italy), the protocol is adjusted so the pulses are no longer delivered at 1/s, but rather in trains of pulses with a pulse repetition rate of 5000/s. For treatments in the thoracic region, the train delivery is synchronized with the patients' electrocardiogram to prevent cardiac arrhythmias. With a pulse repetition rate of 1/s, each of the eight delivered pulses manifests as separate muscle contraction perceived by some patients as painful. By increasing the pulse repetition rate the patient effectively experiences only a single pulse instead of eight, which decreases the unpleasantness of the procedure (Zupanic et al. 2007). The most commonly used electrode models in ECT are the fixed linear and hexagonal needle electrodes for treatment of skin and subcutaneous tumors. Both electrode models come with recommendations for applied pulse parameters, namely the applied voltage, provided by the device manufacturer.

5.2 Irreversible Electroporation Ablation

Irreversible electroporation (IRE) is used as a focal ablation method for various soft tissues and tumors. In IRE ablation, a higher number of monopolar pulses is used compared to ECT. Parameters of electric pulses and supply protocols differ between

studies, but 70–100 electric pulses per electrode pair are most commonly used, and the duration of individual pulses in the train is 50–100μs. The pulse delivery is synchronized with the patients' electrocardiogram, so the pulses are delivered in the absolute refractory period to minimize the risk of triggering arrhythmias. In current pulse generators, sequences of 10 pulses are applied, followed by a short pause to allow recharging of the generator. The pulse amplitude is most often determined by using a fixed ratio between the applied voltage and the distance between paired electrodes.

The most recent development in pulse protocols for IRE is the so-called high-frequency IRE or H-FIRE (Arena et al. 2011; Yao et al. 2017; Ringel-Scaia et al. 2019). In H-FIRE, bursts of bipolar pulses are applied instead of monopolar pulses used in classic electroporation protocols. The bipolar pulses consist of two pulses of opposite polarity. The duration of a single polarity pulse is in the range of a few microseconds and the pulse repetition rate is in the range of 50,000–125,000/s. The short duration of the pulses and higher repetition rates provide several benefits over longer monopolar pulses, such as a higher threshold for nerve and muscle stimulation.

5.3 Gene Electrotransfer

Gene electrotransfer (GET) uses electrical pulses to introduce gene-encoding plasmid DNA into tumor or healthy cells, for example, to induce an antitumor effect or stimulate the immune response (Gothelf and Gehl 2010; Heller and Heller 2015; Rosazza et al. 2016; Lampreht Tratar et al. 2017). As in ECT, the DNA plasmid is injected into the target tissue a few seconds before the pulse application. Unlike the chemotherapeutics in ECT, plasmid DNA molecules are too large to enter the cell by diffusion. Therefore, longer (millisecond range) low-voltage pulses, or a combination of short (microsecond range) high-voltage pulses and long (millisecond range) low-voltage pulses are used to permeabilize the cell membrane and electrophoretically deliver the plasmid to the target location (Gothelf and Gehl 2010). The optimal dose of the plasmids and pulsing protocols are still under development.

5.4 Additional Considerations Regarding Pulse Protocols

Sometimes the recommended pulse parameters cannot be delivered, for instance, due to the limitations of available equipment or specific properties of the experimental setup. Studies have shown that a similar therapeutic outcome can be achieved also with somewhat adjusted pulse parameters (Pucihar et al. 2011; Dermol and Miklavčič 2015; García-Sánchez et al. 2019). For example, at a fixed field amplitude, a similar fraction of electroporated cells can be obtained by using longer pulses or a higher number of pulses. In other words, with longer pulses or higher number of pulses, the critical electric field thresholds shift to lower values (also see Fig. 1). In order to select equivalent pulse parameters, the relations between the pulse

amplitude, duration, and number need to be determined. In their experimental study, Pucihar and colleagues (Pucihar et al. 2011) determined the mathematical relations between amplitude and pulse duration and amplitude and number of delivered pulses with respect to the fraction of electroporated cells in vitro. The results of their study show, that the relations between pulse parameters can be reflected by rather simple mathematical models and that a similar fraction of electroporation can indeed be achieved using carefully selected equivalent parameters.

Pulse delivery dynamics, namely the duration of pulses and pulse repetition rate, also play an important role in tissue heating. The thermal energy W_T is formulated as the product of power (electrical energy converted to thermal energy per unit time) and time:

$$W_T = P \cdot t = \left(\int_V \boldsymbol{J} \cdot \boldsymbol{E} \, dv \right) \cdot t = \left(\int_V \sigma \cdot E^2 \, dv \right) \cdot t \tag{7}$$

where P is power, t is time (pulse duration), J is current density, E is electric field, σ is electrical conductivity of the tissue. The main factor driving the amount of generated thermal energy is the pulse amplitude and the associated Joule heating [$\boldsymbol{J}\cdot\boldsymbol{E}$ term in Eq. (7)] (Lacković et al. 2009). However, as we can see in Eq. (7), the amount of thermal energy also depends on the duration of the applied pulses. There is a considerable difference between the amount of thermal energy generated by a millisecond pulse and a nanosecond pulse of an equivalent amplitude. Living tissue also has the ability to diffuse heat due to micro- and macrovascular perfusion. Tissue cooling effectively happens in the pauses between the individual pulses. If the tissue capacity to effectively diffuse heat after a single pulse is exceeded the tissue temperature will begin to increase. Longer pauses between the pulses (lower pulse repetition rate) result in more effective cooling. However, due to vascular effects of electroporation, the perfusion and therefore the tissue cooling ability is greatly diminished. This is especially important in IRE ablation, where several hundreds of pulses are cumulatively delivered to tissue, resulting in a large amount of generated heat. Strategies for decreasing the thermal effects are being investigated by the use of electrodes with internal cooling or development of protocols, where pulses are delivered in groups with long delays in between (O'Brien et al. 2018; Sano et al. 2020).

6 Numerical Prediction of Treatment Outcome (Treatment Planning)

6.1 Cumulative Coverage of Target Tissue

The volume of tissue that can be effectively treated with a single electrode pair is rather small, therefore, multiple electrodes (electrode pairs) are used in EP-based treatments—be it the fixed electrode models consisting of multiple needles (Figs. 8 and 9) or multiple single needle electrodes (Figs. 10 and 11). In such cases pulses are

delivered to electrode pairs in sequence, therefore, the target tissue volume is cumulatively covered by contributions from all active electrode pairs. The most conservative method for evaluating cumulative coverage of the target tissue is by considering each of the electrode pairs as a separate entity and superimposing their respective contributions as the treatment equivalent field E_{eq}:

$$E_{eq,n} = \begin{cases} \max(E, E_{n-1}); n > 1 \\ E_n; n = 1 \end{cases}; 1 \leq n \leq N, \quad (8)$$

where N is the total number of electrode pairs, $E_{eq,n}$ is the treatment equivalent field after application of pulses to the nth electrode pair, $E_{eq,n-1}$ is the treatment equivalent field from electrode pairs 1 to $n - 1$ and E_n is the actual computed electric field produced by the nth electrode pair. The final electric field distribution in tissue is represented by the equivalent electric field after application of pulses to all N electrode pairs—$E_{eq,N}$. This approach is illustrated in Fig. 13 showing a spherical tumor model treated with four needle electrodes forming a total of six unique electrode pairs. A threshold of 600 V/cm (often used as the threshold for IRE of tumors) is applied to the computed electric field, so the Panels a–f show only tissue covered in electric field strength of or above this threshold. The tumor volume is gradually covered in six segments and the final electric field distribution is shown in panel g. Figure 14 shows the fraction of tumor volume cumulatively covered by pulses sequentially delivered to each of the electrode pairs.

When using more than one electrode pair some areas of tissue will be covered more than once, as can be seen in panels a–f of Fig. 13 (darker blue areas, mainly around the electrodes). This is also very prominent in the hexagonal electrodes, where the central needle serves as the counter electrode in half of all active electrode pairs (6 out of 12 pairs), while each of the outer needles is only used in 4 out of 12 pairs. The tissue surrounding the central electrode will therefore cumulatively experience twice as many pulses as the tissue at the electrode rim.

Considering each pair of electrodes individually will slightly underestimate the extent of the treated region. First, the conductivity increase is not completely independent between the pairs, as a small increase persists also during the time when the pulse delivery is switched to the next pair. Moreover, the required electric field strength to achieve reversible or irreversible electroporation in the target volume reduces with increased exposure duration (see also Fig. 1 and Sect. 5.4). Therefore, in tissue areas that are cumulatively experiencing a higher number of pulses, electroporation may occur already at a lower electric field than expected (Pucihar et al. 2011).

Another possible approach to determine the tissue response is by calculating the probability of effective electroporation in tissue volume. This approach employs the use of statistical models of cell survival, the most commonly used being the Peleg-Fermi model (Peleg 1995; Golberg and Rubinsky 2010; Dermol and Miklavčič 2015). The probability of cell survival S is defined as follows:

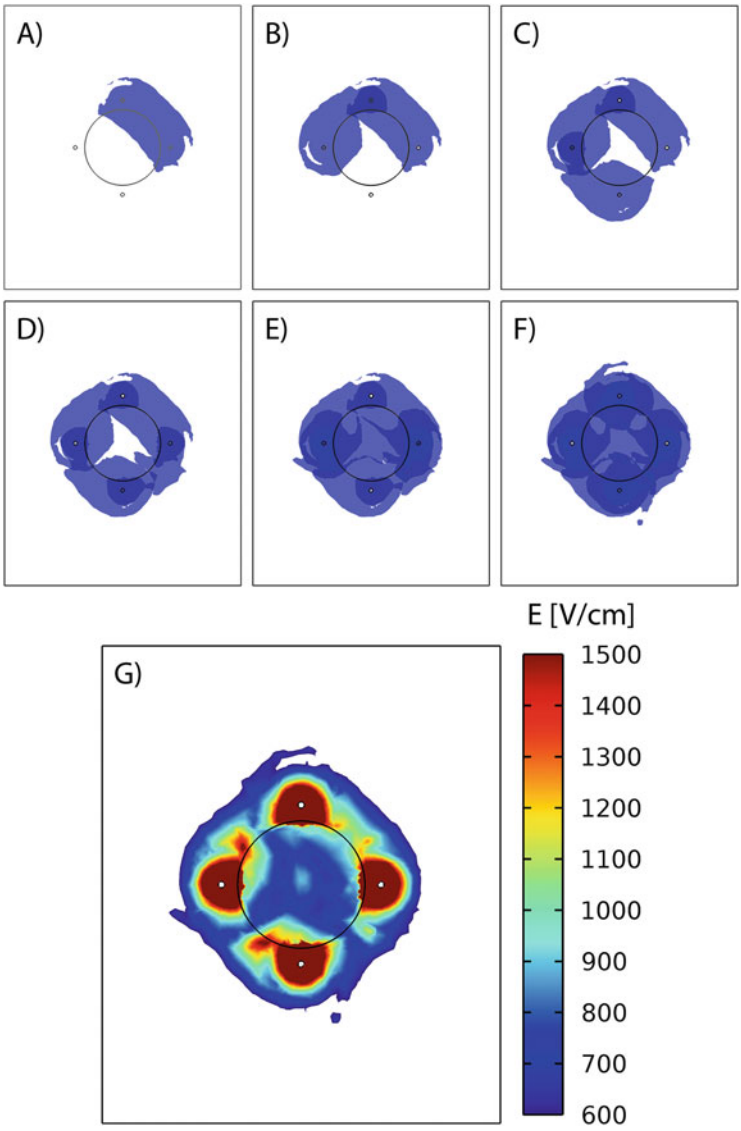

Fig. 13 A simplified model of IRE ablation of a subcutaneous tumor. Four electrodes are used in the model, forming six unique electrode pairs. The tumor volume is gradually covered with electric field at or above the irreversible threshold—in this case 600 V/cm. Panels (**a–f**) show the volume of target tissue covered by at least 600 V/cm after application of pulses to each electrode pair. Panel (**g**) shows the final electric field distribution in the target tissue. The round contour of the tumor can be seen. The color scale is adjusted to the range of 600 to 1500 V/cm for better visibility

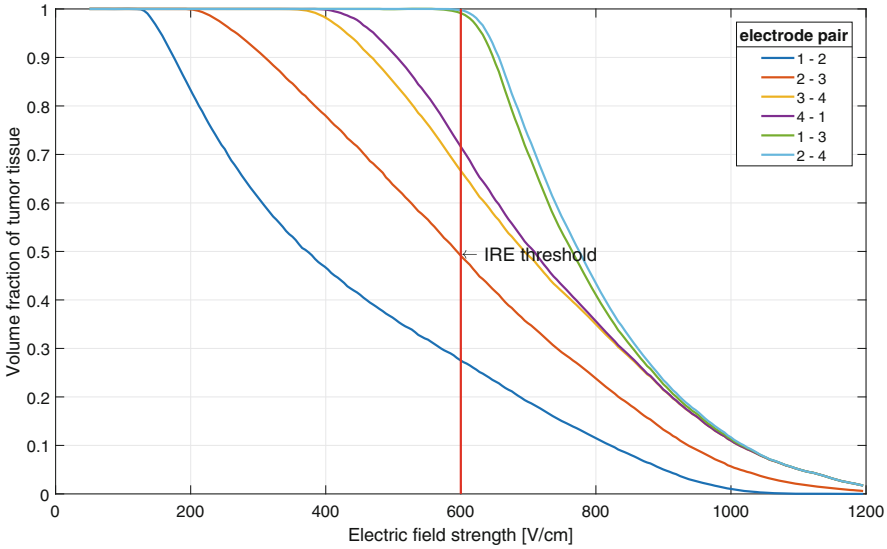

Fig. 14 Cumulative coverage of the tumor volume with electric field. The horizontal axis shows the electric field strength, while the vertical axis shows the fraction of tumor volume covered with electric field of at least the value shown on the horizontal axis. Each curve represents the contribution of a single active electrode pair used in treatment. The critical value for the therapeutic response in this specific case is the IRE threshold of 600 V/cm, however, this value depends on the tissue type and treatment application

$$S(E,m) = \frac{1}{1 + \exp\left(\dfrac{E - E_{\mathrm{c}}(m)}{A(m)}\right)}, \tag{9}$$

where m is the number of pulses, E is the local electric field, $E_{\mathrm{c}}(m)$ is the critical electric field at which 50% of affected cells die, and $A(m)$ is the shape factor defining the size of the transition zone. Both the critical field and shape factor are functions of applied pulse number. There are two main benefits of this approach; the number of applied pulses is included in the calculation, and the nature of probability calculations allows multiplication of different probabilities to reflect the lower probability of survival, where applicable:

$$S = \prod_{N \text{ pairs}} S_n, \tag{10}$$

where S_n is the probability of cell survival after the application of pulses to the nth electrode pair. Considering Eq. (10), the cumulative probability of cell survival will be lower in tissue areas that are covered with multiple pairs.

The last aspect to consider when looking at cumulative coverage is the thermal effects. As the number of delivered pulses increases, the thermal component becomes more pronounced. In IRE ablation, up to 100 pulses are delivered to a

single pair of electrodes. Combining the contributions of all active electrode pairs, certain tissue areas are cumulatively exposed to several hundred pulses. Due to slow thermal diffusion in tissue and ineffective blood perfusion during electroporation a significant thermal component is present (Cornelis et al. 2020). This effect cannot be neglected even in reversible electroporation protocols, e.g., when using the hexagonal electrode. The central needle is active in a total of 6 electrode pairs, so a cumulative 48 pulses are delivered. Undesirable heating and necrosis may therefore occur in the areas around the electrodes (Zmuc et al. 2019), especially at the needle tips, posing a potential risk to anatomical structures that are sensitive to thermal damage, such as bile ducts and nerves. Special care must be taken when placing the electrodes, to avoid damage to sensitive structures near the treatment zone.

6.2 The Basics of Patient-Specific Treatment Planning

As can be inferred from the number of factors affecting the electric field, determining the distribution of the electric field in the target tissue is not a simple task. Since the success of all EP-based treatments depends on complete coverage of the clinical target volume (CTV) with a sufficiently high electric field, it is advisable to use some form of treatment planning to ensure a successful treatment outcome (Miklavčič et al. 2010; Županič et al. 2012; Kos 2017). Treatment planning based on numerical models and computation of electric field has shown promise for guiding the physicians and veterinarians performing electroporation procedures. In 2015, Visifield (www.visifield.com, University of Ljubljana, Slovenia) the first online tool for construction of patient-specific plans for EP-based treatments, was implemented (Pavliha et al. 2013; Marčan et al. 2015). However, the tool is currently intended for research and proof of concept and is not yet available for clinical use.

Patient-specific treatment planning is based on constructing an anatomically accurate numerical model from patients' medical images (MRI, CT scan). First, the patients' preinterventional images are segmented into tissues of interest, namely the tumor mass, surrounding healthy tissue, and other nearby important anatomical structures such as blood vessels and bile ducts. A 3D numerical model is then constructed from the segmented tissue masks and imported into the software for finite element analysis, such as Comsol Multiphysics (www.comsol.com, Comsol Inc., Sweden) and FreeFem++ (https://freefem.org/, UPMC, France) (Hecht 2012). Specific electrical properties are assigned to each tissue in the model to reflect the inhomogeneity in the treated CTV. The next step is to determine the insertion trajectory and placement of the electrodes while considering any anatomical limitations. Finally, we must determine the optimal number and positions of the electrodes and the optimal parameters of applied electric pulses—mainly the amplitude of pulses and, where possible, also the number of applied pulses. Electric field distribution is then computed in the model using the finite element method. The numerical methods for the computation of electric field during electroporation are explained in more detail in Sects. 3.5 and 3.6. The finalized treatment plan provides the physician with a graphical representation of the electrode insertion trajectory and

final placement in the CTV, optimal pulse parameters to be delivered to specified electrode pairs, and a visualization of the expected electric field distribution in tissue and coverage of the CTV (Županič et al. 2012).

When creating a treatment plan, the goal is to ensure complete coverage of the CTV with minimal damage to (surrounding) critical anatomical structures, while ensuring the electrode placement and pulse delivery is technically feasible. In search of the optimal treatment parameters, several difficulties need to be overcome. First of all, we are limited by the hardware specifications of commercially available pulse generators, namely the maximum available voltage supply (3000 V) and maximum allowed electric current (50 A). Even when staying inside the boundaries of available voltage amplitude, increasing the voltage presents a risk of a high current draw in tissues with higher electrical conductivity (note: tissue conductivity also increases during electroporation). Additionally, if the CTV is too large and the electrodes are positioned too far apart it may be impossible to cover the whole CTV with a sufficiently high electric field to ensure successful treatment. The use of optimization algorithms can simplify the search for appropriate electrode positions and voltages. The limitations need to be formulated as a criterion function, which is then minimized during the optimization process, resulting in the best candidate solution for the specified problem (Županič et al. 2012; Kos 2017). Examples of criterion functions for ECT and IRE ablation are as follows:

$$F_{\mathrm{ECT}} = -100 \cdot \mathrm{CTV}_{\mathrm{REV}} + 10 \cdot V_{\mathrm{IRE}} + I_{\mathrm{MAX}}, \tag{11}$$

$$F_{\mathrm{IRE}} = -100 \cdot \mathrm{CTV}_{\mathrm{IRE}} + 10 \cdot V_{\mathrm{IRE}} + 10 \cdot I_{\mathrm{MAX}}, \tag{12}$$

where $\mathrm{CTV}_{\mathrm{ECT}}$ and $\mathrm{CTV}_{\mathrm{IRE}}$ are the volumes of CTV covered in electric field sufficient to cause reversible and irreversible electroporation respectively, V_{IRE} is the volume of surrounding healthy tissue subjected to IRE and I_{MAX} is the maximum delivered electric current. In treatment, we wish to minimize the IRE of healthy tissue and avoid a large current draw; therefore, the contributions from terms V_{IRE} and I_{MAX} increase the value of the fitness function. The optimal solution will have the lowest criterion function and the lowest contributions from "undesired" terms. Eqs. (11) and (12) are just simple examples of criterion functions—there are many ways to formulate the optimization problem.

Treatment plans are currently prepared a few days ahead of intervention using patients' preinterventional images. Their usefulness for the physician is limited since exact electrode placement according to the plan is often difficult to achieve due to anatomical constraints and other technical difficulties. Coupling the preinterventional treatment plan with navigation systems aids in more accurate electrode placement (Grošelj et al. 2015; Fuhrmann et al. 2018). To truly utilize the potential that treatment planning offers, the whole process would need to be translated from preinterventional phase to the interventional phase—meaning, the plan being prepared during the procedure using actual electrode placement and with real-time control of the applied parameters. Nevertheless, treatment planning in its current realization provides a useful guide for the physicians and veterinarians performing EP-based treatments (Kos et al. 2010, 2015; Garcia et al. 2017; Gallinato et al. 2019).

Although treatment planning is currently mainly applied in human procedures, there are a few studies, where the possibilities of treatment planning are also demonstrated for veterinary procedures. In a case report by Kulbacka et al. (2017), a large oral melanoma in a canine patient was treated with a combination of ECT and surgery with promising results. The authors demonstrated the possibility of performing treatment planning using specialized software such as Visifield to increase the efficacy of ECT in veterinary oncology. In another prospective clinical study, seven canine patients were treated with IRE ablation for spontaneous malignant glioma using MRI-based treatment planning (Rossmeisl et al. 2015; Garcia et al. 2017). The authors prepared patient-specific numerical models to determine the pulse parameters used in the procedures and evaluated the predictive power of the treatment planning software using radiologically confirmed clinical outcomes. The results of the study show that the numerical models can evaluate the necessary treatment parameters and effectively predict clinical outcomes. The application of treatment planning in veterinary procedures could result in a more effective treatment.

7 Conclusion

Cell electroporation mainly depends on the local electric field strength and parameters of delivered pulses (pulse number, duration, and delivery rate). Fixed and variable electrode geometries can be used efficiently; however, the depth of electric field penetration and the extent of treated zone around the electrodes is very limited. Therefore, repositioning of fixed electrodes and the use of multiple electrode pairs (in case of single needle electrodes) are suggested to effectively cover the target tissue volume. Increasing the number of pulses can improve the efficacy of treatment to a limited extent; however, it also increases the risk of thermal damage to tissue, which can result in damage to critical anatomical structures (bile ducts, nerves, vessels, etc.) and a longer times to resolve the dead tissue. Numerical modelling has proven to be an indispensable tool in investigating and designing electroporation-based treatments and preparing patient-specific treatment plans. Numerical models can evaluate the necessary treatment parameters and effectively predict clinical outcomes. The application of treatment planning in veterinary procedures could result in more effective treatments.

References

Agnass P, van Veldhuisen E, van Gemert MJC et al (2020) Mathematical modeling of the thermal effects of irreversible electroporation for in vitro, in vivo, and clinical use: a systematic review. Int J Hyperth 37:486–505. https://doi.org/10.1080/02656736.2020.1753828

Ahmed M, Brace CL, Lee FT, Goldberg SN (2011) Principles of and advances in percutaneous ablation. Radiology 258:351–369. https://doi.org/10.1148/radiol.10081634

Arena CB, Sano MB, Rossmeisl JH et al (2011) High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction. Biomed Eng Online 10:102. https://doi.org/10.1186/1475-925X-10-102

Aycock KN, Davalos RV (2019) Irreversible electroporation: background, theory, and review of recent developments in clinical oncology. Bioelectricity 1:214–234. https://doi.org/10.1089/bioe.2019.0029

Ben-David E, Appelbaum L, Sosna J et al (2012) Characterization of irreversible electroporation ablation in in vivo porcine liver. AJR Am J Roentgenol 198:W62–W68. https://doi.org/10.2214/AJR.11.6940

Bertacchini C (2017) Cliniporator: medical electroporation of tumors. In: Miklavcic D (ed) Handbook of electroporation. Springer International Publishing, Cham, pp 1–36

Brock RM, Beitel-White N, Davalos RV, Allen IC (2020) Starting a fire without flame: the induction of cell death and inflammation in electroporation-based tumor ablation strategies. Front Oncol 10:1235. https://doi.org/10.3389/fonc.2020.01235

Campana LG, Edhemovic I, Soden D et al (2019) Electrochemotherapy - emerging applications technical advances, new indications, combined approaches, and multi-institutional collaboration. Eur J Surg Oncol 45:92–102. https://doi.org/10.1016/j.ejso.2018.11.023

Cannon R, Ellis S, Hayes D et al (2013) Safety and early efficacy of irreversible electroporation for hepatic tumors in proximity to vital structures. J Surg Oncol 107:544–549. https://doi.org/10.1002/jso.23280

Čemažar M (2017) Effects of electroporation of mammalian cells on cytoskeleton and intercellular connections. In: Miklavčič D (ed) Handbook of electroporation. Springer International Publishing, Cham, pp 307–321

Cemazar M, Parkins CS, Holder AL et al (2001) Electroporation of human microvascular endothelial cells: evidence for an anti-vascular mechanism of electrochemotherapy. Br J Cancer 84:565–570. https://doi.org/10.1054/bjoc.2000.1625

Cemazar M, Tamzali Y, Sersa G et al (2008) Electrochemotherapy in veterinary oncology. J Vet Intern Med 22:826–831. https://doi.org/10.1111/j.1939-1676.2008.0117.x

Cornelis FH, Cindrič H, Kos B et al (2020) Peri-tumoral metallic implants reduce the efficacy of irreversible electroporation for the ablation of colorectal liver metastases. Cardiovasc Intervent Radiol 43(1):84–93. https://doi.org/10.1007/s00270-019-02300-y

Corovic S, Lackovic I, Sustaric P et al (2013) Modeling of electric field distribution in tissues during electroporation. Biomed Eng Online 12:16. https://doi.org/10.1186/1475-925X-12-16

Davalos RV, Mir ILM, Rubinsky B (2005) Tissue ablation with irreversible electroporation. Ann Biomed Eng 33:223–231

Denzi A, Strigari L, Di Filippo F et al (2015) Modeling the positioning of single needle electrodes for the treatment of breast cancer in a clinical case. Biomed Eng Online 14:S1. https://doi.org/10.1186/1475-925X-14-S3-S1

Dermol J, Miklavčič D (2015) Mathematical models describing Chinese Hamster ovary cell death due to electroporation in vitro. J Membr Biol 248:865–881. https://doi.org/10.1007/s00232-015-9825-6

Dermol-Černe J, Pirc E, Miklavčič D (2020) Mechanistic view of skin electroporation – models and dosimetry for successful applications: an expert review. Expert Opin Drug Deliv 17:689–704. https://doi.org/10.1080/17425247.2020.1745772

Duck FA (2012) Physical properties of tissue: a comprehensive reference book. Institute of Physics and Engineering in Medicine

Dunki-Jacobs EM, Philips P, Martin RCG (2014) Evaluation of thermal injury to liver, pancreas and kidney during irreversible electroporation in an in vivo experimental model. Br J Surg 101:1113–1121. https://doi.org/10.1002/bjs.9536

Edd JF, Horowitz L, Davalos RV et al (2006) In vivo results of a new focal tissue ablation technique: irreversible electroporation. IEEE Trans Biomed Eng 53:1409–1415. https://doi.org/10.1109/TBME.2006.873745

Faroja M, Ahmed M, Appelbaum L et al (2013) Irreversible electroporation ablation: is all the damage nonthermal? Radiology 266:462–470. https://doi.org/10.1148/radiol.12120609

Fuhrmann I, Probst U, Wiggermann P, Beyer L (2018) Navigation systems for treatment planning and execution of percutaneous irreversible electroporation. Technol Cancer Res Treat 17:1533033818791792. https://doi.org/10.1177/1533033818791792

Gabriel S, Lau RW, Gabriel C (1996) The dielectric properties of biological tissues: II. Measurements in the frequency range 10 Hz to 20 GHz. Phys Med Biol 41:2251–2269

Gabriel C, Peyman A, Grant EH (2009) Electrical conductivity of tissue at frequencies below 1 MHz. Phys Med Biol 54:4863–4878. https://doi.org/10.1088/0031-9155/54/16/002

Gallinato O, de Senneville BD, Seror O, Poignard C (2019) Numerical workflow of irreversible electroporation for deep-seated tumor. Phys Med Biol 64:055016. https://doi.org/10.1088/1361-6560/ab00c4

Garcia PA, Rossmeisl JH, Neal RE et al (2011) A parametric study delineating irreversible electroporation from thermal damage based on a minimally invasive intracranial procedure. Biomed Eng Online 10:34. https://doi.org/10.1186/1475-925X-10-34

Garcia PA, Davalos RV, Miklavcic D (2014) A numerical investigation of the electric and thermal cell kill distributions in electroporation-based therapies in tissue. PLoS One 9:e103083. https://doi.org/10.1371/journal.pone.0103083

Garcia PA, Kos B, Rossmeisl JH et al (2017) Predictive therapeutic planning for irreversible electroporation treatment of spontaneous malignant glioma. Med Phys 44:4968–4980. https://doi.org/10.1002/mp.12401

García-Sánchez T, Leray I, Ronchetti M et al (2019) Impact of the number of electric pulses on cell electrochemotherapy in vitro: limits of linearity and saturation. Bioelectrochemistry 129:218–227. https://doi.org/10.1016/j.bioelechem.2019.05.021

Geboers B, Scheffer HJ, Graybill PM et al (2020) High-voltage electrical pulses in oncology: irreversible electroporation, electrochemotherapy, gene electrotransfer, electrofusion, and electroimmunotherapy. Radiology 295:254–272. https://doi.org/10.1148/radiol.2020192190

Gehl J, Sersa G, Matthiessen LW et al (2018) Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol 57:874–882. https://doi.org/10.1080/0284186X.2018.1454602

Golberg A, Rubinsky B (2010) A statistical model for multidimensional irreversible electroporation cell death in tissue. BioMed Eng Online 9:13. https://doi.org/10.1186/1475-925X-9-13

Golberg A, Rubinsky B (2012) Towards electroporation based treatment planning considering electric field induced muscle contractions. Technol Cancer Res Treat 11:189–201. https://doi.org/10.7785/tcrt.2012.500249

Gothelf A, Gehl J (2010) Gene electrotransfer to skin; review of existing literature and clinical perspectives. Curr Gene Ther 10:287–299. https://doi.org/10.2174/156652310791823443

Graybill PM, Davalos RV (2020) Cytoskeletal disruption after electroporation and its significance to pulsed electric field therapies. Cancers (Basel) 12:1132. https://doi.org/10.3390/cancers12051132

Grošelj A, Kos B, Čemažar M et al (2015) Coupling treatment planning with navigation system: a new technological approach in treatment of head and neck tumors by electrochemotherapy. Biomed Eng Online 14(Suppl 3):S2. https://doi.org/10.1186/1475-925X-14-S3-S2

Haemmerich D, Schutt DJ, Wright AW et al (2009) Electrical conductivity measurement of excised human metastatic liver tumours before and after thermal ablation. Physiol Meas 30:459–466. https://doi.org/10.1088/0967-3334/30/5/003

Hecht F (2012) New development in freefem++. J Numer Math 20:251–266. https://doi.org/10.1515/jnum-2012-0013

Heller R, Heller LC (2015) Chapter eight-gene electrotransfer clinical trials. In: Huang L, Liu D, Wagner E (eds) Advances in genetics. Academic, pp 235–262

Hjouj M, Last D, Guez D et al (2012) MRI study on reversible and irreversible electroporation induced blood brain barrier disruption. PLoS One 7:e42817. https://doi.org/10.1371/journal.pone.0042817

Ivorra A, Al-Sakere B, Rubinsky B, Mir LM (2009) In vivo electrical conductivity measurements during and after tumor electroporation: conductivity changes reflect the treatment outcome. Phys Med Biol 54:5949. https://doi.org/10.1088/0031-9155/54/19/019

Jarm T, Cemazar M, Miklavcic D, Sersa G (2010) Antivascular effects of electrochemotherapy: implications in treatment of bleeding metastases. Expert Rev Anticancer Ther 10:729–746. https://doi.org/10.1586/era.10.43

Jiang C, Davalos RV, Bischof JC (2015) A review of basic to clinical studies of irreversible electroporation therapy. IEEE Trans Biomed Eng 62:4–20. https://doi.org/10.1109/TBME.2014.2367543

Kanthou C, Kranjc S, Sersa G et al (2006) The endothelial cytoskeleton as a target of electroporation-based therapies. Mol Cancer Ther 5:3145–3152. https://doi.org/10.1158/1535-7163.MCT-06-0410

Kos B (2017) Treatment planning for electrochemotherapy and irreversible electroporation of deep-seated tumors. In: Miklavčič D (ed) Handbook of electroporation. Springer International Publishing, Cham, pp 1001–1017

Kos B, Županič A, Kotnik T et al (2010) Robustness of treatment planning for electrochemotherapy of deep-seated tumors. J Membr Biol 236:147–153. https://doi.org/10.1007/s00232-010-9274-1

Kos B, Voigt P, Miklavcic D, Moche M (2015) Careful treatment planning enables safe ablation of liver tumors adjacent to major blood vessels by percutaneous irreversible electroporation (IRE). Radiol Oncol 49:234–241. https://doi.org/10.1515/raon-2015-0031

Kotnik T, Pucihar G, Miklavčič D (2011) The cell in the electric field. In: Clinical aspects of electroporation. Springer, New York, NY, pp 19–29

Kotnik T, Frey W, Sack M et al (2015) Electroporation-based applications in biotechnology. Trends Biotechnol 33:480–488. https://doi.org/10.1016/j.tibtech.2015.06.002

Kotnik T, Rems L, Tarek M, Miklavčič D (2019) Membrane electroporation and electropermeabilization: mechanisms and models. Annu Rev Biophys 48:63–91. https://doi.org/10.1146/annurev-biophys-052118-115451

Kulbacka J, Paczuska J, Rembiałkowska N et al (2017) Electrochemotherapy combined with standard and co2 laser surgeries in canine oral melanoma. Slov Vet Res 54:181–186. https://doi.org/10.26873/SVR-322-2017

Lacković I, Magjarević R, Miklavčič D (2009) Three-dimensional finite-element analysis of joule heating in electrochemotherapy and in vivo gene electrotransfer. IEEE Trans Dielectr Electr Insul 16:1338–1347. https://doi.org/10.1109/TDEI.2009.5293947

Lambricht L, Lopes A, Kos S et al (2016) Clinical potential of electroporation for gene therapy and DNA vaccine delivery. Expert Opin Drug Deliv 13:295–310. https://doi.org/10.1517/17425247.2016.1121990

Lampreht Tratar U, Loiacono L, Cemazar M et al (2017) Gene electrotransfer of plasmid-encoding IL-12 recruits the M1 macrophages and antigen-presenting cells inducing the eradication of aggressive B16F10 murine melanoma. Mediat Inflamm 2017:e5285890. https://doi.org/10.1155/2017/5285890

Laufer S, Ivorra A, Reuter VE et al (2010) Electrical impedance characterization of normal and cancerous human hepatic tissue. Physiol Meas 31:995–1009. https://doi.org/10.1088/0967-3334/31/7/009

Maglietti F, Michinski S, Olaiz N et al (2013) The role of Ph fronts in tissue electroporation based treatments. PLoS One 8:e80167. https://doi.org/10.1371/journal.pone.0080167

Mahnič-Kalamiza S, Vorobiev E, Miklavčič D (2014) Electroporation in food processing and biorefinery. J Membr Biol 247:1279–1304. https://doi.org/10.1007/s00232-014-9737-x

Mali B, Gorjup V, Edhemovic I et al (2015) Electrochemotherapy of colorectal liver metastases - an observational study of its effects on the electrocardiogram. Biomed Eng Online 14:S5. https://doi.org/10.1186/1475-925X-14-S3-S5

Maor E, Ivorra A, Mitchell JJ, Rubinsky B (2010) Vascular smooth muscle cells ablation with endovascular nonthermal irreversible electroporation. J Vasc Interv Radiol 21:1708–1715. https://doi.org/10.1016/j.jvir.2010.06.024

Marčan M, Pavliha D, Kos B et al (2015) Web-based tool for visualization of electric field distribution in deep-seated body structures and planning of electroporation-based treatments. Biomed Eng Online 14(Suppl 3):S4. https://doi.org/10.1186/1475-925X-14-S3-S4

Martin RC, Schwartz E, Adams J et al (2015) Intra - operative anesthesia management in patients undergoing surgical irreversible electroporation of the pancreas, liver, kidney, and retroperitoneal tumors. Anesth Pain Med 5:e22786. https://doi.org/10.5812/aapm.22786

Meijerink MR, Scheffer HJ, Narayanan G (eds) (2018) Irreversible electroporation in clinical practice. Springer International Publishing

Mercadal B, Arena CB, Davalos RV, Ivorra A (2017) Avoiding nerve stimulation in irreversible electroporation: a numerical modeling study. Phys Med Biol 62:8060–8079. https://doi.org/10.1088/1361-6560/aa8c53

Miklavčič D, Serša G, Kryžanowski M et al (1993) Tumor treatment by direct electric current-tumor temperature and pH, electrode material and configuration. Bioelectrochem Bioenerg 30:209–220. https://doi.org/10.1016/0302-4598(93)80080-E

Miklavčič D, Pucihar G, Pavlovec M et al (2005) The effect of high frequency electric pulses on muscle contractions and antitumor efficiency in vivo for a potential use in clinical electrochemotherapy. Bioelectrochemistry 65:121–128. https://doi.org/10.1016/j.bioelechem.2004.07.004

Miklavčič D, Čorović S, Pucihar G, Pavšelj N (2006) Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy. Eur J Cancer Suppl 4:45–51. https://doi.org/10.1016/j.ejcsup.2006.08.006

Miklavčič D, Snoj M, Županič A et al (2010) Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. Biomed Eng Online 9:10. https://doi.org/10.1186/1475-925X-9-10

Miklavčič D, Serša G, Brecelj E et al (2012) Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors. Med Biol Eng Comput 50:1213–1225. https://doi.org/10.1007/s11517-012-0991-8

Miklavčič D, Mali B, Kos B et al (2014) Electrochemotherapy: from the drawing board into medical practice. Biomed Eng Online 13:29. https://doi.org/10.1186/1475-925X-13-29

Mir LM, Orlowski S, Belehradek J, Paoletti C (1991) Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. Eur J Cancer 27:68–72

Mir LM, Gehl J, Sersa G et al (2006) Standard operating procedures of the electrochemotherapy: instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator TM by means of invasive or non-invasive electrodes. Eur J Cancer Suppl 4:14–25. https://doi.org/10.1016/j.ejcsup.2006.08.003

Neumann E, Rosenheck K (1972) Permeability changes induced by electric impulses in vesicular membranes. J Membr Biol 10:279–290. https://doi.org/10.1007/BF01867861

O'Brien TJ, Bonakdar M, Bhonsle S et al (2018) Effects of internal electrode cooling on irreversible electroporation using a perfused organ model. Int J Hyperthermia 35(1):44–55. https://doi.org/10.1080/02656736.2018.1473893

O'Rourke AP, Lazebnik M, Bertram JM et al (2007) Dielectric properties of human normal, malignant and cirrhotic liver tissue: in vivo and ex vivo measurements from 0.5 to 20 GHz using a precision open-ended coaxial probe. Phys Med Biol 52:4707–4719. https://doi.org/10.1088/0031-9155/52/15/022

Obermeier B, Daneman R, Ransohoff RM (2013) Development, maintenance and disruption of the blood-brain barrier. Nat Med 19:1584–1596. https://doi.org/10.1038/nm.3407

Olaiz N, Signori E, Maglietti F et al (2014) Tissue damage modeling in gene electrotransfer: the role of pH. Bioelectrochemistry 100:105–111. https://doi.org/10.1016/j.bioelechem.2014.05.001

Pavliha D, Kos B, Marčan M et al (2013) Planning of electroporation-based treatments using Web-based treatment-planning software. J Membr Biol 246:833–842. https://doi.org/10.1007/s00232-013-9567-2

Pavšelj N, Miklavčič D (2008) Numerical modeling in electroporation-based biomedical applications. Radiol Oncol 42:93–101

Pavšelj N, Bregar Z, Cukjati D et al (2005) The course of tissue permeabilization studied on a mathematical model of a subcutaneous tumor in small animals. IEEE Trans Biomed Eng 52:1373–1381. https://doi.org/10.1109/TBME.2005.851524

Peleg M (1995) A model of microbial survival after exposure to pulsed electric fields. J Sci Food Agric 67:93–99. https://doi.org/10.1002/jsfa.2740670115

Pennes HH (1948) Analysis of tissue and arterial blood temperatures in the resting human forearm. J Appl Physiol 85:93–122. https://doi.org/10.1152/jappl.1948.1.2.93

Peyman A, Kos B, Djokić M et al (2015) Variation in dielectric properties due to pathological changes in human liver. Bioelectromagnetics 36:603–612. https://doi.org/10.1002/bem.21939

Potočnik T, Miklavčič D, Maček Lebar A (2019) Effect of electroporation and recovery medium pH on cell membrane permeabilization, cell survival and gene transfer efficiency in vitro. Bioelectrochemistry 130:107342. https://doi.org/10.1016/j.bioelechem.2019.107342

Pucihar G, Krmelj J, Reberšek M et al (2011) Equivalent pulse parameters for electroporation. IEEE Trans Biomed Eng 58:3279–3288. https://doi.org/10.1109/TBME.2011.2167232

Reddy VY, Koruth J, Jais P et al (2018) Ablation of atrial fibrillation with pulsed electric fields: an ultra-rapid, tissue-selective modality for cardiac ablation. J Am Coll Cardiol EP 4:987–995. https://doi.org/10.1016/j.jacep.2018.04.005

Rems L, Miklavčič D (2016) Tutorial: electroporation of cells in complex materials and tissue. J Appl Phys 119:201101. https://doi.org/10.1063/1.4949264

Ringel-Scaia VM, Beitel-White N, Lorenzo MF et al (2019) High-frequency irreversible electroporation is an effective tumor ablation strategy that induces immunologic cell death and promotes systemic anti-tumor immunity. EBioMedicine 44:112–125. https://doi.org/10.1016/j.ebiom.2019.05.036

Rosazza C, Meglic SH, Zumbusch A et al (2016) Gene electrotransfer: a mechanistic perspective. Curr Gene Ther 16:98–129

Rossmanna C, Haemmerich D (2014) Review of temperature dependence of thermal properties, dielectric properties, and perfusion of biological tissues at hyperthermic and ablation temperatures. Crit Rev Biomed Eng 42:467–492

Rossmeisl JH, Garcia PA, Pancotto TE et al (2015) Safety and feasibility of the NanoKnife system for irreversible electroporation ablative treatment of canine spontaneous intracranial gliomas. J Neurosurg 123:1008–1025. https://doi.org/10.3171/2014.12.JNS141768

Rubinsky B (2007) Irreversible electroporation in medicine. Technol Cancer Res Treat 6:255–259. https://doi.org/10.1177/153303460700600401

Sano MB, Petrella RA, Kaufman JD et al (2020) Electro-thermal therapy: microsecond duration pulsed electric field tissue ablation with dynamic temperature control algorithms. Comput Biol Med 121:103807. https://doi.org/10.1016/j.compbiomed.2020.103807

Scheffer HJ, Nielsen K, de Jong MC et al (2014) Irreversible electroporation for nonthermal tumor ablation in the clinical setting: a systematic review of safety and efficacy. J Vasc Interv Radiol 25:997–1011.; quiz 1011. https://doi.org/10.1016/j.jvir.2014.01.028

Schoellnast H, Monette S, Ezell PC et al (2013) The delayed effects of irreversible electroporation ablation on nerves. Eur Radiol 23:375–380. https://doi.org/10.1007/s00330-012-2610-3

Schutt DJ, Haemmerich D (2008) Effects of variation in perfusion rates and of perfusion models in computational models of radio frequency tumor ablation. Med Phys 35:3462–3470. https://doi.org/10.1118/1.2948388

Šel D, Cukjati D, Batiuskaite D et al (2005) Sequential finite element model of tissue electropermeabilization. IEEE Trans Biomed Eng 52:816–827. https://doi.org/10.1109/TBME.2005.845212

Šel D, Maček Lebar A, Miklavčič D (2007) Feasibility of employing model-based optimization of pulse amplitude and electrode distance for effective tumor electropermeabilization. IEEE Trans Biomed Eng 54:773–781. https://doi.org/10.1109/TBME.2006.889196

Serša G, Miklavčič D (2008) Electrochemotherapy of tumours. J Vis Exp 22:1038. https://doi.org/10.3791/1038

Serša G, Čemažar M, Šemrov D, Miklavčič D (1996) Changing electrode orientation improves the efficacy of electrochemotherapy of solid tumors in mice. Bioelectrochem Bioenerg 39:61–66. https://doi.org/10.1016/0302-4598(95)01866-2

Sharabi S, Mardor Y (2016) Effect of electroporation on blood-brain barrier. In: Miklavcic D (ed) Handbook of electroporation. Springer International Publishing, Cham, pp 1–17

Sharabi S, Kos B, Last D et al (2016) A statistical model describing combined irreversible electroporation and electroporation-induced blood-brain barrier disruption. Radiol Oncol 50:28–38. https://doi.org/10.1515/raon-2016-0009

Sharabi S, Bresler Y, Ravid O et al (2019) Transient blood–brain barrier disruption is induced by low pulsed electrical fields in vitro: an analysis of permeability and trans-endothelial electric resistivity. Drug Deliv 26:459–469. https://doi.org/10.1080/10717544.2019.1571123

Stepišnik T, Jarm T, Grošelj A et al (2016) Electrochemotherapy – an effective method for treatment of tumors with combination of chemotherapeutic agent and electric field. Slov Med J 85

Stewart MT, Haines DE, Verma A et al (2019) Intracardiac pulsed field ablation: proof of feasibility in a chronic porcine model. Heart Rhythm 16:754–764. https://doi.org/10.1016/j.hrthm.2018.10.030

Turjanski P, Olaiz N, Abou-Adal P et al (2009) pH front tracking in the electrochemical treatment (EChT) of tumors: experiments and simulations. Electrochim Acta 54:6199–6206. https://doi.org/10.1016/j.electacta.2009.05.062

Turjanski P, Olaiz N, Maglietti F et al (2011) The Role of pH Fronts in Reversible Electroporation. PLoS One 6:e17303. https://doi.org/10.1371/journal.pone.0017303

Vogel JA, van Veldhuisen E, Agnass P et al (2016) Time-dependent impact of irreversible electroporation on pancreas, liver, blood vessels and nerves: a systematic review of experimental studies. PLoS One 11:e0166987. https://doi.org/10.1371/journal.pone.0166987

Wagstaff PGK, de Bruin DM, van den Bos W et al (2015) Irreversible electroporation of the porcine kidney: temperature development and distribution. Urol Oncol 33:168.e1–168.e7. https://doi.org/10.1016/j.urolonc.2014.11.019

Wittkampf FHM, van Es R, Neven K (2018) Electroporation and its relevance for cardiac catheter ablation. JACC Clin Electrophysiol 4:977–986. https://doi.org/10.1016/j.jacep.2018.06.005

Yao C, Dong S, Zhao Y et al (2017) Bipolar microsecond pulses and insulated needle electrodes for reducing muscle contractions during irreversible electroporation. IEEE Trans Biomed Eng 64:2924–2937. https://doi.org/10.1109/TBME.2017.2690624

Yarmush ML, Golberg A, Serša G et al (2014) Electroporation-based technologies for medicine: principles, applications, and challenges. Annu Rev Biomed Eng 16:295–320. https://doi.org/10.1146/annurev-bioeng-071813-104622

Zhao Y, Zheng S, Beitel-White N et al (2020) Development of a multi-pulse conductivity model for liver tissue treated with pulsed electric fields. Front Bioeng Biotechnol 8:396. https://doi.org/10.3389/fbioe.2020.00396

Zmuc J, Gasljevic G, Sersa G et al (2019) Large liver blood vessels and bile ducts are not damaged by electrochemotherapy with bleomycin in pigs. Sci Rep 9:3649. https://doi.org/10.1038/s41598-019-40395-y

Zupanic A, Ribaric S, Miklavcic D (2007) Increasing the repetition frequency of electric pulse delivery reduces unpleasant sensations that occur in electrochemotherapy. Neoplasma 54:246–250

Županič A, Kos B, Miklavčič D (2012) Treatment planning of electroporation-based medical interventions: electrochemotherapy, gene electrotransfer and irreversible electroporation. Phys Med Biol 57:5425–5440. https://doi.org/10.1088/0031-9155/57/17/5425

Part II

Therapeutic Applications with Electroporation in Veterinary Oncology Practice

Electrochemotherapy in Veterinary Oncology

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Abstract

Electrochemotherapy (ECT) has been used in veterinary medicine since the late 1990s and has become increasingly established as a treatment modality for various cutaneous and subcutaneous tumors in dogs. For some tumor types, such as mast cell tumors and perianal tumors, an excellent response can be achieved, while for others, such as oral tumors, palliation of the disease with improvement in quality of life can be expected. In addition to dogs, various tumor types in cats, horses, and exotic pets can be treated with ECT. In this chapter, we have reviewed all the currently available literature describing the use of ECT in different animal species and tumor types. In addition, the treatment procedure for different tumor types in dogs, cats, and horses is presented, describing the selection of appropriate patients, the treatment procedure, and guidelines for follow-up examinations, aftercare, or possible retreatment.

The promising results of published clinical studies place ECT among the successful local therapies for cancer that also provide a good quality of life for the treated animals.

Keywords

Electroporation · Electrochemotherapy · Veterinary oncology · Cisplatin · Bleomycin

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1 History of Electrochemotherapy

The first study describing the facilitated uptake of cytotoxic drugs by means of electroporation date in the year 1988 when they evaluated the cytotoxicity of several anticancer agents on electropermeabilized and non-electropermeabilized cells (Orlowski et al. 1988). The agent exerting the highest increase in cytotoxicity after exposure of cells to short, intense, square-wave electric pulses was bleomycin with a 700-fold increase in toxicity. A few years later, the first study of electrochemotherapy (ECT) in mice was performed, confirming the in vitro observations (Mir et al. 1991). After intramuscular injection of bleomycin, followed by local delivery of electrical pulses, the tumors of the mice were reduced in size and even eradicated. Such results encouraged researchers from several research groups to continue with preclinical studies, which proved the good antitumor effect of ECT on different tumor models (Okino and Mohri 1987; Belehradek et al. 1991; Salford et al. 1993; Serša et al. 1994; Čemažar et al. 1995; Heller et al. 1995). Apart from bleomycin, promising results were achieved using another anticancer agent, cisplatin (Serša et al. 1995).

In 1993, the first phase I–II clinical trial in human medicine was conducted, evaluating the safety and efficacy of ECT with bleomycin for the treatment of head and neck squamous cell carcinoma (Belehradek et al. 1993). No local or systemic side effects were observed, and the treatment was well tolerated. Furthermore, the treatment provided a good antitumor effect with a 72% objective response. The findings were confirmed in several consequent studies on patients with different skin and subcutaneous tumors, treated with single or multiple sessions of ECT with bleomycin (Rudolf et al. 1995; Domenge et al. 1996; Frank Glass et al. 1996; Heller et al. 1996, 1998; Kubota et al. 1998; Serša et al. 2000; Gehl and Geertsen 2000; Rols et al. 2000; Allegretti and Panje 2001; Rodríguez-Cuevas et al. 2001). The complete response rates varied from 9 to 100%, and the treatment was well tolerated in all the observed cases, however, the protocols used for ECT varied significantly between studies (Gothelf et al. 2003). The encouraging results and the need for standardization of the treatment procedure gave rise to ESOPE (European Standard Operating Procedures of Electrochemotherapy), a multicenter European project whose aim was to prepare standard operating procedures (SOP) for ECT that were published in 2006 (Marty et al. 2006; Mir et al. 2006) and updated in 2018 (Gehl et al. 2018). After the publication of the first SOP, an increasing amount of clinical studies describing the use of ECT for treating different superficial tumors was performed. ECT was later incorporated in the National Institute for Health and Care Excellence (NICE) guidelines as a treatment modality for treating several primary and metastatic cutaneous tumors (Campana et al. 2016b). Because of the efficacy and safety, special electrodes were developed for treating deep-seated tumors, such as primary and metastatic liver cancer, colorectal, pancreatic, esophageal and prostatic cancer and metastases of the bone and spinal cord (Edhemović et al. 2014; Coletti et al. 2017; Klein et al. 2017; Djokić et al. 2018; Egeland et al. 2018; Probst et al. 2018; Campana et al. 2019; Cornelis et al. 2019; Falk Hansen et al. 2020). The need for standardized reporting resulted in the publication of a

paper describing recommendations for improving the quality of reporting clinical ECT studies (Campana et al. 2016a).

2 Electrochemotherapy in Veterinary Oncology

Soon after the first clinical studies in human medicine, the first clinical trial using ECT in veterinary medicine was conducted. In this study, Mir et al. (1997) used ECT combined with immunotherapy with interleukin-2 (IL-2) for treating recurrent feline soft tissue sarcomas. In the following years, several research groups continued the work, and ultimately, ECT became a recognized treatment, especially for treating selected cutaneous and subcutaneous tumors in dogs, but also different types of tumors in cats, horses, and exotic pets (Impellizeri et al. 2016).

This chapter focuses on reviewing the current data on the use of ECT in different animal species and tumor types, with an emphasis on the approach for selected tumor types.

2.1 Electrochemotherapy for the Treatment of Cutaneous and Subcutaneous Tumors Dogs

2.1.1 Epithelial Tumors

Perianal Tumors

Tumors arising from the perianal area can be challenging to treat surgically. Especially in cases of larger, infiltrative, or multiple tumor nodules, local tumor control can be difficult to achieve. Because of the high incidence of perianal tumors in dogs and the need for preservation of the surrounding healthy tissues in the area, ECT was used in several studies for the treatment of this histologically different tumor types in the perianal region.

The first clinical case described in the veterinary literature was published by Tozon et al. (2001) as a part of a clinical study of ECT with intratumoral cisplatin injection to treat different tumor types in cats and dogs. A dog with three perianal carcinomas was treated with an intratumoral application of cisplatin (2–12 mg/tumor nodule), followed by ECT. Two smaller tumors were treated with a single ECT session, and a larger tumor (18 cm^3) was treated twice. The smaller tumors regressed completely, while the third tumor was in PR for 4.5 months; the observation time was 7 months (Tozon et al. 2001).

The same group later evaluated the use of ECT on a cohort of 12 dogs with 26 perianal tumors (adenomas and adenocarcinomas) (Tozon et al. 2005). Cisplatin was the chemotherapeutic of choice, whereas bleomycin was used in the tumors that did not respond adequately; both drugs were administered intratumorally. At the end of the observation period for each tumor (1 to 34 months), 92% overall response rate (ORR) (65% complete responses (CR) and 27% partial responses (PR)) and in the rest (8%) of the tumors, no change was observed. In none of the cases, progressive

disease (PD) was noted, and the researchers observed no major local or general side effects. They found the only factor predicting the treatment outcome was the size of the tumor; tumors smaller than 1 cm^3 responded to the treatment better than those measuring 1 cm^3 or more (Tozon et al. 2005). Later, the same group (Tozon et al. 2010) reported results of a clinical trial on a bigger cohort of patients with perianal tumors, where 21 dogs with 66 tumors (adenocarcinomas, adenomas, and epitheliomas) were treated with the intratumoral application of cisplatin or bleomycin. In this study, they evaluated the efficacy of two treatment protocols (Protocol 1: plate electrodes, repetition frequency 1 Hz, amplitude to electrode distance ratio 1300 V/cm; Protocol 2: needle electrodes, repetition frequency 5000 Hz, amplitude to electrode distance ratio 1000 V/cm). At the end of the observation period (median 14 months), an OR was obtained in 62/66 tumors (94%) with an 87.9% CR rate. There were no statistically significant differences in the OR rate based on histological type, previous castration, choice of chemotherapeutic drug and electroporation protocol. The only predictive factor for treatment outcome was the size of the tumor (cut-off value 3 cm^3); this, together with the absence of major local and general side effects, confirmed the findings from the previous study (Tozon et al. 2010). Similar results in the treatment outcome were obtained by Spugnini et al. (2007a), who treated 12 dogs with adenomas and carcinomas of the hepatoid gland by intratumoral injection of bleomycin (dose per tumor not specified), ECT with the modified caliper, needle array and paired needle electrodes for pulse delivery. The OR rate was 91% with an 83% of CR (10/12); 1 dog had a PR that lasted 12 months, and another had PD (Spugnini et al. 2007a).

Mammary Adenocarcinoma

There is an early report of a case of a large, recurrent metastatic mammary adenocarcinoma, treated with ECT with intratumoral cisplatin (Tozon et al. 2001). The tumor was treated with six session, and PR was achieved with prolongation of lifespan for 7 months.

Cutaneous Squamous Cell Carcinoma

A report from Cutrera et al. (2015) describes the use of combined treatment with ECT with intratumoral bleomycin (1 IU/cm^3) or gemcitabine (0.5–10 mg/cm^3) and IL-12 gene electrotransfer (GET). GET is an electroporation-based treatment where pDNA or siRNA molecules can be delivered to various tissues, including tumors (Serša et al. 2015). The aim of IL-12 GET is to enhance the antitumor response by activating the innate and adaptive immunity, mainly through promoting the induction of IFN-γ secretion (Pavlin et al. 2012). In a cohort of dogs with different malignant tumors, three dogs with cutaneous squamous cell carcinoma (SCC) were included. Two of the dogs had previously irradiated SCC of the nasal planum, and the third one had SCC on the limb with metastasis to the prescapular lymph node. Bleomycin was the chemotherapeutic agent of choice and was replaced with gemcitabine in cases with poor clinical response. The size of the tumors reduced 21 days after treatment and, in the cases of nasal planum SCC, SD was achieved. The

dog with SCC on the forelimb was treated 22 times (12 cycles) and responded to the treatment completely (Cutrera et al. 2015).

Other Epithelial Tumors

Recently, dos Anjos et al. (2018) described successful treatment of a canine digital trichoblastoma of the finger. Complete remission was achieved 81 days after the third ECT session, using intravenous bleomycin, with a DFI of 700 days. The authors concluded that ECT might be a better approach than surgery for digital trichoblastomas because it avoids digit amputation and provides a good cosmetic result (Dos Anjos et al. 2018).

2.1.2 Mesenchymal Tumors

Due to the nature of the tumors, the need for large surgical margins and the common anatomical locations (e.g., extremities, trunk) of sarcomas in dogs, complete resection of the tumor can be challenging. New treatment modalities, either as a monotherapy or as an adjuvant treatment to surgery, are sought. In the past years, ECT started gaining attention due to good results in obtaining local tumor control.

The first cases describing the use of ECT for treating mesenchymal tumors in dogs were published by Tozon et al. (2001). Three dogs with different types of mesenchymal tumors (hemangioma, neurofibroma, hemangiosarcoma) were treated with ECT with the intratumoral application of cisplatin. The dogs with hemangioma and neurofibroma were treated with a single ECT session, and the one with hemangiosarcoma was treated twice. While the first two responded completely, in the latter, which was also the largest one (5.2 cm^3), PR was achieved (Tozon et al. 2001).

Spugnini and Porrello (2003) later evaluated the efficacy of ECT with intratumoral bleomycin in dogs with large spontaneous neoplasms, including hemangiopericytoma (HPC, two cases, previously treated with surgery), neurofibrosarcoma (one dog) and liposarcoma (one dog). After four sessions of ECT, a response was noted in all treated dogs: a long-lasting CR (>660 days) in one and PR (580 days) in the other HPC case. The dogs with neurofibrosarcoma and liposarcoma achieved PR (60 and 70 days), however, the authors do not state the cause of the short observation times. A massive necrosis coupled with extensive vascular destruction was seen in all of the patients, possibly due to the large volume of the treated tumors (Spugnini and Porrello 2003).

Later Spugnini et al. (2007e) evaluated the tolerability and efficacy of ECT in the treatment of 22 dogs with incompletely excised high-grade soft tissue sarcoma (STS). The OR rate was 95% (21/22 patients) with a mean time to recurrence of 730 days. The only observed toxicity was wound dehiscence in three patients (Spugnini et al. 2007e). A year later, excellent results were published by the author, describing long-term response (at least 24 months) of a large, high-grade STS, treated with a combination of surgery and ECT (Spugnini et al. 2008d). Recently, the research group published further data describing ECT for the treatment of 30 dogs with incompletely excised STS (Spugnini et al. 2019). Here, the authors used intravenous administration of bleomycin (20 mg/m^2) to increase the likelihood

of drug distribution in the deeper layers of the tumor bed and combined it with cisplatin administration (0.5 mg/cm^2) into the tumor margins to increase the efficacy of the treatment in the superficial layers. A second treatment was performed after 2 weeks. Twenty six/thirty (86.7%) dogs had no evidence of recurrence, 4 (13.3%) had recurrence, and 1/4 recurring dogs died of lung metastases. Median estimated DFI was 857 days. Perivascular wall tumors responded better than other STS, but the difference in outcome was not significant. The treatment was well tolerated, and side effects were minimal (Spugnini et al. 2019).

The largest study describing the use of ECT for canine STS was performed by Torrigiani et al. (2019). They compared the efficacy of different treatment approaches on 52 dogs with 54 tumor nodules: ECT as a single treatment (performed on macroscopic disease, 4 nodules), intraoperative ECT (performed immediately surgical removal of the tumor, 26 nodules) and adjuvant ECT (performed after incomplete tumor removal, 24 nodules). All dogs were treated with intravenous application of bleomycin (15.000 IU/m^2), followed by ECT with type II needle electrodes. The animals were treated with 1–3 ECT sessions, depending on the tumor recurrence. Median time between the first and the second ECT treatment (five cases) was 68 days (range 14–495 days). Median time between the second and the third ECT treatment (three cases) was 82 days (range 27–614 days). In ECT only group, 2/4 (50%) dogs had CR, 1/4 (25%) PR, and 1/4 (25%) SD. The dogs in the other two groups were similar for tumor location, size, and grade, histological margins, treatment toxicity, pulse frequency and voltage. The group compared the treatment outcome when using two different pulse generators with different ECT parameters. They found no differences in the outcome when comparing the groups treated with different pulse generators, however, they observed a higher treatment toxicity score in the group treated with a higher amplitude to electric distance ratio (1200 vs. 1000 V/cm) (Torrigiani et al. 2019).

Apart from the case series, an interesting case of treating a rare condition, aponeurotic fibromatosis, with ECT was published. This large, non-resectable, recurrent tumor was treated with four courses of ECT, the first three with intratumoral cisplatin injection and, because of lack of response to the third session, the fourth was performed using intravenous bleomycin (15 mg/m^2) application. A long-lasting CR was achieved (until the end of the observation period, i.e., 18 months) (Spugnini et al. 2013).

Reed et al. (2010) evaluated the combination of ECT with intratumoral bleomycin, and IL-12 GET for the treatment of different tumors. Among others, a dog with cubital histiocytic sarcoma with metastasis to the spleen was included. The authors report of complete resolution of the primary tumor, although the CR was not histologically confirmed. However, the dog was euthanized 4 months after treatment due to metastatic disease (Reed et al. 2010).

In the report from Cutrera et al. (2015), describing the use of a combined treatment with ECT with intratumoral bleomycin or gemcitabine and IL-12 GET, three dogs with subcutaneous sarcomas of unspecified origin were also included. The authors reported that the combined treatment was not effective in reducing the

size of the tumors, however, when subsequently treated with IL-12 GET only, some response was achieved (two SD and one PD) (Cutrera et al. 2015).

2.1.3 Round Cell Tumors

Mast Cell Tumors

Mast cell tumors (MCT) are currently one of the most frequent tumor types treated with ECT. In this chapter, a brief review of the literature describing the use of ECT in MCT is presented. More information is provided in chapter "Gene Electrotransfer."

The first case of treating canine MCT with ECT was reported by Tozon et al. (2001). One dog with 2 MCT was treated with ECT using the intratumoral injection of cisplatin. Both treated tumors regressed 4 weeks after treatment and remained in CR until the end of the observation period (14 months). The treatment outcome was compared to another dog with 1 MCT, treated with intratumoral cisplatin injection without electroporation. After two cisplatin injections (4 mg/tumor nodule), the dog was in PR for 13 months (Tozon et al. 2001).

Spugnini et al. (2006b) described the use of ECT as an adjuvant treatment for incompletely excised MCT. Twenty-eight dogs with incompletely removed MCT were included in the study and treated with the intratumoral application of bleomycin (1.5 IU/cm^2), followed by ECT using modified caliper electrodes. The OR rate was 85%, with a mean estimated time to recurrence of 52.76 $\pm$ 6.5 months. Median survival time (MST) was not reached, and mean survival time was 52.76 $\pm$ 6.5 months. Three dogs died of metastatic disease that they developed at the same time of local recurrence, one developed multiple cutaneous nodules at different locations, and one with recurrence was retreated and was disease-free after 22 months (Spugnini et al. 2006b). The same group later evaluated the use of ECT with intratumoral application of cisplatin on a larger number of dogs with incompletely excised MCT (Spugnini et al. 2011b). In this prospective study, 37 dogs were first treated with debulking surgery, followed by the injection of cisplatin to the tumor bed and margins (1.5 cm of normal tissue surrounding the surgical scar) and electroporation. A second session was performed 1 or 2 weeks later based on clinical considerations. Twenty nine/thirty seven (78%) had no evidence of disease recurrence over a 6-year observation period, six had tumor recurrence, one died of multiple cutaneous MCT, and one died of unrelated causes (Spugnini et al. 2011b).

Kodre et al. (2009) later performed a clinical study on the use of ECT in a larger cohort of MCT-bearing dogs and compared the treatment outcome with dogs with MCT treated with surgery. Twenty-five dogs with MCT were divided into two treatment groups: surgery (16 dogs with 16 tumors) and ECT group (9 dogs with 12 tumors). The protocol consisted of intratumoral application of cisplatin (1 mg/cm^3) and delivery of pulses with the same parameters as described by Tozon et al. (2001), 1–2 min after chemotherapeutic administration, using plate electrodes. They observed a 62% CR rate 4–5 weeks after treatment. In 2/9 dogs, the disease progressed; both dogs had large tumors (>8 cm^3) and were euthanized 2 and 8 months after treatment. When comparing the treatment outcome between the two groups, they found that ECT with cisplatin as a single treatment had comparable

antitumor effectiveness as standard surgical treatment with a longer duration of local tumor control (not reached) compared to surgery (31.5 months). The authors concluded that ECT is a suitable alternative to surgery, especially in cases of smaller tumors or non-resectable tumors due to the anatomical location (Kodre et al. 2009).

In a retrospective, multi-institutional study by Lowe et al. (2017), 51 dogs with MCT were included and divided into four treatment groups according to ECT procedure: ECT only (15 cases), intraoperative ECT (11 cases), ECT adjuvant to surgery (14 cases) and surgery followed by ECT (recurrent MCT, 11 cases). In all cases, intravenous application of bleomycin was performed, followed by electric pulse application 8 min after. The best CR rates were found for the postoperative (93%) and intraoperative (91%) ECT group. The rates were lower in the ECT only group (80% CR) and in the group of recurrent MCT, treated with surgery, followed by ECT (64% CR). The intraoperative group of dogs showed the best disease-free interval among the groups (Lowe et al. 2017).

To address the systemic antitumor effect, Čemažar et al. (2017) combined ECT with peritumoral interleukin-12 (IL-12) gene electrotransfer (GET) for the treatment of 18 dogs with MCT. Intratumoral injection of cisplatin was used as drug of choice. In case of no or minimal antitumor response at 4 weeks, cisplatin was replaced with bleomycin, either intratumorally or intravenously. IL-12 GET was performed immediately after ECT. Twelve/eighteen patients received single combinational therapy, and in three patients, the procedure was repeated after 1 month. In 3/18 patients, only additional ECT was performed 1 month after the first treatment. At the end of the observation period (median 40 months), CR was achieved in 13/18 (72%) of the treated tumors with another 2/18 nodules (11%) achieving PR, bringing OR of combined therapy to an OR rate of 83%; one dog (6%) achieved stable disease (SD). The response rate in tumors, larger than 2 cm^3 was lower (CR 60% and OR 80%). Another predictive factor was the stage of the disease; both cases with PD (11%) were patients with higher (II and III) clinical stages (Čemažar et al. 2017).

Other Round Cell Tumors

The data on the treatment of round cell tumors other than MCT is limited. An early report from Spugnini and Porrello (2003) includes treatment of two canine cutaneous lymphomas with ECT using the intratumoral injection of bleomycin. The authors achieved a long-lasting (>610 days) CR in one dog after a single session of ECT and PR (100 days) in the other case, treated with four sessions (Spugnini and Porrello 2003). Later, two other cases of cutaneous lymphoma, treated in the same manner, were described, both achieving CR (Spugnini et al. 2007b).

A report of three dogs with transmissible venereal tumor (TVT), refractory to chemotherapy, was published by Spugnini et al. (2008b). After two ECT sessions, all the dogs achieved long-term CR (28–48 months). The therapy allowed the restoration of continence in two dogs and allowed physiological micturition in all the patients within 5 days from the first ECT. Furthermore, in all the patients, ECT reduced the tumor-induced penile bleeding and therefore notably improved the dogs' quality of life (Spugnini et al. 2008b).

The details of the treatments of canine cutaneous and subcutaneous tumors are summarized in Table 1.

2.2 Electrochemotherapy for the Treatment of Oral Tumors in Dogs

2.2.1 Oral Malignant Melanoma

Oral tumors in dogs represent a special entity in veterinary oncology and frequently pose a treatment challenge for the veterinarian. Curative surgery remains the treatment of choice but is commonly not possible, either due to the size of the tumor or the anatomical location. ECT currently represents an alternative treatment option for oral tumors, especially when standard treatments, such as surgery or radiotherapy, are impossible or declined by the owner (Tozon et al. 2016b; Simčič et al. 2020).

ECT in veterinary oncology is most commonly described for the treatment of oral malignant melanoma (OMM). The first case of canine OMM, treated with intratumoral bleomycin application, was described by Spugnini and Porrello (2003). After four treatments, the dog achieved SD. Spugnini et al. (2006a, b) then described a larger case series of ten dogs with OMM, treated with four sessions of ECT using intra- and peritumoral application of bleomycin. The treatment was well tolerated and resulted in an OR rate of 80% with 50% long-term control. Four dogs with initial CR remained in remission for 16–36 months, and all dogs with PR or SD eventually developed PD. The MST for all dogs was 6 months (mean 16.12 months). Interestingly, only one of the dogs died of metastatic disease, and the long-term survivors showed a vitiligo-like discoloration at the site of treatment, potentially suggesting a recruitment of the immune system by the therapy (Spugnini et al. 2006a).

The first report on the use of a combination of ECT with intratumoral bleomycin and IL-12 GET for canine OMM was published by Reed et al. (2010). In a cohort of dogs with different tumors, a dog with metastatic OMM to the lymph nodes and the lung was included. The dog had an initial reduction in tumor size by greater than 50% 21 days after treatment (PR), but was soon euthanized due to progression of the disease and other medical problems (Reed et al. 2010).

Another combinational approach for the treatment of canine OMM was attempted by Kulbacka et al. (2017), who described ECT with intravenous bleomycin, coupled with intratumoral bleomycin and calcium solution ($CaCl_2$), combined with standard and CO_2 laser surgeries. The patient was a 15-year-old dog with a stage IV OMM that was treated with two sessions of ECT: the first one with bleomycin and the second one with calcium ions solution. Treatment planning was performed by means of a web-based electric field visualization tool Visifield (www.visifield.com, University of Ljubljana, Slovenia). The combination of ECT with surgical debulking resulted in rapid recovery and regaining of physiological activities, including normal feeding by the dog. The dog was euthanized 2 months after treatment, however, the authors concluded that the treatment offered successful palliation of the disease (Kulbacka et al. 2017).

Table 1 Use of ECT for cutaneous/subcutaneous tumors in dogs

	Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
Epithelial tumors	Perianal carcinoma	One (three tumor nodules)	Cisplatin (0.5–2.5 mg/100 mm^3 nodule, i/tum)	Plate electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz	Jouan GHT 1287 (Jouan, Saint Herblaine, France)	1–2	CR (2 nodules), PR 4.5 mo (1 nodule); observation time 7 mo	Tozon et al. (2001)
	Metastatic mammary adenocarcinoma	One dog	Cisplatin (0.5–2.5 mg/100 mm^3 nodule, i/tum)	Plate electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz	Jouan GHT 1287 (Jouan, Saint Herblaine, France)	6	PR; observation time 7 mo	Tozon et al. (2001)
	Perianal adenoma and adenocarcinoma	12 dogs (26 tumor nodules)	Cisplatin (1 mg/cm^3, i/tum) or bleomycin (3 mg/cm^3, i/tum)	Plate electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz	Jouan GHT 1287	1–3	ORR 92% (65% CR, 27% PR, NC 8%); observation time 1 to 34 mo	Tozon et al. (2005)
	Perianal adenoma ($n = 8$) and carcinoma ($n = 4$)	12 dogs	Bleomycin (dose unknown, i/tum)	Modified caliper, needle array and paired needle electrodes; 8 (biphasic) pulses, 50 + 50μs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Chemopulse (Center of Bioengineering, Sofia, Bulgaria)	2	ORR 91% (83.3% CR, 8.3% PR), 8.3% PD	Spugnini et al. (2007a)
	Perianal adenocarcinoma ($n = 26$), adenoma, epithelioma (total $n = 40$)	21 dogs (66 tumor nodules)	cisplatin (2 mg/cm^3, i/tum) or bleomycin (3 mg/cm^3, i/tum)	Plate and needle electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz (plate), 8 pulses, 100μs, 1000 V/cm, 5 kHz (needle)	Jouan GHT 1287 (plate elecetrodes), Cliniporator ™ (IGEA S.r.l., Carpi, Italy; needle electrodes)	1–3	ORR 94% (87.9 CR); median observation time 14 mo	Tozon et al. (2010)

	Cutaneous SCC (nasal planum, $n = 2$, limb with ln. metastasis, $n = 1$)	Three dogs	Bleomycin (1 IU/cm^3, i/tum) or gemcitabine (0.5–10 mg/cm^3, i/tum)	Needle electrodes; 2 pulses, 20 ms, 350 V/cm, 10 Hz	ECM 830 pulse generator, BTX® (Harvard Apparatus, Holliston, MA, USA)	Up to 22 treatments (12 cycles) + IL-12 GET (cIL-12, 2 mg/cm tumor diameter, i/tum)	CR (limb SCC), SD (nasal planum SCC)	Cutrera et al. (2015)
	Digital trichoblastoma	One dog	Bleomycin (15.000 IU/m^2, i/v)	Needle electrodes; 8 (unipolar) pulses, 100µs, 1000 V/cm, 1 Hz	LC BK-100 (São Paulo, SP, Brazil)	3	CR, DFI 700 d	Dos Anjos et al. (2018)
Mesenchymal Tumors	Hemangioma ($n = 1$), neurofibroma ($n = 1$), hemangiosarcoma ($n = 1$)	Three dogs	Cisplatin (0.5–2.5 mg/100 mm^3 nodule, i/tum)	Plate electrodes; 8 pulses, 100µs, 1300 V/cm, 1 Hz	Jouan GHT 1287	1–2	CR (hemangioma, neurofibroma), PR (hemangiosarcoma) observation time 2–9 mo	Tozon et al. (2001)
	HPC ($n = 2$), neurofibrosarcoma ($n = 1$), liposarcoma ($n = 1$)	Four dogs	Bleomycin (until saturation-dose unknown, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50µs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Chemopulse	4	HPC: CR (>660 d), PR (580 d)	Spugnini and Porrello (2003)
	Soft tissue sarcoma (incompletely excised)	22 dogs	Bleomycin (dose unknown, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50µs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Chemopulse	2	ORR 95%, mean time to recurrence 730 d	Spugnini et al. (2007e)
	High-grade soft tissue sarcoma	One dog	Bleomycin (10 mg/tumor, i/tum) + hyaluronidase (180 IU/tumor, i/tum)	Caliper and needle electrodes; 8 (biphasic) pulses, 50 + 50µs. 1 ms interpulse interval, 1300 V/cm, 1 Hz (caliper), 8 (biphasic) pulses, 50 + 50µs. 1 ms	Not specified	2 (ECT only, caliper electrodes) + 1 (surgery + intraoperative ECT, needle electrodes)	CR (>24 mo)	Spugnini et al. (2008d)

(continued)

Table 1 (continued)

	Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
				interpulse interval, 800 V/cm, 1 Hz (needle)				
	Cubital histiocytic sarcoma with spleen metastasis	One dog	Bleomycin (0.5–2 IU/treatment, i/tum)	Hexagonal and caliper electrodes; 2 pulses 20 ms, 400 V/cm, 10 Hz	ECM 830	1 + IL-12 GET (fIL-12, 150–400 ug, i/tum)	CR of the primary tumor (not confirmed histologically), euthanasia four mo after treatment (metastatic disease)	Reed et al. (2010)
	Aponeurotic fibromatosis	One dog	Cisplatin (8 mg/tumor, i/tum) or bleomycin (15 mg/m^2)	Clamp electrodes; 8 (biphasic) pulses, 50 + 50μs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Chemipulse III (EU patent application n^0 2221086)	4	CR (18 mo)	Spugnini et al. (2013)
	Subcutaneous sarcoma (origin unknown)	Three dogs	Bleomycin (1 IU/cm^3, i/tum) or gemcitabine (0.5–10 mg/cm^3, i/tum)	Needle electrodes; 2 pulses, 20 ms, 350 V/cm, 10 Hz	ECM 830	Multiple treatments with different combinations + IL-12 GET (cIL-12, 2 mg/cm tumor diameter, i/tum)	ECT + IL-12 GET ineffective; subsequent IL-12 GET only: 2 SD, 1 PD	Cutrera et al. (2015)
	Soft tissue sarcoma (incompletely excised)	30 dogs	Bleomycin (20 mg/m^2, i/v) + cisplatin (0.5 mg/cm^2, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50μs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Onkodisruptor (Biopulse S.r.l., Naples, Italy)	2	86.7% recurrence-free (149–1505 d), 13.3% recurrence (after 93–443 d), DFI 857 d	Spugnini et al. (2019)
	Soft tissue sarcoma	52 dogs (54 tumor nodules)	Bleomycin (15.000 U/m^2, i/v)	Type II needle electrodes; 8 (monophasic)	Cytopulse PA4000 or CytopulseOncovet	1–3; ECT as a single treatment ($n = 4$), intraoperative ECT	ECT only group: CR 50%, PR 25%, SD 25%	Torrigiani et al. (2019)

				pulses, 100μs, 1200 V/cm or 1000 V/cm, 5 kHz (Oncovet) or 1 Hz (PA4000)	(Cyto Pulse Sciences Inc., Holliston, USA)	($n = 26$), ECT adjuvant to surgery ($n = 24$)		
Round cell tumors	MCT	One dog (two tumor nodules)	Cisplatin (0.5–2.5 mg/100 mm^3 nodule, i/tum)	Plate electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz	Jouan GHT 1287	2	CR, observation time 14 mo	Tozon et al. (2001)
	Cutaneous lymphoma	Two dogs	Bleomycin (until saturation-dose unknown, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50μs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Chemopulse	1–4	CR (>610 d), PR (100 d)	Spugnini and Porrello (2003)
	MCT (incompletely excised)	28 dogs	Bleomycin (1.5 IU/cm^2, i/tum)	Modified caliper electrodes; 50 + 50μs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Chemopulse	2	ORR 85%, mean estimated time to recurrence 52.76 ± 6.5 mo, MST not reached	Spugnini et al. (2006b)
	Cutaneous lymphoma	Two dogs	Bleomycin (until saturation-dose unknown, i/tum)	Modified caliper and paired needle electrodes; 8 pulses, 50 + 50μs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Chemopulse	2	CR (20 and 760 d)	Spugnini et al. (2007b)
	Transmissible venereal tumor	Three dogs	bleomycin (until saturation-dose unknown, i/tum)	Modified caliper and paired needle electrodes; 8 pulses, 50 + 50μs. 1 ms interpulse interval, 1300 V/cm, 1 Hz	Chemopulse	2	CR (28–48 mo)	Spugnini et al. (2008b)

(continued)

Table 1 (continued)

	Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
	MCT	Nine dogs (12 tumor nodules)	Cisplatin (1 mg/cm^3, i/tum)	Plate electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz	Jouan GHT 1287	1–3	CR 75% (4–5 weeks after ECT), NC 17%, PD 8%; median observation time 26 mo (2–43 mo)	Kodre et al. (2009)
	MCT (incompletely excised)	37 dogs	Cisplatin (3–15 mg/ tumor, i/tum) + hyaluronidase (mean dose 175 IU/ tumor)	Caliper and needle electrodes; 8 (biphasic) pulses, 50 + 50μs. 1 ms interpulse interval, 1300 V/cm, 1 Hz (caliper), 8 (biphasic) pulses, 50 + 50μs. 1 ms interpulse interval, 800 V/cm, 1 Hz (needle),	Chemopulse	2 + debulking surgery	78% recurrence-free (observation period 6 y), 16% recurrence rate	Spugnini et al. (2011b)
	MCT	51 dogs	Bleomycin (15,000 IU/ m^2, i/v)	Hexagonal and linear needle electrodes; 8 (monophasic) pulses, 100μs, 1000–1200 V/cm, 1 Hz or 5 kHz	Cytopulse PA4000, CytopulseOncovet, Cliniporator	1; ECT as a single treatment ($n = 15$), intraoperative ECT ($n = 11$), ECT adjuvant to surgery ($n = 14$), surgery followed by ECT ($n = 11$)	Postoperative ECT group: CR 93%, intraoperative ECT: CR 91%, ECT only: CR 80%, adjuvant ECT: CR 64%	Lowe et al. (2017)

	MCT	18 dogs	Cisplatin (1 mg/cm^3, i/tum) or bleomycin (1 mg/cm^3, i/tu mor 0.3 mg/kg, i/v)	Plate and needle row electrodes; 8 pulses, 100μs, 1300 V/cm, 5 kHz	Cliniporator	1–3; ± IL-12 GET (hIL-12, 1–2 mg peri/tum)	ORR 83% (CR 72%, PR 11%), SD 6%	Čemažar et al. (2017)
Miscellaneous tumors	Anal melanoma	One dog	Cisplatin (8 mg, i/tum) + hyaluronidase (dose unknown, i/tum)	Caliper electrodes; 8 pulses, 100μs, 1300 V/cm, 5 kHz	Not specified	3	Tumor reduction, euthanasia three mo after ECT (metastatic spread)	Spugnini et al. (2007c)
	Colorectal cancer (lymphoma, $n = 1$, adenocarcinoma, $n = 1$)	Two dogs	Bleomycin (15,000 IU/m^2, i/v)	EndoVE device (Cork Cancer Research Centre, Ireland); parameters?	ePORE (Cork Cancer Research Centre, Ireland)	2	CR (2–2.5 y)	Forde et al. (2016)
	Nasal duct tumors: transmissible venereal tumor ($n = 1$), round cell sarcoma ($n = 1$), adenocarcinoma ($n = 4$), SCC ($n = 3$), solid carcinoma ($n = 1$), fibrosarcoma ($n = 1$)	11 dogs	Bleomycin (15 IU/m^2)	Single needle (SiNE) electrode; 32 pulses, 100μs, 300 V, 1 Hz	BTX ECM 830	1	ORR 91% (CR 27%, PR 64%), SD 9%, median overall survival 16.5 mo	Maglietti et al. (2017)

ECT electrochemotherapy, *GET* gene electrotransfer, *IL-12* interleukin-12, *fIL-12* feline IL-12, *CR* complete response, *PR* partial response, *NC* no change, *SD* stable disease, *PD* progressive disease, *DFI* disease-free interval, *MST* median survival time, *mo* months, *d* days, *y* years, *SCC* squamous cell carcinoma, *HPC* hemangiopericytoma, *MCT* mast cell tumor, *i/v* intravenous, *i/tum* intratumoral, *peri/tum* peritumoral

Later, a case report of treating OMM in a dog model was published Suzuki et al. (2018) as a part of a study investigating the optimization of electroporation parameters/electrodes use by numerical modeling and measuring oral mucosa conductivity during electroporation. The authors report a 15 mm OMM without any other lesions observed, however, no information on staging is described. After a single ECT session with intravenous application of bleomycin, a long-lasting CR (12 months) was achieved (Suzuki et al. 2018).

Recently, our research group (Milevoj et al. 2019) treated nine dogs with OMM (stage I–III) with a combination of cytoreductive surgery, immediately followed by ECT with intravenous bleomycin (0.3 mg/kg) and GET of canine IL-12. The treatment was performed one–five times, depending on the response to the treatment, in 2–4 weeks' intervals. At the end of the observation period, ranging from 2 to 22 months, 8/9 (89%) dogs experienced PD. The achieved MST was 6 months and did not correlate with the stage of the tumor. Interestingly, the three patients with stage III tumors all survived from 5 months to 12 months, and two of them were euthanized due to tumor-unrelated reasons. The treatment was well tolerated with a good cosmetic effect, and no major side effects were noted. We concluded that a combination approach with ECT and IL-12 GET may be beneficial for dogs with OMM, especially when other treatment approaches (surgery, radiotherapy) are not accepted due to their invasiveness or cost (Milevoj et al. 2019).

The most recent and the largest study on the use of ECT in canine OMM, published by Tellado et al. (2020). Sixty-seven dogs with primary OMM of different stages were included in the prospective study. The treatment consisted of intravenous application of bleomycin, followed by application of electrical pulses with needle electrodes. For nasal duct invasion, a specialized single needle electrode was used (Maglietti et al. 2017). The local response and time to progression correlated with the clinical stage of the disease and the presence or absence of bone invasion. The OR rate was 100%, 89.5%, 57.7%, and 36.4%, in stages I, II, III, and IV, respectively. The median time to progression was 11, 7, 4 and 4 months, and MST after the treatment was 16.5, 9.0, 7.5 and 4.5 months for patients in stages I, II, III, and IV, respectively. Significantly better was local response in stage I and II disease ($p = 0.0013$), without the bone involvement ($p = 0.043$). The authors also evaluated the quality of life of the treated dogs, finding that only patients in stages I–III or where CR was achieved experienced improve in their quality of life (Tellado et al. 2020).

2.2.2 Oral Squamous Cell Carcinoma

In the report Reed et al. (2010), two dogs with oral SCC were treated with a combination of ECT with intratumoral bleomycin and IL-12 GET. Both tumors were previously treated with surgery and achieved long-lasting responses (27 and 56 months) after two sessions of the combined treatment approach (10 days apart). In one of the dogs, the authors observed resolution of the bone lysis 1.5 months after the second treatment (Reed et al. 2010).

Cutrera et al. (2015) further evaluated the combination of ECT (gemcitabine or bleomycin) and IL-12 GET in dogs with oral SCC. The authors reported that the

combined treatment was effective in reducing the tumor volume 21 days after treatment, with one dog achieving CR, two PR, and one SD. They concluded that the combined approach could be used as a first-line treatment for oral SCC to reduce tumor volume in sensitive, vital areas or to rapidly debulk tumor volumes for increased efficacy of subsequent treatments, such as surgery or radiotherapy (Cutrera et al. 2015).

A retrospective study on the use of ECT for treating canine oral non-tonsillar SCC was recently published by Simčič et al. (2020). Twelve dogs were included, 11 of them were treated with ECT alone, and one was previously treated with surgery. Most of the dogs were treated with a single session of ECT with intravenous bleomycin, and 2/11 were treated twice. Again, the authors observed that the size of the tumor was a predictive factor for treatment; all six dogs with tumors smaller than 2 cm obtained CR and showed no recurrence (median follow-up 1041 days) of the disease. The response rate was 90.9% (10/11; 8 CR and 2 PR), and the OR rate 27.3%. MST for dogs that died for tumor-related reasons was 110 days, and for dogs dead without tumor 831 days. Among five surviving dogs, one experienced tumor recurrence and four were in CR. DFI and MST for dogs with recurrence were 50 and 115 days, respectively. The authors concluded that ECT could be considered as an alternative treatment to surgery and radiation therapy, mostly when owners have concerns about the financial burden and esthetics outcome that usually follows irradiation and surgery (Simčič et al. 2020).

2.2.3 Other Oral Tumors

Both Spugnini and Porrello (2003) and Reed et al. (2010) demonstrated treatment success in cases of two dogs with acanthomatous ameloblastoma (AA), treated with a combination of ECT with intratumoral bleomycin and IL-12 GET. They achieved CR that lasted until the end of the observation period (>150 days and 9 months) (Spugnini and Porrello 2003; Reed et al. 2010).

An early report of Cutrera et al. (2008) describes the treatment of a dog with a recurrent papillary tumor with an adjacent metastatic bone tumor (origin not specified) with a combination of ECT with intratumoral bleomycin and IL-12 GET. The treatment resulted in complete regression of the visible tumor 2 weeks after the treatment, and the bony lesion regressed 23 weeks after treatment (Cutrera et al. 2008).

In the aforementioned study, combining ECT with IL-12 GET, Cutrera et al. (2015) also treated three dogs with AA, a dog with oral plasma cell tumor (PC) and a dog with oral sarcoma. The sarcoma did not respond to the treatment, which is consistent with observations from other studies (Reed et al. 2010). However, the dogs with AA did not respond completely and were in PR after 4–5 treatment cycles. The dog with PC was treated with 5 cycles (three with gemcitabine and two with bleomycin) that resulted in 27% tumor volume reduction (PR) 21 days after the last treatment. Gemcitabine and bleomycin were equally effective in the treatment of different tumors (Cutrera et al. 2015).

The details of the treatments of canine oral tumors are summarized in Table 2.

Table 2 Use of ECT for oral tumors in dogs

Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
OMM	One dog	bleomycin (until saturation-dose unknown, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50μs. 1 ms interpulse interval, 800 V/cm, 1 Hz	Chemopulse	4	SD (40 d)	Spugnini and Porrello (2003)
OMM	Ten dogs	bleomycin (dose unknown, i/tum and peri/tum)	Modified caliper and needle electrodes; 8 (biphasic) pulses, 50 + 50μs, 800 V/cm, 1000 Hz	Chemopulse	4	CR 70%, PR 10%, SD 20% (1 week after last ECT), MST 6 mo	Spugnini et al. (2006a)
Recurrent papillary tumor with adjacent metastatic bone tumor	One dog	Bleomycin (0.5 U/cm^2, i/tum)	Caliper electrodes; 2 pulses, 25 ms, 450 V/cm	BTS EC830 (Inovio, San Diego, CA)	1 + IL-12 GET (IL-12, 150μg, i/tum)	CR (23 weeks after ECT)	Cutrera et al. (2008)
AA ($n = 1$), SCC ($n = 2$), OMM	Four dogs	Bleomycin (0.5–2 IU/treatment, i/tum)	Hexagonal and caliper electrodes; 2 pulses 20 ms, 400 V/cm, 10 Hz	ECM 830	1 + IL-12 GET (fIL-12, 150–400 μg, i/tum)	SCC, CAA: CR (observation period 9–56 mo) OMM, FSA:	Reed et al. (2010)

($n = 1$), FSA ($n = 1$)						initial PR, soon developed PD	
AA, SCC, PC, sarcoma	Nine dogs	Bleomycin (1 IU/cm^3, i/tum) or gemcitabine (0.5–10 mg/cm^3, i/tum)	Needle electrodes; 2 pulses, 20 ms, 350 V/cm, 10 Hz	ECM 830	Multiple treatments with different combinations + IL-12 GET (cIL-12, 2 mg/cm tumor diameter, i/tum)	27% volume reduction in, SCC and PC (3 weeks after ECT); PD in sarcoma	Cutrera et al. (2015)
OMM	One dog	Bleomycin (0.3 mg/kg, i/v + intratumorally) in the first treatment,	Two-needle array and Petri Pulser electrodes; 8 square-wave pulses, 100μs, 1300 V/cm, 1 Hz	ECM 830	2 + laser surgery + calcium electroporation (Ca ions in the second treatment; 5 mM, 10 ml, i/tum)	PR (1 mo after treatment)	Kulbacka et al. (2017)
OMM	One dog	Bleomycin (15.000 U/m^2, i/v)	type II needle electrodes; 8 pulses, 100μs, 1300 kV/m, 1 Hz	ECM 830	1	CR (observation time 12 mo)	Suzuki et al. (2018)
OMM	Nine dogs	Bleomycin (0.3 mg/kg i/v)	Plate electrodes (ECT) and MEA electrodes (GET); ECT: 8 pulses, 100μs, 1300 V/cm, 5 kHz, GET: 24 pulses, 150 ms, 60 V, 4 Hz	Cliniporator	1–5 + IL-12 GET (cIL-12, 2 mg peri/tum)	CR 11%, PD 89%, observation period 2–22 months, MST 6 months	Milevoj et al. (2019)
SCC	12 dogs	Bleomycin (15.000 U/m^2, i/v)	Type II needle electrodes; 8 (monophasic) pulses, 100μs, 1000 V/cm and	Cytopulse PA4000 or CytopulseOncovet	1–2	ORR 27.3%, calculated response rate 90.9%; MST for dogs that died for	Simčič et al. (2020)

(continued)

Table 2 (continued)

Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
			1 Hz (PA4000) or 1200 V/cm and 5 kHz (Oncovet)			tumor-related reasons 110 d, for dogs dead without tumor 831 d	
OMM	67 dogs	Bleomycin (15.000 U/m^2, i/v)	6-needle electrodes and Single needle Electrode (SiNE, for nasal duct invasion); 8 (6-needle electrodes) or 32 (single needle electrodes) pulses, 100μs, 1000 V/cm, 10 Hz	ECM 830	1–4	Stage I: CR 72.7%, PR 27.3%; MST 16.5 mo; Stage II: CR 21.1%, PR 68.4%, PD 10.5%; MST 9 mo; Stage III: CR 7.7%, PR 50%, SD 26.9%, PD 15.4%; MST 7.5 mo Stage IV: PR 36.4%, SD 36.4%, PD 27.3%; MST 4.5 mo	Tellado et al. (2020)

ECT electrochemotherapy, *GET* gene electrotransfer, *IL-12* interleukin-12, *fIL-12* feline IL-12, *cIL-12* canine IL-12, *ORR* overall response rate, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *DFI* disease-free interval, *MST* median survival time, *mo* months, *d* days, *OMM* oral malignant melanoma, *SCC* squamous cell carcinoma, *AA* acanthomatous ameloblastoma, *PC* plasmacytoma, *FSA* fibrosarcoma, *i/v* intravenous, *i/tum* intratumoral, *peri/tum* peritumoral

2.3 Electrochemotherapy for the Treatment of Miscellaneous Tumors in Dogs

Because of the promising effects of using ECT for cutaneous, subcutaneous, and some oral tumors, an interest in treating deep-seated and intraluminal tumors started to arise. For such reasons, specialized electrodes are currently being produced for accessing tumors in different areas of the body. The progress in advanced diagnostic imaging techniques in veterinary medicine enabled precise treatment planning for tumors in areas, difficult to access. In this chapter, a brief review of currently available literature on the clinical use of ECT in deep-seated and intraluminal tumors in veterinary medicine is offered. Details on the specialized electrodes and treatment planning are provided elsewhere in the book.

An early report of a large anal malignant melanoma, treated with palliative-intent ECT with cisplatin and hyaluronidase, was published by Spugnini et al. (2007c). After three ECT sessions a week apart using caliper electrodes, the dog experienced tumor reduction with the restoration of normal defecation. Unfortunately, even though local control of the disease was achieved, euthanasia was elected 3 months after ECT due to metastatic spread (Spugnini et al. 2007d).

As a part of preclinical evaluation of an endoscopic electroporation system, Forde et al. (2016) evaluated the efficacy and safety of EndoVE device (700209, Mirai Medical, Galway, Ireland), developed for the endoscopic electroporation treatment of gastrointestinal cancers. Two dogs with non-operable colorectal cancers (adenocarcinoma and lymphoma) were treated with two sessions of ECT with intravenous bleomycin, and excellent results were achieved, with both dogs being tumor free for 2–2.5 years after treatment (Forde et al. 2016).

A year later, Maglietti et al. (2017) described the treatment of canine nasal duct tumors with ECT with intravenous bleomycin (15 U/m^2), using a specialized single needle electrode (SiNE). A group of 11 dogs with different malignancies in the nasal duct, treated with ECT, was compared to a control group of 10 dogs, treated with surgery and adjuvant chemotherapy with carboplatin. The results of the study were encouraging, with a 91% OR rate (27% CR, 64% PR) and 9% SD in the SiNE group. The mean overall survival was 16.86 months (4–32 months, median 16.5 months), with a survival rate significantly higher ($p = 0.0008$) when compared with the control group. The only side effect observed was the inflammation of the treated nasal passage, which was managed with a short course of corticosteroid therapy. 1 year after the treatment, 60% of the dogs in the SiNE group vs. 10% of the control group remained alive, and after the 32 months follow-up, the survival rate was 30% and 0%, respectively (Maglietti et al. 2017).

The details of the treatments of canine miscellaneous tumors are summarized in Table 1.

2.4 Electrochemotherapy for the Treatment of Cutaneous and Subcutaneous Tumors in Cats

2.4.1 Epithelial Tumors

Squamous Cell Carcinomas

SCC of the skin is a common malignancy in cats. When standard treatments, such as surgery, radiotherapy, or photodynamic therapy, are not acceptable for the owners due to their invasiveness (including postoperative cosmetic appearance and function) or cost, ECT can be considered as an alternative treatment (Tozon et al. 2014).

The first successful treatment of feline SCC is reported by Spugnini and Porrello (2003), who performed ECT with bleomycin in dogs and cats with large neoplasms. In the study, three cats with four SCC nodules (two ear, two nasal planum) were included. Three nodules responded to the treatment completely (90–465+ days), whereas the cat with SCC on the ear experienced PD. The same cat also had SCC of the nasal planum, which was in CR (Spugnini and Porrello 2003).

The same group later treated nine cats with SCC of the nasal planum (seven cats), pinna (one cat) and eye canthus (one cat) with two sessions of ECT with intratumoral bleomycin, combined with hyaluronidase, 1 week apart. 7/9 cats (77.7%) had a complete response lasting up to 3 years, and minimal side effects (mild erythema) were observed (Spugnini et al. 2009).

The efficacy of ECT for feline SCC was further confirmed by Tozon et al. (2014), who evaluated the efficacy and safety of the treatment in 11 cats with 17 nodules on the nasal planum (6/11 cats), pinnae (3/11 cats) and both locations (2/11 cats). An 87.5% CR rate was achieved, lasting from 2 months to more than 3 years. Two/nine cats in which CR was initially observed had a recurrence 2 and 8 months after ECT. In the remaining two cats with highly infiltrative spread into adjacent tissues, progression of the disease was observed despite ECT, and both were euthanized 4 and 5 months after the procedure. ECT was well tolerated, and no evident local or systemic side effects were observed (Tozon et al. 2014).

Spugnini et al. (2015) later compared the efficacy of intravenous bleomycin with or without electroporation in 21 cats with periocular carcinoma (17 SCC and 4 anaplastic sarcoma) and 26 cats with advanced SCC of the head. ECT treatments (2–8) were performed every other week until CR or PD occurred. In the ECT treated group, the OR rate was significantly better (89%; 21 CR and 2 PR) than in the group treated with bleomycin only (33%; 4 CR, 3 PR). Median time to progression was 30.5 months in the ECT group and 3.9 months in the bleomycin only group. The results confirmed the efficacy of ECT for the treatment of feline cutaneous carcinomas and the potentiation of bleomycin cytotoxicity by means of electroporation (Spugnini et al. 2015).

Mammary Adenocarcinoma

As in dogs, ECT with intratumoral cisplatin was attempted in two cats with recurrent mammary adenocarcinoma (Tozon et al. 2001). The treatment effect varied with one cat being euthanized 4 months after ECT due to metastatic spread to the lung and the

other cat experiencing CR that lasted until the end of the observation period (6 months).

Other Epithelial Tumors

A case of a cat with trichoepithelioma of the forehead, treated with ECT with intratumoral bleomycin is described in a case series by Spugnini and Porrello (2003). After a single session of ECT, PR (120 days) was achieved. In the same study, a cat with multiple skin metastases of adenocarcinoma (probably originating from perianal glands), previously treated with systemic chemotherapy, is included. After three sessions of ECT, the cat experienced PR, however, the authors observed an occurrence of a deep, spontaneously healing, Raynaud-like vasculitis following infiltration of the tumor bed with bleomycin (Spugnini and Porrello 2003).

2.4.2 Mesenchymal Tumors

Soft Tissue Sarcomas

As mentioned above, the first clinical study describing the use of ECT in cats was performed by Mir et al. (1997). Twelve cats with a relapse of soft tissue sarcoma after treatment with conventional therapies were treated with ECT with intravenous bleomycin (0.5 mg/kg), followed by electric pulse delivery from 4 to 15–30 min after chemotherapeutic administration. A combination of external surface and needle electrodes was used. The mean survival time (6.1 months, maximum 18 months) was significantly increased compared to the control group of untreated cats (0.8 months), and CR was observed in 11/12 cats. Tolerance to treatment was excellent without general side effects (Mir et al. 1997).

Later, Spugnini and Porrello (2003) describe the use of ECT in large tumors in dogs and cats, including a cat with FSA of the head that experienced a short-lived PR (14 days). In the article, the authors also report of a cat with a large FSA (not included in the study) that developed fatal pulmonary thromboembolism after ECT. An unusually elevated amount of necrotic tissue evidenced by histopathology suggested that thromboembolic events were triggered by the release of prothrombotic factors from the tumor undergoing lysis. Low dose heparin and diuretics were attempted in the following patients but were inadequate to prevent the occurrence of tumor-associated thrombosis. Those results show that larger tumors should be approached with caution (Spugnini and Porrello 2003).

Soon after that, Spugnini et al. (2007a) further evaluated the efficacy of ECT in 58 cats with high-grade soft tissue sarcomas (STS). The cats were divided into two groups with either microscopic (39 patients) or macroscopic (19 patients) disease. The latter group received surgical treatment together with intraoperative ECT, followed by the second ECT treatment after 1 week. The group in the microscopic arm received two postoperative ECT treatments 1 week apart. The authors observed a longer disease-free interval (DFI) in the postoperative group (19 months) compared to intraoperative group (12 months) or cats, treated with surgery alone (4 months). Moreover, ten patients with recurrence of the disease were retreated and experienced responses lasting from 6 to more than 28 months (Spugnini et al.

2007a). Later, the same group evaluated the use of ECT with intratumoral cisplatin for the treatment of 64 cats with STS. Here, ECT was used as an adjuvant treatment to surgery, and the treatment outcome compared to a control group of 43 cats, treated with surgery only. Oneweek after cytoreductive surgery, the cats received the first ECT treatment, followed by the second one a week apart. The protocol used was the same as described in the last study, used for postoperative ECT. Increased local control was observed in the group, treated with ECT, compared to the group where surgical treatment was performed. The mean DFI was 666 days and 180 days, respectively. Even though cisplatin is considered highly toxic in cats, the authors observed only minimal side effects that were mostly treated symptomatically (Spugnini et al. 2011a). However, in a case of a cat with a recurrent fibrosarcoma (FSA) that was previously treated with radiotherapy, severe erythema was observed at the site of previous irradiation, followed by moist desquamation and ulcer that required debridement and prolonged therapy with steroids and antihistaminic drugs. The symptoms and the response to symptomatic therapy were strongly suggestive of radiation recall, therefore the authors advised that ECT should be used with caution in previously irradiated areas (Spugnini et al. 2008c).

Apart from the case series, several case reports were published, describing the successful use of ECT for various mesenchymal neoplasms (e.g., rhabdomyosarcoma, ganglioneuroblastoma, hemangiopericytoma) in cats (Baldi and Spugnini 2006; Spugnini et al. 2008a, 2010).

2.4.3 Round Cell Tumors

There is not much information on treating round cell tumors in cats with ECT. Similar to dogs, good effect of ECT has been shown in cats with localized lymphoma; all four cats included in the study responded to the treatment completely. However, eventually, all cats died because of the disease; two cats with nasal lymphoma experienced local tumor recurrence 200 and 730 days after treatment, and the other two (one with retrobulbar and the other with cervical lymphoma) died of systemic spread after remissions of 180 and 635 days. Nonetheless, the responses noted were long lasting, and the treatment was safe and well tolerated (Spugnini et al. 2007b).

2.5 Electrochemotherapy for the Treatment of Oral Tumors in Cats

In contrast to their canine counterparts, the information on the treatment of oral tumors in cats is limited, with some anecdotical reports by the authors (Fig. 1). An early report from Spugnini and Porrello (2003) includes treatment of an oral SCC and maxillary anaplastic sarcoma with ECT using intratumoral injection of bleomycin. PR (120 days) was achieved in the first case. In the second, CR was initially observed, however, the tumor relapsed 90 days later; after retreatment, PR (55 days) was achieved. (Spugnini and Porrello 2003).

2.6 Electrochemotherapy for the Treatment of Miscellaneous Tumors in Cats

In cats, the information on the use of ECT in other types of tumors is scarce. An early report describes a long-lasting (>240 days) PR after a single session of ECT in a cat with cutaneous melanoma (Spugnini and Porrello 2003).

A case of ultrasound-guided ECT with intravenous bleomycin for the treatment of a clear cell thymoma was reported (Spugnini et al. 2017). The cat received two sessions of ECT 2 weeks apart using specialized paired needle electrodes. The treatment was performed on a monthly basis afterwards, and PR was achieved, lasting until the end of the observation period (14 months). The treatment was well tolerated and resulted, and a good quality of life was reported by the authors (Fig. 1).

The details of the treatments of feline tumors are summarized in Table 3.

2.7 Procedure

The increasing number of reports using different treatment protocols gave rise to a need for standardization of ECT for treating tumors in cats and dogs. Therefore, in 2016, Operating procedures of ECT for treating canine and feline tumors were published (Tozon et al. 2016b). The following paragraphs are adapted from the recommendations described in the paper.

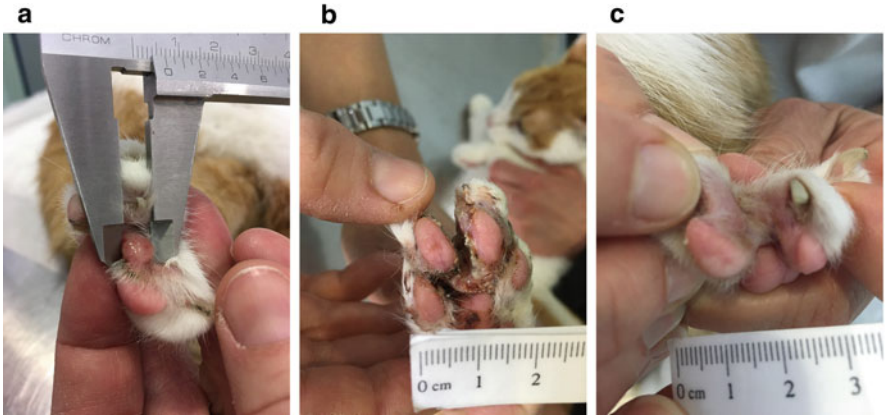

Fig. 1 MCT in a cat treated with ECT: before treatment (**a**), 1 week (**b**) and 4 weeks after treatment (**c**) (Tozon, unpublished clinical case)

Table 3 Use of ECT for tumors in cats

	Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
Epithelial tumors	Mammary adenocarcinoma	Two cats (ten nodules)	Cisplatin (0.5–2.5 mg/100 mm^3 nodule, i/tum)	Plate electrodes; 8 pulses, 100 µs, 1300 V/cm, 1 Hz	Jouan GHT 1287	1–3	CR (6 mo, $n = 1$), euthanasia due to metastasis (4 mo, $n = 1$)	Tozon et al. (2001)
	SCC of the nasal planum ($n = 2$) and ear pinnae ($n = 2$), trichoepithelioma ($n = 1$), skin metastases of adenocarcinoma ($n = 1$)	Five cats (six tumor nodules)	Bleomycin (until saturation-dose unknown, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50 µs. 1 ms interpulse interval, 800 V/cm, 1 Hz	Chemopulse	1–2	SCC: 3 CR (40–465+ d), 1 PD; trichoepithelioma: PR (120 d); adenocarcinoma metastases: PR	Spugnini and Porrello (2003)
	SCC of the nasal planum ($n = 7$), eye canthus ($n = 1$) and ear pinnae ($n = 1$)	Nine cats	Bleomycin (1–1.5 mg/cm^3, i/tum)	Caliper and paired needle electrodes; 8 pulses, 50 + 50 µs. 1 ms interpulse interval, 800 V/cm, 1 Hz	Chemopulse	2	CR 77.7% (90–1613 d), PR 22.2% (70–90 d)	Spugnini et al. (2009)

	SCC of the nasal planum ($n = 6$), ear pinnae ($n = 3$), both locations ($n = 2$)	11 cats (16 tumor nodules)	Bleomycin (30 mg/m^2 BSA, i/v)	Plate electrodes; 8 pulses, 100 μs, 1300 V/cm, 5 kHz	Cliniporator	1–2	CR 81.8% (2 mo-3 y) cats, CR 87.5% tumor nodules	Tozon et al. (2014)
	Periocular carcinoma ($n = 12$), advanced SCC of the head ($n = 14$)	26 cats	Bleomycin (15 mg/m^2, i/v)	Modified caliper electrodes; 8 pulses, 50 + 50 μs. 1 ms interpulse interval, 1 Hz	Chemipulse II	2–9	ORR 89% (CR 81%, PR 8%), median time to progression 30.5 mo	Spugnini et al. (2015)
Mesenchymal tumors	Soft tissue sarcoma	12 cats	Bleomycin (0.5 mg/kg, i/v)	External surface and needle electrodes; series of pulses, 100 μs, 1300 V/cm, 1 Hz	PS15 electropulsator (Nantes, France)	1–5 + IL-2 immunotherapy (30 x 10^6 xenogeneic CHO (IL-2) living cells, peri/tu mor i/tum)	CR 92%, mean survival 6.1 mo (max. 18 mo)	Mir et al. (1997)
	FSA	One cat	Bleomycin (until saturation-dose unknown, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50 μs. 1 ms interpulse interval, 800 V/cm, 1 Hz	Chemopulse	1	PR (14 d)	Spugnini and Porrello (2003)
	HPC	One cat	Bleomycin (4–5 IU, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50 μs. 1 ms interpulse	Chemopulse	2	CR (1 y)	Baldi and Spugnini (2006)

(continued)

Table 3 (continued)

	Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
				interval, 800 V/cm, 1 Hz				
	High-grade soft tissue sarcoma (microscopic disease, $n = 39$, macroscopic disease, $n = 19$)	58 cats	Bleomycin (dose unknown, i/tum) + hyaluronidase (avg. dose 300 IU, i/tum)	Caliper, needle and 6-needle array flexible (intraoperative) electrodes; 8 (biphasic) pulses, 50 + 50 µs. 1 ms interpulse interval, 800 V/cm (intraoperative ECT) or 1300 V/cm, 1 Hz	Chemopulse	2 + surgery in the macroscopic group with intraoperative ECT (first session)	Recurrence rate 46–63%, DFI 19 mo (postoperative ECT) vs. 12 mo (intraoperative ECT)	Spugnini et al. (2007a)
	Ganglioneuroblastoma	One cat	Bleomycin (1.5 IU, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50 µs. 1 ms interpulse	Not specified	4	CR (450 d)	Spugnini et al. (2008a)

				interval, 800 V/cm, 1 Hz				
	Pleomorphic rhabdomyosarcoma	One cat	Cisplatin (0.75 mg, i/tum) + hyaluronidase (175 IU, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50 µs. 1 ms interpulse interval, 800 V/cm, 1 Hz	Chemopulse	2	CR (1 y)	Spugnini et al. (2010)
	Soft tissue sarcoma (incompletely excised)	64 cats	Cisplatin (dose unknown, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50 µs. 1 ms interpulse interval, 800 V/cm, 1 Hz	Chemipulse III	2	DFI 666 d, recurrence rate 29.7%	Spugnini et al. (2011a)
Miscellaneous tumors	Cutaneous melanoma	One cat	Bleomycin (until saturation-dose unknown, i/tum)	Caliper electrodes; 8 (biphasic) pulses, 50 + 50 µs. 1 ms interpulse interval, 800 V/cm, 1 Hz	Chemopulse	1	PR (>240 d)	Spugnini and Porrello (2003)
	Localized lymphoma: nasal ($n = 2$), retrobulbar ($n = 1$), cervical ($n = 1$)	Four cats	Bleomycin (until saturation-dose unknown, i/tum)	Modified caliper and paired needle electrodes; 8 pulses, 50 + 50 µs. 1 ms interpulse	Chemopulse	2	Initial CR rate 100%; local recurrence rate 50% (200 and 730 d), systemic spread 50% (180 and 635 days)	Spugnini et al. (2007b)

(continued)

Table 3 (continued)

	Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
				interval, 1300 V/cm, 1 Hz				
	Clear cell thymoma	One cat	Bleomycin (20 mg/m^2, i/v)	Paired needle electrodes; 8 pulses, 50 + 50 µs. 1 ms interpulse interval, 800 V/cm, 1 Hz	Onkodisruptor	2	PR (14 mo)	Spugnini et al. (2017)

ECT electrochemotherapy, *ORR* overall response rate, *CR* complete response, *PR* partial response, *DFI* disease-free interval, *mo* months, *d* days, *y* years, *SCC* squamous cell carcinoma, *HPC* hemangiopericytoma, *FSA* fibrosarcoma, *i/v* intravenous, *i/tum* intratumoral, *peri/tum* peritumoral

2.7.1 Patient Selection

History

A thorough history of the patient, including general data (age, breed sex, concurrent diseases, tumor type, history of previous treatment of the tumor, recurrence and speed of recurrence, owner's expectations and understanding of the prognosis with the treatment).

Physical Examination and Diagnostic Work-Up

A complete physical examination should be performed prior to the treatment, with a special emphasis on the evaluation of the tumor to be treated. Fine-needle aspiration biopsies for cytological evaluation and, when possible, biopsies of the tumor for histopathological examination should be performed. The latter is especially important in cases of tumors where the histological grade is a prognostic factor (e.g., MCT). In specific cases where selected biomarkers (e.g., Ki-67, c-Kit) are of prognostic importance, immunohistochemistry is recommended (Blackwood et al. 2012). An incisional biopsy, also known as "punch" biopsy, can be performed in the same general anesthesia as ECT before treatment.

Depending on the tumor type, the size of the treated margins should be determined, according to recommendations for any other types of treatment for the tumor type (e.g., surgical margins). Evaluate the number and size of the tumors to prepare a treatment approach. When possible, advanced imaging techniques (e.g., CT or MRI) should be used to aid treatment planning.

Complete blood count (CBC) with a serum biochemistry panel (blood urea nitrogen (BUN), creatinine, alanine-aminotransferase (ALT), alkaline phosphatase (ALP) and calcium) should be performed to exclude concurrent renal and hepatic diseases. Additional specific blood tests should be performed according to the medical history of the patient and tumor type to exclude concurrent diseases and possible paraneoplastic syndromes, which might have a prognostic impact. If the patient is receiving any medicine that could affect the coagulation times, perform a coagulation profile test. When using invasive (needle) types of electrodes, emphasis should be given to the platelet number. Proceed only when platelet numbers are above 100×10^9/L and the coagulation profile (prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, D-dimers) is within normal limits.

General examination should be performed to rule out any concurrent diseases (chronic infections, immune-mediated diseases) that could preclude the treatment. Evaluate whether the patient is suitable to withstand deep sedations and/or general anesthesia. Prior treatment with any of the chemotherapeutics used should be noted. In patients with sensitivity to cisplatin or those that already received a cumulative dose of bleomycin (>400.000 IU/m^2), those drugs should be avoided.

Staging of the disease should be performed according to the tumor type and its potential routes of metastases. Standard (abdominal ultrasound, X-rays of the thorax) or advanced (CT or MRI scans) should be performed for treatment planning and, if necessary, adjuvant treatment after addressing the local disease.

After deciding that the patient is suitable for treatment with ECT, a thorough discussion with the owner of the animal is of crucial importance. Explain the procedure and the expected post-treatment course of recovery, with a special emphasis on wound care. Define the expected number of ECT sessions, total duration of the treatment, costs of treatment, possibility of recurrences. If possible, present graphic material (e.g., pictures, videos) of the treatment in various stages (e.g., before, 1 week, 1 month after ECT). Clarify whether you expect the nature of the treatment will be curative or palliative. Obtain the owner's written consent, if the patient will be enrolled in a clinical trial.

A recent study by our research group showed that the owners of dogs, treated with ECT alone or combined with IL-12 GET and/or surgery, assessed their animals' health-related quality of life (HRQoL) after treatment as good and the general health compared with the initial diagnosis of cancer as improving. However, when comparing the different treatment groups, we found that the owners of animals treated with either ECT and/or IL-12 GET, combined with surgery, assessed their dogs' quality of life (QoL) as worse compared to those that received the treatment without surgery. Also, the owners of dogs that achieved an OR to the treatment assessed the QoL as significantly better compared with those whose dogs did not respond to the treatment. The owners of animals with oral tumors and those larger than 3 cm^3 evaluated the QoL of their dogs as worse compared to those with cutaneous and subcutaneous tumors. The results of the study could aid the veterinarians to clarify the expected quality of life after treatment and to improve the owner-veterinarian interaction (Milevoj et al. 2020).

2.7.2 Treatment Procedure

Define the route and the chemotherapeutic to use. In dogs, use cisplatin (intratumorally) or bleomycin (intratumorally or intravenously). In cats, only bleomycin (intratumorally or intravenously) should be used due to cisplatin toxicity, although some authors also mention the possibility of intratumoral use of cisplatin in cats without detectable toxic effects (Tozon et al. 2001; Spugnini et al. 2008a, 2010, 2011a, Impellizeri, unpublished data).

Before treatment, calculate the tumor volume using the formula $V = a \times b \times c \times \pi/6$, where "a," "b," and "c" are the three perpendicular diameters of the tumor nodule. The dimensions should be measured by using a Vernier caliper.

Patient Preparation

For the procedure, deep sedation (in cases of small tumors) or general anesthesia can be used, the latter being more common. The patients should be evaluated according to The American Society of Anesthesiologists' (ASA) physical status classification. The standard protocols for deep sedation and general anesthesia vary in different treatment facilities and will not be discussed in detail. However, a single dose of a non-steroid analgesic (e.g., carprofen 2–4 mg/kg) is desired during the procedure. The tumor nodules and surrounding safety margins should be clipped and cleaned before treatment.

Working Environment and Safety

Only a trained operator (DVM) should perform ECT. The room for the procedure should be suitable for working with animals under deep sedation or general anesthesia with all the necessary equipment.

Biosafety measures should be followed, complying with national regulations regarding chemotherapeutic drug usage and disposal.

Although cisplatin and bleomycin are not absorbed through intact skin and rarely cause acute problems following droplet exposure, the operator and assistants should be equipped with masks and gloves. The contact of cisplatin with skin can cause local irritation only. In case of skin exposure to either of the used chemotherapeutic drugs, rinse immediately with large quantities of water. When injecting firm tumors with the drug, leakage can occur to the surrounding area. In case of needle stick injury, wash with the abundance of water and perform wound care according to standard medical practice.

Any type of generators producing square-wave pulses that are meeting the EU or FDA standards should be used. The generator generally consists of integrated software, a touch screen interface and a hand-held electrode holder with a pulse-activating button attached to the generator by a cord (usually controlled by a foot switch).

Electrode Selection

The configuration of electrodes affects the electrical field distribution in the treated tissue. Two standard types of electrodes are used: plate (noninvasive) and needle (invasive) electrodes. The plate electrodes consist of two parallel stainless steel plates with variable distances (usually 6–8 mm) between them. The distance depends on the tumor size and the capacity of the pulse generator. Needle electrodes can consist of two rows of needles (parallel array), or they are arranged in hexagonal geometry. The latter is usually used in cases of larger, infiltrative growing tumors (Mir et al. 2006). They have a central needle that is surrounded by six needles, arranged in a circle. The pulses are applied between the electrodes in the circular array and between them and the central needle, in both directions between each electrode pair. The electrode type should be selected individually for each tumor: the depth of the tumor should be considered in order to provide complete coverage of the treated area. The electrodes should also be chosen according to the physical properties of the target tissue. In general, both types of electrodes can be used for treatment, and in cases of heterogeneous tumors, they can be used interchangeably.

Drug Administration

Cisplatin, if in powder form (cis-diamminedichloroplatinum II), should be dissolved in a sterile physiological saline solution to 1 mg/ml (other desired concentrations can be used) and given intratumorally.

Bleomycin is normally dissolved with sterile physiological saline solution to a concentration of 3 mg/ml and can be given intratumorally or intravenously. When the intravenous application is performed, it should be injected in a slow bolus of approximately 30 s.

Table 4 Drug dosing for ECT in dogs and cats

	Tumor size	Intratumoral dose (per cm^3 tumor)	Intravenous dose (per kg BW)
Cisplatin (1 mg/ml)	$<1\ cm^3$	1 mg (1 ml)	
	$>1\ cm^3$	0.5–1 mg (0.5–1 ml)	
Bleomycin (3000 IU/ml)	$<1\ cm^3$	1500 IU (0.5 ml)	300 IU (0.3 ml)
	$>1\ cm^3$	1500–3000 IU (0.5–1 ml)	300 IU (0.3 ml)

Intratumoral applications should be performed slowly to minimize the risk of drug leakage.

The doses of the drugs are presented in Table 4. In a recent study of bleomycin pharmacokinetics in older human patients, a lower dose of intravenous bleomycin has been proposed (10.000 IU/m^2 instead of a standard dose of 15.000 IU/m^2) (Grošelj et al. 2016). The latest research shows that the efficacy of ECT with a reduced dose of bleomycin is comparable to that using a standard dose (Grošelj et al. 2018; Jamšek et al. 2020). However, in veterinary medicine, there are no clinical or pharmacokinetical studies supporting the use of a lower dose of bleomycin so far.

Electroporation

In cases of intratumoral administration of chemotherapeutic, electrical pulse delivery should be started within 1 min after injection. When bleomycin is administered intravenously, the pulse delivery should begin 8 min after injection and should be finished in the next 20 min. This timeframe is extrapolated from a study on human patients (Domenge et al. 1996).

For plate electrodes, the following parameters should be used: 8 trains of unipolar electric pulses with 1 s interval in two perpendicular directions, pulse duration 100μs, amplitude to electrode distance ratio 1300 V/cm, repetition frequency 1 Hz or 5 kHz. For needle row electrodes, the amplitude to electrode distance ratio should be set to 1000 V/cm. If hexagonal needle electrodes are used, there is a fixed voltage of 730 V; the repetition frequency should be set to 5 kHz, pulse duration to 100μs, and the number of pulses depends on the number of needles (e.g., in cases of 7 needles, 24 pulses are applied).

Especially in cases treated with plate electrodes, a water-based gel should be used to achieve optimal contact between the skin and the electrodes and to achieve less heterogeneity in the electric field distribution. In small tumors, the electrodes should be placed in a way that the tumor is placed between them. In cases of plate or needle row electrodes, the electrical pulses should be applied twice in a perpendicular direction to achieve a better electric field distribution. In larger tumors, the application of electrical pulses should start from the tumor margins (depending on the tumor type), move centrally and gradually progress to the central part of the tumor. When using needle electrodes, the application of gel is not necessary. All the needles should be inserted inside the tumor to allow appropriate electric field distribution.

After ECT, all the contaminated material should be disposed of according to national regulations regarding chemotherapeutic use.

2.7.3 Follow-Up Examinations and Retreatments

Follow-up visits are normally scheduled 1, 2, and 4 weeks after treatment to ensure the wound is healing optimally. At each visit, the tumor should be measured with a Vernier caliper. Possible side effects, either reported by the owner or observed upon examination, should be determined according to the Veterinary Cooperative Oncology Group toxicity scale (2016).

The response to the treatment should be evaluated minimally 4 weeks after ECT. The response is determined according to the "Response evaluation criteria in solid tumors" (RECIST) (Nguyen et al. 2015):

- Complete response (CR): the disappearance of all treated lesions.
- Partial response (PR): >30% reduction in the sum of diameters of treated lesions, taking as reference the baseline sum.
- Stable disease (SD): <30% reduction (PR) or <20% increase (PD) in the sum of diameters of treated lesions, taking as reference the smallest sum of diameters while on study.
- Progressive disease (PD): either the appearance of one or more new lesions or ≥20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study.

The treatment should be repeated until CR is achieved, if retreatment is ethically acceptable and with the agreement of the owner. In cases of aggressive oral tumors (e.g., OMM), the interval between treatments can be shortened to 2 weeks to prevent fast regrowth of the tumor mass.

2.8 Electrochemotherapy for the Treatment of Sarcoids in Horses

Sarcoids are one of the most common cutaneous quine neoplasms. Several treatment approaches have been described, including surgery, laser surgery, cryosurgery, photodynamic therapy, immunotherapy with *Bacillus Calmette Guerin* (BCG), brachytherapy, intratumoral chemotherapy with cisplatin oil emulsion or cisplatin beads, as well as numerous topical medications (Lane 1977; Knottenbelt et al. 1995; Théon et al. 1999, 2007; Knottenbelt and Kelly 2000; Martens et al. 2001; Hewes and Sullins 2006; Nogueira et al. 2006; Stadler et al. 2011; Compston et al. 2013; Dobson et al. 2018). Although all the treatment types reported various degrees of success, none of them is universally effective.

The first reports on the treatment of equine sarcoids with ECT date to early 2000s (Tamzali et al. 2001, 2003, 2007; Rols et al. 2002). About a decade later, a study on a large cohort of patients was performed with excellent results. Forty-eight animals (34 horses, 2 ponies, 11 donkeys, and 1 mule) with 194 tumor nodules were treated with ECT using the intratumoral injection of aqueous cisplatin with or without prior excision or debulking. When ECT was used as a single treatment ($n = 110$), the number of ECT treatments required varied from 1–7 sessions (mean 2.6 ± 1.1), depending on tumor size. In cases combined with surgery ($n = 84$), the number of

ECT sessions was not correlated to the size of the tumor (mean number of treatments 2.9 ± 1.4). The authors reported a strong influence of the excision quality on the number of ECT treatments, as a higher number of sessions was required in cases of incomplete tumor resection (with gross residual tumor) than in those with complete resection (mean number of treatments 3.82 ± 1.58 vs. 2.18 ± 0.92). The type of healing also correlated significantly with the number of ECT sessions (number of required sessions was increased in cases of secondary wound closure). The long-term follow-up revealed that 47/48 (97.9%) animals were recurrence-free 4 years after treatment, which is more than reported with treatments using cisplatin oil emulsion (96%) and cisplatin beads (83%) (Hewes and Sullins 2006; Théon et al. 2007). All types of sarcoids responded to the treatment with a 4-year recurrence-free interval for tumors of 99.5%. The treatment was well tolerated by all the animals; the most common adverse effect was a slight edematous reaction for lesions located on thin skin regions (Tamzali et al. 2012).

Later, Tozon et al. (2016a) confirmed the findings by treating 32 animals (31 horses and 1 donkey) with 70 tumor nodules. Also here, ECT was used as a single treatment (18 animals with 52 tumor nodules) or as an adjuvant treatment to marginal surgical excision (14 animals with 18 tumor nodules). In contrast to Tamzali et al. (2012), who performed ECT in 2 weeks' interval, here the authors used a 4-week interval between treatments. Most cases (28/31) were treated with 1–3 sessions, two were treated four times, and one animal was treated with 5 ECT treatments. At the end of observation period (1–7+ years, mean 4 years), CR was obtained in 48/52 (92.3%) nodules and PR in the rest of the nodules (4/52, 7.7%) in the group, treated with ECT only. All the nodules treated with a combination of ECT and surgery experienced CR. In both groups, one recurrence was reported (after 60 months in the ECT only group and 14 months in the group, combined with surgery). In this study, the authors also observed the correlation between the size of the tumor and the number of required treatments. Additionally, tumor type and infiltrative growth were recognized as potential predictive factors for the number of treatment sessions needed and the treatment outcome. The study also confirmed the previous observations regarding minimal side effects observed after the treatment. Typically, mild to moderate edema with mild erythema and ulceration was noted. Local inflammatory reaction was more pronounced in tumor nodules located on the head and limbs compared to the nodules located on the rest of the body and in tumor nodules with infiltrative growth (Tozon et al. 2016a).

The details of the treatments of equine sarcoids are summarized in Table 5.

2.8.1 Procedure

The recommendations, provided below, are adapted from Tozon et al. (2016c).

Patient Selection

A complete physical examination should be performed prior to the treatment, with a special emphasis on the evaluation of the tumor to be treated. Patients with localized and well-circumscribed lesions that are able to withstand repeated anesthesia are suitable for the treatment. After deciding that the patient is suitable for treatment with

Table 5 Use of ECT for tumors in horses

Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
Sarcoid	Three horse (multiple tumor nodules)	Cisplatin (dose unknown, i/tum)	Wire contact electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz	Jouan PS15	1–3	CR 100% (18 mo)	Rols et al. (2002)
Sarcoid	25 horses (46 tumor nodules)	Cisplatin (dose unknown, i/tum)	Wire contact electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz	Jouan PS15	1–4	CR 100% (1 y)	Tamzali et al. (2003)
Sarcoid ($n = 233$), neurofibrosarcoma ($n = 10$), SCC ($n = 1$), anaplastic sarcoma ($n = 4$)	57 animals: horses ($n = 39$), ponies ($n = 3$), donkeys ($n = 14$), mule ($n = 1$); 248 tumor nodules	Cisplatin (dose unknown, i/tum)	Wire contact electrodes; 8 pulses, 100μs, 1300 V/cm, 1 Hz	Jouan PS15	1–13 ± debulking surgery	ORR 94.7% (CR 87.7%); sarcoids: OR 96.2% (CR 90.4%); SCC CR (12 mo); neurofibrosarcoma CR 66,7% (12–35 mo); anaplastic sarcoma PR	Tamzali et al. (2007)

(continued)

Table 5 (continued)

Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
Sarcoid	48 animals: horses ($n = 34$), ponies ($n = 2$), donkeys ($n = 11$), mule ($n = 1$); 194 tumor nodules	Cisplatin (0.3 mg/cm^3, i/tum)	Parallel stainless steel electrodes; 8 pulses, 100μs, 1300 V/cm, 500 Hz	Betatech (Unipolar, L'Union, France)	1–7 ± debulking surgery	97.9% animals (99.5% tumors) recurrence-free 4 y after treatment	Tamzali et al. (2012)
Sarcoid	32 animals: horses ($n = 31$), donkey ($n = 1$); 70 tumor nodules	Cisplatin (2 mg/cm^3, i/tum)	Plate or needle electrodes; 8 pulses, 100μs, 1000 V/cm (needle) or 1300 V/cm (plate), 5 kHz	Jouan GHT 1287 or Cliniporator	1–5 ± debulking surgery	CR 100%; 92.3% nodules recurrence-free at the end of observation period (1–7+, mean 4 y)	Tozon et al. (2016a)

ECT electrochemotherapy, *ORR* overall response rate, *CR* complete response, *PR* partial response, *mo* months, *y* years, *SCC* squamous cell carcinoma, *i/tum* intratumoral

ECT, a thorough discussion with the owner of the animal is of crucial importance. Explain the procedure and the expected post-treatment course of recovery, with a special emphasis on wound care. Define the expected number of ECT sessions, total duration of the treatment, costs of treatment, the possibility of recurrences. If possible, present graphic material (e.g., pictures, videos) of the treatment in various stages. The owners should be aware that their animals would be withdrawn from the human food chain according to most European national regulations. Obtain the owner's written consent, if the patient will be enrolled in a clinical trial.

Treatment Procedure

Define the treatment plan: for T_2–T_4 tumors, surgical debulking prior to ECT is recommended for optimal results (Tamzali et al. 2012). Depending on the characteristics of the tumor, an incomplete tumor resection with evidence of gross residual disease or an incomplete resection with microscopically "dirty" tumor margins can be performed. In cases, where surgery is combined with ECT, the latter should be performed either 2 weeks after surgery or during the perioperative period.

Before treatment, calculate the tumor volume using the formula $V = a \times b \times c \times \pi/6$, where "*a*," "*b*," and "*c*" are the three perpendicular diameters of the tumor nodule. The dimensions should be measured by using a Vernier caliper.

Patient Preparation

For the procedure, a short general anesthesia is mandatory. For the treatment of periorbital lesions under general anesthesia with injectable agents, a sensory block of the eyelids is performed with 2% lidocaine in order to limit the movement of the head as a consequence of electrical pulse delivery. The tumor site is prepared aseptically.

Working Environment and Safety

Only trained operator (DVM/VMD) should perform ECT. The room for the procedure should be suitable for working with horses under general anesthesia with all the necessary equipment.

Biosafety measures should be followed, complying with national regulations regarding chemotherapeutic drug usage and disposal.

Although cisplatin is not absorbed through intact skin and rarely causes acute problems following droplet exposure, the operator and assistants should be equipped with masks and gloves. The contact of cisplatin with the skin can cause local irritation. In case of skin exposure to cisplatin, rinse immediately with large quantities of water. When injecting firm tumors with the drug, leakage can occur to the surrounding area. In case of needle stick injury, wash with the abundance of water and perform wound care according to standard medical practice.

Any type of generators producing square-wave pulses that are meeting the EU or FDA standards should be used. The generator generally consists of integrated software, a touch screen interface and a hand-held electrode holder with a pulse-activating button attached to the generator by a cord (usually controlled by a foot switch).

Electrode Selection

Apart from plate and needle electrodes, described in Sect. 2.7, the specially designed parallel stainless steel electrodes (9 mm wide, 2 mm thick, 9 mm distance between electrodes) can also be used, if available (Mazères et al. 2009).

Drug Administration

Cisplatin, if in powder form (cis-diamminedichloroplatinum II), should be dissolved in a sterile physiological saline solution to 1 mg/ml (other desired concentrations can be used) and given intratumorally and peritumorally to the safety margins 2 cm around the tumor. The theoretical calculated dose of cisplatin is 0.3 mg/cm^3 of the tumor. In cases of tumor excision with primary closure and microscopic residual disease, the skin is infiltrated on 2 cm on both sides of the scar.

Intratumoral applications should be performed slowly to minimize the risk of drug leakage.

Electroporation

Electrical pulse delivery should be started 1–2 (Tozon et al. 2016a) or 5 (Tamzali et al. 2012) min after injection of cisplatin.

In cases, using the electrodes, described by Mazères et al. (2009), two series of eight biphasic electric pulses with a duration of 100μs, amplitude to electrode distance ratio 1300 V/cm and repetition frequency 500 Hz should be applied. When using plate or needle electrodes, refer to the electrical pulse parameters described in Sect. 2.7.

A water-based gel should be used to achieve optimal contact between the skin and the electrodes and to achieve less heterogeneity in the electric field distribution. In cases of plate or needle row electrodes, the electrical pulses should be applied twice in a perpendicular direction to achieve a better electric field distribution. The application of electrical pulses should start from the tumor margins (depending on the tumor type), move centrally and gradually progress to the central part of the tumor. When using needle electrodes, the application of gel is not necessary. All the needles should be inserted inside the tumor to allow appropriate electric field distribution.

After ECT, all the contaminated material should be disposed of according to national regulations regarding chemotherapeutic use.

When possible, the treated area should be bandaged to prevent contamination of the environment with chemotherapeutics. Animals should be kept isolated with restricted and controlled access for at least 24 h after treatment. After the discharge from the hospital, the owners are advised to avoid contact with the treated area and to monitor for the presence of swelling, discharge, erythema, or recurrence of the tumor.

Follow-Up and Retreatments

The treatment should be repeated every 2–4 weeks until CR is achieved, if retreatment is ethically acceptable and with the agreement of the owner. In cases of gross tumor excision with primary closure and microscopic residual disease, two

ECT sessions are usually required. In case of excessive granulation tissue, a biopsy with histopathological evaluation should be performed to ascertain the nature of the tissue.

2.9 Electrochemotherapy for the Treatment of Tumors in Exotic Pets

In the last decade, ECT has also gained increasing attention in the area of veterinary medicine of exotic pets, where complete surgical removal of tumors is often difficult to achieve due to the size of the animals or the healing properties of wounds (Table 6).

Fibropapillomatosis (FP) of sea turtles is a neoplastic disease associated with FP-associated herpesvirus (C-FP-HV), typically presenting with multiple, large, and ulcerated cutaneous masses that compromise both locomotion and feeding. Surgical excision is most commonly attempted for the treatment of FP, but is frequently associated with a high local recurrence rate (Page-Karjian et al. 2014). Brunner et al. (2014) first described the treatment of two green turtles (*Chelonia mydas*) with ECT using intratumoral bleomycin injection and a local lidocaine anesthesia. Both turtles received two treatment sessions 33 days apart and achieved CR without side effects or post-treatment complications. The animals showed no signs of recurrence 1 year after treatment. Recently, Donnelly et al. (2019) confirmed their observations, describing the same treatment protocol in two *C. mydas* turtles with FP. No healing complications or tumor recurrences were observed 3 months after two ECT treatments 6 weeks apart. The authors also performed the first pharmacokinetic study of bleomycin in the treated turtles. Plasma bleomycin reached detectable concentrations, suggesting systemic circulation of the drug, but its concentrations were many orders of magnitude lower than those documented in human pharmacokinetic studies. Interestingly, in one case, an untreated tumor in a distant location appeared to exhibit necrosis within a week of ECT and darkened to a deep purple-black color. This might suggest a systemic antitumor effect of ECT; however, no circulating bleomycin was detected at the time of the ECT session. Therefore, the authors suggest that spontaneous regression, independent from ECT, could also be possible. The untreated tumor was surgically excised shortly after its appearance; thus, longer-term follow-up was not possible. ECT could therefore represent an effective alternative treatment for FP in turtles (Donnelly et al. 2019).

Recently, Račnik et al. (2018) described the treatment of two ferrets (*Mustela putorius furo*) with four different tumor nodules (two MCT, one squamous papilloma, and one sebaceous adenoma) using ECT with intratumoral bleomycin. No local or systemic side effects were noted during or after ECT, and all tumors were in CR 6–70 days after the treatment. No tumor recurrences were observed at the end of the observation period (max. 15 months). The authors concluded that ECT could represent a good treatment of choice instead of surgery for selected skin tumors in ferrets, especially those located on surgically difficult sites (Račnik et al. 2018).

Table 6 Use of ECT for tumors in exotic pets

Tumor type	N° of patients	Chemotherapeutic (dose, route of administration)	Electrodes and parameters (number, duration, amplitude to distance ratio, repetition frequency)	Electric pulse generator	N° of treatments ± adjuvant treatment	Outcome	References
Fibropapillomatosis	Two green turtles (*Chelonia mydas*)	Bleomycin (1 IU/cm^3, i/tum)	Needle electrodes; 1000 V	Vetpulser BK 100 (Cromatica, Sao Paulo, Brazil	2	CR 100% (1 y)	Rols et al. (2002)
Cutaneous SCC	One yellow-bellied slider (*Trachemys scripta scripta*)	Bleomycin (1 mg, i/tum)	Caliper electrodes; 8 pulses, 50 + 50μs. 1200 V/cm, 1 Hz	Onkodisruptor	2	CR 100% (1 y)	Lanza et al. (2015)
Mammary carcinoma (incompletely excised)	Two rats (*Rattus norvegicus*)	Cisplatin (1 mg, i/tum)	Plate electrodes; 8 pulses, 50 + 50μs. 1200 V/cm, 1 Hz	Onkodisruptor	2	CR 100% (10–14 mo)	Lanza et al. (2017)
MCT ($n = 2$), squamous papilloma ($n = 1$), sebaceous adenoma ($n = 1$)	Two ferrets (*Mustela putorius furo*), four tumor nodules	Bleomycin (0.5 mg—volume of 0.1 ml, i/tum)	Plate electrodes; 8 pulses, 100μs, 1300 V/cm, 5 kHz	Cliniporator	1	CR 100%, observation period 8–15 mo	Račnik et al. (2018)

Oral SCC	One African hedgehog (*Atelerix albiventris*)	Bleomycin (1.5 mg, i/tum)	Caliper electrodes; 8 pulses, 50 + 50μs. 1200 V/cm, 1 Hz	Onkodisruptor	monthly ECT until PD	Initial 25% reduction, then SD (5 mo) and PD	Spugnini et al. (2018)
Fibropapillomatosis	Two green turtles (*Chelonia mydas*)	Bleomycin (0.5–1 IU/cm^3, i/tum)	Needle electrodes; 8 pulses, 100μs, anticipated voltage 1000 V (measured voltage 400 V), 5 kH	Vet-ePorator (Evvivax, Rome, Italy) and Electrovet EZ (LeRoy Biotech, Gameville, France)	2	CR 100% (3 mo)	Donnelly et al. (2019)
Periorbital fibroma	One cockatiel (*Nymphicus hollandicus*)	Cisplatin (1 mg/kg, i/tum)	Plate electrodes; 8 pulses, 100μs, 1300 V/cm, 5 kHz	Cliniporator	2	CR (3 y)	Račnik et al. (2019)

ECT electrochemotherapy, *CR* complete response, *PD* progressive disease, *mo* months, *y* years, *SCC* squamous cell carcinoma, *i/tum* intratumoral

Successful reports of complete remissions were also published in cases of a yellow-bellied slider (*Trachemys scripta scripta*) with a cutaneous SCC and two rats (*Rattus norvegicus*) with mammary carcinomas (Lanza et al. 2015, 2017). All of the cases were treated with ECT, adjuvant to surgery. Palliative treatment of an African hedgehog (*Atelerix albiventris*) with an oral SCC was also described, resulting in PR (5 months) (Spugnini et al. 2018). A long-term CR was observed in the treatment of a cockatiel (*Nymphicus hollandicus*) with a non-operable periorbital fibroma. After two sessions of ECT with cisplatin, the tumor was in CR that lasted until the end of the observation period (3 years). No systemic side effects related to cisplatin were noted clinically (Račnik et al. 2019).

References

Allegretti JP, Panje WR (2001) Electroporation therapy for head and neck cancer including carotid artery involvement. Laryngoscope 111:52–56. https://doi.org/10.1097/00005537-200101000-00010

Baldi A, Spugnini EP (2006) Thoracic haemangiopericytoma in a cat. Vet Rec 159:598–600. https://doi.org/10.1136/vr.159.18.598

Belehradek J, Orlowski S, Poddevin B et al (1991) Electrochemotherapy of spontaneous mammary tumours in mice. Eur J Cancer Clin Oncol 27:73–76. https://doi.org/10.1016/0277-5379(91)90065-L

Belehradek M, Domenge C, Luboinski B et al (1993) Electrochemotherapy, a new antitumor treatment. First clinical phase I-II trial. Cancer 72:3694–3700. https://doi.org/10.1002/1097-0142(19931215)72:12<3694::AID-CNCR2820721222>3.0.CO;2-2

Blackwood L, Murphy S, Buracco P et al (2012) European consensus document on mast cell tumours in dogs and cats. Vet Comp Oncol 10:1–29. https://doi.org/10.1111/j.1476-5829.2012.00341.x

Brunner CHM, Dutra G, Silva CB et al (2014) Electrochemotherapy for the treatment of fibropapillomas in Chelonia mydas. J Zoo Wildl Med 45:213–218. https://doi.org/10.1638/2010-0125.1

Campana LG, Clover AJP, Valpione S et al (2016a) Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review. Radiol Oncol 50:1–13. https://doi.org/10.1515/raon-2016-0006

Campana LG, Testori A, Curatolo P, Quaglino P (2016b) Treatment efficacy with electrochemotherapy: a multi- institutional prospective observational study on 376 patients with superficial tumors. Eur J Surg Oncol 42:1914–1923. https://doi.org/10.1016/j.ejso.2016.06.399

Campana LG, Edhemović I, Soden D et al (2019) Electrochemotherapy – emerging applications technical advances, new indications, combined approaches, and multi-institutional collaboration. Eur J Surg Oncol 45:92–102. https://doi.org/10.1016/j.ejso.2018.11.023

Čemažar M, Miklavčič D, Vodovnik L et al (1995) Improved therapeutic effect of electrochemotherapy with cisplatin by intratumoral drug administration and changing of electrode orientation for electropermeabilization on EAT tumor model in mice. Radiol Oncol 29:121–127

Čemažar M, Ambrožič Avguštin J, Pavlin D et al (2017) Efficacy and safety of electrochemotherapy combined with peritumoral IL-12 gene electrotransfer of canine mast cell tumours. Vet Comp Oncol 15:641–654. https://doi.org/10.1111/vco.12208

Coletti L, Battaglia V, De Simone P et al (2017) Safety and feasibility of electrochemotherapy in patients with unresectable colorectal liver metastases: a pilot study. Int J Surg 44:26–32. https://doi.org/10.1016/j.ijsu.2017.06.033

Compston PC, Turner TG, Payne RJ (2013) Laser surgery as a sole treatment of histologically confirmed equine sarcoids: outcome and risk factors for recurrence. Equine Vet J 48:451–456. https://doi.org/10.1111/evj.12145_4

Cornelis FH, Ben Ammar M, Nouri-Neuville M et al (2019) Percutaneous image-guided electrochemotherapy of spine metastases: initial experience. Cardiovasc Intervent Radiol 42:1806–1809. https://doi.org/10.1007/s00270-019-02316-4

Cutrera J, Torrero MN, Shiomitsu K et al (2008) Intratumoral bleomycin and IL-12 electrochemogenetherapy for treating head and neck tumors in dogs. Methods Mol Biol 423:319–325

Cutrera J, King G, Jones P et al (2015) Safe and effective treatment of spontaneous neoplasms with interleukin 12 electro-chemo-gene therapy. J Cell Mol Med 19:664–675. https://doi.org/10.1111/jcmm.12382

Djokić M, Čemažar M, Popović P et al (2018) Electrochemotherapy as treatment option for hepatocellular carcinoma, a prospective pilot study. Eur J Surg Oncol 44:651–657. https://doi.org/10.1016/j.ejso.2018.01.090

Dobson J, de Queiroz GF, Golding JP (2018) Photodynamic therapy and diagnosis: principles and comparative aspects. Vet J 233:8–18. https://doi.org/10.1016/j.tvjl.2017.11.012

Domenge C, Orlowski S, Luboinski B et al (1996) Antitumor electrochemotherapy: New advances in the clinical protocol. Cancer 77:956–963. https://doi.org/10.1002/(SICI)1097-0142(19960301)77:5<956::AID-CNCR23>3.0.CO;2-1

Donnelly KA, Papich MG, Zirkelbach B et al (2019) Plasma bleomycin Concentrations during electrochemotherapeutic treatment of fibropapillomas in green turtles Chelonia mydas. J Aquat Anim Health 31:186–192. https://doi.org/10.1002/aah.10067

Dos Anjos DS, Rossi YA, Magalhães LF et al (2018) Digital trichoblastoma treated with electrochemotherapy in a dog. Vet Rec Case Reports 6:e000671. https://doi.org/10.1136/vetreccr-2018-000671

Edhemović I, Brecelj E, Gašljević G et al (2014) Intraoperative electrochemotherapy of colorectal liver metastases. J Surg Oncol 110:230–327. https://doi.org/10.1002/jso.23625

Egeland C, Baeksgaard L, Johannesen H et al (2018) Endoscopic electrochemotherapy for esophageal cancer: a phase I clinical study. Endosc Int Open 6:727–734. https://doi.org/10.1055/a-0590-4053

Falk Hansen H, Bourke M, Stigaard T et al (2020) Electrochemotherapy for colorectal cancer using endoscopic electroporation: a phase 1 clinical study. Endosc Int Open 8:e124–e132. https://doi.org/10.1055/a-1027-6735

Forde PF, Sadadcharam M, Bourke MG et al (2016) Preclinical evaluation of an endoscopic electroporation system. Endoscopy 48:477–483. https://doi.org/10.1055/s-0042-101343

Frank Glass L, Pepine ML, Fenske NA et al (1996) Bleomycin-mediated electrochemotherapy of metastatic melanoma. Arch Dermatol 132:1353–1357. https://doi.org/10.1001/archderm.132.11.1353

Gehl J, Geertsen PF (2000) Efficient palliation of haemorrhaging malignant melanoma skin metastases by electrochemotherapy. Melanoma Res 10:585–589. https://doi.org/10.1097/00008390-200012000-00011

Gehl J, Serša G, Matthiessen LW et al (2018) Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol (Madr) 57:874–882. https://doi.org/10.1080/0284186X.2018.1454602

Gothelf A, Mir LM, Gehl J (2003) Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. Cancer Treat Rev 29:371–387. https://doi.org/10.1016/S0305-7372(03)00073-2

Grošelj A, Kržan M, Kosjek T et al (2016) Bleomycin pharmacokinetics of bolus bleomycin dose in elderly cancer patients treated with electrochemotherapy. Cancer Chemother Pharmacol 77:939–947. https://doi.org/10.1007/s00280-016-3004-z

Grošelj A, Bošnjak M, Strojan P et al (2018) Efficiency of electrochemotherapy with reduced bleomycin dose in the treatment of nonmelanoma head and neck skin cancer: preliminary results. Head Neck 40:120–125. https://doi.org/10.1002/hed.24991

Heller R, Jaroszeski M, Leo-Messina J et al (1995) Treatment of B16 mouse melanoma with the combination of electropermeabilization and chemotherapy. Bioelectrochem Bioenerg 36:83–87. https://doi.org/10.1016/0302-4598(94)05013-K

Heller R, Jaroszeski MJ, Glass LF et al (1996) Phase I/II trial for the treatment of cutaneous and subcutaneous tumors using electrochemotherapy. Cancer 77:964–971. https://doi.org/10.1002/(SICI)1097-0142(19960301)77:5<964::AID-CNCR24>3.0.CO;2-0

Heller R, Jaroszeski MJ, Reintgen DS et al (1998) Treatment of cutaneous and subcutaneous tumors with electrochemotherapy using intralesional bleomycin. Cancer 83:148–157. https://doi.org/10.1002/(SICI)1097-0142(19980701)83:1<148::AID-CNCR20>3.0.CO;2-W

Hewes CA, Sullins KE (2006) Use of cisplatin-containing biodegradable beads for treatment of cutaneous neoplasia in equidea: 59 Cases (2000-2004). J Am Vet Med Assoc 229:1617–1622. https://doi.org/10.2460/javma.229.10.1617

Impellizeri J, Aurisicchio L, Forde P, Soden DM (2016) Electroporation in veterinary oncology. Vet J 217:18–25. https://doi.org/10.1016/j.tvjl.2016.05.015

Jamšek Č, Serša G, Bošnjak M, Grošelj A (2020) Long term response of electrochemotherapy with reduced dose of bleomycin in elderly patients with head and neck non-melanoma skin cancer. Radiol Oncol 54:79–85. https://doi.org/10.2478/raon-2020-0009

Klein N, Gunther E, Zapf S et al (2017) Prostate cancer infiltrating the bladder sphincter successfully treated with electrochemotherapy: a case report. Clin Case Reports 5:2127–2132. https://doi.org/10.1002/ccr3.1270

Knottenbelt DC, Kelly DF (2000) The diagnosis and treatment of periorbital sarcoid in the horse: 445 cases from 1974 to 1999. Vet Ophthalmol 3:169–191. https://doi.org/10.1046/j.1463-5224.2000.00119.x

Knottenbelt DC, Edwards S, Daniel E (1995) Diagnosis and treatment of the equine sarcoid. In Pract 17:123–129

Kodre V, Čemažar M, Pečar J et al (2009) Electrochemotherapy compared to surgery for treatment of canine mast cell tumours. In Vivo (Brooklyn) 23:55–62

Kubota Y, Mir LM, Nakada T et al (1998) Successful treatment of metastatic skin lesions with electrochemotherapy. J Urol 160:1426. https://doi.org/10.1016/S0022-5347(01)62559-X

Kulbacka J, Paczuska J, Rembiałkowska N et al (2017) Electrochemotherapy combined with standard and CO2 laser surgeries in canine oral melanoma. Slov Vet Res 54:181–186. https://doi.org/10.26873/SVR-322-2017

Lane JG (1977) The treatment of equine sarcoids by cryosurgery. Equine Vet J 9:127–133. https://doi.org/10.1111/j.2042-3306.1977.tb04003.x

Lanza A, Baldi A, Spugnini EP (2015) Surgery and electrochemotherapy for the treatment of cutaneous squamous cell carcinoma in a yellow-bellied slider (*Trachemys scripta scripta*). J Am Vet Med Assoc 246:455–457. https://doi.org/10.2460/javma.246.4.455

Lanza A, Pettorali M, Baldi A, Spugnini EP (2017) Surgery and electrochemotherapy treatment of incompletely excised mammary carcinoma in two male pet rats (*Rattus norvegicus*). J Vet Med Sci 79:623–625. https://doi.org/10.1292/jvms.16-0578

Lowe R, Gavazza A, Impellizeri JA et al (2017) The treatment of canine mast cell tumours with electrochemotherapy with or without surgical excision. Vet Comp Oncol 15:775–784. https://doi.org/10.1111/vco.12217

Maglietti F, Tellado M, Olaiz N et al (2017) Minimally invasive electrochemotherapy procedure for treating nasal duct tumors in dogs using a single needle electrode. Radiol Oncol 51:422–430. https://doi.org/10.1515/raon-2017-0043

Martens A, De Moor A, Vlaminck L et al (2001) Evaluation of excision, cryosurgery and local BCG vaccination for the treatment of equine sarcoids. Vet Rec 149:665–669. https://doi.org/10.1136/vr.149.22.665

Marty M, Serša G, Garbay JR et al (2006) Electrochemotherapy - an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. Eur J Cancer Suppl 4:3–13. https://doi.org/10.1016/j.ejcsup.2006.08.002

Mazères S, Sel D, Golzio M et al (2009) Non invasive contact electrodes for in vivo localized cutaneous electropulsation and associated drug and nucleic acid delivery. J Control Release 134:125–131. https://doi.org/10.1016/j.jconrel.2008.11.003

Milevoj N, Tratar UL, Nemec A et al (2019) A combination of electrochemotherapy, gene electrotransfer of plasmid encoding canine IL-12 and cytoreductive surgery in the treatment of canine oral malignant melanoma. Res Vet Sci 122:40–49. https://doi.org/10.1016/j.rvsc.2018.11.001

Milevoj N, Tozon N, Ličen S et al (2020) Health-related quality of life in dogs treated with electrochemotherapy and/or interleukin-12 gene electrotransfer. Vet Med Sci 6:290–298. https://doi.org/10.1002/vms3.232

Mir LM, Orlowski S, Belehradek J, Paoletti C (1991) Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. Eur J Cancer Clin Oncol 27:68–72. https://doi.org/10.1016/0277-5379(91)90064-K

Mir LM, Devauchelle P, Quintin-Colonna F et al (1997) First clinical trial of cat soft tissue sarcomas treatment by electrochemotherapy. Br J Cancer 76:1617–1622. https://doi.org/10.1038/bjc.1997.606

Mir LM, Gehl J, Serša G et al (2006) Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or non-invasive electrodes. Eur J Cancer Suppl 4:14–25. https://doi.org/10.1016/j.ejcsup.2006.08.003

Nguyen SM, Thamm DH, Vail DM, London CA (2015) Response evaluation criteria for solid tumours in dogs (v1.0): a Veterinary Cooperative Oncology Group (VCOG) consensus document. Vet Comp Oncol 13:176–183. https://doi.org/10.1111/vco.12032

Nogueira SAF, Torres SMF, Malone ED et al (2006) Efficacy of imiquimod 5% cream in the treatment of equine sarcoids: a pilot study. Vet Dermatol 17:259–265. https://doi.org/10.1111/j.1365-3164.2006.00526.x

Okino M, Mohri H (1987) Effects of a high-voltage electrical impulse and an anticancer drug on in vivo growing tumors. Jpn J Cancer Res 78:1319–1321. https://doi.org/10.20772/cancersci1985.78.12_1319

Orlowski S, Belehradek J, Paoletti C, Mir LM (1988) Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. Biochem Pharmacol 37:4727–4733. https://doi.org/10.1016/0006-2952(88)90344-9

Page-Karjian A, Norton TM, Krimer P et al (2014) Factors influencing survivorship in rehabilitating green sea turtles (*Chelonia mydas*) with fibropapillomatosis. J Zoo Wildl Med 45:507–519. https://doi.org/10.1638/2013-0132r1.1

Pavlin D, Čemažar M, Serša G, Tozon N (2012) IL-12 based gene therapy in veterinary medicine. J Transl Med 10:e234. https://doi.org/10.1186/1479-5876-10-234

Probst U, Fuhrmann I, Beyer L, Wiggermann P (2018) Electrochemotherapy as a new modality in interventional oncology: a review. Technol Cancer Res Treat 17:1–12. https://doi.org/10.1177/1533033818785329

Račnik J, Švara T, Zadravec M et al (2018) Electrochemotherapy with bleomycin of different types of cutaneous tumours in a ferret (*Mustela putorius furo*). Radiol Oncol 52:98–104. https://doi.org/10.1515/raon-2017-0057

Račnik J, Švara T, Zadravec M et al (2019) Electrochemotherapy with cisplatin for the treatment of a non-operable cutaneous fibroma in a cockatiel (*Nymphicus hollandicus*). N Z Vet J 67:155–158. https://doi.org/10.1080/00480169.2018.1564393

Reed SD, Fulmer A, Buckholz J et al (2010) Bleomycin/interleukin-12 electrochemogenetherapy for treating naturally occurring spontaneous neoplasms in dogs. Cancer Gene Ther 17:571–578. https://doi.org/10.1038/cgt.2010.13

Rodríguez-Cuevas S, Barroso-Bravo S, Almanza-Estrada J et al (2001) Electrochemotherapy in primary and metastatic skin tumors: phase II trial using intralesional bleomycin. Arch Med Res 32:273–276. https://doi.org/10.1016/S0188-4409(01)00278-8

Rols MP, Bachaud JM, Giraud P et al (2000) Electrochemotherapy of cutaneous metastases in malignant melanoma. Melanoma Res 10:468–474. https://doi.org/10.1097/00008390-200010000-00009

Rols MP, Tamzali Y, Teissié J (2002) Electrochemotherapy of horses. A preliminary clinical report. Bioelectrochemistry 55:101–105. https://doi.org/10.1016/S1567-5394(01)00156-6

Rudolf Z, Štabuc B, Čemažar M et al (1995) Electrochemotherapy with bleomycin. The first clinical experience in malignant melanoma patients. Radiol Oncol 29:229–235

Salford LG, Persson BRR, Brun A et al (1993) A new brain tumor therapy combining bleomycin with in vivo electropermeabilization. Biochem Biophys Res Commun 194:938–943. https://doi.org/10.1006/bbrc.1993.1911

Serša G, Čemažar M, Miklavčič D, Mir LM (1994) Electrochemotherapy: variable anti-tumor effect on different tumor models. Bioelectrochem Bioenerg 35:23–27. https://doi.org/10.1016/0302-4598(94)87006-3

Serša G, Čemažar M, Miklavčič D (1995) Antitumor effectiveness of electrochemotherapy with cis-diamminedichloroplatinum(II) in mice. Cancer Res 55:3450–3455

Serša G, Čufer T, Čemažar M et al (2000) Electrochemotherapy with bleomycin in the treatment of hypernephroma metastasis: case report and literature review. Tumori 86:163–165. https://doi.org/10.1177/030089160008600211

Serša G, Teissie J, Čemažar M et al (2015) Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. Cancer Immunol Immunother 64:1315–1327. https://doi.org/10.1007/s00262-015-1724-2

Simčič P, Lowe R, Granziera V et al (2020) Electrochemotherapy in treatment of canine oral non-tonsillar squamous cell carcinoma. A case series report. Vet Comp Oncol 18(3):428–432. https://doi.org/10.1111/vco.12530

Spugnini EP, Porrello A (2003) Potentiation of chemotherapy in companion animals with spontaneous large neoplasms by application of biphasic electric pulses. J Exp Clin Cancer Res 22:571–580

Spugnini EP, Dragonetti E, Vincenzi B et al (2006a) Pulse-mediated chemotherapy enhances local control and survival in a spontaneous canine model of primary mucosal melanoma. Melanoma Res 16:23–27. https://doi.org/10.1097/01.cmr.0000195702.73192.a0

Spugnini EP, Vincenzi B, Citro G, Baldi A (2006b) Adjuvant electrochemotherapy for the treatment of incompletely resected canine mast cell tumors. Anticancer Res 26:4585–4589

Spugnini EP, Dotsinsky I, Mudrov N et al (2007a) Biphasic pulses enhance bleomycin efficacy in a spontaneous canine perianal tumors model. J Exp Clin Cancer Res 26:483–487

Spugnini EP, Citro G, Mellone P et al (2007b) Electrochemotherapy for localized lymphoma: a preliminary study in companion animals. J Exp Clin Cancer Res 26:343–346

Spugnini EP, Baldi A, Vincenzi B, Citro G (2007c) Intraoperative versus postoperative electrochemotherapy in high grade soft tissue sarcomas: a preliminary study in a spontaneous feline model. Cancer Chemother Pharmacol 59:375–381. https://doi.org/10.1007/s00280-006-0281-y

Spugnini EP, Filipponi M, Romani L et al (2007d) Local control and distant metastasis after electrochemotherapy of a canine anal melanoma. In Vivo (Brooklyn) 21:897–900

Spugnini EP, Vincenzi B, Citro G et al (2007e) Adjuvant electrochemotherapy for the treatment of incompletely excised spontaneous canine sarcomas. In Vivo (Brooklyn) 21:819–822

Spugnini EP, Citro G, Dotsinsky I et al (2008a) Ganglioneuroblastoma in a cat: A rare neoplasm treated with electrochemotherapy. Vet J 178:291–293. https://doi.org/10.1016/j.tvjl.2007.08.014

Spugnini EP, Dotsinsky I, Mudrov N et al (2008b) Biphasic pulses enhance bleomycin efficacy in a spontaneous canine genital tumor model of chemoresistance: sticker sarcoma. J Exp Clin Cancer Res 27:7–10. https://doi.org/10.1186/1756-9966-27-58

Spugnini EP, Dotsinsky I, Mudrov N et al (2008c) Electrochemotherapy-induced radiation recall in a cat. In Vivo (Brooklyn) 22:751–753
Spugnini EP, Vincenzi B, Betti G et al (2008d) Surgery and electrochemotherapy of a high-grade soft tissue sarcoma in a dog. Vet Rec 162(6):186–188. https://doi.org/10.1136/vr.162.6.186
Spugnini EP, Vincenzi B, Citro G et al (2009) Electrochemotherapy for the treatment of squamous cell carcinoma in cats: a preliminary report. Vet J 179:117–120. https://doi.org/10.1016/j.tvjl.2007.08.011
Spugnini EP, Filipponi M, Romani L et al (2010) Electrochemotherapy treatment for bilateral pleomorphic rhabdomyosarcoma in a cat. J Small Anim Pract 51:330–332. https://doi.org/10.1111/j.1748-5827.2010.00913.x
Spugnini EP, Renaud SM, Buglioni S et al (2011a) Electrochemotherapy with cisplatin enhances local control after surgical ablation of fibrosarcoma in cats: an approach to improve the therapeutic index of highly toxic chemotherapy drugs. J Transl Med 9:152. https://doi.org/10.1186/1479-5876-9-152
Spugnini EP, Vincenzi B, Citro G, Dotsinsky I (2011b) Evaluation of cisplatin as an electrochemotherapy agent for the treatment of incompletely excised mast cell tumors in dogs. J Vet Intern Med 25:407–411. https://doi.org/10.1111/j.1939-1676.2011.0678.x
Spugnini EP, Di Tosto G, Salemme S et al (2013) Electrochemotherapy for the treatment of recurring aponeurotic fibromatosis in a dog. Can Vet J 54:606–609
Spugnini EP, Pizzuto M, Filipponi M et al (2015) Electroporation enhances bleomycin efficacy in cats with periocular carcinoma and advanced squamous cell carcinoma of the head. J Vet Intern Med 29:1368–1375. https://doi.org/10.1111/jvim.13586
Spugnini EP, Menicagli F, Pettorali M, Baldi A (2017) Ultrasound guided electrochemotherapy for the treatment of a clear cell thymoma in a cat. Open Vet J 7:57–60. https://doi.org/10.4314/ovj.v7i1.8
Spugnini EP, Lanza A, Sebasti S, Baldi A (2018) Electrochemotherapy palliation of an oral squamous cell carcinoma in an African hedgehog (*Atelerix albiventris*). Vet Res Forum 9:379–381. https://doi.org/10.30466/vrf.2018.33109
Spugnini EP, Vincenzi B, Amadio B, Baldi A (2019) Adjuvant electrochemotherapy with bleomycin and cisplatin combination for canine soft tissue sarcomas: a study of 30 cases. Open Vet J 9:88–93. https://doi.org/10.4314/ovj.v9i1.15
Stadler S, Kainzbauer C, Haralambus R et al (2011) Successful treatment of equine sarcoids by topical aciclovir application. Vet Rec 168:187. https://doi.org/10.1136/vr.c5430
Suzuki DOH, Berkenbrock JA, Frederico MJS et al (2018) Oral mucosa model for electrochemotherapy treatment of dog mouth cancer: ex vivo, in silico, and in vivo experiments. Artif Organs 42:297–304. https://doi.org/10.1111/aor.13003
Tamzali Y, Teissie J, Rols MP (2001) Cutaneous tumor treatment by electrochemotherapy: Preliminary clinical results in horse sarcoids. Rev Med Vet (Toulouse) 152:605–609
Tamzali Y, Teissie J, Rols MP (2003) First horse sarcoid treatment by electrochemotherapy: preliminary experimental results. In: 49th annual convention of the AAEP, New Orleans, LA
Tamzali Y, Teissie J, Golzio M, Rols MP (2007) Electrochemotherapy of equids cutaneous tumors: a 57 case retrospective study 1999-2005. In: IFMBE proceedings, New York, pp 610–613
Tamzali Y, Borde L, Rols MP et al (2012) Successful treatment of equine sarcoids with cisplatin electrochemotherapy: a retrospective study of 48 cases. Equine Vet J 44:214–220. https://doi.org/10.1111/j.2042-3306.2011.00425.x
Tellado MN, Maglietti FH, Michinski SD et al (2020) Predictive factors of response to electrochemotherapy in canine oral malignant melanoma. Radiol Oncol 54:68–78. https://doi.org/10.1101/727164
Théon AP, Pascoe JR, Galuppo LD et al (1999) Comparison of perioperative versus postoperative intratumoral administration of cisplatin for treatment of cutaneous sarcoids and squamous cell carcinomas in horses. J Am Vet Med Assoc 215:1655–1660

Théon AP, Wilson WD, Magdesian KG et al (2007) Long-term outcome associated with intratumoral chemotherapy with cisplatin for cutaneous tumors in equidae: 573 cases (1995-2004). J Am Vet Med Assoc 230:1506–1513. https://doi.org/10.2460/javma.230.10.1506

Torrigiani F, Pierini A, Lowe R et al (2019) Soft tissue sarcoma in dogs: a treatment review and a novel approach using electrochemotherapy in a case series. Vet Comp Oncol 17:234–241. https://doi.org/10.1111/vco.12462

Tozon N, Serša G, Čemažar M (2001) Electrochemotherapy: potentiation of local antitumour effectiveness of cisplatin in dogs and cats. Anticancer Res 21:2483–2488

Tozon N, Kodre V, Serša G, Čemažar M (2005) Effective treatment of perianal tumors in dogs with electrochemotherapy. Anticancer Res 25:839–845. https://doi.org/10.1016/j.ijggc.2012.10.004

Tozon N, Kodre V, Juntes P et al (2010) Electrochemotherapy is highly effective for the treatment of canine perianal hepatoid adenoma and epithelioma. Acta Vet Brno 60:285–302. https://doi.org/10.2298/AVB1003285T

Tozon N, Pavlin D, Serša G et al (2014) Electrochemotherapy with intravenous bleomycin injection: an observational study in superficial squamous cell carcinoma in cats. J Feline Med Surg 16:291–299. https://doi.org/10.1177/1098612X13507071

Tozon N, Kramarič P, Kos Kadunc V et al (2016a) Electrochemotherapy as a single or adjuvant treatment to surgery of cutaneous sarcoid tumours in horses: a 31-case retrospective study. Vet Rec 179:1–6. https://doi.org/10.1136/vr.103867

Tozon N, Lampreht Tratar U, Žnidar K et al (2016b) Operating procedures of the electrochemotherapy for treatment of tumor in dogs and cats. J Vis Exp 9:e54760. https://doi.org/10.3791/54760

Tozon N, Tamzali Y, Čemazar M (2016c) Handbook of electroporation. In: Miklavčič D (ed) Handbook of electroporation. Springer, Cham, pp 1953–1967

Veterinary Cooperative Oncology Group (2016) Common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. Vet Comp Oncol 14:417–446. https://doi.org/10.1111/vco.283

Treating Mast Cell Tumors with Electrochemotherapy

Petra Simčič, Alessio Pierini, and George Lubas

Abstract

Mastocytoma (MCT) is the most common cutaneous tumor of dogs. Several prognostic factors and treatment strategies have been described, for effective management, however, a subset of MCTs remain difficult to treat. Surgery and radiation therapy often cure these tumors. When necessary, chemotherapy and/or tyrosine kinase inhibitors have been reported to improve the disease-free interval and overall survival time in dogs with non-resectable MCTs or distant metastasis. However, several reasons may bring owners to refuse "traditional" therapies, including financial constraints. In addition, the lack of access to highly specialized facilities (i.e., radiation therapy) is limited in many areas. Additional therapeutic strategies have been reported, including electrochemotherapy (ECT). This chapter focuses on the application of ECT coupled with intravenous or intralesional chemotherapy in the treatment of canine MCT. Additionally, the use of gene-electrotransfer therapy in the treatment of canine MCT and the use of ECT in feline MCT will be discussed briefly.

Keywords

Mast cell tumor · Electrochemotherapy · Gene-electrotransfer therapy · Canine · Feline · Local treatment

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1 Canine Cutaneous Mast Cell Tumors

1.1 General Information

Mast cell tumor or mastocytoma (MCT) refers to the neoplastic proliferation of mast cells and represents the most common malignancy of the dog's skin accounting for 16–21% of all cutaneous tumors (Bostock 1986; Finnie and Bostock 1979; Rothwell et al. 1987; Brodey 1970). The mast cell precursors migrate from the bone marrow to various tissues and organs where they become mature mast cells. There are several mediators inside the granules of mast cells, which are released into the extracellular environment during degranulation and play a key role in the inflammatory process. Among these mediators, proteases (i.e., tryptase), histamine, proteoglycans (mainly heparin), lysosomal enzymes, cytokines (i.e., TNF-alfa, BFGF, IL-4, and SCF), eicosanoids (PGD2, PAF, LTC4, and TXA2) have been identified (Moon et al. 2014; Stone et al. 2010; Kumar and Sharma 2010). When activated, mast cells can rapidly release or produce the mediators of several biologic activities, including allergic reactions, anaphylaxis, induction of immune tolerance, wound healing, angiogenesis, antiparasitic activity, and reaction to insect venoms (da Silva et al. 2014; Kumar and Sharma 2010; Metz et al. 2006).

MCTs frequently occur in older dogs (8–9 years) (Pierini et al. 2019; Bostock 1986; Finnie and Bostock 1979; Rothwell et al. 1987). Several breeds have been reported to be more at risk for MCTs, mainly Boxer and Bulldog-related breeds. However, Labrador and Golden Retrievers, Staffordshire terriers, Beagles, Chinese Shar-peis, and others are also considered at increased risk of MCTs (Bostock 1986; Rothwell et al. 1987; Patnaik et al. 1984; Peters 1969; White et al. 2011; Pierini et al. 2019).

The molecular alterations in MCTs involve the *c-kit* gene and its encoded receptor tyrosine kinase KIT. KIT (also known as CD117 and stem cell factor receptor, SCFR) is normally expressed on the surface of a variety of cells, mainly hematopoietic stem cells (Galli et al. 1994; Roskoski 2005a, b; Yarden et al. 1987). When SCF, the ligand of KIT, binds this receptor, induces KIT dimerization that activates its intrinsic tyrosine kinase activity, generating intracellular signal cascades that promote cell survival, proliferation, and differentiation (Galli et al. 1994; Roskoski 2005a, b). Dysregulation of KIT function is reported in canine MCTs, including aberrant localization and increased phosphorylation. Up to 30% of MCTs show mutations in the *c-kit* gene resulting in an independent activation and dimerization of KIT that turns out in intracellular signal transduction (Kiupel et al. 2004; London et al. 1996, 1999, 1996, 1999; Morini et al. 2004; Reguera et al. 2000; Halsey et al. 2017; Downing et al. 2002; Jones et al. 2004; Letard et al. 2008). Several studies reported an increased recurrence rate, metastasis, and worse outcome in dogs diagnosed with MCTs presenting *c-kit* mutations (Downing et al. 2002; London et al. 1999; Webster et al. 2006, 2008; Zemke et al. 2002).

1.2 Clinical Presentation

MCTs can develop in all tissue, however, cutaneous, and subcutaneous tissues are mostly affected. Cutaneous MCTs frequently affect the skin of trunk and limbs, with the head and neck regions less frequently involved. The clinical appearance of MCTs is variable and can be misdiagnosed with a variety of other cutaneous tumors. Dogs with MCTs usually present a solitary lesion, although up to 20% of dogs present multiple nodules (Mullins et al. 2006; Pelt et al. 1986; Pierini et al. 2019). Low-grade well-differentiated MCTs tend to be small, slow-growing tumors localized in the dermis (London and Thamm 2020). Some of them show temporary spontaneous regression ("wax and wane" presentation) that can delay the time of diagnosis. In contrast, high-grade undifferentiated MCTs tend to be rapid-growing tumors, larger in size, and more likely to be associated with cutaneous ulcerations. Moreover, highly malignant MCTs can be presented with small satellite nodules in the proximity of the primary tumor. Sentinel lymph node(s) should be clinically and microscopically assessed to rule-in/out metastasis, especially in dogs having high-risk MCTs (Ferrari et al. 2018). However, the regional lymph node (RLN) is more often evaluated rather than sentinel lymph node because of the lack of possibility to perform lymphography in basic clinical practice (Marconato et al. 2020). The palpation of the RLNs is not sensitive for early metastasis, thus cytological or even better histopathological evaluation is needed. When RLNs are enlarged because of the overt metastasis, lymphatic drainage failure can occur, contributing to tissue swelling, especially for MCTs localized in the extremities. The most frequent well-known clinical complaint is the Darier's sign (Tams and Macy 1981). With mechanical manipulation (i.e., during physical examination) or sometimes spontaneously, neoplastic mast cells can release vasoactive amines (mainly histamine) with subsequent erythema and swelling that usually resolves by itself. Some dogs, usually affected by high-grade undifferentiated MCTs, are presented with a history of GI disorders, coagulation abnormalities, fever, pain, tumor ulceration, and rarely collapse (London and Thamm 2020). Since these clinical signs are referred to as the degranulation of mast cells and subsequent releasing of several mediators into the bloodstream, dogs with more serious presentation of the disease (large tumors or metastasis) are more likely to develop these clinical signs.

1.3 Prognostic Factors

The biological behavior of MCTs is unpredictable and a single prognostic factor cannot predict the clinical outcome of the disease. Dogs presented without any complaints and with solitary or multiple slow-growing tumors without significant change in months or even years are usually associated with a benign behavior. Dogs presented with systemic or localized clinical signs may have a higher histological grade or higher stage of the disease (presence of regional or distant metastasis), therefore they can be associated with a worse prognosis (Mullins et al. 2006; O'Keefe et al. 1987; Thamm et al. 1999). Local recurrence of MCTs after surgical

excision has been also associated with a worse prognosis (Seguin et al. 2006; Thamm et al. 1999).

Some breeds are reported to be more frequently associated with a benign course of the disease (i.e., Boxer and Pug) while others (McNiel et al. 2006; Mochizuki et al. 2017) tend to be associated with a worse prognosis (i.e., Shar-pei) (Şmiech et al. 2018).

Some authors stated that MCTs located in the inguinal/preputial and axillary area, subungual space and mucocutaneous regions are associated with an aggressive course (Mullins et al. 2006; Turrel et al. 1988; Cahalane et al. 2004; Sfiligoi et al. 2005; Hillman et al. 2010; Thamm et al. 2006).

Tumor size does not strongly correlate with the prognosis. However, larger tumors are more difficult to excise with wide margins, especially those located on the extremities, inguinal/perineal area, and head-neck region. Since incomplete margins are considered as an independent prognostic factor for local recurrence of MCTs, tumor size may indirectly influence the outcome in some cases (Pierini et al. 2019; Moore et al. 2020).

Some studies report a worse prognosis in dogs with ulcerated tumors (Thamm et al. 1999). However, as stated above, no clinical sign alone can predict the behavior of the MCT.

The clinical staging system for MCT proposed by the World Health Organization is of limited usefulness for prognosis, even with high-grade tumors, because of the wide range of uncertainties about the tumor progression. Dogs presented with distant metastasis are known to be associated with a worse prognosis (Pizzoni et al. 2018). MCTs metastasize initially to lymph node(s) and then to visceral organs (mainly liver and spleen) and/or to bone marrow. Therefore, the RLNs often play a pivotal role in decision-making, especially for further diagnostic tests and treatment options. In the last two decades, several studies have reported long-term survival for dogs with lymph node metastasis that received treatment to the lymph node itself (surgical excision and/or radiation therapy) (Dobson et al. 2004; Marconato et al. 2020; Mendez et al. 2020). However, there are some limitations in interpretation due to the retrospective nature of the studies, the lack of randomization, and standardization of the treatments. In addition, nodal mapping in dogs with MCTs, which can recognize the sentinel from the regional lymph node in up to 40% of cases, is not commonly performed (Worley 2014). Finally, lymph node palpation is not sensitive to predict metastasis indeed some dogs with metastasis have normal-sized lymph nodes (Ferrari et al. 2018).

Histological grading is one of the most reliable prognostic factors for dogs with MCTs. The initial classification of cutaneous MCTs, made by Patnaik et al. in 1984, was revisited in 2011 by Kiupel et al. The former classification, which divided MCTs into three groups based on microscopic features and deep invasiveness, was for a long time, considered excellent to distinguish highly malignant MCTs (grade III) from more benign behaving tumors (grade I). However, the intermediate grade (grade II) diagnosed quite frequently was inaccurate to predict prognosis. The more recent Kiupel classification was arranged to improve the prediction of survival in grade II MCTs and to minimize the disagreement between the pathologists using the

Patnaik system. The new two-tier Kiupel grading system classified MCTs in high or low grade based on four cellular features. Although the Kiupel system seemed to better predict survival for dogs with MCTs than the Patnaik system, especially for dogs with grade II MCTs, both grading systems should be used and are often reported together for tumor evaluation (Patnaik et al. 1984; Kiupel et al. 2011; Horta et al. 2018; Sabattini et al. 2015). Recently, subcutaneous MCTs required a separate classification, since they have shown a less aggressive behavior compared to cutaneous MCTs (Thompson et al. 2011a).

Several markers of proliferation have been evaluated to improve the prediction of the outcome for dogs diagnosed with a MCT. The mitotic index (MI) evaluated in hematoxylin and eosin-stained tissue sections is easy and inexpensive and is also an independent factor for survival. MI >5 for cutaneous and MI >4 for subcutaneous MCTs are considered negative prognostic factors (Kiupel et al. 2011; Thompson et al. 2011a, b; Romansik et al. 2007; Elston et al. 2009; Preziosi et al. 2007). Ki-67, argyrophilic nucleolar organizer regions (AgNOR), and proliferating cell nuclear antigen (PCNA) have been associated with MI, histological grade, and patient survival (Scase et al. 2006; Bostock et al. 1989). However, their prognostic importance in localized and well-differentiated tumors requires further investigation.

Alteration of KIT expression, assessed with immunohistochemical staining, identifies three types of staining pattern: membrane (pattern I), focal/stippled (pattern II), and diffuse cytoplasmatic (pattern III) (Kiupel et al. 2004). Although pattern III has been associated with worse prognosis, until now the role of KIT expression pattern in predicting the outcome of the tumor needs further investigations. *C-kit* gene mutations have been associated with an increased recurrence rate, metastasis, and worse outcome in several studies (Downing et al. 2002; London et al. 1999; Webster et al. 2006, 2008; Zemke et al. 2002).

1.4 Staging and Diagnostic Workup

Since MCTs occur primarily in older dogs, general evaluation of a dog diagnosed with MCT should include complete blood count, biochemical and coagulation profile, and urinalysis. Direct evaluation of the nodule(s) often represents the first step of diagnosis. Cytological examination not only can diagnose MCTs in almost all cases, but it can also provide some prognostic information (Scarpa et al. 2016). Well-differentiated low-grade Kiupel MCTs exfoliate with a high number of round cells densely stippled of metachromatic granules. Usually, the nuclei are not easy to evaluate because of granules overlapping, but when they are clearly visible, no sign of atypia (anisokaryosis, macrokaryosis, and mitosis) can be seen. Infiltration of eosinophils and reactive mesenchymal cells are frequently observed. In contrast, undifferentiated high-grade Kiupel MCTs exfoliate with a high number of round cells with a moderate amount of clear/gray cytoplasm and with few visible or without granules (Raskin 2010). Some authors report a "fried-egg" appearance of the cells. Cells can show a variety of cellular and nuclear atypia which can also be used to predict histopathological Kiupel grade with good sensitivity and specificity

(Scarpa et al. 2016). In some cases, undifferentiated MCTs need further diagnostic investigations to be distinguished from other round cell tumors. Although not specific, eosinophils, when present, can help to hypothesize MCT as a definitive diagnosis. Presurgical biopsy and histopathological examination could be useful when a wide margin surgery is not possible and other treatment options (radiation therapy, chemotherapy, tyrosine kinase inhibitor, electrochemotherapy, intralesional triamcinolone, corticosteroids, tiginalol tiglate) that can achieve complete remission, should be used in a neoadjuvant setting. As seen above, histopathological grade and the possibility to test other prognostic factors with immunohistochemistry are needed to offer the best treatment option to dogs with MCTs.

The second step should be to locate and sample the sentinel lymph node. As seen above, the lack of routine lymphography in most facilities heightens the role of RLNs in the staging process. RLNs can be detected with palpation during a physical examination, or via imaging (ultrasound and CT scan are most used). However, normal size RLNs do not exclude metastasis (Ferrari et al. 2018). Cytological examination is recommended for all cases, but also RLN excision and histopathological examination are highly recommended with high-risk MCT, in dogs presented with enlarged RLN, and when it is feasible, even in dogs with non-high-risk MCT and normal-sized RLN. Until today, lymph nodes can be pathologically staged from HN0 (absence of metastasis) to HN3 (overt metastasis) with some evidence to be prognostic (Weishaar et al. 2014).

Abdominal ultrasound is highly recommended in all cases for the staging of MCT, especially when tumors are located in the hind limbs or perineal/inguinal region. Abdominal ultrasound helps to rule out other diseases that commonly occur in old dogs and that sometimes need to be managed simultaneously or before the treatment of the tumor. Since MCTs metastasizes to lymph nodes and then primarily to liver and spleen, ultrasound is a noninvasive and low-cost procedure able to assess the structure and texture of abdominal lymph nodes and organs. Moreover, since fine-needle aspiration biopsy of liver and spleen is considered more sensitive and specific than ultrasound to detect metastasis of MCTs, ultrasound is usually used to guide the needle into the organs (Book et al. 2011).

Currently, bone marrow evaluation is requested only in few cases, mostly to evaluate peripheral blood alterations that need further investigations (Endicott et al. 2007). The buffy-coat evaluation can help in the detection of peripheral mastocytosis but it is not specific for MCTs, and therefore, not performed routinely (McManus 1999). Thoracic radiographs are more commonly used to exclude other cardiopulmonary disorders that can influence the priority of treatment (i.e., pleural effusion) or complicate anesthesia but are not considered useful in the staging MCTs in dogs since pulmonary metastasis is uncommon (London and Thamm 2020; Warland et al. 2014).

1.5 Treatment

Treatment decisions should be evaluated case by case, considering the presence or absence of prognostic factors. Surgery is the treatment of choice for cutaneous MCTs localized in areas amenable to wide surgical excision. Since the majority of MCTs are low to intermediate grade (Patnaik grade I or II and Kiupel low grade) associated with no RLN metastasis, they can mostly be cured locally with surgery alone (London and Thamm 2020). When tumor size does not allow a wide excision or there is an increased risk for surgical complications, tumor biopsy and neoadjuvant therapy are suggested for the shrinkage of the tumor.

A variety of neoadjuvant treatments are described, all of them having pros and cons. Radiation therapy is one of the most reported neoadjuvant treatments usually used with palliative intent (Tollett et al. 2016; Dobson et al. 2004). Although radiation therapy has advantages and is considered the best alternative treatment to surgery as sole therapy for the treatment of low-grade MCTs, it is associated with some limitations. Multiple anesthesia, costs, and the lack of mainstream availability in all countries are the major limitations (Gordon 2020).

Systemic chemotherapy has been used to treat MCTs in neoadjuvant and adjuvant settings with incompletely excised MCT (Olsen et al. 2018; Thamm et al. 1999, 2006; Davies et al. 2004; Hosoya et al. 2009). However, its benefit is yet to be confirmed. Currently, chemotherapy is frequently applied in dogs with metastatic or high-risk MCTs (Marconato et al. 2020; Pizzoni et al. 2018; Thamm et al. 2006).

In the last 15 years, two veterinary-approved tyrosine kinase inhibitors (TKI) became commercially available on the market (masitinib mesylate and toceranib phosphate). *C-kit* mutations play an important role in the proliferation and survival of neoplastic mast cells. Masitinib and toceranib demonstrated different grades of clinical activity, with a greater response rate in *c-kit* mutant MCTs (London et al. 2009; Hahn et al. 2008). Both are indicated as neoadjuvant treatment in recurrent or non-resectable Patnaik grade II or grade III MCTs. Although combined therapy with radiation therapy or chemotherapy and TKI's have been reported, the role of TKI's as neoadjuvant treatment to surgery, still needs to be further investigated (Olsen et al. 2018; Carlsten et al. 2012).

Multimodal treatments are needed for metastatic, high-grade (Patnaik grade III/Kiupel high grade) or high-risk (presence of negative prognostic factors) MCTs.

Oral or intra-tumoral administration of corticosteroids has been associated with a response rate of up to 70%. However, long-term control with corticosteroids is not common and usually a combination with other treatment options, such as surgery, are needed (dos Santos et al. 2018; Case and Burgess 2018; Stanclift and Gilson 2008).

Recently, intralesional tigilanol tiglate, a novel diterpene ester extracted from an Australian native plant (*Fontainea picrosperma*), has been proposed as local therapy for nonmetastatic non-resectable, and non-ulcerated small MCTs (tumor volume inferior or equal to 8 cm^3). This drug can destroy tumor cells by modification of cell signaling process, inducing rapid hemorrhagic necrosis of the treated nodule. Furthermore, tigilanol tiglate is a strong protein kinase C activator and primarily targets

tumor vasculature. When applied intratumorally, this drug induces mitochondrial swelling and plasma membrane destruction in neoplastic cells. Response rate to this drug was 90%, at a dose of 1 mg/mL (0.5 mL per cm^3 of tumor volume), with mild and transient adverse effects (Miller et al. 2019). However, this treatment modality still needs further investigation to better understand its clinical benefit compared with the other treatments.

Electrochemotherapy (ECT) or electroporation coupled with intratumoral or intravenous chemotherapy is a novel, encouraging and promising treatment that could replace or support other therapeutic strategies. ECT in the treatment of MCTs was previously described in a few published studies (Spugnini et al. 2006, 2007, 2011; Kodre et al. 2009; Suzuki et al. 2015; Lowe et al. 2017).

In the next sections, the current knowledge on ECT, the use of ECT for the treatment of MCTs as a sole therapy or prior to surgery, and ECT combined with surgical resection intra- and postoperative will be discussed to help the clinician in the evaluation and selection of the candidates for this procedure. We will conclude with the use of gene-electrotransfer therapy (GET) in the treatment of MCTs.

1.5.1 Neoadjuvant Electrochemotherapy (Sole, Pre-surgery)

Currently, neoadjuvant electrochemotherapy may be considered an alternative to traditional treatment options in selected cases. A few studies already focused on the effectiveness of ECT in dogs with macroscopic MCTs. ECT has been reported to obtain complete response mainly in small tumors, regardless of the histological grade, suggesting ECT as an alternative option to surgery or radiation therapy, as a sole therapy for tumors localized in anatomical regions where wide excision is challenging. However, local treatment as a sole therapy for MCTs in dogs should be reserved only to selected patients based on various prognostic factors, not only on size and localization. Thus, we suggest a full staging of all dogs and the investigation of MCTs with biopsy to obtain prognostic information before ECT is pursued. Moreover, ECT can be used as a neoadjuvant treatment before surgery, since even large tumors may respond.

In a paper by Spugnini et al., the authors focused on the histological modifications that occur during the ECT procedure, in a group of 127 dogs and cats with different tumor types. Over a 7-year period, tumor biopsies were collected before treatment, after the first ECT and at the end of the treatment. The patients were treated with two cycles of ECT (bleomycin at a dose of 1.5 mg/mL, 8 biphasic electric pulses 50 + 50 μs each, with 1 ms interpulse intervals, and caliper electrodes), one week apart with intratumoral application of bleomycin (Spugnini et al. 2007).

After the first ECT, the histology showed an acute inflammatory response involving neutrophils, lymphocytes, and plasma cells, accompanied with an extensive necrosis. After the second ECT, the number of tumor cells was distinctively lower. They were mainly in apoptosis without any signs of inflammation and were mostly replaced by scar tissue. All these changes in the histological architecture seemed to be a consequence of ECT and happened in all treated tumors regardless of the tumor type. This article interestingly showed the microscopic changes in the tissue, caused by ECT and reported that high levels of necrosis (>55%) and apoptosis (>9%)

detected after the first ECT were independent factors associated with longer survival. Although twenty-eight dogs included in the study were affected by MCTs, treatment outcome was not evaluated for each tumor type separately (Spugnini et al. 2007). Readers should interpret survival analysis results with caution when referring to canine MCT.

In 2009, a Slovenian research group compared the outcome of dogs with MCTs treated with surgery or ECT coupled with intratumoral administration of cisplatin (Kodre et al. 2009). The ECT protocol consisted of an intratumoral administration of cisplatin (diluted in distilled water at a concentration of 2 mg/mL and administered intratumorally at dose ~1 mg/cm^3 of tumor volume) followed, by the application of electric pulses with plate electrodes 1–2 min after (8 pulses of 100 μm each, 1300 V/cm and 1 Hz). The pulses were delivered concentrically to reduce the blood flow, starting from the margins toward the tumor center. If the treatment did not give a complete response (CR), it was repeated 2–4 weeks after, without any additional medications.

Sixteen dogs with 16 tumors were treated with surgery and 9 dogs with 12 tumors, whose owners refused surgical treatment, were treated with ECT alone. Median tumor size in the surgery group was 5.2 cm^3, mostly located on the head, while in the ECT group, median size was 2.9 cm^3, and the tumors mostly located on the hindlegs. The median time of follow-up was greater than 18 months in both groups. Overall disease-free interval (DFI) was 31.5 months in the surgery group, however, in 5/7 dogs with Patnaik grade III MCTs the DFI for recurred tumors was only 8.5 months. Four to five weeks after ECT, 62.5% of the population achieved CR, but DFI was not reached. Two dogs did not respond to ECT, though both tumors were larger than 8 cm^3. Although, in the group treated with ECT, the histological grading was not performed, there was no significant difference between the two treatment methods when comparing CRs. The side effects after ECT were limited to partial necrosis of the tumor nodules and no degranulation of neoplastic cells was noticed. The authors hypothesized that the reduction in the local blood flow caused by the concentrically pulse delivering might prevent reactions caused by mast cell degranulation.

In conclusion, the study by Kodre et al. showed that ECT could achieve long-term local tumor control in dogs with MCTs, proposing ECT as an alternative treatment option to surgery, especially in cases when owners refuse surgical excision or other procedures. However, the interpretation of results can be biased by both the lack of histological grading of MCTs in the ECT group and the difference in volume of the tumors treated in the two groups. Nowadays, the histological grade, especially in MCTs, is an essential prognostic factor to predict the outcome of the disease. To highlight, almost half of the tumors in the surgery group were grade III (7/16) and the recurrence rate was quite high (5/7). Moreover, one of the tumors that recurred after surgery was a 0.7 cm^3 Patnaik grade I MCT, making the interpretation of results quite difficult. Nevertheless, the study properly described the outcome and toxicity of the ECT group, and it should be consulted if clinicians have the intention to pursue ECT for the treatment of MCTs (Kodre et al. 2009).

A study by Suzuki et al. aimed to investigate the influence of tumor position and tissue thickness on the local electric field distribution and to compare the tumor model with a canine MCT treated with ECT (Suzuki et al. 2015).

The authors proposed ECT with plate electrodes as an effective treatment for tumors located in the dermis (deepness 0.8–1.5 mm) but also in subcutaneous tumors that are not deeper than 3 mm. These results are very interesting and promising but can be applied only to very small and superficial tumors using plate electrodes. As for the efficacy on larger tumors, this study does not give any information to be used in practice (Suzuki et al. 2015).

In a study conducted by Lowe et al. ECT was offered as a sole treatment, when owners refused treatment with surgery and/or radiation therapy of MCT (group ECT alone), but also as a rescue treatment when surgery was already performed, and dogs had macroscopic local tumor recurrence (group ECT recur) (Lowe et al. 2017).

Tumors were mostly Patnaik grade II MCTs, with the mitotic index less than 5 in all samples. The pulses delivered were 8 monophasic square pulses of 0.1 ms each, with a frequency of 1 Hz to 5 kHz, 1000–1200 V/cm with different types of needle electrodes. Bleomycin was used in all patients at a dose of 15,000 IU/m^2 IV, 8 min before the application of electric pulses. The group ECT alone included 15 patients with a median tumor size of 1 cm, mostly located on the head region. ECT was applied on the tumor surface and margins (1–2 cm). The time between surgery and ECT in the ECT recur group of 11 dogs, was longer than a month and the median size of the recurred tumors was 4.1 cm, mostly located in the ischial and thigh regions. The dogs in both groups responded to the treatment. In the ECT alone group, 80% and 20% experienced CR and partial response (PR), respectively. Whereas in the recur group, 64% and 36% of the dogs experienced CR and PR, respectively. Median DFI was greater than 500 days and did not differ between the two groups. The local toxicity for both groups was low, and it ranged from 1 to 4 on a scale from 0 to 5, where grade 0 meant no toxicity at all, followed by grade 1 = slight swelling, grade 2 = swelling and necrosis <1 cm, grade 3 = severe swelling, grade 4 = deep necrosis, and grade 5 = severe swelling and tissue loss.

The study groups and the inclusion criteria described by Lowe et al. get close to what could be considered as the perfect candidate for ECT. The small median tumor size, tumors located where surgery would be difficult to perform or cause aesthetical changes that owners are not eager to accept, but also low-grade tumors are the main criteria when ECT should be considered as a first-line treatment (sole or neoadjuvant treatment). Moreover, three dogs in the ECT alone group that experienced PR, required additional surgical treatment, in order to obtained a full local control of the disease, justifying the use of ECT as a neoadjuvant option in some patients. Finally, the study by Lowe et al. suggested that dogs with local recurrence should be considered good candidates for ECT when other options are refused by owners.

1.5.2 Adjuvant Electrochemotherapy (Post- and Intraoperative)

The locoregional tumor control is mandatory to reach a good outcome with long DFI and prolonged survivals in dogs diagnosed with MCT. However, even when well planned in some cases a wide margin surgery cannot be performed without the high

risk of cosmetic or functional side effects. In such cases, a debulking surgery is often discussed as the initial step of a multimodal treatment followed by adjuvant therapy (ies) (radiation therapy most commonly) for the treatment of the inevitable incomplete margins. In other cases, although a "macroscopic" wide margin surgery is performed, margins result microscopically incomplete, making a second surgery challenging or impossible. When the evaluation of the absence or presence of prognostic factors and/or the owners will push the clinicians to consider further therapies to control the microscopic residual disease, ECT could be an adjuvant therapy to be taken into consideration. For this reason, ECT has been proposed when radiation therapy was refused by owners and when the systemic chemotherapy was believed unnecessary to treat a localized disease. In particular two modalities have already been described. The first one is intraoperative ECT performed when the tumor has to be removed with marginal surgery. In this case ECT is applied on the surgical bed, immediately after the mass excision and before the closure of the wound. On the other hand, the second modality is the postoperative ECT, performed on the area around the surgical scar, with incomplete margins.

The first paper considering adjuvant ECT included twenty-eight dogs with nonmetastatic incompletely excised MCTs treated with postsurgical ECT (Spugnini et al. 2006). Dogs were treated with two cycles of ECT coupled with bleomycin one week apart (bleomycin at a concentration of 1.5 IU/mL, 8 biphasic pulses lasting 50 + 50 μs, frequency 1 Hz, voltage 1300 V/cm, and modified caliper type electrodes). Bleomycin was injected in the treated area, which included the surgical scar and 1 cm in the surrounding tissue. Five minutes after the injection of the drug, the area was treated with electroporation. Electroporation field ranged from 5 to 400 cm^2 (average 28 cm^2). Most of the MCTs were Patnaik grade II, located on the limbs. The recurrence rate was 18% (5 out of 28 dogs) with an estimated mean survival time of months. Two of the five dogs with recurrence had Patnaik grade III MCT. The overall local toxicity, defined as cutaneous changes within the treatment field, was uncommon and no dogs experienced gastrointestinal toxicity. Thirty minutes after treatment two dogs showed local edema with mild erythema. One dog experienced wound dehiscence and prolonged healing. The good outcome of MCTs treated with incomplete excision and postoperative ECT observed in this study proposed adjuvant ECT as an alternative treatment to radiation therapy (Spugnini et al. 2006).

A few years later, Spugnini and his coworkers evaluated the tolerability and efficacy of ECT coupled with cisplatin in thirty-seven dogs with nonmetastatic incompletely or un-excised MCTs, Patnaik grade II located on the limbs (Spugnini et al. 2011).

The ECT protocol (cisplatin at a concentration of 0.5 mg/mL, 8 biphasic pulses, 50 + 50 μs, frequency 1 Hz, 1300 V/cm or 800 V/cm, and modified needle electrodes) included two sessions 1–2 weeks apart with intratumoral injection of cisplatin, 1.5 cm around the surgical scar. Before the treatment dogs also received a dose of hyaluronidase in the area to be treated to increase the absorption of cisplatin. The mean dose of cisplatin and hyaluronidase was 4.5 mg and 175 IU, respectively. Electroporation field ranged from 4 to 40 cm^2.

Response to treatment without any recurrence of the disease and a follow-up period (median 365 days) was reached in 78% of dogs. No specific information regarding the clinical benefit or response rate of dogs with gross disease was reported. Interestingly, none of the 6 dogs with Patnaik grade III MCTs developed recurrence during the follow-up. According to the authors, 32% of dogs developed ECT-induced edema and erythema, 10 min after the procedure and lasted for about 30 min. No systemic toxicity was noticed. Even though, this latter study resulted in a well-tolerated treatment, the lack of more detailed findings does not allow the complete understanding of its clinical application.

A recent study described the use of ECT coupled with systemic bleomycin in intraoperative and postoperative settings (bleomycin at a dose of 15,000 IU/m^2, 8 monophasic square pulses of 0.1 ms each, frequency 1 Hz to 5 kHz, 1000–1200 V/cm, and different types of needle electrodes) (Lowe et al. 2017). The decision to treat the tumors intraoperatively was made since only a marginal surgery was feasible. Intraoperative ECT was applied on the tumor bed and 1–2 cm in the lateral margins, immediately after the mass excision and before the wound closure. On the other hand, postoperative ECT was offered to dogs with incompletely excised MCTs and was applied 2–4 weeks after surgery, on the healed surgical scar, with a lateral margin of 1–1.5 cm.

Eleven dogs, mostly with Patnaik grade II MCTs located on the limb, were included in the intraoperative group and tumor size ranged from 0.7 to 6 cm with a median of 2.8 cm. However, 14 dogs with MCTs of a median tumor size 2.2 cm received postoperative ECT. Ninety-one percent and 93% of the intraoperative and postoperative ECT groups, respectively, showed no local recurrence during the follow-up period (follow-up duration not reported). When compared with neoadjuvant and intraoperative settings, postoperative ECT showed significantly longer DFI. However, the lack of a rate of incomplete surgical margins in the intraoperative group, and the lack of data regarding follow-up duration make further consideration, in this paper, challenging (Lowe et al. 2017).

Even though postoperative and intraoperative ECT coupled with bleomycin or cisplatin showed promising results, future prospective, randomized, case-control studies are necessary to better highlight the role of ECT as adjuvant treatment for (presumably) incompletely excised MCTs.

1.5.3 Gene-Electrotransfer Therapy

The following section will focus on the results obtained with a newer treatment modality in immunotherapy already described in previous chapters. Although only a few studies reported the combination of ECT and gene-electrotransfer (GET, also known as electrogene therapy) with IL-12 to treat MCTs in dogs, they represent a new frontier of treatment using the antitumoral mechanism of action different from conventional treatments (Pavlin et al. 2011; Čemažar et al. 2017; Salvadori et al. 2017; Impellizeri et al. 2014).

The first study by Pavlin et al. (2011) aimed to evaluate the local and systemic effectiveness of IL-12 applied with GET and the possible side effects in treatment of MCTs. The study included 8 patients who received an intratumoral injection of

plasmid encoding IL-12 followed by the release of electric pulses. The pulse parameters were consistent with an initial high voltage pulse (1 × 1200 V/cm, 100 μs) followed by 8 low voltage pulses (8 × 50 ms, 140 V/cm, 2 Hz). Some of the dogs included in the study were scheduled for surgery and had GET before, some had recurrent disease and tried all the other available options without success, and lastly some refused other treatment options and GET was the only one left. GET was delivered as the only treatment in some patients, applied in one or more sessions, followed by surgery, systemic chemotherapy, or ECT with intratumoral cisplatin. The success of the treatment was evaluated at each visit by measurement of the treated area and by the measurement of the amount of IL-12 and IFN-γ in the patients' blood to evaluate the systemic response. The progress of the treatment reached its maximum effect 1–2 weeks after the last GET procedure (from 1 to 4 sessions were performed). Most of the tumors (9/11) showed a major reduction in size already 1 week after the last GET before any other treatment procedure was applied. The initial tumor size, before treatment ranged from 0.03 to 25.4 cm^3 and was reduced significantly 1–2 weeks after the last GET, when the tumor size ranged from 0.005 to 18 cm^3. These measurements were made after the last GET and before any other procedure was performed. Two dogs treated only with GET with two and four sessions, respectively, achieved a partial response for a minimum of 3 years. Tumors were biopsied before and after the last GET session. After the treatment, the number of mast cells, estimated with tissue histology, was evidently lower compared to the tissue sampled before treatment. Moreover, in the posttreatment biopsies, clusters of leukocytes were found. The presence of human IL-12 and canine IFN-γ in the serum collected after GET was confirmed in 4 patients. No side effects to the treatment were identified.

This type of treatment showed a promising outcome, without any side effects. The infiltration of lymphocytes, plasma cells, and degranulation of the residual mast cells in the treated areas, after the local application of IL-12, was the most important finding, suggesting an antitumoral effect due to the stimulation of the immune system against the tumor (Pavlin et al. 2011).

A few years later, the same research group evaluated the efficacy and safety of ECT coupled with GET with IL-12 in canine MCTs (Čemažar et al. 2017). The authors' hypothesis was that the combined treatment could lead to increased activation of the immune system, which is greater when the two treatments are combined. They focused on the side effects of the therapy and the safety of using plasmid DNA delivered IL-12. The GET treatment consisted of intradermal injection of IL-12 in two locations around the tumor and then the delivery of electric pulses (1 × 1200 V/cm, 100 μs, followed by 1 × 400 ms, 140 V/cm). The ECT treatment consisted of intratumoral injection of cisplatin (1 mg cm^{-1}) and bleomycin (1 mg cm^{-1}), or intravenous bleomycin (0.3 mg kg^{-1}) in some cases coupled with electrical impulses (8 electric pulses of 100 μs duration, 1300 V/cm, and 5 kHz, using plate electrodes). The levels of cytokines in the patients' blood were measured before and up to 3 months after treatment. To test the presence of residual plasmid DNA, skin swabs were taken from the different spots and from the treated area to evaluate the

possibility of a horizontal gene transfer. The included tumors had a median size of 2.1 cm^3 (Čemažar et al. 2017).

At the end of the study period, 72% of the patients achieved CR (13/18) and two achieved PR (2/18) with an overall response rate of 83%. The CR was influenced by the size of the tumor since most nodules that achieved CR were <2 cm^3 in size. In larger nodules (>2 cm^3) CR was 60%. As seen in other studies, 4 weeks after treatment, the reduction in the tumor tissue was histologically noticed. Also, the number of microvessels was lower than before treatment and infiltrates of lymphocytes were present. No side effects to the treatment were noticed (Čemažar et al. 2017).

Similar findings have been reported later in another study by the same group of researchers. The authors investigated the histopathological changes in low-grade MCT samples collected by biopsy before treatment and then 4 and 8 weeks after ECT plus GET treatment (Salvadori et al. 2017).

The tissue samples collected 4 weeks after the treatment in 7 patients with a CR showed a substitution of the neoplastic tissue with fibrotic tissue related to inflammatory infiltration with lymphocytes and macrophages. Three dogs with a partial response had still some evidence of neoplastic tissue and in one case the situation was not much different as before the treatment. Eight weeks after treatment, the samples of the 7 dogs that were already free of neoplastic cells 4 weeks after the procedure, were infiltrated with mononuclear cells and free from neoplastic cells. In the remaining 3 dogs, 2 PRs, and one stable disease (SD), the histological situation was similar. All three of them had connective tissue infiltrated with small amounts of neoplastic cells. In one case with PR, the situation did not change and remained as it was before the treatment (Salvadori et al. 2017). Even though the statistics was not significant, the authors noted an increased number of T-lymphocytes 4 weeks after treatment, but the numbers of lymphocytes lowered 8 weeks after treatment. In subjects who achieved CR, the number of CD3+ lymphocytes were higher at 4 and 8 weeks after the procedure, compared to dogs that had achieved a PR, progressive disease (PD) or SD. They also noticed a higher number of macrophages 8 weeks after treatment and high T-reg lymphocytes 4 weeks after treatment. In addition, 4 and 8 weeks after treatment, proliferation of neoplastic cells was reduced, and was lower in patients that achieved a CR. The expression of the anti-apoptotic Bcl-2 protein was higher 4 weeks after treatment and then lowered down. The microvessel density was highly reduced in the posttreatment time (Salvadori et al. 2017).

Though the results regarding GET are quite optimistic, they are not different from historical results obtained with ECT alone (see neoadjuvant ECT session). Thus, the interpretation of the response was due to GET, ECT, or the combination of both is not possible. However, the activation of immune system is very interesting, and more studies are needed to prove its antitumoral effect. If proven, GET could be considered for the activation of the immune system when treating dogs with metastasis or with multiple MCTs, in order to reduce the progression of the disease in distant organs and to reduce the risk of new tumor development (Salvadori et al. 2017).

2 Feline Cutaneous Mast Cell Tumor

Feline MCTs can occur in three different forms, overlapping one another, i.e., splenic/visceral, intestinal, and cutaneous. As in dogs also in cats, the involvement of mutations in *c-kit* is present but less well studied and defined (Saar et al. 1969; Isotani et al. 2010; Berger et al. 2018).

Cats affected by splenic MCT tend to have an enlarged spleen and/or liver, accompanied by clinical signs as vomiting, anorexia, and weight loss. Systemic signs caused by the release of vasoactive substances by mastocytes can also occur (Carpenter et al. 1987; Feinmehl et al. 1992). The splenic MCT is the most common differential diagnosis for splenic diseases in older cats (Carpenter et al. 1987; Litster and Sorenmo 2006; Spangler and Culbertson 1992). Even though the spleen is mostly affected, other organs, such as liver, visceral lymph nodes, bone marrow, lung, and intestine may also be involved. Peritoneal and pleural effusion with eosinophils and mast cells is common as well as the presence of mast cells in the peripheral blood (Carpenter et al. 1987; Feinmehl et al. 1992; Skeldon et al. 2010). MCT of the spleen can occur in two forms, diffuse or nodular, the last one being less common (Sato and Solano 2004; Hanson et al. 2001). The diagnosis should include abdominal ultrasound with FNA, and complete blood work (Carpenter et al. 1987; Feinmehl et al. 1992). The treatment of choice is splenectomy, even if the release of mast cell mediators, that could cause intraoperative death is probable (Sato and Solano 2004; Hanson et al. 2001).

The intestinal form of MCT is the third most common intestinal tumor in cats. As before, older cats are more at risk. The cats are presented with vomiting, diarrhea, anorexia, and a palpable abdominal mass at the clinical examination. Lesions can be solitary or multiple and more commonly located in the small intestine (Carpenter et al. 1987; Bortnowski and Rosenthal 1992; Halsey et al. 2010). As in the splenic form, peritoneal effusion of eosinophils and mast cells can be present. The disease is typically metastatic to the lymph nodes and liver. Unlike the splenic form, mast cell infiltration of the peripheral blood is less common. The prognosis of this form is poor, mostly due to the late discovery of the disease that is already metastatic at the time of diagnosis (Carpenter et al. 1987; Bortnowski and Rosenthal 1992; Halsey et al. 2010; Sabattini et al. 2016*)*. The treatment of choice is surgery with wide surgical margins (5–10 cm), because microscopically the spread of the tumor is greater than what is seen macroscopically (Carpenter et al. 1987; Bortnowski and Rosenthal 1992). The use of chemotherapy is limited, but lomustine showed some good responses. As in the splenic MCT, c-KIT inhibitors showed some efficacy (Rassnick et al. 2008; Berger et al. 2018).

The cutaneous form of MCT is the second most common skin tumor in cats with different incidence (e.g., accounting for 20% of all feline cutaneous tumors in the USA and only for 8% in the United Kingdom) (Bostock 1986; Carpenter et al. 1987; Miller et al. 1991; Brodey 1970). Cutaneous MCT in cats is usually a solitary mass with alopecia, white in color, sometimes erythematosus, or with superficial ulceration, mostly not bigger than 3 cm. Some studies reported that 20% of cats can present multiple nodules (Bostock 1986; Carpenter et al. 1987; Miller et al. 1991; Burger

and Scott 1987; Wilcock et al. 1986). Cats from 8 to 9 years are more prone to develop a MCT and Siamese cats have a slight breed predisposition (Carpenter et al. 1987; Miller et al. 1991; Chastain et al. 1988; Burger and Scott 1987; Wilcock et al. 1986). The most common anatomical regions for MCTs to appear are the head and neck region, rarely in the oral cavity, followed by the trunk and limbs (Carpenter et al. 1987; Miller et al. 1991; Burger and Scott 1987; Macy and Reynolds 1981). The cutaneous form can be divided into the mastocytic subtype, subdivided into a compact, well-differentiated form (50–90% of cats) or diffuse anaplastic form, and the histiocytic subtype, less common and usually spontaneously regressing (Carpenter et al. 1987; Chastain et al. 1988; Wilcock et al. 1986; Holzinger 1973). The well-differentiated compact form has a benign behavior and metastases are rare (Burger and Scott 1987; Litster and Sorenmo 2006; Molander-McCrary et al. 1998). On the other hand, the anaplastic form may be presented with a high MI and infiltrative nature (Wilcock et al. 1986). The mastocytic subtype, histologically similar to canine MCT, can cause pruritus and erythema, so self-trauma and ulceration are also very common. The Darier's sign described in dogs is also seen in cats during tumor manipulation (Isotani et al. 2010; Chastain et al. 1988; Macy and Reynolds 1981; Wilcock et al. 1986). Most of the tumors can be diagnosed with simple cytology, except for the rare histiocytic form which is more complicated to diagnose (Chastain et al. 1988; Wilcock et al. 1986). When diagnosing a cutaneous MCT, a detailed clinical examination should be performed to rule out other nodules, lymph nodes, and spleen involvement. One study demonstrated that cats with multiple cutaneous MCT usually have also the splenic MCT (Litster and Sorenmo 2006; Garrett et al. 2007). Even if the grading system for dogs is not prognostically adequate for cats, a high MI is a predicting factor for the biological behavior of the tumor (Macy and Reynolds 1981; Wilcock et al. 1986; Johnson et al. 2002; Dobromylskyj et al. 2015; Melville et al. 2015).

Nowadays, the treatment of choice for feline cutaneous MCT is excisional surgery (Molander-McCrary et al. 1998). Sometimes surgery can be combined with radiation therapy or cryotherapy (Montgomery et al. 2010). The data collected suggest that feline MCT are mostly benign and wide surgical margins required in dogs, are not necessary. Also, the systemic infiltration after surgery seems to be from 0 to 22%, with most of the metastatic tumors being anaplastic (Burger and Scott 1987; Wilcock et al. 1986; Litster and Sorenmo 2006; Molander-McCrary et al. 1998; Johnson et al. 2002). These tumors, that also tend to have a higher MI, require more attention and an aggressive treatment (Johnson et al. 2002; Lepri et al. 2003). If tumors cannot be resected completely, radiation therapy is the treatment of choice to follow surgery (Turrel et al. 2006). Chemotherapy is a field that still needs to be investigated in treatment of feline cutaneous MCT. Prednisone seems to be less effective than in canine MCT, but lomustine has shown some efficacy (Chastain et al. 1988; Rassnick et al. 2008). The use of masitinib and toracenib has shown some effects but still needs further evaluation (Isotani et al. 2006, 2010; Lachowicz et al. 2005; Schulman 1987; Bellamy et al. 2009; Daly et al. 2011; Berger et al. 2018; Harper and Blackwood 2017). ECT could be offered as an additional choice to avoid surgery, even if marginal surgery seems curative in almost all mastocytic MCTs.

Indeed, when coming across owners who are not keen for surgery, ECT could be a good second option to propose. Also, ECT could be used as a palliative treatment for cutaneous metastasis of visceral MCTs when ulceration, pain, or discomfort reduce cat's quality of life.

Bibliography

Bellamy F, Bader T, Moussy A, Hermine O (2009) Pharmacokinetics of masitinib in cats. Vet Res Commun 33(8):831–837. https://doi.org/10.1007/s11259-009-9231-6

Berger EP, Johannes CM, Post GS et al (2018) Retrospective evaluation of toceranib phosphate (Palladia) use in cats with mast cell neoplasia. J Feline Med Surg 20(2):95–102. https://doi.org/10.1177/1098612X17695898

Book AP, Fidel J, Wills T, Bryan J, Sellon R, Mattoon J (2011) Correlation of ultrasound findings, liver and spleen cytology, and prognosis in the clinical staging of high metastatic risk canine mast cell tumors. Vet Radiol ultrasound Off J Am Coll Vet Radiol Int Vet Radiol Assoc 52 (5):548–554. https://doi.org/10.1111/j.1740-8261.2011.01839.x

Bortnowski HB, Rosenthal RC (1992) Gastrointestinal mast cell tumors and eosinophilia in two cats. J Am Anim Hosp Assoc 28:271–275

Bostock DE (1986) Neoplasms of the skin and subcutaneous tissues in dogs and cats. Br Vet J 142 (1):1–19. https://doi.org/10.1016/0007-1935(86)90002-3

Bostock DE, Crocker J, Harris K, Smith P (1989) Nucleolar organiser regions as indicators of post-surgical prognosis in canine spontaneous mast cell tumours. Br J Cancer 59(6):915–918. https://doi.org/10.1038/bjc.1989.193

Brodey RS (1970) Canine and feline neoplasia. Adv Vet Sci Comp Med 14:309–354

Burger R, Scott D (1987) Cutaneous mast cell neoplasia in cats: 14 cases (1975-1985). J Am Vet Med Assoc 190:1440–1444

Cahalane AK, Payne S, Barber LG et al (2004) Prognostic factors for survival of dogs with inguinal and perineal mast cell tumors treated surgically with or without adjunctive treatment: 68 cases (1994-2002). J Am Vet Med Assoc 225(3):401–408. https://doi.org/10.2460/javma.2004.225.401

Carlsten KS, London CA, Haney S, Burnett R, Avery AC, Thamm DH (2012) Multicenter prospective trial of hypofractionated radiation treatment, toceranib, and prednisone for measurable canine mast cell tumors. J Vet Intern Med 26(1):135–141. https://doi.org/10.1111/j.1939-1676.2011.00851.x

Carpenter JL, Andrews LK, Holzworth J (1987) Tumors and tumor-like lesions. In: Holzworth J (ed) Diseases of the cat: medicine and surgery. WB Saunders, Philadelphia, pp 406–596

Case A, Burgess K (2018) Safety and efficacy of intralesional triamcinolone administration for treatment of mast cell tumors in dogs: 23 cases (2005–2011). J Am Vet Med Assoc 252 (1):84–91

Čemažar M, Ambrožič Avgustin J, Pavlin D et al (2017) Efficacy and safety of electrochemotherapy combined with peritumoral IL-12 gene electrotransfer of canine mast cell tumours. Vet Comp Oncol 15(2):641–654. https://doi.org/10.1111/vco.12208

Chastain CB, Turk MA, O'Brien D (1988) Benign cutaneous mastocytomas in two litters of Siamese kittens. J Am Vet Med Assoc 193(8):959–960

da Silva EZM, Jamur MC, Oliver C (2014) Mast cell function: a new vision of an old cell. J Histochem Cytochem 62(10):698–738. https://doi.org/10.1369/0022155414545334

Daly M, Sheppard S, Cohen N et al (2011) Safety of masitinib mesylate in healthy cats. J Vet Intern Med 25(2):297–302. https://doi.org/10.1111/j.1939-1676.2011.0687.x

Davies DR, Wyatt KM, Jardine JE, Robertson ID, Irwin PJ (2004) Vinblastine and prednisolone as adjunctive therapy for canine cutaneous mast cell tumors. J Am Anim Hosp Assoc 40 (2):124–130. https://doi.org/10.5326/0400124

Dobromylskyj MJ, Rasotto R, Melville K, Smith KC, Berlato D (2015) Evaluation of minichromosome maintenance protein 7 and c-KIT as prognostic markers in feline cutaneous mast cell tumours. J Comp Pathol 153(4):244–250. https://doi.org/10.1016/j.jcpa.2015.08.005

Dobson J, Cohen S, Gould S (2004) Treatment of canine mast cell tumours with prednisolone and radiotherapy. Vet Comp Oncol 2(3):132–141. https://doi.org/10.1111/j.1476-5810.2004.00048.x

dos Santos HR, Eunice Lavalle G, Narducci Monteiro L et al (2018) Evaluation of histological, immunohistochemical, clinical and genetic prognostic factors associated with the response of canine mast cell tumours to glucocorticotherapy. J Comp Pathol 165:72–81. https://doi.org/10.1016/j.jcpa.2018.10.001

Downing S, Chien MB, Kass PH, Moore PE, London CA (2002) Prevalence and importance of internal tandem duplications in exons 11 and 12 of c-kit in mast cell tumors of dogs. Am J Vet Res 63(12):1718–1723. https://doi.org/10.2460/ajvr.2002.63.1718

Elston LB, Sueiro FAR, Cavalcanti JN, Metze K (2009) The importance of the mitotic index as a prognostic factor for survival of canine cutaneous mast cell tumors: a validation study. Vet Pathol 46(2):362–364., author reply 364–5. https://doi.org/10.1354/vp.46-2-362

Endicott MM, Charney SC, McKnight JA, Loar AS, Barger AM, Bergman PJ (2007) Clinicopathological findings and results of bone marrow aspiration in dogs with cutaneous mast cell tumours: 157 cases (1999-2002). Vet Comp Oncol 5(1):31–37. https://doi.org/10.1111/j.1476-5829.2006.00115.x

Feinmehl R, Matus R, Mauldin GN, Patnaik AK (1992) Splenic mast cell tumours in 43 cats (1975–1992). In: Proceedings annual conference of the veterinary cancer society, vol 12, p 50

Ferrari R, Marconato L, Buracco P et al (2018) The impact of extirpation of non-palpable/normal-sized regional lymph nodes on staging of canine cutaneous mast cell tumours: a multicentric retrospective study. Vet Comp Oncol 16(4):505–510. https://doi.org/10.1111/vco.12408

Finnie JW, Bostock DE (1979) Skin neoplasia in dogs. Aust Vet J 55(12):602–604. https://doi.org/10.1111/j.1751-0813.1979.tb07068.x

Galli SJ, Zsebo KM, Geissler EN (1994) The kit ligand, stem cell factor. Adv Immunol 55:1–96. https://doi.org/10.1016/s0065-2776(08)60508-8

Garrett LD, Craig CL, Szladovits B, Chun R (2007) Evaluation of buffy coat smears for circulating mast cells in healthy cats and ill cats without mast cell tumor-related disease. J Am Vet Med Assoc 231(11):1685–1687. https://doi.org/10.2460/javma.231.11.1685

Gordon I (2020) Radiation facilities. http://vetcancersociety.org/vcs-members/links-of-interest-2/radiation-facilities, Update May 2020

Hahn KA, Oglivie G, Rusk T et al (2008) Masitinib is safe and effective for the treatment of canine mast cell tumors. J Vet Intern Med 22(6):1301–1309. https://doi.org/10.1111/j.1939-1676.2008.0190.x

Halsey CHC, Powers BE, Kamstock DA (2010) Feline intestinal sclerosing mast cell tumour: 50 cases (1997-2008). Vet Comp Oncol 8(1):72–79. https://doi.org/10.1111/j.1476-5829.2009.00206.x

Halsey CHC, Thamm DH, Weishaar KM et al (2017) Expression of phosphorylated KIT in canine mast cell tumor. Vet Pathol 54(3):387–394. https://doi.org/10.1177/0300985816688943

Hanson JA, Papageorges M, Girard E, Menard M, Hebert P (2001) Ultrasonographic appearance of splenic disease in 101 cats. Vet Radiol Ultrasound Off J Am Coll Vet Radiol Int Vet Radiol Assoc 42(5):441–445. https://doi.org/10.1111/j.1740-8261.2001.tb00967.x

Harper A, Blackwood L (2017) Toxicity and response in cats with neoplasia treated with toceranib phosphate. J Feline Med Surg 19(6):619–623. https://doi.org/10.1177/1098612X16643124

Hillman LA, Garrett LD, de Lorimier L-P, Charney SC, Borst LB, Fan TM (2010) Biological behavior of oral and perioral mast cell tumors in dogs: 44 cases (1996-2006). J Am Vet Med Assoc 237(8):936–942. https://doi.org/10.2460/javma.237.8.936

Holzinger EA (1973) Feline cutaneous mastocytomas. Cornell Vet 63(1):87–93

Horta RS, Lavalle GE, Monteiro LN, Souza MCC, Cassali GD, Araujo RB (2018) Assessment of canine mast cell tumor mortality risk based on clinical, histologic, immunohistochemical, and molecular features. Vet Pathol 55(2):212–223. https://doi.org/10.1177/0300985817747325

Hosoya K, Kisseberth WC, Alvarez FJ et al (2009) Adjuvant CCNU (lomustine) and prednisone chemotherapy for dogs with incompletely excised grade 2 mast cell tumors. J Am Anim Hosp Assoc 45(1):14–18. https://doi.org/10.5326/0450014

Impellizeri JA, Ciliberto G, Aurisicchio L (2014) Electro-gene-transfer as a new tool for cancer immunotherapy in animals. Vet Comp Oncol 12(4):310–318. https://doi.org/10.1111/vco.12006

Isotani M, Tamura K, Yagihara H et al (2006) Identification of a c-kit exon 8 internal tandem duplication in a feline mast cell tumor case and its favorable response to the tyrosine kinase inhibitor imatinib mesylate. Vet Immunol Immunopathol 114(1-2):168–172. https://doi.org/10.1016/j.vetimm.2006.07.004

Isotani M, Yamada O, Lachowicz JL et al (2010) Mutations in the fifth immunoglobulin-like domain of kit are common and potentially sensitive to imatinib mesylate in feline mast cell tumours. Br J Haematol 148(1):144–153. https://doi.org/10.1111/j.1365-2141.2009.07926.x

Johnson TO, Schulman FY, Lipscomb TP, Yantis LD (2002) Histopathology and biologic behavior of pleomorphic cutaneous mast cell tumors in fifteen cats. Vet Pathol 39(4):452–457. https://doi.org/10.1354/vp.39-4-452

Jones CLR, Grahn RA, Chien MB, Lyons LA, London CA (2004) Detection of c-kit mutations in canine mast cell tumors using fluorescent polyacrylamide gel electrophoresis. J Vet Diagn Investig 16(2):95–100. https://doi.org/10.1177/104063870401600201

Kiupel M, Webster JD, Kaneene JB, Miller R, Yuzbasiyan-Gurkan V (2004) The use of KIT and tryptase expression patterns as prognostic tools for canine cutaneous mast cell tumors. Vet Pathol 41(4):371–377. https://doi.org/10.1354/vp.41-4-371

Kiupel M, Webster JD, Bailey KL et al (2011) Proposal of a 2-tier histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behavior. Vet Pathol 48(1):147–155. https://doi.org/10.1177/0300985810386469

Kodre V, Čemazar M, Pečar J, Serša G, Cor A, Tozon N (2009) Electrochemotherapy compared to surgery for treatment of canine mast cell tumours. In Vivo 23(1):55–62

Kumar V, Sharma A (2010) Mast cells: emerging sentinel innate immune cells with diverse role in immunity. Mol Immunol [Internet] 48(1–3):14–25. http://dx.doi.org/10.1016/j.molimm.2010.07.009

Lachowicz JL, Post GS, Brodsky E (2005) A phase I clinical trial evaluating imatinib mesylate (Gleevec) in tumor-bearing cats. J Vet Intern Med 19(6):860–864. https://doi.org/10.1892/0891-6640(2005)19[860:apicte]2.0.co;2

Lepri E, Ricci G, Leonardi L, Sforna M, Mechelli L (2003) Diagnostic and prognostic features of feline cutaneous mast cell tumours: a retrospective analysis of 40 cases. Vet Res Commun 27(Suppl 1):707–709. https://doi.org/10.1023/b:verc.0000014253.07296.0c

Letard S, Yang Y, Hanssens K et al (2008) Gain-of-function mutations in the extracellular domain of KIT are common in canine mast cell tumors. Mol Cancer Res 6(7):1137–1145. https://doi.org/10.1158/1541-7786.MCR-08-0067

Litster AL, Sorenmo KU (2006) Characterisation of the signalment, clinical and survival characteristics of 41 cats with mast cell neoplasia. J Feline Med Surg 8(3):177–183. https://doi.org/10.1016/j.jfms.2005.12.005

London C, Thamm D (2020) Mast cell tumors. In: Vail D, Thamm D, Liptak J (eds) *Withrow & MacEwen's small animal clinical oncology*, 6th edn. Elsevier, St. Louis, MO, pp 382–403

London CA, Kisseberth WC, Galli SJ, Geissler EN, Helfand SC (1996) Expression of stem cell factor receptor (c-kit) by the malignant mast cells from spontaneous canine mast cell tumours. J Comp Pathol 115(4):399–414. https://doi.org/10.1016/s0021-9975(96)80074-0

London CA, Galli SJ, Yuuki T, Hu ZQ, Helfand SC, Geissler EN (1999) Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-kit. Exp Hematol 27(4):689–697. https://doi.org/10.1016/s0301-472x(98)00075-7

London CA, Malpas PB, Wood-Follis SL et al (2009) Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. Clin Cancer Res 15(11):3856–3865. https://doi.org/10.1158/1078-0432.CCR-08-1860

Lowe R, Gavazza A, Impellizeri JA, Soden DM, Lubas G (2017) The treatment of canine mast cell tumours with electrochemotherapy with or without surgical excision. Vet Comp Oncol 15 (3):775–784. https://doi.org/10.1111/vco.12217

Macy DW, Reynolds HA (1981) The incidence, characteristics and clinical management of skin tumors of cats. J Am Anim Hosp Assoc 17:1026–1034

Marconato L, Stefanello D, Kiupel M et al (2020) Adjuvant medical therapy provides no therapeutic benefit in the treatment of dogs with low-grade mast cell tumours and early nodal metastasis undergoing surgery. *Vet Comp Oncol* 18(3):409–415. https://doi.org/10.1111/vco.12566

McManus PM (1999) Frequency and severity of mastocytemia in dogs with and without mast cell tumors: 120 cases (1995-1997). J Am Vet Med Assoc 215(3):355–357

McNiel EA, Prink AL, O'Brien TD (2006) Evaluation of risk and clinical outcome of mast cell tumours in pug dogs. Vet Comp Oncol 4(1):2–8. https://doi.org/10.1111/j.1476-5810.2006.00085.x

Melville K, Smith KC, Dobromylskyj MJ (2015) Feline cutaneous mast cell tumours: a UK-based study comparing signalment and histological features with long-term outcomes. J Feline Med Surg 17(6):486–493. https://doi.org/10.1177/1098612X14548784

Mendez SE, Drobatz KJ, Duda LE, White P, Kubicek L, Sorenmo KU (2020) Treating the locoregional lymph nodes with radiation and/or surgery significantly improves outcome in dogs with high-grade mast cell tumours. Vet Comp Oncol 18(2):239–246. https://doi.org/10.1111/vco.12541

Metz M, Piliponsky AM, Chen C-C et al (2006) Mast cells can enhance resistance to snake and honeybee venoms. Science 313(5786):526–530. https://doi.org/10.1126/science.1128877

Miller MA, Nelson SL, Turk JR et al (1991) Cutaneous neoplasia in 340 cats. Vet Pathol 28 (5):389–395. https://doi.org/10.1177/030098589102800506

Miller J, Campbell J, Blum A et al (2019) Dose characterization of the investigational anticancer drug tigilanol tiglate (EBC-46) in the local treatment of canine mast cell tumors. *Front Vet Sci* 6:106. https://doi.org/10.3389/fvets.2019.00106

Mochizuki H, Motsinger-Reif A, Bettini C, Moroff S, Breen M (2017) Association of breed and histopathological grade in canine mast cell tumours. Vet Comp Oncol 15(3):829–839. https://doi.org/10.1111/vco.12225

Molander-McCrary H, Henry CJ, Potter K, Tyler JW, Buss MS (1998) Cutaneous mast cell tumors in cats: 32 cases (1991-1994). J Am Anim Hosp Assoc 34(4):281–284. https://doi.org/10.5326/15473317-34-4-281

Montgomery KW, van der Woerdt A, Aquino SM, Sapienza JS, Ledbetter EC (2010) Periocular cutaneous mast cell tumors in cats: evaluation of surgical excision (33 cases). Vet Ophthalmol 13(1):26–30. https://doi.org/10.1111/j.1463-5224.2009.00751.x

Moon TC, Befus AD, Kulka M (2014) Mast cell mediators: their differential release and the secretory pathways involved. *Front Immunol* 5:569. https://doi.org/10.3389/fimmu.2014.00569

Moore AS, Frimberger AE, Taylor D, Sullivan N (2020) Retrospective outcome evaluation for dogs with surgically excised, solitary Kiupel high-grade, cutaneous mast cell tumours. Vet Comp Oncol 18(3):402–408. https://doi.org/10.1111/vco.12565

Morini M, Bettini G, Preziosi R, Mandrioli L (2004) C-kit gene product (CD117) immunoreactivity in canine and feline paraffin sections. J Histochem Cytochem 52(5):705–708. https://doi.org/10.1177/002215540405200515

Mullins MN, Dernell WS, Withrow SJ, Ehrhart EJ, Thamm DH, Lana SE (2006) Evaluation of prognostic factors associated with outcome in dogs with multiple cutaneous mast cell tumors treated with surgery with and without adjuvant treatment: 54 cases (1998-2004). J Am Vet Med Assoc 228(1):91–95. https://doi.org/10.2460/javma.228.1.91

O'Keefe DA, Couto CG, Burke-Schwartz C, Jacobs RM (1987) Systemic mastocytosis in 16 dogs. J Vet Intern Med 1(2):75–80. https://doi.org/10.1111/j.1939-1676.1987.tb01990.x

Olsen JA, Thomson M, O'Connell K, Wyatt K (2018) Combination vinblastine, prednisolone and toceranib phosphate for treatment of grade II and III mast cell tumours in dogs. Vet Med Sci 4 (3):237–251. https://doi.org/10.1002/vms3.106

Patnaik AK, Ehler WJ, MacEwen EG (1984) Canine cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. Vet Pathol 21(5):469–474. https://doi.org/10.1177/030098588402100503

Pavlin D, Cemazar M, Cör A, Sersa G, Pogacnik A, Tozon N (2011) Gene-electrotransfer therapy with interleukin-12 in canine mast cell tumors. Radiol Oncol 45(1):30–39. https://doi.org/10.2478/v10019-010-0041-9

Pelt DR, Fowler JD, Leighton FA (1986) Multiple cutaneous mast cell tumors in a dog: a case report and brief review. Can Vet J = La Rev Vet Can 27(7):259–263

Peters JA (1969) Canine mastocytoma: excess risk as related to ancestry. J Natl Cancer Inst 42 (3):435–443

Pierini A, Lubas G, Gori E, Binanti D, Millanta F, Marchetti V (2019) Epidemiology of breed-related mast cell tumour occurrence and prognostic significance of clinical features in a defined population of dogs in West-Central Italy. Vet Sci 6(2):53. https://doi.org/10.3390/vetsci6020053

Pizzoni S, Sabattini S, Stefanello D et al (2018) Features and prognostic impact of distant metastases in 45 dogs with de novo stage IV cutaneous mast cell tumours: a prospective study. Vet Comp Oncol 16(1):28–36. https://doi.org/10.1111/vco.12306

Preziosi R, Sarli G, Paltrinieri M (2007) Multivariate survival analysis of histological parameters and clinical presentation in canine cutaneous mast cell tumours. Vet Res Commun 31 (3):287–296. https://doi.org/10.1007/s11259-006-3427-9

Raskin R (2010) Skin and subcutaneous tissues. In: Raskin R, Meyer D (eds) Canine and feline cytology - a color atlas and interpretation guide, 2nd edn. Elsevier, St. Louis, MO, pp 26–76

Rassnick KM, Williams LE, Kristal O et al (2008) Lomustine for treatment of mast cell tumors in cats: 38 cases (1999-2005). J Am Vet Med Assoc 232(8):1200–1205. https://doi.org/10.2460/javma.232.8.1200

Reguera MJ, Rabanal RM, Puigdemont A, Ferrer L (2000) Canine mast cell tumors express stem cell factor receptor. Am J Dermatopathol 22(1):49–54. https://doi.org/10.1097/00000372-200002000-00010

Romansik EM, Reilly CM, Kass PH, Moore PF, London CA (2007) Mitotic index is predictive for survival for canine cutaneous mast cell tumors. Vet Pathol 44(3):335–341. https://doi.org/10.1354/vp.44-3-335

Roskoski RJ (2005a) Signaling by Kit protein-tyrosine kinase-- stem cell factor receptor. Biochem Biophys Res Commun 337(1):1–13. https://doi.org/10.1016/j.bbrc.2005.08.055

Roskoski RJ (2005b) Structure and regulation of Kit protein-tyrosine kinase--the stem cell factor receptor. Biochem Biophys Res Commun 338(3):1307–1315. https://doi.org/10.1016/j.bbrc.2005.09.150

Rothwell TLW, Howlett CR, Middleton DJ, Griffiths DA, Duff BC (1987) Skin neoplasms of dogs in Sydney. Aust Vet J 64(6):161–164. https://doi.org/10.1111/j.1751-0813.1987.tb09673.x

Saar C, Opitz M, Lange W et al (1969) Mast cell reticulosis in cats. I. Berl Munch Tierarztl Wochenschr 82(22):438–444

Sabattini S, Scarpa F, Berlato D, Bettini G (2015) Histologic grading of canine mast cell tumor: is 2 better than 3? Vet Pathol 52(1):70–73. https://doi.org/10.1177/0300985814521638

Sabattini S, Giantin M, Barbanera A et al (2016) Feline intestinal mast cell tumours: clinicopathological characterisation and KIT mutation analysis. J Feline Med Surg 18(4):280–289. https://doi.org/10.1177/1098612X15581205

Salvadori C, Švara T, Rocchigiani G et al (2017) Effects of electrochemotherapy with cisplatin and peritumoral IL-12 gene electrotransfer on canine mast cell tumors: a histopathologic and

immunohistochemical study. Radiol Oncol 51(3):286–294. https://doi.org/10.1515/raon-2017-0035

Sato AF, Solano M (2004) Ultrasonographic findings in abdominal mast cell disease: a retrospective study of 19 patients. Vet Radiol Ultrasound Off J Am Coll Vet Radiol Int Vet Radiol Assoc 45 (1):51–57. https://doi.org/10.1111/j.1740-8261.2004.04008.x

Scarpa F, Sabattini S, Bettini G (2016) Cytological grading of canine cutaneous mast cell tumours. Vet Comp Oncol 14(3):245–251. https://doi.org/10.1111/vco.12090

Scase TJ, Edwards D, Miller J et al (2006) Canine mast cell tumors: correlation of apoptosis and proliferation markers with prognosis. *J Vet Intern Med* 20(1):151–158. https://doi.org/10.1892/0891-6640(2006)20[151,cmctco]2.0.co;2

Schulman A (1987) Splenic mastocytosis in a cat. Calif Vet 17:17–18

Seguin B, Besancon MF, McCallan JL et al (2006) Recurrence rate, clinical outcome, and cellular proliferation indices as prognostic indicators after incomplete surgical excision of cutaneous grade II mast cell tumors: 28 dogs (1994-2002). *J Vet Intern Med* 20(4):933–940. https://doi.org/10.1892/0891-6640(2006)20[933,rrcoac]2.0.co;2

Sfiligoi G, Rassnick KM, Scarlett JM, Northrup NC, Gieger TL (2005) Outcome of dogs with mast cell tumors in the inguinal or perineal region versus other cutaneous locations: 124 cases (1990-2001). J Am Vet Med Assoc 226(8):1368–1374. https://doi.org/10.2460/javma.2005.226.1368

Skeldon NCA, Gerber KL, Wilson RJ, Cunnington SJ (2010) Mastocytaemia in cats: prevalence, detection and quantification methods, haematological associations and potential implications in 30 cats with mast cell tumours. J Feline Med Surg 12(12):960–966. https://doi.org/10.1016/j.jfms.2010.08.003

Şmiech A, Ślaska B, Łopuszyński W, Jasik A, Bochyńska D, Da˛browski R (2018) Epidemiological assessment of the risk of canine mast cell tumours based on the Kiupel two-grade malignancy classification. Acta Vet Scand 60(1). doi:https://doi.org/10.1186/s13028-018-0424-2

Spangler WL, Culbertson MR (1992) Prevalence and type of splenic diseases in cats: 455 cases (1985-1991). J Am Vet Med Assoc 201(5):773–776

Spugnini EP, Vincenzi B, Baldi F, Citro G, Baldi A (2006) Adjuvant electrochemotherapy for the treatment of incompletely resected canine mast cell tumors. *Anticancer Res* 26(6 B):4585–4589

Spugnini EP, Baldi F, Mellone P et al (2007) Patterns of tumor response in canine and feline cancer patients treated with electrochemotherapy: preclinical data for the standardization of this treatment in pets and humans. J Transl Med 5(1):48. https://doi.org/10.1186/1479-5876-5-48

Spugnini EP, Vincenzi B, Citro G, Dotsinsky I, Mudrov T, Baldi A (2011) Evaluation of cisplatin as an electrochemotherapy agent for the treatment of incompletely excised mast cell tumors in dogs. J Vet Intern Med 25(2):407–411. https://doi.org/10.1111/j.1939-1676.2011.0678.x

Stanclift RM, Gilson SD (2008) Evaluation of neoadjuvant prednisone administration and surgical excision in treatment of cutaneous mast cell tumors in dogs. J Am Vet Med Assoc 232(1):53–62. https://doi.org/10.2460/javma.232.1.53

Stone KD, Prussin C, Metcalfe DD (2010) IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* 125(2 Suppl. 2):S73–S80. https://doi.org/10.1016/j.jaci.2009.11.017

Suzuki DOH, Anselmo J, de Oliveira KD et al (2015) Numerical model of dog mast cell tumor treated by electrochemotherapy. Artif Organs 39(2):192–197. https://doi.org/10.1111/aor.12333

Tams TR, Macy DW (1981) Canine mast cell tumors. Compend Contin Educ Pr Vet 27:259–263

Thamm DH, Mauldin EA, Vail DM (1999) Prednisone and vinblastine chemotherapy for canine mast cell tumor--41 cases (1992-1997). J Vet Intern Med 13(5):491–497. https://doi.org/10.1892/0891-6640(1999)013<0491:pavcfc>2.3.co;2

Thamm DH, Turek MM, Vail DM (2006) Outcome and prognostic factors following adjuvant prednisone/vinblastine chemotherapy for high-risk canine mast cell tumour: 61 cases. J Vet Med Sci 68(6):581–587. https://doi.org/10.1292/jvms.68.581

Thompson JJ, Pearl DL, Yager JA, Best SJ, Coomber BL, Foster RA (2011a) Canine subcutaneous mast cell tumor: characterization and prognostic indices. Vet Pathol 48(1):156–168. https://doi.org/10.1177/0300985810387446

Thompson JJ, Yager JA, Best SJ et al (2011b) Canine subcutaneous mast cell tumors: cellular proliferation and KIT expression as prognostic indices. Vet Pathol 48(1):169–181. https://doi.org/10.1177/0300985810390716

Tollett MA, Duda L, Brown DC, Krick EL (2016) Palliative radiation therapy for solid tumors in dogs: 103 cases (2007–2011). J Am Vet Med Assoc 248(1):72–82. https://doi.org/10.2460/javma.248.1.72

Turrel JM, Kitchell BE, Miller LM, Theon A (1988) Prognostic factors for radiation treatment of mast cell tumor in 85 dogs. J Am Vet Med Assoc 193(8):936–940

Turrel JM, Farrelly J, Page RL, McEntee MC (2006) Evaluation of strontium 90 irradiation in treatment of cutaneous mast cell tumors in cats: 35 cases (1992-2002). J Am Vet Med Assoc 228 (6):898–901. https://doi.org/10.2460/javma.228.6.898

Warland J, Amores-Fuster I, Newbury W, Brearley M, Dobson J (2014) The utility of staging in canine mast cell tumours. Vet Comp Oncol 12(4):287–298. https://doi.org/10.1111/vco.12012

Webster JD, Yuzbasiyan-Gurkan V, Kaneene JB, Miller R, Resau JH, Kiupel M (2006) The role of c-KIT in tumorigenesis: evaluation in canine cutaneous mast cell tumors. Neoplasia 8 (2):104–111. https://doi.org/10.1593/neo.05622

Webster JD, Yuzbasiyan-Gurkan V, Thamm DH, Hamilton E, Kiupel M (2008) Evaluation of prognostic markers for canine mast cell tumors treated with vinblastine and prednisone. BMC Vet Res 4:32. https://doi.org/10.1186/1746-6148-4-32

Weishaar KM, Thamm DH, Worley DR, Kamstock DA (2014) Correlation of nodal mast cells with clinical outcome in dogs with mast cell tumour and a proposed classification system for the evaluation of node metastasis. J Comp Pathol 151(4):329–338. https://doi.org/10.1016/j.jcpa.2014.07.004

White CR, Hohenhaus AE, Kelsey J, Procter-Gray E (2011) Cutaneous MCTs: associations with spay/neuter status, breed, body size, and phylogenetic cluster. J Am Anim Hosp Assoc 47 (3):210–216. https://doi.org/10.5326/JAAHA-MS-5621

Wilcock BP, Yager JA, Zink MC (1986) The morphology and behavior of feline cutaneous mastocytomas. Vet Pathol 23(3):320–324. https://doi.org/10.1177/030098588602300313

Worley DR (2014) Incorporation of sentinel lymph node mapping in dogs with mast cell tumours: 20 consecutive procedures. Vet Comp Oncol 12(3):215–226. https://doi.org/10.1111/j.1476-5829.2012.00354.x

Yarden Y, Kuang WJ, Yang-Feng T et al (1987) Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J 6(11):3341–3351. https://doi.org/10.1002/j.1460-2075.1987.tb02655.x

Zemke D, Yamini B, Yuzbasiyan-Gurkan V (2002) Mutations in the juxtamembrane domain of c-KIT are associated with higher grade mast cell tumors in dogs. Vet Pathol 39(5):529–535. https://doi.org/10.1354/vp.39-5-529

Electrochemotherapy for the Treatment of Transitional Cell Carcinoma in Dogs

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Abstract

Electrochemotherapy (ECT) involves the application of specific electrical pulses, which reversibly increase the permeability of the cell membranes and the administration of anticancer agents (Cemazar M, Tamzali Y, Sersa G, J Vet Intern Med 22:826–31, 2008; Gehl J, Sersa G, Matthiessen LW, et al., Acta Oncol 57 (7):874–882, 2018; Rangel MMM, Luz JCS, Oliveira KD, Austral J Vet Sci 51:45–51, 2019). The objective response rate for different histological types is approximately 70–80% (Miklavčič D, Mali B, Kos B, Biomed Eng Online 13:29, 2014; Gehl J, Sersa G, Matthiessen LW, et al., Acta Oncol 57(7):874–882, 2018). ECT is increasingly being used for subcutaneous tumors; however, its use for intracavitary organs has not been standardized to date (Kos B, Zupanic A, Kotnik T, J Membr Biol 236:147–153, 2010; Miklavcic D, Snoj M, Zupanic A, et al. Biomed Eng Online 9: 10, 2010; Mali B, Gorjup V, Edhemovic I, et al. Biomed Eng Online 14 Suppl 3: S5, 2015). The treatment of tumors of the bladder and urethra is mainly clinical with stabilization of the disease by chemotherapy and a median survival >1-year has been noted with this treatment. A surgical approach is not ever feasible as the involvement of the vesical trigone and urethra is observed in >50% of the dogs (Knapp DW, Ramos-Vara JA, Moore GE, ILAR J 55:100–118, 2014; Fulkerson CM, Knapp DW, Vet J 205:217–225, 2015). The selectivity of ECT, when bleomycin is used, opens the possibility of addressing bladder tumors more conservatively (Mir LM, Eur J Cancer Suppl 4:38, 2006; Campana LG, Marconato R, Sieni E, Recent Prog Med

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107:422–433, 2016; Gehl J, Sersa G, Matthiessen LW, et al., Acta Oncol 57 (7):874–882, 2018). The high response rate can lead to total local control of the tumor.

Keywords

Cancer · Electroporation · Bladder · Tumors · Electrochemotherapy

1 Classical Treatment of Tumors of the Bladder and Urethra in Dogs

Approximately 2% of all cancers in dogs are tumors of the bladder and urethra. Approximately 78% of dogs present with T2 clinical staging and 20% with T3 staging (according to WHO criteria, Table 1). The clinical signs associated with bladder tumors are mistaken with those associated with cystitis, namely, pollakiuria, stranguria, and hematuria, and total obstruction of the urine outflow tract may be observed. The trigone and urethra are affected in >50% of the cases of bladder tumors; hence, a surgical approach is difficult for the local control of the disease (Fulcher et al. 2006). Therefore, the main treatment for most patients with bladder tumors is based on chemotherapy, COX inhibitors (NSAID), or a combination of these modalities. Clinical treatment may provide the patient with a median survival that can exceed one year while maintaining a good quality of life. Distant metastases were documented in 58% of 137 dogs by necropsy. Any, distant and nodal metastases, lung abdominal, pelvic inguinal nodes are the most common metastasis locations. It is recommended that a systemic treatment be maintained as the main approach while the tumor is being controlled (Knapp et al. 2014; Fulkerson and Knapp 2015).

Table 1 TNM Clinical staging system for canine bladder cancer (Meuten 2016; Vail et al. 2019)

T—Primary Tumor	
T_{is}	Carcinoma in situ
T0	No evidence of a primary tumor
T1	Superficial papillary tumor
T2	Tumor invading the bladder wall, with induration
T3	Tumor invading neighboring organs (prostate, uterus, vagina, and pelvic canal)
N—Regional Lymph Node (Internal and External lilac Lymph Node)	
N0	No regional lymph node involvement
N1	Regional lymph node involved
N2	Regional lymph node and juxta regional lymph node involved
M—Distant Metastases	
M0	No evidence of metastasis
M1	Distant metastasis present

2 Electrochemotherapy for the Treatment of Tumors in the Bladder and Urethra

A major difficulty in the surgical treatment of bladder and urethra tumors is the need to preserve the region of the trigone through which critical vessels and nerves pass, maintaining voluntary control of the patient's urination and the viability of the organ (Saulnier-Troff et al. 2008; Rangel et al. 2018). Wide surgical resection of tumors in this region is technically unfeasible (Fulkerson and Knapp 2015).

There are two key benefits of electrochemotherapy (ECT), namely, high objective response rate (approximately 70–80% for different tumors) (Cemazar et al. 2008; Sersa and Miklavcic 2008; Suzuki et al. 2015; Cemazar and Sersa 2019; Rangel et al. 2019) and its selectivity for tumor tissue, at least when bleomycin is administered during ECT (Mir 2006). These two characteristics suggest that the performance of ECT in the bladder could attain good results, achieving a good local control of the disease and preserving the important structures present in the trigone. One relevant concern when performing ECT in the bladder and urethra is dehiscence and consequently uroperitoneum. The tumor cells are electroporated by applied electric fields allowing access of bleomycin into cells. This high bleomycin concentration in the cytosol may produce tumor necrosis as part of this mechanism of action. Consequently, wound dehiscence may result from tumor necrosis. Accordingly, some exclusion criteria were established for patients to be eligible for the procedure, which include:

- T3 Staging, tumor are invading neighboring organs.
- T2 Staging with involvement of the serous membrane.
- Pregnant or lactating, medications may affect fetuses and infants.

Given that the application of ECT in bladder cancer is still being established, the criteria used are still preliminary and have been postulated in order to avoid the formation of fistulas in the bladder and a subsequent uroperitoneum. We have analyzed all patients undergoing bladder ECT with transoperative biopsy by freezing sections to determine if the tumor had invaded the serous membrane (Rangel et al. 2018).

3 ECT Protocol for the Treatment of Bladder Tumors

The electrical parameters used for ECT in the bladder were as follows (Mir et al. 2006; Gehl et al. 2018; Suzuki et al. 2018):

Electric field amplitude: 1000 V/cm
Frequency: 5 kHz
Pulse duration: 100μs
Number of pulses: 8

These parameters were selected based on the treatment of epithelial tumors using parallel needle electrode arrays. The established dose of bleomycin was 15,000 U/m^2. The route of administration was intravenous. Electrical pulses were initiated 7 min after the drug was administered (Mir et al. 2006; Impellizeri et al. 2016; Gehl et al. 2018).

4 ECT Procedure for the Treatment of Bladder Tumors

Patients undergoing bladder ECT received general anesthesia, after which they underwent caudal laparotomy to access the bladder and a subsequent cystotomy. The cystotomy procedure should be performed with utmost care to minimize the chances of tumor implantation within the abdominal cavity. These precautions include careful manipulation of the bladder, instruments that may contact the tumor inside the bladder should be changed, and gloves should be replaced if there is manipulation of the interior of the bladder or direct contact with the tumor. In case of eversion of the bladder wall, utmost care should be taken to avoid contact with the perivesical fat or any other structure external to the inner wall of the bladder. Care should be taken when triggering the electrical pulses whenever the internal wall of the bladder is attached to the electrode needles (Fig. 1). After the procedure, it is recommended to keep the patient hospitalized for 48 h to monitor the urinary output, presence of fluid in the abdomen, and other general care of a postoperative patient (similar to laparotomy and cystotomy).

Currently, we are recommending three sessions of electrochemotherapy with an interval of 1 month. The decision for additional sessions arises after an abdominal ultrasound procedure showing macroscopic evidence of the disease (Hanazono et al. 2014; Rangel et al. 2018). No complications were observed in patients treated with this technique. Adhesions have been observed after the procedures in fat around the bladder. Ideal guidelines for a reliable assessment of bladder tumors include: the same professional make the assessment; same decubitus position; same ultrasound equipment and patient with a full bladder (Fulkerson and Knapp 2015).

5 Follow-Up of Patients Who Underwent ECT for the Treatment of Bladder Tumors

We recommended to patients undergo abdominal ultrasound, and full blood and biochemical tests 1 week and 2 weeks after the procedure. After 2 weeks, further evaluation was done. If the patient is submitted to another procedure after this period, the same regimen was repeated; if no other procedure was performed, the tests were done every 30 days. After 4 months of follow-up, patients were monitored every 3 months for clinical signs of the disease (Knapp et al. 2014; Fulkerson and Knapp 2015; Rangel et al. 2018).

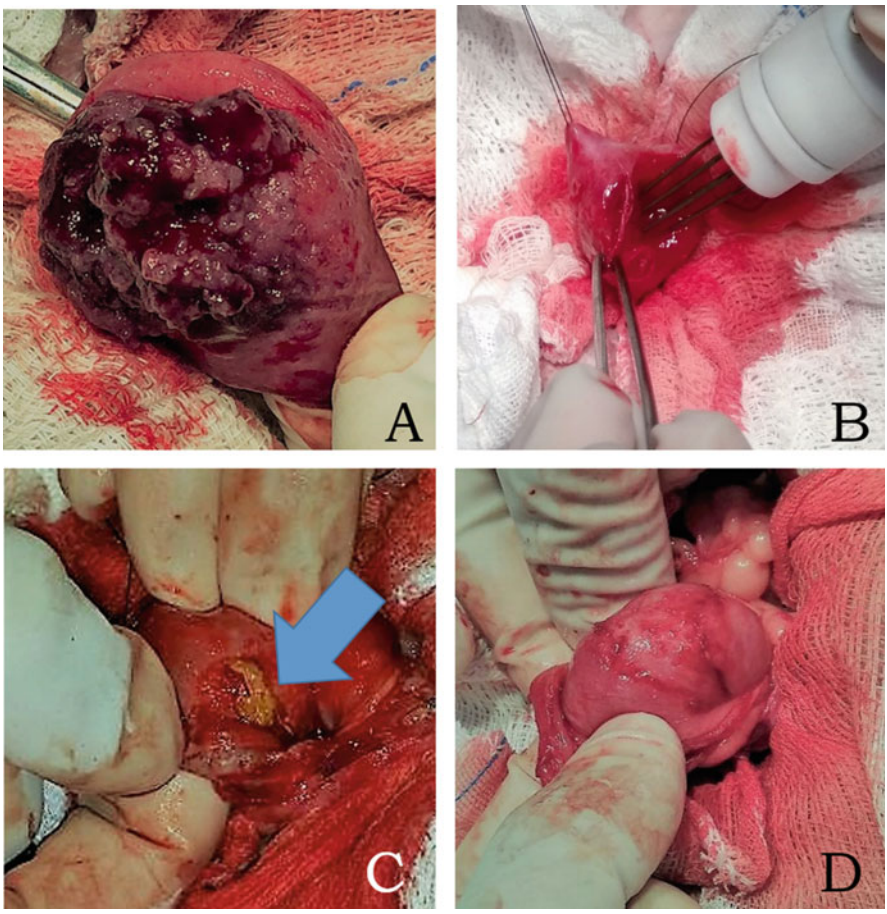

Fig. 1 Bladder electrochemotherapy during cystotomy. (**a**) Bladder with translational cell carcinoma involvement of the trigone. (**b**) Electrochemotherapy performed on the bladder. (**c**) Histopathology analysis by frozen section provided confirmation of tumor evidence, second ECT procedure was applied. Necrosis in bladder tumor treated with ECT. (**d**) Bladder with no clinical evidence of tumor after 2 electrochemotherapies 70 days after the first one application (Rangel et al. 2018)

6 Treatment with COX Inhibitors in Patients Undergoing ECT for the Treatment of Bladder Tumors

One major problem associated with bladder tumors is the development of distant metastases. Approximately 20% of patients already presented metastases at diagnosis and 58% of patients had distant metastases at necropsy (Fulkerson and Knapp 2015). The abscopal effect of ECT is known, but its effectiveness has not yet been determined to the point of excluding the need for adjuvant systemic therapies (Mir et al. 1995; Mali et al. 2013; Calvet and Mir 2016). For this reason, even if bladder

ECT fully controls the disease locally, adjuvant chemotherapy is recommended. Some drugs used after obtaining this local control were carboplatin and mitoxantrone. The use of COX inhibitors can be instituted before surgical procedure with ECT (similar to the traditional treatment), with the exception of patients who present contraindications such as kidney failure. It is noteworthy that when local control of the disease is achieved, not all owners accept to continue with the systemic treatment (Knapp et al. 2014; Fulkerson and Knapp 2015).

7 ECT Outcomes for Bladder Tumors

The treatment of bladder tumors with electrochemotherapy is a new approach. The first study with this approach followed 19 patients treated since December 2016 (who are still being monitored) with the initial data reported as promising:

- No patient died during the bladder ECT procedure.
- No patient died in the postoperative and post ECT due to the procedure.
- No patient presented suture dehiscence after the bladder ECT procedure.
- No patient had serious complications after the bladder ECT procedure.

These results serve as initial evidence that the technique, when applied under the established criteria, is safe. Some side effects were observed in patients undergoing treatment and these were similar to those presented before the procedure, such as pollakiuria, stranguria, or dysuria. A side effect observed after the procedure that differed from those presented before the procedure is temporary urinary incontinence, which was observed in all patients and disappeared within 7–86 days (Rangel et al. 2018). None of the patients had distant metastasis. Some patients presented with local recurrence. These tumor recurrences were treated with another application of ECT.

Currently, electrochemotherapy is applied to human visceral, and deep-seated tumors. Phase I clinical study of electrochemotherapy on treatment of colorectal cancer using endoscopy (Falk Hansen et al. 2020) and esophageal cancer (Egeland et al. 2018). Phase I/II study of local pancreatic cancer with ECT demonstrated no damage to surrounding viscera, no major complications with good functional result (Granata et al. 2015). A prospective pilot study on hepatocellular carcinomas showed a feasible and safe treatment, with an overall response of 88% (15/17) of treated lesions (Djokic et al. 2018).

References

Calvet CY, Mir LM (2016) The promising alliance of anti-cancer electrochemotherapy with immunotherapy. Cancer Metastasis Rev 35:165–177. https://doi.org/10.1007/s10555-016-9615-3

Cemazar M, Sersa G (2019) Recent advances in electrochemotherapy. Bioelectricity 1:204–213. https://doi.org/10.1089/bioe.2019.0028

Cemazar M, Tamzali Y, Sersa G et al (2008) Electrochemotherapy in veterinary oncology. J Vet Intern Med 22:826–831. https://doi.org/10.1111/j.1939-1676.2008.0117.x

Djokic M, Cemazar M, Popovic P et al (2018) Electrochemotherapy as treatment option for hepatocellular carcinoma, a prospective pilot study. Eur J Surg Oncol 44:651–657. https://doi.org/10.1016/j.ejso.2018.01.090

Egeland C, Baeksgaard L, Johannesen HH et al (2018) Endoscopic electrochemotherapy for esophageal cancer: a phase I clinical study. Endosc Int open 6:E727

Falk Hansen H, Bourke M, Stigaard T et al (2020) Electrochemotherapy for colorectal cancer using endoscopic electroporation: a phase 1 clinical study. Endosc Int open 8:E124–E132. https://doi.org/10.1055/a-1027-6735

Fulcher RP, Ludwig LL, Bergman PJ et al (2006) Evaluation of a two-centimeter lateral surgical margin for excision of grade I and grade II cutaneous mast cell tumors in dogs. J Am Vet Med Assoc 228:210–215

Fulkerson CM, Knapp DW (2015) Management of transitional cell carcinoma of the urinary bladder in dogs: a review. Vet J 205:217–225. https://doi.org/10.1016/j.tvjl.2015.01.017

Gehl J, Sersa G, Matthiessen LW et al (2018) Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol 57(7):874–882. https://doi.org/10.1080/0284186X.2018.1454602

Granata V, Fusco R, Piccirillo M et al (2015) Electrochemotherapy in locally advanced pancreatic cancer: preliminary results. Int J Surg 18:230–236. https://doi.org/10.1016/j.ijsu.2015.04.055

Hanazono K, Fukumoto S, Endo Y et al (2014) Ultrasonographic findings related to prognosis in canine transitional cell carcinoma. Vet Radiol Ultrasound 55:79–84. https://doi.org/10.1111/vru.12085

Impellizeri J, Aurisicchio L, Forde P, Soden DM (2016) Electroporation in veterinary oncology. Vet J 217:18–25

Knapp DW, Ramos-Vara JA, Moore GE et al (2014) Urinary bladder cancer in dogs, a naturally occurring model for cancer biology and drug development. ILAR J 55:100–118. https://doi.org/10.1093/ilar/ilu018

Kos B, Zupanic A, Kotnik T et al (2010) Robustness of treatment planning for electrochemotherapy of deep-seated tumors. J Membr Biol 236:147–153. https://doi.org/10.1007/s00232-010-9274-1

Mali B, Jarm T, Snoj M et al (2013) Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis. Eur J Surg Oncol 39:4–16. https://doi.org/10.1016/j.ejso.2012.08.016

Meuten DJ (2016) Tumors in domestic animals, 4th edn. Iowa, Ames, IA

Mir LM (2006) Bases and rationale of the electrochemotherapy. Eur J Cancer Suppl 4:38–44. https://doi.org/10.1016/j.ejcsup.2006.08.005

Mir LM, Roth C, Orlowski S et al (1995) Systemic antitumor effects of electrochemotherapy combined with histoincompatible cells secreting interleukin-2. J Immunother Emphas Tumor Immunol 17:30–38

Mir LM, Gehl J, Sersa G et al (2006) Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator TM by means of invasive or non-invasive electrodes. Eur J Cancer Suppl 4:14–25. https://doi.org/10.1016/j.ejcsup.2006.08.003

Rangel MMM, De Oliveira KD, Freytag JO et al (2018) Electrochemotherapy on bladder-preliminary results. Biomed J Sci Tech Res 5:11–14. https://doi.org/10.26717/BJSTR.2018.12.002221

Rangel MMM, Luz JCS, Oliveira KD et al (2019) Electrochemotherapy in the treatment of neoplasms in dogs and cats. Aust J Vet Sci 51:45–51. https://doi.org/10.4067/S0719-81322019000200045

Saulnier-Troff FG, Busoni V, Hamaide A (2008) A technique for resection of invasive tumors involving the trigone area of the bladder in dogs: preliminary results in two dogs. Vet Surg 37:427–437. https://doi.org/10.1111/j.1532-950X.2008.00406.x

Sersa G, Miklavcic D (2008) Electrochemotherapy of tumours. JoVE e 15(22):1038. https://doi.org/10.3791/1038

Suzuki DOH, Anselmo J, de Oliveira KD et al (2015) Numerical model of dog mast cell tumor treated by electrochemotherapy. Artif Organs 39:192–197. https://doi.org/10.1111/aor.12333

Suzuki DOH, Berkenbrock JA, Frederico MJS et al (2018) Oral mucosa model for electrochemotherapy treatment of dog mouth cancer: ex vivo, in silico, and in vivo experiments. Artif Organs 42:297–304. https://doi.org/10.1111/aor.13003

Vail DM, Thamm D, Liptak J (2019) Withrow and MacEwen's small animal clinical oncology. Elsevier Health Sciences

Calcium Electroporation in Veterinary Medicine

Stine K. Frandsen, Martin S. Thoefner, and Julie Gehl

Abstract

Calcium electroporation is a novel anticancer treatment where calcium is injected intratumorally followed by electroporation, which transiently permeabilizes the plasma membrane leading to high intracellular calcium and subsequent cell death. The treatment induces cell death in all tested cancer cell lines and tumor types across histologies but with a difference in sensitivity while normal surrounding tissue is relatively spared. Calcium electroporation is a simple procedure, inexpensive, and safe, with good response rates, which may be an extra tool in the armamentarium of anticancer treatments available at veterinary clinics.

In this book chapter, calcium electroporation is described, starting with a description of how the treatment could be performed followed by background information about the treatment based on the preclinical and clinical research. Results from the three published human clinical trials are described in this chapter followed by a more thorough description of the four published studies on calcium electroporation in veterinary medicine—two trials on equine sarcoids and two case reports on a canine and a feline patient, respectively. The perspectives and

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conclusion finalize the chapter about this novel, simple, and inexpensive treatment that may be of great interest for many veterinary clinics.

Keywords

Calcium electroporation · Feline · Canine · Equine · Sarcoid

1 Introduction

Treatment of malignant tumors in veterinary patients is a challenging problem. Careful considerations are made to minimize suffering and limit functional impairment and disfigurement. To this end it is important to expand the treatment choices available.

Calcium electroporation is a novel anticancer treatment where lethally high calcium concentrations are introduced into cells by electroporation (Frandsen et al. 2012, 2020a). Short, high voltage pulses permeabilize the plasma membrane allowing for uptake of non-permeant molecules and ions, e.g., calcium. The high intracellular calcium concentration induces ATP depletion and cell death in malignant cells while normal cells are relatively spared (Frandsen et al. 2012, 2017; Hansen et al. 2015).

This book chapter begins with a description of how calcium electroporation is carried out followed by a thorough introduction to calcium electroporation including the mechanism of action, effects on different cancer histologies and normal cells, as well as immunological systemic effects. This novel treatment has been studied in vitro and in vivo on mice, and only 5 years after the first preclinical study the first human clinical study was published showing good results. These preclinical studies and human clinical studies are described followed by a thorough description of the first veterinary studies using calcium electroporation—two studies on equine sarcoids as well as two case reports on a feline and a canine patient.

2 Calcium Electroporation and How It Is Carried Out

Calcium electroporation is a local treatment where a calcium solution is injected intratumorally immediately followed by electroporation (Fig. 1). The following description of how the treatment is carried out is based on murine studies (Frandsen et al. 2012, 2017), human clinical studies (Falk et al. 2017a, b; Plaschke et al. 2019), and veterinary studies (Galant et al. 2019; Frandsen et al. 2020b) using calcium electroporation as well as the standard operating procedure for electrochemotherapy (Mir et al. 2006; Gehl et al. 2018).

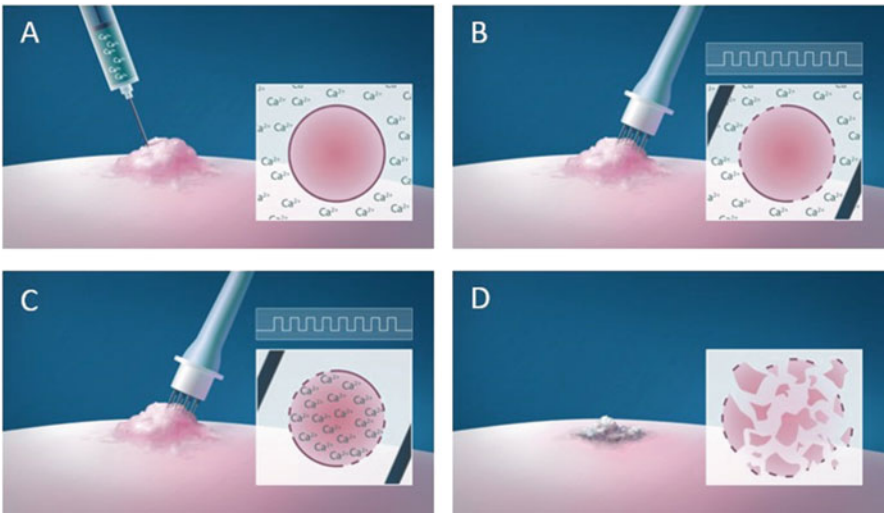

Fig. 1 Calcium electroporation treatment. High concentration of calcium is injected intratumorally (**a**) increasing the extracellular calcium concentration (small image) followed by application of short, high voltage pulses (electroporation) inducing transient permeabilization of the plasma membrane (**b**). Due to the concentration gradient calcium diffuses into the cells (**c**) leading to cell death (**d**). From Frandsen et al., Cancers 2020 (2020a)

2.1 Preparation

The treatment is performed under general anesthesia due to muscle contractions induced by the electric pulses (Fig. 2 and Sect. 2.2.1). When the veterinary patient is anesthetized following standard procedure, the treatment area is shaved, if needed and cleaned before the treatment. Surgical preparation including aseptic preparation of the treatment area is not deemed necessary since the treatment only includes local calcium injection and use of electrodes (e.g., needle electrodes, which is further described in Sect. 3.4.2). The size of the lesion is determined by measuring the longest diameter (a) and the longest diameter perpendicular to a (b), and in case of very thin lesions the height of the lesion can be measured or estimated. Based on the size measurements the volume of the lesion is calculated by $ab^2\pi/6$ (or $abc\pi/6$ in case of thin lesions) and the volume of calcium needed (see Table 1). In most cases, calcium has been injected in a volume equivalent to around 50% of the tumor volume and using a calcium concentration of 168–225 mM $CaCl_2$ but a large range of calcium concentrations, injection volumes, and calcium sources may be used, as further described in Sect. 3.4.1. Calcium may be diluted to the desired concentration, and physiological saline is suggested but sterile water has also been used for dilution of the calcium source.

Fig. 2 Calcium electroporation treatment of equine sarcoid. The horse is treated under general anesthesia (**a**, **b**) and the treatment area is shaved and cleaned before treatment (**b**–**d**). Calcium is injected into the tumor tissue and a small margin around the tumor (**c**) followed by application of the pulsed electric fields (**b**, **d**). Note that it is two different sarcoids being treated on images (**c**, **d**). From Frandsen et al., Animals 2020 (2020b)

2.2 Treatment

Calcium is injected intratumorally (Fig. 2) often in a volume equivalent to 50% of the tumor volume as described in Table 1 and Sect. 3.4.1. It is important to ensure that calcium is present in all of the tumor area before applying the electric pulses, and it is suggested to inject calcium (and apply the electric fields) in the margin area as well to secure treatment of all the malignant tissue and since normal surrounding tissue is less affected than tumor tissue (see Sect. 3.3). To secure calcium being evenly distributed in the entire treatment area, calcium may be injected in a grid pattern. It is recommended not to perform biopsies before the treatment, if possible since the injected calcium solution may spill from the biopsy hole. If this happens, additional calcium may be injected to secure enough calcium being present in the tissue before electroporation. In case of large tumors, the tumor may be treated in sections to secure calcium present in the area when applying the electric pulses.

Immediately after the calcium injection, the electric pulses are applied (Fig. 2). Most often 8 square wave pulses of 100 μs, 1000 V/cm, and 1 Hz are applied and

Table 1 Example of calculations of tumor volume and calcium volume needed for calcium electroporation treatment of different size tumors, both spherical tumors and flat tumors. Diameter *a* is the longest diameter of the tumor and diameter *b* is the longest diameter perpendicular to *a*. The two diameters are not always the same but in this example perfect spherical and round tumors are shown. Note that the suggested calcium volume is not exactly 50% of the tumor volume but around 50%, and more calcium may be needed to ensure calcium being distributed in the entire treatment area before electroporation

Shape		Diameter, *a* (cm)	Diameter, *b* (cm)	Height, *c* (for thin tumors) (cm)	Tumor volume, $ab^2\pi/6$ or $abc\pi/6$ (cm^3)	Calcium volume, e.g., around 50% of tumor volume (ml)
Spherical		2	2		4.2	2–2.5
		4	4		33.5	17
		6	6		113.1	57
Round, thin		2	2	1	2.1	1
		4	4	1	8.4	4–4.5
		6	6	1	18.8	9–10

needle electrodes are used but other pulsing conditions and electrode types can be used (see Sect. 3.4.2). The electric pulses should be applied to the entire tumor area and the margin area by moving the electrode and applying the pulses in slightly overlapping sections, to secure permeabilization of all tumor cells.

2.2.1 Muscle Contractions

Electroporation treatment of human patients may be performed under local or general anesthesia depending on the size, number, and placement of treated tumors and the decision of the clinician. When applying the electric pulses, contraction of underlying musculature will occur. This may be reduced by lifting the treatment area from the underlying musculature using a gauze pad (Gehl et al. 2018). In the veterinary studies using calcium electroporation, general anesthesia has been used due to the brief muscle contractions occurring during application of the electric pulses, which could result in a stress reaction in the animal, if not performed under general anesthesia. In the two equine studies, muscle contractions were local muscle fasciculations in most cases but were sometimes stronger if the lesion were above nerves. Note that it may be needed to hold smaller animals when applying the field due to the muscle contractions and precautions may be needed when treating larger animals due to these contractions. Further research may show that treatment of some lesions in veterinary studies may be performed in local anesthesia with sedation but at the moment general anesthesia is recommended in all veterinary studies when treating with calcium electroporation.

2.3 Follow-Up

In general, the treatment is well-tolerated and safe, and normally, none or very few reactions after the treatment are observed. No changes in electrocardiogram (ECG) have been observed in trials where ECG was monitored as standard protocol for anesthetized horses undergoing electroporation, even where lesions were located in the chest region close to the heart. Minor to moderate local swelling may be observed after treatment, which normally diminishes within a few days. Scab formation has also been observed after treatment. In a few cases, infection of the treatment area has been observed, which has been treated effectively with antibiotics. Patients can normally leave the clinic in their normal habitus a few hours after the procedure. Calcium electroporation is a once-only treatment, however, tumors may be treated more than once, if needed, which has been done in one study where the second treatment was performed $\geq$4 weeks after the first treatment (Frandsen et al. 2020b).

3 Calcium Electroporation: Background

3.1 Mechanisms of Action

As described, calcium electroporation is a treatment modality where high calcium concentrations are injected intratumorally immediately followed by application of electric pulses (electroporation) (Frandsen et al. 2012; Falk et al. 2017b). The electric pulses induce permeabilization of the plasma membrane, which allows uptake of calcium resulting in high intracellular calcium concentration (Frandsen et al. 2017). Due to the concentration gradient across the plasma membrane, the transient permeabilization of the membrane also induces an influx of other ions and molecules, e.g., sodium as well as loss of intracellular ion and molecules, such as potassium and ATP (see Fig. 3) (Gehl 2003; Neumann et al. 1998; Rols and Teissie 1990). When the membrane reseals, the cell will begin to re-establish cellular homeostasis. Calcium is a ubiquitous second messenger involved in numerous intracellular processes including cell death, and because of the importance of this ion in cellular signaling, it is tightly regulated (Carafoli et al. 2001; Clapham 2007; Zhivotovsky and Orrenius 2011). Intracellular calcium is extruded by ATP-dependent plasma membrane calcium ATPase's (PMCA) and ATP-independent exchangers (sodium–calcium exchanger—NCX and sodium–potassium–calcium exchanger—NCKX) and stored by different organelles, including the endoplasmic reticulum (ER) and mitochondria (Carafoli et al. 2001; Clapham 2007).

Calcium electroporation induces ATP depletion (Frandsen et al. 2012; Hansen et al. 2015), which may be due to (1) direct loss of ATP through the permeabilized cell membrane (Rols and Teissie 1990), (2) increased usage of ATP by ion pumps, e.g., PMCA and sodium–potassium ATPase, to re-establish the ion homeostasis (Cerella et al. 2010), and (3) loss of ATP production due to mitochondrial dysfunction (Gibot et al. 2020), see Fig. 3. Furthermore, calcium electroporation reduces

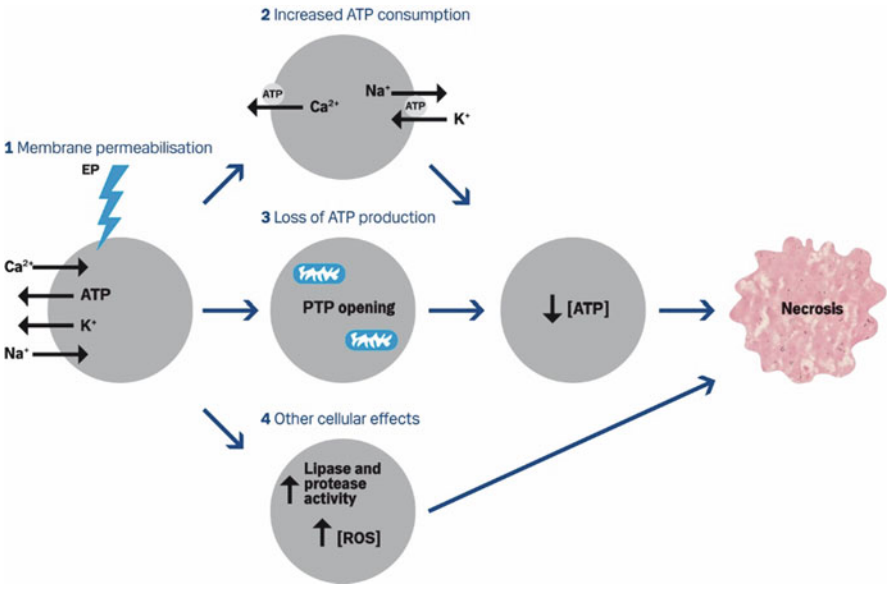

Fig. 3 Proposed mechanism of action behind calcium electroporation published in 2012. Calcium electroporation induces an influx of calcium and sodium and loss of ATP and potassium due to the concentration gradients (1). Following resealing, the cell will try to re-establish the ion homeostasis using plasma membrane calcium ATPase (PMCA) and sodium–potassium ATPase, which leads to increased ATP consumption (2). The high intracellular calcium concentration results in mitochondrial dysfunction, maybe due to opening of permeability transition pores (PTP) in the mitochondrial membrane, leading to loss of ATP production (3). This will lead to ATP depletion and cell death. Other cellular effects are likely also involved (4). From Frandsen et al., Cancer Research 2012 (Frandsen et al. 2012)

cytoskeleton organization and disrupts microtubules (Thompson et al. 2014; Szewczyk et al. 2018; Graybill and Davalos 2020). Finally, calcium electroporation leads to cell death where necrosis and apoptosis were identified in cell lines in vitro after treatment (Szewczyk et al. 2018; Zielichowska et al. 2016; Staresinic et al. 2018) and necrosis was identified in tumors in vivo (Frandsen et al. 2012, 2017), in a human clinical trial (Falk et al. 2017b) and in a veterinary trial (Galant et al. 2019). Thus, calcium electroporation might induce a mix of necrosis and apoptosis, and/or the type of cell death induced by calcium electroporation may depend on cell type, calcium concentration, time after treatment, or other factors.

3.2 Effects Across Histologies

The dramatic increase in the intracellular calcium concentration resulting from calcium electroporation induces cell death, which has been shown in several cancer cell lines in vitro (Frandsen et al. 2012; Hansen et al. 2015; Szewczyk et al. 2018; Zielichowska et al. 2016; Staresinic et al. 2018; Frandsen and Gehl 2017; Falk et al.

Table 2 Overview of response rates after calcium electroporation treatment in different tumor types in murine, human, and veterinary trials

Study type	Cancer type	Response	References
Murine	Human small cell lung cancer	89% complete response (8 of 9 treated mice)	Frandsen et al. (2012)
Murine	Human breast, bladder, and colon cancer	36–65% necrosis in tumors 2 days after treatment	Frandsen et al. (2017)
Murine	Human rhabdomyosarcoma	45% decrease of tumor volume 10 days after treatment compared with control	Szewczyk et al. (2018)
Murine	Murine colon cancer	100% complete response (12 of 12 treated mice)	Falk et al. (2017c)
Murine	Murine melanoma (GFP labelled)	Complete loss of fluorescence 1 day after treatment but regrowth at day 7	Staresinic et al. (2018)
Human	Cutaneous metastases (breast cancer and malignant melanoma)	66% complete response (12 of 18 treated metastases)	Falk et al. (2017b)
Human	Mucosal head and neck cancer	50% objective response (3 of 6 treated patients), one of six treated patients with complete response	Plaschke et al. (2019)
Human	Cutaneous metastases (breast cancer and malignant melanoma)	22% complete response (4 of 18 treated metastases)	Agoston et al. (2020)
Canine	Oral melanoma	Case report: 42% decrease of primary tumor, complete response of treated lymph node metastases	Kulbacka et al. (2017)
Feline	Cutaneous melanoma	Case report: 100% complete response (2 of 2 treated lymph node metastases)[a]	Dos Anjos et al. (2019)
Equine	Sarcoid	>50% necrosis in 9 of 16 treated sarcoids	Galant et al. (2019)
Equine	Sarcoid	22% complete response (6 of 27 treated sarcoids)	Frandsen et al. (2020b)

[a]Treated with bleomycin and calcium in combination with electroporation

2017c; Hoejholt et al. 2019), tumor types in vivo in murine studies (Frandsen et al. 2012, 2017; Szewczyk et al. 2018; Staresinic et al. 2018; Falk et al. 2017c), in human clinical trials (Falk et al. 2017b; Plaschke et al. 2019; Agoston et al. 2020) and in veterinary trials (Galant et al. 2019; Frandsen et al. 2020b; Kulbacka et al. 2017; Dos Anjos et al. 2019). Across all tested cancer cells and tissues, calcium electroporation effectively induces cell death, however, with a difference in sensitivity between cell lines and tumor types. In Table 2, an overview of tested cancer types in preclinical

murine studies, human clinical trials, and veterinary trials as well as the response to calcium electroporation is shown. As described, calcium is a ubiquitous second messenger involved in multiple cellular processes from cell division to cell death (Carafoli et al. 2001). Calcium electroporation affects the homeostasis of this essential ion and thereby affects all cells across tumor histology, however, sensitivity to treatment differs and interestingly normal cells and tissues seem least affected, as discussed in detail in Sect. 3.3.

3.3 Effects on Normal Versus Malignant Cells

Interestingly, normal cells and tissues seem much less affected than cancer cells and tissues (Frandsen et al. 2015, 2017; Falk et al. 2017b; Galant et al. 2019). This was observed when normal and cancer cells were treated as spheroids, a 3D tumor-like in vitro model, where all three tested types of cancer cell spheroids dramatically decreased in size after treatment with calcium electroporation as well as electrochemotherapy. Spheroids of primary normal fibroblasts did not decrease in size after treatment with calcium electroporation nor electrochemotherapy (Frandsen et al. 2015). Similarly, surrounding normal tissues (muscle and skin) were less affected than the tumor tissue after calcium electroporation treatment of human breast cancer tumors placed subcutaneously on mice (Frandsen et al. 2017). When normal muscle and skin tissue were directly treated with calcium electroporation lower effect was also observed compared with treated tumor tissue (Frandsen et al. 2017). In another in vivo study treating murine melanoma tumors, changes were observed in the normal surrounding tissue including scab formation after calcium electroporation and effects were observed in both tumor vasculature and normal vasculature after treatment (Staresinic et al. 2018). In the first human clinical trial, effects on normal tissue were not directly investigated, however, clinical observations showed that only the tumor tissue was necrotic not the surrounding normal tissue indicating that normal tissue is spared (Falk et al. 2017b). These observations were verified in a study investigating the effect of calcium electroporation on equine sarcoids (see Sect. 5.1) where necrosis was not seen in the normal surrounding tissue unlike the sarcoids where necrosis was observed in 13 of 16 treated sarcoids (Galant et al. 2019).

3.3.1 Mechanisms of Action: Normal and Malignant Cells

The mechanism behind this difference in sensitivity between normal and malignant cells and tissues has been investigated showing some differences that might affect the sensitivity to treatment. When cells and tissues are electroporated in the presence of calcium, the intracellular calcium concentration increases immediately in both normal and malignant cells and tissues (Frandsen et al. 2017; Szewczyk et al. 2018; Guionet et al. 2018), however, not significantly in surrounding normal muscle tissue in a murine study, which might be related to the efficient calcium homeostasis in this cell type (Frandsen et al. 2017). In normal skin located above tumors treated with calcium electroporation the intracellular calcium concentration decreased over time,

and 4 h after treatment the level was similar to the calcium level before treatment. This was not the case for tumor tissue where the intracellular calcium concentration was significantly higher than before treatment for more than 4 h after treatment. Thus, normal skin and muscle tissue are able to re-establish the calcium homeostasis within 4 h unlike the tumor tissue (Frandsen et al. 2017). Permeabilization of the plasma membrane induced by electroporation is likely affected by the lipid composition and heat capacity of the membrane, which thereby might affect the efficiency of calcium electroporation (Hoejholt et al. 2019). Shortly after permeabilization the plasma membrane reseals. The plasma membrane of normal cells repairs faster than of cancer cells, as observed in an in vitro study, and this might affect the differences in effect of calcium electroporation, since normal cells may be able to start re-establishing the calcium homeostasis faster than cancer cells (Frandsen et al. 2016). As described previously, intracellular calcium is removed from the cells by PMCA and exchangers (NCX and NCKX). Studies have shown that the total PMCA expression was significantly lower in five cancer cell lines compared with normal dermal fibroblasts and normal muscle cells (Frandsen et al. 2017; Szewczyk et al. 2018). Expression of NCX in cancer and normal muscle cells was similar, however, when treated with calcium electroporation the expression of NCX increased in normal muscle cells while decreased in cancer muscle cells (Szewczyk et al. 2018). These differences in the expression of calcium transporters might as well affect the cell's sensitivity to calcium electroporation. As described above, the cytoskeleton organization decreases after calcium electroporation which was observed in cancer muscle cells but interestingly cytoskeleton organization increased in normal muscle cells after calcium electroporation (Szewczyk et al. 2018).

Despite the many cellular effects of calcium electroporation, it has been shown that calcium electroporation does not induce any genotoxic effects (Gibot et al. 2020). Thus, also on a longer term, normal surviving cells seem to be less affected after calcium electroporation, which is important in cancer treatment where the normal surrounding cells are also treated to secure treatment of the entire tumor.

3.4 Calcium Doses and Pulse Application

3.4.1 Injection Volume, Calcium Concentration, and Calcium Solution

The effect of calcium electroporation using different injection volumes (equivalent to 20–80% of the tumor volume with a concentration of 168 mM $CaCl_2$) and different calcium concentrations (100–500 mM in a volume equivalent to 50% of tumor volume) were investigated in murine studies showing similar treatment effect within the tested range of doses (Frandsen et al. 2017; Staresinic et al. 2018). In most of the murine studies, 168 mM $CaCl_2$ was used (Frandsen et al. 2012, 2017; Szewczyk et al. 2018; Staresinic et al. 2018; Falk et al. 2017c) while a slightly higher concentration (220–225 mM) was used in the human clinical trials based on the much larger dilution volume (Falk et al. 2017b; Plaschke et al. 2019; Agoston et al. 2020). In the veterinary studies (see Sect. 5), different doses have been used in

all studies—in a canine patient 5 mM calcium in volume equivalent to less than 8% of the tumor volume were used (Kulbacka et al. 2017); in a feline patient two tumors were treated with 9 mM calcium together with bleomycin in a volume equivalent to 350% and 18% of the tumor volume, respectively (Dos Anjos et al. 2019); in two equine sarcoid studies 168 and 220 mM $CaCl_2$ in a volume equivalent to 50% of the tumor volume were used, respectively (Galant et al. 2019; Frandsen et al. 2020b). These first studies show that calcium electroporation is effective within a range of calcium doses (different injection volumes and calcium concentrations), thus the exact calcium concentration and injection volume are not essential for the effect of the treatment. It is believed to be more important that calcium is present in the entire treatment area before application of the electric pulses.

Importantly, in a human clinical study on recurrent head and neck cancer patients, the calcium level in serum was measured before treatment and 30 min and 6 h after treatment showing no increase in serum calcium after treatment with calcium electroporation using up to 10 ml 220 mM $CaCl_2$ (Plaschke et al. 2019).

An in vitro study has shown that the effect of calcium electroporation is independent on the calcium source, where calcium chloride and calcium glubionate were compared (Frandsen et al. 2014). Furthermore, the effect of calcium together with bleomycin in combination with electroporation was investigated showing an additive effect, but no synergistic effect of the two drugs (Dos Anjos et al. 2019; Frandsen et al. 2014).

3.4.2 Generators, Electrodes, and Pulsing Parameters

For electroporation-based treatments, electric pulses are applied using electrodes and a pulse generator. Different types and geometry of electrodes are available and used, e.g., plate, contact, and needle electrodes. The choice of electrode depends on the generator used (should be able to apply the desired electric field for the type and geometry of electrode) and the treatment area. For further description of the choice of electrode type see Gehl et al. (2018). Different generators certified for veterinary use are also available for electroporation-based treatments. Most often generators applying square wave pulses are used because this allows independent control of pulse amplitude and pulse length (Gehl 2003).

For calcium electroporation treatment, reversible electroporation is the mechanism, thus pulsing parameters resulting in a transient permeabilization of the plasma membrane. According to the European Standard Operating Procedures of Electrochemotherapy (ESOPE) pulsing parameters of 8 pulses of 100 μs, 1000–1300 V/cm, and 1–5000 Hz are used, depending on the electrode used, where 1000 V/cm is used for needle electrodes and 1300 V/cm is used for plate and contact electrodes (Mir et al. 2006; Gehl et al. 2018; Marty et al. 2006). These pulsing parameters are also used in all of the murine studies, human clinical trials, and veterinary trials described in this chapter, however, an in vitro study has shown that ESOPE equivalent pulsing conditions, such as applying a higher number of pulses with lower electric field amplitude than the ESOPE standard protocol, could also be used for calcium electroporation (Romeo et al. 2018). Intratumoral calcium injection may also enhance the effect of treatment using other electric field

applications, such as irreversible electroporation (IRE) and nanosecond pulsed electric fields (nsPEF). For IRE, very high electric fields and higher number of pulses are used, which result in permanent disruption of the plasma membrane, however, in the margin of the treatment area the applied field is lower, resulting in reversible electroporation. Thus, high extracellular calcium in the treatment area will expand the area with cell death (Wasson et al. 2020). When applying shorter (in the nanosecond range), higher voltage pulses (nsPEF) the plasma membrane as well as internal organelle membranes transiently permeabilize resulting in increased intracellular calcium from the extracellular space and from the intracellular storages resulting in cell death. By increasing the extracellular calcium concentration the effect of nsPEF may increase (Morotomi-Yano et al. 2014; Pakhomova et al. 2014).

3.5 Systemic Effect

Calcium electroporation is a local anticancer treatment but it might also activate a systemic immune response against the treated tumor cells. In a murine study, immunocompetent Balb/c mice with subcutaneously murine colorectal cancer tumors were treated with calcium electroporation or electrochemotherapy using bleomycin. All treated tumors were completely eliminated and when the mice were rechallenged with the same cancer cells no tumor grew, indicating an activated immune response against the cancer cells after calcium electroporation as well as after electrochemotherapy. When the mice were rechallenged with other cancer cells tumors started to grow, thus no immune response was activated against other cancer cells. When a similar experiment was performed on immune-incompetent NMRI-Fox1nu mice, a lower complete response rate was observed and when rechallenged no activated immune response was observed. The study also showed increased release of HMGB1, a danger signal known to induce activation of an immune response, and proinflammatory cytokines (Falk et al. 2017c). Thus, calcium electroporation has the ability to induce a systemic immune response in vivo.

In the first human clinical trial where cutaneous metastases were randomized to treatment with calcium electroporation or electrochemotherapy (see Sect. 4), one patient experienced a systemic immune response with complete response of treated as well as untreated tumors. The patient had numerous cutaneous metastases from malignant melanoma and more than 100 of these were treated with electrochemotherapy with good response. But 4 months later new metastases appeared and the patient was included in the calcium electroporation trial, where two of the new metastases were treated, one with calcium electroporation and one with electrochemotherapy. Both metastases were completely eliminated 2 months after treatment and 9 months after treatment leveling of untreated cutaneous metastases was observed as well as decrease of lymph node metastases, indicating a systemic immune response (Fig. 4). After 17 months new subcutaneous metastases and several brain metastases were observed (Falk et al. 2017a). To our knowledge, electrochemotherapy has not previously been reported to have induced a systemic immune response in human clinical trials indicating that calcium electroporation

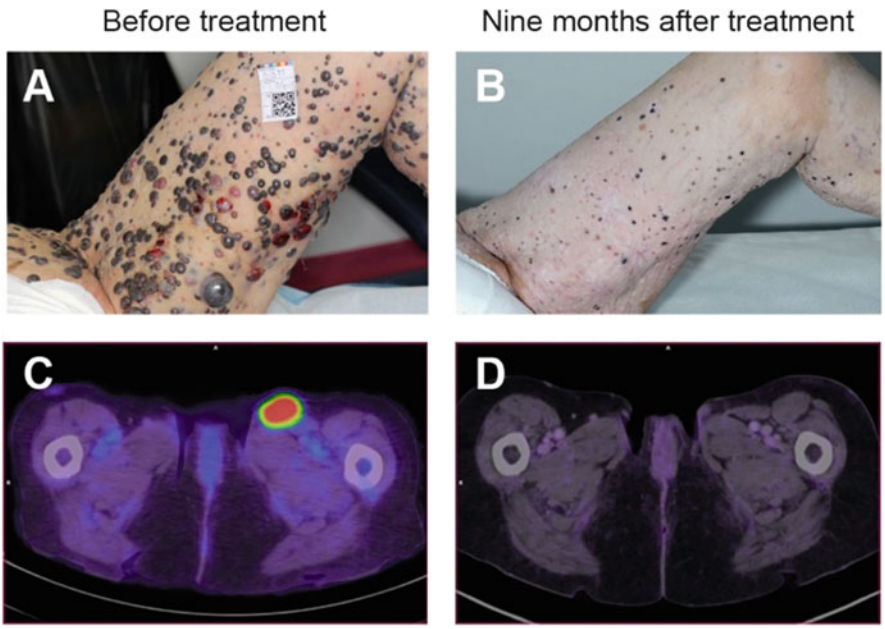

Fig. 4 Systemic immune response in a patient with malignant melanoma treated with calcium electroporation. A malignant melanoma patient with cutaneous metastases (**a**) and lymph node metastases (**c**) was treated with electrochemotherapy using bleomycin with good response but 4 months later new metastases appeared. Two of these metastases were treated, one with calcium electroporation and one with electrochemotherapy, and with complete response two months after treatment. Nine months after treatment levelling of treated as well as untreated cutaneous metastases (**b**) and decrease of lymph node metastases (**d**) were observed. (**a**, **c**) show metastases before treatment with calcium electroporation and **b** and **d** show metastases 9 months after treatment with calcium electroporation. Adapted from Falk et al., Acta Oncologica 2017 (2017a) and Frandsen et al., Cancers 2020 (2020a)

and/or the retreatment with calcium electroporation after electrochemotherapy has induced the systemic immune response.

A systemic immune response may also have been observed in a case report describing calcium electroporation treatment of a canine patient with oral melanoma with metastatic spread to lymph nodes (see Sect. 5.2) (Kulbacka et al. 2017).

These studies indicate that calcium electroporation may induce a systemic immune response in a subset of patients. More studies are warranted to confirm these results and to further investigate the mechanism behind this immune activation.

4 Human Clinical Trials

Three human clinical studies with calcium electroporation are published, two double-blinded, randomized phase II studies including patients with cutaneous metastases (Falk et al. 2017b; Agoston et al. 2020) and a phase II study with head

and neck cancer patients (Plaschke et al. 2019). The two studies with calcium electroporation of cutaneous metastases showed promising results. In each study, seven patients with small cutaneous metastases (<3 cm in diameter) from breast cancer or malignant melanoma were treated once by intratumoral injection of either 220 mM $CaCl_2$ or bleomycin immediately followed by electroporation with eight pulses of 100 μs, 1000 V/cm, and 1 Hz. The effect of the treatment was followed for 6 months showing that calcium electroporation was safe and deemed non-inferior to electrochemotherapy (Falk et al. 2017b; Agoston et al. 2020). In one of the studies, the complete response rate was 66% for calcium electroporation and 68% for electrochemotherapy (Falk et al. 2017b). In the other study, the complete response rates were lower, 22% for calcium electroporation and 40% for electrochemotherapy (Agoston et al. 2020). This might be due to differences in the type of needle electrodes used in the two studies, but also that both studies included a small number of patients. In the clinical study investigating calcium electroporation treatment of head and neck cancer patients, six patients with recurrent mucosal head and neck cancer where no other treatment options were available, were treated once with 225 mM $CaCl_2$ immediately followed by electroporation of eight pulses of 100 μs, 1000 V/cm and 1 Hz. This study also showed safety of the treatment and three of the patients had a partial response 2 months after treatment. One of these three patients experienced a complete remission and was without sign of cancer for more than 12 months after treatment (Plaschke et al. 2019).

Five studies with calcium electroporation are ongoing, two studies on colorectal cancer, two studies on cutaneous metastases and one on keloids. One of the studies with colorectal cancer investigates safety of the treatment for patients in palliative care (clinicaltrials.gov #NCT03542214) and in the other study on colorectal cancer, patients are treated with calcium electroporation 1–2 weeks prior to planned curative surgical removal (clinicaltrials.gov #NCT03694080) in hope of inducing a systemic immune response (see Sect. 3.5) before surgical removal, which may lower micrometastatic disease and risk of recurrence. In two clinical studies with patients with cutaneous metastases, tumor response (clinicaltrials.gov #NCT04225767), and histopathological effects of the treatment are investigated including changes in the amount of tumor infiltrating lymphocytes and many other immune-related signals (clinicaltrials.gov #NCT04259658). Finally, a study on patients with keloids, proliferative scars, treated with calcium electroporation is completed and manuscript submitted (clinicaltrials.gov #NCT01941914).

5 Veterinary Studies with Calcium Electroporation

Calcium electroporation may also be relevant for use in veterinary medicine (see Table 2) and recently two studies on equine sarcoids have been published. Furthermore, calcium electroporation may also be used for smaller domestic animals and two case reports on a feline and a canine patient, respectively, have been published showing a potential of this novel anticancer treatment for veterinary use.

5.1 Equine Studies

Two studies are published showing safety and response of calcium electroporation treatment of equine sarcoids (Galant et al. 2019; Frandsen et al. 2020b). Sarcoids are the most common equine skin tumors where several different treatment options have been investigated, however, most without high success rate and often with relapse. Thus, other treatment options are warranted and calcium electroporation could be an effective option.

Galant et al. published the first study on calcium electroporation treatment for sarcoids in 2019 where they studied safety and short-term effects of calcium electroporation (Galant et al. 2019). Sixteen sarcoids on ten horses were treated with calcium electroporation using 168 mM $CaCl_2$. Eight pulses of 100 μs, 1300 V/cm, and 500 Hz were applied using two parallel contact electrodes (10 mm between the electrodes) immediately after calcium injection. The treatment was well tolerated by all horses in the study and the only side effects observed were edema and pain to palpation in the first hours after treatment, which disappeared 2–5 days after treatment. Furthermore, crust formation was observed in 14 of 16 treated sarcoids. Treated sarcoids were surgically excised after 7, 14, or 21 days to perform histological analyses of the treatment effect. Analyses verified the clinical diagnose of sarcoid and determined the microscopic changes of the tumor and the surrounding normal tissue. In the 16 treated tumors findings included necrosis in 13, ulceration in 13, hemorrhages in 14, and calcification in 11. Of the 13 sarcoids showing necrosis, nine contained a fraction of necrosis higher than 50%. The authors indicate, based on the few data that the fraction of necrosis is higher after 21 days than earlier and that the fraction of necrosis may depend on the tumor volume before treatment. In the surrounding normal tissue, inflammation were observed in all 16 cases while hemorrhages were observed in eight and calcification in ten cases but importantly no necrosis was observed (Galant et al. 2019).

In the second study published by Frandsen et al. in 2020, safety and long-term effect of calcium electroporation on sarcoids were investigated (Frandsen et al. 2020b). Thirty-two sarcoids on eight horses were treated once or twice with 220 mM calcium chloride and application of eight pulses of 100 μs, 1000 V/cm and 1 Hz using 8-needle electrode with 4 mm between the rows. Six of the included horses were treated twice with 4–13 weeks between treatments. This study confirmed that calcium electroporation is well tolerated by horses. Response of the sarcoids was followed for 12–38 weeks after the first treatment and showed 22% complete response, 22% partial response, and 30% progressive disease (Fig. 5). The response seemed not to be related to location of the sarcoid, sarcoid type nor sarcoid size. Biopsies of most of the sarcoids were performed before the treatment (s) verifying the clinical diagnose of sarcoid and only few changes were observed before the second treatment likely due to the time span between treatments. Four of the lesions were not biopsied before any of the treatments and those four lesions all showed complete response. Biopsies have previously been shown to exacerbate sarcoid growth and this might affect the response of study (Frandsen et al. 2020b).

Fig. 5 Equine sarcoids treated with calcium electroporation. Two sarcoids on the same horse treated twice with calcium electroporation. Top row (**a**) shows sarcoid placed on eye lid and bottom row (**b**) shows sarcoid placed on ventral abdomen. Images show sarcoids before the first treatment (**a1**, **b1**), before the second treatment 4 weeks after the first treatment (**a2**, **b2**—note that biopsy was performed before picture was taken of sarcoid on ventral abdomen), and at follow-up 8 weeks (**a3**, **b3**), and 25 weeks (**a4**, **b4**) after the first treatment. The sarcoid on the eye lid (**a**) was completely eliminated at 25 weeks follow-up where only scar tissue remained (**a4**). The sarcoid on the ventral abdomen reacted to the treatments (**b3**) but regrowth was observed at 25 weeks follow-up (**b4**). From Frandsen et al., Animals 2020 (2020b)

These two studies on equine sarcoids show that calcium electroporation was well tolerated and with response of some of the treated lesions. The first study (Galant et al. 2019) showed better short-term response than the long-term response observed in the second study (Frandsen et al. 2020b), however, these results are based on a rather small number of treated sarcoids. The response to treatment may be affected by the electrodes used, the time of observation and pre-treatment.

5.2 Smaller Domestic Animals: Case Reports

Two case reports describing use of calcium electroporation for treatment of tumors in a feline and a canine patient, respectively, are published.

Kulbacka et al. published in 2017 a case report describing treatment of a 15-year-old canine patient with oral melanoma (Kulbacka et al. 2017). The patient was diagnosed with a 140-cm^3 malignant melanoma in the oral cavity with metastatic spread to lymph nodes and was unable to eat due to the disease. The owner approved the suggested palliative treatment with a combination of electrochemotherapy and laser surgery. The dog was treated in general anesthesia where debulking of the tumor in the oral cavity was performed using a CO_2 laser. The surgical debulking was followed by treatment with electrochemotherapy using bleomycin (administered

both intravenously and intratumorally) and two different needle electrodes containing 2 needles with a distance of 5 mm and 13 needles with a distance of 2 mm, respectively. The electroporation parameters used were eight pulses of 100 μs, 1300 V/cm, and 1 Hz. Electrochemotherapy stopped the bleeding caused by the surgery and was added to enhance the effect of the surgery for local control. The dog resumed eating the day after the treatment, and 10 days after treatment decrease of the tumor was observed. Fourteen days after treatment, enlargement of the metastatic spread in the lymph nodes as well as the treated tumor were detected. Based on the findings and the condition of the dog, affected lymph nodes and the previously treated tumor were treated with calcium electroporation using 10 ml 5 mM $CaCl_2$ and the two-needle electrode. This treatment caused strong inflammation in the lymph nodes 5 days after treatment, which was treated with the anti-inflammatory drug dexamethasone for 2 days. One month after the second treatment, the primary tumor was decreased and no metastatic disease was observed why the authors indicate activation of an immune response after treatment with calcium electroporation. The dog was euthanized shortly after due to seizure possibly caused by metastatic spread of the melanoma to the brain (Kulbacka et al. 2017).

In 2019, Dos Anjos et al. published a case report describing the treatment of a 9-year-old feline patient with a 0.19-cm^3 cutaneous melanoma and spread to a submandibular lymph node (0.5 cm^3) (Dos Anjos et al. 2019). The primary tumor as well as the lymph node metastasis were treated with electrochemotherapy using intravenous administration of bleomycin. Eight pulses of 100 μs, 1000 V/cm, and 1 Hz were applied using a 6-needle parallel electrode with 3 mm between the rows. Complete remission of the primary tumor and the metastasis in the lymph node were detected 28 days after treatment but 120 days after treatment tumor recurrence was confirmed in two lymph nodes. These lymph nodes were treated with a combination of electrochemotherapy and calcium using 9 mM calcium gluconate injected intratumorally followed by antibiotic and anti-inflammatory drug treatment due to mild edema and ulceration. As described above (Sect. 3.4.1), treatment with bleomycin and calcium in combination with electroporation in vitro showed additive effect but no synergistic effect (Frandsen et al. 2014). Twenty-eight days after the second treatment, complete response was observed and verified histologically but with recurrence in a lymph node 5 months after the second treatment. This was surgically removed but unfortunately, the cat died during the surgery due to cardio-respiratory arrest (Dos Anjos et al. 2019).

To our knowledge at this time only casuistic use of calcium electroporation in smaller domestic animals is described, and larger studies are needed to further characterize treatment effects.

6 Perspectives

Calcium electroporation is a novel anticancer treatment that, within a short period of time has been tested in preclinical studies, human clinical trials, and recently for use in veterinary medicine. Studies have shown a good effect of the treatment, however,

with differences in sensitivity between tumor types and further studies are warranted to clarify the mechanisms of action in order to optimize the treatment.

This anticancer treatment is simple and inexpensive, and no chemotherapeutic agents are used which is a great advantage since it is safer for the veterinary personnel treating the patient and there is no (expensive) handling of biological hazard waste. To perform this treatment, electroporation equipment is needed, and is now commercially available.

Some of the studies with calcium electroporation have shown activation of systemic immune response. More studies are warranted to investigate when and how the systemic response is activated.

7 Conclusion

Calcium electroporation is a safe, simple, and effective anticancer treatment, and the first studies in veterinary medicine are now published. Preclinical murine studies, human clinical trials, and veterinary trials have investigated safety and efficacy of this novel treatment with good results. Calcium electroporation is effective on all tested tumor types with differences in sensitivity while normal surrounding tissue is relatively spared. In some cases, calcium electroporation has activated a systemic immune response against the treated cancer. Calcium electroporation is a novel anticancer treatment with promising results and further studies are warranted to fully elucidate the potential of this treatment.

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References

Agoston D, Baltas E, Ocsai H, Ratkai S, Lazar PG, Korom I et al (2020) Evaluation of calcium electroporation for the treatment of cutaneous metastases: a double blinded randomised controlled phase II trial. Cancers (Basel) 12(1):179

Carafoli E, Santella L, Branca D, Brini M (2001) Generation, control, and processing of cellular calcium signals. Crit Rev Biochem Mol Biol 36(2):107–260

Cerella C, Diederich M, Ghibelli L (2010) The dual role of calcium as messenger and stressor in cell damage, death, and survival. Int J Cell Biol 2010:546163–546176

Clapham DE (2007) Calcium signaling. Cell 131(6):1047–1058

Dos Anjos DS, Rodriguese CG, Silva NC, De Nardi AB, Fonseca-Alves CE (2019) Electrochemotherapy associated with calcium electroporation in metastatic feline cutaneous malignant melanoma. Acta Sci Vet 47(Suppl 1):435

Falk H, Lambaa S, Johannesen HH, Wooler G, Venzo A, Gehl J (2017a) Electrochemotherapy and calcium electroporation inducing a systemic immune response with local and distant remission of tumors in a patient with malignant melanoma—a case report. Acta Oncol 56(8):1126–1131

Falk H, Matthiessen LW, Wooler G, Gehl J (2017b) Calcium electroporation for treatment of cutaneous metastases; a randomized double-blinded phase II study, comparing the effect of calcium electroporation with electrochemotherapy. Acta Oncol 57:311–319

Falk H, Forde PF, Bay ML, Mangalanathan UM, Hojman P, Soden DM et al (2017c) Calcium electroporation induces tumor eradication, long-lasting immunity and cytokine responses in the CT26 colon cancer mouse model. Oncoimmunology 6:e1301332

Frandsen SK, Gehl J (2017) Effect of calcium electroporation in combination with metformin in vivo and correlation between viability and intracellular ATP level after calcium electroporation in vitro. PLoS One 12(7):e0181839

Frandsen SK, Gissel H, Hojman P, Tramm T, Eriksen J, Gehl J (2012) Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis. Cancer Res 72 (6):1336–1341

Frandsen SK, Gissel H, Hojman P, Eriksen J, Gehl J (2014) Calcium electroporation in three cell lines: a comparison of bleomycin and calcium, calcium compounds, and pulsing conditions. Biochim Biophys Acta 1840(3):1204–1208

Frandsen SK, Gibot L, Madi M, Gehl J, Rols MP (2015) Calcium electroporation: evidence for differential effects in normal and malignant cell lines, evaluated in a 3D spheroid model. PLoS One 10(12):e0144028

Frandsen SK, McNeil AK, Novak I, McNeil PL, Gehl J (2016) Difference in membrane repair capacity between cancer cell lines and a normal cell line. J Membr Biol 249(4):569–576

Frandsen SK, Kruger MB, Mangalanathan UM, Tramm T, Mahmood F, Novak I et al (2017) Normal and malignant cells exhibit differential responses to calcium electroporation. Cancer Res 77(16):4389–4401

Frandsen SK, Vissing M, Gehl J (2020a) A comprehensive review of calcium electroporation—A novel cancer treatment modality. Cancers 12(2):290

Frandsen SK, Gehl J, Tramm T, Thoefner MS (2020b) Calcium electroporation of equine sarcoids. Animals (Basel) 10(3):517

Galant L, Delverdier M, Lucas MN, Raymond-Letron I, Teissie J, Tamzali Y (2019) Calcium electroporation: the bioelectrochemical treatment of spontaneous equine skin tumors results in a local necrosis. Bioelectrochemistry 129:251–258

Gehl J (2003) Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. Acta Physiol Scand 177(4):437–447

Gehl J, Sersa G, Matthiessen LW, Muir T, Soden D, Occhini A et al (2018) Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol 57(7):874–882

Gibot L, Montigny A, Baaziz H, Fourquaux I, Audebert M, Rols MP (2020) Calcium delivery by electroporation induces in vitro cell death through mitochondrial dysfunction without DNA damages. Cancers (Basel) 12(2):425

Graybill PM, Davalos RV (2020) Cytoskeletal disruption after electroporation and its significance to pulsed electric field therapies. Cancers (Basel) 12(5):1132

Guionet A, Moosavi Nejad S, Teissie J, Sakugawa T, Katsuki S, Akiyama H et al (2018) Spatiotemporal dynamics of calcium electrotransfer during cell membrane permeabilization. Drug Deliv Transl Res 8(5):1152–1161

Hansen EL, Sozer EB, Romeo S, Frandsen SK, Vernier PT, Gehl J (2015) Dose-dependent ATP depletion and cancer cell death following calcium electroporation, relative effect of calcium concentration and electric field strength. PLoS One 10(4):e0122973

Hoejholt KL, Muzic T, Jensen SD, Dalgaard LT, Bilgin M, Nylandsted J et al (2019) Calcium electroporation and electrochemotherapy for cancer treatment: importance of cell membrane composition investigated by lipidomics, calorimetry and in vitro efficacy. Sci Rep 9(1):4758

Kulbacka JPJ, Rembiałkowska N, Saczko J, Kiełbowicz Z, Kinda W, Liszka B, Kotulska M, Kos B, Miklavčič D, Tozon N, Čemažar M (2017) Electrochemotherapy combined with standard and CO_2 laser surgeries in canine oral melanoma. Slov Vet Res 54(4):181–186

Marty M, Sersa G, Garbay JR, Gehl J, Collins CG, Snoj M et al (2006) Electrochemotherapy—an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. EJC Suppl 4 (11):3–13

Mir LM, Gehl J, Sersa G, Collins CG, Garbay JR, Billard V et al (2006) Standard operating procedures of the electrochemotherapy: instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator (TM) by means of invasive or non-invasive electrodes. Eur J Cancer Suppl 4(11):14–25

Morotomi-Yano K, Akiyama H, Yano K (2014) Different involvement of extracellular calcium in two modes of cell death induced by nanosecond pulsed electric fields. Arch Biochem Biophys 555–556:47–54

Neumann E, Toensing K, Kakorin S, Budde P, Frey J (1998) Mechanism of electroporative dye uptake by mouse B cells. Biophys J 74(1):98–108

Pakhomova ON, Gregory B, Semenov I, Pakhomov AG (2014) Calcium-mediated pore expansion and cell death following nanoelectroporation. Biochim Biophys Acta 1838(10):2547–2554

Plaschke CC, Gehl J, Johannesen HH, Fischer BM, Kjaer A, Lomholt AF et al (2019) Calcium electroporation for recurrent head and neck cancer: A clinical phase I study. Laryngoscope Investig Otolaryngol 4(1):49–56

Rols MP, Teissie J (1990) Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon. Biophys J 58(5):1089–1098

Romeo S, Sannino A, Scarfi MR, Vernier PT, Cadossi R, Gehl J et al (2018) ESOPE-equivalent pulsing protocols for calcium electroporation: an in vitro optimization study on 2 cancer cell models. Technol Cancer Res Treat 17:1533033818788072

Staresinic B, Jesenko T, Kamensek U, Krog Frandsen S, Sersa G, Gehl J et al (2018) Effect of calcium electroporation on tumour vasculature. Sci Rep 8(1):9412

Szewczyk A, Gehl J, Daczewska M, Saczko J, Frandsen SK, Kulbacka J (2018) Calcium electroporation for treatment of sarcoma in preclinical studies. Oncotarget 9(14):11604–11618

Thompson GL, Roth CC, Dalzell DR, Kuipers M, Ibey BL (2014) Calcium influx affects intracellular transport and membrane repair following nanosecond pulsed electric field exposure. J Biomed Opt 19(5):055005

Wasson EM, Alinezhadbalalami N, Brock RM, Allen IC, Verbridge SS, Davalos RV (2020) Understanding the role of calcium-mediated cell death in high-frequency irreversible electroporation. Bioelectrochemistry 131:107369

Zhivotovsky B, Orrenius S (2011) Calcium and cell death mechanisms: a perspective from the cell death community. Cell Calcium 50(3):211–221

Zielichowska A, Daczewska M, Saczko J, Michel O, Kulbacka J (2016) Applications of calcium electroporation to effective apoptosis induction in fibrosarcoma cells and stimulation of normal muscle cells. Bioelectrochemistry 109:70–78

Irreversible Electroporation Applications

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Abstract

In veterinary medicine, irreversible electroporation (IRE) applications have been evaluated in several early phase clinical trials evaluating the safety and feasibility of treatment of solid tumors located within different organ systems. These clinical trials support the use of IRE as a safe and effective alternative treatment option for tumors that are otherwise not amenable to current standard of care therapy. The recent development of high-frequency irreversible electroporation (H-FIRE) has made treatment delivery even more feasible for veterinary clinical patients. Here, we provide an overview of treatment planning and techniques while acknowledging current limitations associated with treatment delivery, followed by a review of IRE applications in veterinary clinical trials of spontaneous cancers. Lastly, we review the application of IRE in preclinical normal animal models or experimentally induced tumors as results of these studies may help facilitate translation of IRE into standard clinical practice.

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1 Irreversible Electroporation Theory and Techniques

Irreversible electroporation (IRE) is an attractive nonthermal ablation method that uses high voltage (500–3000 V) pulsed electric fields (PEFs) of short duration (100 μs) to increase the transmembrane potential of cells and cause cell membrane disruption (Fig. 1a and Table 1). In response to a high-voltage PEF, the cell membrane forms electrically conducting nanopores to stabilize the transmembrane potential (Weaver and Chizmadzhev 1996). As the transmembrane potential is proportional to the locally applied electric field, the PEF protocol dictates whether the electroporation effects are transient or will lead to irrevocable damage. Under certain pulsing protocols, this process is reversible and has historically been applied in this manner to introduce genes and drugs into cells that would normally be impermeant to the cell membrane. However, once the applied electric field exceeds the threshold required to induce irreversible electroporation, the exposed cells will undergo cell death (Rubinsky 2007; Arena et al. 2011a; DeBruin and Krassowska 1999). As the electric fields which induce electroporation follow a sharp sigmoidal response, tissue ablation with IRE resolves within a submillimeter demarcation between treated and nontreated tissues (Potocnik et al. 2019). A major advantage of IRE compared to other ablation methods is that treatment results in minimal thermal damage due to negligible Joule heating of treated tissue, except for that lying immediately adjacent (<~1 mm) to the electrode edges where the electric field is greatest (Arena et al. 2011a). Contrary to thermal ablation methods, IRE is not prone to the phenomenon known as the "heat sink effect," in which larger blood vessels act to dissipate heat from surrounding tissue by means of conduction and convection heat transfer (Ahmed et al. 2011). Rather, cell death occurs at nearly discrete electric field thresholds and thus, treatment volumes are predictable and represented by a well-defined ablation zone following IRE delivery (Edd and Davalos 2007). Thus, its nonthermal cell death mechanism allows for treatment in otherwise inoperable locations, such as near major blood vessels and nerves. Taken together, the clinical advantages afforded from IRE therapy have resulted in improved overall survival in patients with pancreatic cancer (Kwon et al. 2014; Martin et al. 2013; Narayanan et al. 2012; Scheffer et al. 2014a, b). The promising early responses have led to the Food and Drug Administration granting an Expedited Access Pathway (EAP) designation to the IRE-based NanoKnife System to commercialize this therapy for widespread use.

IRE has demonstrated high efficacy for treatment of unresectable tumors, though improvements have recently been identified and engineered. Traditional IRE uses monophasic/unipolar pulses characterized by low frequency components; it is known that low frequency electric currents preliminarily traverse the extracellular

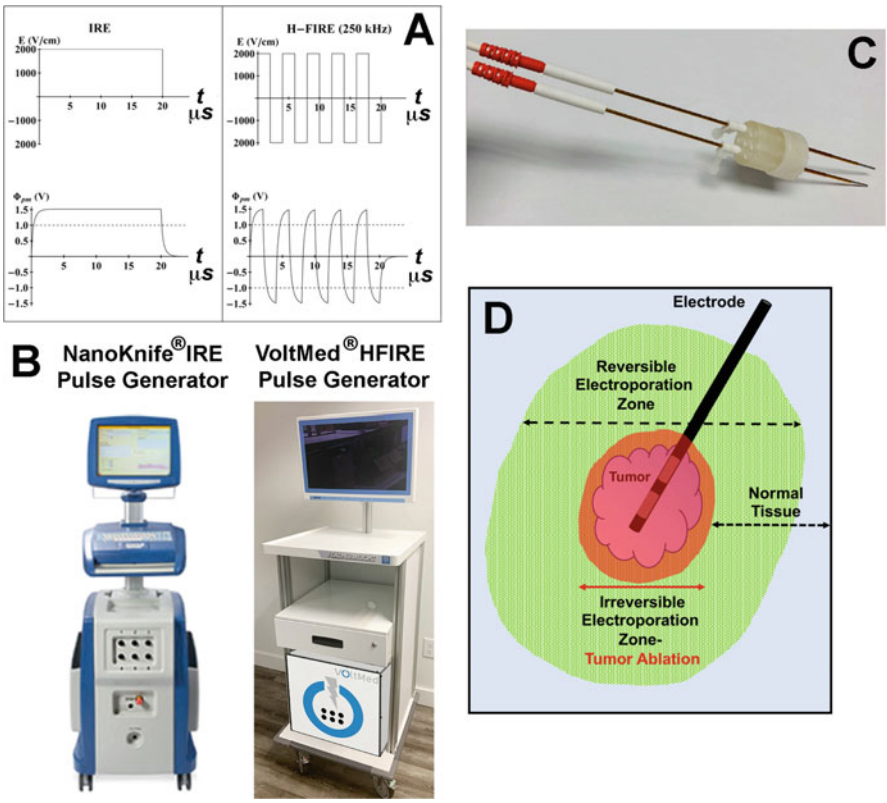

Fig. 1 (**a**) Comparison between IRE (left) and H-FIRE (right) pulses. IRE employs monopolar pulses of <100μs duration. H-FIRE pulse cycles consist of a series of ultrashort 0.5–2μs pulses of alternating polarity separated by 0.5–5μs of no energy delivery. Cycles are repeatedly delivered (10–100 cycles) to form bursts which are delivered at a ~1 Hz frequency. Amplitude of voltage delivery ranges from 0.25 to 5.0 kV. (**b**) NanoKnife IRE and INSPIRE H-FIRE pulse generators. (**c**) Blunt-tipped monopolar H-FIRE electrodes for use in the brain. (**d**) Schematic representation of electroporation treatment paradigm. Electrodes are inserted into the target tissue and used to deliver pulses that expose target tissue to the critical electrical field threshold that results in irreversible electroporation and tumor ablation (red). Cells further away from the electrodes and exposed to a smaller electrical field will undergo reversible electroporation

domain prior to cell electroporation. Thus, IRE pulses may be more susceptible to large impedance changes due to electroporation and may become disrupted by the impedance barrier of poorly conductive epithelial layers, including the skin (Bhonsle et al. 2015). High-frequency irreversible electroporation (H-FIRE) is a next generation IRE method that uses bursts of ultrashort (<10 μs) bipolar (Fig. 1a) PEFs to selectively ablate cells within a target volume (Arena et al. 2011b). As the characteristic frequencies associated with ultrashort PEFs (biphasic 1 μs pulses = 500 kHz) are higher than that of IRE (pulsed DC), H-FIRE is less susceptible to field distortions caused by heterogenous tissues and can overcome the impedance barrier

Table 1 Summary of treatment-related characteristics unique to ECT, IRE, and H-FIRE

Method	Pulse parameters	Treatment planning/ delivery	Limitations
ECT	Electric field threshold: <1000 V/cm Pulse duration: 50–100μs Pulse polarity: unipolar/ monophasic Pulse delivery rate: 1 Hz or 5 kHz	Flat plate electrodes[a,b] Linear array of needle electrodes Hexagonal array of needle electrodes Finger electrodes: axial or perpendicular needle electrodes At least 2 monopolar probes placed parallel to each other Neuroparalytic/muscle relaxant: local anesthetic administered	Requires multiple probes to be precisely placed for treatment of deep-seated tumors Tetany requires paralytics for treatment of deep-seated tumors Cardiac asynchrony requires cardiac synchronization for treatment of deep-seated tumors Difficulty overcoming impedance of epithelial tissues Limited by tumor size Potentially contra-indicated near metal implants for treatment of deep-seated tumors
IRE	Electric field threshold: >1000 V/cm Pulse duration: 50–100μs Pulse polarity: unipolar/ monophasic Pulse delivery rate: ~1 Hz or ECG synced	At least 2 monopolar probes placed parallel to each other Single needle-dual electrode Neuroparalytic/muscle relaxant: required	Requires multiple probes to be precisely placed for treatment of deep-seated tumors Tetany requires paralytics for treatment of deep-seated tumors Cardiac asynchrony requires cardiac synchronization for treatment of deep-seated tumors Difficulty overcoming impedance of epithelial tissues Limited by tumor size Contra-indicated near metal implants
H-FIRE	Electric field threshold: • Ablation: >1000 V/cm • BBBD: 113.5 V/cm Pulse width: 1–10μs Pulse polarity: bipolar/biphasic Burst delivery rate: ~1 Hz	At least 2 monopolar probes placed parallel to each other Single needle-dual electrode Single needle and surface electrode Neuroparalytic/muscle relaxant: clinicians' discretion	Requires multiple probes to be precisely placed for treatment of deep-seated tumors Limited by tumor size Contra-indicated near metal implants

[a]Miklavcic et al. (2005)
[b]Miklavcic et al. (2014)

posed by epithelial layers, thereby increasing treatment precision and minimizing the risk of skip lesions (Arena et al. 2011a). Additionally, the use of charge-balanced bipolar pulses minimizes nerve and muscle stimulation, thereby negating the need for paralytics and cardiac synchronization required during IRE delivery (Mercadal et al. 2017; Arena et al. 2011a, b).

Currently, an IRE platform is commercially available as the NanoKnife (Fig. 1b; AngioDynamics. Latham, NY, USA). The device is composed of a high-voltage

pulse generator with capabilities of pulsing across up to 6 electrode probes (not simultaneously) and can be used with a cardiac synchronization device. The generator delivers high voltage, pulsed direct current (DC) monophasic waveforms between the tips of two monopolar probes (Fig. 1c), or between the poles of a single insertion bipolar probe. Depending on the size of the lesion, insertion of multiple probes (>2) may be required to achieve complete ablation of the target tissue. IRE pulse delivery is synchronized with the patient's ECG, specifically to the absolute refractory period of the cardiac cycle, so that each pulse delivery occurs 50 ms after each R wave, to reduce the risk of inducing ventricular arrhythmias observed in earlier studies involving IRE (Thomson et al. 2011; Deodhar et al. 2011). For H-FIRE delivery, the authors use a generator commercially available through VoltMed, LLC (INSPIRE; Blacksburg, VA, USA), though many iterations of biphasic pulse generators have been proposed (Rebersek and Miklavcic 2011; Redondo et al. 2019; Elgenedy et al. 2017). Since the risk of tetany and cardiac arrhythmia induction may be reduced with H-FIRE compared to traditional IRE, cardiac synchronization is not required though remains a capability of commercial biphasic pulse generators.

In clinical settings, IRE and H-FIRE may be delivered through electrodes inserted percutaneously, endoscopically, trans-rectally, or directly into target tissues following exposure through an open surgical approach (Fig. 1d). Electrode placement is often performed under ultrasound, CT, or more recently, MRI guidance to ensure accurate placement (Collettini et al. 2019). The pulsing protocol (i.e., the number of pulses, energized time per pulse, and the locally applied electric field) dictates the electroporation effects incurred (Bhonsle et al. 2016; Pucihar et al. 2011), with reversible and irreversible electroporation treatment regimens having been described (Fig. 1d). Additionally, zones of tissue ablation can be visualized by posttreatment imaging using magnetic resonance imaging (MRI), computed tomography (CT), or ultrasonography. Due to the inherent nonthermal nature of IRE, tumor antigens and tissue components released from dying cells remain in their native form and are therefore capable of inducing a unique and robust systemic immune response. This hypothesis has been supported by evidence of DAMP signaling detected in rodent and canine models following IRE for treatment of various tumors (Ringel-Scaia et al. 2019; Brock et al. 2019).

Development of novel equipment and delivery techniques to improve treatment feasibility, efficacy, and safety have been evolving in parallel with the use of IRE for an increasing number of clinical indications. IRE delivery to cutaneous tumors historically involved the use of plate electrodes placed on either side of the tumor. Subsequent development of clinically relevant single-needle electrodes, including those with customized features such as blunted tips for use in the brain, has minimized invasiveness and improved feasibility of IRE delivery, such that these electrodes have become a standard part of the equipment used during treatment (Neal et al. 2010a). An endoscopic needle-electrode has been more recently evaluated in the porcine pancreas and determined to be feasible and effective when utilized in an endoscopic ultrasound-guided procedure. Although additional studies evaluating the safety and efficacy of this procedure are warranted, it would make endoscopic

treatment of pancreatic cancer under real-time monitoring possible and negate the need for more invasive surgical procedures. A newer technique using two spoon-shaped parallel plate electrodes (paddles) in lieu of IRE needles successfully ablated porcine pancreatic tissue when the paddles were placed around the target tissue. This technique was capable of creating a homogenous electrical field without the need for needle insertion, reducing the risk of pancreatic fistula, which has been reported in up to 18% of human patients secondary to needle tracks (Rombouts et al. 2017).

2 Treatment Planning

Treatment outcome with IRE therapies is directly influenced by exposure of target tissue to threshold electric fields. The applied electric field distribution (EFD) can be fine-tuned by altering either the PEF amplitude (500–3000 V) or the electrode configuration in which the PEFs are administered. Electrode positioning and number of electrodes, as well as length of active tip exposure, affect the EFD and therefore the ablation zone and overall treatment outcome (Wendler et al. 2016). Furthermore, though not directly affecting the EFD, parameters such as number of pulses, energized time per pulse, and pulse delivery rate can be tuned to increase the tissue's exposure to these therapeutic electric fields. An increased exposure to high electric fields is linked to a decrease in the IRE electric field threshold (Latouche et al. 2017; Siddiqui et al. 2016), therefore larger volumes of IRE can be produced. Other factors affecting treatment outcome, not under the control of the operator, include the cell type, morphology, age, and size (Gehl and Mir 1999; Miller et al. 2005). In addition, the distribution of tissue impedance can promote distortions in the applied electric fields and therefore affect ablation outcomes (Edd and Davalos 2007). Thus, pre-treatment planning using numerical methods can be used to predict the EFD throughout the target lesion prior to IRE delivery, and greatly enhance the efficacy and precision of IRE tissue ablation (Kos et al. 2015; Zupanic et al. 2012).

The EFD during electroporation can be estimated by solving a modified Laplace equation:

$$\nabla \cdot (\sigma \nabla \varphi) = 0.$$

Here, σ is the tissue electrical conductivity and φ is the electric potential. During electroporation, creation of electrically conducting defects on the cell membrane alter the bulk tissue conductivity. The change in conductivity caused by electroporation is therefore proportional to the applied PEF amplitude, which follows a sigmoidal behavior. This relationship has been characterized in skeletal muscle (Corovic et al. 2012), liver (Miklavcic et al. 2000; Zhao et al. 2018a; Bhonsle et al. 2018; Ivorra et al. 2009), prostate (Neal et al. 2014; Campelo et al. 2017), pancreas (O'Brien et al. 2019; Beitel-White et al. 2018), brain (Lorenzo et al. 2019; Garcia et al. 2010), kidney tissue (Neal et al. 2012), and other tissues (Ivorra et al. 2009; Cindric et al. 2018). Incorporation of this sigmoid–conductivity relationship captures electric field redistribution effects due to electroporation, thus informing an

accurate numerical model. Thereafter, Joule heating effects from PEF treatment are captured using a modified Pennes Bioheat transfer equation:

$$\nabla(k\nabla T) - \omega_b C_b \rho_b (T - T_a) + q''' + \frac{\sigma \cdot |E|^2 \cdot p}{\tau} = \rho C_p \frac{\partial T}{\partial t}.$$

Here, k is the thermal conductivity, ω_b is the blood perfusion rate, C_b is the blood-specific heat capacity, ρ_b is the density of blood, T_a is the arterial blood temperature, q''' is the metabolic heat generation, ρ is the density of the tissue, and C_p is the specific heat capacity of the tissue. Joule heating, resistive heating incurred from high-voltage pulsing, is dependent on the EFD as well as the on-time per pulse, p, and the pulsing period τ. Altogether, an approximation for the EFD with Joule heating effects can be accomplished using numerical methods, for example, using a finite element package like COMSOL Multiphysics (COMSOL Inc., Stockholm, Sweden). This platform can be used to develop IRE treatment plans to ensure the treatment protocol will not yield any adverse thermal effects (Edd and Davalos 2007). It should be noted, this methodology does not account for microscopic heterogeneities found in biological tissue. Tumor calcifications, tumor necrosis, large vasculature, and other critical structures embedded within the target tissue must be incorporated into the model to account for potential field distortions (Golberg et al. 2015; Ben-David et al. 2013). Lastly, once the EFD is approximated, the anticipated IRE ablation regions are determined as discrete electric field thresholds within the EFD. These thresholds can be approximated a priori using in vitro cell suspensions (Vizintin et al. 2020; Pavlin et al. 2005), in vitro tumor constructs (Dettin et al. 2019; Ivey et al. 2019; Arena et al. 2012), or determined using in vivo and ex vivo results (Corovic et al. 2012; Neal et al. 2015; Miklavcic et al. 2004). Following pulse delivery, increased cell membrane permeabilization equivalently results in increased bulk tissue conductivity; as the difference in conductivity is detectable using electrical impedance spectroscopy (EIS) methods, this change in conductivity has been the target for real-time peri-operative monitoring of treatment progression. Proposed methods for monitoring impedance changes include: (1) electrical impedance tomography, which utilizes an array of electrodes placed in/around the target tissue to reconstruct an impedance map following pulse delivery (Davalos et al. 2004); (2) electrical impedance spectroscopy to measure bulk impedance changes (Bonakdar et al. 2015; Ivorra et al. 2009); (3) using the absolute change in tissue resistance to indicate treatment cessation (Dunki-Jacobs et al. 2014); and (4) approximating the electric field distribution during pulse delivery using magnetic resonance electrical impedance tomography (MREIT) (Kranjc et al. 2017); and (5) more recently, the use of rapid EIS for continually measuring changes in tissue impedance to monitor an ablation endpoint and delineate change due to thermal and electroporation effects (Lorenzo et al. 2020). IRE-mediated cell membrane disruption results in shunting of the applied electric current throughout the target lesion, therefore successful IRE ablation will decrease tissue impedance as a result of an impaired membrane (Zhao et al. 2018b).

Altogether, the electric field distribution and the temperature distribution for a representative monopolar electrode configuration and single-insertion bipolar configuration is seen in Fig. 2 as performed in COMSOL Multiphysics v5.5. This numerical simulation was solved using electrical conductivity values of human pancreatic cancer tissue (Beitel-White et al. 2018), IRE threshold (500 V/cm) for pancreatic ductal adenocarcinoma (Arena et al. 2012) and thermal properties of healthy pancreatic tissue (O'Brien et al. 2019). The monopolar configuration was simulated as 1.5 cm spacing and 1.5 cm electrode exposure with pulsing parameters of 100 IRE pulses, applied voltage of 2500 V, 70 μs energized-time per pulse, delivered at 1 pulse per second (Fig. 2a). The bipolar configuration was simulated as 0.8 cm spacing and 0.7 cm electrode exposure, with pulsing parameters similar to the monopolar-electrode model (Fig. 2b). In this numerical model, the IRE ablation far exceeds the volume of tissue exposed to >50 °C. This numerical simulation serves to show how the electrode configuration can be modified to ablate large volumes of tissue, with minimal heating, to treat desired patient-specific tumor geometries.

Patient-specific IRE/H-FIRE treatment planning requires cross-sectional imaging of the target tissue acquired via computed tomography (CT) or magnetic resonance imaging (MRI) to generate a three-dimensional (3D) model of the target lesion (i.e., tumor) (Zupanic et al. 2012). The 3D model is then used to estimate the electric field distribution and generate a patient-specific treatment plan. Although numerical modeling computations can be completed manually, it is time consuming, so commercially available treatment planning software interfaces are often used to facilitate this process. The NanoKnife System incorporates its own touch screen software interface for basic treatment planning (not including patient imaging), procedure set-up, and real-time visualization of changes in voltage and electric current during treatment delivery. ApiVizTEP is a downloadable software application capable of generating rough estimates of the electric field distribution in real time. Web-based treatment planning software using algorithms for tissue segmentation and 3D modeling is also available that generates a downloadable treatment plan for the user (Pavliha et al. 2013). Recently, an online platform was developed to further streamline treatment planning for use with IRE treatments (Perera-Bel et al. 2020). Treatment plans provide users with information regarding probe location and spacing, electrode exposure, and delivered voltage required to achieve optimal tumor ablation while sparing adjacent normal tissue (Byron et al. 2019).

3 Limitations of IRE and H-FIRE

One of the clinical limitations of IRE is the high level of technical skill required for accurate electrode placement. Additionally, electrode positioning must be maintained during pulse delivery to ensure precise and complete treatment (Linecker et al. 2016). Risks associated with treatment are location-dependent and include hemorrhage, acute fulminant necrosis of the target tissue/organ, infection, muscle excitation, and cardiac arrhythmias. Compared to other surgical techniques,

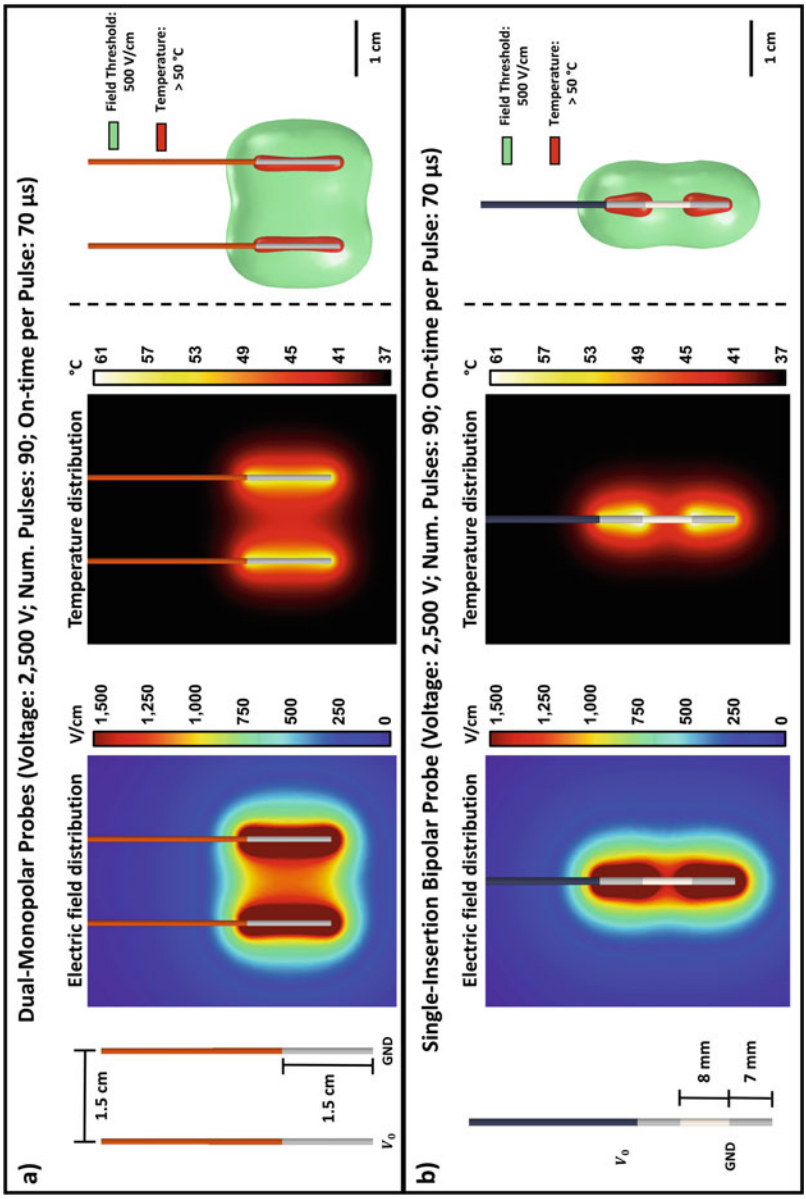

Fig. 2 Electric field and temperature distributions in monopolar and bipolar probe electrode configurations. The electric field distribution between (**a**) two monopolar electrodes and (**b**) a single-insertion bipolar probe is highest at the electrode tips and dissipates with increased distance. The temperature increase

complications are drastically lowered due to the nonthermal nature of the ablation modality with preservation of nerve function and major vasculature (Onik and Rubinsky 2010). However, the serious potential for muscle excitation and cardiac asynchrony necessitates the need for paralytics and cardiac synchronization during IRE delivery and restricts treatment eligibility to patients without underlying heart disease. In the absence of adequate neuromuscular blockade, muscle contractions associated with IRE delivery may displace implanted electrodes, thereby increasing the risk of delivering unintended electric fields to tissues and collateral damage to adjacent critical structures. These have potential for deleterious consequences, such as inadvertent damage to surrounding normal tissues (Kingham et al. 2012; Thomson et al. 2011). Furthermore, cardiac synchrony is required to avoid arrythmias. Lastly, the presence of metal, such as a stent or implant, within the ablation zone results in higher temperatures around the electrodes due to changes in electric field distribution, and is therefore contraindicated at this time (Scheffer et al. 2016).

In order to overcome some of these limitations, high-frequency irreversible electroporation (H-FIRE) was developed. H-FIRE uses ultrashort (<10 μs) bipolar PEFs that minimize nerve and muscle stimulation (Table 1). This negates the need for paralytics and cardiac synchronization, and shortens the duration of anesthetic events required for treatment procedures (Arena et al. 2011a; Ball et al. 2010). Development of a dual electrode-single needle probe compatible with currently available generators for H-FIRE delivery helped mitigate some of the technical challenges associated with multiple electrode insertions required by traditional IRE (O'Brien et al. 2019). Despite improvements in feasibility, efficacy in treating very large tumors remains limited, and research is ongoing to determine a size threshold for effective treatment.

4 Clinical Applications of IRE and H-FIRE in Companion Animals with Naturally Occurring Cancers

Veterinary patients with a variety of spontaneous cancers have served as large animal models for investigation into the feasibility and safety of IRE and H-FIRE for tumor ablation. In aggregate, these studies have indicated that IRE and H-FIRE are viable options for treatment of tumors in veterinary patients with superficial tumors (Neal et al. 2010b; Byron et al. 2019), primary liver tumors (Partridge et al. 2020), prostate cancer (Neal et al. 2014; Onik et al. 2007), and brain tumors (Garcia et al. 2010, 2012; Latouche et al. 2018; Rossmeisl et al. 2015), although clinical

Fig. 2 (continued) (resistive heating) is proportional to the applied field and is also highest near the electrode surface. At an IRE threshold of 500 V/cm, the ablation volume far exceeds a volume of tissue encompassing 50 °C. The configuration and pulsing parameters are tunable to maximize tumor cell death while sparing healthy tissue and critical structures

evidence supporting the efficacy of IRE and H-FIRE in veterinary oncology is currently limited. However, what has been demonstrated to date has been promising, and several clinical trials are ongoing. Porcine and rodent models have also been used to evaluate these technologies for treatment of pancreatic cancer (Clark 2017; Fritz et al. 2015; Charpentier et al. 2010) and mammary cancer (Ringel-Scaia et al. 2019), respectively (Table 2). Collectively, these studies support the use of IRE and H-FIRE for safe treatment of tumors located near critical structures, and particularly those that are not amenable to surgery or have failed conventional therapies.

4.1 Superficial Soft Tissue Tumors

Soft tissue sarcomas are a group of tumors arising from mesenchymal tissue that share many biological and clinical characteristics. These tumors are relatively common among veterinary patients but rarely encountered in human medicine, however, their behavior is similar across species. Important prognostic factors include tumor location, histologic subtype, tumor grade, and size (Pasquali et al. 2018). In general, these tumors tend to be very locally aggressive, forming finger-like projections, called tendrils that infiltrate surrounding tissue. The metastatic potential of these tumors greatly depends on tumor grade, with grade 1 and 2 (low-grade) tumors metastasizing in less than 15% of cases, and grade 3 (high-grade) tumors metastasizing in up to 50% of cases. Regardless of grade, most patients succumb to complications from local disease if the tumor cannot be adequately controlled via some combination of complete surgical removal and radiation therapy. Unfortunately, these tumors often form in locations not amendable to wide surgical excision, such as on the distal extremity, or in close proximity to vital structures, making wide surgical excision difficult with a high risk of morbidity and/or mortality. Thus, a nonthermal ablation method, like IRE, provides an attractive treatment option for these tumors given its ability to precisely ablate tumor tissue while sparing adjacent critical structures (Vailas et al. 2019). Current evidence supporting its application for treatment of soft tissue sarcomas in both veterinary and human patients is limited to a few case reports, making it difficult to draw significant conclusions about safety and efficacy of IRE for this purpose (Qin et al. 2017; Neal et al. 2010b; Usman et al. 2012).

IRE was successful at alleviating clinical signs associated with histiocytic sarcoma, a round cell tumor with behavior that resembles that of sarcomas, located within the left coxofemoral region in a canine patient (Neal et al. 2010b). The primary tumor was intimately associated with the sciatic nerve and femoral artery, resulting in compartment syndrome of the left thigh. Due to severe osteoarthritis, aggressive surgery involving hemipelvectomy and limb amputation was contraindicated. In this case, CT imaging was used to generate a geometric model of the target region, and a numerical model was used to determine predicted ablation zones, which were used to develop a treatment plan and pulsing protocol. IRE electrodes were placed under CT guidance, and all neurophysiologic and vascular function remained intact following treatment. Presenting clinical signs of sciatic

Table 2 Clinical applications of irreversible electroporation (IRE and H-FIRE) in veterinary patients and animal models

Application	Treatment plan	Histologic features	Adverse events	Histologic subtypes	Animal models
Superficial Soft Tissue Tumors *IRE and H-FIRE	CT-guided; percutaneous; 2-5-2 waveform in 100μs bursts; 1500–3100 V	Preservation of neurovascular structures	Depigmentation (melanoma); no clinically significant adverse effects	Histiocytic sarcoma, cutaneous tumors, melanoma	Canine[a], equine
Liver Cancer *IRE and H-FIRE	US-guided; percutaneous 1–360 pulses; 20–100μs duration; 360–3000 V/cm 2-5-2 waveform; single bipolar probe	Vasculitis; endothelial necrosis; preservation of bile ducts/vessels; well-defined ablation zones with T-cell infiltration; chronic fibrosis within ablation zone	Elevations in ALT, ALP, AST, and GGT; no clinically significant adverse effects	Normal liver, hepatocellular carcinoma	Canine, porcine, murine (rat), caprine, rabbit
Brain Cancer *IRE and H-FIRE	CT- and MR-guided; stereotactic electrode placement; intracranial via craniectomy 0–2000 V; various waveforms	Well-defined ablation zone characterized by malacia and hemorrhage; vascular preservation; microglial activation; transient BBB disruption	Coagulative necrosis and brain herniation at 2000 V/cm; seizures; peri-operative GI upset; aspiration pneumonia; hemorrhage	Normal brain, glioblastoma, meningioma	Murine, canine
Pancreatic Cancer *IRE and H-FIRE	US-guided; percutaneous; endoscopic; 100μs bursts (1/s); 80–200 s total duration; <3000 V; various waveforms; single-needle delivery	Well-defined ablation zone—interstitial hemorrhage, necrosis and edema; vascular and duct preservation; chronic—glandular atrophy and fibrosis	Elevations in ALT, amylase, and lipase; transient hypoglycemia	Normal pancreas, adenocarcinoma	Porcine

Prostate Cancer *IRE	Percutaneous or transrectal under US guidance; high-voltage steep-pulse therapy device—70 pulses of 2250 V	Distinct, well-defined lesion—complete necrosis; preservation of adjacent critical structures; resolution by 2 weeks	Muscle contraction above 1500 V	Normal prostate	Canine
Mammary Cancer *IRE and H-FIRE	200 bursts (1/s); 100μs duration 2-5-2 waveform	Well-defined ablation zone—necrosis and apoptosis; preservation of critical structures; chronic fibrosis; systemic antitumor response	Transient changes in skin color; pectoralis muscle injury	Orthotopic models (4T1), carcinoma	Porcine, rabbit, murine

[a]Single case report (Neal et al. 2010b)

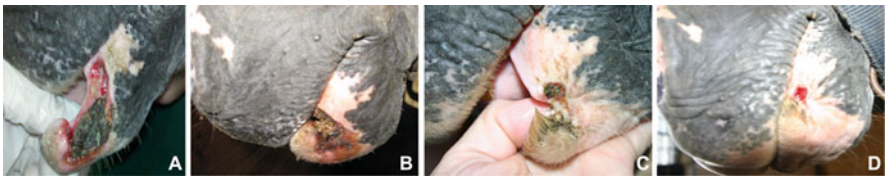

Fig. 3 Regression of equine oral squamous cell carcinoma following IRE treatment. (**a**) Prior to treatment, the tumor appears as a multilobular ulcerated and necrotic lesion involving an extensive section of the buccal mucosa. There is sloughing of the necrotic ablated tumor with subsequent contraction and healing of the wound bed on days 14 (**b**), 32 (**c**), and 50 (**d**) after treatment

neuropathy and pain improved within 24 h of treatment. The ability to predict electric field distribution produced by IRE pulses through numerical modeling prior to treatment enables treatment planning for precise ablation of large, bulky tumors while sparing adjacent healthy tissue. Thus, additional research into safety and efficacy for treatment of non-resectable soft tissue tumors is warranted.

IRE and H-FIRE have been used to safely and effectively treat infiltrative superficial tumors in awake, standing horses (Byron et al. 2019). *Cutaneous tumors* are extremely common in horses, with sarcoids, squamous cell carcinoma (Fig. 3), and melanoma representing the predominant histologic subtypes. As with soft tissue sarcomas, tumors may become locally aggressive and infiltrate around critical structures, making complete surgical removal difficult (Byron et al. 2019). The current standard of care involves a combination of surgery, cryoablation, and/or intralesional chemotherapy with 5-fluoruracil or cisplatin. Despite aggressive multimodal therapy, many patients with advanced disease succumb to complications associated with their local tumor. In the aforementioned phase I clinical trial, five horses with spontaneously occurring multifocal cutaneous tumors were treated with H-FIRE every 2 weeks for 2–4 outpatient visits. H-FIRE was delivered as a 2-5-2 μs burst scheme (positive phase-, intraphase delay-, negative phase) with each burst energized for 100 μs and an applied voltage between 1500 and 3100 V. Treatment was successfully delivered to all horses on an outpatient basis, without the need for general anesthesia or neuromuscular blockade. All patients tolerated treatment well with no reported complications. A significant decrease in tumor volume was noted in all horses, and tumor ablation was confirmed on ultrasound and histologic examination. Additionally, depigmentation of skin occurred within treatment zones of melanomas, suggesting effective ablation of melanocytes. Thus, H-FIRE appears to be a clinically feasible, safe, and potentially effective outpatient treatment option for management of superficial tumors, however, multiple treatments may be necessary to adequately treat large tumors.

4.2 Liver Cancer

Primary liver tumors are the third most common cause of cancer related death in people. Surgical resection is the most effective treatment for the predominant

histologic subtype, hepatocellular carcinoma (HCC), however, this is only feasible for about 40% of patients due to the presence of advanced disease at the time of diagnosis in the majority of cases (Balogh et al. 2016). Aside from liver transplantation, effective alternative treatment options are limited, thus non-resectable tumors have historically carried a grave prognosis.

HCC is also fairly common in dogs and represents the predominant histologic subtype of primary liver tumors. Compared to HCC in people, tumors are resectable in about 60% of canine patients; however, non-resectable tumors mirror their human counterparts (Kinsey et al. 2015; Liptak et al. 2004). The majority of canine HCCs arise from a single liver lobe (massive form), however, large tumors arising from the right side of the liver and/or those in close proximity to the hilus can rarely be removed completely without compromising vital structures. Thus, limited alternative treatment options exist for these patients despite the absence of disseminated disease. Canine HCC has served as a model for investigation into novel therapies, such as IRE, specifically H-FIRE, for treatment of non-resectable liver tumors. Given the potential for IRE to induce a unique, yet robust, antitumor immune response, the liver became of particular interest because of its immunologically rich but tolerogenic environment (Robinson et al. 2016).

Percutaneous irreversible electroporation (IRE) has been evaluated in people bearing primary and secondary liver tumors and appears to be well tolerated (Scheffer et al. 2014b; Narayanan 2015). IRE has been successfully used to ablate tumors intimately associated with the biliary tract without damaging bile ducts (Silk et al. 2014). This makes it an attractive treatment option for centrally located liver tumors, which typically lie next to major bile ducts. Likewise, safety of IRE for treatment of tumors near or encasing major vessels has been demonstrated (Narayanan et al. 2014). Adverse effects reported in the literature include liver abscess formation (4–5%), hemorrhage (typically self-limiting), subcapsular hematoma formation, kidney failure, pneumothorax, mild pleural effusion, hepatic arteriovenous shunt formation (3.5%), partial portal vein thrombosis, atrial fibrillation, and transient neurologic signs affecting the right thoracic limb (2.3%) (Kalra et al. 2019; Mafeld et al. 2019; Dollinger et al. 2015; Bhutiani et al. 2016). Significant, yet transient, elevations in alanine transaminase (ALT) and aspartate transaminase (AST) have been reported 1–2 days following treatment, returning to baseline within approximately 2 weeks. Similarly, significant elevations in total and direct bilirubin levels have been detected 8–10 days following treatment, returning to baseline within 2 weeks (Alnaggar et al. 2018).

Based on completed studies, treatment efficacy and clinical outcome are dependent on completeness of tumor ablation, tumor recurrence, local recurrence-free survival (LRFS), and progression-free survival (Tameez Ud Din et al. 2019). Complete ablation rates vary by study, but are generally higher in medium-sized tumors (66%) compared to large tumors (25%) (Kalra et al. 2019; Zeng et al. 2017). Local recurrence has been observed in 21% of patients at 3 months and 31–34% of patients at 6 months and beyond (Niessen et al. 2017; Fruhling et al. 2017; Saini et al. 2018). The overall median survival time of patients experiencing local recurrence is 26.3 months. Based on currently available data, 87–97% of patients remain disease

free at 3 months, 78–94% remain disease free at 6 months, and 59–75% of patients remain disease free at 12 months following treatment. LRFS improved significantly for tumors measuring less than 3 cm as 100% of patients were free of disease at 3 and 6 months, and 98% of patients free of disease at 12 months (Cannon et al. 2013; Niessen et al. 2016). The median overall progression-free survival following IRE alone is 7–9 months, longer for smaller tumors (Kalra et al. 2019; Sutter et al. 2017). The reported median survival time following IRE is approximately 26.8 months for primary liver tumors and 19.9 months for secondary liver tumors, with overweight patients and those with tumors >3 cm or >3 lesions having a worse prognosis (Saini et al. 2018). Tumors larger than 5 cm often fail to respond favorably to IRE (Thomson et al. 2011). Thus, the utility of IRE may involve treating smaller (<3 cm), residual lesions within the liver following resection of the primary tumor. Additionally, IRE has been successful at down-staging patients after initial surgery to facilitate the second portion of a two-staged hepatectomy (Langan et al. 2017).

Preliminary studies evaluating the effects of IRE on liver tissue were performed using various animal models, including pigs, rats, goats, and rabbits. All animals in all studies survived the immediate treatment and posttreatment period (Vogel et al. 2019). Applied pulse parameters varied from 1 to 360 pulses of 20–100 μs duration delivered at 360–3000 V/cm. Similar to people, acute transient increases in ALT and AST were observed starting as early as 1 h after treatment and resolving within 2 weeks after treatment (Charpentier et al. 2011; Au et al. 2011). IRE produced well-demarcated ablation zones of variable diameter as early as 90 min after treatment (Appelbaum et al. 2012, 2014; Ben-David et al. 2012). Ablation zone size appears to be significantly associated with electrode size, inter-electrode distance, and electric field strength (Miklavcic et al. 2000). Vasculitis and endothelial necrosis with preservation of nearby bile ducts and vessels were observed in gross and histologic liver samples following treatment, which is consistent with IRE-induced changes described in other organs (Liu et al. 2012; Schmidt et al. 2012). Progression of histologic events resembles that observed in other organs, with necrosis, hemorrhage, vascular congestion, and mononuclear inflammation predominating over the first 24–48 h after treatment, which resolve by two weeks post-treatment and become completely replaced by fibrous scar tissue (Guo et al. 2011; Lee et al. 2012).

High-frequency irreversible electroporation (H-FIRE) has been evaluated in a porcine liver model in which it induced rapid, predictable ablations without the need for intraoperative paralytics or cardiac synchronization. H-FIRE electrodes were inserted 1.5 cm apart with ultrasound guidance, and ablations were performed using 100, 200, or 300 bursts with a 2-5-2 μs burst scheme and an applied voltage 2250 V. Hepatic ablations were intentionally planned across, or adjacent to, critical vascular and biliary structures. Minor muscle twitching was observed but no other clinically significant adverse effects. Porcine livers were resected 6 h after treatment for histologic evaluation. Reproducible ablation volumes were observed at necropsy, with apoptosis predominating within the lesions treated with <200 pulses. Necrosis predominated in lesions treated with >200 pulses (Siddiqui et al. 2016). In all lesions, minor endothelial damage was observed, while vascular and biliary

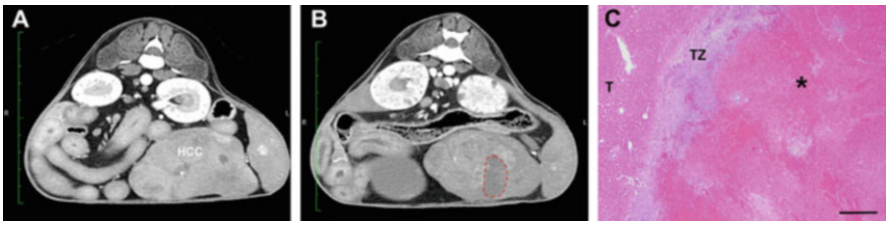

Fig. 4 H-FIRE ablation of canine hepatocellular carcinoma (HCC). Post-contrast CT scans of the HCC prior to (**a**) and following H-FIRE treatment (**b**), with a well-demarcated ablative lesion appearing as a hypoattenuating region in **b** (red dashes). Photomicrograph (**c**) illustrating the well-demarcated ablation zone (*) separated from untreated tumor (T) by a transition zone (TZ); H&E, bar = 500μm

structures remained structurally intact. Given the improved clinical feasibility of H-FIRE delivery compared to IRE it may be the preferred ablation method for various tumor types that are not amenable to surgical removal (Siddiqui et al. 2016).

The safety and feasibility of percutaneous H-FIRE were evaluated in a pilot study involving three canine HCC patients. H-FIRE was delivered through a bipolar probe that was percutaneously placed into the center of tumor under ultrasound guidance. Treatment delivery involved a 2-5-2 μs H-FIRE burst scheme, 300 bursts delivered at 1 burst per second, 100 μs energized time per burst, and an applied voltage of 2250 V. Treatment delivery was completed under general anesthesia without the use of paralytics or cardiac synchronization. No treatment-related adverse effects were observed during treatment delivery or the immediate posttreatment period. CT imaging performed 4 days post-treatment revealed a well-defined ablation zone within the tumor that corresponded with gross findings following tumor resection (Fig. 4). Histologically, H-FIRE produced a well-defined ablation/tumor interface characterized by CD3+ lymphocyte infiltration. Interestingly, infiltrating lymphocytes were negative for CD4 and CD8, and CD79a+ lymphocytes were not observed. CD3+/CD4-/CD8- lymphocytes have previously been associated with immune regulation and tolerance (Martina et al. 2015; Ford et al. 2002). Given that the liver is an immunologically rich microenvironment, these lymphocytes may serve to recognize tumor antigens released in their native form following H-FIRE-induced cell death (Partridge et al. 2020).

Overall, no clinically significant adverse effects were observed throughout the study period, supporting the safety and feasibility of H-FIRE for treatment of non-resectable liver tumors. Elevations in liver enzymes (ALT and ALP) above baseline occurred following H-FIRE delivery but resolved over time following tumor removal. Additional investigation into the efficacy of H-FIRE as a treatment for non-resectable liver tumors is currently ongoing. In general, canine HCC patients present with relatively large primary tumors unless identified incidentally while small. Given the risk of tumor rupture and subsequent life-threatening hemorrhage if left in place, a more practical approach may involve H-FIRE delivery to residual gross and/or microscopic disease following incomplete resection of bulky disease.

Investigation into the efficacy of H-FIRE for residual and/or non-resectable liver tumors is currently ongoing. Additionally, further characterization of the systemic immune response to H-FIRE delivery within the liver is warranted (Partridge et al. 2020).

4.3 Brain Cancer

Glioblastoma (GBM) is the most common and deadliest of the malignant primary brain tumors affecting humans. These tumors are incredibly aggressive and neuroinvasive, making complete surgical removal nearly impossible. Additionally, its location behind the blood–brain barrier prevents effective delivery of most therapeutics. Thus, GBM carries a grave prognosis with median survival times following a combination of aggressive surgery, radiation, and chemotherapy of only 14–16 months, and a 5-year survival rate of about 5% (Stupp et al. 2005; Tamimi and Juweid 2017). Canine and humans are the only species that commonly develop malignant gliomas, and a number of clinicopathological and genomic characteristics of these tumors are shared across species, thus dogs are an excellent large animal model for investigation into novel therapies for malignant glioma.

Glioblastomas are capable of creating a highly immunosuppressive microenvironment, allowing them to evade the immune system, further promoting tumor progression. Decreased MHC expression by tumor cells limits self-presentation of antigens, and PDL1 expression binds to PD1 on effector cells and silences immune system activation. Tumor cells also produce chemokines that recruit regulatory T-cells, tumor-associated macrophages and myeloid-derived suppressor cells, which further suppress the immune system and promote tumor growth. Lastly, resident microglial cells within the microenvironment express immunosuppressive factors, such as TGFβ and IL-10, that suppress local and systemic immune responses, ultimately disrupting systemic tumor antigen detection and immune system activation (Chen and Hambardzumyan 2018; Lim et al. 2018). IRE seems capable of transforming the immunosuppressive, tumor-promoting microenvironment associated with these tumors to an antitumor pro-inflammatory microenvironment by inducing damage-associated molecular pattern (DAMP) signaling in ablated cells. In contrast to thermal ablation methods, the nonthermal nature of IRE allows for release of tumor antigens in their native form, unaltered by heat or cold, and is, therefore, more likely to induce a robust systemic immune response. Such local and systemic immune system activation may be capable of inducing tumor cell death within distant lesions (Ringel-Scaia et al. 2019).

Following a single treatment, IRE induces a central zone of irreversible electroporation surrounded by a zone of reversible electroporation, providing an opportunity to therapeutically target the penumbra of microscopic disease, which is a major source of disease recurrence (Arena et al. 2011a). More specifically, in the needle electrode configuration, the reversible regime occurs at lower electric field thresholds and thus will always encase the IRE tissue ablation volume. This penumbra extends centimeters beyond the zone of IRE and coincides with the outer zone of

reversible electroporation, thus following a single IRE delivery, microscopic cells within this zone undergo transient membrane permeabilization, whereas cells comprising the gross tumor are ablated. Thus, this technology may be exploited further with adjuvant electrochemotherapy to target microscopic cells within the penumbra (outside the ablation zone) and potentially improve long-term outcomes. Furthermore, additional improvements in clinical outcomes may be achieved by delivering adjuvant electrochemotherapy to microscopic tumor cells residing within adjacent grossly normal brain tissue following tumor resection. Lastly, this technology could be used to intentionally and reversibly disrupt the blood–brain barrier to deliver therapeutic agents that would otherwise be impermeant to the CNS (Sharabi et al. 2020). As the thresholds for BBB permeabilization have been shown to be significantly lower than that of irreversible electroporation, the opportunity to disrupt the BBB while fine-tuning the desired extent of tissue ablation is feasible with IRE and H-FIRE therapies. In addition, a delineation between reversible electroporation and BBB disruption has been demonstrated in vitro, thereby presenting the opportunity for BBB disruption at very low electric fields with minimal to no reversible electroporation effects (Sharabi et al. 2019).

To date, research investigating IRE and H-FIRE for treatment of brain tumors has been limited to rodent and canine models as further characterization of its local and systemic effects is required prior to translating it for treatment of primary brain tumors in people (Garcia et al. 2012). The safety and feasibility of intracranial IRE were first evaluated in five normal research bred dogs (Ellis et al. 2011). IRE was delivered to the cerebrum at various pulse parameters followed by intra-operative ultrasound and postoperative MRI to visualize the ablation zones and treatment associated blood–brain barrier disruption (BBBD; Fig. 5a). In order to study the upper safety limit of the procedure, one dog received a higher total voltage, and subsequently experienced coagulative necrosis of all tissues within the treatment field and clinical signs of brain herniation within 14 h of treatment. This dog was humanely euthanized, but no other dogs experienced significant deterioration in neurologic function from baseline or seizure activity. All dogs were humanely euthanized 3 days after treatment to evaluate IRE-induced histologic changes. Gross pathologic findings included a well-demarcated region of malacia and intraparenchymal hemorrhage, which corresponded to changes present on posttreatment MRI. Histologically, a sharp demarcation between the ablation zone and transition zone, the submillimeter region located between the ablation zone and normal brain, was evident, with preservation of major blood vessels located within the ablation zone itself (Rossmeisl et al. 2015). Additionally, parenchymal vacuolization and astrogliosis was noted following delivery of higher voltage. The DAMP protein, high mobility group box 1 (HMGB1) was detectable in all dogs with increased amounts present within the IRE lesion itself compared to the adjacent transition zone, increasing directly with electric field strength (Fig. 5b). Immunohistochemistry with IBA-1 for resident microglia revealed an increase in microglial size within the zone of transition compared to normal brain, which has previously been associated with microglial activation. Evidence of a cell-mediated immune response was limited as minimal T-cell infiltration occurred within the transition zone, but this

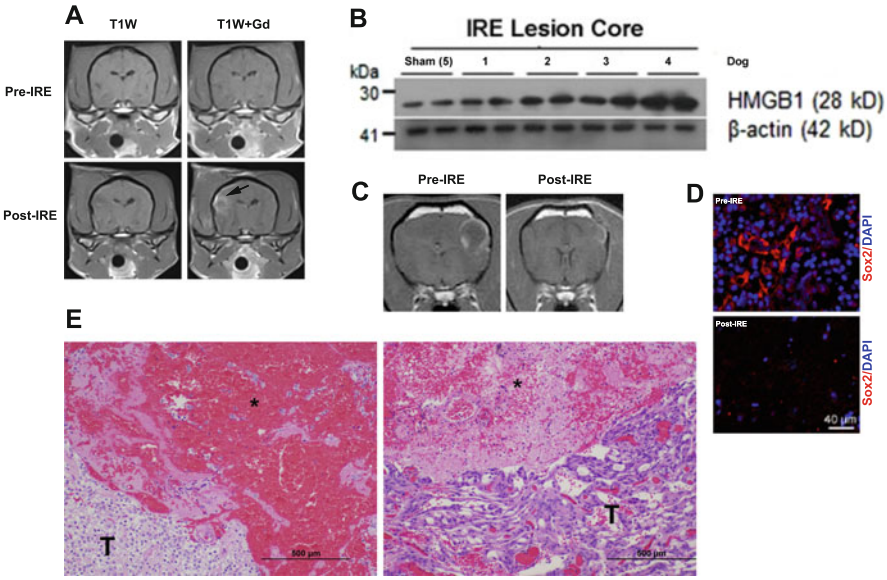

Fig. 5 Effects of IRE and H-FIRE treatments in the canine brain. (**a**) IRE treatment of normal brain results in blood–brain barrier disruption as indicated by the presence of contrast-enhancement of the brain parenchyma (arrow) in the treated region on MRI. (**b**) Western blot demonstrating increased expression of the DAMP protein, high mobility group box 1 (HMGB1), in the brains of all dogs treated with IRE compared to sham operated control. (**c**) MRI demonstrating ring-enhancing astrocytoma in the brain (left) with complete response after IRE treatment, as evidenced by complete disappearance of the tumor (right). (**d**) Pretreatment biopsy (top) of a canine glioblastoma revealing SOX2 positive glioma stem-like cells (GSC); following IRE treatment (bottom) the GSC are ablated. (**e**) IRE (left; GBM) and H-FIRE (right; meningioma) produce ablations that are sharply demarcated from untreated tumor (T); H&E, bar = 500µm

is likely due to the temporal confines of the study preventing detection of a robust cell-mediated immune response (unpublished data). Results of this study suggest that when used at appropriate pulse parameters, IRE is a safe and feasible ablation method for use within the brain and capable of inducing DAMP signaling.

A subsequent clinical trial was completed to evaluate the safety and feasibility of IRE for the treatment of canine intracranial gliomas (Rossmeisl et al. 2015). Seven client-owned dogs with spontaneous gliomas were treated with IRE delivered under CT-guidance. A posttreatment CT was performed immediately after treatment to visualize tumor ablation. Significant adverse events occurred in two dogs, one of which had aspiration pneumonia that was unlikely directly related to IRE and more likely related to the craniectomy, as it is a known risk of this procedure. The remaining dog received an unexpectedly high dose of energy due to pulse delivery to the ventricular system and increased conductivity of the cerebrospinal fluid compared to tumor tissue. The median duration of hospitalization was 4 days, and Karnofsky Performance Scores, which assesses functional impairment, improved in all dogs that survived to discharge by two weeks following treatment. Likewise,

improvement in seizure control was noted in five dogs. Based on RANO criteria, 80% of dogs with a quantifiable target lesion responded to IRE treatment, defined as stable disease (1), partial response (2), and complete remission (1, Fig. 5c) (Garcia et al. 2017). The overall median survival time of these patients was 119 days with two patients completing the 12-month follow-up. Histologic evaluation of the treated lesion from the dog that unexpectedly died of aspiration pneumonia revealed a sharp delineation between ablated tumor and surrounding brain (Fig. 5e) with a visible transition zone between the lesion and normal brain characterized by vacuolated neuropil and perivascular inflammatory cuffing. Additionally, immunofluorescent staining with the neural stem cell marker, SOX2, revealed selective ablation of glioma stem-like cells following IRE treatment compared to pre-treatment biopsy samples, suggesting that IRE may be capable of eliminating glioma-like stem cells which are often implicated in treatment failure and subsequent tumor recurrence (Fig. 5d). These findings provide evidence that the treatment plan designed to ablate the entire tumor was effective at treating the tumor without causing significant collateral brain damage. Given the results of this study, IRE seems to be a safe and feasibly treatment option for canine gliomas.

We have shifted our research focus toward H-FIRE technology since its development. H-FIRE was able to successfully ablate brain tissue and transiently disrupt the blood–brain barrier within the penumbra of tissue surrounding the ablation zone in rodent models without causing muscular contractions (Arena et al. 2011a, b). This novel technique was introduced as a potential treatment for spontaneous brain tumors in a feasibility study evaluating H-FIRE in dogs with intracranial meningioma (Latouche et al. 2018). Three dogs with intracranial meningioma were treated using patient-specific treatment plans generated based on MRI imaging. H-FIRE was stereotactically delivered to all patients followed by a posttreatment MRI and tumor resection to characterize histologic changes. H-FIRE delivery was successful in all patients without any adverse events directly related to H-FIRE treatment. One dog experienced intraoperative hemorrhage and subsequent hypotension during tumor resection and developed worsening of neurologic signs postoperatively, but recovered and was discharged with improvement in neurologic signs by 2 weeks posttreatment. No intra- or postoperative adverse effects were observed in either of the remaining dogs. Posttreatment MRI revealed a well-demarcated homogenous region of ablation characterized by complete disruption of tumor architecture with tumor necrosis volumes measuring 0.25–1.29 cm^3. In one dog, posttreatment histopathology revealed nonuniform ablations with viable tumor cells persisting around foci of intra-tumoral mineralization. This suggests that intra-tumoral mineralization may limit the ability of H-FIRE to produce complete, homogenous tumor ablation. Results of this study support the use of H-FIRE for the treatment of brain tumors based on its ability to safely produce clinically relevant volumes of tumor ablation without muscular contraction or cardiac arrhythmias.

Current research into the mechanism of H-FIRE mediated blood–brain barrier disruption (BBBD) as well as the local and systemic immune response to H-FIRE in normal brain and those affected by glioma are ongoing. A study evaluating H-FIRE in normal rat brains provided evidence of H-FIRE induced transient blood–brain

barrier disruption at electrical field strengths significantly less than those required to ablate tumor tissue (Lorenzo et al. 2019). MRI performed at various time points following H-FIRE delivery revealed an increase in contrast enhancement at the site of H-FIRE delivery 1-hour post-treatment compared to the sham control, followed by a gradual decrease in contrast enhancement over time consistent with blood–brain barrier repair. This correlated with gross pathologic findings, where an increase in Evan's Blue Dye was noted within the brain parenchyma 1 hour following treatment compared to the sham control, followed by a gradual decrease over time. Histologically, H-FIRE lesions were limited to mechanical damage associated with the electrode insertion tract, where lesions did not differ between treatment and control groups. An influx of inflammatory cells was noted at 48 and 96 h but there was no visible damage to the surrounding brain parenchyma. In contrast, large, necrotic lesions were present within the brain parenchyma surrounding lesions created by higher, more ablative doses. Results of these preliminary studies indicate that H-FIRE transiently permeates the blood–brain barrier without disrupting adjacent normal brain tissue. A more realistic application of this technology is for the treatment of residual microscopic disease following surgical removal of gross tumor tissue. The ability of H-FIRE to induce transient BBB disruption and create a surrounding zone of reversible electroporation that coincides with the penumbra of microscopic disease may be exploited to treat residual microscopic disease and delay tumor recurrence. Additionally, H-FIRE induced BBBD could aid in the delivery of chemotherapeutics that would otherwise be excluded from the CNS by an intact BBB.

Intracranial H-FIRE has been successfully delivered under MRI guidance in canine patients with gliomas without any treatment-associated complications. An increase in gadolinium contrast enhancement following H-FIRE treatment was observed, which corresponded to the amount of BBBD in these patients. Thus, H-FIRE induced BBBD has been successfully achieved in tumor-bearing dogs, but further investigations into its safety and efficacy are ongoing. Additionally, ongoing experiments aim to better characterize the local and systemic immune responses to intracranial H-FIRE and assess its efficacy when used alone or with a molecular adjuvant as a combinatorial approach for treatment of gliomas.

5 Clinical Applications of IRE and H-FIRE in Preclinical Normal Animal Models or Experimentally Induced Tumors

5.1 Pancreatic Cancer

Pancreatic adenocarcinoma is the deadliest of cancers affecting the gastrointestinal tract with less than 5% of people surviving 5 years despite aggressive therapy (Siegel et al. 2017). Many people are asymptomatic during early stages of the disease, making early detection difficult. At the time of diagnosis, 50% of patients have metastatic disease and only 20% of patients are candidates for surgery (Callery et al. 2009). Given its location within the pancreas, locally advanced disease often

involves nearby structures, such as major vessels. Surgical resection in these cases is associated with high perioperative morbidity and mortality, while only minimally impacting survival (Kato et al. 2013; Chua and Saxena 2010). The prognosis for people with non-resectable tumors is grave, with an overall median survival time of 9–13 months despite treatment with chemotherapy and/or radiation therapy (Kane and Knox 2018).

Pancreatic carcinomas are uncommonly diagnosed in veterinary medicine, but their clinical presentation and response to treatment parallels that described in people (Priester 1974). Clinical signs are non-specific and may include some combination of lethargy, anorexia, vomiting, diarrhea, polyuria, polydipsia, and/or a palpable mid-abdominal mass. By the time veterinary patients become clinical for their disease, it is often advanced with local invasion into adjacent structures and metastasis to the regional lymph nodes, liver, intestine, and/or lungs. Surgical resection is rarely possible at the time of diagnosis due to advanced stage of disease; so many patients are humanely euthanized without further treatment. Reported survival times vary, with most patients succumbing to disease within days to months following diagnosis despite treatment with chemotherapy and/or radiation therapy (Aupperle-Lellbach et al. 2019; Pinard et al. 2020). No currently available treatments have been shown to significantly prolong survival of veterinary patients with pancreatic carcinoma, thus the need for novel treatment options is undeniable.

IRE has emerged as an attractive ablation method for treatment of non-resectable pancreatic tumors. Since IRE does not rely on heat for effective tumor ablation, adjacent critical structural components are spared and it can be used in proximity to large vessels without compromising efficacy due to the "heat sink effect" (Davalos et al. 2015). Given the proximity of the pancreas to critical vascular structures, including but not limited to arterial and venous vessels, bile and pancreatic ducts, nerves, and adjacent organs, IRE may be used to effectively ablate locally advanced tumors without the unintentional collateral damage that typically occurs during attempts at surgical resection. Unlike IRE ablations in other organs, real-time ultrasound imaging during IRE delivery to the pancreas is limited due to formation of edema, which obscures the field (Rubinsky et al. 2007). Recent development of pre-treatment planning models helps predict ablation volumes and ensures precise IRE delivery and subsequent tumor ablation (Latouche et al. 2017).

Clinical trials evaluating the use of IRE in patients with late-stage, locally advanced pancreatic adenocarcinoma have shown promising results. The reported median overall survival time is 7–23 months post-treatment with longest survival times reported in patients undergoing a combination of IRE and surgical resection (Ansari et al. 2017). These survival times surpass those reported for patients receiving traditional therapies and treatment-associated complication rates are significantly lower than those associated with other ablation methods (Scheffer et al. 2014a). IRE is not only capable of safely ablating locally invasive tumors, but appears to induce a pro-inflammatory microenvironment that promotes immune cell infiltration into the tumor, further halting tumor progression. Additionally, since IRE ablates cancer cells without heat, tumor antigens released from the

dying cells are preserved and may induce an antitumor immune response against residual and metastatic cells (Brock et al. 2019).

Veterinary patients have historically been utilized as models to investigate the feasibility and safety of IRE in pancreatic tissue. Porcine models predominate as their pancreatic anatomy and physiology parallels that of humans and small animal veterinary patients. Studies evaluating IRE in pancreatic porcine models have shown this technique to be feasible and safe (Fritz et al. 2015; Bower et al. 2011; Charpentier et al. 2010; Wimmer et al. 2013). A study evaluating the safety and ablation volume of IRE in a porcine pancreas model showed that IRE is well tolerated when performed at an optimal voltage of 3 kV. Ultrasound was utilized to guide electrodes within 1 mm of the portal vein or mesenteric artery. All animals recovered with only mild adhesions but no pancreatic necrosis, ascites, or hemorrhage, which have been reported in other studies (Bower et al. 2011; Fritz et al. 2015; Wimmer et al. 2013). Elevations in ALT levels and transient hypoglycemia were also noted in these patients immediately after treatment but resolved over 24 h. IRE resulted in a 3 cm × 2.8 cm ablation zone characterized by significant destruction of pancreatic tissue with the exception of spared, intact vasculature (Bower et al. 2011). These histologic findings parallel those described in other studies, particularly with regards to preservation of blood vessels and pancreatic ducts among hemorrhagic necrosis of the pancreatic interstitium (Charpentier et al. 2010; Fritz et al. 2015). In another study, IRE was delivered to the pancreatic tail in pigs that were terminated after 60 min, whereas it was delivered to the head of the pancreas in a separate cohort of pigs that were observed for 7 days. In this study, no cardiac abnormalities, signs of acute pancreatitis nor other clinically significant adverse effects were observed. CT performed 60 min after treatment revealed the presence of a hypodense lesion corresponding with the ablation zone (Fritz et al. 2015). Transient increases in amylase and lipase is frequently reported as a side effect of IRE treatment within the pancreas, typically peaking after 24 h and resolving within 2–3 days (Fritz et al. 2015; Bower et al. 2011; Vogel et al. 2019). Histologically, acute lesions observed within 1 hour of treatment appear sharply demarcated with interstitial edema and mild hemorrhage. Over time, inflammatory infiltrates contribute to fibrosis and glandular atrophy and signs of apoptosis and necrosis become evident. Autolytic changes become apparent approximately two weeks after treatment with progression to extensive fibrosis and acinar atrophy within the ablation zone after 1 month.

H-FIRE was evaluated for pancreatic tissue ablation using 100 μs energized bursts delivered at 1 burst/second for 80–200 bursts to successfully ablate porcine pancreatic tissue without inducing muscle tetany or cardiac arrhythmias (Clark 2017). As with IRE, treatment zones were visible on intraoperative ultrasound and posttreatment CT. Another study successfully delivered H-FIRE through a single-needle delivery approach in swine pancreas without concurrent use of intraoperative paralytics or cardiac synchronization (O'Brien et al. 2019). Three different waveforms (1-5-1, 2-5-2, and 5-5-5 μs burst schemes) were used with a total energized time of 100 μs per burst resulting in a significant increase in mean ablation area with increasing pulse width. No clinically significant adverse effects were noted and the lethal threshold for pancreatic tissue varied between voltage waveforms, but

ranged from 693 to 1114 V/cm. Immunohistochemical staining for cleaved caspase-3 was absent from the ablation zone; however, the extent of positive staining was significantly different between voltage waveforms. These findings suggest H-FIRE-induced cell death likely occurs through mechanisms other than apoptosis, which have been highlighted through various studies evaluating IRE in other organs.

Immunotherapy has shown significant promise in cancer treatment for many tumor types, but its efficacy in pancreatic cancer is limited by the presence of a highly fibrotic stroma, which creates an immunosuppressive tumor microenvironment by physically preventing cytotoxic T-cell infiltration (Thind et al. 2017). IRE appears capable of transforming this immunosuppressive tumor microenvironment to a pro-inflammatory antitumor microenvironment by inducing immunogenic cell death and dendritic cell activation (Zhao et al. 2019). When combined with anti-programmed cell death protein 1 (anti-PD1) immune checkpoint blockade, tumor infiltration by CD8+ T-cells occurs and a long-term memory immune response has been documented in a murine orthotopic pancreatic cancer model (Zhao et al. 2019). Thus, IRE may be exploited to improve response to checkpoint inhibitors and overall outcome in people with pancreatic cancer. Currently, checkpoint inhibitors are not available for use in small animal veterinary patients, but development is ongoing and response to combination therapy is expected to yield similar results.

5.2 Prostate Cancer

The incidence of *prostate cancer* in people is on the rise with more than a million new cases diagnosed each year. Men aged 65 years and older have the highest risk, thus annual screening is recommended at ages 45 years and above (Rawla 2019; Guenther et al. 2019). Currently, the available treatment options include radical prostatectomy, radiation therapy of the entire prostate, and chemotherapy. Treatment frequently results in physically and emotionally debilitating side effects, such as impotence and incontinence, with damage to the rectum and bladder also possible (Guenther et al. 2019). The survival benefit of aggressive multimodal therapy is modest compared to active surveillance alone, thus a need for effective, well-tolerated treatment options currently exists for management of this disease (Bill-Axelson et al. 2018). The incidence of prostate cancer in veterinary patients is much lower but these patients often present with advanced disease, including osteoblastic bone metastases, which are common in people. Similar challenges are faced when treating prostate cancer in veterinary patients, with most patients succumbing to local disease progression within a year despite aggressive therapy (Leroy and Northrup 2009). Noninvasive thermal ablation methods, such as radiofrequency ablation and high intensity focused ultrasound (HIFU) are increasing in popularity, but the efficacy and adverse effects are influenced by the “heat sink” effect. In contrast, the nonthermal mechanism by which IRE induces cell death allows improved treatment precision and spares adjacent critical structures. Given the prostate is closely associated with many critical structures, including the components

of the urinary tract, rectum, and neurovascular bundles, treatment precision is imperative to eliminate adverse effects.

In people, the clinical feasibility and safety of IRE for treatment of prostatic carcinoma have been established (van den Bos et al. 2016). Outcomes following treatment with IRE for prostatic carcinoma parallel those following radical prostatectomy but are associated with less urogenital dysfunction. A study evaluating transrectal IRE in 34 human patients with prostatic carcinoma reported only mild (grades 1 and 2) complications within a 6-month follow-up period. All patients remained continent and only 5% of patients experienced impotence (Valerio et al. 2014). The utility of IRE ranges from focal tumor to whole-gland ablation, and may provide a safe, alternative treatment option for large tumors that are no longer amenable to surgery and radiation therapy (Guenther et al. 2019).

IRE treatment of prostatic tissue was first evaluated in 6 normal dogs. IRE probes were placed percutaneously or transrectally under ultrasound guidance for delivery and patients were followed for up to 2 weeks. The rectum, urethra and neurovascular bundle were intentionally targeted in a single dog to assess the impact of treatment on these structures. Patients experienced variable degrees of muscle contraction, with the severity of contractions increasing with applied voltage, however, voltages less than 1.5 kV produced minimal contraction. This likely reflects the voltage that would be applied in a clinical setting, as it appears to successfully ablate prostatic tissue without adverse effects. IRE resulted in a distinct, well-demarcated lesion characterized by complete necrosis and a narrow zone of transition from the ablation zone to normal tissue. Adjacent critical structures appeared unaffected by treatment, and near resolution of the lesions occurred within two weeks. Similar histologic lesions with preservation of adjacent neurovasculature have been reported in human patients following IRE for treatment of prostatic carcinoma, thus IRE appears to be a safe and potentially effective treatment option for prostate cancer (Onik et al. 2007; van den Bos et al. 2016).

Traditionally, IRE has been delivered through the commercially available NanoKnife system. Recently, a novel high-voltage steep-pulse therapy device was developed for ablation of prostatic tissue and evaluated in normal dogs. Two electrode probes were used to deliver IRE under real-time ultrasound guidance. Seventy pulses of 2250 V were delivered to each dog with no serious complications. Treatment resulted in a sharply demarcated ablation zone with preservation of the urethra and adjacent blood vessels, consistent with previous studies (Han 2019). Research into the efficacy of IRE for treatment of canine prostatic carcinoma is currently lacking, but based on its safety and efficacy in human patients, promising results are to be expected.

5.3 Mammary Cancer

Breast cancer is the second most common cancer in women, following closely behind skin cancer. Treatment and prognosis are frequently influenced by the presence of estrogen receptor alpha (ERa), progesterone receptor (PR), and human

epidermal growth factor receptor 2 (HER2) expressions (Rakha et al. 2010). Triple-negative breast cancers (TNBCs), which fail to express ERa, PR, and HER2 have been associated with a poor prognosis due to the current lack of effective targeted therapy (Bianchini et al. 2016). Mammary carcinomas in veterinary medicine share a number of clinical, epidemiologic, and pathologic features with their human counterpart. In dogs, triple-negative mammary carcinomas predominate (76%) and the aggressive biological behavior of these tumors parallels those TNBCs in people (Nguyen et al. 2018).

The current standard of care for treatment of mammary carcinoma in people involves some combination of surgery, radiation therapy, chemotherapy, hormone therapy, and targeted therapy (Dhankhar et al. 2010). Surgery typically involves a unilateral or bilateral mastectomy, which is invasive and frequently associated with cosmetic deformities. Radiation therapy may cause decreased sensation to the areas as well as moderate to severe skin irritation characterized by erythema, moist desquamation, and subsequent pruritus (Akram and Siddiqui 2012; Nounou et al. 2015). Hormone therapies, such as GnRH agonists, ER antagonists (tamoxifen), and aromatase inhibitors, are typically reserved for patients with tumors that express ER and/or PR, and significantly prolongs survival in these patients. Although the severity of side effects associated with these therapies is less than those caused by chemotherapy, common side effects include hot flashes, fatigue, gastrointestinal upset, weight gain, and severe mood swings characterized by depression and anxiety, which can be debilitating. Additionally, long-term treatment has been associated with osteoporosis, putting patients at risk for bone fracture and/or chronic pain (Burstein and Griggs 2010).

Chemotherapy remains the treatment of choice for TNBC. Chemotherapy protocols using some combination of anthracyclines and taxanes decrease the risk of cancer-related death by about 33% (Early Breast Cancer Trialists' Collaborative Group et al. 2012). Most chemotherapy protocols are administered over multiple weeks and are frequently associated with debilitating side effects, such as cardiotoxicity (anthracyclines), hair loss, nausea, and severe bone marrow suppression with subsequent sepsis, among others (Valero 1997). HER2-directed therapy (Trastuzumab, Herceptin) has recently become the standard of care for patients with HER2+ breast cancer as it decreases the risk of disease reoccurrence by about 50% compared to chemotherapy alone (Callahan and Hurvitz 2011; Swain et al. 2014). Although direct therapies appear to be less debilitating, flu-like symptoms and cardiotoxicity occur with relative frequency (Ewer and Ewer 2008).

Regardless of treatment, early detection is the key to achieving remission and long-term survival. Approximately 20–30% of women diagnosed during the early stages of disease will develop distant metastasis. Despite aggressive treatment with current standard of care therapy, historically the median survival time for patients with stage 4 breast cancer was 3 years, with only 22% of patients living 5 years (Selzner et al. 2000). Development of novel targeted therapies is ongoing and results are promising with significant improvements in survival times, however, the prognosis remains grim for those patients who are not good candidates for such therapies, such as TNBC patients. Thus, ablative techniques, like IRE, have become an

attractive treatment option for these patients, as efficacy is not dependent on hormone receptor expression. Additionally, electric fields >600 V/cm applied to TNBC cells appear capable of downregulating thymic stromal lymphopoietin (TSLP) signaling, a promoter of the pro-cancer phenotype. This results in an antitumor immune response capable of transforming a "pro-cancer" microenvironment into one that is "anti-cancer." Given the predominance of TNBC in veterinary patients and the limited availability (due to accessibility and cost) of recently developed directed therapies, IRE offers a new and alternative treatment option that may be capable of improving long-term outcomes in these patients as well (Goswami et al. 2017).

Traditional breast cancer ablative techniques, such as radiofrequency ablation and high-intensity focused ultrasound (HIFU) have been associated with damage to the skin overlying breast tissue, particularly in patients with large and/or superficial tumors (Li et al. 2016; Palussiere et al. 2012). The feasibility and safety of IRE treatment of breast tissue have been demonstrated using animal models (Zhang et al. 2017; Li et al. 2016). Cutaneous effects, and gross and histopathologic changes associated with IRE delivery to breast tissue have been evaluated in a porcine model where the untreated contralateral breast tissue served as controls (Li et al. 2016). Transient changes in skin color were observed, corresponding with the skin-electrode distance and absent at distances greater than 5 mm. A rabbit orthotopic breast cancer model was also used to characterize IRE-induced skin damage and subsequent repair. IRE was able to successfully ablate all targeted breast tissue or tumor followed by complete regeneration of mammary tissue and overlying skin in the absence of fibrosis or mass formation (Li et al. 2016). Pectoralis muscle injury, despite skin preservation following IRE delivery to breast tissue, was reported in a safety and feasibility study utilizing normal rabbit models. Histologic lesions were similar to those described previously with regards to the presence of necrosis and apoptosis and preservation of critical interstitial components, but contained marked fibrous and granulation tissue following repair (Zhang et al. 2017). Results of these preliminary studies suggest IRE provides a treatment option for breast cancer that maintains esthetics.

IRE has been shown to effectively ablate local tumors regardless of hormone receptor expression, resulting in an immunologic cell death that provides the unique benefit of promoting systemic antitumor immunity capable of targeting distant lesions. This response was demonstrated in a study evaluating high-frequency irreversible electroporation (H-FIRE) in an orthotopic mouse 4T1 mammary model (Ringel-Scaia et al. 2019). H-FIRE delivery consisted of 200 bursts of bipolar PEFs at a frequency of 1 burst per second for an energized time of 100 μs per burst using a (2-5-2 μs) burst scheme. H-FIRE delivery resulted in local tumor ablation and shifted the tumor microenvironment to a pro-inflammatory state. Additionally, a significant decrease in the number of circulating metastatic cells was observed in mice that had their mammary tumors treated with H-FIRE compared to untreated mice. 4T1 cells were treated in vitro with H-FIRE or cryoablation and the resulting cell-free supernatant was injected IV into mice 10 days prior to injecting cancer cells into a mammary fat pad. Mice that received lysates from cells treated with H-FIRE

demonstrated a significant decrease in tumor size compared to both the cryoablation lysate and the control groups. Mice treated with either cryoablation lysate or H-FIRE lysate also demonstrated a significant decrease in circulating metastatic cancer cells compared to control groups. Results of this study suggest that H-FIRE activates the local innate immune system and generates neoantigens from cancer cells in their native form capable of stimulating the adaptive immune system and attenuating mammary tumor progression (Ringel-Scaia et al. 2019).

6 Summary

Irreversible electroporation applications in veterinary medicine have been limited to clinical trials evaluating the safety and feasibility of this technology for the treatment of solid tumors that are not otherwise amenable to standard of care therapy. The nonthermal ablation induced by IRE results in a more predictable ablation volume compared to thermal ablation techniques, further reducing damage to adjacent critical structures. Additionally, tumor neoantigens released following IRE remain in their native form, unaltered by temperature, and appear capable of inducing a robust systemic antitumor immune response, essentially serving as an "in-situ" vaccine. The recent development of next-generation IRE, H-FIRE, has further improved treatment precision and feasibility by overcoming some important limitations of IRE, such as treatment-induced muscle tetany and cardiac asynchrony. Patient-specific pre-treatment planning using numerical models based on CT images to predict electric field distribution throughout the target lesion has greatly enhanced the precision and efficacy of IRE ablation. Furthermore, the development of minimally invasive delivery techniques makes IRE even more attractive for treatment of solid tumors that would otherwise require highly invasive therapies. Companion animals with naturally occurring cancers continue to serve as large animal models for further investigation into the safety and efficacy of various IRE applications, including ablation of superficial and deep-seated tumors as well as blood–brain barrier disruption to enhance drug delivery to brain tumors. Clinical trials investigating the application of H-FIRE for treatment of canine pancreatic and lung tumors are currently ongoing. Results of these clinical trials will be imperative prior to translating this technology into human clinical trials. Future veterinary studies should aim to define the size threshold for effective treatment, as large tumors remain a significant challenge despite advancements in IRE delivery.

References

Ahmed M, Brace CL, Lee FT Jr, Goldberg SN (2011) Principles of and advances in percutaneous ablation. Radiology 258(2):351–369. https://doi.org/10.1148/radiol.10081634

Akram M, Siddiqui SA (2012) Breast cancer management: past, present and evolving. Indian J Cancer 49(3):277–282. https://doi.org/10.4103/0019-509X.104486

Alnaggar M, Qaid AM, Chen J, Niu L, Xu K (2018) Irreversible electroporation of malignant liver tumors: effect on laboratory values. Oncol Lett 16(3):3881–3888. https://doi.org/10.3892/ol.2018.9058

Ansari D, Kristoffersson S, Andersson R, Bergenfeldt M (2017) The role of irreversible electroporation (IRE) for locally advanced pancreatic cancer: a systematic review of safety and efficacy. Scand J Gastroenterol 52(11):1165–1171. https://doi.org/10.1080/00365521.2017.1346705

Appelbaum L, Ben-David E, Sosna J, Nissenbaum Y, Goldberg SN (2012) US findings after irreversible electroporation ablation: radiologic-pathologic correlation. Radiology 262 (1):117–125. https://doi.org/10.1148/radiol.11110475

Appelbaum L, Ben-David E, Faroja M, Nissenbaum Y, Sosna J, Goldberg SN (2014) Irreversible electroporation ablation: creation of large-volume ablation zones in in vivo porcine liver with four-electrode arrays. Radiology 270(2):416–424. https://doi.org/10.1148/radiol.13130349

Arena CB, Sano MB, Rossmeisl JH Jr, Caldwell JL, Garcia PA, Rylander MN, Davalos RV (2011a) High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction. Biomed Eng Online 10:102. https://doi.org/10.1186/1475-925X-10-102

Arena CB, Sano MB, Rylander MN, Davalos RV (2011b) Theoretical considerations of tissue electroporation with high-frequency bipolar pulses. IEEE Trans Biomed Eng 58(5):1474–1482. https://doi.org/10.1109/TBME.2010.2102021

Arena CB, Szot CS, Garcia PA, Rylander MN, Davalos RV (2012) A three-dimensional in vitro tumor platform for modeling therapeutic irreversible electroporation. Biophys J 103 (9):2033–2042. https://doi.org/10.1016/j.bpj.2012.09.017

Au JT, Wong J, Mittra A, Carpenter S, Haddad D, Carson J, Jayaraman S, Monette S, Solomon SB, Ezell P, Fong Y (2011) Irreversible electroporation is a surgical ablation technique that enhances gene transfer. Surgery 150(3):474–479. https://doi.org/10.1016/j.surg.2011.07.007

Aupperle-Lellbach H, Torner K, Staudacher M, Muller E, Steiger K, Klopfleisch R (2019) Characterization of 22 Canine pancreatic carcinomas and review of literature. J Comp Pathol 173:71–82. https://doi.org/10.1016/j.jcpa.2019.10.008

Ball C, Thomson KR, Kavnoudias H (2010) Irreversible electroporation: a new challenge in "out of operating theater" anesthesia. Anesth Analg 110(5):1305–1309. https://doi.org/10.1213/ANE.0b013e3181d27b30

Balogh J, Victor D 3rd, Asham EH, Burroughs SG, Boktour M, Saharia A, Li X, Ghobrial RM, Monsour HP Jr (2016) Hepatocellular carcinoma: a review. J Hepatocell Carcinoma 3:41–53. https://doi.org/10.2147/JHC.S61146

Beitel-White N, Bhonsle S, Martin RCG, Davalos RV (2018) Electrical characterization of human biological tissue for irreversible electroporation treatments. Conf Proc IEEE Eng Med Biol Soc 2018:4170–4173. https://doi.org/10.1109/EMBC.2018.8513341

Ben-David E, Appelbaum L, Sosna J, Nissenbaum I, Goldberg SN (2012) Characterization of irreversible electroporation ablation in in vivo porcine liver. AJR Am J Roentgenol 198(1): W62–W68. https://doi.org/10.2214/AJR.11.6940

Ben-David E, Ahmed M, Faroja M, Moussa M, Wandel A, Sosna J, Appelbaum L, Nissenbaum I, Goldberg SN (2013) Irreversible electroporation: treatment effect is susceptible to local environment and tissue properties. Radiology 269(3):738–747. https://doi.org/10.1148/radiol.13122590

Bhonsle S, Arena C, Sweeney D, Davalos R (2015) Mitigation of impedance changes due to electroporation therapy using bursts of high-frequency bipolar pulses. Biomed Eng Online 14 (3):S3. https://doi.org/10.1186/1475-925X-14-S3-S3

Bhonsle S, Bonakdar M, Neal RE 2nd, Aardema C, Robertson JL, Howarth J, Kavnoudias H, Thomson KR, Goldberg SN, Davalos RV (2016) Characterization of irreversible electroporation ablation with a validated perfused organ model. J Vasc Interv Radiol 27(12):1913–1922. https://doi.org/10.1016/j.jvir.2016.07.012

Bhonsle S, Lorenzo MF, Safaai-Jazi A, Davalos RV (2018) Characterization of nonlinearity and dispersion in tissue impedance during high-frequency electroporation. IEEE Trans Biomed Eng 65(10):2190–2201. https://doi.org/10.1109/TBME.2017.2787038

Bhutiani N, Philips P, Scoggins CR, McMasters KM, Potts MH, Martin RC (2016) Evaluation of tolerability and efficacy of irreversible electroporation (IRE) in treatment of Child-Pugh B (7/8) hepatocellular carcinoma (HCC). HPB (Oxford) 18(7):593–599. https://doi.org/10.1016/j.hpb.2016.03.609

Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L (2016) Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. Nat Rev Clin Oncol 13(11):674–690. https://doi.org/10.1038/nrclinonc.2016.66

Bill-Axelson A, Holmberg L, Garmo H, Taari K, Busch C, Nordling S, Haggman M, Andersson SO, Andren O, Steineck G, Adami HO, Johansson JE (2018) Radical prostatectomy or watchful waiting in prostate cancer - 29-year follow-up. N Engl J Med 379(24):2319–2329. https://doi.org/10.1056/NEJMoa1807801

Bonakdar M, Latouche EL, Mahajan RL, Davalos RV (2015) The feasibility of a smart surgical probe for verification of ire treatments using electrical impedance spectroscopy. IEEE Trans Biomed Eng 62(11):2674–2684. https://doi.org/10.1109/TBME.2015.2441636

Bower M, Sherwood L, Li Y, Martin R (2011) Irreversible electroporation of the pancreas: definitive local therapy without systemic effects. J Surg Oncol 104(1):22–28. https://doi.org/10.1002/jso.21899

Brock R, White N, Ringel-Scaia V, Coutermarsh-Ott S, Eden K, Coutri J, Manuchehrabadi N, Davalos R, Allen I (2019) Irreversible electroporation stimulates a pro-inflammatory tumor microenvironment in pancreatic cancer. J Immunol 202(1):194

Burstein HJ, Griggs JJ (2010) Adjuvant hormonal therapy for early-stage breast cancer. Surg Oncol Clin N Am 19(3):639–647. https://doi.org/10.1016/j.soc.2010.03.006

Byron CR, DeWitt MR, Latouche EL, Davalos RV, Robertson JL (2019) Treatment of infiltrative superficial tumors in awake standing horses using novel high-frequency pulsed electrical fields. Front Vet Sci 6:265. https://doi.org/10.3389/fvets.2019.00265

Callahan R, Hurvitz S (2011) Human epidermal growth factor receptor-2-positive breast cancer: current management of early, advanced, and recurrent disease. Curr Opin Obstet Gynecol 23(1):37–43. https://doi.org/10.1097/gco.0b013e3283414e87

Callery MP, Chang KJ, Fishman EK, Talamonti MS, William Traverso L, Linehan DC (2009) Pretreatment assessment of resectable and borderline resectable pancreatic cancer: expert consensus statement. Ann Surg Oncol 16(7):1727–1733. https://doi.org/10.1245/s10434-009-0408-6

Campelo S, Valerio M, Ahmed HU, Hu Y, Arena SL, Neal RE 2nd, Emberton M, Arena CB (2017) An evaluation of irreversible electroporation thresholds in human prostate cancer and potential correlations to physiological measurements. APL Bioeng 1(1):016101. https://doi.org/10.1063/1.5005828

Cannon R, Ellis S, Hayes D, Narayanan G, Martin RC 2nd (2013) Safety and early efficacy of irreversible electroporation for hepatic tumors in proximity to vital structures. J Surg Oncol 107(5):544–549. https://doi.org/10.1002/jso.23280

Charpentier KP, Wolf F, Noble L, Winn B, Resnick M, Dupuy DE (2010) Irreversible electroporation of the pancreas in swine: a pilot study. HPB (Oxford) 12(5):348–351. https://doi.org/10.1111/j.1477-2574.2010.00174.x

Charpentier KP, Wolf F, Noble L, Winn B, Resnick M, Dupuy DE (2011) Irreversible electroporation of the liver and liver hilum in swine. HPB (Oxford) 13(3):168–173. https://doi.org/10.1111/j.1477-2574.2010.00261.x

Chen Z, Hambardzumyan D (2018) Immune microenvironment in glioblastoma subtypes. Front Immunol 9:1004. https://doi.org/10.3389/fimmu.2018.01004

Chua TC, Saxena A (2010) Extended pancreaticoduodenectomy with vascular resection for pancreatic cancer: a systematic review. J Gastrointest Surg 14(9):1442–1452. https://doi.org/10.1007/s11605-009-1129-7

Cindric H, Kos B, Tedesco G, Cadossi M, Gasbarrini A, Miklavcic D (2018) Electrochemotherapy of spinal metastases using transpedicular approach: a numerical feasibility study. Technol Cancer Res Treat 17:1533034618770253. https://doi.org/10.1177/1533034618770253

Clark C (2017) Safety of next generation high frequency irreversible electroporation (H-FIRE) in a porcine pancreatic cancer treatment model. HPB. https://doi.org/10.1016/j.hpb.2017.02.366

Collettini F, Enders J, Stephan C, Fischer T, Baur ADJ, Penzkofer T, Busch J, Hamm B, Gebauer B (2019) Image-guided irreversible electroporation of localized prostate cancer: functional and oncologic outcomes. Radiology 292(1):250–257. https://doi.org/10.1148/radiol.2019181987

Corovic S, Mir LM, Miklavcic D (2012) In vivo muscle electroporation threshold determination: realistic numerical models and in vivo experiments. J Membr Biol 245(9):509–520. https://doi.org/10.1007/s00232-012-9432-8

Davalos RV, Otten DM, Mir LM, Rubinsky B (2004) Electrical impedance tomography for imaging tissue electroporation. IEEE Trans Biomed Eng 51(5):761–767. https://doi.org/10.1109/TBME.2004.824148

Davalos RV, Bhonsle S, Neal RE 2nd (2015) Implications and considerations of thermal effects when applying irreversible electroporation tissue ablation therapy. Prostate 75(10):1114–1118. https://doi.org/10.1002/pros.22986

DeBruin KA, Krassowska W (1999) Modeling electroporation in a single cell. II. Effects of ionic concentrations. Biophys J 77(3):1225–1233. https://doi.org/10.1016/S0006-3495(99)76974-2

Deodhar A, Dickfeld T, Single GW, Hamilton WC Jr, Thornton RH, Sofocleous CT, Maybody M, Gonen M, Rubinsky B, Solomon SB (2011) Irreversible electroporation near the heart: ventricular arrhythmias can be prevented with ECG synchronization. AJR Am J Roentgenol 196(3):W330–W335. https://doi.org/10.2214/AJR.10.4490

Dettin M, Sieni E, Zamuner A, Marino R, Sgarbossa P, Lucibello M, Tosi AL, Keller F, Campana LG, Signori E (2019) A novel 3D Scaffold for cell growth to assess electroporation efficacy. Cells 8(11). https://doi.org/10.3390/cells8111470

Dhankhar R, Vyas SP, Jain AK, Arora S, Rath G, Goyal AK (2010) Advances in novel drug delivery strategies for breast cancer therapy. Artif Cells Blood Substit Immobil Biotechnol 38(5):230–249. https://doi.org/10.3109/10731199.2010.494578

Dollinger M, Beyer LP, Haimerl M, Niessen C, Jung EM, Zeman F, Stroszczynski C, Wiggermann P (2015) Adverse effects of irreversible electroporation of malignant liver tumors under CT fluoroscopic guidance: a single-center experience. Diagn Interv Radiol 21(6):471–475. https://doi.org/10.5152/dir.2015.14442

Dunki-Jacobs EM, Philips P, Martin RC 2nd (2014) Evaluation of resistance as a measure of successful tumor ablation during irreversible electroporation of the pancreas. J Am Coll Surg 218(2):179–187. https://doi.org/10.1016/j.jamcollsurg.2013.10.013

Early Breast Cancer Trialists' Collaborative Group, Peto R, Davies C, Godwin J, Gray R, Pan HC, Clarke M, Cutter D, Darby S, McGale P, Taylor C, Wang YC, Bergh J, Di Leo A, Albain K, Swain S, Piccart M, Pritchard K (2012) Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. Lancet 379(9814):432–444. https://doi.org/10.1016/S0140-6736(11)61625-5

Edd JF, Davalos RV (2007) Mathematical modeling of irreversible electroporation for treatment planning. Technol Cancer Res Treat 6(4):275–286. https://doi.org/10.1177/153303460700600403

Elgenedy MA et al (2017) A transition arm modular multilevel universal pulse-waveform generator for electroporation applications. IEEE Trans Power Electronics 32(12):8979–8991

Ellis TL, Garcia PA, Rossmeisl JH Jr, Henao-Guerrero N, Robertson J, Davalos RV (2011) Nonthermal irreversible electroporation for intracranial surgical applications. Laboratory investigation. J Neurosurg 114(3):681–688. https://doi.org/10.3171/2010.5.JNS091448

Ewer SM, Ewer MS (2008) Cardiotoxicity profile of trastuzumab. Drug Saf 31(6):459–467. https://doi.org/10.2165/00002018-200831060-00002

Ford MS, Young KJ, Zhang Z, Ohashi PS, Zhang L (2002) The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. J Exp Med 196(2):261–267

Fritz S, Sommer CM, Vollherbst D, Wachter MF, Longerich T, Sachsenmeier M, Knapp J, Radeleff BA, Werner J (2015) Irreversible electroporation of the pancreas is feasible and safe in a porcine survival model. Pancreas 44(5):791–798. https://doi.org/10.1097/MPA.0000000000000331

Fruhling P, Nilsson A, Duraj F, Haglund U, Noren A (2017) Single-center nonrandomized clinical trial to assess the safety and efficacy of irreversible electroporation (IRE) ablation of liver tumors in humans: short to mid-term results. Eur J Surg Oncol 43(4):751–757. https://doi.org/10.1016/j.ejso.2016.12.004

Garcia PA, Rossmeisl JH Jr, Neal RE 2nd, Ellis TL, Olson JD, Henao-Guerrero N, Robertson J, Davalos RV (2010) Intracranial nonthermal irreversible electroporation: in vivo analysis. J Membr Biol 236(1):127–136. https://doi.org/10.1007/s00232-010-9284-z

Garcia PA, Rossmeisl JH Jr, Robertson JL, Olson JD, Johnson AJ, Ellis TL, Davalos RV (2012) 7.0-T magnetic resonance imaging characterization of acute blood-brain-barrier disruption achieved with intracranial irreversible electroporation. PLoS One 7(11):e50482. https://doi.org/10.1371/journal.pone.0050482

Garcia PA, Kos B, Rossmeisl JH Jr, Pavliha D, Miklavcic D, Davalos RV (2017) Predictive therapeutic planning for irreversible electroporation treatment of spontaneous malignant glioma. Med Phys 44(9):4968–4980. https://doi.org/10.1002/mp.12401

Gehl J, Mir LM (1999) Determination of optimal parameters for in vivo gene transfer by electroporation, using a rapid in vivo test for cell permeabilization. Biochem Biophys Res Commun 261 (2):377–380. https://doi.org/10.1006/bbrc.1999.1014

Golberg A, Bruinsma BG, Uygun BE, Yarmush ML (2015) Tissue heterogeneity in structure and conductivity contribute to cell survival during irreversible electroporation ablation by "electric field sinks". Sci Rep 5:8485. https://doi.org/10.1038/srep08485

Goswami I, Coutermarsh-Ott S, Morrison RG, Allen IC, Davalos RV, Verbridge SS, Bickford LR (2017) Irreversible electroporation inhibits pro-cancer inflammatory signaling in triple negative breast cancer cells. Bioelectrochemistry 113:42–50. https://doi.org/10.1016/j.bioelechem.2016.09.003

Guenther E, Klein N, Zapf S, Weil S, Schlosser C, Rubinsky B, Stehling MK (2019) Prostate cancer treatment with Irreversible Electroporation (IRE): safety, efficacy and clinical experience in 471 treatments. PLoS One 14(4):e0215093. https://doi.org/10.1371/journal.pone.0215093

Guo Y, Zhang Y, Nijm GM, Sahakian AV, Yang GY, Omary RA, Larson AC (2011) Irreversible electroporation in the liver: contrast-enhanced inversion-recovery MR imaging approaches to differentiate reversibly electroporated penumbra from irreversibly electroporated ablation zones. Radiology 258(2):461–468. https://doi.org/10.1148/radiol.10100645

Han Y (2019) Safety and feasibility of prostatic tissue ablation in dogs by percutaneous irreversible electroporation (IRE) using a newly developed high-voltage steep-pulse-therapy device. Int J Clin Exp Med 12(7):8014–8023

Ivey JW, Wasson EM, Alinezhadbalalami N, Kanitkar A, Debinski W, Sheng Z, Davalos RV, Verbridge SS (2019) Characterization of ablation thresholds for 3D-cultured patient-derived glioma stem cells in response to high-frequency irreversible electroporation. Research (Wash D C). https://doi.org/10.34133/2019/8081315

Ivorra A, Al-Sakere B, Rubinsky B, Mir LM (2009) In vivo electrical conductivity measurements during and after tumor electroporation: conductivity changes reflect the treatment outcome. Phys Med Biol 54(19):5949–5963. https://doi.org/10.1088/0031-9155/54/19/019

Kalra N, Gupta P, Gorsi U, Bhujade H, Chaluvashetty SB, Duseja A, Singh V, Dhiman RK, Chawla YK, Khandelwal N (2019) Irreversible electroporation for unresectable hepatocellular carcinoma: initial experience. Cardiovasc Intervent Radiol 42(4):584–590. https://doi.org/10.1007/s00270-019-02164-2

Kane GMO, Knox JJ (2018) Locally advanced pancreatic cancer: an emerging entity. Curr Probl Cancer 42(1):12–25. https://doi.org/10.1016/j.currproblcancer.2017.10.006

Kato H, Usui M, Isaji S, Nagakawa T, Wada K, Unno M, Nakao A, Miyakawa S, Ohta T (2013) Clinical features and treatment outcome of borderline resectable pancreatic head/body cancer: a

multi-institutional survey by the Japanese Society of Pancreatic Surgery. J Hepatobiliary Pancreat Sci 20(6):601–610. https://doi.org/10.1007/s00534-013-0595-1

Kingham TP, Karkar AM, D'Angelica MI, Allen PJ, Dematteo RP, Getrajdman GI, Sofocleous CT, Solomon SB, Jarnagin WR, Fong Y (2012) Ablation of perivascular hepatic malignant tumors with irreversible electroporation. J Am Coll Surg 215(3):379–387. https://doi.org/10.1016/j.jamcollsurg.2012.04.029

Kinsey JR, Gilson SD, Hauptman J, Mehler SJ, May LR (2015) Factors associated with long-term survival in dogs undergoing liver lobectomy as treatment for liver tumors. Can Vet J 56 (6):598–604

Kos B, Voigt P, Miklavcic D, Moche M (2015) Careful treatment planning enables safe ablation of liver tumors adjacent to major blood vessels by percutaneous irreversible electroporation (IRE). Radiol Oncol 49(3):234–241. https://doi.org/10.1515/raon-2015-0031

Kranjc M, Kranjc S, Bajd F, Sersa G, Sersa I, Miklavcic D (2017) Predicting irreversible electroporation-induced tissue damage by means of magnetic resonance electrical impedance tomography. Sci Rep 7(1):10323. https://doi.org/10.1038/s41598-017-10846-5

Kwon D, McFarland K, Velanovich V, Martin RC (2014) Borderline and locally advanced pancreatic adenocarcinoma margin accentuation with intraoperative irreversible electroporation. Surgery 156(4):910–922. https://doi.org/10.1016/j.surg.2014.06.058

Langan RC, Goldman DA, D'Angelica MI, DeMatteo RP, Allen PJ, Balachandran VP, Jarnagin WR, Kingham TP (2017) Recurrence patterns following irreversible electroporation for hepatic malignancies. J Surg Oncol 115(6):704–710. https://doi.org/10.1002/jso.24570

Latouche EL, Sano MB, Lorenzo MF, Davalos RV, Martin RCG 2nd (2017) Irreversible electroporation for the ablation of pancreatic malignancies: a patient-specific methodology. J Surg Oncol 115(6):711–717. https://doi.org/10.1002/jso.24566

Latouche EL, Arena CB, Ivey JW, Garcia PA, Pancotto TE, Pavlisko N, Verbridge SS, Davalos RV, Rossmeisl JH (2018) High-frequency irreversible electroporation for intracranial meningioma: a feasibility study in a spontaneous canine tumor model. Technol Cancer Res Treat 17:1533033818785285. https://doi.org/10.1177/1533033818785285

Lee YJ, Lu DS, Osuagwu F, Lassman C (2012) Irreversible electroporation in porcine liver: short- and long-term effect on the hepatic veins and adjacent tissue by CT with pathological correlation. Invest Radiol 47(11):671–675. https://doi.org/10.1097/RLI.0b013e318274b0df

Leroy BE, Northrup N (2009) Prostate cancer in dogs: comparative and clinical aspects. Vet J 180 (2):149–162. https://doi.org/10.1016/j.tvjl.2008.07.012

Li S, Chen F, Shen L, Zeng Q, Wu P (2016) Percutaneous irreversible electroporation for breast tissue and breast cancer: safety, feasibility, skin effects and radiologic-pathologic correlation in an animal study. J Transl Med 14(1):238. https://doi.org/10.1186/s12967-016-0993-7

Lim M, Xia Y, Bettegowda C, Weller M (2018) Current state of immunotherapy for glioblastoma. Nat Rev Clin Oncol 15(7):422–442. https://doi.org/10.1038/s41571-018-0003-5

Linecker M, Pfammatter T, Kambakamba P, DeOliveira ML (2016) Ablation strategies for locally advanced pancreatic cancer. Dig Surg 33(4):351–359. https://doi.org/10.1159/000445021

Liptak JM, Dernell WS, Monnet E, Powers BE, Bachand AM, Kenney JG, Withrow SJ (2004) Massive hepatocellular carcinoma in dogs: 48 cases (1992-2002). J Am Vet Med Assoc 225 (8):1225–1230

Liu Y, Xiong Z, Zhou W, Hua Y, Li C, Yao C (2012) Percutaneous ultrasound-guided irreversible electroporation: a goat liver study. Oncol Lett 4(3):450–454. https://doi.org/10.3892/ol.2012.781

Lorenzo MF, Thomas SC, Kani Y, Hinckley J, Lee M, Adler J, Verbridge SS, Hsu FC, Robertson JL, Davalos RV, Rossmeisl JH Jr (2019) Temporal characterization of blood-brain barrier disruption with high-frequency electroporation. Cancers (Basel) 11(12). https://doi.org/10.3390/cancers11121850

Lorenzo MF, Bhonsle S, Arena CB, Davalos RV (2020) Rapid impedance spectroscopy for monitoring tissue impedance, temperature, and treatment outcome during electroporation-based therapies. IEEE Trans Biomed Eng. https://doi.org/10.1109/TBME.2020.3036535

Mafeld S, Wong JJ, Kibriya N, Stenberg B, Manas D, Bassett P, Aslam T, Evans J, Littler P (2019) Percutaneous irreversible electroporation (IRE) of hepatic malignancy: a bi-institutional analysis of safety and outcomes. Cardiovasc Intervent Radiol 42(4):577–583. https://doi.org/10.1007/s00270-018-2120-z

Martin RC, McFarland K, Ellis S, Velanovich V (2013) Irreversible electroporation in locally advanced pancreatic cancer: potential improved overall survival. Ann Surg Oncol 20(3):S443–S449. https://doi.org/10.1245/s10434-012-2736-1

Martina MN, Noel S, Saxena A, Rabb H, Hamad AR (2015) Double negative (DN) alphabeta T cells: misperception and overdue recognition. Immunol Cell Biol 93(3):305–310. https://doi.org/10.1038/icb.2014.99

Mercadal B, Arena CB, Davalos RV, Ivorra A (2017) Avoiding nerve stimulation in irreversible electroporation: a numerical modeling study. Phys Med Biol 62(20):8060–8079. https://doi.org/10.1088/1361-6560/aa8c53

Miklavcic D, Semrov D, Mekid H, Mir LM (2000) A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. Biochim Biophys Acta 1523(1):73–83. https://doi.org/10.1016/s0304-4165(00)00101-x

Miklavcic D, Sel D, Cukjati D, Batiuskaite D, Slivnik T, Mir L (2004) Sequential finite element model of tissue electropermeabilisation. Conf Proc IEEE Eng Med Biol Soc 2004:3551–3554. https://doi.org/10.1109/IEMBS.2004.1403998

Miklavcic D, Pucihar G, Pavlovec M, Ribaric S, Mali M, Macek-Lebar A, Petkovsek M, Nastran J, Kranic S, Cemazar M, Sersa G (2005) The effect of high frequency electric pulses on muscle contractions and anti-tumor efficiency in vivo for a potential use in clinical electrochemotherapy. Bioelectrochemistry 65(2):121–128. https://doi.org/10.1016/j.bioelechem.2004.07.004

Miklavcic D, Mali B, Kos B, Heller R, Sersa G (2014) Electrochemotherapy: from the drawing board into medical practice. Biomed Eng Online 13(1):29. https://doi.org/10.1186/1475-925X-13-29

Miller L, Leor J, Rubinsky B (2005) Cancer cells ablation with irreversible electroporation. Technol Cancer Res Treat 4(6):699–705. https://doi.org/10.1177/153303460500400615

Narayanan G (2015) Irreversible electroporation. Semin Intervent Radiol 32(4):349–355. https://doi.org/10.1055/s-0035-1564706

Narayanan G, Hosein P, Arora G, Barbery K, Froud T, Livingstone A, Franceschi D, Rocha Lime CM, Yrizarry J (2012) Percutaneous irreversible electroporation for downstaging and control of unresectable pancreatic adenocarcinoma. J Vasc Interven Radiol 23(12):1613–1612. https://doi.org/10.1016/j.jvir.2012.09.012

Narayanan G, Bhatia S, Echenique A, Suthar R, Barbery K, Yrizarry J (2014) Vessel patency post irreversible electroporation. Cardiovasc Intervent Radiol 37(6):1523–1529. https://doi.org/10.1007/s00270-014-0988-9

Neal RE, Singh R, Hatcher HC, Kock ND, Torti SV, Davalos RV (2010a) Treatment of breast cancer through the application of irreversible electroporation using a novel minimally invasive single needle electrode. Breast Cancer Res Treat 123(1):295–301. https://doi.org/10.1007/s10549-010-0803-5

Neal RE, Garcia PA, Rossmeisl JH, Davalos RV (2010b) A study using irreversible electroporation to treat large, irregular tumors in a canine patient. Conf Proc IEEE Eng Med Biol Soc 2010:2747–2750. https://doi.org/10.1109/IEMBS.2010.5626372

Neal RE, Garcia PA, Robertson JL, Davalos RV (2012) Experimental characterization and numerical modeling of tissue electrical conductivity during pulsed electric fields for irreversible electroporation treatment planning. IEEE Trans Biomed Eng 59(4):1076–1085. https://doi.org/10.1109/TBME.2012.2182994

Neal RE, Millar JL, Kavnoudias H, Royce P, Rosenfeldt F, Pham A, Smith R, Davalos RV, Thomson KR (2014) In vivo characterization and numerical simulation of prostate properties for non-thermal irreversible electroporation ablation. Prostate 74(5):458–468. https://doi.org/10.1002/pros.22760

Neal RE, Garcia PA, Kavnoudias H, Rosenfeldt F, McLean CA, Earl V, Bergman J, Davalos RV, Thomson KR (2015) In vivo irreversible electroporation kidney ablation: experimentally correlated numerical models. IEEE Trans Biomed Eng 62(2):561–569. https://doi.org/10.1109/TBME.2014.2360374

Nguyen F, Pena L, Ibisch C, Loussouarn D, Gama A, Rieder N, Belousov A, Campone M, Abadie J (2018) Canine invasive mammary carcinomas as models of human breast cancer. Part 1: natural history and prognostic factors. Breast Cancer Res Treat 167(3):635–648. https://doi.org/10.1007/s10549-017-4548-2

Niessen C, Beyer LP, Pregler B, Dollinger M, Trabold B, Schlitt HJ, Jung EM, Stroszczynski C, Wiggermann P (2016) Percutaneous ablation of hepatic tumors using irreversible electroporation: a prospective safety and midterm efficacy study in 34 patients. J Vasc Interv Radiol 27(4):480–486. https://doi.org/10.1016/j.jvir.2015.12.025

Niessen C, Thumann S, Beyer L, Pregler B, Kramer J, Lang S, Teufel A, Jung EM, Stroszczynski C, Wiggermann P (2017) Percutaneous irreversible electroporation: long-term survival analysis of 71 patients with inoperable malignant hepatic tumors. Sci Rep 7:43687. https://doi.org/10.1038/srep43687

Nounou MI, ElAmrawy F, Ahmed N, Abdelraouf K, Goda S, Syed-Sha-Qhattal H (2015) Breast cancer: conventional diagnosis and treatment modalities and recent patents and technologies. Breast Cancer (Auckl) 9(Suppl 2):17–34. https://doi.org/10.4137/BCBCR.S29420

O'Brien TJ, Passeri M, Lorenzo MF, Sulzer JK, Lyman WB, Swet JH, Vrochides D, Baker EH, Iannitti DA, Davalos RV, McKillop IH (2019) Experimental high-frequency irreversible electroporation using a single-needle delivery approach for nonthermal pancreatic ablation in vivo. J Vasc Interv Radiol 30(6):854–862. https://doi.org/10.1016/j.jvir.2019.01.032

Onik G, Rubinsky B (2010) Irreversible electroporation: first patient experience focal therapy of prostate cancer. In: Rubinsky B (ed) Irreversible electroporation, Series in biomedical engineering. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-05420-4_10

Onik G, Mikus P, Rubinsky B (2007) Irreversible electroporation: implications for prostate ablation. Technol Cancer Res Treat 6(4):295–300. https://doi.org/10.1177/153303460700600405

Palussiere J, Henriques C, Mauriac L, Asad-Syed M, Valentin F, Brouste V, Mathoulin-Pelissier S, Tunon de Lara C, Debled M (2012) Radiofrequency ablation as a substitute for surgery in elderly patients with nonresected breast cancer: pilot study with long-term outcomes. Radiology 264(2):597–605. https://doi.org/10.1148/radiol.12111303

Partridge BR, O'Brien TJ, Lorenzo MF, Coutermarsh-Ott SL, Barry SL, Stadler K, Muro N, Meyerhoeffer M, Allen IC, Davalos RV, Dervisis NG (2020) High-frequency irreversible electroporation for treatment of primary liver cancer: a proof-of-principle study in canine hepatocellular carcinoma. J Vasc Interv Radiol. https://doi.org/10.1016/j.jvir.2019.10.015

Pasquali S, Hadjinicolaou AV, Chiarion Sileni V, Rossi CR, Mocellin S (2018) Systemic treatments for metastatic cutaneous melanoma. Cochrane Database Syst Rev 2:CD011123. https://doi.org/10.1002/14651858.CD011123.pub2

Pavliha D, Kos B, Marcan M, Zupanic A, Sersa G, Miklavcic D (2013) Planning of electroporation-based treatments using Web-based treatment-planning software. J Membr Biol 246(11):833–842. https://doi.org/10.1007/s00232-013-9567-2

Pavlin M, Kanduser M, Rebersek M, Pucihar G, Hart FX, Magjarevic R, Miklavcic D (2005) Effect of cell electroporation on the conductivity of a cell suspension. Biophys J 88(6):4378–4390. https://doi.org/10.1529/biophysj.104.048975

Perera-Bel E, Yague C, Mercadal B, Ceresa M, Beitel-White N, Davalos R, Gonzalez B, Ivorra A (2020) EView: an electric field visualization web platform for electroporation-based therapies. Computer Methods Prog Biomed. https://doi.org/10.1016/j.cmpb.2020.105682

Pinard CJ, Hocker SE, Weishaar KM (2020) Clinical outcome in 23 dogs with exocrine pancreatic carcinoma. Vet Comp Oncol. https://doi.org/10.1111/vco.12645

Potocnik T, Miklavcic D, Lebar AM (2019) Effect of electroporation and recovery medium pH on cell membrane permeabilization, cell survival and gene transfer efficiency in vitro. Bioelectrochemistry 130. https://doi.org/10.1016/j.bioelechem.2019.107342

Priester WA (1974) Data from eleven United States and Canadian colleges of veterinary medicine on pancreatic carcinoma in domestic animals. Cancer Res 34(6):1372–1375

Pucihar G, Krmelj J, Rebersek M, Napotnik TB, Miklavcic D (2011) Equivalent pulse parameters for electroporation. IEEE Trans Biomed Eng 58(11):3279–3288. https://doi.org/10.1109/TBME.2011.2167232

Qin Z, Zeng J, Liu G, Long X, Fang G, Li Z, Xu K, Niu L (2017) Irreversible electroporation ablation of an unresectable fibrous sarcoma with 2 electrodes: a case report. Technol Cancer Res Treat. https://doi.org/10.1177/1533034617711530

Rakha EA, Reis-Filho JS, Ellis IO (2010) Combinatorial biomarker expression in breast cancer. Breast Cancer Res Treat 120(2):293–308. https://doi.org/10.1007/s10549-010-0746-x

Rawla P (2019) Epidemiology of prostate cancer. World J Oncol 10(2):63–89. https://doi.org/10.14740/wjon1191

Rebersek M, Miklavcic D (2011) Advantages and disadvantages of different concepts of electroporation pulse generation. Automatika 52(1):12–19

Redondo LM, Zahyka M, Kandratsyeu A (2019) Solid-state generation of high-frequency burst of bipolar pulses for medical applications. IEEE Trans Plasma Sci 47(8):4091–4095

Ringel-Scaia VM, Beitel-White N, Lorenzo MF, Brock RM, Huie KE, Coutermarsh-Ott S, Eden K, McDaniel DK, Verbridge SS, Rossmeisl JH Jr, Oestreich KJ, Davalos RV, Allen IC (2019) High-frequency irreversible electroporation is an effective tumor ablation strategy that induces immunologic cell death and promotes systemic anti-tumor immunity. EBioMedicine 44:112–125. https://doi.org/10.1016/j.ebiom.2019.05.036

Robinson MW, Harmon C, O'Farrelly C (2016) Liver immunology and its role in inflammation and homeostasis. Cell Mol Immunol 13(3):267–276. https://doi.org/10.1038/cmi.2016.3

Rombouts SJE, van Dijck WPM, Nijkamp MW, Derksen TC, Brosens LAA, Hoogwater FJH, van Leeuwen MS, Borel Rinkes IHM, van Hillegersberg R, Wittkampf FH, Molenaar IQ (2017) Clinical and pathological outcomes after irreversible electroporation of the pancreas using two parallel plate electrodes: a porcine model. HPB (Oxford) 19(12):1058–1065. https://doi.org/10.1016/j.hpb.2017.02.443

Rossmeisl JH, Garcia PA, Pancotto TE, Robertson JL, Henao-Guerrero N, Neal RE 2nd, Ellis TL, Davalos RV (2015) Safety and feasibility of the NanoKnife system for irreversible electroporation ablative treatment of canine spontaneous intracranial gliomas. J Neurosurg 123(4):1008–1025. https://doi.org/10.3171/2014.12.JNS141768

Rubinsky B (2007) Irreversible electroporation in medicine. Technol Cancer Res Treat 6(4):255–260. https://doi.org/10.1177/153303460700600401

Rubinsky B, Onik G, Mikus P (2007) Irreversible electroporation: a new ablation modality – clinical implications. Technol Cancer Res Treat 6(1):37–48. https://doi.org/10.1177/153303460700600106

Saini A, Breen I, Alzubaidi S, Pershad Y, Sheth R, Naidu S, Knuttinen MG, Albadawi H, Oklu R (2018) Irreversible electroporation in liver cancers and whole organ engineering. J Clin Med 8(1). https://doi.org/10.3390/jcm8010022

Scheffer HJ, Nielsen K, de Jong MC, van Tilborg AA, Vieveen JM, Bouwman AR, Meijer S, van Kuijk C, van den Tol PM, Meijerink MR (2014a) Irreversible electroporation for nonthermal tumor ablation in the clinical setting: a systematic review of safety and efficacy. J Vasc Interv Radiol 25(7):997–1011. https://doi.org/10.1016/j.jvir.2014.01.028

Scheffer HJ, Nielsen K, van Tilborg AA, Vieveen JM, Bouwman RA, Kazemier G, Niessen HW, Meijer S, van Kuijk C, van den Tol MP, Meijerink MR (2014b) Ablation of colorectal liver metastases by irreversible electroporation: results of the COLDFIRE-I ablate-and-resect study. Eur Radiol 24(10):2467–2475. https://doi.org/10.1007/s00330-014-3259-x

Scheffer HJ, Vogel JA, van den Bos W, Neal RE 2nd, van Lienden KP, Besselink MG, van Gemert MJ, van der Geld CW, Meijerink MR, Klaessens JH, Verdaasdonk RM (2016) The influence of

a metal stent on the distribution of thermal energy during irreversible electroporation. PLoS One 11(2):e0148457. https://doi.org/10.1371/journal.pone.0148457

Schmidt CR, Shires P, Mootoo M (2012) Real-time ultrasound imaging of irreversible electroporation in a porcine liver model adequately characterizes the zone of cellular necrosis. HPB (Oxford) 14(2):98–102. https://doi.org/10.1111/j.1477-2574.2011.00409.x

Selzner M, Morse MA, Vredenburgh JJ, Meyers WC, Clavien PA (2000) Liver metastases from breast cancer: long-term survival after curative resection. Surgery 127(4):383–389. https://doi.org/10.1067/msy.2000.103883

Sharabi S, Bresler Y, Ravid O, Shemesh C, Atrakchi D, Schnaider-Beeri M, Gosselet F, Dehouck L, Last D, Guez D, Daniels D, Mardor Y, Cooper I (2019) Transient blood-brain barrier disruption is induced by low pulsed electric fields in vitro: an analysis of permeability and trans-endothelial electric resistivity. Drug Deliv 26(1):459–469. https://doi.org/10.1080/10717544.2019.1571123

Sharabi S, Guez D, Daniels D, Cooper I, Atrakchi D, Liraz-Zaltsman S, Last D, Mardor Y (2020) The application of point source electroporation and chemotherapy for the treatment of glioma: a randomized controlled rat study. Sci Rep 10(1):2178. https://doi.org/10.1038/s41598-020-59152-7

Siddiqui IA, Latouche EL, DeWitt MR, Swet JH, Kirks RC, Baker EH, Iannitti DA, Vrochides D, Davalos RV, McKillop IH (2016) Induction of rapid, reproducible hepatic ablations using next-generation, high frequency irreversible electroporation (H-FIRE) in vivo. HPB (Oxford) 18(9):726–734. https://doi.org/10.1016/j.hpb.2016.06.015

Siegel RL, Miller KD, Jemal A (2017) Cancer statistics, 2017. CA Cancer J Clin 67(1):7–30. https://doi.org/10.3322/caac.21387

Silk MT, Wimmer T, Lee KS, Srimathveeravalli G, Brown KT, Kingham PT, Fong Y, Durack JC, Sofocleous CT, Solomon SB (2014) Percutaneous ablation of peribiliary tumors with irreversible electroporation. J Vasc Interv Radiol 25(1):112–118. https://doi.org/10.1016/j.jvir.2013.10.012

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO, European Organisation for Research, Treatment of Cancer Brain Tumor, Radiotherapy Groups, National Cancer Institute of Canada Clinical Trials Group (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352(10):987–996. https://doi.org/10.1056/NEJMoa043330

Sutter O, Calvo J, N'Kontchou G, Nault JC, Ourabia R, Nahon P, Ganne-Carrie N, Bourcier V, Zentar N, Bouhafs F, Sellier N, Diallo A, Seror O (2017) Safety and efficacy of irreversible electroporation for the treatment of hepatocellular carcinoma not amenable to thermal ablation techniques: a retrospective single-center case series. Radiology 284(3):877–886. https://doi.org/10.1148/radiol.2017161413

Swain SM, Im YH, Im SA, Chan V, Miles D, Knott A, Clark E, Ross G, Baselga J (2014) Safety profile of Pertuzumab with Trastuzumab and Docetaxel in patients from Asia with human epidermal growth factor receptor 2-positive metastatic breast cancer: results from the phase III trial CLEOPATRA. Oncologist 19(7):693–701. https://doi.org/10.1634/theoncologist.2014-0033

Tameez Ud Din A, Tameez-Ud-Din A, Chaudhary FMD, Chaudhary NA, Siddiqui KH (2019) Irreversible electroporation for liver tumors: a review of literature. Cureus 11(6):e4994. https://doi.org/10.7759/cureus.4994

Tamimi AF, Juweid M (2017) Epidemiology and outcome of glioblastoma. In: De Vleeschouwer S (ed) Glioblastoma. Codon Publications, Brisbane. https://doi.org/10.15586/codon.glioblastoma.2017.ch8

Thind K, Padrnos LJ, Ramanathan RK, Borad MJ (2017) Immunotherapy in pancreatic cancer treatment: a new frontier. Therap Adv Gastroenterol 10(1):168–194. https://doi.org/10.1177/1756283X16667909

Thomson KR, Cheung W, Ellis SJ, Federman D, Kavnoudias H, Loader-Oliver D, Roberts S, Evans P, Ball C, Haydon A (2011) Investigation of the safety of irreversible electroporation in humans. J Vasc Interv Radiol 22(5):611–621. https://doi.org/10.1016/j.jvir.2010.12.014

Usman M, Moore W, Talati R, Watkins K, Bilfinger TV (2012) Irreversible electroporation of lung neoplasm: a case series. Med Sci Monit 18(6):CS43–CS47. https://doi.org/10.12659/msm.882888

Vailas M, Syllaios A, Hashemaki N, Sotiropoulou M, Schizas D, Papalampros A, Felekouras E, Pikoulis E (2019) Irreversible electroporation and sarcomas: where do we stand? J BUON 24 (4):1354–1359

Valerio M, Stricker PD, Ahmed HU, Dickinson L, Ponsky L, Shnier R, Allen C, Emberton M (2014) Initial assessment of safety and clinical feasibility of irreversible electroporation in the focal treatment of prostate cancer. Prostate Cancer Prostatic Dis 17(4):343–347. https://doi.org/10.1038/pcan.2014.33

Valero V (1997) Docetaxel and cyclophosphamide in patients with advanced solid tumors. Oncology (Williston Park) 11(6 Suppl):21–23

van den Bos W, de Bruin DM, Jurhill RR, Savci-Heijink CD, Muller BG, Varkarakis IM, Skolarikos A, Zondervan PJ, Laguna-Pes MP, Wijkstra H, de Reijke TM, de la Rosette JJ (2016) The correlation between the electrode configuration and histopathology of irreversible electroporation ablations in prostate cancer patients. World J Urol 34(5):657–664. https://doi.org/10.1007/s00345-015-1661-x

Vizintin A, Vidmar J, Scancar J, Miklavcic D (2020) Effect of interphase and interpulse delay in high-frequency irreversible electroporation pulses on cell survival, membrane permeabilization and electrode material release. Bioelectrochemistry 134:107523. https://doi.org/10.1016/j.bioelechem.2020.107523

Vogel JA, van Veldhuisen E, Alles LK, Busch OR, Dijk F, van Gulik TM, Huijzer GM, Besselink MG, van Lienden KP, Verheij J (2019) Time-dependent impact of irreversible electroporation on pathology and ablation size in the porcine liver: a 24-hour experimental study. Technol Cancer Res Treat 18:1533033819876899. https://doi.org/10.1177/1533033819876899

Weaver JC, Chizmadzhev YA (1996) Theory of electroporation: a review. Bioelectrochem Bioenergetics 41(2):135–160

Wendler JJ, Fischbach K, Ricke J, Jurgens J, Fischbach F, Kollermann J, Porsch M, Baumunk D, Schostak M, Liehr UB, Pech M (2016) Irreversible Electroporation (IRE): standardization of terminology and reporting criteria for analysis and comparison. Pol J Radiol 81:54–64. https://doi.org/10.12659/PJR.896034

Wimmer T, Srimathveeravalli G, Gutta N, Ezell PC, Monette S, Kingham TP, Maybody M, Durack JC, Fong Y, Solomon SB (2013) Comparison of simulation-based treatment planning with imaging and pathology outcomes for percutaneous CT-guided irreversible electroporation of the porcine pancreas: a pilot study. J Vasc Interv Radiol 24(11):1709–1718. https://doi.org/10.1016/j.jvir.2013.05.056

Zeng J, Liu G, Li ZH, Yang Y, Fang G, Li RR, Xu KC, Niu L (2017) The safety and efficacy of irreversible electroporation for large hepatocellular carcinoma. Technol Cancer Res Treat 16 (1):120–124. https://doi.org/10.1177/1533034616676445

Zhang W, Wang W, Chai W, Luo X, Li J, Shi J, Bi L, Niu L (2017) Breast tissue ablation with irreversible electroporation in rabbits: a safety and feasibility study. PLoS One 12(7):e0181555. https://doi.org/10.1371/journal.pone.0181555

Zhao Y, Bhonsle S, Dong S, Lv Y, Liu H, Safaai-Jazi A, Davalos RV, Yao C (2018a) Characterization of conductivity changes during high-frequency irreversible electroporation for treatment planning. IEEE Trans Biomed Eng 65(8):1810–1819. https://doi.org/10.1109/TBME.2017.2778101

Zhao Y, Liu H, Bhonsle SP, Wang Y, Davalos RV, Yao C (2018b) Ablation outcome of irreversible electroporation on potato monitored by impedance spectrum under multi-electrode system. Biomed Eng Online 17(1):126. https://doi.org/10.1186/s12938-018-0562-9

Zhao J, Wen X, Tian L, Li T, Xu C, Wen X, Melancon MP, Gupta S, Shen B, Peng W, Li C (2019) Irreversible electroporation reverses resistance to immune checkpoint blockade in pancreatic cancer. Nat Commun 10(1):899. https://doi.org/10.1038/s41467-019-08782-1

Zupanic A, Kos B, Miklavcic D (2012) Treatment planning of electroporation-based medical interventions: electrochemotherapy, gene electrotransfer and irreversible electroporation. Phys Med Biol 57(17):5425–5440. https://doi.org/10.1088/0031-9155/57/17/5425

Electrochemotherapy as a Multi-Modality Component of Cancer Treatment: Combinations with Surgery, Cryosurgery, Radiation Therapy, and Chemotherapy

M. Tellado, F. Maglietti, and J. Impellizeri

Abstract

Cancer treatments are based on a combination of different treatment modalities with different mechanisms of action allowing oncologists to improve survival times, maintain a good quality of life, and in many situations, provide long-term control, and sometimes cure. This chapter is provided to educate veterinarians about electrochemotherapy (ECT) as another valuable therapy available in veterinary oncology. The amalgamation of ECT in combination with surgery is advantageous and may be underutilized in both veterinary and human medicine. Combinations of ECT with other standard procedures in the existing armamentarium of treatments (surgery, systemic chemotherapy, cryosurgery, immunotherapy, and radiotherapy) are less common, but could potentially achieve even better results than any single modality currently being offered.

Keywords

Cancer · Electroporation · Combination · Tumors · ECT

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1 Combination with Surgery

Today, surgery is the most common therapy for local management of cancer. Progressive improvements and advancing expertise of the surgical technique, allow excellent tumoral resection with complete removal of the neoplasm and its margin, leading to minimal local recurrence rates and many times cure (Aiken 2003; Fasel et al. 2007).

Although it is well known that many cancers are not completely resolved by surgery alone. It is important to review some basic concepts regarding oncological surgery and how it may relate to the addition of electrochemotherapy (ECT), to assess where the combination could be most beneficial. First, a complete patient evaluation is imperative to choose among the most effective treatment modalities. This planning should always include a discussion with the owner about pre-operative diagnostic tests, cancer stage, surgical options, associated treatments, prognosis, and costs. It is important to obtain the correct oncologic diagnosis through cytology or histopathology, to develop an accurate strategy (Cohen et al. 2003; Eich et al. 2000). Once surgery has been considered, it is important to emphasize that margins must be assessed beforehand and not intra- or post-surgically, meaning that the surgeon must evaluate and measure the lesion, taking into consideration adjacent structures. Additional tests such as MRI and CT-Scan allow tridimensional analysis of the involved structures, aiding in further planning (Wallack et al. 2002; Kondo et al. 2008; Willcox et al. 2020) "A chance to cut is a chance to cure" but only when the pre-surgical planning is factored in before the patient is even anesthetized.

Surgical margins are based on the volume of normal tissue surrounding the tumor and the invasiveness of the tumor type. When the main lesion has adjacent satellite lesions, sometimes only visible with advanced imaging, the space in between them is not considered a safe margin (Fasel et al. 2007). Histopathology post extirpation of the tumor is critical to margin evaluation and thus local control (Liptak 2020). Often in these studies, tissue margins are reported as "clean" with margins reported in mm. However, it is vital that margins are interpreted in the context of the grade of the disease (pathologists can provide additional review of cell and nuclear size, mitotic rate, "cell bizarreness" along with lymphatic and vascular invasion, guiding a better understanding of the tumor's behavior). If margins are compromised, it will factor into post-surgery recommendations and necessary follow-up treatment. As a general rule, the histological staging of the margins must be defined as in Table 1. To achieve a histopathologically clean margin, the surgical margin must be of at least 2 to 5 cm depending on the case (Simpson et al. 2004; Fulcher et al. 2006; Phelps et al. 2011; Risselada et al. 2015; Meuten 2016).

Table 1 Histological staging of margins

M1	Infiltrate margin (focal or diffuse)
M2	Narrow margin (<2 mm)
M3	Clean margin (2–5 mm)
M4	Clean margin (>5 mm)

Fig. 1 Patient with multiple squamous cell carcinoma lesions, an adequate candidate for pinna surgery in a first step, followed by ECT covering the nose, the auriculopalpebral area and the eyelid 1 month later

With incomplete margins, whether known prior to surgery or afterward, ECT should be considered an important adjunctive treatment with surgery in several situations including:

- When extirpation with the proper margins is not possible due to compromise of vital structures, secondary organ dysfunction, or rejection by the owner due to aesthetics. In those examples, ECT may be effectively used to treat incomplete margins. In one study, 28 dogs with cutaneous mastocytoma and incomplete resection were treated with ECT obtaining a response rate of 85% with a mean estimated time to recurrence of 52.76 $\pm$ 6.5 months (Spugnini et al. 2006). In a second study with 37 dogs with incompletely excised mast cell tumors treated with ECT utilizing cisplatin a local response rate of 62% was observed (Spugnini et al. 2011). Another study compared the responses with ECT alone, or combined with surgery, concluding that ECT can be combined with surgery either intraoperatively or post-operatively for larger lesions without significant toxicity (Lowe et al. 2017). Finally, a study comparing the intraoperative or post-operative use of ECT in dogs with soft tissue sarcomas showed similar recurrence rates (23% Vs 25%) with minimal toxicity (Torrigiani et al. 2019). Meaning that ECT can improve results used either in a intraoperative or post-operative way.
- When an oncological surgery is planned with an anticipated non-resectable margin (e.g., feline squamous cell carcinoma, that compromises both pinna, eyelid and nose (see Fig. 1). ECT's role is complementary in treating the surgical sites that were unable to completely remove all tumor cells (Gehl et al. 2018).
- When the patient has multiple, or hemorrhagic, or ulcerated lesions, as in cutaneous metastasis. ECT's role in that setting is palliative, allowing potential

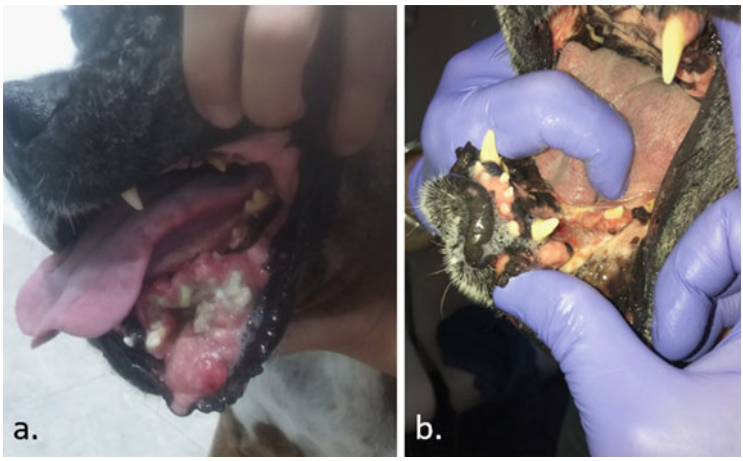

Fig. 2 Image of a patient with a squamous cell carcinoma that extensively involves the lower lip, maxilla, and mucosa. A complete response in the lip and a partial response in the mucosa were obtained after ECT. The residual lesion can be resected by hemimandibulectomy preserving the lip to be used in the reconstruction

control of the disease, improving the patient's quality of life. The application of electric pulses induces an immediate spasm of the blood vessels, attenuating bleeding, by a phenomenon called "vascular-lock" (Markelc et al. 2012; Gehl and Geertsen 2000; Jarm et al. 2010).

- When the patient has a very large lesion. ECT can be used as a neoadjuvant treatment, reducing tumor size, and thus improving results of subsequent surgery. In these particular cases, with ECT, as well as with other neoadjuvant treatments including chemotherapy or radiotherapy, the original surgical plan must be followed. We should not reduce the surgical resection because of the reduction of tumor's size. Therefore, if surgery with planned margins is designed and a neoadjuvant ECT is elected, that surgery performed at a later time on significantly smaller lesions, must be the same as the originally planned surgical approach. Therefore, neoadjuvant ECT would not reduce the resection but it is outcome would be considerably better, allowing much longer recurrence-free periods (Mozzillo et al. 2012; Cabula 2013; Gehl et al. 2018). On the contrary, in human medicine some authors suggest that neoadjuvant ECT could help reduce the size of the resection, sparing healthy tissue without compromising treatment effectiveness (Sersa and Miklavcic 2008; Mozzillo et al. 2012; Perrone et al. 2018) (Fig. 2).

It is well known that ECT provides better results with smaller tumors than in larger ones (Mali et al. 2013) and when appropriate, oncological surgery must be the first therapeutic option, as it provides better disease control but is clearly more invasive.

1.1 Excision of the Tumor and Treatment of the Site

When surgical planning, incomplete surgery may be performed anticipating that ECT will follow in the treatment of these margins. One example of this is surgery for tumor removal in the distal region of the limbs. When neoplasms are very large, complete removal may only be accomplished by amputation. Not all patients are appropriate candidates due to obesity or concurrent orthopedic disease or their owners do not approve because of the aggressiveness of this option.

In these cases, marginal excision of the mass is considered palliative management by reducing pain, ulceration, bleeding, and a decrease in tumor load, which may allow a more successful adjuvant treatment. In these cases, the treatment of the surgical site by ECT must include the lateral margin calculated from the original tumor and the deep margin, which in many cases is bone tissue (see Figs. 3 and 4) (Spugnini et al. 2006). It should be noted that bone involvement worsens the prognosis as their is the limitation of extending the electric field through bone (Tellado et al. 2020).

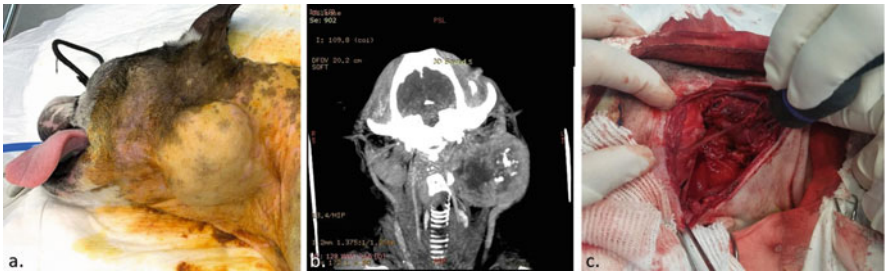

Fig. 3 Patient with a solid carcinoma in the parotid gland. (**a**) The patient the day of the ECT. (**b**) The CT-scan of the patient showing the extension of the mass. (**c**) ECT procedure during surgery

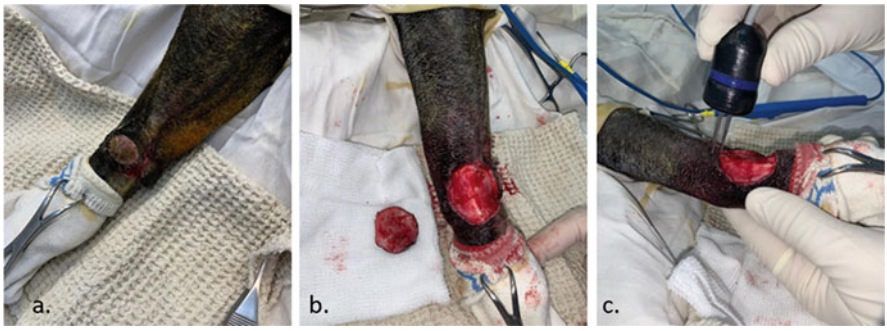

Fig. 4 Patient with a soft tissue sarcoma. (**a**) Resection of the tumor with 1 cm margin. (**b**) After excision, the margin should be expanded with ECT, adding an additional centimeter and the entire basal margin in depth until contacting the bone. (**c**) ECT procedure during surgery

External beam radiation therapy (RT) has the advantage of blanketing a wider treatment area than ECT, however, ECT does not have acute or late side effects as seen with RT (Bujko et al. 1993).

1.2 Flap Reconstruction and ECT

When planning an oncological intervention involving extensive tissue removal and a flap repairing surgery, the latter must be made out of tissue beyond the safe margin to be free of neoplastic cells.

In certain cases, where the aforementioned plan cannot be guaranteed, ECT can be performed on the flap, with the aim of eliminating residual tumor cells, without any risk of affecting tissue viability. However, if the flap was taken from within the safe margins, the complementary use of the ECT becomes mandatory. ECT may be used here due to the fact that ECT preserves the extracellular matrix, vasculature, and even the cells that are not actively replicating (Mir 2006), without affecting the flap's viability. Nevertheless, in order to preserve these advantages, the technique must be performed correctly, avoiding overtreatment, which possibly compromises the viability of healthy tissues and the flap (Gehl et al. 2006).

It is important to clarify that when performing a treatment on a flap, it should not contain macroscopic neoplastic tissue.

1.3 Scar Tissue Treatment

Treatment of surgical scars by ECT is extremely useful in several scenarios. Fundamentally, for post-surgical recurrences, or even before recurrence is noted when it is known that the tumor or its margin was not adequately resected. This is specifically recommended in post-inoculation feline sarcomas or injection site fibrosarcomas (ISFSA), when adjuvant radiotherapy is not available (Spugnini et al. 2006), or when the side effects of RT are not acceptable to the owner (Bujko et al. 1993).

In our experience, the indications for ECT in these cases are as follows:

- Surgical resection scar with compromised margins.
- When a recurrence occurs on top of the scar, and another surgery is not achievable.

In general, when complete removal is not possible, intraoperative ECT is recommended, and should not be postponed.

In cases where it cannot be performed intraoperatively, surgical scars may be treated later after healing, although this approach will naturally be less effective. In extensive surgeries that produce large scars or that require reconstructive procedures, the original anatomical relations of the tumor are lost. For that reason, the original tumoral bed may not be exactly under the scar. Thus, treating the scar may not

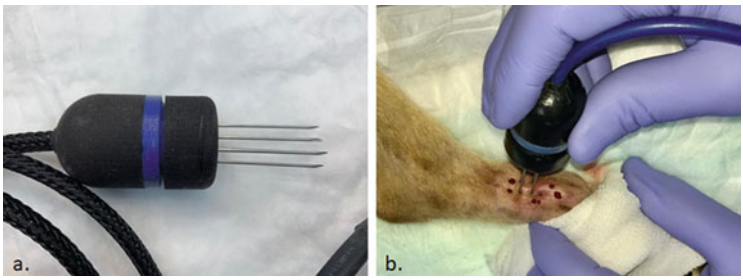

Fig. 5 (**a**) 20G, 6 needle single-use electrode for EPV-100 electroporator (BIOTEX, Buenos Aires, Argentina). (**b**) Soft tissue sarcoma resection scar in a limb, during ECT treatment. Note the blood spots corresponding to the insertions of the electrode covering the whole scar plus a safety margin

guarantee that all remaining neoplastic cells are treated. However, in small scars, the risk of treating the wrong area is reduced.

ECT can become an additional treatment combined with surgery increasing the effectiveness of surgery. ECT has become a very useful procedure for the surgical oncologist (Torrigiani et al. 2019).

From a practical standpoint, scar tissue is fibrous and therefore has a hard consistency. Based on that, it is preferred to use thick needle electrodes such as (20G, BIOTEX, Buenos Aires, Argentina) instead of thin needle electrodes such as (25G, BIOTEX, Buenos Aires, Argentina). The entire length of the scar must be treated, placing the electrode directly in it. Then, include a wide margin (1–2 cm) around the scar to ensure maximum coverage of the compromised area (see Fig. 5).

Also, given the fact that the scar tissue is poorly vascularized, the local bioavailability of the drug administered intravenously might not be optimal. Therefore, consideration for combining intralesional and intravenous bleomycin may improve response (Maglietti et al. 2016).

A surgical approach allows the use of ECT to internal organs extending applications and providing a very useful therapeutic option to the surgical oncologist. This approach will not be discussed in this chapter, as it is not always a true combination of both techniques, but sometimes is only ECT, using a different pathway to access the tumor.

2 Combination with Chemotherapy

There are only a few studies evaluating the effectiveness and disease-free survival using the combination of systemic chemotherapy and ECT. When chemotherapy is used as an adjuvant to ECT, one should consider the role of ECT as an activator of the immune system, which may be impaired by the immunosuppressive effect of systemic chemotherapy (Sersa et al. 2015). It must be carefully assessed whether the risks outweigh the benefits for this combination of neoadjuvant, concomitant, or adjuvant chemotherapy with ECT (Allegra et al. 2015). Mast cell tumors are a good

example. Neoadjuvant treatment of the primary tumor (or tumors) is very common with these neoplasms, allowing a significant reduction in tumor burden and inflammation. ECT effectiveness will be much greater due to the significant reduction in tumor burden at the time of treatment (Tozon et al. 2016).

Another combination of chemotherapy and ECT is with metronomic chemotherapy. One example is using a regular interval dosage of a COX-2 inhibitor associated with an antineoplastic agent at very low doses (Gaspar et al. 2018). This treatment inhibits tumor neo-angiogenesis and modulates the immune response by acting directly on T lymphocytes and the endothelium, decreasing the metastatic potential. This is an advantage for ECT since it requires a complete immune system to provide the best possible responses (Mir 2006).

3 Combination with Radiotherapy

Radiation therapy works directly affecting DNA of cells, or indirectly through the ionization of water, forming free radicals, in particular hydroxyl radicals, which then damage DNA. This damage is endured by all irradiated cells. On the contrary, ECT increases the permeability of electroporated cells to the chemotherapeutic agent used (bleomycin in most of the cases), but the impact of this phenomenon will be minimal in cells with a slow cell cycle, and maximal in highly replicating cells. This is because bleomycin breaks DNA strands impeding the formation of chromosomes, which induces mitotic cell death when the cells try to divide. ECT has a significant advantage; preserving healthy tissue, allowing it to treat large margins with minimum side effects.

Radiotherapy has multiple indications in oncology, providing a key role in the treatment of scars of lesions with a high rate of local recurrence, deep nasal neoplasms, oral cavity neoplasms, head and neck neoplasms, CNS neoplasms, and some radiosensitive neoplasms that have not been controlled with chemotherapy (as is the case of resistant transmissible venereal tumors) (LaRue and Custis 2014).

There are just a few studies describing the concomitant use of ECT with radiotherapy, so the indications for their use together have not been fully elucidated. Previously irradiated areas with recurrences are the most likely scenario for ECT—specifically, radio-resistant primary and metastatic lesions (Gehl et al. 2018).

Lack of availability of radiotherapy equipment is common in veterinary medicine worldwide, however, more machines are available in North America. In many cases, ECT can be used in lieu of radiation therapy as has been investigated with nasal tumors (Maglietti et al. 2017, 2020). The use of ECT was shown to be as effective as external beam radiation therapy (historical control) with equivalent overall survival.

The combination of ECT with radiotherapy, has not been reported in veterinary medicine. However, the use of radiation therapy symbiotically with ECT is another area of interest and future research. Unpublished data (MT/FM) supports that this combination can be successful in patients with nasal SCC (see Fig. 6) and perianal adenocarcinomas.

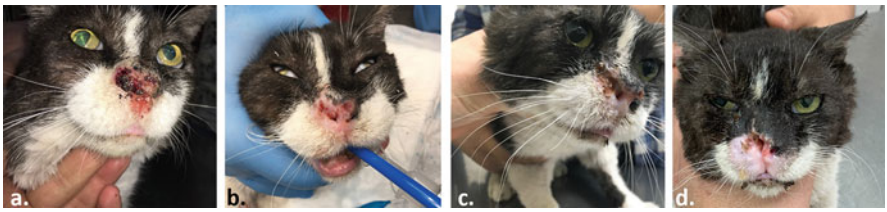

Fig. 6 (**a**) The day of the first ECT for treating feline SCC. (**b**) A relapse was observed and the second session of ECT was performed. (**c**) A partial response was obtained, and the tumor continues to grow. (**d**) After an orthovoltage treatment with 12 fractions of 3.3 Gy a complete response was obtained with an excellent quality of life

4 Combination with Cryosurgery

Cryosurgery is a local ablation technique that usually involves the application of liquid nitrogen (which reaches extremely low temperatures of approximately −196 °C) in viable tissues, with the goal of causing cell destruction (cryonecrosis) in a controlled manner (O'Donoghue et al. 2019). The main indication for combining ECT with cryosurgery is when a partial response or incipient relapse has been obtained after one ECT session, and a second session cannot be performed. Likewise, a time of at least 6 weeks in between should always be considered when performing ECT and cryosurgery, to allow the full response of the ECT treatment. Otherwise a still responding lesion will be unnecessarily treated.

We have obtained beneficial results using cryosurgery in recurrent or partially respondent lesions after ECT, in terms of tolerance, response, and outcome. The decision whether to choose to perform cryosurgery or another ECT session is based on the lesion's size and depth. Lesions larger than 5 cm at their longest axis or deeper than 5 mm, should be treated with ECT. In small residual lesions, we can use cryosurgery to achieve a complete response. It must be taken into account that cryosurgery has a lower cost than ECT, making this combination of techniques accessible to owners (see Fig. 7). It is equally effective but less expensive than a second ECT session when treating very small tumors. It is very useful for patients who for some reason cannot be anesthetized or cannot receive chemotherapy, (pregnant or lactating females, patients with uncompensated kidney disease and animals destined for human consumption) (Lana et al. 1997).

ECT is a valuable tool to be used as a stand-alone therapy or in combination. As ECT has the advantage to not damage healthy tissue, it is an outstanding option to be combined with surgery by allowing an extended safety margin and thus extending time to tumor recurrence. When combining ECT with chemotherapy, more research is needed as some patients may display a good systemic response. Radiotherapy provides excellent response rates and is especially useful to treat large areas, but it is too expensive for most clients but when a relapse is seen, ECT may be the only option as a rescue therapy that can still provide excellent results. Finally, cryotherapy can be combined with ECT, mainly for small relapses or when a second ECT session

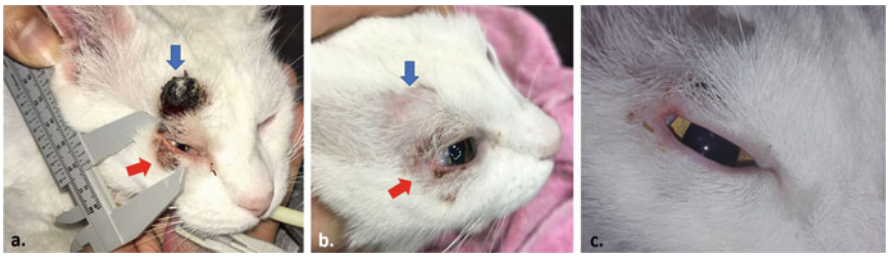

Fig. 7 (**a**) The day of the ECT for treating squamous cell carcinoma main lesion (blue arrow) and satellite lesions (red arrow). (**b**) A complete response was obtained in the main lesion (blue arrow), and a partial response in the satellite lesions (red arrow). (**c**) A complete response was obtained in the remaining satellite lesions after cryosurgery

can be avoided. In conclusion, ECT is an important therapeutic that can improve results when combined with many other oncology treatments.

References

Aiken SW (2003) Principles of surgery for the cancer patient. Clin Tech Small Anim Pract 18:75–81

Allegra E, Domanico R, Trapasso S et al (2015) Electrochemotherapy in combination with chemoradiotherapy in the treatment of oral carcinomas in advanced stages of disease: efficacy, safety, and clinical outcomes in a small number of selected cases. Drug Des Dev Therapy 9:1185–1191

Bujko K, Suit HD, Springfield DS, Convery K (1993) Wound healing after preoperative radiation for sarcoma of soft tissues. Surg Gynecol Obstet 176:124–134

Cabula C (2013) Neoadjuvant electrochemotherapy of breast cancer: our experience on first case treated in Italy. Updat Surg 65:325–328. https://doi.org/10.1007/s13304-012-0170-3

Cohen M, Bohling MW, Wright JC et al (2003) Evaluation of sensitivity and specificity of cytologic examination: 269 cases (1999-2000). J Am Vet Med Assoc 222:964–967

Eich CS, Whitehair JG, Moroff SD, Heeb LA (2000) The accuracy of intraoperative cytopathological diagnosis compared with conventional histopathological diagnosis. J Am Anim Hosp Assoc 36:16–18

Fasel JHD, DembÉ J-C, Majno PE (2007) Fascia: a pragmatic overview for surgeons. Am Surg 73:451–453

Fulcher RP, Ludwig LL, Bergman PJ et al (2006) Evaluation of a two-centimeter lateral surgical margin for excision of grade I and grade II cutaneous mast cell tumors in dogs. J Am Vet Med Assoc 228:210–215. https://doi.org/10.2460/javma.228.2.210

Gaspar TB, Henriques J, Marconato L, Queiroga FL (2018) The use of low-dose metronomic chemotherapy in dogs-insight into a modern cancer field. Vet Comp Oncol 16:2–11. https://doi.org/10.1111/vco.12309

Gehl J, Geertsen PF (2000) Efficient palliation of haemorrhaging malignant melanoma skin metastases by electrochemotherapy. Melanoma Res 10:585–589

Gehl J, Sersa G, Garbay J et al (2006) Results of the ESOPE (European Standard Operating Procedures on Electrochemotherapy) study: efficient, highly tolerable and simple palliative treatment of cutaneous and subcutaneous metastases from cancers of any histology. J Clin Oncol 24:8047–8047

Gehl J, Sersa G, Matthiessen LW et al (2018) Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol 57:874–882. https://doi.org/10.1080/0284186X.2018.1454602

Jarm T, Cemazar M, Miklavcic D, Sersa G (2010) Antivascular effects of electrochemotherapy: implications in treatment of bleeding metastases. Expert Rev Anticancer Ther 10:729–746. https://doi.org/10.1586/era.10.43

Kondo Y, Matsunaga S, Mochizuki M et al (2008) Prognosis of canine patients with nasal tumors according to modified clinical stages based on computed tomography: a retrospective study. J Vet Med Sci 70:207–212

Lana SE, Ogilvie GK, Withrow SJ, Straw RC, Rogers KS (1997) Feline cutaneous squamous cell carcinoma of the nasal planum and the pinnae: 61 cases. J Am Anim Hosp Assoc 33:329–332

LaRue SM, Custis JT (2014) Advances in veterinary radiation therapy: targeting tumors and improving patient comfort. Vet Clin North Am Small Anim Pract 44:909–923. https://doi.org/10.1016/j.cvsm.2014.05.010

Liptak JM (2020) Histologic margins and the residual tumour classification scheme: is it time to use a validated scheme in human oncology to standardise margin assessment in veterinary oncology? Vet Comp Oncol 18:25–35. https://doi.org/10.1111/vco.12555

Lowe R, Gavazza A, Impellizeri JA et al (2017) The treatment of canine mast cell tumours with electrochemotherapy with or without surgical excision. Vet Comp Oncol 15:775–784

Maglietti F, Tellado M, Olaiz N et al (2016) Combined local and systemic bleomycin administration in electrochemotherapy to reduce the number of treatment sessions. Radiol Oncol 50:58–63. https://doi.org/10.1515/raon-2016-0015

Maglietti F, Tellado M, Olaiz N et al (2017) Minimally invasive electrochemotherapy procedure for treating nasal duct tumors in dogs using a single needle electrode. Radiol Oncol 51:422–430. https://doi.org/10.1515/raon-2017-0043

Maglietti F, Tellado M, De Robertis M et al (2020) Electroporation as the immunotherapy strategy for cancer in veterinary medicine: state of the art in Latin America. Vaccines (Basel) 8. https://doi.org/10.3390/vaccines8030537

Mali B, Miklavcic D, Campana LG et al (2013) Tumor size and effectiveness of electrochemotherapy. Radiol Oncol 47

Markelc B, Bellard E, Sersa G et al (2012) In vivo molecular imaging and histological analysis of changes induced by electric pulses used for plasmid DNA electrotransfer to the skin: a study in a dorsal window chamber in mice. J Membr Biol 245:545–554

Meuten DJ (2016) Tumors in domestic animals. Wiley, Boca Raton, FL

Mir LM (2006) Bases and rationale of the electrochemotherapy. Eur J Cancer Suppl 4:38–44. https://doi.org/10.1016/j.ejcsup.2006.08.005

Mozzillo N, Caracò C, Mori S et al (2012) Use of neoadjuvant electrochemotherapy to treat a large metastatic lesion of the cheek in a patient with melanoma. J Transl Med 10:131

O'Donoghue N, Mowatt D, Sykes AJ (2019) Electrochemotherapy and ablative therapies in non-melanoma skin cancer. Clin Oncol 31(11):e1–e9

Perrone AM, Galuppi A, Borghese G et al (2018) Electrochemotherapy pre-treatment in primary squamous vulvar cancer. Our preliminary experience. J Surg Oncol 117:1813–1817. https://doi.org/10.1002/jso.25072

Phelps HA, Kuntz CA, Milner RJ et al (2011) Radical excision with five-centimeter margins for treatment of feline injection-site sarcomas: 91 cases (1998-2002). J Am Vet Med Assoc 239:97–106. https://doi.org/10.2460/javma.239.1.97

Risselada M, Mathews KG, Griffith E (2015) Surgically planned versus histologically measured lateral tumor margins for resection of cutaneous and subcutaneous mast cell tumors in dogs: 46 cases (2010–2013). J Am Vet Med Assoc 247:184–189

Sersa G, Miklavcic D (2008) Electrochemotherapy of tumours. J Vis Exp. https://doi.org/10.3791/1038

Sersa G, Teissie J, Cemazar M et al (2015) Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. Cancer Immunol Immunother 64:1315–1327

Simpson AM, Ludwig LL, Newman SJ et al (2004) Evaluation of surgical margins required for complete excision of cutaneous mast cell tumors in dogs. J Am Vet Med Assoc 224:236–240. https://doi.org/10.2460/javma.2004.224.236

Spugnini EP, Baldi A, Vincenzi B et al (2006) Intraoperative versus postoperative electrochemotherapy in high grade soft tissue sarcomas: a preliminary study in a spontaneous feline model. Cancer Chemother Pharmacol 59:375–381

Spugnini EP, Vincenzi B, Citro G et al (2011) Evaluation of cisplatin as an electrochemotherapy agent for the treatment of incompletely excised mast cell tumors in dogs. J Vet Intern Med 25:407–411. https://doi.org/10.1111/j.1939-1676.2011.0678.x

Tellado MN, Maglietti FH, Michinski SD et al (2020) Electrochemotherapy in treatment of canine oral malignant melanoma and factors influencing treatment outcome. Radiol Oncol 54:68–78. https://doi.org/10.2478/raon-2020-0014

Torrigiani F, Pierini A, Lowe R et al (2019) Soft tissue sarcoma in dogs: a treatment review and a novel approach using electrochemotherapy in a case series. Vet Comp Oncol 17:234–241. https://doi.org/10.1111/vco.12462

Tozon N, Lampreht Tratar U, Znidar K et al (2016) Operating procedures of the electrochemotherapy for treatment of tumor in dogs and cats. J Vis Exp. https://doi.org/10.3791/54760

Wallack ST, Wisner ER, Werner JA et al (2002) Accuracy of magnetic resonance imaging for estimating intramedullary osteosarcoma extent in pre-operative planning of canine limb-salvage procedures. Vet Radiol Ultrasound 43:432–441. https://doi.org/10.1111/j.1740-8261.2002.tb01030.x

Willcox JL, Spriet M, Zwingenberger AL et al (2020) Evaluation of accuracy for F-FDG positron emission tomography and computed tomography for detection of lymph node metastasis in canine oral malignant melanoma. Vet Comp Oncol. https://doi.org/10.1111/vco.12651

Part III

Gene-Electrotransfer and Immunotherapy Applications with Electroporation

Gene Electrotransfer

Loree C. Heller and Richard Heller

Abstract

Gene therapies can be used in many ways for cancer therapies. The most technologically advanced form of nonviral gene delivery is electroporation or "gene electrotransfer." This chapter reviews the basic principles of gene electrotransfer and describes the preclinical development of the first therapy to advance to human or veterinary clinical trials. Published clinical and veterinary oncology trials are described.

Keywords

Gene therapy · Gene electrotransfer · Oncology applications · Interleukin · Plasmid DNA

1 Introduction

Gene therapy has the potential to be an effective treatment for many diseases. This treatment can compensate for a missing or mutated protein, induce immune responses to infectious diseases via vaccines, or treat cancers by various mechanisms. Significant progress has been made in moving potential therapies to veterinary and clinical testing. In recent years, regulatory approval has been obtained for a few indications, initially in China but also in the European Union and the USA.

There are two major delivery approaches for successful and effective gene transfer. One approach is to use a genetically engineered biological vector, often a virus. Alternatively, nonviral plasmid DNA can be delivered by simple injection or with chemical or physical assistance. Typically, when a therapeutic approach

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requires long-term and/or high expression, viral vectors are preferable (Vannucci et al. 2013; Wirth et al. 2013; Kaufmann et al. 2013). However, if the transgene encodes a potentially toxic protein or a protein designed to modulate the immune response, the short-term and/or low expression levels of the nonviral approach may be appropriate (Ramamoorth and Narvekar 2015; Yin et al. 2014; Alsaggar and Liu 2015; Li and Huang 2006). There are other clear advantages of using a nonviral approach. Removing the need for a biological vector can improve the safety profile of the therapy by reducing the risk of insertional mutagenesis, an anti-vector immune response, or environmental spread.

Gene therapy development is often hampered by the concept that optimal therapy is one with the highest and longest transgene expression. This is not always the case. As with drugs, the dose of expressed protein should be appropriate to correct the disease. While a low dose may be ineffective, a high dose may be toxic or may set off signaling cascades that regulate the response to or activity of the protein. Transgene expression should also be tailored so the therapeutic protein is located in the appropriate tissue and cellular location.

Advances in nonviral delivery have increased both protein expression levels and reproducibility, reducing the differences between viral and nonviral gene therapies. These advances are related to improvements in delivery approaches such as lipid or polymer conjugation, particle-mediated delivery, hydrodynamic delivery, ultrasound, and electroporation (Song et al. 2011; Noble et al. 2013; Bonamassa et al. 2011; Suda and Liu 2007; Young and Dean 2015; Bodles-Brakhop et al. 2009; Heller and Heller 2006). The physical approach that has demonstrated the highest efficacy thus far is electroporation, and this delivery method has advanced the farthest in clinical trials.

2 Mechanism of Gene Electrotransfer

Electroporation or electropermeabilization (EP) introduces molecules through the cell membrane by exposing cells to controlled electric pulses (electrotransfer). The technique was first described by Neumann in 1982 (Neumann et al. 1982). Since then, EP has been used to transport a variety of molecules such as ions, drugs, dyes and tracers, and nucleic acids across the cell membrane. This method of gene transfer was initially used for simple in vitro applications but is now used for a variety of in vivo preclinical and clinical applications to deliver chemotherapeutic agents and nucleic acids such as plasmid DNA.

Gene electrotransfer (GET) has been demonstrated in a variety of tissue types, including skin, kidney, liver, testis, brain, cartilage, arteries, prostate, cornea, heart and skeletal muscle, as well as many tumor types (Heller and Heller 2015; Heller 2016). The specific characteristics of the tissue and its cells must be considered for successful in vivo electroporation. In general, cell types in vivo are not homogenous, they may not have consistent shape or size, and they may be densely packed in the tissue surrounded by other cells and extracellular matrix. These factors may influence the distribution of the electric fields and the nucleic acid injected into the tissue,

which are important considerations in determining the pulse parameter and electrode choice.

While the name of the technique implies the existence of physical pores or permeabilized areas in a cell's membrane, these structures have not been visualized. Theoretically, these areas have a diameter of 1–10 nm (Tsong 1991). Small molecules such as chemotherapeutic agents diffuse freely through the plasma membrane during or after electrotransfer (Rols and Teissie 1998). Oligonucleotides and siRNAs are approximately 6 kilodaltons in size and freely enter a cell's cytoplasm when present during electrotransfer but do not enter when added after pulse application (Paganin-Gioanni et al. 2011). The concepts behind electrotransfer and its use in small molecule delivery are overviewed in other chapters.

Plasmid DNA is a large molecule. The DNA superhelix diameter is approximately 10 nm in physiological saline (Rybenkov et al. 1997; Hammermann et al. 1998), while supercoiling and hybridization can produce a variety of topoisomers greater than 100 nm in diameter (Voordouw et al. 1978; Ledley 1996; Teissie 2013). Plasmid DNA enters the cell via an endocytosis-like mechanism (Young and Dean 2015; Wu and Yuan 2011; Rosazza et al. 2012, 2013, 2016; Mao et al. 2017; Wang et al. 2018); how pDNA escapes the endosomes to enter the nucleus for transcription is poorly understood.

Pulse variables can be used not only for delivery but to tailor the consequent protein expression levels and kinetics. These variables include pulse applied voltage, length, number, and frequency. For in vivo applications, the pulse voltage-to-distance ratio is generally $<$1500 V/cm. The individual pulse length can vary between 100 μs to 200 ms. In general, the total pulse number ($<$10) and frequency ($<$10 Hz) are low. Variations in electric pulse parameters can modulate the level of plasmid expression as well as the cell's response to the entry of the DNA itself (Heller and Coppola 2002; Heller et al. 2010, 2013; Znidar et al. 2016; Znidar et al. 2018; Bosnjak et al. 2018; Semenova et al. 2019).

3 Preclinical Use of GET

GET delivery has been demonstrated in multiple tissues, which enables its use for many therapeutic applications. A key advantage of GET is the ability to manipulate delivery parameters as well as the specific tissue target to obtain a specific protein expression pattern (Shirley et al. 2015). When developing protocols for clinical translation, increased control over the expression pattern can be instrumental in obtaining the desired clinical response. During the past decade, the increase in clinical trials utilizing GET (clinicaltrials.gov) may be directly linked to this flexibility. Naturally, the number of preclinical studies has also increased. This delivery approach has been used in single and multiple plasmid protocols as well as in combination with other agents. For example, multiple studies have evaluated the combination of electrochemotherapy (drug delivery with electrotransfer) with GET delivery of immunostimulators and other combinations (Sersa et al. 2015; Savarin et al. 2017; Milevoj et al. 2019). GET delivery strategies have included immune

stimulation, agents that induce apoptosis, inhibition of angiogenesis and toxic molecules that could reduce tumor size (Young and Dean 2015; Cemazar et al. 2009; Heller and Heller 2010). Many of the protocols tested both in preclinical and clinical applications have targeted cutaneous or subcutaneous tumors such as melanoma, squamous cell carcinoma, head and neck cancer and breast cancer. This has been predominately due to available electrodes and direct and minimally invasive accessibility to the tumors. Procedures have been developed to treat internal tumors that typically combine therapy with surgical procedures. In addition, new electrode designs utilize catheters, transcutaneous electrodes or other devices that enable treatment of internal tumors using minimally invasive tools (Soden et al. 2004, 2006; Agerholm-Larsen et al. 2011; Edhemovic et al. 2014).

3.1 Preclinical Development for Oncology Applications

Development of cancer therapies has been one of the primary focuses of GET research. Several therapeutic approaches have been tested in preclinical models. These approaches included testing the delivery of immune modulators, cell cycle regulators, suicide genes, antiangiogenic genes and genes encoding toxins (Heller and Heller 2006). Tumors are typically induced via injection subcutaneously or at the orthotopic site. Treatment is then administered either through intratumor injection to induce local expression or via the muscle for systemic dissemination of the expressed protein. Both approaches have shown the ability to induce a localized regression of a single existing tumors. Some approaches have also shown the ability to induce a local response as well as a protection from recurrence as well as an abscopal effect (Young and Dean 2015; Heller and Heller 2006).

In addition to identifying the appropriate tissue for delivery, it is also important to understand the level of expression required to obtain the desired effect (Shirley et al. 2015). Pulse parameter selection is important in controlling expression. This is particularly important when inducing an immune response. The use of GET for immunotherapy approaches for cancer has included both vaccine approaches using specific antigenic targets or the delivery of plasmids encoding cytokines to induce an upregulation of the immune response (Shirley and Heller 2016). Vaccines typically use intramuscular or intradermal routes of delivery, while the cytokine approach has been more focused on intratumor delivery. Both of these approaches can be combined with radiation therapy, electrochemotherapy, or other approaches to enhance the anti-tumor effect (Shirley and Heller 2016).

Interleukin 12 (IL-12) is a potent stimulator of both innate and adaptive immunity (Trinchieri 1995). This cytokine specifically acts through the stimulation of IFN-γ as well as the activation of T-cells and NK cells (Trinchieri 2003; Del Vecchio et al. 2007). When delivered in the form of recombinant protein, IL-12 stimulated a potent anti-tumor response in preclinical and human clinical trials (Cocco et al. 2009). However, significant toxicity was noted in many of these studies. On the positive side, an increase in the presentation of tumor antigens which lead to a specific response was observed when IL-12 was within the tumor (Cavallo et al. 1999). It

is highly likely that the toxicity observed was related to the high doses required when delivered in the form of recombinant protein. Therefore, studies were conducted to determine if a gene-based approach that controlled dosing could reduce or eliminate toxicity while preserving the anti-tumor effects.

3.2 Preclinical Studies of IL-12 GET in the B16.F10 Mouse Model

GET delivery of IL-12 for the treatment of melanoma was the first clinical trial utilizing GET. Therefore, in this section, we present the preclinical studies that led to the initiation of those trials. When developing a protocol to translate to human or veterinary clinical use, it is important to select an appropriate model to test both the efficacy and potential adverse effects of the approach. Based on these criteria, the poorly immunogenic B16.F10 C57Bl/6 mouse melanoma model was selected for therapeutic studies. To determine efficacy, it was critical to treat fully established tumors in the mouse model of at least 4–5 mm in diameter. Tumors were induced 6–7 days prior to initiating treatment by injection of 10^6 cells subcutaneously into the left flank. Various treatment regimens were tested, including a two-treatment (treating on days 0 and 7) and a three-treatment protocol (treating on days 0, 4 and 7). Treatment (day 0) consisted of an intratumor injection of a plasmid encoding IL-12 followed by GET at a field strength of 1300 V/cm, 100μs pulse width and 6 pulses (Lucas et al. 2002; Lucas and Heller 2003). Control groups included no treatment, injection of IL-12 plasmid alone, or empty plasmid (vector) injection with pulses. Tumors were measured twice weekly, and animals humanely euthanized if tumors reached 1000 mm^3 or if animals displayed discomfort.

The three-treatment protocol produced the best response. Complete regression was observed in 80% of mice, while only 60% of mice achieved complete regression when treated with the two-treatment protocol (Fig. 1). Mice were followed for 100 days, and those that remained disease-free were considered cured. These disease-free mice were then challenged subcutaneously with B16.F10 cells on the opposite flank and followed an additional 50 days. All 12 of the mice in the three-treatment group and 8 of the mice in the two-treatment group were resistant to secondary tumor growth, suggesting the development of an immune memory response.

The premise of this successful treatment was based on the induction of a robust immune response. To evaluate this concept, histological evaluation of the tumors was performed to assess the tumor microenvironment. Microscopic analysis of tumor sections showed that within 5 days following the first treatment, tumors from mice receiving IL-12 GET showed extensive infiltration of lymphocytes, macrophages and, to a lesser extent, polymorphonuclear cells. Few or no lymphocytes were observed in untreated tumors or tumors from mice that received plasmid injections without GET. Further analysis of the cellular infiltrate from the GET treated tumors revealed both $CD4^+$ and $CD8^+$ cells were present (Lucas and Heller 2003). Evaluation of tumors, peripheral blood mononuclear cells and spleens also revealed a proinflammatory response with IL-12 GET. An increase in activated

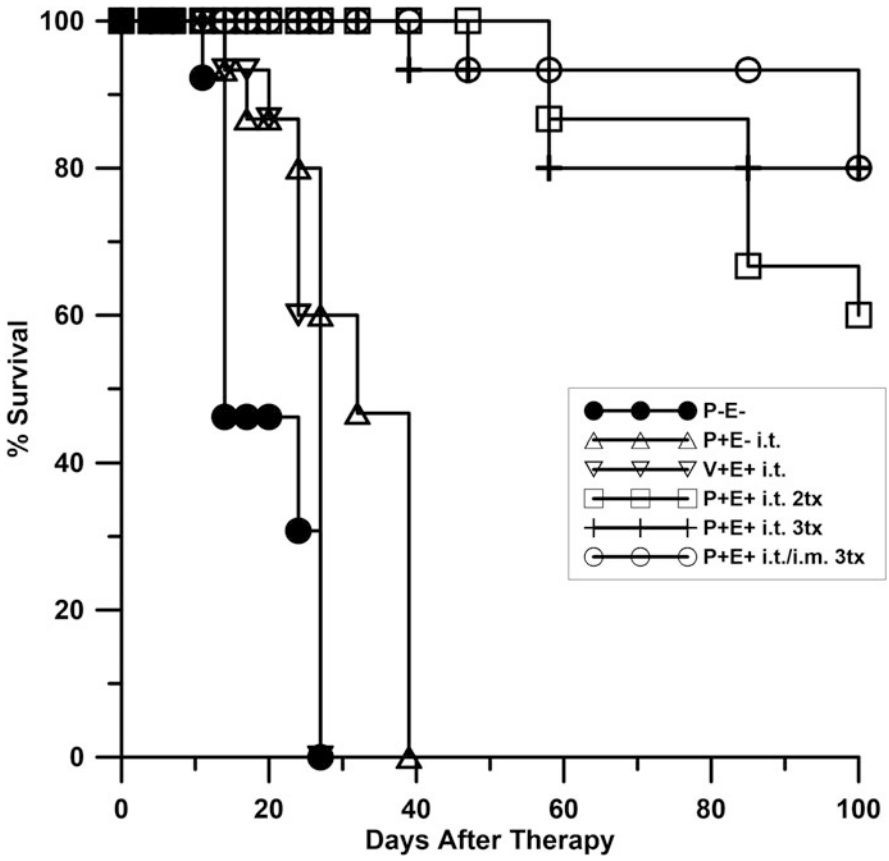

Fig. 1 B16.F10 murine melanoma survival. Percent survival of mice following treatment. P = pIRES IL-12; V = control plasmid, pND2Lux; E = electroporation. Day 0 is the day of the initial treatment. Mice were retreated on days 4 and 7. Results represent the combined data from three replicate experiments for a total number of samples per treatment group of 15. Data is expressed for surviving mice on each day. Reproduced with permission (Lucas and Heller 2003)

effector and memory cells and a decrease in T-regulatory cells and myeloid-derived suppressor cells was observed (Shi et al. 2018).

To translate a cancer immune therapy to clinical trials, it is also critical to demonstrate that the effect is not limited to inducing a response in treated tumors only. To explore this concept, a two-tumor model was utilized. Three days after mice received an injection of B16.F10 cells in the left flank, a second injection of B16.F10 cells was administered to the right flank. Only the tumor on the left flank was treated. Mice were evaluated for regression of both tumors. Only mice receiving two or three IT IL-12 GET treatments showed a significant increase ($p < 0.05$) in survival when compared to the control groups (Fig. 2) (Lucas and Heller 2003).

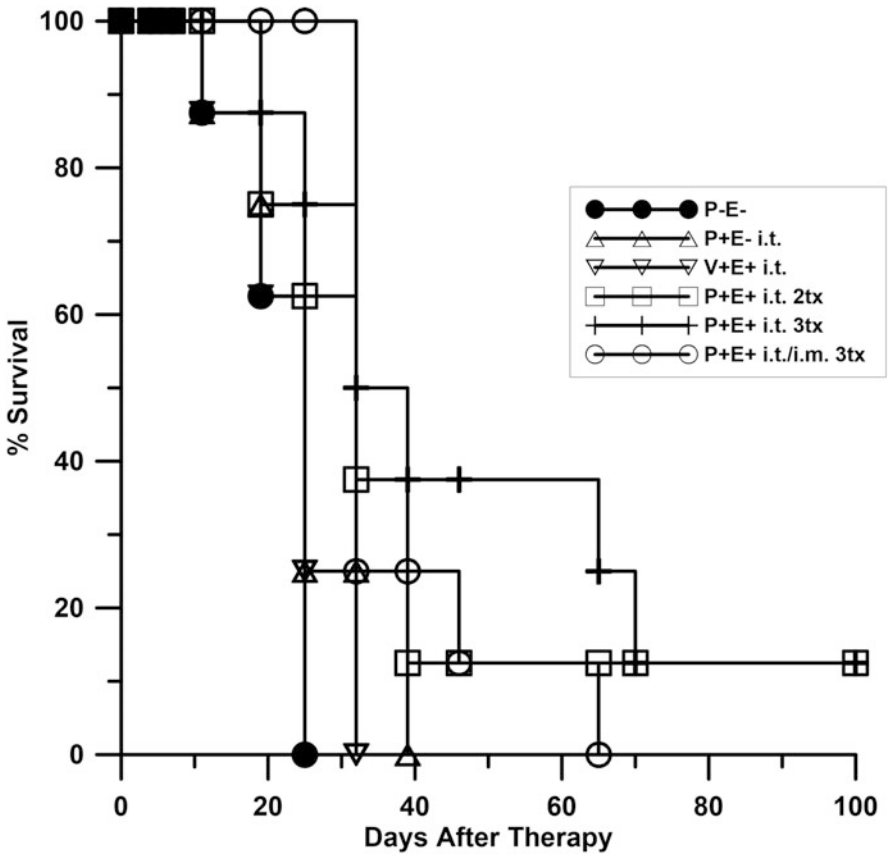

Fig. 2 Prevention of second tumor induced prior to initiation of therapy. Percent survival of mice following treatment. Two tumors were established (one on each flank); only the tumor on the left flank was treated. P = pIRES IL-12; V = control plasmid, pND2Lux; E = electrotransfer. Reproduced with permission (Lucas and Heller 2003)

To evaluate effect on distant disease, mice were injected via the tail vein with 10^5 B16-F10 cells. Immediately after the injection, mice were administered intramuscular IL-12 GET and again 4 days later. At 21 days, mice were humanely euthanized, and their lungs examined for tumor nodules. Growth of lung colonies was seen in only 37.5% (3 out of 8) of mice receiving IL-12 GET. In contrast, 87.5% (7 out of 8) untreated control mice developed lung colonies, and 75% (6 out of 8) of mice receiving an intramuscular injection of plasmid encoding IL-12 without GET or mice receiving intramuscular injection of a control plasmid with GET developed nodules (Lucas and Heller 2003).

In summary, IL-12 GET induced complete tumor regression and a specific adaptive immune response in a poorly immunogenic mouse melanoma model. At this point, this therapy was translated to Phase I clinical trials.

4 Clinical Development for Oncology Applications

The use of GET in clinical trials has increased since the initiation of the first trial (Daud et al. 2008). More than 200 clinical trials utilizing pulse electric fields are currently registered on clinicaltrials.gov, including irreversible electroporation and electrochemotherapy as well as GET, which encompasses more than half of these studies. GET also has potential in the development of effective therapies beyond cancer, including metabolic diseases, cardiovascular diseases as well as prophylactic or therapeutic vaccines for infectious diseases (Heller 2017).

The steady increase in GET clinical trials is related to the ability to develop protocols that can achieve reproducible expression, which enhances the ability to achieve the desired clinical effects with relatively low adverse effects. As described in Sect. 3, GET has been utilized successfully to deliver plasmid DNA to a variety of tissues. However, thus far, GET delivery in clinical trials has been predominate to muscle, tumors, and skin. The translation of the potential therapy to clinical testing requires demonstration of efficacy as well as safety. Typically, safety studies will determine potential toxicity by evaluating both hematological and histological data. The evaluation should assess multiple time points and include relevant doses and number of treatments as well as all relevant controls (Heller et al. 2006). Prior to submitting for regulatory approval to initiate a new clinical trial, it is also important to determine the biodistribution of the plasmid.

GET is currently utilized in several gene therapy clinical trials testing potential cancer therapies (Table 1). Various approaches are being tested. One approach is to stimulate an immune response against precancerous conditions or existing disease utilizing DNA vaccines (Heller and Heller 2015). A few trials have evaluated the delivery of antiangiogenic factors (Spanggaard et al. 2013, 2017). This concept has been tested utilizing either an intramuscular or intratumor injections. Another approach designed to stimulate a robust immune response has been to use GET to deliver plasmids encoding immunostimulating molecules such as cytokines. This has also been tested by injections directly into the tumor or via an intramuscular route. Finally, another approach is to perform the plasmid delivery ex vivo and then inject the transformed cells to achieve the desired effect. Cell types that have been utilized include dendritic cells, T-cells as well as cancer cells (Van Nuffel et al. 2012; Wilgenhof et al. 2015; Beatty et al. 2014).

4.1 IL-12 Gene Electrotransfer (GET) Clinical Trials

Intratumor IL-12 GET for the treatment of melanoma was the first-in-human clinical trial utilizing GET. This clinical trial was based on the experimental design and testing in the B16.F10 mouse melanoma model as described in Sect. 3.2 (Shirley et al. 2015; Lucas et al. 2002; Lucas and Heller 2003; Shi et al. 2018; Heller et al. 2006; Marrero et al. 2014). In the trial, a plasmid encoding IL-12 was delivered IT into metastatic melanomas using GET. This therapy, ImmunoPulse® IL-12, tavokinogene telseplasmid electroporation or "Tavo" (OncoSec Medical Inc. San

Table 1 Published in vivo gene electrotransfer oncology clinical trials

Cancer type	GET intervention	Highlights	Study	References
Metastatic melanoma	IT interleukin 12 (IL-12)	Safe and tolerable; 15% complete response; 42% disease stabilization or partial response	Daud et al. (2008)	Daud et al. (2008)
	IT IL-12	Safe and tolerable; objective response rate 35.7% in the main study with a complete response rate of 17.9%; increased immune infiltration with adaptive immune resistance	Algazi et al. (2020a)	Algazi et al. (2020a)
	IT IL-12	Systemic and intratumoral T-cell responses	Greaney et al. (2020)	Greaney et al. (2020)
	IT IL-12 plus PD-1 blockade	Safe and tolerable; 41% with 36% complete responses; increased IT immune cells	Algazi et al. (2020b)	Algazi et al. (2020b)
Prostate cancer	IT and IM prostate specific membrane antigen (PSMA)	Safe and tolerable	Low et al. (2009)	Low et al. (2009)
	IM PSMA	Safe and tolerable; PSMA specific immune response	Chudley et al. (2012)	Chudley et al. (2012)
	ID PSMA	Safe and tolerable; PSMA specific immune response	Eriksson et al. (2013)	Eriksson et al. (2013)
Human papillomavirus (HPV) associated diseases (cervical intraepithelial neoplasia)	IM HPV E6 + E7	Safe and tolerable; HPV 16/18 specific immune response	Bagarazzi et al. (2012)	Bagarazzi et al. (2012)
	IM HPV E6 + E7	Safe and tolerable; HPV 16/18 specific immune response; 78% complete response	Kim et al. (2014)	Kim et al. (2014)
	IM HPV E6 + E7	Histopathological regression in 49.5% treated patients and 30.6% placebo recipients	Trimble et al. (2015)	Trimble et al. (2015)

(continued)

Table 1 (continued)

Cancer type	GET intervention	Highlights	Study	References
HPV-associated diseases (head and neck cancer)	IM HPV E6 + E7 plus IL-12	Safe and tolerable; HPV 16/18 specific immune response	Aggarwal et al. (2019)	Aggarwal et al. (2019)
Disseminated melanoma	IT antiangiogenic metargidin peptide (AMEP)	Safe and tolerable; stable disease in treated lesions	Spanggaard et al. (2013)	Spanggaard et al. (2013)
Solid tumors	IM HER2/CEA	Safe and tolerable; no immune response to HER2 or CEA	Diaz et al. (2013)	Diaz et al. (2013)
	IM AMEP	Safe and tolerable; no objective responses	Spanggaard et al. (2017)	Spanggaard et al. (2017)
Merkel cell carcinoma	IT IL-12	Safe and tolerable; 25% overall response rate in metastatic disease	Bhatia et al. (2020)	Bhatia et al. (2020)

IT intratumoral; *IM* intramuscular; *ID* intradermal

Diego, CA), induced complete regression in the treated tumors via a tumor-targeting adaptive immune response due to expression of IL-12 locally within the tumor. In the Phase I dose escalation study, 24 patients were treated. Over 70% of the treated lesions regressed, and no systemic treatment-related adverse events were observed. This localized treatment also induced an abscopal effect. Three of the 19 patients (15%) who had additional lesions that were not treated experienced complete regression of all metastases, both treated and untreated. Disease stabilization and/or partial response were observed in 42% of treated patients (Daud et al. 2008). After this successful Phase I trial, the therapy was tested in Phase II clinical trials (Daud et al. 2008; Algazi et al. 2020a, b; Greaney et al. 2020). Four of the 29 patients enrolled achieved complete responses of all treated and untreated lesions. In addition, 14 of the 29 had an objective response in at least one untreated lesion. Interestingly, patients who did not respond to the Tavo therapy were observed to have elevated levels of PD-1 and PD-L1 in their tumors (Algazi et al. 2020a; Greaney et al. 2020).

The tumors of many patients are initially refractory to checkpoint inhibitor therapy or acquire resistance (O'Donnell et al. 2017). The results observed during the Phase II Tavo trial suggested that this therapy could be used to turn cold tumors (low or no immune cell infiltrate) into hot tumors (increased levels of immune cell infiltrate and increased levels of PD1 and PDL1), potentiating the use of checkpoint inhibitors (Algazi et al. 2020a). A phase II clinical trial using the combination of Tavo and pembrolizumab (Keytruda, Merck & Co., Inc) in immunologically quiescent melanomas was associated with a higher than expected response rate (Algazi et al. 2020b). Objective responses were achieved in 9 of 22 patients (41%), and

complete response rate (all tumors responding) was 36% (Algazi et al. 2020b). This combination therapy is being evaluated in a registration trial enrolling patients refractory to checkpoint inhibitor therapy. Finally, Tavo has demonstrated clinical benefit in other solid tumor types (Bhatia et al. 2020).

5 GET Veterinary Clinical Trials

Several GET-based interventions have been tested for naturally occurring cancers in companion dogs (Table 2) with varying success. The types of intervention include both direct immune activation, for example, via IL-12 plasmid delivery, and cancer antigen vaccines, sometimes in combination with forms of chemotherapy or

Table 2 Published in vivo gene electrotransfer veterinary oncology clinical trials

Cancer type	GET Intervention	Highlights	Study	References
Cancer-associated anemia	IM growth hormone-releasing hormone	Safe and tolerable; increased survival	Bodles-Brakhop et al. (2008)	Bodles-Brakhop et al. (2008)
B-cell lymphoma	IM telomerase reverse transcriptase plus adenovirus plus chemotherapy	Safe and tolerable; dTERT-specific immune response; increased survival	Peruzzi et al. (2010), Gavazza et al. (2013), Impellizeri et al. (2018)	Peruzzi et al. (2010), Gavazza et al. (2013), Impellizeri et al. (2018)
Solid tumors	IT interleukin 12 alone or with simultaneous combination with ECT	Safe and tolerable; sample size too low for statistical analysis	Cutrera et al. (2015a, b)	Cutrera et al. (2015a, b)
Mast cell tumors	IT interleukin 12	Safe and tolerable; local anti-tumor effect	Pavlin et al. (2011)	Pavlin et al. (2011)
	PT interleukin 12 plus ECT	Safe and tolerable; 72% complete responses, prevented recurrences or distant metastases	Cemazar et al. (2017)	Cemazar et al. (2017)
Oral malignant melanoma	IM human chondroitin sulfate proteoglycan 4	Safe and tolerable; increased disease-free survival	Riccardo et al. (2014), Piras et al. (2017)	Riccardo et al. (2014), Piras et al. (2017)
	Submucosal interleukin 12 plus ECT plus cytoreductive surgery	Safe and tolerable; 67% objective response	Milevoj et al. (2019)	Milevoj et al. (2019)

IT intratumoral; *IM* intramuscular; *ID* intradermal; *PT* peritumoral

cytoreductive surgery. In an interesting trial, cancer-associated anemia was significantly ameliorated by growth hormone-releasing hormone delivery. The clinical trials of several of these therapies are discussed in detail in other chapters.

6 Summary

Effective and safe delivery is a critical aspect of developing effective gene therapy. Preclinical and clinical data have emerged, which highlights the potential of GET as an important tool in delivering DNA. GET is useful in the delivery of plasmids to any tissue that can be accessed. The versatility of GET is a major reason why clinical testing has significantly increased during the last several years. Manipulating the electrical parameters of the pulses as well as the specific tissue target can allow for designing protocols to achieve specific expression profiles. This has the potential to result in successful clinical use. It is conceivable that this approach will continue to mature with new technological advances. Enhanced control over the administration of pulsing and enhanced targeting of the delivery will further expand the utilization of this approach.

The efficacy of cancer gene therapies utilizing electrotransfer has been demonstrated in both humans and companion animals. This form of gene therapy is safe and tolerable to both groups.

References

Agerholm-Larsen B, Iversen HK, Ibsen P, Moller JM, Mahmood F, Jensen KS et al (2011) Preclinical validation of electrochemotherapy as an effective treatment for brain tumors. Cancer Res 71(11):3753–3762

Aggarwal C, Cohen RB, Morrow MP, Kraynyak KA, Sylvester AJ, Knoblock DM et al (2019) Immunotherapy targeting HPV16/18 generates potent immune responses in HPV-associated head and neck cancer. Clin Cancer Res 25(1):110–124

Algazi A, Bhatia S, Agarwala S, Molina M, Lewis K, Faries M et al (2020a) Intratumoral delivery of tavokinogene telseplasmid yields systemic immune responses in metastatic melanoma patients. Ann Oncol 31(4):532–540

Algazi AP, Twitty CG, Tsai KK, Le M, Pierce R, Browning E et al (2020b) Phase II trial of IL-12 plasmid transfection and PD-1 blockade in immunologically quiescent melanoma. Clin Cancer Res

Alsaggar M, Liu D (2015) Physical methods for gene transfer. Adv Genet 89:1–24

Bagarazzi ML, Yan J, Morrow MP, Shen X, Parker RL, Lee JC et al (2012) Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. Sci Transl Med 4 (155):155ra38

Beatty GL, Haas AR, Maus MV, Torigian DA, Soulen MC, Plesa G et al (2014) Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies. Cancer Immunol Res 2(2):112–120

Bhatia S, Longino NV, Miller NJ, Kulikauskas R, Iyer JG, Ibrani D et al (2020) Intratumoral delivery of plasmid IL12 via electroporation leads to regression of injected and noninjected tumors in Merkel cell carcinoma. Clin Cancer Res 26(3):598–607

Bodles-Brakhop AM, Brown PA, Pope MA, Draghia-Akli R (2008) Double-blinded, placebo-controlled plasmid GHRH trial for cancer-associated anemia in dogs. Mol Ther 16(5):862–870

Bodles-Brakhop AM, Heller R, Draghia-Akli R (2009) Electroporation for the delivery of DNA-based vaccines and immunotherapeutics: current clinical developments. Mol Ther 17 (4):585–592

Bonamassa B, Hai L, Liu D (2011) Hydrodynamic gene delivery and its applications in pharmaceutical research. Pharm Res 28(4):694–701

Bosnjak M, Jesenko T, Kamensek U, Sersa G, Lavrencak J, Heller L et al (2018) Electrotransfer of different control plasmids elicits different antitumor effectiveness in B16.F10 melanoma. Cancers 10(2):37

Cavallo F, Di Carlo E, Butera M, Verrua R, Colombo MP, Musiani P et al (1999) Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin 12. Cancer Res 59(2):414–421

Cemazar M, Golzio M, Sersa G, Hojman P, Kranjc S, Mesojednik S et al (2009) Control by pulse parameters of DNA electrotransfer into solid tumors in mice. Gene Ther 16(5):635–644

Cemazar M, Ambrozic Avgustin J, Pavlin D, Sersa G, Poli A, Krhac Levacic A et al (2017) Efficacy and safety of electrochemotherapy combined with peritumoral IL-12 gene electrotransfer of canine mast cell tumours. Vet Comp Oncol 15(2):641–654

Chudley L, McCann K, Mander A, Tjelle T, Campos-Perez J, Godeseth R et al (2012) DNA fusion-gene vaccination in patients with prostate cancer induces high-frequency CD8(+) T-cell responses and increases PSA doubling time. Cancer Immunol Immunother 61(11):2161–2170

Cocco C, Pistoia V, Airoldi I (2009) New perspectives for melanoma immunotherapy: role of IL-12. Curr Mol Med 9(4):459–469

Cutrera J, King G, Jones P, Kicenuik K, Gumpel E, Xia X et al (2015a) Safety and efficacy of tumor-targeted interleukin 12 gene therapy in treated and non-treated, metastatic lesions. Curr Gene Ther 15(1):44–54

Cutrera J, King G, Jones P, Kicenuik K, Gumpel E, Xia X et al (2015b) Safe and effective treatment of spontaneous neoplasms with interleukin 12 electro-chemo-gene therapy. J Cell Mol Med 19 (3):664–675

Daud AI, DeConti RC, Andrews S, Urbas P, Riker AI, Sondak VK et al (2008) Phase I trial of Interleukin-12 plasmid electroporation in patients with metastatic melanoma. J Clin Oncol 26 (36):5896–5903

Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G et al (2007) Interleukin-12: biological properties and clinical application. Clin Cancer Res 13(16):4677–4685

Diaz CM, Chiappori A, Aurisicchio L, Bagchi A, Clark J, Dubey S et al (2013) Phase 1 studies of the safety and immunogenicity of electroporated HER2/CEA DNA vaccine followed by adenoviral boost immunization in patients with solid tumors. J Transl Med 11:62

Edhemovic I, Brecelj E, Gasljevic G, Marolt Music M, Gorjup V, Mali B et al (2014) Intraoperative electrochemotherapy of colorectal liver metastases. J Surg Oncol 110(3):320–327

Eriksson F, Totterman T, Maltais AK, Pisa P, Yachnin J (2013) DNA vaccine coding for the rhesus prostate specific antigen delivered by intradermal electroporation in patients with relapsed prostate cancer. Vaccine 31(37):3843–3848

Gavazza A, Lubas G, Fridman A, Peruzzi D, Impellizeri JA, Luberto L et al (2013) Safety and efficacy of a genetic vaccine targeting telomerase plus chemotherapy for the therapy of canine B-cell lymphoma. Hum Gene Ther 24(8):728–738

Greaney SK, Algazi AP, Tsai KK, Takamura KT, Chen L, Twitty CG et al (2020) Intratumoral plasmid IL12 electroporation therapy in patients with advanced melanoma induces systemic and intratumoral T-cell responses. Cancer Immunol Res 8(2):246–254

Hammermann M, Brun N, Klenin KV, May R, Toth K, Langowski J (1998) Salt-dependent DNA superhelix diameter studied by small angle neutron scattering measurements and Monte Carlo simulations. Biophys J 75(6):3057–3063

Heller LC (2016) Principles of electroporation for gene therapy. In: Miklavcic D (ed) Handbook of electroporation. Springer, Cham, pp 1–16

Heller R (2017) Gene electrotransfer. In: Akiyama H, Heller R (eds) Bioelectrics. Springer, Tokyo

Heller L, Coppola D (2002) Electrically mediated delivery of vector plasmid DNA elicits an antitumor effect. Gene Ther 9(19):1321–1325
Heller LC, Heller R (2006) In vivo electroporation for gene therapy. Hum Gene Ther 17 (9):890–897
Heller LC, Heller R (2010) Electroporation gene therapy preclinical and clinical trials for melanoma. Curr Gene Ther 10(4):312–317
Heller R, Heller LC (2015) Gene electrotransfer clinical trials. Adv Genet 89:235–262
Heller L, Merkler K, Westover J, Cruz Y, Coppola D, Benson K et al (2006) Evaluation of toxicity following electrically mediated interleukin-12 gene delivery in a B16 mouse melanoma model. Clin Cancer Res 12(10):3177–3183
Heller LC, Cruz YL, Ferraro B, Yang H, Heller R (2010) Plasmid injection and application of electric pulses alter endogenous mRNA and protein expression in B16.F10 mouse melanomas. Cancer Gene Ther 17(12):864–871
Heller L, Todorovic V, Cemazar M (2013) Electrotransfer of single-stranded or double-stranded DNA induces complete regression of palpable B16.F10 mouse melanomas. Cancer Gene Ther 20(12):695–700
Impellizeri JA, Gavazza A, Greissworth E, Crispo A, Montella M, Ciliberto G et al (2018) Tel-eVax: a genetic vaccine targeting telomerase for treatment of canine lymphoma. J Transl Med 16(1):349
Kaufmann KB, Buning H, Galy A, Schambach A, Grez M (2013) Gene therapy on the move. EMBO Mol Med 5(11):1642–1661
Kim TJ, Jin HT, Hur SY, Yang HG, Seo YB, Hong SR et al (2014) Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. Nat Commun 5:5317
Ledley FD (1996) Pharmaceutical approach to somatic gene therapy. Pharm Res 13(11):1595–1614
Li SD, Huang L (2006) Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene Ther 13(18):1313–1319
Low L, Mander A, McCann KJ, Dearnaley D, Tjelle TE, Mathiesen I et al (2009) DNA vaccination with electroporation induces increased antibody responses in patients with prostate cancer. Hum Gene Ther 20:1269–1278
Lucas ML, Heller R (2003) IL-12 gene therapy using an electrically mediated nonviral approach reduces metastatic growth of melanoma. DNA Cell Biol 22(12):755–763
Lucas ML, Heller L, Coppola D, Heller R (2002) IL-12 plasmid delivery by in vivo electroporation for the successful treatment of established subcutaneous B16.F10 melanoma. Mol Ther 5 (6):668–675
Mao M, Wang L, Chang CC, Rothenberg KE, Huang J, Wang Y et al (2017) Involvement of a Rac1-dependent macropinocytosis pathway in plasmid DNA delivery by electrotransfection. Mol Ther 25(3):803–815
Marrero B, Shirley S, Heller R (2014) Delivery of Interleukin-15 to B16 melanoma by electroporation leads to tumor regression and long-term survival. Technol Cancer Res Treat 13(6):551–560
Milevoj N, Tratar UL, Nemec A, Brozic A, Znidar K, Sersa G et al (2019) A combination of electrochemotherapy, gene electrotransfer of plasmid encoding canine IL-12 and cytoreductive surgery in the treatment of canine oral malignant melanoma. Res Vet Sci 122:40–49
Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J 1(7):841–845
Noble ML, Kuhr CS, Graves SS, Loeb KR, Sun SS, Keilman GW et al (2013) Ultrasound-targeted microbubble destruction-mediated gene delivery into canine livers. Mol Ther 21(9):1687–1694
O'Donnell JS, Long GV, Scolyer RA, Teng MW, Smyth MJ (2017) Resistance to PD1/PDL1 checkpoint inhibition. Cancer Treat Rev 52:71–81
Paganin-Gioanni A, Bellard E, Escoffre JM, Rols MP, Teissie J, Golzio M (2011) Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells. Proc Natl Acad Sci U S A 108(26):10443–10447

Pavlin D, Cemazar M, Cör A, Sersa G, Pogacnik A, Tozon N (2011) Electrogene therapy with interleukin-12 in canine mast cell tumors. Radiol Oncol 45:30–39

Peruzzi D, Gavazza A, Mesiti G, Lubas G, Scarselli E, Conforti A et al (2010) A vaccine targeting telomerase enhances survival of dogs affected by B-cell lymphoma. Mol Ther 18(8):1559–1567

Piras LA, Riccardo F, Iussich S, Maniscalco L, Gattino F, Martano M et al (2017) Prolongation of survival of dogs with oral malignant melanoma treated by en bloc surgical resection and adjuvant CSPG4-antigen electrovaccination. Vet Comp Oncol 15(3):996–1013

Ramamoorth M, Narvekar A (2015) Non viral vectors in gene therapy - an overview. J Clin Diagn Res 9(1):GE01–GE06

Riccardo F, Iussich S, Maniscalco L, Lorda Mayayo S, La Rosa G, Arigoni M et al (2014) CSPG4-specific immunity and survival prolongation in dogs with oral malignant melanoma immunized with human CSPG4 DNA. Clin Cancer Res 20(14):3753–3762

Rols MP, Teissie J (1998) Electropermeabilization of mammalian cells to macromolecules: control by pulse duration. Biophys J 75(3):1415–1423

Rosazza C, Phez E, Escoffre JM, Cezanne L, Zumbusch A, Rols MP (2012) Cholesterol implications in plasmid DNA electrotransfer: evidence for the involvement of endocytotic pathways. Int J Pharm 423(1):134–143

Rosazza C, Buntz A, Riess T, Woll D, Zumbusch A, Rols MP (2013) Intracellular tracking of single-plasmid DNA particles after delivery by electroporation. Mol Ther 21(12):2217–2226

Rosazza C, Deschout H, Buntz A, Braeckmans K, Rols MP, Zumbusch A (2016) Endocytosis and endosomal trafficking of DNA after gene Electrotransfer in vitro. Mol Therapy Nucl Acids 5: e286

Rybenkov VV, Vologodskii AV, Cozzarelli NR (1997) The effect of ionic conditions on the conformations of supercoiled DNA. I Sedimentation analysis. J Mol Biol 267(2):299–311

Savarin M, Kamensek U, Cemazar M, Heller R, Sersa G (2017) Electrotransfer of plasmid DNA radiosensitizes B16F10 tumors through activation of immune response. Radiol Oncol 51 (1):30–39

Semenova N, Bosnjak M, Markelc B, Znidar K, Cemazar M, Heller L (2019) Multiple cytosolic DNA sensors bind plasmid DNA after transfection. Nucleic Acids Res 47(19):10235–10246

Sersa G, Teissie J, Cemazar M, Signori E, Kamensek U, Marshall G et al (2015) Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. Cancer Immunol Immunother 64(10):1315–1327

Shi G, Edelblute C, Arpag S, Lundberg C, Heller R (2018) IL-12 gene electrotransfer triggers a change in immune response within mouse tumors. Cancers 10(12):498

Shirley SA, Heller R, Heller LC (2016) Electroporation gene therapy. In: Lattime EC, Gerson SL (eds) Gene therapy of cancer: translational approaches from preclinical studies to clinical implementation, 3rd edn. Academic Press, New York, pp 93–106

Shirley SA, Lundberg CG, Li F, Burcus N, Heller R (2015) Controlled gene delivery can enhance therapeutic outcome for cancer immune therapy for melanoma. Curr Gene Ther 15(1):32–43

Soden D, Larkin J, Collins C, Piggott J, Morrissey A, Norman A et al (2004) The development of novel flexible electrode arrays for the electrochemotherapy of solid tumour tissue. (Potential for endoscopic treatment of inaccessible cancers). Conference proceedings: Annual International Conference of the IEEE Engineering in Medicine and Biology Society 5:3547–3550

Soden DM, Larkin JO, Collins CG, Tangney M, Aarons S, Piggott J et al (2006) Successful application of targeted electrochemotherapy using novel flexible electrodes and low dose bleomycin to solid tumours. Cancer Lett 232(2):300–310

Song S, Shen Z, Chen L, Brayman AA, Miao CH (2011) Explorations of high-intensity therapeutic ultrasound and microbubble-mediated gene delivery in mouse liver. Gene Ther 18 (10):1006–1014

Spanggaard I, Snoj M, Cavalcanti A, Bouquet C, Sersa G, Robert C et al (2013) Gene electrotransfer of plasmid antiangiogenic metargidin peptide (AMEP) in disseminated melanoma: safety and efficacy results of a phase I first-in-man study. Hum Gene Ther Clin Dev 24 (3):99–107

Spanggaard I, Dahlstroem K, Laessoee L, Hansen RH, Johannesen HH, Hendel HW et al (2017) Gene therapy for patients with advanced solid tumors: a phase I study using gene electrotransfer to muscle with the integrin inhibitor plasmid AMEP. Acta Oncol 56(7):909–916

Suda T, Liu D (2007) Hydrodynamic gene delivery: its principles and applications. Mol Ther 15 (12):2063–2069

Teissie J (2013) Electrically mediated gene delivery: basic and translational concepts. Novel gene therapy approaches. IntechOpen Limited, London

Trimble CL, Morrow MP, Kraynyak KA, Shen X, Dallas M, Yan J et al (2015) Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. Lancet 386(10008):2078–2088

Trinchieri G (1995) Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 13:251–276

Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3(2):133–146

Tsong TY (1991) Electroporation of cell membranes. Biophys J 60(2):297–306

Van Nuffel AM, Benteyn D, Wilgenhof S, Pierret L, Corthals J, Heirman C et al (2012) Dendritic cells loaded with mRNA encoding full-length tumor antigens prime CD4+ and CD8+ T cells in melanoma patients. Mol Ther 20(5):1063–1074

Vannucci L, Lai M, Chiuppesi F, Ceccherini-Nelli L, Pistello M (2013) Viral vectors: a look back and ahead on gene transfer technology. New Microbiol 36(1):1–22

Voordouw G, Kam Z, Borochov N, Eisenberg H (1978) Isolation and physical studies of the intact supercoiled, the open circular and the linear forms of ColE1-plasmid DNA. Biophys Chem 8 (2):171–189

Wang L, Miller SE, Yuan F (2018) Ultrastructural analysis of vesicular transport in electrotransfection. Microsc Microanal 24(5):553–563

Wilgenhof S, Corthals J, Van Nuffel AM, Benteyn D, Heirman C, Bonehill A et al (2015) Long-term clinical outcome of melanoma patients treated with messenger RNA-electroporated dendritic cell therapy following complete resection of metastases. Cancer Immunol Immunother 64 (3):381–388

Wirth T, Parker N, Yla-Herttuala S (2013) History of gene therapy. Gene 525(2):162–169

Wu M, Yuan F (2011) Membrane binding of plasmid DNA and endocytic pathways are involved in electrotransfection of mammalian cells. PLoS One 6(6):e20923

Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG (2014) Non-viral vectors for gene-based therapy. Nat Rev Genet 15(8):541–555

Young JL, Dean DA (2015) Electroporation-mediated gene delivery. Adv Genet 89:49–88

Znidar K, Bosnjak M, Cemazar M, Heller LC (2016) Cytosolic DNA sensor upregulation accompanies DNA electrotransfer in B16.F10 melanoma cells. Mol Therapy Nucl Acids 5(6): e322

Znidar K, Bosnjak M, Semenova N, Pakhomova O, Heller L, Cemazar M (2018) Tumor cell death after electrotransfer of plasmid DNA is associated with cytosolic DNA sensor upregulation. Oncotarget 9(27):18665–18681

Immunotherapy Applications (Telomerase and HER2) with Gene Electrotransfer

Antonella Conforti, Joseph Impellizeri, and Luigi Aurisicchio

Abstract

Electroporation is a delivery technique that is gaining popularity among the veterinary community due to its low cost, ease of application, and flexibility. It combines the administration of pharmaceutical compounds such as chemotherapy agents, antisense, and plasmids to the application of permeabilizing pulses. This chapter reviews the preclinical and clinical results obtained, both for humans and companion animals, through gene-electrotransfer (GET) in cancer treatment. Recent delivery techniques such as the gene gun and in vivo electroporation (EP) have completely changed the efficiency of DNA vaccines in humans. The two central factors are most likely the increased DNA uptake due to the transient membrane destabilization and the local tissue damage is acting as an adjuvant. To date, several studies in humans have used in vivo EP to deliver DNA. Some of these results have been quite promising, with strong T-cell responses and/or therapeutic effects on cancer progression. The development of vaccines against cancer in human oncology is gaining increasing importance as a therapeutic approach, which can complement standard chemotherapy and/or targeted therapies to achieve increased survival and improved quality of life for patients. In the specialty of veterinary oncology, there is an unmet need for additional therapeutic interventions such as immunotherapy, because of the increased

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demand of owners seeking advanced options for cancer treatment for their pet, so the evaluation of genetic cancer vaccines and delivery technologies in pet dogs is gaining increasing interest both as a predictive model for human clinical trials and also as tools to provide novel therapeutic opportunities in veterinary oncology. In addition, since the role of therapeutics against naturally occurring cancers in domestic animals is an attractive prelude to human studies and drug approvals, several veterinary trials are discussed in this chapter. To this end, we expect that veterinary oncology research will follow human medicine, and many tumor types will be investigated as targets for tumor vaccination.

Keywords

Cancer vaccine · DNA · Gene-electro-transfer · TAA

1 Cancer Vaccines and GET

In recent years, several remarkable breakthroughs, such as cancer vaccines, inhibitory T-cell checkpoint molecules and ex vivo engineered tumor-specific T-cells, have driven notable success in the field of cancer immunotherapy, leading to a dramatic control of previously refractory tumors (Yang 2015). Besides this, to provide a full therapeutic armamentarium in cancer treatment. Immunotherapy can complement traditional therapies such as surgery, chemotherapy, and radiation therapy both in human and in veterinary oncology. The concept of cancer vaccines is an appealing immunotherapeutic approach, potentially translatable to the clinics, for many reasons, such as ease of administration, the possibility to target pathways involved in tumor development and a "whole" approach to treating a patient wherever cancer may develop or metastasize. Moreover, cancer vaccines are more -effective than other vaccination platforms, such as recombinant protein, tumor cells, or viral vectors, and they allow for multiple administrations while keeping safe and immunogenic (Lopes et al. 2019).

New delivery technologies have recently put acceleration to the rapidly growing sector of genetic cancer vaccines. One of these vaccination tools is Gene-electrotransfer (GET). GET is a powerful method of DNA delivery currently employed for several medical applications, such as DNA vaccination and gene therapy for cancer treatment. Indeed, due to its safety, efficacy, and ease of application, GET is the ideal candidate vaccine platform as a substitute for viral gene delivery methods. This procedure consists of exposing a cell or a tissue to an external electric field that increases cell membrane permeability to molecules that otherwise would cross the plasma membrane with low efficiency, and for this reason, it has been widely used in different biomedical applications (Kotnik et al. 2015). First, it has been employed for the delivery of anti-cancer drugs into cutaneous and subcutaneous tumor nodules and this procedure, termed electrochemotherapy, is now applied regularly in clinical practice for treatment of cutaneous tumors (Marty et al. 2006). In addition to chemotherapeutic drugs, larger molecules such as DNA

can be introduced into cells by means of electroporation; thus electro-gene-therapy is now successfully performed on many tissues in the context of medical care (Lambricht et al. 2016). Since gene therapy may be performed on different tissues, to achieve a better outcome, host tissue should be chosen carefully. Due to rapid turnover of tumor cells and to non-integration of the plasmid, a short-lived and low transgene expression may be observed when electroporating tumor cells, whereas a longer expression, in the order of a few weeks, may be expected when transferring genes to skin and, even at higher expression levels, to myocytes (Gehl 2014).

Therefore, in cancer immunotherapy, DNA vaccines may be administered by the intramuscular or intradermal injection of plasmid DNA encoding an antigen of interest. Initially, although presenting a number of advantages, such as easy and standardized production and absence of anti-vector immunity, still DNA vaccines presented some drawbacks to efficient immunization, with naked DNA injection failing to show strong vaccine immunogenicity (Trimble et al. 2009), possibly due to the combination of inefficient cellular delivery of DNA plasmids, low levels of antigen production and lack of stimulation of the innate immune system. Thus, genetic vaccination technology has been further optimized by introducing GET, which may enhance immune responses through increased protein expression, secretion of inflammatory chemokines and cytokines, and recruitment of antigen-presenting cells at the site of electroporation (Babiuk et al. 2004; Liu et al. 2008). Indeed, in vivo electroporation causes transient and reversible cell damage resulting in local inflammation and the release of cytokines acting as a danger signal, which further facilitates the induction of adaptive immune responses and innate immunity, therefore enhancing vaccine potency. As a result, antigen-specific humoral and cellular immune responses are increased by electroporation-mediated delivery of plasmid DNA in comparison with levels achieved by intramuscular injection of DNA alone (Aurisicchio and Ciliberto 2012). Furthermore, in vivo gene-electro transfer of plasmid DNA (DNA-GET) has been shown to be a safe methodology, resulting in greater DNA cell uptake, enhanced protein expression and concomitant increases in longer term immune responses against the target antigen compared to naked DNA injection in a variety of species, including large animals such as dogs, pigs, cattle, and nonhuman primates (Cappelletti et al. 2003; Capone et al. 2006; Fowler et al. 2012; Luckay et al. 2007; Reed and Li 2009). Skin is another excellent target for GET: it is the largest organ in the body and, due to the presence of antigen-presenting cells, is an extremely immunocompetent site and an excellent target for vaccinations. Another advantage of the skin is its easy accessibility and the possibility to develop devices where the electrodes are minimally invasive, do not penetrate the skin and can operate at low voltage (Ansaldi et al. 2011).

2 Genetic Cancer Vaccines and Tumor-Associated Antigens

Genetic vaccines, both viral and plasmid DNA, offer a highly versatile and powerful platform technology. There are several advantages to consider: they are able (in particular viral vectors) to induce elevated levels of proinflammatory cytokines

(IL-1, IL-6, TNF-α, IFN-γ, IL-17) at the site of injection, thus promoting homing and activation of dendritic cells; they allow for a prolonged antigen expression and consequently higher epitope display on MHC class I molecules and induction of CD8+ responses; by means of easy genetic engineering practices, it is possible to manipulate the antigen of interest to generate more immunogenic versions or fusion genes between the TAAs and epitopes known to facilitate the generation of a T-helper memory response; nucleic acid vaccines have the potential to be produced in large scale as a generic manufacturing platform. Indeed, viral vectors can elicit more powerful immune responses than plasmid DNA, although DNA vaccines present all the features required to become an optimal technology platform for therapeutic cancer vaccination, such as easy and economic GMP production, high level of stability and safety, due to the absence of viral elements. Furthermore, DNA vaccines can be administered repeatedly because they do not elicit specific anti-vector immunity, and there are not preexisting antibodies against DNA (Aurisicchio et al. 2009; Peruzzi et al. 2010a).

Genetic cancer vaccines can elicit immune responses against a wide variety of tumor antigens, mainly tumor-specific antigens (TSA), found only on cancer cells but not on healthy cells, and tumor-associated antigens (TAA), which have elevated levels on tumor cells but are also expressed at lower levels on healthy cells (Buonaguro et al. 2010). After delivering in vivo an expression cassette carrying (in part or entirely, and usually controlled by a strong constitutive promoter) the coding region of one or more antigens of choice in the subject to be vaccinated, the genetic material is uptaken by resident cells (e.g., muscle cells, fibroblasts and dendritic cells) and this results in the endogenous production of the selected antigen(s). It is well known that the extent of gene expression is proportional to the amount of transduced cells and of intact plasmid copies entering the nucleus (Wolff and Budker 2005). Once produced and secreted by transduced cells (usually myocytes or dermal fibroblasts, depending on the injection route), the antigen is endocytosed by host professional antigen-presenting cells (APCs), processed, and cross-presented to the immune system in the draining lymph nodes (Ulmer and Otten 2000). The inoculation of a plasmid DNA coding for a protein antigen by means of a simple intradermal or intramuscular injection is the simplest and cheapest gene-based in vivo delivery approach (Liu 2011).

Strong evidences show that vaccination schedules comprising more than one delivery method against the same antigen(s) achieve a higher degree of immune responses and protection from disease (Ranasinghe and Ramshaw 2009). Heterologous regimens focus CMI response on the insert and away from the vector. This concept is being applied with increasing attention to the development of prophylactic vaccines against infectious diseases. The sequential administration of plasmid DNA and adenoviral (Ad) vector in different combinations results in synergistic immune activation and a higher degree of protection from tumor development. Both in preclinical murine and primate models, this heterologous prime-boost regimen induces 10- to 100-fold higher frequencies of T-cells than do naked DNA or recombinant viral vectors alone (Aurisicchio et al. 2007; Facciabene et al. 2006; Mennuni et al. 2008). A further advantage of heterologous prime-boost protocols

comprising the sequential use of Ad and plasmid DNA is that one can exploit the strong immunogenicity of Ad as the best priming agent to break tolerance, whereas DNA can be used for repeated boosting because of the lack of anamnestic responses against the vector.

Tumor-associated antigens can cause highly specific T-lymphocyte responses against cancer cells while sparing normal cells. These observations form the rationale behind the use of telomerase and Her2 as potential antigens for cancer immunotherapy. HER2 and neu are the human and rodent homologs of an oncogenic growth factor receptor that were identified and named independently in the early 1980s from rodent and human models, but soon found to be homologs of each other. HER-2/neu oncoprotein is a tyrosine kinase receptor overexpressed in several human and animal tumors and associated with poor prognosis (Moasser 2007). Considering this, HER-2/neu has become an optimal target for therapeutic intervention. Indeed, Trastuzumab (Herceptin), a humanized monoclonal antibody directed against HER-2, and pertuzumab (Omnitarg), a novel antibody acting as HER dimerization inhibitor, are currently used for the treatment of HER-2-expressing breast cancer in humans (Cesca et al. 2020).

Telomerase reverse transcriptase (TERT), a ribonucleoprotein comprising an RNA component and a catalytic protein component, is an attractive target for cancer immunotherapy given its broad expression in human tumors and its demonstrated immunogenicity (Meyerson et al. 1997; Nakamura et al. 1997). As telomerase confers immortality to cells, telomerase activity has been detected in cancerous cell lines and in a diverse range of tumor types (Kim et al. 1994). Conversely, telomerase is inactive or only transiently expressed at low levels in normal human tissues and normal somatic cell cultures. Thus, the combination of telomerase overexpression in most tumor types and low or absent expression in normal cells makes TERT a tumor-associated antigen and a suitable target for cancer immunotherapy. In these conditions, TERT is processed and presented in the context of class I major histocompatibility complex molecules, and tumors are recognized by T lymphocytes specific against telomerase (Vonderheide 2007; Chen et al. 2007). These findings have justified vaccination trials in cancer patients based either on autologous dendritic cells transfected or loaded with human TERT-derived peptides (Su et al. 2005; Domchek et al. 2007).

3 Preclinical Studies

Many preclinical studies have demonstrated the robust immunological and therapeutic effect exerted by DNA electroporation (DNA-EP) in small and large animal models. Moreover, combinations of heterologous modalities of immunization, comprising both DNA and Ad administrations, have proven to induce superior immune reactions as compared to single modality vaccines.

In a preclinical study performed in BALB/NeuT mice (a murine model that develops spontaneously mammary tumors driven by HER-2/neu overexpression) Aurisicchio et al. showed that the combination of a genetic vaccine targeting HER-2/

neu with a TLR9 agonist has a significant therapeutic effect in leading to tumor regression/stabilization (Aurisicchio et al. 2009). Moreover, this effect was associated with immunologic parameters involving innate and adaptive immunity. After being vaccinated at 13 weeks of age in the presence of relatively small mammary hyperplasia and in situ carcinomas, only the group of mice that received the DNA-EP vaccine in combination with a TLR9 agonist produced a marked and long-lasting antitumor response, disease stabilization, and tumor shrinkage. Immunohistochemistry analysis showed normal mammary glands or hyperplasia in treated mice and no detectable lung metastases. Importantly, these mice received the last treatment at week 43 and were subsequently disease-free for their lifespan (>70 weeks), suggesting the induction of durable immune memory. In DNA-EP treated mice, the antitumoral effect correlated with the induction of Th1-type cytokine secretion and the activation of NK cells, the increase in adaptive T-cell responses and a significant increase in antigen-specific antibody responses with IgG2a isotype switch, the induction of ADCC activity against antibody-coated tumor cells and the development of high-titer antibodies against the HER-2/neu dimerization domain, which may play a relevant role in the inhibition of signaling and tumor growth.

A genetic vaccine targeting murine TERT (mTERT) based on DNA electroporation (DNA-EP) and adenovirus serotype 6 (Ad6) have elicited significant immune responses against mouse TERT (Mennuni et al. 2008) and therapeutic effects in two different murine models of spontaneous carcinogenesis (Conforti et al. 2010). Besides these encouraging results in mouse models, a DNA-EP and Ad6 vaccine targeting human TERT (hTERT) was evaluated for innate humoral and cell-mediated immune responses also in rhesus monkeys (Dharmapuri et al. 2009), given that human and rhesus TERT protein sequence share 96% identity and tolerance is expected to play a major role in determining vaccination efficacy also in this animal model. The first two DNA-EP injections appeared to be sufficient to break immune tolerance in immunized animals, and the subsequent two Ad6 injections dramatically increased the T-cell response, further demonstrating the efficacy of the DNA-EP/Ad heterologous vaccination. The T-cell reactivity fell by 30% a week later but remarkably held steady when measured after 4 months, and one single DNA-EP injection restored the T-cell reactivity to almost that obtained a week after the second Ad6 injection. This observation suggests that a vaccination regimen with multiple DNA-EP injections at appropriate intervals can maintain an optimal level of antigen-specific T-cell activity. This was further supported by the data from T-cell memory analysis. Based on a flow cytometric study used for the detection of degranulating cells in humans that was adapted to rhesus macaques, memory T-cell populations were identified. The increase in effector memory (EM) and terminally differentiated effector memory (TDEM) cells strongly suggested that the DNA-EP/Ad6 vaccination regimen can stimulate durable immune responses. These data indicated that DNA-EP/Ad6 could break immune tolerance to TERT and achieve a durable immune response in nonhuman primates (NHPs), thus paving the way for clinical applications of DNA-EP also in humans.

4 DNA-GET in Human Clinical Trials

Since the first phase I clinical trial of gene electrotransfer, conducted in patients with metastatic melanoma (Daud et al. 2008; Muramatsu et al. 1997), several clinical studies for DNA vaccination in cancer patients have been performed or are currently ongoing (see Table 1). DNA electrotransfer in vivo is in many cases more efficient than other nonviral methods of gene delivery, such as gene gun in the liver (Muramatsu et al. 1997), liposomes in the brain or the cornea (Tsujie et al. 2001; Blair-Parks et al. 2002), sonoporation in the muscle (Sheyn et al. 2008), or cationic lipids in the synovial tissue (Ohashi et al. 2002). Moreover, since gene expression can be transient, it is possible to repeat the electrotransfer procedure to reach same levels of transfection as following the first treatment (Hoover and Magne 2000). Electrotransfer of multiple genes in parallel is also feasible, and by adapting the procedure to the target tissue, electrotransfer has been successfully applied to various tissues, including skeletal muscle, skin, tumors, liver, lungs, kidneys, brain, retina, cornea, and heart with minimal tissue damage (Trezise 2002). Although it effectively delivers plasmids to a wide variety of tissue in preclinical animal studies, translation of GET applications has thus far focused on muscle, skin, and directly to tumors. The skeletal muscle is the most widely used tissue for GET in clinics (Prud'homme et al. 2006) because of its dimensions, accessibility, and its organization in long parallel fibers, which offers an optimal orientation relative to the direction of the electric field, promoting maximum delivery across the entire length of the fibers. Since skeletal muscle cells do not divide, gene expression following electrotransfer is stable for a long period. Most importantly, skeletal muscle produces biologically active proteins and releases them into the bloodstream, and consequently, muscle can be used as a protein delivery system for distant targets. The skin is the second most broadly used tissue for GET (Gothelf and Gehl 2010). It is accessible for treatment over large areas, and some epidermal cells (keratinocytes) can also produce and release proteins into the bloodstream. Indeed, the first human clinical trial, based on GET, was for the treatment of skin cancer (Heller and Heller 2010). However, it must be taken into account that therapeutic applications of GET concern not only cancers but also cardiovascular diseases (Adachi et al. 2002), autoimmune diseases (Bloquel et al. 2004), monogenic diseases (Gollins et al. 2003), organ-specific disorders (Tanaka et al. 2002) and vaccination (Bakker et al. 2004; Perez et al. 2004; Vasan 2014). Comparison between DNA injection alone and DNA-EP has demonstrated an increase in both cellular and humoral response after electric fields were applied. It has been demonstrated that the addition of electroporation provides a 10–100 fold augmentation of immune response and defense against pathogens in humans and numerous animal models of diseases such as HIV/SIV, malaria, hepatitis B and C, human papilloma virus (HPV), anthrax and influenza (Heller and Heller 2015). An interesting human clinical trial, which has collected promising results, concerned the treatment of HPV16/18 delivering VGX-3100 vaccine using electroporation (Bagarazzi et al. 2012). In this study, a significant increase of antibody titer and of specific T-cells were achieved for both HPV16 and

Table 1 Some human clinical trials currently ongoing about DNA-EP in cancer treatment (last updated on June 2020 on clinicaltrials.gov)

Trial	Status	Conditions	Interventions	Location
INO-3107 with electroporation (EP) in participants with HPV-6- and/or HPV-11-associated recurrent respiratory Papillomatosis (RRP)	Not yet recruiting	Respiratory papillomatosis	Drug: INO-3107 Device: CELLECTRA™ 2000	USA
HPV DNA vaccine via electroporation for HPV16 positive Cervical neoplasia	Not yet recruiting	– Human papillomavirus type 16 – Cervical intraepithelial neoplasia grade II – Cervical intraepithelial neoplasia, grade III	Drug: pNGVL4aCRTE6E7L2	USA
INO-5401 + INO-9012 in combination with Atezolizumab in locally advanced Unresectable or metastatic/recurrent Urothelial carcinoma	Active, not recruiting	Urothelial carcinoma	Biological: INO-5401 Biological: INO-9012 Drug: Atezolizumab Device: CELLECTRA™ 2000	USA
INO-5401 and INO-9012 delivered by electroporation (EP) in combination with Cemiplimab (REGN2810) in newly-diagnosed glioblastoma (GBM)	Active, not recruiting	Glioblastoma	– Biological: INO-5401 – Biological: INO-9012 – Biological: Cemiplimab – Radiation: Radiation therapy – Drug: Temozolomide	USA
DNA plasmid encoding Interleukin-12/HPV DNA plasmids Therapeutic vaccine INO-3112 and Durvalumab in treating patients with recurrent or metastatic human papillomavirus associated cancers	Recruiting	– Human Papillomavirus-16 positive – Human Papillomavirus-18 positive – Metastatic malignant neoplasm – Recurrent Anal Canal carcinoma – Recurrent cervical carcinoma – Recurrent malignant neoplasm – Recurrent penile carcinoma – Recurrent vaginal carcinoma – Recurrent vulvar carcinoma – Refractory malignant neoplasm	– Biological: DNA plasmid encoding IL-12/HPV DNA plasmids therapeutic vaccine MEDI0457 – Biological: Durvalumab	USA

hTERT immunotherapy alone or in combination with IL-12 DNA followed by electroporation in adults with solid tumors at high risk of relapse	Completed	– Breast cancer – Lung cancer – Pancreatic cancer – Head and neck cancer – Ovarian cancer – ColoRectal Cancer – Gastric cancer – Esophageal cancer – Hepato Cellular carcinoma	– Biological: INO-1400 – Biological: INO-9012 – Biological: INO-1401	USA
INVAC-1 anti-Cancer hTERT DNA immunotherapy	Completed	Solid tumors	Biological: INVAC-1	France
Safety of GX-188E DNA therapeutic vaccine administered by electroporation to cervical intraepithelial neoplasia grade 3	Completed	Cervical intraepithelial neoplasia 3	Genetic: GX-188E administered by electroporation	South Korea
Safety study of DNA vaccine delivered by intradermal Electroporation to treat Colorectal Cancer	Unknown	Colorectal Cancer	– Biological: tetwtCEA DNA (wt CEA with tetanus toxoid Th epitope) – Device: Derma vax (electroporation device) – Biological: GM-CSF – Drug: Cyclophosphamide	Sweden
Phase I trial of Intratumoral pIL-12 electroporation in malignant melanoma	Completed	Malignant melanoma	– Biological: IL-12p DNA – Procedure: Intratumoral electroporation	USA
A study of V934/V935 vaccine in Cancer participants with selected solid tumors (V934-002)	Completed	– Non-small cell lung carcinoma – Breast cancer – Melanoma – Upper GI tract carcinoma – Colon carcinoma – Renal cell carcinoma – Bladder carcinoma – Prostate Cancer	Biological: V935 Biological: V934-EP	USA

HPV 18, persisting over several months. The results confirmed that DNA-GET is safe and able to significantly elicit the immune response.

As mentioned above, the first clinical trial using GET was performed in patients with metastatic melanoma, and the protocol was based on extensive preclinical studies that demonstrated both a therapeutic response and a resistance to new tumor growth following reinjection of cells (Daud et al. 2008). The major objective of this Phase I trial was safety and tolerability, following electroporation of a plasmid encoding human interleukin-12 (IL-12). Patients had metastatic melanoma with the accessible cutaneous disease that was surgically unresectable, and they were enrolled with multiple cutaneous or subcutaneous lesions, with the treatment being administered directly into two to four of these sites (Daud et al. 2014). In another phase 1 study, designed to evaluate the safety and immunogenicity of a DNA vaccine in melanoma patients at risk for disease progression or recurrence, patients received intramuscular DNA-EP of a plasmid encoding tyrosinase (Heller and Heller 2015).

Another clinical study was designed to co-target two well-known TAAs, namely HER2 and CEA (carcinoembryonic antigen), through the coinjection of two plasmids carrying expression cassettes for codon usage optimized versions of the respective cDNAs. The vaccine had been previously tested in mice double transgenic for both human HER2 and human CEA, where it was able to break tolerance and to induce strong antibody and CMI (Aurisicchio et al. 2014). The vaccine, called V930, was administered in a dose-escalation protocol to a total of 28 patients in 5 biweekly injections by muscle electroporation using a Medpulser TM DDS device (Inovio Pharmaceuticals). The clinical trials were only designed to assess safety, tolerability, and immunogenicity. The vaccine was, indeed, safe and well tolerated with no major adverse events. However, immunogenicity data were not encouraging: none of the 28 patients vaccinated with V930 developed a detectable immune response against CEA and HER2, probably because of the co-delivery of two plasmids bearing expression cassettes for the two antigens may have interfered with each other. Indeed, preclinical studies had already shown a lower level of immune responses in mice co-injected with the two plasmids versus mice injected with a single plasmid; thus it is reasonable that scale up from mice to human muscles might have magnified this phenomenon (Aurisicchio et al. 2013).

A therapeutic cancer vaccine brought into Phase I/II, which utilized the above mentioned Inovio technology, was V934/V935, directed against the protein component of human TERT and based on a heterologous prime-boost administration of Ad vector and plasmid DNA by GET in 37 patients with selected solid tumors. Results of this trial demonstrated the safety and feasibility of this vaccination strategy (Aurisicchio et al. 2020). Another Phase I study, based on a vaccine targeting human TERT and administered by intradermal route followed by electroporation or by Tropis (a needle-free injection system), proved to be safe and immunogenic in patients with relapsed or refractory solid tumors (Teixeira et al. 2020). In conclusion, albeit requiring complete validation in Phase II randomized efficacy studies, GET-based DNA vaccines demonstrate to be promising vaccination platforms in cancer immunotherapy treatments.

5 DNA-GET in Veterinary Clinical Trials

The expansion of immunotherapy as the fourth arm of veterinary therapeutics is an exciting approach. The most appropriate endpoints in veterinary oncology are overall survival (OS), but also a better quality of life. As observed in human clinical trials, the "build up" of an immune response leading to disease stabilization and improved survival requires evaluation of cell-mediated and antibody responses, since target therapies are not expected to shrink tumors, but inhibit metastasis and have an impact on the quality of life and survival. Local application of electroporation, in combination with certain chemotherapeutic agents (ECT, electro-chemotherapy), is an effective tool for the control of some types of primary and metastatic disease. The treatment can be provided with curative intent or as an adjuvant treatment to surgery. Although no prospective comparative studies have been documented, the low toxicity and minimal damage to surrounding healthy tissues due to the non-thermal nature of the treatment is a significant advantage, along with the efficient delivery and reasonable cost of treatment. However, it must be taken into account that the delivery of electroporation to veterinary patients requires sedation and/or general anesthesia, which removes the simplicity and speed of treatment. The electrodes available are largely limited to cutaneous tumors, with improved devices for intraluminal and laparoscopic approaches under development. The next generation of electroporation generators promises to overcome some of these issues through the employment of more complex pulse waveforms (Impellizeri et al. 2016). Several devices are available for in vivo DNA-GET. Nowadays, the most advanced technologies are those being developed by Inovio Pharmaceuticals (USA), Ichor Medical Systems (USA) and IGEA (Italy). Nevertheless, compared with transdermal devices, DNA-GET technology suffers two main limitations: (1) it is available only in specialized hospitals and veterinary oncology centers and (2) it requires general sedation of the treated animals. The two different delivery routes for DNA vaccines have been recently compared in laboratory beagles inoculated with transmissible venereal tumors. Using a xenogeneic chicken HSP70 DNA vaccine. The needle-free transdermal route was less potent both in evoking an immune response and a clinical response than the electroporation-based DNA delivery method (Yu et al. 2011).

The translational relevance of cancer immunotherapy is strictly dependent on the preclinical models employed (mostly rodents, to date) and suffers from the lack of a suitable therapeutic large animal model. As for humans, companion animals naturally develop tumors in a chronologically relevant time and in an immunocompetent environment and for these reasons, tumors arising in companion dogs are becoming an increasingly recognized tool with which to study the therapeutic potential of anticancer treatments. This is particularly true for some types of tumors, for which physiological, anatomical, biological, and clinical features are shared by canine and human diseases as well. Examples include non-Hodgkin lymphoma (NHL), osteosarcoma, melanoma, prostate carcinoma, lung carcinoma, head and neck carcinoma, mammary carcinoma, and soft tissue sarcoma. Moreover, also in pets, cancer is characterized by interindividual and intratumoral heterogeneity, development of

recurrent or resistant disease and metastasis to relevant distant sites. Thanks to their large population size, the cancer rate in pets is sufficient to power clinical trials, including the assessment of new drugs, including cancer vaccines. To this end, a large number of cancers in dogs and cats seem to be remarkably stronger models for counterpart human tumors than currently available murine models. Moreover, as several cancer-associated genetic alterations that influence cancer progression in humans have also been identified in canine cancer (Cutrera et al. 2008), testing new targeted therapies for cancer treatment holds great translational value for proof-of-concept and proof-of-target efficacy. On this basis, in recent years, several groups have performed veterinary studies to test their innovative strategies in a high translational setting against a wide range of comparative tumors, such as lymphomas, melanoma, osteosarcomas, and many others (a list of current and completed veterinary clinical trials is available at ebusiness.avma.org; see Table 2).

Canine mammary tumors (CMT) share many characteristics with human breast cancer, including histological appearance, biological behavior, hormone dependence, frequent oncogene Her-2/neu activation and response to conventional treatments (Abdelmegeed and Mohammed 2018). Similarly, feline mammary

Table 2 Some canine clinical trials currently ongoing in USA about DNA-EP in cancer treatment (last updated on June 2020 on ebusiness.avma.org)

Trial	Status	Conditions	Interventions	Location
Combination of electrochemotherapy and gene therapy with canine IL-12	Recruiting	Skin/oral tumors	Biological: DNA plasmid encoding IL-12	VOS (NY, USA)
Evaluating a targeted her2/neu cancer vaccine for the stimulation of antitumor immunity and prolonging survival times in dogs with transitional cell carcinoma (bladder or prostate cancer).	Recruiting	Transitional cell carcinoma	Device: Vet-eporator	GVS (NY, USA)
Evaluating a targeted her2/neu cancer vaccine for the stimulation of antitumor immunity and prolonging survival times in dogs with osteosarcoma (bone cancer).	Recruiting	Osteosarcoma	Device:Vet-eporator	GVS (NY, USA)
Evaluating a targeted telomerase vaccine to stimulate antitumor immunity and prolong survival times in dogs and cats with various cancers.	Recruiting	Lymphoma	– Device:Vet-eporator	GVS (NY, USA)
Preclinical evaluation of intratumoral plasmid IL-12 + electroporation in dogs with spontaneous soft tissue sarcoma	Completed	Soft tissue sarcoma	– Biological: DNA plasmid encoding IL-12	University of California (USA)

tumors (FMT) show protein and gene expression profiles that are comparable to human cancers (Adega et al. 2016). No standard chemotherapy protocol is known to be effective by itself, and standard therapies include surgical extirpation of the gland (dog) versus radical bilateral mastectomies (cat) followed by chemotherapy. Mammary tumors are associated with a high risk of metastatic disease, especially in cats, and several studies indicate that Her-2/neu expression is similar in human breast carcinoma (Soares et al. 2013). For all these reasons, CMT and FMT are ideal preclinical models with which to evaluate Her-2/neu immunotherapy. A genetic vaccine based on a combination of DNA-EP and adenovirus targeting Her2/neu has been shown to be efficacious in inducing safe, and log-lasting immune responses in healthy dogs (Peruzzi et al. 2010a) and clinical trials testing antitumoral efficacy of anti-Her2/neu DNA-EP vaccination in dogs and cats affected by Her2/neu-expressing tumors are currently ongoing. Recent evaluation of canine transitional cell carcinomas by 2 of the authors (JI and LA) supported overexpression of Her2/neu with immunohistochemistry, questioning a role for immunotherapy as an additional target for these difficult tumors (Millanta et al. 2018).

An important breakthrough in the field of tumor vaccination and in the treatment of canine melanoma was achieved with a DNA vaccine encoding the human tyrosinase (TYR) gene, called Oncept (Merial). Currently, this is the only veterinary therapeutic tumor vaccine licensed by the United States Department of Agriculture (USDA) for the treatment of oral melanoma, following a clinical study that had demonstrated prolonged survival compared to historical control dogs (Bergman et al. 2006). The vaccine targets the human tyrosinase protein, a molecule that is very similar though not identical to the canine tyrosinase protein. The plasmid encoding the xenogenic TYR is administered by intradermal immunization using a needle-free injection system in dogs with stage II and III melanoma. In fact, needle-free injection systems, such as Bioinjector 2000 (iHealthNet, USA) and Vitajet (Bioject, USA), which use high pressure to force the dissolved DNA vaccine into the dermis or muscle, resulting in a far better immune response than the traditional intramuscular or intradermal route of administration (Impellizeri et al. 2014). An antibody response against human TYR was present in 3/9 tested dogs, two of which were also positive for antibodies against canine TYR (Liao et al. 2006), and a correlation between antibody response and clinical response was observed. Recently, the efficacy of Oncept has been questioned, and therefore further prospective studies are necessary (Ottnod et al. 2013).

Malignant lymphosarcoma (LSA) is the most common hematopoietic malignancy in dogs, caused by clonal proliferation of lymphocytes in solid tissue with distinctive morphologic and immunophenotypic features. In a clinical study evaluating the antitumoral effect of a genetic vaccine targeting dog TERT and based on adenovirus (Ad) and DNA gene-electro-transfer (DNA-GET), a strong immune response specific for dog TERT was observed in dogs affected by B-cell LSA. Most importantly, a standard chemotherapy regimen did not interfere with the effects of the immunotherapy, and survival of LSA dogs was significantly augmented in comparison to historical controls of chemotherapy-treated subjects (Peruzzi et al. 2010b). This observation was further extended in a double-arm trial of canine patients affected

by B-cell LSA, where the safety/toxicology profile and the impact on overall survival of this combined vaccine–chemotherapy treatment was evaluated (Gavazza et al. 2013). In this study, the primary end point of the study was to assess the efficacy of dTERT vaccination in combination with COP therapy on the progression and evolution of canine LSA and overall survival. Results from double-arm study showed that the dTERT vaccine was safe and had a significant impact on LSA canine patients' survival when combined with COP chemotherapy, thus supporting the evaluation of Ad6/DNA-EP-based cancer vaccine in a phase III canine trial as well as in human lymphoma patients. In a double-arm study evaluating the antitumoral effect of the same vaccination strategy, targeting dog TERT, in combination with CHOP therapy, this genetic vaccine proved to be safe, immunogenic, and efficient in achieve a prolonged overall survival in dogs affected by multicentric Diffuse Large B-cell Lymphoma (DLBCL) in comparison to dogs treated only with COP therapy (Impellizeri et al. 2018).

Other tumor antigens have been utilized in recent canine tumor vaccine studies. A vaccine prepared from heat-shock proteins (HSPs) obtained from allogeneic tumor cells has been evaluated in dogs and found to be safe and associated with a significant increased survival times and times to progression, compared to a placebo vaccinated population (Marconato et al. 2014). A new DNA vaccine expressing the chondroitin sulfate proteoglycan 4 (CSPG4) has been proposed for the treatment of dogs with oral malignant melanoma. CSPG4 is an early cell surface progression marker involved in tumor cell proliferation, migration and invasion, and expressed in ~80% of human melanomas and ~ 60% of canine melanomas. The vaccine is a DNA plasmid encoding the human CSPG4 sequence, administered monthly through GET. When tested in dogs with surgically resected stage II-III CSPG4-positive oral melanomas, it extended the overall and disease-free survival times of vaccinated dogs compared to control dogs. All vaccinated dogs developed antibodies against both human and canine CSPG4, showing that xenogeneic vaccination was able to overcome host unresponsiveness to the self-antigen (Riccardo et al. 2015).

6 Conclusions

To date, cancer vaccines delivered by gene-electro-transfer have reached Phase I/II human clinical trials showing safety, tolerability, and immunogenicity and have proven to be a reliable anti-cancer strategy rapidly becoming a first line of intervention in veterinary medicine as well. However, appropriate design of cancer vaccine clinical trials, defining clear therapeutic endpoints, is crucial to demonstrate DNA-GET clinical efficacy. As for human clinical trials, the most appropriate endpoints in veterinary oncology are overall survival and a better quality of life, besides the evaluation of cell-mediated and antibody responses. Moreover, the next generation of electroporators promises to overcome some of the current issues related to in vivo GET, improving the speed and simplicity of treatment, especially for animals. As a more general consideration, we believe that the delivery of genetic cancer vaccines is expected to demonstrate clinical efficacy in randomized trials

within the next few years, thus becoming soon a marketed product in veterinary oncology.

References

Abdelmegeed SM, Mohammed S (2018) Canine mammary tumors as a model for human disease. Oncol Lett 15(6):8195–8205

Adachi O, Nakano A, Sato O et al (2002) Gene transfer of Fc-fusion cytokine by in vivo electroporation: application to gene therapy for viral myocarditis. Gene Ther 9(9):577–583

Adega F, Borges A, Chaves R (2016) Cat mammary tumors: genetic models for the human counterpart. Vet Sci 3(3):17

Ansaldi F, Durando P, Icardi G (2011) Intradermal influenza vaccine and new devices: a promising chance for vaccine improvement. Expert Opin Biol Ther 11(3):415–427

Aurisicchio L, Ciliberto G (2012) Genetic cancer vaccines: current status and perspectives. Expert Opin Biol Ther 12(8):1043–1058

Aurisicchio L, Mennuni C, Giannetti P et al (2007) Immunogenicity and safety of a DNA prime/adenovirus boost vaccine against rhesus CEA in nonhuman primates. Int J Cancer 120:2290–2300

Aurisicchio L, Peruzzi D, Conforti A et al (2009) Treatment of mammary carcinomas in HER-2 transgenic mice through combination of genetic vaccine and an agonist of toll-like receptor 9. Clin Cancer Res 15:1575–1584

Aurisicchio L, Mancini R, Ciliberto G (2013) Cancer vaccination by electro-gene-transfer. Expert Rev Vaccines 12(10):1127–1137

Aurisicchio L, Peruzzi D, Koo G, Wei WZ, La Monica N, Ciliberto G (2014) Immunogenicity and therapeutic efficacy of a dual-component genetic cancer vaccine cotargeting carcinoembryonic antigen and HER2/neu in preclinical models. Hum Gene Ther 25(2):121–131

Aurisicchio L, Fridman A, Mauro D et al (2020) Safety, tolerability and immunogenicity of V934/V935 hTERT vaccination in cancer patients with selected solid tumors: a phase I study. J Transl Med 18(1):39

Babiuk S, Baca-Estrada ME, Foldvari M et al (2004) Increased gene expression and inflammatory cell infiltration caused by electroporation are both important for improving the efficacy of DNA vaccines. J Biotechnol 110(1):1–10

Bagarazzi ML, Yan J, Morrow MP et al (2012) Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. Sci Transl Med 4(155):155ra38

Bakker JM, Bleeker WK, Parren PWHI (2004) Therapeutic antibody gene transfer: an active approach to passive immunity. Mol Ther 10(3):411–416

Bergman PJ, Camps-Palau MA, McKnight JA et al (2006) Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. Vaccine 24 (21):4582–4585

Blair-Parks K, Weston BC, Dean DA (2002) High-level gene transfer to the cornea using electroporation. J Gene Med 4(1):92–100

Bloquel C, Bessis N, Boissier MC et al (2004) Gene therapy of collagen induced arthritis by electrotransfer of human tumor necrosis factor alpha soluble receptor I variants. Hum Gene Ther 15(2):189–201

Buonaguro L, Petrizzo A, Tornesello ML, Buonaguro FM (2010) Translating tumor antigens into cancer vaccines. Clin Vaccine Immunol 18(1):23–34

Capone S, Zampaglione I, Vitelli A et al (2006) Modulation of the immune response induced by gene electrotransfer of a hepatitis C virus DNA vaccine in nonhuman primates. J Immunol 177 (10):7462–7471

Cappelletti M, Zampaglione I, Rizzuto G et al (2003) Gene electro-transfer improves transduction by modifying the fate of intramuscular DNA. J Gene Med 5(4):324–332

Cesca MG, Vian L, Cristóvão-Ferreira S, Pondé N, de Azambuja E (2020) HER2-positive advanced breast cancer treatment in 2020. Cancer Treat Rev 88:102033

Chen DY, Vance BA, Thompson LB, Domchek SM, Vonderheide RH (2007) Differential lysis of tumors by polyclonal T cell lines and T cell clones specific for hTERT. Cancer Biol Ther 6:1991–1996

Conforti A, Cipriani B, Peruzzi D et al (2010) A TLR9 agonist enhances therapeutic effects of telomerase genetic vaccine. Vaccine 28(20):3522–3530

Cutrera J, Torrero M, Shiomitsu K, Mauldin N, Li S (2008) Intratumoral bleomycin and IL-12 electrochemogenetherapy for treating head and neck tumors in dogs. In: Li S (ed) Electroporation protocols. Methods in molecular biology™, vol 423. Humana Press, Totowa

Daud AI, DeConti RC, Andrews S et al (2008) Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. J Clin Oncol 26(36):5896–5903

Daud A, Algazi AP, Ashworth MT et al (2014) Systemic antitumor effect and clinical response in a phase 2 trial of intratumoral electroporation of plasmid interleukin-12 in patients with advanced melanoma. J Clin Oncol 32:9025

Dharmapuri S, Peruzzi D, Mennuni C et al (2009) Coadministration of telomerase genetic vaccine and a novel TLR9 agonist in nonhuman primates [published correction appears in Mol Ther. 2010 Feb;18(2):447]. Mol Ther 17(10):1804–1813

Domchek SM, Recio A, Mick R, Clark CE, Carpenter EL, Fox KR et al (2007) Telomerase-specific T-cell immunity in breast cancer: effect of vaccination on tumor immunosurveillance. Cancer Res 67:10546–10555

Facciabene A, Aurisicchio L, Elia L et al (2006) DNA and adenoviral vectors encoding carcinoembryonic antigen fused to immunoenhancing sequences augment antigen-specific immune response and confer tumor protection. Hum Gene Ther 17:81–92

Fowler V, Robinson L, Bankowski B et al (2012) A DNA vaccination regime including protein boost and electroporation protects cattle against foot-and-mouth disease. Antivir Res 94 (1):25–34

Gavazza A, Lubas G, Fridman A et al (2013) Safety and efficacy of a genetic vaccine targeting telomerase plus chemotherapy for the therapy of canine B-cell lymphoma. Hum Gene Ther 24 (8):728–738

Gehl J (2014) Gene electrotransfer in clinical trials. Methods Mol Biol 1121:241–246

Gollins H, McMahon J, Wells KE et al (2003) High-efficiency plasmid gene transfer into dystrophic muscle. Gene Ther 10(6):504–512

Gothelf A, Gehl J (2010) Gene electrotransfer to skin; review of existing literature and clinical perspectives. Curr Gene Ther 10(4):287–299

Heller LC, Heller R (2010) Electroporation gene therapy preclinical and clinical trials for melanoma. Curr Gene Ther 10(4):312–317

Heller R, Heller LC (2015) Gene electrotransfer clinical trials. Adv Genet 89:235–262

Hoover F, Magne KJ (2000) A double-injection DNA electroporation protocol to enhance in vivo gene delivery in skeletal muscle. Anal Biochem 285(1):175–178

Impellizeri JA, Ciliberto G, Aurisicchio L (2014) Electro-gene-transfer as a new tool for cancer immunotherapy in animals. Vet Comp Oncol 12(4):310–318

Impellizeri J, Aurisicchio L, Forde P, Soden DM (2016) Electroporation in veterinary oncology. Vet J 217:18–25

Impellizeri JA, Gavazza A, Greissworth E et al (2018) Tel-eVax: a genetic vaccine targeting telomerase for treatment of canine lymphoma. J Transl Med 16(1):349

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL et al (1994) Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011–2015

Kotnik T, Frey W, Sack M et al (2015) Electroporation-based applications in biotechnology. Trends Biotechnol 33(8):480–488

Lambricht L, Lopes A, Kos S et al (2016) Clinical potential of electroporation for gene therapy and DNA vaccine delivery. Expert Opin Drug Deliv 13(2):295–310
Liao JC, Gregor P, Wolchok JD et al (2006) Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. Cancer Immun 6:8
Liu MA (2011) DNA vaccines: an historical perspective and view to the future. Immunol Rev 239:62–84
Liu J, Kjeken R, Mathiesen I et al (2008) Recruitment of antigen-presenting cells to the site of inoculation and augmentation of human immunodeficiency virus type 1 DNA vaccine immunogenicity by in vivo electroporation. J Virol 82:5643–5649
Lopes A, Vandermeulen G, Préat V (2019) Cancer DNA vaccines: current preclinical and clinical developments and future perspectives. J Exp Clin Cancer Res 38(1):146
Luckay A, Sidhu MK, Kjeken R et al (2007) Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. J Virol 81(10):5257–5269
Marconato L, Frayssinet P, Rouquet N, Comazzi S, Leone VF, Laganga P et al (2014) Randomized, placebo-controlled, double-blinded chemo-immunotherapy clinical trial in a pet dog model of diffuse large B-cell lymphoma. Clin Cancer Res 20:668–677
Marty M, Sersa G, Garbay JR et al (2006) Electrochemotherapy – an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: results of ESOPE (European standard operating procedures of Electrochemotherapy) study. Eur J Cancer Suppl 4(11):3–13
Mennuni C, Ugel S, Mori F et al (2008) Preventive vaccination with telomerase controls tumor growth in genetically engineered and carcinogen-induced mouse models of cancer. Cancer Res 68:9865–9874
Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD et al (1997) hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 90:785–795
Millanta F, Impellizeri J, McSherry L, Rocchigiani G, Aurisicchio L, Lubas G (2018) Overexpression of HER-2 via immunohistochemistry in canine urinary bladder transitional cell carcinoma - a marker of malignancy and possible therapeutic target. Vet Comp Oncol 16 (2):297–300. https://doi.org/10.1111/vco.12345
Moasser MM (2007 Oct 4) The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene 26(45):6469–6487
Muramatsu T, Shibata O, Ryoki S et al (1997) Foreign gene expression in the mouse testis by localized in vivo gene transfer. Biochem Biophys Res Commun 233(1):45–49
Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J et al (1997) Telomerase catalytic subunit homologs from fission yeast and human. Science 277:955–959
Ohashi S, Kubo T, Kishida T et al (2002) Successful genetic transduction in vivo into synovium by means of electroporation. Biochem Biophys Res Commun 293(5):1530–1535
Ottnod JM, Smedley RC, Walshaw R, Hauptman JG, Kiupel M, Obradovich JE (2013) A retrospective analysis of the efficacy of Oncept vaccine for the adjunct treatment of canine oral malignant melanoma. Vet Comp Oncol 11(3):219–229
Perez N, Bigey P, Scherman D et al (2004) Regulatable systemic production of monoclonal antibodies by in vivo muscle electroporation. Genet Vaccines Ther 2(1):2
Peruzzi D, Mesiti G, Ciliberto G et al (2010a) Telomerase and HER-2/neu as targets of genetic cancer vaccines in dogs. Vaccine 28:1201–1208
Peruzzi D, Gavazza A, Mesiti G et al (2010b) A vaccine targeting telomerase enhances survival of dogs affected by B-cell lymphoma. Mol Ther 18(8):1559–1567
Prud'homme GJ, Glinka Y, Khan AS et al (2006) Electroporation enhanced non viral gene transfer for the prevention or treatment of immunological, endocrine and neoplastic diseases. Curr Gene Ther 6(2):243–273
Ranasinghe C, Ramshaw IA (2009) Genetic heterologous prime-boost vaccination strategies for improved systemic and mucosal immunity. Expert Rev Vaccines 8(9):1171–1181
Reed SD, Li S (2009) Electroporation advances in large animals. Curr Gene Ther 9(4):316–326

Riccardo F, Aurisicchio L, Impellizeri JA, Cavallo F (2015) The importance of comparative oncology in translational medicine. Cancer Immunol Immunother 64(2):137–148

Sheyn D, Kimelman-Bleich N, Pelled G et al (2008) Ultrasound-based nonviral gene delivery induces bone formation in vivo. Gene Ther 15(4):257–266

Soares M, Correia J, Rodrigues P, Simoes M, de Matos A, Ferreira F (2013) Feline HER2 protein expression levels and gene status in feline mammary carcinoma: optimization of immunohistochemistry (IHC) and in situ hybridization (ISH) techniques. Microsc Microanal 19:876–882

Su Z et al (2005) Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer. J Immunol 174:3798–3807

Tanaka T, Ichimaru N, Takahara S et al (2002) In vivo gene transfer of hepatocyte growth factor to skeletal muscle prevents changes in rat kidneys after 5/6 nephrectomy. Am J Transplant 2 (9):828–836

Teixeira L, Medioni J, Garibal J et al (2020) A first-in-human phase I study of INVAC-1, an optimized human telomerase DNA vaccine in patients with advanced solid tumors. Clin Cancer Res 26(3):588–597

Trezise AE (2002) In vivo DNA electrotransfer. DNA Cell Biol 21(12):869–877

Trimble CL, Peng S, Kos F et al (2009) A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. Clin Cancer Res 15(1):361–367

Tsujie M, Isaka Y, Nakamura H et al (2001) Electroporation-mediated gene transfer that targets glomeruli. J Am Soc Nephrol 12(5):949–954

Ulmer JB, Otten GR (2000) Priming of CTL responses by DNA vaccines: direct transfection of antigen presenting cells versus cross-priming. Dev Biol 104:9–14

Vasan S (2014) Electroporation-mediated administration of candidate DNA vaccines against HIV-1. Methods Mol Biol 1121:291–307

Vonderheide RH (2007) Universal tumor antigens for cancer vaccination: targeting telomerase for immunoprevention. Discov Med 7:103–108

Wolff JA, Budker V (2005) The mechanism of naked DNA uptake and expression. Adv Genet 54:3–20

Yang Y (2015) Cancer immunotherapy: harnessing the immune system to battle cancer. J Clin Invest 125(9):3335–3337

Yu W, Chuang TF, Guichard C, El-Garch H, Tierny D, Laio AT, Lin CS, Chiou KH, Tsai CL, Liu CH, Fisher L, Chu RM (2011) Chicken HSP70 DNA vaccine inhibits tumor growth in a canine cancer model. Vaccine 29:3489–3500

Interleukin-12 Gene Electrotransfer in Veterinary Oncology

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Abstract

Interleukin-12 has been a promising candidate for cancer treatment for over 20 years. And from its infamous start, the gene electrotransfer delivery has regained assurance of the safe and effective use of interleukin-12 in cancer treatment. Here, basic facts about interleukin-12 are presented with a focus on its antitumor action and the benefit of electrotransfer delivery. In veterinary oncology, interleukin-12 gene electrotransfer has been used in preclinical and clinical studies on dogs for over 10 years. In this chapter studies of interleukin-12 immunotherapy alone or in combination with electrochemotherapy or surgery in different types of dogs' tumors are presented with emphasis on the antitumor effect on primary tumors as well as on distant metastasis. Also, the impact of interleukin-12 gene electrotransfer on the immune system is discussed as well as the safety of the procedure and possible improvement of the procedure.

Keywords

Gene electrotransfer · Interleukin-12 · Dog · Oncology · Clinical study · Immune response

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1 Introduction

Cancer is a systemic disease, and its development and progress are highly connected to malfunctions of the immune system. Cancer cells interact with cells of the immune system on different levels via a process known as immunoediting or the 3Es: immune Escape, Elimination, and Equilibrium (Vinay et al. 2015). "Escape" is responsible for cancer progression and immune evasion of cancer cells. Cancer cells have the potential to evade the immune system by loss of antigenicity, loss of immunogenicity or by creating an immunosuppressive environment. Based on different mechanisms of immune evasion, new therapies are focused on induction, reactivation, or enhancement of antitumor immune responses and/or blockade of immunosuppressive signals (Finn 2012; Gonzalez-Gugel et al. 2016). In addition, many immunotherapies are focused on antigen-presenting cells and cytotoxic T cells and not on tumor cells.

1.1 Antigen Presenting Cells

Antigen-presenting cells are immune cells, such as dendritic cells, macrophages, and B cells. Their maturation is triggered by pro-inflammatory cytokines and pattern recognition receptor (PRR) engagement by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). Antigens are processed and presented via four pathways: exogenous or endogenous pathway, cross-presentation, and autophagic presentation (Wculek et al. 2020). Macrophages play an especially important role in cancer immunotherapy because they can play dual roles. These cells suppress (M1) or encourage (M2) tumor cell growth (Kim et al. 2016). The main antitumor action of M1 macrophages is the presentation of tumor antigen through MHC II receptors and releasing pro-inflammatory molecules, which induce CD4+ Th1 and Th17 responses, including cytotoxic action toward tumor cells. In contrast, M2 macrophages release cytokines and chemokines that recruit regulatory T cells (T_{regs}) and CD4+ Th2 cells to promote tumor growth. Macrophages are weak antigen presenters, but they promote tumor growth through inhibiting inflammation, stimulating angiogenesis, and promoting tissue remodeling (Achyut and Arbab 2016; Yang and Zhang 2017).

1.2 Cytotoxic Immune Cells

Cytotoxic immune cells, including cytotoxic T cells and natural killer (NK) cells, are the most important cells in tumor elimination as they directly kill tumor cells. These cells are activated by cross-presentation of tumor antigens via MHC I receptors on antigen-presenting cells. Three signals are required for cytotoxic T cell activation. The first signal is provided by binding of the antigen processed by antigen-presenting cells and TCR-CD3 complex on a T cell. The second signal, which is also referred to as a costimulatory signal, is the interaction between CD28 on a T cell

and B7–1 on an antigen-presenting cell. The third signal (interleukin-12 (IL-12) release from dendritic cells) enables proliferation of activated cytotoxic T cells. In the case of a NK cell, the activation is dependent on the balance between activating and inhibitory receptors on NK cells and ligands on tumor cells (Martínez-Lostao et al. 2015). The cytotoxic function of activated cells is based on two enzymes: perforin and granzyme B. The perforin enzyme creates the pores in the target cells, through which granzyme B enters the cells and causes cell death (Chávez-Galán et al. 2015).

Due to the pivotal role of the immune system in tumor promotion and progression, preclinical, and clinical oncology studies are focused on immunotherapy strategies. One strategy is IL-12 gene electrotransfer, which is discussed in this chapter.

1.3 Interleukin-12

Interleukin-12 is a proinflammatory cytokine that was discovered in 1989 by Trinchieri and colleagues as a "natural killer cell stimulating factor" (Kobayashi et al. 1989) and in 1990 as a "cytotoxic lymphocyte maturation factor" by Gately and colleagues (Stern et al. 1990). Interleukin-12 is a heterodimeric protein composed of two subunits: a light chain with a molecular weight of 35 kDa (also known as p35 or IL-12α) and a heavy chain with a molecular weight of 40 kDa (also known as p40 or IL-12β). Three characteristics of IL-12 were initially determined: induction of IFNγ production, intensification of NK cell-mediated cytotoxicity and enhancement of the mitogenic response of T cells (Kobayashi et al. 1989). To assess the antitumor effect of recombinant IL-12 protein on different tumor models and metastases, the human and murine genes for IL-12 were cloned shortly after the discovery of IL-12 (Wolf et al. 1991; Schoenhaut et al. 1992). Recombinant IL-12 seemed to be a perfect candidate for tumor immunotherapy as it activates both arms of the immune response directly or indirectly through IFNγ. The main characteristic is that it enhances the infiltration of cytotoxic immune cells, such as cytotoxic T cells or NK cells (Del Vecchio et al. 2007). Interleukin-12 also has an antiangiogenic function through activation of IFNγ, which activates interferon-inducible protein 10 (IP10; CXCL10) and monokine induced by IFNγ, (MIG; CXCL9) (Voest et al. 1995).

Several clinical studies were started to examine the safety and antitumor effect of recombinant IL-12 (rIL-12) (Atkins et al. 1997; Motzer et al. 1998; Robertson et al. 1999; Gollob et al. 2000; Lenzi et al. 2002; Younes et al. 2004), and a clinical phase I study was initiated only 8 years after the initial discovery of IL-12 (Atkins et al. 1997). Primarily patients with renal carcinoma and melanoma were included. Recombinant IL-12 was administered intravenously. The enthusiasm about this cytokine was quickly hampered due to low antitumor effectiveness and severe side effects when it was used systemically. The most common adverse effects of systemic treatment with rIL-12 included pyrexia, headache, weakness, and gastrointestinal toxicity (Atkins et al. 1997). Moreover, in phase II human clinical trials, systemic

rIL-12 therapy also resulted in death in two of 17 treated patients (Leonard et al. 1997).

2 IL-12 Gene Electrotransfer

Gene therapy introduced a new route of IL-12 administration to patients using plasmids encoding IL-12 to achieve therapeutic long-term secretion of the cytokine, which would result in minimal or no side effects. Several viral and nonviral gene delivery methods were used, including IL-12 gene electrotransfer. The delivery system of gene electrotransfer is the same as used in electrochemotherapy (ECT), namely, electroporation. However, in the case of gene electrotransfer, the molecule used is plasmid DNA instead of a chemotherapeutic drug (Cemazar et al. 2010). Plasmid DNA is a circular DNA that can be constructed artificially and used as a vector to carry transgenes. These transgenes are then produced by the cell after the plasmid DNA is introduced into the cell. Depending on the target tissue and gene electrotransfer parameters, the transgene acts locally or systemically. Currently, there are several transgenes used in cancer immunotherapies, including immune-eliciting transgenes (IL-2, IL-15, GM-CSF), vessel-targeting transgenes (VEGF, anti-endoglin, endostatin), and DNA vaccines (human papillomavirus) (Ferraro et al. 2011; Gothelf and Gehl 2012; Yarmush et al. 2014). However, one of the most investigated cytokines used by gene electrotransfer is IL-12. Several preclinical studies have been published using IL-12 gene electrotransfer, mainly in induced tumor models in mice.

2.1 IL-12 Gene Electrotransfer Studies on Mice

Interleukin-12 gene electrotransfer is an effective, safe, and feasible treatment method of established tumors and metastases of many different histologies as demonstrated in preclinical studies in mice (reviewed in Cemazar et al. 2010) (Cemazar et al. 2010). The first IL-12 gene electrotransfer study was published by Yamashita et al. (2001). Since then, several studies have been performed using different tumor models and different routes of plasmid DNA application. The most effective route of administration is intratumoral with up to 100% of local tumors cured in certain tumor types, such as melanoma and fibrosarcoma (Lampreht Tratar et al. 2017; Lucas et al. 2002; Pavlin et al. 2009) followed by intramuscular and peritumoral application (Hanna et al. 2001; Lucas et al. 2002; Pavlin et al. 2009).

In addition to the local effect, a systemic effect was also observed. In the case of intratumoral application, antitumor immunity after IL-12 gene electrotransfer was observed when cured mice were rechallenged with tumor cells. Specifically, the tumor did not develop in 71% of mice (Lucas et al. 2002). In the case of intramuscular application, the systemic effect was observed as an effect on tumor establishment. Tumor cells were inoculated the same day as IL-12 gene electrotransfer, and 40% of mice did not develop tumors (Hanna et al. 2001). Furthermore, both

intratumoral and peritumoral applications had systemic antitumor effects as demonstrated by the reduced growth of untreated fibrosarcoma tumors growing simultaneously at a distant site in the same animal compared with tumors in untreated mice (Pavlin et al. 2009).

The systemic effect was also monitored by measuring IL-12 and IFNγ cytokines in the blood. The results of different studies showed that IL-12 and IFNγ levels were increased with all three routes of administration of the plasmid (intratumoral, peritumoral, and intramuscular) (Hanna et al. 2001; Lampreht Tratar et al. 2018; Lucas et al. 2002; Pavlin et al. 2009; Shirley et al. 2015). The systemic effect of the treatment was also confirmed by an increase in memory CD4+ and CD8+ cells in the blood (Shirley et al. 2015; Shi et al. 2018) and an increase in granzyme B cells in the spleen (Lampreht Tratar et al. 2018).

Furthermore, studies also monitored the presence of immune cells in the tumor after the IL-12 gene electrotransfer. The results of numerous studies in different tumor models show an increased immune cell infiltration in tumors, such as antigen-presenting cells, M1 macrophages, helper T cells, cytotoxic T cells, and NK cells, and decreased infiltration of regulatory T cells after intratumoral application (Li et al. 2002; Shirley et al. 2015; Lampreht Tratar et al. 2017; Shi et al. 2018). In addition to the immune response, the antiangiogenic effect of IL-12 was also demonstrated. The number of vessels in tumors were statistically reduced upon intratumoral IL-12 gene electrotransfer compared with control groups (Li et al. 2002; Lampreht Tratar et al. 2018).

The IL-12 gene electrotransfer in mice was also performed in combination with ECT. Combined intratumoral application of bleomycin and IL-12 followed by electric pulses resulted in significant tumor growth delay in B16 melanoma tumors, and reduction of induced lung metastases was observed in 38% of mice (Kishida et al. 2003). In murine sarcoma and carcinoma tumor models, adjuvant intramuscular IL-12 gene electrotransfer potentiated specific tumor growth delay of ECT by a factor of 1.8 and 2 and increased tumor cure rates by approximately 20% (Sedlar et al. 2012). The IL-12 gene electrotransfer in mice was also combined with radiotherapy, and a synergistic antitumor effect of intramuscular IL-12 gene electrotransfer and local tumor irradiation was observed in two fibrosarcoma tumor models. The IL-12 gene electrotransfer alone resulted in complete responses in 13% of LPB tumors (murine sarcoma cell line that is a clonal derivative of TBL.CI2, a methylcholanthrene-induced murine sarcoma tumor in C57Bl/6 mice (Belehradek et al. 1972)); however, in combination with local tumor irradiation, which alone resulted in 60% tumor cures, the complete responses increased up to 100% (Tevz et al. 2009). On the other hand, in a SA-1 tumor model, which is a more radioresistant model with no tumor cures at an irradiation dose of 10 Gy, IL-12 gene electrotransfer alone was more effective, resulting in 28% tumor cures; moreover, combination with irradiation increased the percentage of tumor cures to 44% (Tevz et al. 2009). In addition to intramuscular administration, intratumoral IL-12 gene electrotransfer was combined with irradiation in radioresistant SA-1 tumors. This combination proved to be very promising given that the combination of triple intratumoral IL-12 gene electrotransfer with local tumor irradiation (10 Gy) resulted

in 87% tumor cures, while the dose modifying factor of a single irradiation treatment was 2.16 (tumor control dose 50 (TCD_{50}) of a single irradiation was 29.8 Gy, while TCD_{50} of IL-12 gene electrotransfer and irradiation was 13.8 Gy). Importantly, these cured mice were also resistant to secondary challenge, demonstrating that effective immune memory was developed against tumor cells (Sedlar et al. 2013). The promising and convincing results obtained in preclinical studies in mice that demonstrate high antitumor efficacy support the translation of the therapy to clinical studies of veterinary and human oncology.

2.2 IL-12 Gene Electrotransfer Studies in Companion Dogs with Cancer

As mentioned, good results in preclinical studies on mice have established a great platform to start studies in canine oncology.

2.2.1 Effectiveness of IL-12 gene Electrotransfer ALONE or in Combination with ECT

In 2009, Chuang et al. published the results of an experimental study of intratumoral application of a plasmid encoding human IL-12 (hIL-12) followed by electric pulses on induced transmissible venereal tumors (1–2 cm of diameter) in six experimental beagle dogs. Fifty days after the induction of tumors, the mean volume of the group receiving the treatment was 3.82 ± 1.09 cm^3 compared to 181.1 ± 21 cm^3 in the nontreated group. Twelve of 16 tumors completely regressed. In contrast, four became liquefied; comprised dead tumor cells and inflammatory cells, mainly neutrophils; and were subsequently absorbed with complete regression approximately 14 days after the beginning of the treatment. Importantly, no systemic side effects (toxicity) were observed, and a strong systemic effect was induced, which prevented new tumor growth and reduced the growth of distant untreated tumors. This study is the only study in dogs using experimentally induced tumors. All other studies have been performed in companion dogs with spontaneous tumors. In that first study reported by Chuang et al., the treatment significantly inhibited tumor growth, leading to complete tumor regression (Chuang et al. 2009).

A few years later, our group published a paper on intramuscular gene electrotransfer with a plasmid encoding hIL-12 in spontaneous tumors in companion dogs (Cemazar et al. 2011). The treatment protocol was selected based on our previous study published in 2008 where intramuscular IL-12 gene electrotransfer was performed in experimental beagle dogs (Pavlin et al. 2008). In the study of Pavlin et al., four different pulse protocols were tested. All protocols proved to be safe, and two of them also led to detectable serum IL-12 and IFNγ concentrations without any systemic toxicity (Pavlin et al. 2008). Six companion dogs with four different neoplasms were included in the study by Cemazar et al. Three dogs had mast cell tumors, one had osteosarcoma of the iliac bone, another one had pulmonary histiocytic sarcoma and one had mammary adenocarcinoma that recurred after surgical treatment. Intramuscular gene electrotransfer using one mg of plasmid

DNA was performed once into semitendinosus muscle. The treatment with IL-12 gene electrotransfer was performed as an adjuvant treatment to surgery and/or chemotherapy, except in the patient with osteosarcoma, where no other treatment was applied. The treatment did not lead to tumor regression. Nevertheless, tumors that were not surgically removed remained stable throughout the observation time. Additionally, the survival times after IL-12 gene electrotransfer were longer than survival times of standard treatment associated with specific tumor types described in the literature (Withrow and Vail 2007). The only side effect observed was small local inflammation at the site of therapy application (Cemazar et al. 2011).

To achieve better local response, we evaluated intratumoral application of IL-12 gene electrotransfer instead of intramuscular delivery (Pavlin et al. 2011). The treatment was performed in eight client-owned dogs with 11 spontaneous mast cell tumors. The treatment was applied as a one-time treatment or repeated up to four times. Tumor size before IL-12 gene electrotransfer ranged from 0.05 to 25.4 cm^3. The results showed that the tumor volume regressed from 13% to 83% (median 50%). Even in large tumors (25.4 cm^3), a reduction in volume (to 18 cm^3) was observed following the gene electrotransfer treatment (Pavlin et al. 2011); however, intratumoral IL-12 gene electrotransfer treatment alone did not lead to complete tumor regression. Again, no systemic or local toxicity was observed. Furthermore, in 2015, Cutrera et al. published a study that included four dogs. Specifically, three dogs were enrolled in a dose-escalating study, and one was treated therapeutically. All four patients had spontaneous squamous cell carcinoma in different locations: nasal planum, ventral abdomen, and oral cavity. A plasmid encoding hIL-12 was administered subcutaneously near the tumor or intratumorally followed by the application of electric pulses. Because the first three dogs were enrolled in the dose-escalating study, they did not monitor the antitumor effect. Regardless, a reduction in tumor size was noted in one patient that was receiving 11.8 mg of plasmid DNA distributed throughout five treatments. The dog had more than 100 metastatic squamous cell carcinoma lesions, and the reduction was observed in five treated lesions (two PR, two SD, and one PD) and in some nontreated distant tumors (two CR and one PR with 72% size reduction). Patient 4 was treated with 6.6. mg of plasmid DNA distributed throughout five treatment cycles, and the tumor volume was reduced to approximately 50% of the volume before the treatments. Moreover, the patient also had lung metastases that seemed to become less opaque, smaller, and difficult to identify via chest radiographs during the treatment (Cutrera et al. 2015a). The latest study using IL-12 gene electrotransfer as a monotherapy in companion dogs was published in 2017 by Cicchelero et al. They performed intratumoral gene electrotransfer with a plasmid encoding hIL-12 in nine dogs with histologically different tumors (two schwannomas, two fibrosarcomas, osteosarcoma, tubulopapillar complex carcinoma, adenocarcinoma, mast cell tumor, and squamous cell carcinoma). Dogs were treated two to three times with one mg of plasmid DNA, and the treatment showed no effect on the primary tumor. However, in one of two dogs with metastases, a transient decrease in the size of one of the metastases and slower growth of the other metastases were noted. One possible reason why the study did not show any effect on primary tumors is that the tumors in

this study were very large with an average volume of 45 cm^3 (Cicchelero et al. 2017). The other possible reason is that the treatment was used as a monotherapy and not as adjuvant treatment to standard therapies.

We propose that the full therapeutic potential of IL-12 gene electrotransfer was exploited when used as an adjuvant to a local ablative therapy. The idea is that when an ablative conventional therapy, such as ECT or radiotherapy, is administered, part of the tumor cells dies. Then, tumor-associated antigens (TAA) and DAMPs are shed from dying cells (in situ vaccination). The IL-12 gene electrotransfer creates a pro-inflammatory microenvironment that leads to recruitment of circulating immune cells. When the released TAAs are captured by antigen-presenting cells, which are present in large numbers due to IL-12, they migrate to local lymph nodes. There, TAAs prime naïve T cells to become effector and memory T cells. Then, cytotoxic T cells and Th1 cells, which now recognize tumor antigens, are released and can infiltrate the primary tumor site or distant metastases via circulation where they exert their antitumor actions (Sersa et al. 2015).

Few papers have been published using the combination of ECT and IL-12 gene electrotransfer for the treatment of spontaneous tumors in dogs to date. In 2008, Cutrera et al. published the first report on the combination of ECT and IL-12 gene electrotransfer in a dog. A dog with a poorly determined "recurrent papillary tumor with adjacent metastatic bone tumor" was treated with a combination of 150 μg of plasmid DNA encoding IL-12 and 0.5 units of BLM per cm^2 of tumor, which was 3–4 cm in diameter. Electric pulses were delivered immediately after the injection using a caliper plate electrode (450 V/cm, two pulses of 25 ms). In less than two weeks, the visible "papillary tumor" was completely eradicated, and the adjacent bone tumor was not present 23 weeks after the treatment which was analyzed by multiple CT scans and indicates on bystander effect of therapy (Cutrera et al. 2008). In 2010, the same group published results of the combination treatment from a previous case report with additional five dogs with different spontaneous tumors: one with squamous cell carcinoma (SCC), one with acanthomatous ameloblastoma (AA), one with mandibular melanoma with pulmonary and lymph node metastases, one with cubital histiocytic sarcoma with spleen metastases and one with soft palate fibrosarcoma. Treatment included the combination of intratumoral application of bleomycin (0.5–2.0 IU) and a plasmid encoding feline IL-12 (fIL-12; 150–400 μg) followed by application of electric pulses. With the exception of the first dog with "papillary tumor" that was treated by caliper electrodes (Cutrera et al. 2008), the remaining dogs were treated with a hexagonal array of 6-needle electrodes surrounding the injection point (trans-lesionally) (two 20 ms pulses 100 ms apart at 400 V/cm were delivered). Three dogs (SCCs and AA) had complete responses, and the other three dogs had partial responses (approximately 50% size reduction). Bone lysis repair was also observed in dogs, and an overall improved quality of life was noted for dogs with partial responses with an extended overall survival time (Reed et al. 2010; Nemec et al. 2020).

In 2015, Cutrera et al. published a study cohort involving 13 dogs with 19 malignant tumors, including AA, SCC, and sarcoma. Repetitive treatments using the combination of electroporation with a plasmid encoding canine IL-12 (caIL-12)

and chemotherapeutic drugs bleomycin or gemcitabine were applied, and gemcitabine dose-escalation studies were also conducted. A good antitumor response was noted in SCC and AA with 27% tumor volume reduction. In contrast, sarcoma did not respond to the treatment, and a 165% volume increase was observed. In addition, the combination of ECT with IL-12 gene electrotransfer was equally effective regardless of the drug (bleomycin or gemcitabine) used. Additionally, the antitumor effect was noted in patients with small and large tumors, and there was no statistically significant change in the effectiveness of the treatments between these groups. In one patient, progressive growth of a recurrent SCC lesion was noted after treatment that included bleomycin; thus, the treatment was switched to gemcitabine. The lesion was eradicated after five treatments. The authors concluded that the use of combined treatment could be considered as a first-line treatment for cutaneous and oral SCC in sensitive and vital areas to reduce tumor volume and thus allow subsequent treatment with surgery or radiation (Cutrera et al. 2015b).

In 2016, our group published the first study using the combination of ECT and IL-12 gene electrotransfer on a single tumor histotype, namely, mast cell tumors. The study enrolled 18 dogs with 18 mast cell tumors of different histopathological grades and clinical stages. The treatment combined peritumoral application of a plasmid encoding hIL-12 and intratumoral application of cisplatin or bleomycin. In two cases, the ECT consisted of an intravenous application of bleomycin to prevent chemotherapeutic drug leakage due to a bleeding tumor. At the end of the observation period (median 40 months), complete responses were achieved in 72% of dogs, and objective responses were noted in 83%. The response to the treatment correlated with tumor size. In cases where tumors were smaller than 2 cm^3, complete response was achieved in all of the treated tumors. The response was reduced in larger tumors with a complete response of 60%. However, with repetitive treatments, complete responses were achieved even in tumors as big as 12 cm^3 and 16 cm^3. In addition to tumor volume, the clinical stage was also relevant for predictions of treatment efficacy. Both dogs that had progressive disease were classified as clinical stages 2 and 3 according to the WHO classification. Dogs showed good responses immediately after the treatment, achieving stable disease and even complete responses; however, the disease progressed further after the observation period (Cemazar et al. 2016).

One of our latest studies published in 2019 (Milevoj et al. 2019) describes the treatment of oral melanoma (OM) in companion dogs with the combination of cytoreductive surgery and/or ECT with intravenous bleomycin and peritumoral gene electrotransfer of a plasmid encoding canine caIL-12. In this study, nine dogs were enrolled with clinical stages ranging from I to III according to WHO staging criteria for oral tumors (Owen 1980). The treatments were repeated continually according to the treatment response in two- to four-week intervals. Up to five treatments were performed, but most dogs (five of nine) received four treatments. The objective response 1 month after the last treatment was 67% (six of nine). At the end of the observation period (2–22 months; median: 6 months), the disease progressed in all but one dog. In this dog, radiographic evidence of a new mandibular bone formation was observed after the last treatment, which was consistent with a

complete clinical response to the treatment. However, 7 months after the last treatment, the disease progressed, and the dog developed lung metastases. The dog was euthanized 9 months after initial presentation due to tumor-unrelated causes (development of a histologically different cutaneous tumor). The results of this study show an extended median survival time in dogs with aggressive tumors, such as OM. In addition, with this treatment no invasive surgery was needed; thus, there were no functional or cosmetic consequences. In conclusion, this type of treatment would be beneficial in cases where major maxillofacial surgery or radiotherapy is declined by the client due to high financial costs or expected functional impairment after extensive surgery (Milevoj et al. 2019).

2.2.2 Immune Response after IL-12 Gene Electrotransfer Alone or in Combination with ECT

Interleukin-12 is an immune stimulator. Thus, several studies focused on detecting immune response following IL-12 gene electrotransfer. Early research studies from Pavlin et al. (2008) and Chuang et al. (2009) focused on the detection of IL-12 in tumors and serum. Pavlin et al. showed that intramuscular IL-12 gene electrotransfer caused increased IL-12 serum levels in one of six dogs and increased IFNγ levels in three of six dogs as determined by ELISA (Pavlin et al. 2008). In experimentally induced tumors in six beagle dogs, Chuang et al. (2009) showed that intratumoral IL-12 gene electrotransfer significantly increased IL-12 levels in tumors. In contrast, only a trace amount of IL-12 was noted in the serum. Local IL-12 gene electrotransfer increased lymphocyte infiltration in the tumor and MHC I and MHC II expression in the tumor cells (Chuang et al. 2009). Furthermore, preclinical studies in dogs using IL-12 gene electrotransfer also assessed IL-12 and IFNγ levels after the treatment. In our study from 2011, increased IL-12 serum levels were observed in one dog, while IFNγ expression, which is induced by IL-12, was increased in four of six dogs treated with intramuscular application of IL-12 gene electrotransfer; a similar response was noted in an experimental study of intramuscular IL-12 application (Cemazar et al. 2011).

When intratumoral IL-12 gene electrotransfer was used, elevated IL-12 serum levels were detected in 33% to 38% of treated dogs; moreover, increased IFNγ serum levels were noted in 25% of the dogs in one study and none of the dogs in another study (Cicchelero et al. 2017; Pavlin et al. 2011). In tumor tissue, transient intratumoral increases in hIL-12 were observed in four of six dogs, and increased IFNγ levels in the tumor were only noted in one of six dogs (Cicchelero et al. 2017). However, morphological changes in the tumor microenvironment were observed after intratumoral IL-12 gene electrotransfer. Namely, the number of viable malignant cells was reduced and replaced by infiltration of leukocyte clusters that mainly consisted of lymphocytes and plasma cells (Pavlin et al. 2011).

The immune response after peritumoral hIL-12 gene electrotransfer was also monitored in our previous study using this therapy in combination with ECT. The results showed the presence of hIL-12 in the serum in 14 of 18 dogs (78%). In some cases, increased hIL-12 levels were detected up to 3 months after the treatment, and

two of these dogs achieved complete responses despite having large tumors. In addition, canine IFNγ was detected in 11 of 18 dogs (61%) (Cemazar et al. 2016).

Histological analyses of the tumors were also performed before and after therapy, describing changes in the tumor microenvironment and focusing on immune cell infiltration, proliferation, apoptosis, and angiogenesis (Salvadori et al. 2017). Tissue biopsies were collected before the treatment, 1 month and 2 months after the treatment from 11 dogs with mast cell tumors. In biopsies collected before the treatment, a small amount of immune cells infiltrating the tumor tissue, mainly consisting of T lymphocytes and macrophages, was observed. One month after the treatment, an increase in T lymphocytes was observed but was not significant. In contrast, the number of T_{reg} was significantly increased 1 month after the treatment, and the number of macrophages was significantly increased 2 months after the treatment. In addition, the number of tumors cells was reduced in seven of 11 biopsies at both time points after the treatment. However, in the other four cases, small clusters of neoplastic cells remained. Additionally, 1 month after the treatment, the expression of anti-apoptotic protein Bcl-2 and viable tumor cell proliferation were significantly reduced. The reduction of tumor cell proliferation was also evident in the samples taken 2 months after the treatment. Importantly, the antiangiogenic effect of the combined therapy was observed; specifically, microvessel density was significantly reduced in all samples obtained after the treatment (Salvadori et al. 2017). Similar results were obtained in the study of Cicchelero et al. that evaluated the effect of intratumoral gene electrotransfer on tumor neoangiogenesis by ultrasound. The findings showed a significant decrease in relative blood volume and blood flow in the tumor after treatment compared with baseline (Cicchelero et al. 2017).

In addition to local induction of immune responses, which are characterized as an infiltration of immune cells into tumors, systemic immune responses can also be evaluated by measuring circulating immune cells. In our recent study, the number of immune cells before and after the treatment (ECT and peritumoral IL-12 gene electrotransfer) of OM was measured by flow cytometry. The focus was on T_{reg}, which is known to be related to a poor prognosis (Lasek et al. 2014). The results showed a statistically significant decrease in the percentage of T_{reg} at the end of observation period compared with that prior to treatment (Milevoj et al. 2019).

2.2.3 Safety of IL-12 Gene Electrotransfer Alone or in Combination with ECT

Due to the history of recombinant IL-12 treatment, a significant focus was placed on the safety of the treatment, especially possible undesired side effects. In most of the studies on dogs described in this chapter, one or more tests were performed to determine the treatment's safety. Blood biochemistry and hematology blood panels were analyzed to determine any deviations from normal values. In addition, the overall clinical condition and specific changes in other organ systems (e.g., skin and gastrointestinal tract) were also evaluated (Cutrera et al. 2008, 2015b; Reed et al. 2010). Some studies used the Veterinary Cooperative Oncology Group toxicity scale

(VCOG-CTCAE) at each posttreatment examination (Cemazar et al. 2011, 2016; Cicchelero et al. 2017; Cutrera et al. 2015b; Milevoj et al. 2019; Pavlin et al. 2011).

The first two studies published in 2009 that were more experimentally oriented showed no noticeable side effects as determined by physical exam, complete blood count, and biochemistry panel values. The only side effect associated with the therapy was transitory tissue swelling at the site of electric pulses application (Chuang et al. 2009; Pavlin et al. 2008). These results paved the way for the use of IL-12 gene electrotransfer in veterinary clinical studies. In our studies, when using IL-12 gene electrotransfer as a monotherapy, no deviations from normal physiological values were detected, and no visible side effects were observed (Cemazar et al. 2011; Pavlin et al. 2011). Similar results were also observed in a dose-escalation study where patients received 600 to 11,800 μg plasmid DNA distributed to several tumors during the same treatment session (Cutrera et al. 2015a).

In the study by Cicchelero et al., IL-12 gene electrotransfer was initially performed three times on days one, three, and five. However, because one dog developed transient immune-mediated anemia and another dog developed fatal thrombocytopenia, these schedules were changed to include one-week intervals between the treatments. Additionally, a temporary decrease in leucocytes in all nine patients included in the study was observed after the treatment. Specifically, the number of lymphocytes, eosinophils, and basophils initially decreased, but the levels subsequently increased above the baseline during the course of follow-up. The only observed local side effect of the treatment was erythema and swelling that appeared at the site of application merely on days two and three after first gene electrotransfer and later on resolved (Cicchelero et al. 2017).

When combining IL-12 gene electrotransfer with ECT, more side effects could be expected due to the addition of ECT (Figs. 1 and 2). Indeed, in the study by Reed et al., minimal side effects, such as swollen limbs or transient lethargy, were observed. Additionally, one dog developed diarrhea 48 hours after the treatment; however, whether this episode resulted from the treatment or a concurrent disease remains inconclusive (Reed et al. 2010). Similarly, no severe side effects were reported in the study by Cutrera et al. (2015b). The only minor side effects were transient bleeding after treatment and ulceration due to tumor necrosis. In these studies, plasmid DNA was administered intratumorally (Reed et al. 2010; Cutrera et al. 2015b). When IL-12 gene electrotransfer was performed peritumorally, no local or systemic side effects were observed. Additionally, all examined blood parameters (hematology and biochemistry) did not show any deviations from normal physiological values (Cemazar et al. 2016; Milevoj et al. 2019).

2.2.4 Health-Related Quality of Life

Recently, our group published a paper offering a different point of view regarding safety and health-related quality of life (HRQoL) and the combined treatment of ECT and IL-12 gene electrotransfer. The study describes the results of a questionnaire distributed among the owners of dogs that received ECT and/or IL-12 gene electrotransfer with or without surgery. The aim was to investigate how the owners perceived the HRQoL of their dogs after treatment. The questionnaire was

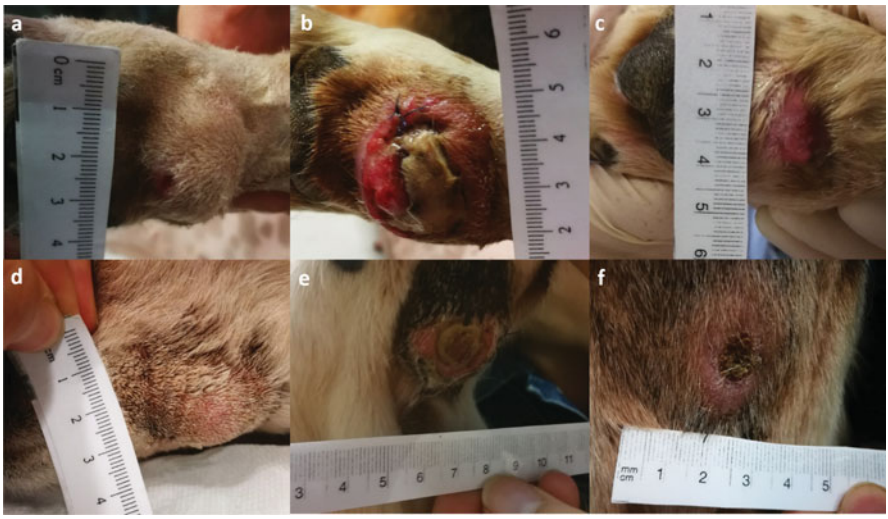

Fig. 1 An example of two patients (basset hound (**a, b**, **c**) and golden retriever (**d**, **e**, **f**)) with mast cell tumors (locations: right hind leg and chin) that received electrochemotherapy with intratumoral cisplatin and intratumoral application of IL-12 gene electrotransfer. The pictures are showing the time of the treatment (**a**, **d**), one week (**b**, **e**) and 1 month (**c**, **f**) after the treatment. The pictures taken one week after the treatment (**b**, **e**) are displaying edema and necrosis of the tumor mass. This was already replaced by crust and scar tissue 1 month after the treatment, which is seen in pictures (**e**) and (**f**). The response of the first patient (**a**) was partial. The size of the tumor reduced from 2.6 cm^3 to 0.4 cm^3. After the two additional combined therapies consisting of electrochemotherapy with intravenous bleomycin and intratumoral application of IL-12 gene therapy the tumor responded completely. The response of the second patient (**d**) was complete. The initial volume of the tumor was 0.9 cm^3 and after 1 month the tumor regressed completely

completed by 44 owners of dogs with histologically different tumors within 1 month after the treatment. The results demonstrated that the owners assessed their dogs' HRQoL after the treatment as good, and owners reported that the dogs' general health had improved compared with the initial diagnosis of cancer. Unsurprisingly, when comparing the assessments of HRQoL with groups where more invasive (i.e., surgery in addition to ECT and gene electrotransfer) treatment was employed, the HRQoL was statistically significantly worse compared with the group where less invasive (ECT and/or gene electrotransfer) treatment was used. Additionally, most owners (86%) would choose this treatment again regardless of the financial costs. This was the first study evaluating HRQoL using a questionnaire in veterinary oncology when treating dogs with ECT and gene electrotransfer. Client's satisfaction with the treatments of their dogs further supports the use of ECT and IL-12 gene electrotransfer as relevant methods for the treatment of selected tumors in veterinary medicine (Milevoj et al. 2020).

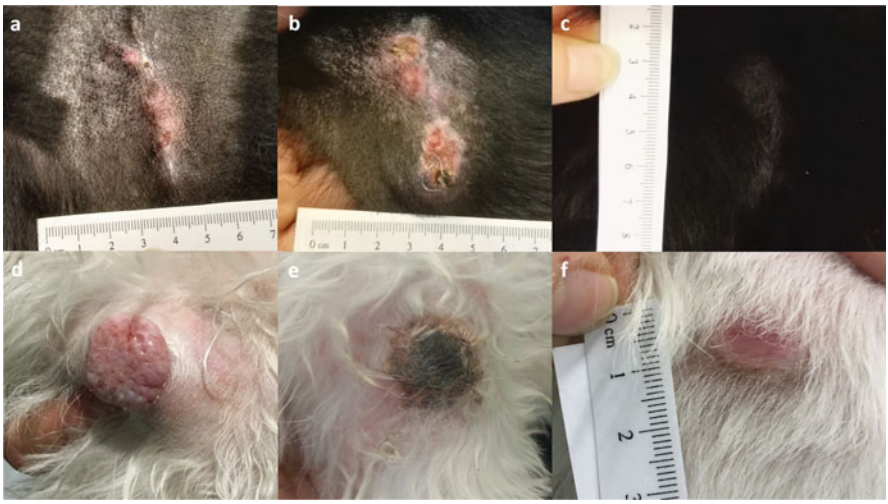

Fig. 2 An example of two patients (flat-coated retriever (**a**, **b**, **c**) and West Highland white terrier (**d**, **e**, **f**)) with mast cell tumors (locations of both patients were on right flank) that received electrochemotherapy with intratumoral cisplatin and peritumoral application of IL-12 gene electrotransfer. The pictures are showing the time of the treatment (**a**, **d**), one week (**b**, **e**) and one month (**c**, **f**) after the treatment. One week after the treatment necrosis of tumor tissue was observed (**b**, **c**) and 1 month after the treatment scar tissue was present (**c**, **f**). The response of the first patient (**a**) was complete. The size of the tumor was 1.7 cm^3 and after 1 month, the tumor regressed completely. The response of the second patient (**d**) was partial. The initial volume of the tumor was 1.15 cm^3 and after 1 month, the tumor volume reduced to 0.34 cm^3

2.2.5 Plasmid Shedding

Another safety aspect that must be taken into the account is the effect of gene therapy on the environment. Thus, the aim of our previous study was to evaluate plasmid DNA shedding from the treatment site after treatment and subsequent release into the environment. Additionally, the possibility of horizontal gene transfer to bacteria constituting the normal skin microbiota in dogs was tested. Horizontal gene transfer of antibiotic resistance genes could represent a significant obstacle for gene therapy using plasmid DNA. The results showed that the maximal concentration of plasmid DNA at the site of injection was 40 ng/mL immediately after treatment. One week after the treatment, the concentration was reduced to 0.13 ng/mL and only one of 18 patients had a detectable plasmid DNA levels (0.01 ng/mL) at four weeks. To evaluate the possibility of horizontal transfer, commensal skin bacteria from dogs were isolated prior to treatment, and these bacterial strains were later subjected to transformation with therapeutic plasmid in in vitro conditions. Horizontal transfer was not observed for any of the bacteria, except for an *E. coli* strain isolated from the skin of one patient. To eliminate any potential transfer of genes carrying antibiotic resistance, future studies should use plasmid DNA without an antibiotic resistance gene (Cemazar et al. 2016).

3 Future Directions and Conclusions

3.1 Optimization of Plasmid DNA

One of the future goals in improving IL-12 gene electrotransfer in dogs is the development of optimized plasmid DNA. First, the transgene used in the majority of studies was human or feline IL-12 (Tables 1 and 2). The homology between canine and human or feline IL-12 is 86% and 82%, respectively (Buttner et al. 1998). Although the homology between cytokines is high, there is still a possibility that a heterogenic IL-12 elicits allergic reactions and has low efficiency. Thus, plasmids encoding caIL-12 could contribute to increased specificity. To date, two studies using a plasmid encoding caIL-12 were published (Cutrera et al. 2015b; Milevoj et al. 2019).

Another important goal is the development and use of plasmid DNA without the antibiotic resistance gene to achieve safer treatment. Plasmids that were primarily used in published studies to date encoded a gene for ampicillin resistance. As ampicillin is commonly used as a wide-spectrum antibiotic in the veterinary and human clinical setting (Solensky 2003), the use of plasmids with genes for antibiotic resistance possesses a risk and is, therefore, one of the main obstacles to start with human clinical studies of plasmid-based gene therapies (Vandermeulen et al. 2011). Thus, the development and use of plasmid DNA without antibiotic resistance genes are crucial to achieve safer treatment. A plasmid encoding caIL-12 without the gene for antibiotic resistance was developed, and its efficacy in gene electrotransfer in in vitro and in vivo conditions was tested in our recent study. We demonstrated comparable efficiency to plasmids used in previous clinical trials. In addition, the results of the study showed that the plasmid is safe and capable of inducing local and systemic immune responses (Lampreht Tratar et al. 2018).

3.2 Course of Treatment

The procedure itself could also be optimized. The amount of plasmid DNA, the number of treatments, the frequency of treatments, and the treatment electric pulse parameters could be optimized.

To date, published studies used different protocols, which are listed in Tables 1 and 2. The plasmid dose ranged from 150 μg up to 3.6 mg per treatment. Although no adverse effect was noted with the highest dose, the plasmid dose should be optimized to achieve the highest immune response possible, which is not always linearly dependent on the dose of plasmid DNA. Moreover, excessive use of IL-12 could have a negative effect on antitumor immune responses due to increased activation of regulatory mechanisms, which promote immunosuppression and consequent tumor progression (Lasek et al. 2014). Next, the frequency of the treatments also varied in published studies with 1-day to 1-month intervals between the treatments. Precaution should be made on too many treatments in a short period of time due to possibility of side effects. In the study by Cicchelero et al., the gene

Table 1 Studies using IL-12 gene electrotransfer as monotherapy

	Type of treatment	Number of treatments	Cytostatic used	Plasmid DNA (mg) + administration	Electrodes + voltage	Generator of electric pulses	Tumor type	Number of patients	Size cm^3 (median; average)	Effectiveness	References
1	GET	One time	/	Human IL-12 (1 mg) intramuscular	Needle electrodes; 1 pulse 600 V/cm, 100μs 4 pulses 80 V/cm, 100 ms, 1 Hz	Cliniporator ™	Different types	6	N/A	33% CR 33% SD 33% PD	Cemazar et al. (2011)
2	GET	1–2 treatments (1 week or 4 weeks interval)	/	Human IL-12 (0.5 to 1 g/cm^3) intratumorally	Needle electrodes; 1 pulse 1200 V/cm, 100μs 8 pulses 140 V/cm, 50 ms, 2 Hz	Cliniporator ™	Mast cell tumor	8	0,6 3,1	Size reduction 13% to 83% (median 50%) Local response: 40% CR 40% SD 20% PD	Pavlin et al. (2011)
3	GET	Up to 5 cycles of 13 treatments with different intervals	/	Human IL-12 intratumorally (patient 2, 3,4) or subcutaneously (patient 1) Different doses: From 300 ug up to 3,6 mg of plasmid DNA per treatment	Needle electrode; 2 pulses 350 V/cm, 20 ms, 10 Hz	ECM 830 pulse generator, BTX®	Squamous cell carcinoma (3) Melanoma (1)	4 patients (3 in dose-escalating study, 1 treated therapeutically)	Patient 3 multiple tumors size unknown patient 4 1,9 cm3	Patient 3 (1 PR 2 SD 2 PD) Patient 4 PR (40% reduction in size) metastases less opaque, smaller, and difficult to identify	Cutrera et al. (2015a)

4	GET	2–3 treatments (1 day–1 week interval)	/	Human IL-12 intratumorally (1 mg per treatment)	Needle electrodes 2 pulses of 750 V/cm, 0,05 ms, 5 kHz 8 pulses of 183 V/cm, 10 mc, 50 Hz	Agile pulse generator, BTX®	Different types	9 dogs	12,5 44,8 + two tumors in the skull with 13–15% of volume invasion	Softening of the tumor but no effect on tumor growth; a significant decrease of tumor blood volume	Cicchelero et al. (2017)

Table 2 Studies using IL-12 gene electrotransfer in combination with electrochemotherapy

	Type of treatment	Number of treatments	Cytostatic used	Plasmid DNA (mg) + administration	Electrodes + voltage	Generator of electric pulses	Tumor type	Number of patients	Size cm3 (median; average)	Effectiveness	References
1	GET + ECT	NA	Bleomycin (0.5 units per cm3)	IL-12 intratumorally (150 ug)	Caliper electrode 2 pulses 450 V/cm 25 ms	BTS EC830 pulse generator	Head and neck tumors	1 dog	NA	Complete response of papillary tumor and bone tumor 23 weeks after treatment	Cutrera et al. (2008)
2	GET + ECT	1–3 treatments (10 day interval)	Bleomycin (0.5–2 IU per treatment)	Feline IL-12 intratumorally (150 ug – 400 ug)	Hexagonal electrodes 2 pulses 400 V/cm 20 msec 10 Hz	ECM 830 pulse generator, BTX®	Different types	6 dogs	3 cm 3,4 cm (diameter)	50% CR 17% PR (euthanize due to poor QoL) 33% PD (euthanize due to metastatic disease and decision by owner)	Reed et al. (2010)
3	GET +/− ECT	Multiple treatment with different frequency and combinations	Bleomycin (100ul, 1 IU per cm^3) or gemcitabine (0.5–10 mg/cm^3)	Canine IL-12 (2 mg/cm tumor diameter) intratumoral	Needle electrode, two 350 V/cm 20 msec, 10 Hz	ECM 830 pulse generator, BTX®	2 AA 4 sarcomas 7 SCC	13	NA	27% volume reduction in, SCC and PC, 165% volume increase in sarcomas	Cutrera et al. (2015b)
4	GET + ECT	1–2 treatments (4 weeks interval)	Bleomycin intravenously (0.3 mg/kg) intratumorally	Human IL-12 (1–2 mg per patient per treatment)	Plate or needle electrodes ECT (81,300 V/cm, 100 μs, 5 kHz)	Cliniporator ™	Mast cell tumor	18	2,1 3,5	CR 72% PR 11% SD 6% PD 11%	Cemazar et al. (2016)

			(1 mg/cm3) or cisplatin (1 mg/cm^3) intratumorally	peritumorally (intradermally)	GET (1 HV 1200 V/cm 100 µs, 1 LV 140 V/cm 400 ms)						
5	GET + ECT (cytoreductive surgery beforehand)	1–5 treatments (2–4 weeks interval)	Bleomycin intravenously (0.3 mg/kg)	Canine IL-12 (2 mg per treatment) peritumorally (intradermally)	Plate or needle electrodes ECT (81,300 V/cm, 100 µs, 5 kHz) Multielectrode array (MEA) GET (24 pulses, 60 V, 150 ms, 4 Hz	Cliniporator ™	OMM	9	8,4 10,7	One month after treatment: CR 33% PR 33% PD 33% End of observation period (2–22 months): CR 11% PD 89% Median survival time 6 months	Milevoj et al. (2019)

electrotransfer schedule was altered from a one-day interval to a one-week interval because one dog developed transient immune-mediated anemia and another developed fatal thrombocytopenia (Cicchelero et al. 2017). However, whether repetitive treatments are necessary and benefit patients by reducing relapses should be assessed.

The most common route of plasmid DNA administration is intratumoral, but there is no consistency among the published studies with regards to pulse parameters, which range from one to two high voltage (750–1200 V/cm) and eight low voltage (140–183 V/cm) or using two pulses of 350–450 V/cm.

Data obtained from studies using different plasmid doses, treatment frequencies, and electroporation parameters make it difficult to draw conclusions of the most appropriate treatment protocol. Not to mention that due to different tumor types and immunological statuses of the tumor IL-12 gene electrotransfer treatment is beneficial only for certain patients with certain tumors. In human oncology, IL-12 gene electrotransfer was so far performed in melanoma and Merkle cell carcinoma (Canton et al. 2017; Bhatia et al. 2020) which are known to be immunogenic tumors (Vandeven and Nghiem 2016; Passarelli et al. 2017). The results of these studies showed that in both tumor types local and systemic immune response was achieved and the treatment was clinically beneficial for patients (Canton et al. 2017; Bhatia et al. 2020). Local IL-12 gene electrotransfer in cutaneous melanoma metastases also led to abscopal effect of non-treated tumors (Canton et al. 2017). In order to recommend this treatment for certain types of tumors the correlation between the antitumor response, properties of tumor microenvironment and immune status of the tumor as well as the patient should be determined. This is only possible in larger study cohorts with focus on one type of tumor. Additionally, as performed in preclinical testing, the immune status of the animal and the tumor before and after the treatment should be elucidated to better predict which patient would most benefit from IL-12 gene electrotransfer treatment. Thus, large international clinical trials with clearly defined criteria to improve the quality of reporting clinical studies from this field are critical for elucidation of which tumor types and which patients can benefit from IL-12 gene electrotransfer treatment the most.

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References

Achyut BR, Arbab AS (2016) Myeloid cell signatures in tumor microenvironment predicts therapeutic response in cancer. Onco Targets Ther 9:1047–1055. https://doi.org/10.2147/OTT.S102907

Atkins MB, Robertson MJ, Gordon M et al (1997) Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. Clin Cancer Res 3(3):409–417

Belehradek J, Barski G, Thonier M (1972) Evolution of cell-mediated antitumor immunity in mice bearing a syngeneic chemically induced tumor. Influence of tumor growth, surgical removal and

treatment with irradiated tumor cells. Int J Cancer 9:461–469. https://doi.org/10.1002/ijc.2910090302

Bhatia S, Longino NV, Miller NJ et al (2020) Intratumoral delivery of plasmid IL12 via electroporation leads to regression of injected and noninjected tumors in Merkel cell carcinoma. Clin Cancer Res 26:598–607. https://doi.org/10.1158/1078-0432.CCR-19-0972

Buttner M, Belke-Louis G, Rziha HJ et al (1998) Detection, cDNA cloning and sequencing of canine interleukin 12. Cytokine 10:241–248. https://doi.org/10.1006/cyto.1997.0284

Canton DA, Shirley S, Wright J et al (2017) Melanoma treatment with intratumoral electroporation of tavokinogene telseplasmid (pIL-12, tavokinogene telseplasmid). Immunotherapy 9:1309–1321. https://doi.org/10.2217/imt-2017-0096

Cemazar M, Ambrozic Avgustin J, Pavlin D et al (2016) Efficacy and safety of electrochemotherapy combined with peritumoral IL-12 gene electrotransfer of canine mast cell tumours. Vet Comp Oncol 15(2):641–654. https://doi.org/10.1111/vco.12208

Cemazar M, Jarm T, Sersa G (2010) Cancer electrogene therapy with interleukin-12. Curr Gene Ther 10:300–311

Cemazar M, Sersa G, Pavlin D, Tozon N (2011) Intramuscular IL-12 Electrogene therapy for treatment of spontaneous canine tumors. In: Yongping Y (ed) targets in gene therapy, IntechOpen, doi: https://doi.org/10.5772/20734. https://www.intechopen.com/books/targets-in-gene-therapy/intramuscular-il-12-electrogene-therapy-for-treatment-of-spontaneous-canine-tumors. Accessed 3 May 2020

Chávez-Galán L, Olleros ML, Vesin D, Garcia I (2015) Much more than M1 and M2 macrophages, there are also CD169(+) and TCR(+) macrophages. Front Immunol 6:263. https://doi.org/10.3389/fimmu.2015.00263

Chuang T-F, Lee S-C, Liao K-W et al (2009) Electroporation-mediated *IL-12* gene therapy in a transplantable canine cancer model. Int J Cancer 125:698–707. https://doi.org/10.1002/ijc.24418

Cicchelero L, Denies S, Haers H et al (2017) Intratumoural interleukin 12 gene therapy stimulates the immune system and decreases angiogenesis in dogs with spontaneous cancer. Vet Comp Oncol 15(4):1187–1205. https://doi.org/10.1111/vco.12255

Cutrera J, King G, Jones P et al (2015a) Safety and efficacy of tumor-targeted interleukin 12 gene therapy in treated and non-treated, metastatic lesions. Curr Gene Ther 15:44–54

Cutrera J, King G, Jones P et al (2015b) Safe and effective treatment of spontaneous neoplasms with interleukin 12 electro-chemo-gene therapy. J Cell Mol Med 19:664–675. https://doi.org/10.1111/jcmm.12382

Cutrera J, Torrero MN, Shiomitsu K et al (2008) Intratumoral bleomycin and IL-12 electrochemogenetherapy for treating head and neck tumors in dogs. Methods Mol Biol 423:319–325. https://doi.org/10.1007/978-1-59745-194-9_24

Del Vecchio M, Bajetta E, Canova S et al (2007) Interleukin-12: biological properties and clinical application. Clin Cancer Res 13:4677–4685. https://doi.org/10.1158/1078-0432.CCR-07-0776

Ferraro B, Morrow MP, Hutnick NA et al (2011) Clinical applications of DNA vaccines: current progress. Clin Infect Dis 53:296–302. https://doi.org/10.1093/cid/cir334

Finn OJ (2012) Immuno-oncology: understanding the function and dysfunction of the immune system in cancer. Ann Oncol 23(Suppl 8):viii6–viii9. https://doi.org/10.1093/annonc/mds256

Gollob JA, Mier JW, Veenstra K et al (2000) Phase I trial of twice-weekly intravenous interleukin 12 in patients with metastatic renal cell cancer or malignant melanoma: ability to maintain IFN-γ induction is associated with clinical response. Clin Cancer Res 6(5):1678–1692

Gonzalez-Gugel E, Saxena M, Bhardwaj N (2016) Modulation of innate immunity in the tumor microenvironment. Cancer Immunol Immunother 65:1261–1268

Gothelf A, Gehl J (2012) What you always needed to know about electroporation based DNA vaccines. Hum Vaccin Immunother 8:1694–1702. https://doi.org/10.4161/Hv.22062

Hanna E, Zhang X, Woodlis J et al (2001) Intramuscular electroporation delivery of IL-12 gene for treatment of squamous cell carcinoma located at distant site. Cancer Gene Ther 8:151–157. https://doi.org/10.1038/sj.cgt.7700287

Kim J, Kim J, Bae JS (2016) ROS homeostasis and metabolism: a critical liaison for cancer therapy. Exp Mol Med 48(11):e269. https://doi.org/10.1038/emm.2016.119

Kishida T, Asada H, Itokawa Y et al (2003) Electrochemo-gene therapy of cancer: intratumoral delivery of interleukin-12 gene and bleomycin synergistically induced therapeutic immunity and suppressed subcutaneous and metastatic melanomas in mice. Mol Ther 8:738–745

Kobayashi M, Fitz L, Ryan M et al (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med 170(3):827–845. https://doi.org/10.1084/jem.170.3.827

Lampreht Tratar U, Kos S, Kamensek U et al (2018) Antitumor effect of antibiotic resistance gene-free plasmids encoding interleukin-12 in canine melanoma model. Cancer Gene Ther 25 (9–10):260–273. https://doi.org/10.1038/s41417-018-0014-5

Lampreht Tratar U, Loiacono L, Cemazar M et al (2017) Gene Electrotransfer of plasmid-encoding IL-12 recruits the M1 macrophages and antigen-presenting cells inducing the eradication of aggressive B16F10 murine melanoma. Mediat Inflamm 2017:5285890. https://doi.org/10.1155/2017/5285890

Lasek W, Zagożdżon R, Jakobisiak M (2014) Interleukin 12: still a promising candidate for tumor immunotherapy? Cancer Immunol Immunother 63:419–435. https://doi.org/10.1007/s00262-014-1523-1

Lenzi R, Rosenblum M, Verschraegen C et al (2002) Phase I study of intraperitoneal recombinant human interleukin 12 in patients with Müllerian carcinoma, gastrointestinal primary malignancies, and mesothelioma. Clin Cancer Res 8(12):3686–3695

Leonard JP, Sherman ML, Fisher GL et al (1997) Effects of single-dose interleukin-12 exposure on interleukin-12 associated toxicity and interferon-γ production. Blood 90(7):2541–2548. https://doi.org/10.1182/blood.V90.7.2541

Li S, Zhang X, Xia X (2002) Regression of tumor growth and induction of long-term antitumor memory by interleukin 12 electro-gene therapy. J Natl Cancer Inst 94:762–768. https://doi.org/10.1093/jnci/94.10.762

Lucas ML, Heller LC, Coppola D, Heller R (2002) IL-12 plasmid delivery by in vivo electroporation for the successful treatment of established subcutaneous B16.F10 melanoma. Mol Ther 5:668–675. https://doi.org/10.1006/mthe.2002.0601

Martínez-Lostao L, Anel A, Pardo J (2015) How do cytotoxic lymphocytes kill Cancer cells? Clin Cancer Res 21(22):5047–5056. https://doi.org/10.1158/1078-0432.CCR-15-0685

Milevoj N, Tozon N, Licen S et al (2020) Health-related quality of life in dogs treated with electrochemotherapy and/or interleukin-12 gene electrotransfer. Vet Med Sci 6(3):290–298. https://doi.org/10.1002/vms3.232

Milevoj N, Tratar UL, Nemec A et al (2019) A combination of electrochemotherapy, gene electrotransfer of plasmid encoding canine IL-12 and cytoreductive surgery in the treatment of canine oral malignant melanoma. Res Vet Sci 122:40–49. https://doi.org/10.1016/j.rvsc.2018.11.001

Motzer RJ, Rakhit A, Schwartz LH et al (1998) Phase I trial of subcutaneous recombinant human interleukin-12 in patients with advanced renal cell carcinoma. Clin Cancer Res 4(5):1183–1191

Nemec A, Milevoj N, Lampreht Tratar U et al (2020) Electroporation-based treatments in small animal veterinary Oral and maxillofacial oncology. Front Vet Sci 7:575911. https://doi.org/10.3389/fvets.2020.575911

Owen LN (1980) TNM classification of tumours in domestic animals. World Health Organization. Veterinary public health unit & WHO collaborating Center for Comparative Oncology. Edited by L.N. Owen. World Health Organization. https://apps.who.int/iris/handle/10665/68618; Assessed on 26.10.2020

Passarelli A, Mannavola F, Stucci LS et al (2017) Immune system and melanoma biology: a balance between immunosurveillance and immune escape. Oncotarget 8(62):106132–106142. https://doi.org/10.18632/oncotarget.22190

Pavlin D, Cemazar M, Cor A et al (2011) Electrogene therapy with interleukin-12 in canine mast cell tumors. Radiol Oncol 45:31–39. https://doi.org/10.2478/v10019-010-0041-9

Pavlin D, Cemazar M, Kamensek U et al (2009) Local and systemic antitumor effect of intratumoral and peritumoral IL-12 electrogene therapy on murine sarcoma. Cancer Biol Ther 8:2114–2122

Pavlin D, Tozon N, Sersa G et al (2008) Efficient electrotransfection into canine muscle. Technol Cancer Res Treat 7(1):45–54. https://doi.org/10.1177/153303460800700106

Reed SD, Fulmer A, Buckholz J et al (2010) Bleomycin/interleukin-12 electrochemogenetherapy for treating naturally occurring spontaneous neoplasms in dogs. Cancer Gene Ther 17:571–578. https://doi.org/10.1038/cgt.2010.13

Robertson MJ, Cameron C, Atkins MB et al (1999) Immunological effects of interleukin 12 administered by bolus intravenous injection to patients with cancer. Clin Cancer Res 5 (1):9–16

Salvadori C, Svara T, Rocchigiani G et al (2017) Effects of electrochemotherapy with cisplatin and peritumoral IL-12 gene electrotransfer on canine mast cell tumors: a histopathologic and immunohistochemical study. Radiol Oncol 51:286–294. https://doi.org/10.1515/raon-2017-0035

Schoenhaut DS, Chua AO, Wolitzky AG et al (1992) Cloning and expression of murine IL-12. J Immunol 148(11):3433–3440

Sedlar A, Dolinsek T, Markelc B et al (2012) Potentiation of electrochemotherapy by intramuscular IL-12 gene electrotransfer in murine sarcoma and carcinoma with different immunogenicity. Radiol Oncol 46:302–311. https://doi.org/10.2478/v10019-012-0044-9

Sedlar A, Kranjc S, Dolinsek T et al (2013) Radiosensitizing effect of intratumoral interleukin-12 gene electrotransfer in murine sarcoma. BMC Cancer 13:38. https://doi.org/10.1186/1471-2407-13-38

Sersa G, Teissie J, Cemazar M et al (2015) Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. Cancer Immunol Immunother 64:1315–1327. https://doi.org/10.1007/s00262-015-1724-2

Shi G, Edelblute C, Arpag S et al (2018) IL-12 gene electrotransfer triggers a change in immune response within mouse tumors. Cancers (Basel). 10(12):E498. https://doi.org/10.3390/cancers10120498

Shirley SA, Lundberg CG, Li F et al (2015) Controlled gene delivery can enhance therapeutic outcome for cancer immune therapy for melanoma. Curr Gene Ther 15:32–43

Solensky R (2003) Hypersensitivity reactions to beta-lactam antibiotics. Clin Rev Allergy Immunol 24:201–220. https://doi.org/10.1385/criai:24:3:201

Stern AS, Podlaski FJ, Hulmes JD et al (1990) Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc Natl Acad Sci U S A 87(17):6808–6812. https://doi.org/10.1073/pnas.87.17.6808

Tevz G, Kranjc S, Cemazar M et al (2009) Controlled systemic release of interleukin-12 after gene electrotransfer to muscle for cancer gene therapy alone or in combination with ionizing radiation in murine sarcomas. J Gene Med 11:1125–1137. https://doi.org/10.1002/jgm.1403

Vandermeulen G, Marie C, Scherman D, Préat V (2011) New generation of plasmid backbones devoid of antibiotic resistance marker for gene therapy trials. Mol Ther 19:1942–1949. https://doi.org/10.1038/mt.2011.182

Vandeven NA, Nghiem P (2016) Merkel cell carcinoma: an unusually immunogenic cancer proves ripe for immune therapy. J Oncol Pract 12(7):649–650. https://doi.org/10.1200/JOP.2016.014498

Vinay DS, Ryan EP, Pawelec G et al (2015) Immune evasion in cancer: mechanistic basis and therapeutic strategies. Semin Cancer Biol 35:S185–S198. https://doi.org/10.1016/j.semcancer.2015.03.004

Voest EE, Kenyon BM, O'reilly MS et al (1995) Inhibition of angiogenesis in vivo by interleukin 12. J Natl Cancer Inst 87(8):581–586. https://doi.org/10.1093/jnci/87.8.581

Wculek SK, Cueto FJ, Mujal AM et al (2020) Dendritic cells in cancer immunology and immunotherapy. Nat Rev Immunol 20:7–24

Withrow SJ, Vail DM (2007) Specific malignancies in the small animal patient. In: Vail D (ed) Small Animal Clinical Oncology, 4th edn. Elsevier, Netherlands, pp 305–715

Wolf SF, Temple PA, Kobayashi M et al (1991) Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J Immunol 146(9):3074–3081

Yamashita YI, Shimada M, Hasegawa H et al (2001) Electroporation-mediated interleukin-12 gene therapy for hepatocellular carcinoma in the mice model. Cancer Res 61:1005–1012

Yang L, Zhang Y (2017) Tumor-associated macrophages: from basic research to clinical application. J Hematol Oncol 10:58

Yarmush ML, Golberg A, Sersa G et al (2014) Electroporation-based technologies for medicine: principles, applications, and challenges. Annu Rev Biomed Eng 16:295–320. https://doi.org/10.1146/annurev-bioeng-071813-104622

Younes A, Pro B, Robertson MJ et al (2004) Phase II clinical trial of interleukin-12 in patients with relapsed and refractory non-Hodgkin's lymphoma and Hodgkin's disease. Clin Cancer Res 10 (16):5432–5438. https://doi.org/10.1158/1078-0432.CCR-04-0540

Canine Melanoma and Osteosarcoma Immunotherapy by Means of In Vivo DNA Electroporation

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Abstract

Despite significant advancements in diagnostic and treatment options, cancer management remains challenging and ever evolving. Conventional treatments, such as surgery, radiotherapy, and chemotherapy are rarely effective against the most aggressive tumors, including malignant melanoma (MM) and osteosarcoma (OSA). Therefore, great efforts have focused on the identification of novel treatments with immunotherapy emerging as the fourth pillar of the anticancer arsenal. In this chapter, we introduce relevant immunotherapeutic approaches for the management of oncological canine patients, with a focus on MM and OSA. Among the immune-based strategies discussed, DNA vaccination arises as one of the most appealing thanks to its mechanism of a safe, specific, and long-lasting stimulation of the patients' own immune system against cancer cells. The design and delivery methods have been optimized to increase the power of DNA immunization, with in vivo electroporation emerging as one of the most attractive approaches. This chapter will provide an overview of our promising veterinary studies using adjuvant DNA vaccination through in vivo electroporation, targeting the chondroitin sulfate proteoglycan (CSPG)4 for the management of oral MM (OMM) and OSA canine patients.

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Abbreviations

CARs	Chimeric antigen receptors
CSC	Cancer stem cells
CSPG4	Chondroitin sulfate proteoglycan 4
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cells
DFI	Disease-free interval
DTH	Delayed-type hypersensitivity
EGFR	Epidermal growth factor receptor
FAK	Focal adhesion kinase
FDA	Food and drug administration
HER2	Human epidermal growth factor receptor 2
Hu-CSPG4	Human CSPG4
huTyr	Human tyrosinase
IDO	Indolamine-2,3-dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
Lm	Listeria monocytogenes
L-MTP-PE	Liposome-encapsulated lipophilic derivative of muramyl dipeptide
mAb	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MM	Malignant melanoma
MST	Median survival time
OMM	Oral malignant melanoma
OSA	Osteosarcoma
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death receptor-1
PD-L1	Programmed death-ligand 1
RTK	Receptor tyrosine kinases
TAA	Tumor-associated antigen
TLR9	Toll-like receptor 9
TSA	Tumor-specific antigens
USDA	United States Department of Agriculture

1 Canine Malignant Melanoma and Osteosarcoma: An Overview

Cancer is one of the most frequent and deleterious diseases in dogs, with 50% incidence in older dogs and an estimated mortality rate of 30% (Davis and Ostrander 2014; Ostrander et al. 2019). While the surgical resection of the primary tumor is still of paramount importance for local tumor control, recurrences and metastasis remain the main cause of mortality in canine oncological patients (Kudnig and Séguin 2013; Sandru et al. 2014; Withrow et al. 2012). Even though the addition of radio- and chemotherapy has demonstrated to be better tolerated in dogs than in humans, they may negatively affect the quality of life of the canine patient, in the face of a limited benefit in some instances, with disease progression occurring often within the year (Klingemann 2018).

Among all, malignant melanoma (MM) and osteosarcoma (OSA) are two of the most common canine cancers.

Both are characterized by high frequency, aggressiveness, and fatal behavior and are generally strongly resistant to conventional therapies (Hernandez et al. 2018; Riccardo et al. 2014a, b; Tarone et al. 2019; Varshney et al. 2016; Fan and Khanna 2015).

MM accounts for up to 100,000 diagnoses/year in the United States alone (Bosenberg et al. 2014; Hernandez et al. 2018). It affects a broad range of anatomical sites, preferentially including eye, lips, skin, digit/footpad, and mucosae (Prouteau and André 2019; Tarone et al. 2019). By comprising about 60% of MM cases, oral MM (OMM) represents the most common subtype (Prouteau and André 2019; Almela and Ansón 2019) (Table 1). OMM affects several breeds among which Scottish Terriers, Golden and Labrador Retrievers, Poodles, Dachshunds, Cocker Spaniel, Miniature Poodle, Chow Chow, Gordon Setter, and Anatolian Sheepdog, with a higher prevalence in elderly dogs, without any gender predilection (Nishiya et al. 2016). OMM is a locally aggressive disease with a high metastatic propensity (Bergman 2007; Nishiya et al. 2016). The first-line therapy for localized OMM is the surgical resection of the primary tumor, but in most cases, local invasion of the bone is already present at the time of surgery, making the complete removal of the tumor less likely.

Both neoadjuvant and adjuvant radiotherapies have been demonstrated to be a good option for local control of the tumor, with complete remission in up to 70% of treated cases (Bergman 2007). However, OMM is characterized by high recurrence rate and metastasis development. Indeed, a rapid progression from localized to advanced-stage disease commonly occurs, with the formation of metastasis mainly in the regional lymph nodes (58–74%), lungs (14–67%), and tonsils (65%) (Bowlt Blacklock et al. 2019). For these patients, the 1-year survival rate does not exceed 30% even when surgery and/or radiotherapy are applied (Bowlt Blacklock et al. 2019; Boston et al. 2014). In these cases, chemotherapy is attempted as an adjuvant treatment to control the systemic tumor spreading. However, it barely increases the overall survival: a median survival time (MST) of 335 days was reported for OMM patients after en bloc surgery alone, and 352 days when adjuvant chemotherapy was

Table 1 Canine OMM and OSA features

	Oral malignant melanoma	Osteosarcoma
Incidence	Up to 60.000 diagnoses/year in the USA (Hernandez et al. 2018; Bosenberg et al. 2014)	Up to 10.000 diagnoses/year in the USA (Makielski et al. 2019; Fan and Khanna 2015; Rodriguez 2014)
Breed	Scottish Terriers, Golden Retrievers, Poodles, Dachshunds, Cocker Spaniel, Miniature Poodle, Chow Chow, Gordon Setter, Anatolian Sheepdog, and mixed-breed dogs (Nishiya et al. 2016)	Saint Bernard, Great Dane, Irish setter, Doberman pinscher, Rottweilers, German Shepherds, Golden Retriever (Lazarides et al. 2017)
Age of Patients	Median age 10–11 years (Prouteau and André 2019)	Median age 7 years (Lazarides et al. 2017)
Causes	The major risk factors include: – Consanguinity, pigmentation characteristics – Environmental carcinogens (mucosal melanomas) – Sun exposure (cutaneous melanomas) Major genetic mutations – NRAS, KRAS, PTEN, KIT, TP53 – $BRAF^{V600E}$ mutation not detected (Prouteau and André 2019)	Etiology generally unknown Possible risk factors include: – Genetic alterations – Mutagenic effects of ionizing radiation – Multiple minor trauma Major genetic mutations: - TP53, RB1, PTEN, MYC
Anatomic site	Any portion of the oral cavity Gingival mucosa, mandibular labial mucosa, tongue, skin, digit/footpad (Prouteau and André 2019; Almela and Ansón 2019) Metastasis: lymph nodes, lungs, tonsils, other distant organs (Nishiya et al. 2016; Bergman et al. 2006)	Mostly metaphysis of long bones Forelimbs, hind limbs Distal radius, proximal humerus, distal femur, proximal tibia, distal tibia, diaphyseal ulna (Morello et al. 2011) Metastasis: Lungs, bones, soft tissues, regional lymph nodes (Morello et al. 2011)
Conventional treatments	– Surgery and/or radiation therapy – Chemotherapy (carboplatin)	– Surgery – Postoperative chemotherapy (doxorubicin and cisplatin)
Survival	1-year survival rate: < 30% (Bowlt Blacklock et al. 2019; Boston et al. 2014)	1-year survival rate: <45% (Simpson et al. 2017; Lazarides et al. 2017; Selmic et al. 2014)
*Surgery alone**	335 days (Boston et al. 2014)	Without metastasis to regional lymph nodes at diagnosis: 318 days With metastasis to regional lymph nodes at diagnosis: 49–57 days
*Surgery and radiotherapy or chemotherapy**	352 days (Boston et al. 2014)	235–540 days (Selmic et al. 2014; Simpson et al. 2017)

*Indicated as Median Survival Times (MST)

also applied (Boston et al. 2014). As a consequence, it is clear that the highly aggressive behavior of OMM could be associated with strong resistance to standard therapies. In this regard, genetic alterations in canine MM have not been fully evaluated, and the molecular mechanisms of therapy resistance still need to be clarified (Nishiya et al. 2016).

Also, OSA is a frequently occurring cancer in canine patients, with around 10,000 dogs diagnosed each year in the United States (Rodriguez 2014; Fan and Khanna 2015; Makielski et al. 2019) (Table 1). OSA represents the predominant form of primary bone cancer, mainly occurring in middle-aged to older dogs, with a median age at diagnosis of 7 years (Lazarides et al. 2017). Predisposing factors such as increased weight and height appear to be related to OSA onset (Morello et al. 2011; Lazarides et al. 2017). Indeed, a wider peak of incidence is observed in larger and giant breed dogs such as the Saint Bernard, Great Dane, Irish setter, Doberman Pinscher, Rottweilers, German Shepherds, and Golden Retriever (Lazarides et al. 2017). In approximately 75% of cases, OSA arises in the appendicular skeleton, usually affecting the metaphysis of long bones, such as distal radius, proximal humerus, distal femur, proximal and distal tibia, and ulna (Morello et al. 2011).

The primary tumor is initially restricted to the local bone microenvironment but rapidly evolves into a very aggressive form. Standard therapy includes limb amputation or limb–sparing for the primary tumor control followed by platinum or doxorubicin-based chemotherapy. Dogs treated with amputation and chemotherapy exhibit a MST of 235–540 days, with less than 15% of patients achieving a complete remission (Selmic et al. 2014; Simpson et al. 2017). These data mirror the high heterogeneity in both tumor biology and response to therapy. Patients with a localized tumor are characterized by a 1-year survival rate of less than 45% (Simpson et al. 2017), while a worse scenario is evident for OSA patients displaying metastatic lesions at the diagnosis, typically dying within few months because of severe and progressive metastatic spreading, with the lungs the most affected site (Simpson et al. 2017; Lazarides et al. 2017; Riccardo et al. 2014a, b).

Due to the limited response to the established standard-of-care treatments, including adjuvant radiotherapy and chemotherapy, survival times for OMM- and OSA-affected dogs have not improved in the last two-three decades (Simpson et al. 2017; Mochel et al. 2019). Hence, the pursuit of more efficacious and tolerable treatments has spurred the finding of novel anticancer approaches for improving the long-term prognosis of canine cancer patients. Emerging evidence, supports a key role of the immune system in the treatment of cancer. By flanking surgery, radiotherapy, and chemotherapy, immunotherapy has emerged as the fourth pillar of the anticancer arsenal, even in veterinary oncology.

2 Immunotherapeutic Approaches in Canine Malignant Melanoma and Osteosarcoma Oncology

Excitement in the immunotherapy field has risen in the last years, with the achievement of several remarkable breakthroughs in human oncology (Kruger et al. 2019). In this panorama, the study of the potential of immunotherapy as an alternative therapeutic approach has extended also in veterinary medicine with promising results (Gardner et al. 2016; Regan et al. 2016; Anderson and Modiano 2015), especially for dogs, that among pets are those more exposed to medical surveillance and preventative health care (Ostrander et al. 2019). Nevertheless, a significant hindrance exists to veterinary research since the canine immune system is not well studied and characterized, as compared to the human system. Therefore, canine cancer patients have limited immunotherapeutic options that should be studied through a deeper investigation of the immune system–tumor relationship.

Currently, explored immune treatments for canine cancer patients range from those that nonspecifically boost the immune system, to those that aid the immune system to specifically target cancer cells. The most relevant immunotherapeutic strategies for the treatment of canine oncological patients, with a focus on MM and OSA, will be summarized in the following paragraphs and in Table 2.

2.1 Nonspecific Immunotherapy

Nonspecific immunotherapy is perhaps one of the oldest of all immunotherapy concepts, based on pioneering works started more than 100 years ago by Coley, who introduced the first successful anticancer immunotherapy by treating human sarcoma patients with a mixture of bacterial products called "Coley's toxin" (Coley 1991). Since then, the idea of a nonspecific boosting of the immune system became an appealing approach to treat cancer patients.

Different strategies have been exploited in the veterinary field to potentiate patient's immune cells to attack cancer. Among them, several studies have investigated the antitumor potential of recombinant cytokines, such as interferon-gamma (IFN-γ), interleukin (IL)-2, IL-12, IL-15, and others, administered as soluble factors, encapsulated in liposomes, or delivered by using viral and nonviral gene vectors (Whitley et al. 1995; Finocchiaro et al. 2008; Cutrera et al. 2008; Finocchiaro and Glikin 2012; Dow et al. 2005; Finocchiaro et al. 2018; Kamstock et al. 2006; Cemazar et al. 2010; Pavlin et al. 2012; Kruth 1998; Khanna et al. 1996). Overall, either as local intratumoral or as systemic treatments, these approaches promise to be safe and effective for large animals with various spontaneous tumor types, including MM and OSA.

Also, activated monocytes and macrophages have been of interest for the treatment of cancer-bearing dogs. The activation of these immune effector cells may lead to tumor cells elimination by both direct lysis and the release of tumoricidal pro-inflammatory cytokines. This nonspecific antitumor immunity proved to be strongly effective in veterinary medicine when a randomized, double-blinded

Table 2 Most relevant immunotherapy trials in canine MM and OSA patients

	Therapy	Tumor type	Study	References
Recombinant cytokines	Lipid-complexed plasmid DNA+cGM-CSF/cIL-2	Malignant melanoma	Phase I/II prospective trial	Dow (1998)
	hIL-2+hGM-CSF HSV-tk suicide gene therapy	Melanoma	Phase I/II prospective non-randomized trial	Finocchiaro et al. (2008, Finocchiaro and Glikin 2012)
Bacteria-based	L-MTP-PE	Osteosarcoma	Randomized trial	Kurzman et al. (1995)
		Melanoma	Phase II prospective trial	MacEwen et al. (1999)
Immune-modulation	TLR9 agonist + IDO inhibitor	Malignant melanoma and soft tissue sarcoma	Pilot trial	Monjazeb et al. (2016)
Checkpoint inhibitors	Anti-PD-L1	Oral malignant melanoma and undifferentiated sarcoma	Pilot trial	Maekawa et al. (2017)
	Anti-PD-1	Multiple tumor types	Phase I trial (Ongoing)	Dow (2020); Coy (2017)
Vaccines	Allogeneic whole tumor cell vaccine expressing hgp100	Malignant melanoma	Phase II prospective trial	Alexander et al. (2006)
	Autologous DC + hgp100	Malignant melanoma	Pilot study	Gyorffy et al. (2005)
	Autologous DC loaded with canine MM cell lysate	Malignant melanoma	Undefined	Tamura et al. (2008)
	Listeria monocytogenes (Lm)-based vaccine expressing a chimeric human HER2 fusion protein	Osteosarcoma	Phase I dose escalation trial	Mason et al. (2016)
	DNA vaccine huTyr	Oral malignant melanoma	Retrospective study	Ottnod et al. (2013)
	DNA vaccine huCSPG4	Oral malignant melanoma	Phase I prospective trial	Riccardo et al. (2014a, b); Piras et al. (2017)

clinical trial demonstrated the efficacy of the immunomodulatory agent liposome-encapsulated lipophilic derivative of muramyl dipeptide (L-MTP-PE) for the treatment of OSA-bearing dogs after surgical resection of the primary tumor (Kurzman et al. 1995; Lascelles et al. 2005). When combined with chemotherapy, L-MTP-PE resulted effective in counteracting metastatic spread and in improving dogs' survival as compared to placebo-treated controls, enhancing both monocyte activation and macrophage cytotoxic activity against OSA cells (Kleinerman 1995; Morello et al. 2011; Macewen et al. 1989; Shi et al. 1993; Kurzman et al. 1995).

Similarly, when administered following surgery in spontaneous stage I OMM dogs, L-MTP-PE alone was successful in significantly prolonging canine patients' survival (Almela and Ansón 2019; MacEwen et al. 1999). Interestingly, on the basis of the positive results obtained in veterinary oncology, this immunotherapeutic strategy was approved as a possible adjuvant treatment for human pediatric OSA patients (Meyers et al. 2008).

A more recent veterinary study demonstrated the efficacy of a combined immune-modulation approach for the treatment of sarcoma and MM canine patients. The treatment consists of radiotherapy associated with the intra-tumor delivery of a conventional immune-stimulatory toll-like receptor 9 (TLR9) agonist, CpG oligodeoxynucleotide (CpG), and the systemic blockade of the indolamine-2,3-dioxygenase (IDO), a molecule able to suppress T and NK cells and to sustain T-regulatory and myeloid-derived suppressor cells (Monjazeb et al. 2016). This study highlighted the importance of combining the stimulation of the immune system and the parallel inhibition of immunosuppressive pathways, in order to achieve a stronger clinical effect.

Among the nonspecific immunotherapeutic approaches, a special mention is dedicated to monoclonal antibodies (mAb) directed against the immune checkpoint molecules.

2.2 Checkpoint Inhibitors

Immune checkpoints including the cytotoxic T lymphocyte antigen-4 (CTLA-4), the programmed cell death receptor-1 (PD-1), and its ligand (PD-L1), are surface molecules that strictly regulate T-cell priming and activation. Briefly, during T-cell priming, a key co-stimulatory signal is provided by the interaction between B7–1 and B7–2 molecules expressed on antigen-presenting cells with the CD28 receptor on T-cells. Soon after T-cells are activated, when they are still in the secondary lymphoid organ, they also upregulate CTLA-4, which is necessary to attenuate and regulate T-cell function. The competitive binding of CTLA-4 to B7 molecules results in the inhibition of T-cell activation. Consequently, anti-CTLA-4 mAb may restore T-cell stimulation mainly in the secondary lymphoid organs (Wei et al. 2018). Also, following long-term stimulation, the PD-1 receptor is upregulated by T cells, while its ligand PD-L1 can be highly expressed on cancer cells, leading to T-cell inhibition following receptor–ligand interaction. Therefore, mAb targeting the PD-1/PD-L1 axis may enhance the functional properties of effector T cells inside the

tumor (Wei et al. 2018). As such, immune checkpoint inhibitors, i.e., mAb against these molecules, could play a critical role in fighting against cancer-associated immune suppression and evasion.

Considering the clinical relevance of these molecules for human oncology, the expression of these checkpoints has been investigated also in canine tumors (Maekawa et al. 2014; Tagawa et al. 2016). Although their functions in veterinary medicine still need to be entirely clarified, the use of immune checkpoint inhibitors for canine cancer treatment is currently under investigation, considering the revolution they have brought in the human immune-oncology field (Darvin et al. 2018).

Interestingly, an elevated CTLA-4 expression has been observed in canine sarcoma patients compared to healthy controls (Tagawa et al. 2016), as well as a high PD-L1 expression has been found in canine tumor samples of different types, including MM, OSA, and hemangiosarcoma; while PD-1 upregulation has been determined on tumor-infiltrating lymphocytes (Maekawa et al. 2014; Maekawa et al. 2017; Anderson and Modiano 2015). Overall, these results provided the first evidence of the possible therapeutic benefit of immune checkpoint blockade in canine cancer patients (Maekawa et al. 2017). On the basis of the results obtained in human clinical trials using the Food and Drug Administration (FDA)-approved mAb against CTLA-4 (ipilimumab) and against PD-1 (nivolumab, pembrolizumab, cemiplimab) or PD-L1 (atezolizumab, avelumab, and durvalumab) (Vaddepally et al. 2020; Darvin et al. 2018; Wilky 2019), veterinary studies have started focusing on the blockade of the PD-1/PD-L1 axis, considered as the more promising. Recently, a chimeric rat-dog anti-PD-L1 mAb has been generated (Maekawa et al. 2017) and a pilot study has been conducted in OMM and sarcoma-bearing dogs. The treatment was well-tolerated and safe, with neither toxicity nor autoimmunity evidence. Furthermore, treated dogs showed a slight increase in the MST as compared to historical controls (Maekawa et al. 2017; Borgatti et al. 2020). Additionally, a fully canine anti-PD-1 mAb (ca-4F12-E6) has been developed and its efficacy has been tested both in vitro and in vivo in stage IV OMM-bearing dogs enrolled in a pilot clinical study (Igase et al. 2020). Hence, its possible approval is expected (Dow 2020).

2.3 Monoclonal Antibodies Against Tumor-Associated Antigens

Due to their target specificity, therapeutic mAb have rapidly become a milestone of cancer treatment. From the first FDA-approved mAb, the anti-CD-20 Rituximab, more than 75 mAb have been approved for the treatment of human cancers until now (Bergman and Clifford 2019). Spurred by the significant results obtained following Rituximab administration in human clinics, several veterinary groups have focused on the development of similar therapeutics for the benefit of cancer-bearing dogs. However, as very few tumor antigens have been characterized in dogs, just a few mAb for veterinary oncology have been developed until now, but no one is suitable for the treatment of either MM or OSA. Nevertheless, it is worthy to mention here the clinical relevance of two mAb labeled for the treatment of other tumor types. The

first is a caninized anti-CD20 antibody (Blontress®, AT-004), developed since Rituximab did not show to cross-react with the canine CD20 (Impellizeri et al. 2006), and licensed in 2015 for the treatment of dogs with B-cell lymphoma (Bergman and Clifford 2019). However, no proof supports that this mAb exerts a real survival benefit in treated dogs as compared to conventionally treated controls. Secondly, in 2012, the significant homology between canine and human epidermal growth factor receptor (EGFR) was described, demonstrating that the canine protein contains highly conserved epitopes for the antibody Cetuximab, a chimeric human-mouse anti-EGFR mAb. Canine EGFR targeting by means of Cetuximab proved to inhibit canine mammary carcinoma tumor cells in vitro (Singer et al. 2012). However, this antibody could be possibly recognized as foreign by the canine immune system and induce hypersensitivity when administered to dogs. Therefore, to prevent adverse effects, a caninized Cetuximab-like "225 IgG" antibody was generated. This so-called can225IgG was obtained by fusing the variable regions of murine precursor of Cetuximab to canine constant gamma-regions. This mAb showed a strong tumor-inhibitory potential against EGFR-overexpressing canine cancer cells, comparable to the results previously obtained with Cetuximab. Furthermore, can225IgG was also effective in mediating antibody-dependent cellular phagocytosis (Fazekas-Singer et al. 2017).

Spurred by these positive results, several groups have been focusing on the identification of relevant antigens involved in canine MM and OSA progression that could be used as target for mAb treatment.

2.4 CAR-T Cells

The ex vivo engineering of T cells to target Tumor-Associated Antigens (TAA) is one of the most promising strategies currently under investigation in the field of cancer immunotherapy. With the aim of enhancing target specificity, patients-derived T cells are modified in order to express Chimeric Antigen Receptors (CAR). Nevertheless, the use of CAR-T cells in human clinic is still difficult and highly expensive (Townsend et al. 2020) and their application into veterinary medicine is even more challenging. However, the high parallelism that exists between human and canine cancer patients has allowed the assessment of the first-ever CAR-T-cell therapy in a canine clinical trial in leukemia- and B-cell lymphoma-affected dogs. In this small pilot trial, Panjwany and coworkers have demonstrated the feasibility of generating permanently transduced canine CD20-CAR-T cells that are well tolerated in dogs and are able to selectively kill CD20+ target cells (Panjwani et al. 2016; Panjwani et al. 2020).

Following this promising wave, a CAR-T cell therapy has been investigated for the treatment of Human Epidermal Growth Factor Receptor 2 (HER2) + canine OSA, even if till now only by means of in vitro experiments (Mata et al. 2014). Canine T cells expressing HER2-CAR have been demonstrated to recognize and kill

human and canine HER2+ target cells in an antigen-dependent manner (Mata et al. 2014). Preliminary results are encouraging, but improvements to enhance the persistence and the function of canine CAR-T cells in dogs are still needed.

2.5 Vaccines

Among all the immunotherapeutic approaches designed to fight cancer, a special regard should be dedicated to antitumor vaccination that represents a very attractive tool thanks to its potential safety, specificity, and long-lasting response (Iezzi et al. 2012; Hollingsworth and Jansen 2019). Actually, major limitations are shared by most of the immunotherapeutic strategies described above, including the onset of toxicities and the short-term efficacy of the clinical response, due to acquired mechanisms of resistance (Wilky 2019; Tran and Theodorescu 2020; Manis 2019). Instead, the concept behind antitumor vaccination is to properly induce a patient's immune system to safely develop his/her own specific humoral and cellular response against cancer cells, with the aspiring goal to develop a life-long immune memory able to protect from recurrences and metastasis. Although the development of an effective immunotherapeutic vaccine is challenging, many different vaccination strategies have been investigated, including cell-, protein-, or peptide-, dendritic cell (DC)-, viral-, bacterial-, and nucleic acid-based vaccines (Lopes et al. 2019; Guo et al. 2013; Song et al. 2018). All these strategies have been successfully tested in preclinical mouse models and a few effectively translated into human clinical trials, although with less impressive achievements (Olson et al. 2018; Pound and Ritskes-Hoitinga 2018). Some of these vaccination strategies are under investigation also in large companion animals, with important results for MM and OSA-bearing dogs (Table 2).

In this regard, an allogeneic whole tumor cell vaccine was tested in a Phase II trial in spontaneously occurring MM-bearing dogs. The vaccine was represented by a canine melanoma cell line transfected with the human gp100 melanoma-associated antigen and killed by irradiation. This approach resulted in a modest overall response rate and tumor control because of the induction of anti-hgp100 antibodies and enhanced peripheral blood mononuclear cell (PBMC) cytotoxicity. An enhanced post-vaccination delayed-type hypersensitivity (DTH) response was correlated with the positive clinical response to the treatment. However, the induced tumor response was not further characterized (Alexander et al. 2006).

DC-based vaccines currently represent a powerful immunization strategy in the setting of canine MM. By using this approach, bone marrow-derived autologous DC loaded ex vivo with relevant antigens, following reinjection in the patient are supposed to traffic to the lymphoid tissues and activate an antigen-specific T-cell response (Sabado et al. 2017). As an important example, evidence of the antitumor potential of autologous canine DC transfected ex vivo with an adenovirus expressing

the human melanoma antigen gp100 and administered in MM-bearing dogs, was reported by Gyoffry and coworkers (Gyorffy et al. 2005).

Tamura and colleagues supported the potential of using autologous DC in the treatment of canine MM. Following vaccination with autologous DC that were pulsed with lysates from a canine MM cell line, the induction of T cell-mediated immunity was demonstrated by delayed-type hypersensitivity skin testing in vaccinated dogs. Recruitment of both CD8 and CD4 T cells was detected, confirming that the vaccination was effective in eliciting a T cell-mediated response (Tamura et al. 2008; Atherton et al. 2016).

Among the bacterial-based immunization strategies, the most successful example in the veterinary field till now is directed against one of the most relevant OSA-associated antigens, the tyrosine kinase receptor (RTK) HER2. HER2 is overexpressed in 40% of canine OSA. Its overexpression is linked to a reduced response to chemotherapy, higher metastatic rates, and shorter survival (Flint et al. 2004; Mason et al. 2016). For this reason, a recombinant *Listeria monocytogenes* (*Lm*)-based vaccine expressing a chimeric human HER2 fusion protein was developed and tested in a phase I clinical trial, with the aim of preventing metastatic spreading in OSA-bearing dogs. The vaccine hampered lung metastasis development and prolonged the overall survival of treated OSA canine patients. On the basis of these positive results, a lyophilized formulation of the live *Listeria* vector vaccine (the canine OSA vaccine-live Listeria vector; COV-LLV) received a conditional license by the United States Department of Agriculture (USDA) in 2017 for the adjuvant treatment of dogs with OSA (Mason et al. 2016; Flickinger et al. 2018). However, the safety of this vaccine has been recently contested. The occurrence of important adverse events, such as *Listeria* abscess and severe infections following administration, was reported in some treated dogs. Considering also the potential hazard of zoonotic spread of the disease, not only for canine patients receiving the vaccine, but also for the health care workers and family caring for the pet, the vaccine license has been terminated by the company (Musser et al. 2019; Musser et al. 2021).

Therefore, despite some promising attempts, the application of these types of vaccines is still limited due to a number of logistical challenges. For example, as mentioned above, the potential dangers associated with a live virus or bacteria use or the presence of preexisting neutralizing anti-vector immune responses in the patients are among the limitations in the use of viral and bacterial vaccines (Saxena et al. 2013; Agrawal 2019). In the case of DC-based strategies, that require the preparation of patient-specific vaccines, the high production costs represent an additional limitation.

On the contrary, the high versatility, ease of production, and low cost of manufacturing combined with the high stability and easy scalability of nucleic acid-based vaccines, in particular DNA plasmids, may provide important advantages to further develop this approach into effective cancer therapies. For this reason, a specific session will be dedicated to this type of vaccines.

3 DNA Vaccines

Antitumor vaccines based on gene transfer have received great attention in the immune-oncology field over the last decades. A DNA vaccine basically consists of a bacterial DNA plasmid containing multiple cloning sites. This allows the insertion of the desired antigen-coding sequences which induce the immune response, and possibly also sequences coding for immune-modulating molecules. High expression of the sequences is allowed by the presence of a ubiquitarian enhancer-promoter and a transcription termination site. The presence of a bacterial replication origin and an antibiotic selection gene, which confers the antibiotic resistance, allows the efficient plasmid replication in bacterial cells and their selection (Iezzi et al. 2012; Riccardo et al. 2017).

For these properties, as compared to other vaccine platforms, DNA vaccines have the advantage of being simple and relatively inexpensive to produce (Hollingsworth and Jansen 2019) thanks to the use of large-scale bacterial cultures; they are temperature-stable and produced in large quantities conferring an advantage for both transport and storage. Of note, DNA plasmids are able to ensure antigen expression over long periods of time in vivo (Li and Petrovsky 2016). Together, these aspects make DNA vaccines an appealing strategy for veterinary medicine applications (Table 2).

Ideally, an anticancer vaccine should be specific enough to discriminate between cancer and normal cells, able to eliminate small or large numbers of tumor cells and be effective in preventing recurrences and metastasis over time (Cavallo et al. 2007; Riccardo et al. 2017). In this context, significant efforts must be focused on the choice of the best tumor antigen to target (Cavallo et al. 2007; Riccardo et al. 2017; Rolih et al. 2017). Tumor antigens are proteins that display significant expression in the tumor tissue but not in healthy cells and play a key role in cancer progression (Cavallo et al. 2007; Iezzi et al. 2012; Rolih et al. 2017). Based on their different nature, these antigens can be classified into two main types. Those derived from mutated self-proteins, named tumor-specific antigens (TSA), or non-mutated self-proteins that are aberrantly expressed in cancer cells, defined as tumor associated antigens (TAA; Lopes et al. 2019; Cavallo et al. 2007; Iezzi et al. 2012; Cavallo et al. 2014).

However, it must be noted that TAA derives from naturally occurring self-proteins and as such, the development of effective immune-targeting is challenging. By means of complex mechanisms regulating central and peripheral immune tolerance, B and T cells are tightly controlled to ensure effective elimination of foreign antigens while maintaining immune tolerance to self-antigens (Romagnani 2006). Therefore, with the aim of eliciting a proper immune response, circumventing self-tolerance, exploiting orthologous sequences from different species is required for the design of effective DNA vaccines. Immunization with DNA vaccines coding for xenogeneic antigens that share a significant homology with the self-antigen have shown to trigger a better immune response as compared to self-homologous ones, as it was first demonstrated by Naftzger and colleagues in the setting of murine melanoma studies (Naftzger et al. 1996). Xenogeneic antigens act as “altered self”

proteins (Cavallo et al. 2014) and are recognized by the immune system as non-self-antigens as they are not identical to the self-antigen at the sequence level. The subtle differences in one or more epitopes between the xenogeneic and the native sequence of the antigen could be enough to overcome immune tolerance and induce both B and T cell responses against the foreign protein as well as being able to cross-react with the self-homologous TAA (Cavallo et al. 2014; Riccardo et al. 2017; Quaglino et al. 2011).

The efficacy of this strategy has been extensively demonstrated in several murine models of cancer both in prophylactic and therapeutic settings. Moreover, this xenovaccination approach has recently been tested also in veterinary cancer patients (Riccardo et al. 2014a, b; Bergman et al. 2006; Grosenbaugh et al. 2011; Liao et al. 2006; Kamstock et al. 2007; Peruzzi et al. 2010; Manley et al. 2011).

In this context, a relevant achievement was obtained in 2010, when the xenogeneic DNA vaccine ONCEPT® was approved by the USDA for the treatment of OMM-bearing dogs (Bergman and Clifford 2019). ONCEPT® is a DNA-plasmid vaccine encoding the human tyrosinase (huTyr), which has a 91% similarity with the canine protein (Bergman et al. 2003). It was licensed as an adjuvant treatment for stage II and III OMM-bearing dogs, and reported to induce both cellular and antibody responses. The antibody response was quite unexpected, given the intracellular nature of tyrosinase glycoprotein, normally inaccessible to antibodies. The authors postulated that the canine tyrosinase could be expressed at low levels on the cell surface, thus representing a target for the vaccine-induced antibodies (Liao et al. 2006). Of note, a correlation between the antibody titer and clinical response was observed. Tyrosinase targeting by means of xenogeneic DNA vaccination has been also proposed for the treatment of canine digit MM, where its efficacy in improving the survival of vaccinated patients as compared to controls treated with surgery alone was reported (Manley et al. 2011).

However, the reliability of ONCEPT® has been questioned (Ottnod et al. 2013) and several studies reported its efficacy with contradictory results (Almela and Ansón 2019). Moreover, some discrepancies in the setting of the study that led to USDA approval have been highlighted, including the inclusion criteria, the low number of dogs enrolled, and the fact that 50% of dogs were censored from the analysis (Almela and Ansón 2019).

Nevertheless, to date ONCEPT® is the first and only approved anticancer DNA-based treatment, raising up a renewed enthusiasm for the development of novel DNA vaccines against different TAA.

The possibility to elicit an antibody response is particularly attractive when the target antigen is a surface antigen. In this context, the very low-affinity antibody production induced by xenogeneic vaccines is an important limitation for their application purposes (Quaglino et al. 2011; Riccardo et al. 2017). The use of hybrid DNA plasmids could overcome this limitation. Hybrid plasmids are designed to code for chimeric TAA, composed of both heterologous and homologous domains (Riccardo et al. 2017). The innovation and the potential efficacy of these plasmids relie on the hybrid nature of the TAA sequence, encompassing both a homologous

portion, which ensures the specificity of the immune response, and a xenogeneic portion, which is instrumental in circumventing the immune tolerance.

The slight differences in the amino acid sequence and in the tertiary structure of the chimeric protein, encoded by the hybrid plasmids, may result in the exposition of subdominant and/or new conformational epitopes, triggering an even more efficient humoral and cellular immune response than that induced by the fully xenogeneic or homologous vaccines. The effectiveness of this approach has been investigated till now in murine models (Quaglino et al. 2010; Jacob et al. 2010; Riccardo et al. 2017), however, the first application of this chimeric concept also in veterinary oncology is ongoing (Riccardo et al., unpublished results).

Besides plasmid optimization, the antitumor efficacy of a DNA vaccine relies on the application of the most efficient plasmid delivery method as well.

4 Application of Electroporation to DNA Vaccination

Besides plasmid optimization, the efficacy of a DNA vaccine relies on the delivery method as well. Indeed, naked plasmid DNA injection has revealed to be a major issue in making DNA vaccination an efficient procedure. This is especially evident when DNA vaccines are translated from smaller species, such as mice, to large animals, such as dogs. It has been postulated that the different composition of the extracellular matrix of the target cells among species could impair the efficacy of naked DNA vaccines (Impellizeri et al. 2014). Moreover, the volume of the DNA solution injected has shown to play a major role as well, being small volumes unable to produce an extent of hydrodynamic pressure strong enough to allow a high plasmid transfection efficiency and trigger a proper immune response (Wolff and Budker 2005). However, the precise mechanisms by which naked DNA injection in large animal species fails to induce an effective immune response still need to be exhaustively clarified (Impellizeri et al. 2014).

To overcome this limitation, to date, several delivery methods for DNA vaccines have been investigated, and some of them found successful clinical application. This is the case of the intradermal delivery via high-pressure needle-free devices, which led to the veterinary development of the ONCEPT® vaccine described above. DNA tattooing or gene gun methods are other examples of delivery methods that have effectively shown to potentiate immunogenicity of DNA vaccines (Lopes et al. 2019). Finally, among the delivery systems that are clinically available, electroporation is one of the most promising.

DNA vaccination through in vivo electroporation (electrovaccination) has been used since 1970 and represents a great ally for improving the efficacy of antitumor immunization.

Basically, electroporation is a transfection method consisting in the delivery of short electric pulses by means of electrodes, which causes the formation of transient pores in the cell membrane**.** In the case of electrovaccination, the delivery of a pulse immediately after the plasmid injection—either in dermis or muscles—and the consequent transient and reversible permeabilization of the cellular membrane,

leads to a significant plasmid uptake (Sardesai and Weiner 2011; Kraynyak et al. 2017). In this way, the plasmid route through the cell membrane is facilitated as compared to simple naked DNA administration. Indeed, the electrical gradient inside the tissue allows the electrophoretic movement of negatively charged DNA molecules into the target cell nucleus, where the coding antigen cloned inside the plasmid can be transcribed into mRNA and subsequently translated into protein. As the electrical field ends, the pores on the membrane rapidly close (Aurisicchio et al. 2013; Impellizeri et al. 2014; Quaglino et al. 2011; Riccardo et al. 2017). Depending on the type of transfected tissue and type of electrodes used, the optimal pulse parameters need to be properly chosen. Indeed, an efficient gene delivery into muscle cells is easier as compared to skin or tumors, because a lower electric field is required (Lambricht et al. 2016).

The delivery of electric pulses following DNA plasmid injection not only improves target cells transfection and antigen production, but also participates in the generation of a stronger immune response as compared to naked DNA injection alone. This is because electroporation can act as an adjuvant by locally affecting the tissue integrity and eventually attracting an inflammatory infiltrate (Iezzi et al. 2012). Indeed, the local tissue damage that is initially caused by electric pulses promotes the secretion of inflammatory cytokines and chemokines. Particularly, in the case of intramuscular injection, the combination of needle insertion and electric pulses induces a great extent of local cell death and inflammation. This event leads to the infiltration of both granulocytes and monocytes to the injured site and subsequently, differentiated macrophages and DC gain access to the necrotic fibers. When transfected cells start to express the DNA vaccine-encoded gene, local DC, B- and T-cell recruitment occurs with the final induction of both adaptive and innate immune responses (Iezzi et al. 2012; Riccardo et al. 2017).

Overall, it is clear that electroporation appears to positively affect DNA plasmid efficacy even when low doses of DNA vaccine are used, by significantly increasing antigen delivery of 100- to 1000-folds, and its immunogenicity as compared to naked DNA delivery alone, and actually in general to the other delivery systems exploited till now (Sardesai and Weiner 2011; Kraynyak et al. 2017).

Nonetheless, there are also some concerns on electrovaccination that must be taken into account. In order to target the right cells, electrovaccination requires the optimization of many parameters. If the electric pulses are not adequate to the specific features of the target tissue, cell death can occur. To avoid these issues, the electric field strength and the resulting transmembrane voltage, and the number and duration of the applied electric pulses are key factors that must be properly balanced in order to achieve the right extent of membrane permeabilization. Moreover, the choice of the appropriate pulse generator and electrodes must be adequate (Lambricht et al. 2016).

Overall, in vivo electrovaccination has widely demonstrated to be effective in inducing a strong and long-lasting antigen-specific immune response in both humans (Daud et al. 2008; Low et al. 2009; Heller and Heller 2015; Aurisicchio et al. 2020) and animals, including dogs (Riccardo et al. 2014a, b; Piras et al. 2017; Cutrera et al. 2008; Peruzzi et al. 2010; Impellizeri et al. 2018). The cost-effectiveness and the

safety profile of this approach allow repeated low-dose administrations over long periods, thus assuring a long-term protection (Lambricht et al. 2016). This has led to the introduction of electrovaccination as a tool for cancer treatment in several veterinary studies, including ours.

5 Case Study: DNA Vaccination with In Vivo Electroporation in Canine Oral Malignant Melanoma and Osteosarcoma

Over the last years, we have focused our studies on testing the clinical relevance of electrovaccination in canine OMM and OSA. To this end we analyzed the possible shared targets between the two tumor types, identifying the chondroitin sulfate proteoglycan (CSPG)4 as one of the most promising.

5.1 CSPG4

CSPG4 is a cell surface type I transmembrane protein covalently modified with chondroitin sulfate glycosaminoglycans (Price et al. 2011). It retains a limited expression on pericytes during neovascularization, whereas it is lowly expressed or absent in quiescent and stable vessels and in adult healthy tissues (Price et al. 2011; Rolih et al. 2017; Nicolosi et al. 2015). In contrast, it is highly expressed in several solid tumors where it orchestrates multiple tumor-related processes. Indeed, CSPG4 functions as a co-receptor for several RTK (Price et al. 2011) sustaining the activation of downstream oncogenic signaling pathways, i.e., the MAPK cascade. Moreover, CSPG4 can cooperate with integrin signaling through focal adhesion kinase (FAK), whose activation plays a key role in the survival and growth of tumor cells (Yang et al. 2019; Price et al. 2011).

CSPG4 was initially found overexpressed on over 85% of human melanomas (Wilson et al. 1981). Later, it was proved to be expressed in a wide range of solid tumors and on cancer stem cells (CSC) as well (Wang et al. 2010a, b; Beard et al. 2014; Nicolosi et al. 2015; Tarone et al. 2019; Riccardo et al. 2019). Moreover, CSPG4 could play an important role also in the tumor microenvironment, being highly expressed on activated pericytes and promoting tumor vascularization by mediating pericyte interactions with endothelial cells (Schlingemann et al. 1990; Huang et al. 2010; You et al. 2014). Notably, CSPG4 is highly abundant and homogeneously distributed also in metastasis of some tumors including soft tissue sarcoma and triple-negative breast cancer (Cattaruzza et al. 2013; Nicolosi et al. 2015). Therefore, its targeting could provide a strong therapeutic potential to fight against the tumor at three different levels: against the primary lesions, against its metastatic spread and against the CSC compartment, considered responsible for recurrences, metastasis, and treatment resistance (Fig.1).

CSPG4 targeting by using different immunotherapeutic strategies, including mAbs, CAR-T cells, and anti-idiotypic antibodies, has already been documented in several studies (Jamil et al. 2016; Rivera et al. 2012; Wang et al. 2005, 2010a, b;

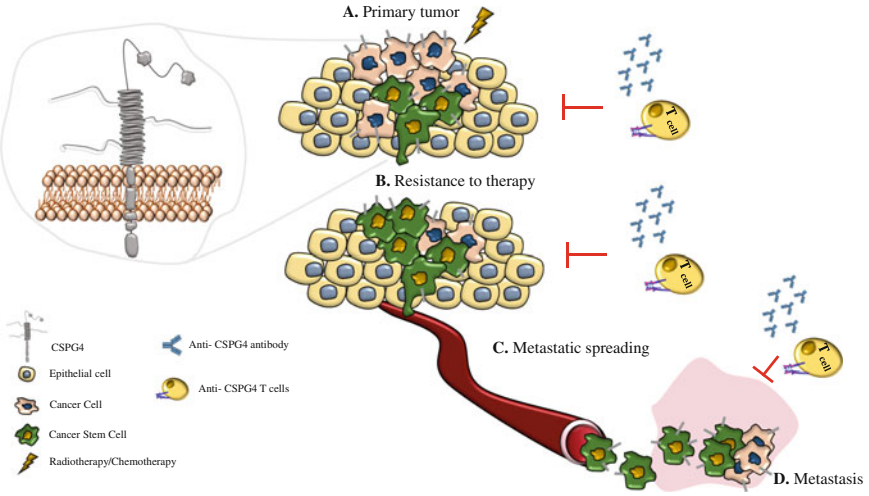

Fig. 1 Anti-CSPG4 immune-targeting potential in fighting against cancer progression. When a primary tumor is detected, conventional therapies are exploited (**a**). Mainly CSC results resistant to conventional treatments (**b**) and through mechanisms of extravasation could enter into the blood-stream (**c**) and reach distant organs where they can give rise to metastasis (**d**). CSPG4 is overexpressed on both cancer cells and CSC, therefore its immune-targeting could contribute to the killing of cancer cells in the primary tumors (**a**), could impair the therapeutic resistance (**b**) and the spreading of CSC (**c**), and could act against the metastatic lesions (**d**)

Mittelman et al. 1992, 1995). A first human clinical study reports the success of anti-CSPG4 immunization by means of anti-idiotypic antibody (MK2–23) vaccination in advanced melanoma patients. This strategy was effective in both reducing metastasis and in prolonging patients' survival because of the eliciting of a strong immune response. Of note, it was later suggested that the great success of this method was also because of the presence of already existing anti-CSPG4 autoantibodies (Mittelman et al. 1995; Nicolosi et al. 2015). This evidence shows that CSPG4 represents an attractive antigen to target by means of immunotherapy in the human setting.

5.2 CSPG4 in canine OMM and OSA

Until 10 years ago nothing was known about CSPG4 expression and function in canine tumors. CSPG4 overexpression was identified for the first time by our group in 57% canine OMM samples analyzed in a cohort of 65 dogs by means of immunohistochemistry (Mayayo et al. 2011). Actually, the percentage of CSPG4 expression in canine OMM was underrated in that study for different reasons, and our recent, still unpublished, data indicate a percentage of expression in OMM cases of more than 80%. Undoubtedly, the use of an anti-human CSPG4 antibody for its detection, that could not strongly cross-react with the canine orthologous, could have

been responsible for a reduced signal in some samples. However, since CSPG4 expression in tumor samples was comparable to the well-established Melan A (Koenig et al. 2001) and PNL2 (Giudice et al. 2010) canine MM markers, we suggested CSPG4 as both a potential marker and a good target for immunotherapy in canine MM (Mayayo et al. 2011).

Recently, we have shown that anti-CSPG4 immuno-targeting could represent a successful strategy also for the treatment of canine OSA (Riccardo et al. 2019). Indeed, CSPG4 is overexpressed in about 80% of OSA samples we analyzed. Furthermore, its overexpression is strictly related to patients' poor prognosis. In fact, canine patients affected by CSPG4-positive OSA showed a shorter survival as compared with CSPG4-negative ones, with a MST of 271 and 440 days, respectively (Riccardo et al. 2019).

5.3 CSPG4 Electrovaccination in Canine OMM and OSA

Since CSPG4 is a self-antigen with relatively poor immunogenicity in autologous hosts (Wang et al. 2005), the aforementioned advantages of using xenogeneic DNA vaccination in breaking immune tolerance against self-target antigens led us to consider the potentiality of using this strategy at first in CSPG4+ OMM-bearing dogs (Tarone et al. 2019; Riccardo et al. 2014a, b; Piras et al. 2017). Of note, since the amino acid sequence of CSPG4 is highly evolutionarily conserved, with 88% similarity between human and canine proteins, we supposed this approach would have been effective in breaking host self-tolerance and triggering an effective immune response able to cross-react against the canine antigen. With this idea, we investigated the anti-tumor potential of a xenogeneic human (Hu)-CSPG4 DNA vaccine in association with in vivo electroporation as an adjuvant therapeutic strategy for the treatment of client-owned dogs affected by spontaneous stage II-III, CSPG4+ OMM (Fig. 2).

A strong humoral immune response against both the human and the canine CSPG4 antigen was detected in electrovaccinated dogs as compared to controls only treated with surgery (Riccardo et al. 2014a, b). Recently, we have demonstrated that the Hu-CSPG4 vaccine is effective in inducing not only anti-CSPG4 IgG but also a certain degree of anti-CSPG4 IgA, fundamental to block recurrences in the oral cavity by inducing mucosal protection (Tarone et al. 2019). On the contrary, a low frequency of circulating canine CSPG4 reactive T cells was detected following anti-Hu-CSPG4 electrovaccination (Riccardo et al. 2014a, b). However, this could be due also to the limited efficacy of T-cell-based assays in veterinary oncology at the time of the investigation.

Overall, from the clinical point of view, this approach resulted in a significant increase of the MST and disease-free interval (DFI) of vaccinated dogs (653 days and 477 days, respectively) as compared to those treated with surgery alone (220 days and 180 days, respectively) (Riccardo et al. 2014a, b). The 1-year survival of dogs was significantly longer in patients that received the adjuvant Hu-CSPG4 vaccination as compared to conventionally treated controls, with 6 treated patients

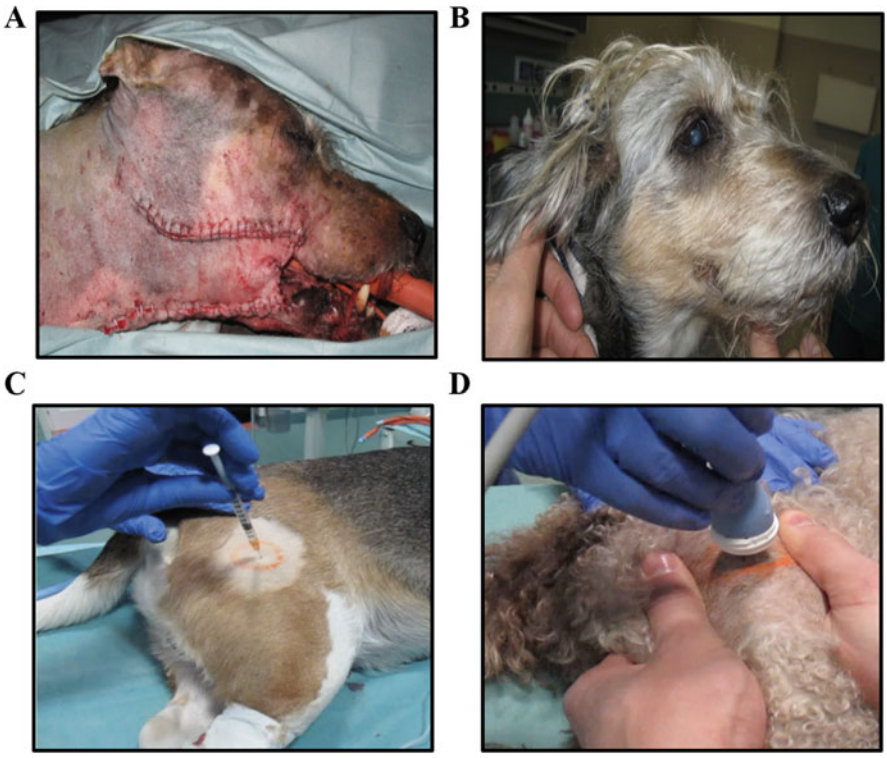

Fig. 2 Adjuvant electrovaccination procedure in MM-bearing dogs. Postsurgical appearance of a MM-bearing dog after resection of the cheek and reconstruction with an advancement flap (**a**). The same dog after 4 months from surgery (**b**). 3–4 weeks after the surgical removal of the primary tumor the adjuvant electrovaccination started. Intramuscular injection of 500 μg of Hu-CSPG4 DNA plasmid was performed in the posterior thigh leg muscles (**c**). In vivo electroporation through electrodes by using the Cliniporaotor (IGEA, Carpi, Italy) device was performed (**d**). Electrical pulses applied: 1 high voltage HV, 450 V, length 50 μs, frequency 3 HZ, followed by 8 low voltage LV, 110 V, length 20 ms, interval 300 ms

still alive at the end of the observation period (1694 days), suggesting that the vaccination was able to confer a long-term immune protection against tumor recurrences and metastasis (Riccardo et al. 2014a, b; Piras et al. 2017; Tarone et al. 2019). It has also to be noted that the clinical efficacy of the vaccine was lower when the primary tumor expressed low levels of CSPG4 antigen. This could be probably linked to the presence of CSPG4-negative tumor clones within the lesion, which eventually could be responsible for the progression of the disease (Piras et al. 2017). Despite the relevant clinical efficacy of this strategy, some Hu-CSPG4-treated canine patients died because of metastasis and tumor relapse, therefore the improvement of this strategy could be of paramount importance to reach more effective clinical achievements.

As a step forward in this direction, with the idea to exploit our expertise (Quaglino et al. 2010, 2011; Riccardo et al. 2017) and the advantages described above regarding the application of a chimeric DNA vaccination, we developed a second-generation anti-CSPG4 DNA vaccine that codes for a chimeric human/dog protein. A new prospective, multicentric veterinary clinical trial has been approved by the Italian Ministry of Health and started in order to evaluate the potential chimeric benefit of the human/dog-CSPG4 DNA electrovaccination for the adjuvant treatment of client-owned dogs affected by CSPG4+ OMM. Promising results have been collected until now, opening up an appealing leading edge for the clinical development of such a immunotherapeutic strategy for OMM-bearing dogs (Riccardo in preparation; Giacobino et al. 2021).

Moreover, recently we demonstrated the potential of CSPG4 immune targeting also in the canine OSA setting. Indeed, our data suggested that anti-CSPG4 mAb and Hu-CSPG4-DNA vaccine-induced antibodies, alone or in combination with chemotherapy, are effective in hampering canine OSA cell tumorigenicity in vitro, in terms of both proliferation and migration (Riccardo et al. 2019). Moreover, when the potential of anti-CSPG4 antibodies was investigated in vitro against CSPG4-expressing OSA-derived CSC, a strong inhibition of osteospheres generation and chemoresistance was observed (Riccardo et al. 2019), with appealing implications for the fighting against the "stem compartment" of the tumor, considered responsible for metastasis and therapeutic failure.

Overall, these findings hold the promise to have a great impact on the treatment of canine OSA, and all the CSPG4-expressing tumors, raising the possibility of an effective combination with standard of care and anti-CSPG4 electrovaccination. On this positive wave, a new prospective veterinary clinical trial has been approved by the Italian Ministry of Health and is ongoing for the evaluation of the antitumor potential of adjuvant anti-CSPG4 DNA electrovaccination in association with chemotherapy for the treatment of client-owned dogs affected by spontaneous CSPG4-positive, stage I-II, OSA.

6 Conclusions

MM and OSA are among the most common and fatal cancer types arising in pet dogs. Despite the great efforts of veterinarians and researchers, the standard of care for these oncological diseases has not significantly changed in the last decades, prompting an urgent need for novel and more effective anticancer therapies.

As a step forward in this direction, immunotherapy has resurfacing over the last years, thanks to the encouraging results obtained in the fight against cancer in human patients. This shifted a renewed interest in cancer immunotherapy in veterinary oncology as antitumor vaccination raises an appealing approach for the treatment of canine patients, resulting in the advancement of several immunization strategies.

In the last years, we have been focusing on the development of an effective adjuvant DNA-based vaccination protocol for the treatment of both OMM and OSA canine patients, with the final aim to induce a specific and long-lasting immune

response, effective in protecting against recurrences and metastasis, the principal cancer-related cause of death. With this in mind, CSPG4 arises as an ideal oncoantigen to be immunologically targeted in canine OMM and OSA. For the optimization of this anti-CSPG4 DNA vaccination, in vivo electroporation clearly shines as one of the most appealing approaches, being dramatically effective in improving DNA vaccine immunogenicity, thanks to its ability to promote i) the entering of DNA plasmids inside the cells, ii) the long-lasting expression of the target antigen, and iii) a pro-inflammatory environment, that finally potentiates the specific immune response.

Overall, our investigations highlight the safety, immunogenicity and the clinical relevance of anti-CSPG4 electrovaccination, laying the foundation to include this strategy as a future standard of care for the treatment of all CSPG4-positive canine tumors.

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References

Agrawal B (2019) Heterologous immunity: role in natural and vaccine-induced resistance to infections. Front Immunol:10

Alexander AN et al (2006) Development of an allogeneic whole-cell tumor vaccine expressing xenogeneic gp100 and its implementation in a phase II clinical trial in canine patients with malignant melanoma. Cancer Immunol Immunother 55(4):433–442

Almela RM, Ansón A (2019) A review of immunotherapeutic strategies in canine malignant melanoma. Vet Sci 6(1)

Anderson KL, Modiano JF (2015) Progress in adaptive immunotherapy for cancer in companion animals: success on the path to a cure. Vet Sci 2(4):363–387

Atherton MJ et al (2016) Cancer immunology and canine malignant melanoma: a comparative review. Vet Immunol Immunopathol 169:15–26

Aurisicchio L, Mancini R, Ciliberto G (2013) Cancer vaccination by electro-gene-transfer. Exp Rev Vaccines 11(10):1–11. Available from http://www.ncbi.nlm.nih.gov/pubmed/24066796

Aurisicchio L et al (2020) Safety, tolerability and immunogenicity of V934/V935 hTERT vaccination in cancer patients with selected solid tumors: a phase I study. J Transl Med 18(1):39

Beard RE et al (2014) Multiple chimeric antigen receptors successfully target chondroitin sulfate proteoglycan 4 in several different cancer histologies and cancer stem cells. J Immunotherapy Cancer 2(1):25. Available from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4155770&tool=pmcentrez&rendertype=abstract

Bergman PJ (2007) Canine Oral Melanoma. Clin Tech Small Anim Pract 22(2):55–60

Bergman PJ, Clifford CA (2019) Recent advancements in veterinary oncology. Vet Clin North Am Small Anim Pract 49(5):xiii–xiv

Bergman PJ et al (2003) Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin Cancer Res 9(4):1284–1290

Bergman PJ et al (2006) Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the animal medical center. Vaccine 24(21):4582–4585

Borgatti A, Dickerson EB, Lawrence J (2020) Emerging therapeutic approaches for canine sarcomas: pushing the boundaries beyond the conventional. Vet Comp Oncol 18(1):9–24

Bosenberg M, Arnheiter H, Kelsh R (2014) Melanoma in mankind's best friend. Pigment Cell Melanoma Res 27(1):1
Boston SE et al (2014) Efficacy of systemic adjuvant therapies administered to dogs after excision of oral malignant melanomas: 151 cases (2001–2012). J Am Vet Med Assoc 245(4):401–407. https://doi.org/10.2460/javma.245.4.401
Bowlt Blacklock KL et al (2019) Genome-wide analysis of canine oral malignant melanoma metastasis-associated gene expression. Sci Rep 9(1)
Cattaruzza S et al (2013) NG2/CSPG4-collagen type VI interplays putatively involved in the microenvironmental control of tumour engraftment and local expansion. J Mol Cell Biol 5 (3):176–193
Cavallo F, Calogero RA, Forni G (2007) Are oncoantigens suitable targets for anti-tumour therapy? Nat Rev Cancer 7(9):707–713. https://doi.org/10.1038/nrc2208
Cavallo F et al (2014) Xenogene vaccination in the therapy of cancer. Expert Opin Biol Ther 14 (10):1427–1442
Cemazar M, Jarm T, Sersa G (2010) Cancer Electrogene therapy with Interleukin-12. Curr Gene Ther 10(4):300–311
Coley WB (1991) The treatment of malignant tumors by repeated inoculations of erysipelas: with a report of ten original cases. Clin Orthop Relat Res 262:3–11
Coy J (2017) PD-1 expression by canine T cells and functional effects of PD-1 blockade. Vet Comp Oncol 15(4):1487–1502
Cutrera J et al (2008) Intratumoral bleomycin and IL-12 electrochemogenetherapy for treating head and neck tumors in dogs. Method Mol Biol (Clifton, N.J.) 423:319–325
Darvin P et al (2018) Immune checkpoint inhibitors: recent progress and potential biomarkers. Exp Mol Med 50(12):165
Daud AI et al (2008) Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. J Clin Oncol 26(36):5896–5903
Davis BW, Ostrander EA (2014) Domestic dogs and cancer research: a breed-based genomics approach. ILAR J 55(1):59–68
Dow SW (1998) In vivo tumor transfection with superantigen plus cytokine genes induces tumor regression and prolongs survival in dogs with malignant melanoma. J Clin Invest 101(11):2406–2414
Dow S (2020) A role for dogs in advancing Cancer immunotherapy research. Front Immunol 10:2935
Dow S et al (2005) Phase I study of liposome-DNA complexes encoding the interleukin-2 gene in dogs with osteosarcoma lung metastases. Hum Gene Ther 16(8):937–946
Fan TM, Khanna C (2015) Comparative aspects of osteosarcoma pathogenesis in humans and dogs. Vet Sci 2(3):210–230
Fazekas-Singer J et al (2017) Development of a radiolabeled caninized anti-EGFR antibody for comparative oncology trials. Oncotarget 8(47):83128–83141
Finocchiaro LME, Glikin GC (2012) Cytokine-enhanced vaccine and suicide gene therapy as surgery adjuvant treatments for spontaneous canine melanoma: 9 years of follow-up. Cancer Gene Ther 19(12):852–861
Finocchiaro LME et al (2008) Suicide gene and cytokines combined nonviral gene therapy for spontaneous canine melanoma. Cancer Gene Ther 15(3):165–172
Finocchiaro LME et al (2018) Combination of suicide and cytokine gene therapies as surgery adjuvant for canine mammary carcinoma. Vet Sci 5(3):70
Flickinger JC, Rodeck U, Snook AE (2018) Listeria monocytogenes as a vector for cancer immunotherapy: current understanding and progress. Vaccine 6(3):48
Flint AF et al (2004) Overexpression of the erbB-2 proto-oncogene in canine osteosarcoma cell lines and tumors. Vet Pathol 41(3):291–296
Gardner HL, Fenger JM, London CA (2016) Dogs as a model for Cancer. Ann Rev Anim Biosci 4 (1):199–222

Giacobino D et al (2021) Difference in outcome between curative intent vs marginal excision as a first treatment in dogs with oral malignant melanoma and the impact of adjuvant CSPG4-DNA electrovaccination: a retrospective study on 155 cases. Vet Comp Oncol. https://doi.org/10.1111/vco.12690

Giudice C et al (2010) Immunohistochemical investigation of PNL2 reactivity of canine melanocytic neoplasms and comparison with Melan a. J Vet Diagn Investig 22(3):389–394

Grosenbaugh DA et al (2011) Safety and efficacy of a xenogeneic DNA vaccine encoding for human tyrosinase as adjunctive treatment for oral malignant melanoma in dogs following surgical excision of the primary tumor. Am J Vet Res 72(12):1631–1638

Guo C et al (2013) Therapeutic cancer vaccines. Past, present, and future. In: Advances in Cancer Research, pp 421–475

Gyorffy S et al (2005) Bone marrow-derived dendritic cell vaccination of dogs with naturally occurring melanoma by using human gp100 antigen. J Vet Intern Med 19(1):56–63

Heller R, Heller LC (2015) Gene Electrotransfer clinical trials. Adv Genet 89:235–262

Hernandez B et al (2018) Naturally occurring canine melanoma as a predictive comparative oncology model for human mucosal and other triple wild-type melanomas. Int J Mol Sci 19 (2):394

Hollingsworth RE, Jansen K (2019) Turning the corner on therapeutic cancer vaccines. npj Vaccines 4(1):7

Huang FJ et al (2010) Pericyte deficiencies lead to aberrant tumor vascularizaton in the brain of the NG2 null mouse. Dev Biol 344(2):1035–1046

Iezzi M et al (2012) DNA vaccination against oncoantigens: a promise. OncoImmunology 1 (3):316–325

Igase M et al (2020) A pilot clinical study of the therapeutic antibody against canine PD-1 for advanced spontaneous cancers in dogs. Sci Rep 10(1):18311

Impellizeri JA, Ciliberto G, Aurisicchio L (2014) Electro-gene-transfer as a new tool for cancer immunotherapy in animals. Vet Comp Oncol 12(4):310–318

Impellizeri JA et al (2006) The role of rituximab in the treatment of canine lymphoma: an ex vivo evaluation. Vet J 171(3):556–558

Impellizeri JA et al (2018) Tel-eVax: a genetic vaccine targeting telomerase for treatment of canine lymphoma. J Transl Med 16(1):349

Jacob JB et al (2010) Combining human and rat sequences in Her-2 DNA vaccines blunts immune tolerance and drives antitumor immunity. Cancer Res 70(1):119–128

Jamil NSM et al (2016) Functional roles of CSPG4/NG2 in chondrosarcoma. Int J Exp Pathol 97 (2):178–186

Kamstock D et al (2006) Liposome-DNA complexes infused intravenously inhibit tumor angiogenesis and elicit antitumor activity in dogs with soft tissue sarcoma. Cancer Gene Ther 13 (3):306–317

Kamstock D et al (2007) Evaluation of a xenogeneic VEGF vaccine in dogs with soft tissue sarcoma. Cancer Immunol Immunother 56(8):1299–1309

Khanna C et al (1996) Aerosol delivery of interleukin 2 liposomes is nontoxic and biologically effective: canine studies. Clin Cancer Res 2(4):721–734

Kleinerman ES (1995) Biologic therapy for osteosarcoma using liposome-encapsulated muramyl tripeptide. Hematol Oncol Clin North Am 9(4):927–938

Klingemann H (2018) Immunotherapy for dogs: running behind humans. Front Immunol 9:133

Koenig A et al (2001) Expression of S100a, vimentin, NSE, and Melan a/MART-1 in seven canine melanoma cell lines and twenty-nine retrospective cases of canine melanoma. Vet Pathol 38 (4):427–435

Kraynyak KA, Bodles-Brakhop A, Bagarazzi M (2017) Tapping the potential of DNA delivery with electroporation for cancer immunotherapy. In: *Current topics in microbiology and immunology*, pp 55–78

Kruger S et al (2019) Advances in cancer immunotherapy 2019–latest trends. J Exp Clin Cancer Res 38(1):268

Kruth SA (1998) Biological response modifiers: interferons, interleukins, recombinant products, liposomal products. Vet Clin North Am Small Anim Pract 28(2):269–295
Kudnig ST, Séguin B (2013) Veterinary surgical oncology,
Kurzman ID et al (1995) Adjuvant therapy for osteosarcoma in dogs: results of randomized clinical trials using combined liposome-encapsulated Muramyl tripeptide and cisplatin. Clin Cancer Res 1(12):1595–1601
Lambricht L et al (2016) Clinical potential of electroporation for gene therapy and DNA vaccine delivery. Expert Opin Drug Deliv 13(2):295–310
Lascelles BDX et al (2005) Improved survival associated with postoperative wound infection in dogs treated with limb-salvage surgery for osteosarcoma. Ann Surg Oncol 12(12):1073–1083
Lazarides AL et al (2017) A dog in the Cancer fight: comparative oncology in osteosarcoma. In: Osteosarcoma–Biology, behavior and mechanisms
Li L, Petrovsky N (2016) Molecular mechanisms for enhanced DNA vaccine immunogenicity. Expert Rev Vaccines 15(3):313–329
Liao JCF et al (2006) Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. Cancer Immunity 6:8
Lopes A, Vandermeulen G, Préat V (2019) Cancer DNA vaccines: current preclinical and clinical developments and future perspectives. J Exp Clin Cancer Res 38(1):146
Low L et al (2009) DNA vaccination with electroporation induces increased antibody responses in patients with prostate cancer. Hum Gene Ther 20(11):1269–1278
Macewen EG et al (1989) Therapy for osteosarcoma in dogs with intravenous injection of liposome-encapsulated muramyl tripeptide. J Natl Cancer Inst 81(12):935–938
MacEwen EG et al (1999) Adjuvant therapy for melanoma in dogs: results of randomized clinical trials using surgery, liposome-encapsulated muramyl tripeptide, and granulocyte macrophage colony-stimulating factor. Clin Cancer Res 5(12):4249–4258
Maekawa N et al (2014) Expression of PD-L1 on canine tumor cells and enhancement of IFN-γ production from tumor-infiltrating cells by PD-L1 blockade. PLoS One 9(6):e98415
Maekawa N et al (2017) A canine chimeric monoclonal antibody targeting PD-L1 and its clinical efficacy in canine oral malignant melanoma or undifferentiated sarcoma. Sci Rep 7(1):8951
Makielski KM et al (2019) Risk factors for development of canine and human osteosarcoma: a comparative review. Vet Sci 6(2):48
Manis JP (2019) Overview of therapeutic monoclonal antibodies. UpToDate:1–24
Manley CA et al (2011) Xenogeneic murine Tyrosinase DNA vaccine for malignant melanoma of the digit of dogs. J Vet Intern Med 25(1):94–99
Mason NJ et al (2016) Immunotherapy with a HER2-targeting listeria induces HER2-specific immunity and demonstrates potential therapeutic effects in a phase I trial in canine osteosarcoma. Clin Cancer Res 22(17):4380–4390
Mata M et al (2014) Toward immunotherapy with redirected T cells in a large animal model: ex vivo activation, expansion, and genetic modification of canine T cells. J Immunother 37 (8):407–415
Mayayo SL et al (2011) Chondroitin sulfate proteoglycan-4: a biomarker and a potential immunotherapeutic target for canine malignant melanoma. Vet J 190(2):e26–e30
Meyers PA et al (2008) Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival–a report from the children's oncology group. J Clin Oncol 26 (4):633–638
Mittelman A et al (1992) Human high molecular weight melanoma-associated antigen (HMW-MAA) mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: induction of humoral anti-HMW-MAA immunity and prolongation of survival in patients with stage IV melanoma. Proc Natl Acad Sci 89(2):466–470
Mittelman A et al (1995) Human high molecular weight-melanoma associated antigen mimicry by mouse anti-Idiotypic monoclonal antibody mk2-23: modulation of the immunogenicity in patients with malignant melanoma. Clin Cancer Res 1(7):705–713

Mochel JP et al (2019) CAR T cell immunotherapy in human and veterinary oncology: changing the odds against hematological malignancies. AAPS J 21(3):50
Monjazeb AM et al (2016) Blocking indolamine-2,3-dioxygenase rebound immune suppression boosts antitumor effects of radio-immunotherapy in murine models and spontaneous canine malignancies. Clin Cancer Res 22(17):4328–4340
Morello E, Martano M, Buracco P (2011) Biology, diagnosis and treatment of canine appendicular osteosarcoma: similarities and differences with human osteosarcoma. Vet J 189(3):268–277
Musser ML et al (2019) Vaccine strain Listeria monocytogenes abscess in a dog: a case report. BMC Vet Res 15(1)
Musser ML et al (2021) Safety evaluation of the canine osteosarcoma vaccine, live Listeria vector. Vet Comp Oncol 19(1):92–98
Naftzger C et al (1996) Immune response to a differentiation antigen induced by altered antigen: a study of tumor rejection and autoimmunity. Proc Natl Acad Sci U S A 93(25):14809–14814
Nicolosi PA, Dallatomasina A, Perris R (2015) Theranostic impact of NG2/CSPG4 proteoglycan in cancer. Theranostics 5(5):530–544
Nishiya A et al (2016) Comparative aspects of canine melanoma. Vet Sci 3(1):7. Available at: http://www.mdpi.com/2306-7381/3/1/7
Olson B et al (2018) Mouse models for cancer immunotherapy research. Cancer Discov 8 (11):1358–1365
Ostrander EA, Dreger DL, Evans JM (2019) Canine Cancer genomics: lessons for canine and human health. Ann Rev Animal Biosci 7(1):449–472
Ottnod JM et al (2013) A retrospective analysis of the efficacy of Oncept vaccine for the adjunct treatment of canine oral malignant melanoma. Vet Comp Oncol 11(3):219–229
Panjwani MK et al (2016) Feasibility and safety of RNA-transfected CD20-specific chimeric antigen receptor T cells in dogs with spontaneous B cell lymphoma. Mol Ther 24(9):1602–1614
Panjwani MK et al (2020) Establishing a model system for evaluating CAR T cell therapy using dogs with spontaneous diffuse large B cell lymphoma. OncoImmunology 9(1):1676615
Pavlin D et al (2012) IL-12 based gene therapy in veterinary medicine. J Transl Med 10(1):234
Peruzzi D et al (2010) A vaccine targeting telomerase enhances survival of dogs affected by B-cell lymphoma. Mol Ther 18(8):1559–1567
Piras LA et al (2017) Prolongation of survival of dogs with oral malignant melanoma treated by en bloc surgical resection and adjuvant CSPG4-antigen electrovaccination. Vet Comp Oncol 15 (3):996–1013
Pound P, Ritskes-Hoitinga M (2018) Is it possible to overcome issues of external validity in preclinical animal research? Why most animal models are bound to fail. J Transl Med 16(1):304
Price M a et al (2011) CSPG4, a potential therapeutic target, facilitates malgnant progression of melanoma. Pigment Cell Melanoma Res 24(6):1148–1157
Prouteau A, André C (2019) Canine melanomas as models for human melanomas: clinical, histological, and genetic comparison. Genes 10(7)
Quaglino E et al (2010) A better immune reaction to Erbb-2 tumors is elicited in mice by DNA vaccines encoding rat/human chimeric proteins. Cancer Res 70(7):2604–2612
Quaglino E et al (2011) Chimeric DNA vaccines against ErbB2+ carcinomas: from mice to humans. *Cancers* 3(3):3225–3241. https://doi.org/10.3390/cancers3033225
Regan D et al (2016) Cancer immunotherapy in veterinary medicine: current options and new developments. Vet J 207:20–28
Riccardo F, Aurisicchio L et al (2014a) The importance of comparative oncology in translational medicine. Cancer Immunol Immunother 64(2):137–148
Riccardo F, Iussich S et al (2014b) CSPG4-specific immunity and survival prolongation in dogs with oral malignant melanoma immunized with human CSPG4 DNA. Clin Cancer Res 20 (14):3753–3762
Riccardo F et al (2017) Chimeric DNA vaccines: an effective way to overcome immune tolerance. Curr Top Microbiol Immunol 405:99–122

Riccardo F et al (2019) Identification of CSPG4 as a promising target for translational combinatorial approaches in osteosarcoma. Therapeutic Adv Med Oncol 11:1758835919855491
Rivera Z et al (2012) CSPG4 as a target of antibody-based immunotherapy for malignant mesothelioma. Clin Cancer Res 18(19):5352–5363
Rodriguez CO (2014) Using canine osteosarcoma as a model to assess efficacy of novel therapies: can old dogs teach us new tricks? Adv Exp Med Biol 804:237–256
Rolih V et al (2017) CSPG4: a prototype oncoantigen for translational immunotherapy studies. J Transl Med 15(1):151
Romagnani S (2006) Immunological tolerance and autoimmunity. Intern Emerg Med 1(3):187–196
Sabado RL, Balan S, Bhardwaj N (2017) Dendritic cell-based immunotherapy. Cell Res 27 (1):74–95
Sandru A et al (2014) Survival rates of patients with malignant melanoma. J Med Life 7(4):572–576
Sardesai NY, Weiner DB (2011) Electroporation delivery of DNA vaccines: prospects for success. Curr Opin Immunol 23(3):421–429
Saxena M et al (2013) Pre-existing immunity against vaccine vectors–friend or foe? Microbiology (United Kingdom) 159(1):1–11
Schlingemann RO et al (1990) Expression of the high molecular weight melanoma-associated antigen by pericytes during angiogenesis in tumors and in healing wounds. Am J Pathol 136 (6):1393–1405. Available from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1877594&tool=pmcentrez&rendertype=abstract
Selmic LE et al (2014) Comparison of carboplatin and doxorubicin-based chemotherapy protocols in 470 dogs after amputation for treatment of appendicular osteosarcoma. J Vet Intern Med 28 (2):554–563
Shi F, MacEwen EG, Kurzman ID (1993) In vitro and in vivo effect of doxorubicin combined with liposome-encapsulated Muramyl tripeptide on canine monocyte activation. Cancer Res 53 (17):3986–3991
Simpson S et al (2017) Comparative review of human and canine osteosarcoma: morphology, epidemiology, prognosis, treatment and genetics. *Acta veterinaria Scandinavica* 59(1):71
Singer J et al (2012) Comparative oncology: ErbB-1 and ErbB-2 homologues in canine cancer are susceptible to cetuximab and trastuzumab targeting. Mol Immunol 50(4):200–209
Song Q, Zhang CD, Wu XH (2018) Therapeutic cancer vaccines: from initial findings to prospects. Immunol Lett 196:11–21
Tagawa M et al (2016) Evaluation of costimulatory molecules in peripheral blood lymphocytes of canine patients with histiocytic sarcoma. PLoS One 11(2):e0150030
Tamura K et al (2008) Induction of dendritic cell-mediated immune responses against canine malignant melanoma cells. Vet J 175(1):126–129
Tarone L et al. (2019) Naturally occurring cancers in pet dogs as pre-clinical models for cancer immunotherapy. Cancer Immunology, Immunotherapy
Townsend MH et al (2020) Paving the way towards universal treatment with allogenic T cells. Immunol Res 68(1):63–70
Tran L, Theodorescu D (2020) Determinants of resistance to checkpoint inhibitors. Int J Mol Sci 21 (5):1594
Vaddepally RK et al (2020) Review of indications of FDA-approved immune checkpoint inhibitors per NCCN guidelines with the level of evidence. Cancers 12(3):738
Varshney J et al (2016) Understanding the osteosarcoma pathobiology. Veterinary sciences 3(1):3. https://www.scopus.com/inward/record.uri?partnerID=HzOxMe3b&scp=85008317850&origin=inward
Wang X et al (2005) Human high molecular weight melanoma-associated antigen mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: enhancement of immunogenicity of anti-idiotypic monoclonal antibody MK2-23 by fusion with interleukin 2. Cancer Res 65 (15):6976–6983
Wang X et al (2010a) CSPG4 protein as a new target for the antibody-based immunotherapy of triple-negative breast cancer. J Natl Cancer Inst 102(19):1496–1512

Wang X-X et al (2010b) CSPG4 in cancer: multiple roles. *Current molecular medicine* 10 (4):419–429. Available from http://www.ncbi.nlm.nih.gov/pubmed/20455858

Wei SC, Duffy CR, Allison JP (2018) Fundamental mechanisms of immune checkpoint blockade therapy. Cancer Discov 8(9):1069–1086

Whitley EM et al (1995) Modulation by canine interferon-γ of major histocompatibility complex and tumor-associated antigen expression in canine mammary tumor and melanoma cell lines. Anticancer Res 15(3):923–929

Wilky BA (2019) Immune checkpoint inhibitors: the linchpins of modern immunotherapy. Immunol Rev 290(1):6–23

Wilson BS et al (1981) Distribution and molecular characterization of a cell-surface and a cytoplasmic antigen detectable in human melanoma cells with monoclonal antibodies. Int J Cancer 28(3):293–300

Withrow SJ, Vail DM, Page RL (2012) Withrow and MacEwen's small animal clinical oncology, 5th edn

Wolff JA, Budker V (2005) The mechanism of naked DNA uptake and expression. Adv Genet 54:1–20

Yang J et al (2019) Chondroitin sulfate proteoglycan 4 enhanced melanoma motility and growth requires a cysteine in the core protein transmembrane domain. Melanoma Res 29(4):365–375

You WK et al (2014) NG2 proteoglycan promotes tumor vascularization via integrin-dependent effects on pericyte function. Angiogenesis 17(1):61–76

Part IV

Evolution of the Field and New Applications with Electroporation-Based Treatments: Outlook

Electrodes for Unique Anatomical Access in Electroporation

F. Maglietti, M. Tellado, and J. Impellizeri

Abstract

Electroporation-based treatments have consistently proven their usefulness in the treatment of superficial tumors with high response rates, regardless of the histology and virtual absence of side effects. The natural evolution of the field moves toward extending the applications to internal organs, and for that purpose, novel electrodes are being designed. Most of them focus on the idea of minimal invasion to preserve its virtual absence of side effects. Of course, this comes with a price, reaching the tumor and delivering an adequate electric field to it increases the difficulty of the procedure when compared with standard approaches. For this reason, treatment planning and image guidance are mandatory for reaching maximum response rates. In this chapter, we present the following electrodes: SiNE, SN-HFIRE, Endo-Ve, Brain Electrode, NeLIe, Pulsed Field Ablation System, Curved Electrode, Finger Electrode, and the independent placing of needles. Between all of them, a great number of new applications for electroporation-based treatments are possible. Most of the research for developing electrodes for human medicine is performed in animals. In this sense, these developments are closer to its application in veterinary oncology than in human oncology, giving veterinary medicine an advantage of innovation for extended applications of ECT.

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Electrochemotherapy · Irreversible electroporation · Probes · Oncology · Cancer · Deep-seated tumors · Tumor · Minimal invasion · Electroporation

1 Introduction

Electroporation-based treatments have proven their effectivity and applicability in tissues that are easily reached, such as the skin, muscle, or even the mouth (Mali et al. 2013). They have been combined with surgery to access internal organs, increasing its applications (Spugnini et al. 2006; Cemazar et al. 2008; Edhemovic et al. 2014; Lowe et al. 2017).

Surgical access enables the user to treat virtually any organ using the standard electrodes available for electroporation, i.e., arrays of needles or plates. But to avoid complications derived from the surgery or reduce the recovery time, minimally invasive electrodes are available. Designing electrodes to deliver the electric field in a minimally invasive way, requires creativity, accurate electric field simulations, and advanced knowledge of the anatomical area to be treated (Kotnik et al. 2012). The main challenge of this approach is to cover the whole tumor and its margin with an electric field above the electroporation threshold. Any area left untreated will lead to relapse (Gehl et al. 2018). The best way to assess the electric field distribution generated by the novel electrode is by computer simulations. When the simulations are combined with imaging procedures from a patient, a very precise treatment planning can be performed. Again, during the actual procedure, image guidance can be very useful to reproduce the treatment planning.

Preparing to be utilized in human medicine, all novel designs of electrodes are tested in animals, as preclinical models for human disease. For veterinary medicine, these studies are valid, and though they are not veterinary clinical trials, they provide very valuable information.

Another very interesting fact is that human patients are mostly consistent anatomically with variations in size as the main variable whereas veterinary medicine encompasses various anatomies and various sizes. The veterinarian can treat a small cat or a horse. This requires not only needles or plates but also needles of different lengths and thicknesses. These different designs improve results of the treatment but may not add any different capability to access a certain organ and will not be discussed here.

In this chapter, we are going to explore different and very original electrode designs for unique anatomical access.

2 The Electrodes

Electrochemotherapy, Irreversible Electroporation, Calcium Electroporation, and Gene Electrotransfer are treatments based on electroporation. Even with the differences in optimum pulse parameters (which require different pulse generator capabilities), they share a common fundamental aspect of delivering an adequate electric field to the area intended to be treated. Even if a particular electrode was developed for a specific electroporation-based treatment, they can be used with any of the techniques above, provided the adequate pulse parameters for each are known. Among them, electrochemotherapy has a very interesting characteristic that is its selectivity toward dividing cells. This makes the therapy very appealing for treating extended margins, reducing the risk of relapse without compromising healthy tissue. The selectivity is obtained by the mechanism of action of the two drugs that can be used in this therapy, bleomycin and cisplatin (Mir 2006).

In this chapter, information regarding electrode characteristics, target organ, and for which electroporation treatment is intended, is presented (see Fig. 1).

3 Single Needle Electrode

Nasal tumors in canine patients are uncommon and are usually diagnosed late in the course of the disease when the patient reveals common clinical signs which may include epistaxis, mucopurulent nasal discharge, facial deformity, sneezing, dyspnea, local pain, stertorous breathing, exophthalmos, and ocular discharge. All of

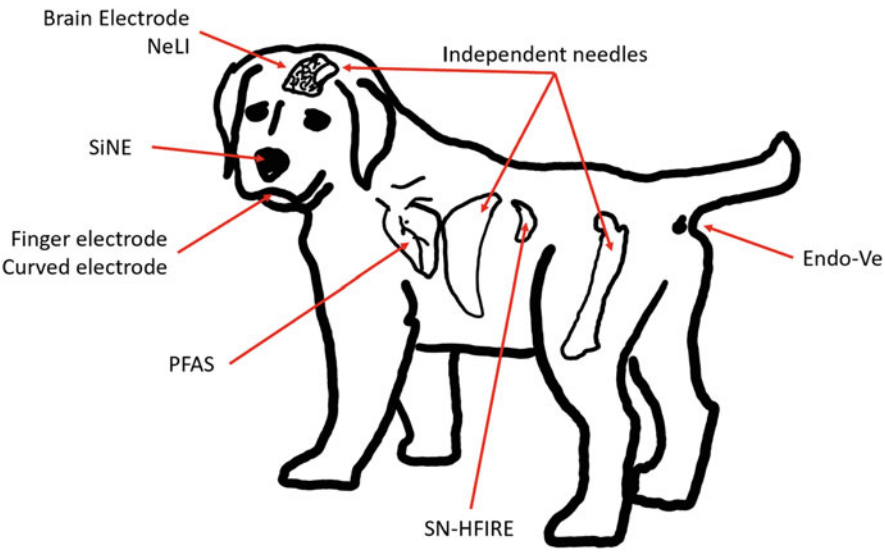

Fig. 1 Image depicting organs treatable with electroporation-based treatments using specially designed electrodes

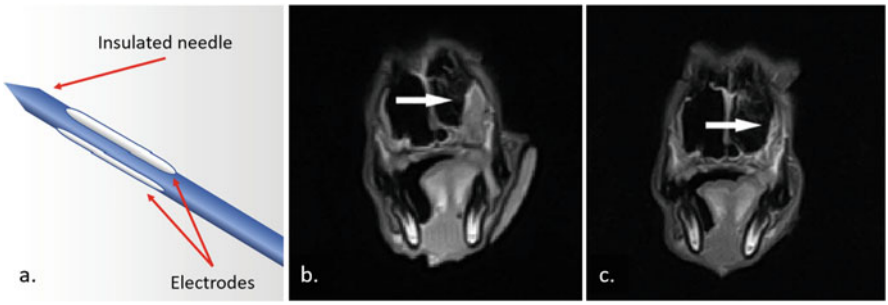

Fig. 2 (**a**) SiNE electrode. (**b**) MRI of a canine patient with an adenocarcinoma pre-treatment with electrochemotherapy using the SiNE, the white arrow shows the tumor in the lateral side of the nasal cavity. In (**c**) after a Complete Response

them greatly affect the quality of life of the patients. The first-line treatment for these tumors is surgery plus radiotherapy or radiotherapy alone, but this therapy is not readily available, or the cost is beyond acceptance to most pet owners as an out-of-pocket expense (Withrow et al. 2019). In addition, radiotherapy has many acute and chronic side effects such as oral mucositis, rhinitis, keratoconjunctivitis, and blepharitis, keratoconjunctivitis, anterior uveal hemorrhage, brain necrosis, seizures, optic nerve degeneration, and osteonecrosis. Acute complications can last up to eight weeks, and many of the chronic ones are not treatable negatively affecting quality of life (Maruo et al. 2011).

The Single Needle Electrode (SiNE) is an electrode developed for treating nasal tumors in canine patients with electrochemotherapy. It was validated in canine patients with spontaneous tumors of the nasal duct obtaining an OR rate of 91% (see Fig. 2a). The electrode consists of two plates mounted on an insulated needle (see Fig. 2b and c). The area that can be treated is an elliptical cylinder, centered in the electrode providing a treatment radius extending 0.85 cm. Electrical treatment parameters for this electrode are 32 pulses 100 μs long of 1000 V/cm at 10 Hz. The authors reported that the procedure was very well tolerated, the only side effect observed was the inflammation of the treated nasal passage, which was very well controlled with corticosteroid therapy for one week. Median survival was reported as 16.5 months (range 4–32) which is comparable to the results obtained with external beam radiotherapy (Maglietti et al. 2017).

This electrode was also used to treat extended margins with oral malignant melanoma with nasal invasion, reducing the chances of relapse (Tellado et al. 2020).

A similar electroporation system for the nasal cavity is the Nasal-Cath, and a flexible version for treating urethral tumors, the Uro-Cath both electrodes of the EPV-100 electroporator (BIOTEX SRL, Buenos Aires, Argentina).

4 The Curved Electrode

Oral tumors account for 3–12% of all tumors in dogs, and for 6% of all tumors in cats. The most common are malignant melanoma, squamous cell carcinoma, fibrosarcoma, and acanthomatous ameloblastoma in dogs, and squamous cell carcinomas and fibrosarcomas in cats. In all cases, surgery is the preferred treatment option, and in combination with radiotherapy, improves tumor control (Withrow et al. 2019). But its combination radiotherapy is not always an option. Electrochemotherapy is now a very common treatment for tumors in the oral cavity. Treating tumors in this location can pose a great challenge as there is very little space to place the electrodes, besides the interference of the teeth and bone along with the possibility of receiving a bite when the pulses are delivered as secondary muscle contraction is expected with the technique.

The Curved Electrode Handle from the EPV-100 electroporator (BIOTEX SRL, Buenos Aires, Argentina) is an electrode that can be fitted either with needles or with plates at the tip, designed for electrochemotherapy. The handle is curved 90 degrees to improve the access to the caudal parts of the oral cavity (see Fig. 3). Reaching better the tumors allows the user to adequately cover the whole tumor and margin improving the ability for local control. The optimal pulse parameters for this electrode are 8 pulses 100 μs long of 1000 V/cm at 5 kHz.

4.1 Finger Electrode

Another very interesting approach for the treatment of oral cavity tumors is the finger electrode of the Cliniporator/Clinivet (IGEA, Carpi, Italy), designed for treating mucosal tumors (Gehl et al. 2018). It consists of a thimble with 4 needles which can be either on the tip or on the finger pad. With this electrode, the operator can reach easily different parts of the mouth (Miklavčič et al. 2014). It is mostly used in human

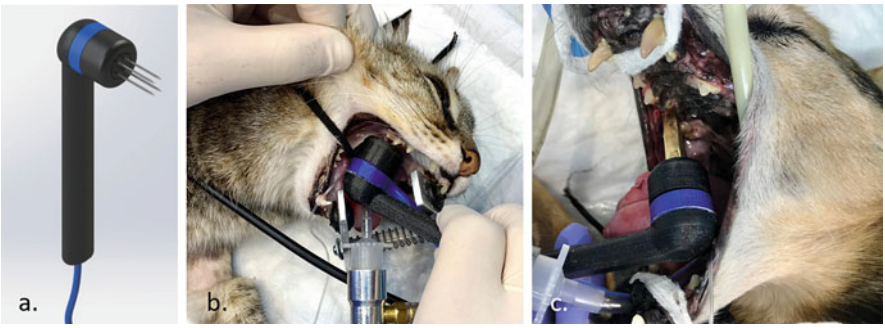

Fig. 3 In (**a**) the Curved Electrode (*image courtesy of BIOTEX SRL*), mainly intended for the treatment of the caudal part of the oral cavity. In (**b**) the electrode handle fitted with needles, and in (**c**) the same electrode handle fitted with the plate electrode

medicine, where it is applied in several locations, at the user's discretion (Bertino et al. 2016).

5 SN-HFIRE Electrode

Pancreatic tumors are a very rare disease in dogs and cats accounting for less than 0.5% of all cancers. Surgical treatment by total pancreatectomy has been described but it has very high morbidity and mortality. The prognosis of this disease is very poor because of its critical location and advanced stage at the time of diagnosis.

Hepatic tumors are also uncommon accounting for less than 1.5% of all tumors in dogs and 1–2.9% of all tumors in cats. The prognosis is determined by histology and morphology, and except for the massive hepatocellular carcinomas and benign tumors, generally, prognosis is not good. Most symptoms are related to the mass effect of the tumor and are nonspecific. The treatment of choice is surgery for most of the cases (Withrow et al. 2019).

The SN-HFIRE (single needle, dual-electrode device for high-frequency irreversible electroporation, AngioDynamics Inc., Latham, NY, USA) is an 18-gauge probe with two electrodes separated with an insulating gap, one in the tip, and the other after the gap. Initially, this electrode was developed for performing a minimally invasive irreversible electroporation treatment and tested in the porcine liver (Wandel et al. 2016). After successful tests, it was used with High-Frequency Irreversible Electroporation (HFIRE), a variation of irreversible electroporation with many advantages: the use of paralytic medications is not required as it does not induce muscle contractions and there is no need to be synchronized with the electrocardiogram, as electric activity of the heart is not affected. A preclinical validation study made in porcine pancreas revealed that the optimum parameters for this electrode are: 300 bursts of 5 μs ON 5 μs OFF 5 μs ON, total ON time 100 μs, 2250 V. The advantage of placing only one needle for the treatment may be diminished by the reduced electric field distribution in the surroundings of the electrode. Because of this, treating a tumor larger than 32 cm^2 would require multiple insertions of the electrode (O'Brien et al. 2019).

6 Brain Electrode

Dogs, cats, and humans are the only mammals that frequently present brain tumors. Studies indicate that intracranial neoplasms are present in necropsies of 2–4.5% of dogs and 2% of cats. In dogs, most commonly seen are meningiomas, gliomas, and tumors of the choroid plexus. In cats, the most common intracranial tumor is the meningioma. Clinical signs and symptoms are due to direct compression or invasion of brain tissue, or due to secondary effects such as edema, neuroinflammation, obstructive hydrocephalus, and intracranial hemorrhage. Regarding treatment, there is a lack of large rigorously designed clinical trials to determine the best treatment modality (Withrow et al. 2019).

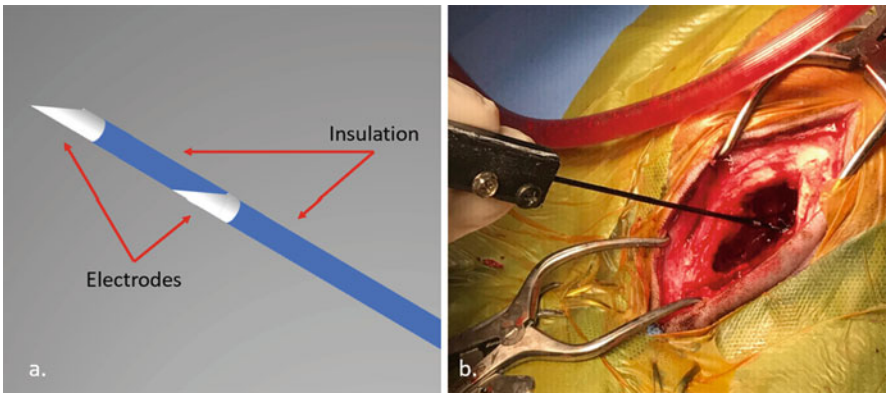

Fig. 4. (**a**) The NeLI electrode. (**b**) The electrode insertion during the treatment of a canine patient with a grade III oligodendroglioma. *Image courtesy by Dr. Impellizeri*

Brain tumors are a very interesting field to extend the applications of electrochemotherapy, as it is quick, effective, localized, and preserves healthy tissue, in this case, normal neurons. The brain electrode is a novel electrode designed for treating brain tumors which consists of a probe that extends the electrodes from its tip, adopting the shape of a cone. After careful treatment planning using MRI images, the electrode is positioned by means of stereotactic equipment and inserted through a burr hole. A clinical trial for brain metastases was performed, but it was terminated due to slow patient recruitment, and only one patient was treated. The patient treated presented no adverse effects, but due to disease progression, the response could not be evaluated. Different ways of using this electrode are being studied, such as in combination with surgery or radiotherapy, or for calcium electroporation. Calcium electroporation is virtually identical to electrochemotherapy, with the only difference of using calcium instead of bleomycin or cisplatin. The therapy preserves selectivity toward dividing cells, allowing to treat margins without compromising healthy tissue, but is less effective (Frandsen et al. 2020).

Patients with brain tumors generally have an unfavorable prognosis and this new approach can provide a better option when other treatments have failed (Linnert et al. 2012, 2014).

Another interesting brain electrode is the NeLIe (Needle Linear Electrodes), developed by Dr. Michinski et al. It is a similar design to the SN-HFIRE but modified for ECT in brain tissue (see Fig. 4). It consists of a conductive tip and base, separated by an insulating material. The distance between electrodes can be varied according to patient's needs and generator capabilities. The area treated is an oval between the conductive parts of the electrode.

7 Endo-Ve

Cancer of the colon and rectum is very common in human patients that consume processed red-meat (Chan et al. 2011; Fernández-Villa et al. 2020), it is the fourth most common cancer in the United States, after breast, lung, and prostate (2015). In contrast, the incidence of colon cancer in dogs and cats is much less common, and cancer is more frequently observed in the small intestine. Surgical excision is the treatment of choice, which may be combined with chemotherapy. Endoscopic removal of the benign lesion has been reported for rectal tumors, with good results improving patient's quality of life (Withrow et al. 2019).

Endo-Ve is an electroporation system developed at the Cork Cancer Center of Ireland. It consists of an electroporation chamber designed to perform endoluminal cancer treatment. It is used with its pulse generator, the ePore (Mirai Medical, Cork, Ireland). It is validated for electrochemotherapy and gene electro transfer in murine, porcine, and canine patients (Forde et al. 2011; Forde et al. 2016). A preclinical validation study has been performed (Wezgowiec et al. 2016).

In human medicine, the Endo-Ve can be also used with the Cliniporator (IGEA, Carpi, Italy), though it was reported that the first version of this device may lack the power needed to use this kind of electrode.

The device consists of a chamber that is attached to the end of an endoscopic device, such as a colonoscope. The treatment chamber uses an outside surgical suction device to draw the lesion inside of the chamber and make contact with the electrodes.

Electrical pulses are delivered 8 min after the IV infusion of bleomycin, or immediately after local injection of the drug. The tissue inside of the chamber and underneath of it is treated with a sufficiently intense electric field (500–1400 V/cm), successfully permeabilizing the exposed cells. As mentioned before, electrochemotherapy is selective towards cancer cells sparing the normal ones. The procedure is performed with general anesthesia both in human and in veterinary medicine, to reduce the discomfort produced by the procedure and by the muscle contractions. It does not require a hospital stay and the patient usually returns home the same day after the procedure. Complications were not reported in preclinical studies, but when it becomes more commonly available for treatment, it is reasonable to expect the same complications as for other endoscopic procedures, i.e., perforation, infection, or bleeding.

The first phase I study in human patients with esophageal cancer was published in 2018. Six patients were recruited and treated with electrochemotherapy using intravenous bleomycin, following the ESOPE recommendations and 8 pulses 100 μs long of 760 V/cm at 5 kHz were delivered. The procedure was very well tolerated, but three out of the six patients required hospitalization due to adverse events. A nasal feeding tube was placed in case edema caused appetite suppression, impeding intake of adequate nutrition of the patients. Not all the tumors were completely treated due to inadequate coverage of the lesion with the electric field, and for that reason, relapses were seen (Egeland et al. 2018). The inadequate coverage may be due to human mistakes rather than a problem with the technology.

In 2020, a multicenter phase I study in patients with colorectal cancer was published with very promising results. Among the seven patients treated they reported only minor side effects. The response was monitored with CT scan, MRI, or endoscopically and was 43% complete response and 57% partial response, meaning 100% objective responses. Some patients required two sessions while the most only one. Alleviation of bleeding was a remarkable result of the procedure that greatly improved the quality of life of the patients. Authors report that some of the lesions could not have been covered with the adequate electric field and for that reason, four of the treated patient experimented relapse (Hansen et al. 2020).

This device was also tested with calcium electroporation showing very promising results. The first patient treated with sigmoid colon cancer experienced an improvement in pain, the bleeding stopped, and the obstruction was relieved (Broholm et al. 2019).

8 Pulsed-Field Ablation System for Cardiac Ablation

Atrial fibrillation is the most commonly diagnosed arrhythmia in dogs. It can produce signs and symptoms of cardiac failure because of decreased cardiac output. Its treatment is based on controlling heart rate by using medication, and if not enough more aggressive treatment may be needed (Saunders et al. 2009). Atrial fibrillation treatment by ablation can be performed by radiofrequency, cryoablation, laser, or ultrasound. All of them can provoke similar complications because of the undesired extension of the ablation area, the most critical being the atrioesophageal fistula (Nair et al. 2015; Kapur et al. 2017). Pulsed-field ablation, as a nonthermal procedure, does not have this problem and virtually eliminates the risks associated to thermal diffusion. An endovascular electrode and a biphasic pulse generator were developed (Farapulse Inc., CA, USA). The catheter itself has 5 splines, each containing 4 electrodes, and is advanced over a guidewire. A study compared it with traditional radiofrequency ablation and showed that animals treated with pulsed-field ablation system induced no chronic histopathologic esophageal changes, while radiofrequency demonstrated a spectrum of esophageal lesions including fistula and deep esophageal ulcers and abscesses (Koruth et al. 2020a). This device can produce ablations of a maximum depth of 9.4 mm and a width of 28.6 mm (Koruth et al. 2020b).

8.1 Placing independent needles

Some tumors arise in very complex locations. They can deep-seated and close to large blood vessels, and surgical removal may not be possible. In these cases, irreversible electroporation or electrochemotherapy can be great options, provided adequate treatment planning is performed. An adequate treatment plan utilizes computer-assisted simulation of the electric field distribution for determining the best placement location for the needles. Some very important aspects of this

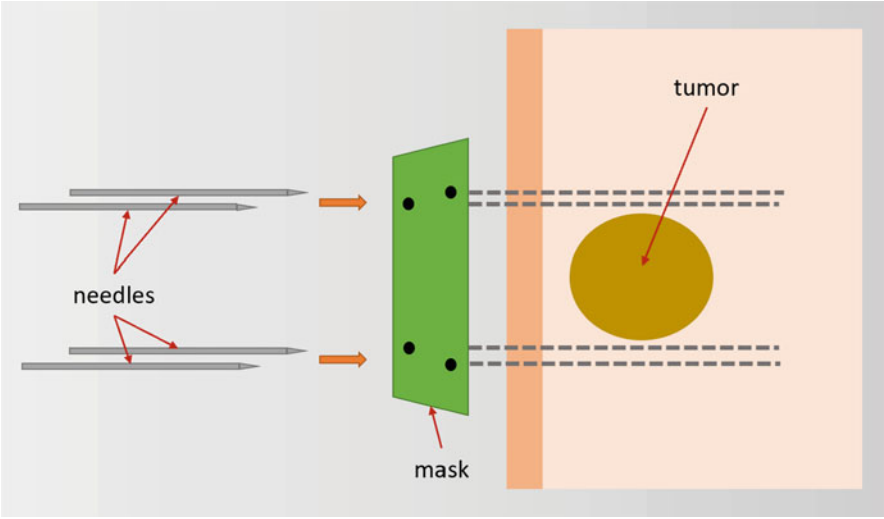

Fig. 5 Scheme of the insertion of needles using a mask to improve parallelism and location in the tumor

approach are to keep needles parallel, to observe the distance between needles and proximity to critical organs. Any deviation will alter the electric field distribution and may leave areas of the tumor untreated, or if the treatment is performed too close to the heart, i.e., less than 1.7 cm, the procedure may induce cardiac arrhythmia. To address this, patient-specific masks or templates can be used for guiding the insertion of the needles into the tissue (see Fig. 5), guaranteeing its parallelism, and combining with ECG synchronization is recommended (Jiang et al. 2015). Image guidance is valuable to confirm the electrode's location, before pulse delivery (Lee et al. 2010b). By this approach, many tumors in many organs can be successfully treated using electrochemotherapy or irreversible electroporation, such as the liver (Lee et al. 2010a; Edhemovic et al. 2014), the pancreas (Martin et al. 2015; Tafuto et al. 2015), the lungs (Deodhar et al. 2011), the brain, the prostate (Onik et al. 2007), and the kidneys (Buijs et al. 2019).

Another location suitable for using independent needles is bone. The nature of bone makes it impossible for the insertion of regular needle electrodes and requires the use of very hard and sharp electrodes. Typically inserted using a surgical drill, in a position previously determined by the treatment planning. As mentioned before, the parallelism of the needles is very important, and the distance between them is determined by the maximum power of the generator used. This option of treating bone tumors proved to be very effective for pain control, as 84% of the treated patients showed improvement in pain control and could reduce by more than 50% overall narcotics consumption (Bianchi et al. 2016). Another study showed similar results for treating spine metastasis, where it improved disability and provided a better quality of life (Cornelis et al. 2019) (Cornelis et al. 2019).

Developing new electrodes is a challenging and exciting task, many of the best ideas come from users who demand ways to access certain tumors, giving rise to new designs. As new electrodes are developed, careful validation and reproducible operating procedures are needed to establish treatment standards, extending the frontiers of electroporation-based treatments and providing a great benefit to the patients both human and animal.

References

Bertino G, Sersa G, De Terlizzi F et al (2016) European research on Electrochemotherapy in head and neck Cancer (EURECA) project: results of the treatment of skin cancer. Eur J Cancer 63:41–52. https://doi.org/10.1016/j.ejca.2016.05.001

Bianchi G, Campanacci L, Ronchetti M, Donati D (2016) Electrochemotherapy in the treatment of bone metastases: a phase II trial. World J Surg 40:3088–3094. https://doi.org/10.1007/s00268-016-3627-6

Broholm M, Stigaard T, Bulut M et al (2019) Calcium electroporation for the treatment of colorectal cancer calcium endove–preliminary results. Eur J Surg Oncol 45:e119

Buijs M, Zondervan PJ, de Bruin DM et al (2019) Feasibility and safety of irreversible electroporation (IRE) in patients with small renal masses: results of a prospective study. Urol Oncol 37:183.e1–183.e8. https://doi.org/10.1016/j.urolonc.2018.11.008

Cemazar M, Tamzali Y, Sersa G et al (2008) Electrochemotherapy in veterinary oncology. J Vet Intern Med 22:826–831. https://doi.org/10.1111/j.1939-1676.2008.0117.x

Chan DSM, Lau R, Aune D et al (2011) Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. PLoS One 6:e20456. https://doi.org/10.1371/journal.pone.0020456

Cornelis FH, Ben Ammar M, Nouri-Neuville M et al (2019) Percutaneous image-guided Electrochemotherapy of spine metastases: initial experience. Cardiovasc Intervent Radiol 42:1806–1809. https://doi.org/10.1007/s00270-019-02316-4

Deodhar A, Monette S, Single GW et al (2011) Percutaneous irreversible electroporation lung ablation: preliminary results in a porcine model. Cardiovasc Intervent Radiol 34:1278–1287

Edhemovic I, Brecelj E, Gasljevic G et al (2014) Intraoperative electrochemotherapy of colorectal liver metastases. J Surg Oncol 110:320–327. https://doi.org/10.1002/jso.23625

Egeland C, Baeksgaard L, Johannesen HH et al (2018) Endoscopic electrochemotherapy for esophageal cancer: a phase I clinical study. Endoscopy International Open 06:E727–E734. https://doi.org/10.1055/a-0590-4053

Fernández-Villa T, Álvarez-Álvarez L, Rubín-García M et al (2020) The role of dietary patterns in colorectal cancer: a 2019 update. Expert Rev Gastroenterol Hepatol 14:281–290. https://doi.org/10.1080/17474124.2020.1736043

Forde PF, Bourke MG, Salwa S et al (2011) Minimally invasive intraluminal tumor ablation. Clin Aspects Electroporation:137–141

Forde PF, Sadadcharam M, Bourke MG et al (2016) Preclinical evaluation of an endoscopic electroporation system. Endoscopy 48(05):477–483. https://doi.org/10.1055/s-0042-101343

Frandsen SK, Vissing M, Gehl J (2020) A comprehensive review of calcium electroporation—a novel Cancer treatment modality. Cancers:12–290. https://doi.org/10.3390/cancers12020290

Gehl J, Sersa G, Matthiessen LW et al (2018) Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol 57:874–882. https://doi.org/10.1080/0284186X.2018.1454602

Hansen HF, Bourke M, Stigaard T et al (2020) Electrochemotherapy for colorectal cancer using endoscopic electroporation: a phase 1 clinical study. Endoscopy International Open 08:E124–E132. https://doi.org/10.1055/a-1027-6735

Jiang C, Davalos RV, Bischof JC (2015) A review of basic to clinical studies of irreversible electroporation therapy. IEEE Trans Biomed Eng 62:4–20. https://doi.org/10.1109/TBME.2014.2367543

Kapur S, Barbhaiya C, Deneke T, Michaud GF (2017) Esophageal injury and Atrioesophageal fistula caused by ablation for atrial fibrillation. Circulation 136:1247–1255. https://doi.org/10.1161/CIRCULATIONAHA.117.025827

Koruth JS, Kuroki K, Iwasawa J et al (2020b) Endocardial ventricular pulsed field ablation: a proof-of-concept preclinical evaluation. Europace 22:434–439. https://doi.org/10.1093/europace/euz341

Koruth JS, Kuroki K, Kawamura I et al (2020a) Pulsed field ablation versus radiofrequency ablation: esophageal injury in a novel porcine model. Circ Arrhythm Electrophysiol 13:e008303. https://doi.org/10.1161/CIRCEP.119.008303

Kotnik T, Kramar P, Pucihar G et al (2012) Cell membrane electroporation- part 1: the phenomenon. IEEE Electr Insul Mag 28:14–23

Lee EW, Chen C, Prieto VE et al (2010a) Advanced hepatic ablation technique for creating complete cell death: irreversible electroporation. Radiology 255:426–433. https://doi.org/10.1148/radiol.10090337

Lee EW, Thai S, Kee ST (2010b) Irreversible electroporation: a novel image-guided cancer therapy. Gut Liver 4(Suppl 1):S99–S104. https://doi.org/10.5009/gnl.2010.4.S1.S99

Linnert M, Agerholm-Larsen B, Mahmood F et al (2014) Treatment of brain tumors: Electrochemotherapy. Tumors of the Central Nervous System:247–259

Linnert M, Iversen HK, Gehl J (2012) Multiple brain metastases - current management and perspectives for treatment with electrochemotherapy. Radiol Oncol 46:271–278. https://doi.org/10.2478/v10019-012-0042-y

Lowe R, Gavazza A, Impellizeri JA et al (2017) The treatment of canine mast cell tumours with electrochemotherapy with or without surgical excision. Vet Comp Oncol 15:775–784. https://doi.org/10.1111/vco.12217

Maglietti F, Tellado M, Olaiz N et al (2017) Minimally invasive Electrochemotherapy procedure for treating nasal duct tumors in dogs using a Single needle electrode. Radiol Oncol 51:422–430. https://doi.org/10.1515/raon-2017-0043

Mali B, Jarm T, Snoj M et al (2013) Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis. Eur J Surg Oncol 39:4–16. https://doi.org/10.1016/j.ejso.2012.08.016

Martin RCG, Kwon D, Chalikonda S et al (2015) Treatment of 200 locally advanced (stage III) pancreatic adenocarcinoma patients with irreversible electroporation: safety and efficacy. Ann Surg 262:486–494.; Discussion 492–4. https://doi.org/10.1097/SLA.0000000000001441

Maruo T, Shida T, Fukuyama Y et al (2011) Retrospective study of canine nasal tumor treated with Hypofractionated radiotherapy. J Vet Med Sci 73:193–197

Miklavčič D, Mali B, Kos B et al (2014) Electrochemotherapy: from the drawing board into medical practice. Biomed Eng Online 13:29. https://doi.org/10.1186/1475-925X-13-29

Mir L (2006) Bases and rationale of the electrochemotherapy. Eur J Cancer Supp 4(11):38–44. https://doi.org/10.1016/j.ejcsup.2006.08.005

Nair KKM, Danon A, Valaparambil A et al (2015) Atrioesophageal fistula: a review. J Atr Fibrillation 8:1331. https://doi.org/10.4022/jafib.1331

O'Brien TJ, Passeri M, Lorenzo MF et al (2019) Experimental high-frequency irreversible electroporation using a Single-needle delivery approach for nonthermal pancreatic ablation in vivo. J Vasc Interv Radiol 30:854–862.e7. https://doi.org/10.1016/j.jvir.2019.01.032

Onik G, Mikus P, Rubinsky B (2007) Irreversible electroporation: implications for prostate ablation. Technol Cancer Res Treat 6:295–300. https://doi.org/10.1177/153303460700600405

Saunders A, Gordon S, Miller M (2009) Canine atrial fibrillation. Compend Contin Educ Vet 31:E1–E9. quiz E10

Spugnini EP, Baldi A, Vincenzi B et al (2006) Intraoperative versus postoperative electrochemotherapy in high grade soft tissue sarcomas: a preliminary study in a spontaneous feline model. Cancer Chemother Pharmacol 59:375–381

Tafuto S, von Arx C, De Divitiis C et al (2015) Electrochemotherapy as a new approach on pancreatic cancer and on liver metastases. Int J Surg 21(Suppl 1):S78–S82. https://doi.org/10.1016/j.ijsu.2015.04.095

Tellado MN, Maglietti FH, Michinski SD et al (2020) Electrochemotherapy in treatment of canine oral malignant melanoma and factors influencing treatment outcome. Radiol Oncol 54:68–78. https://doi.org/10.2478/raon-2020-0014

Wandel A, Ben-David E, Ulusoy BS et al (2016) Optimizing irreversible electroporation ablation with a bipolar electrode. J Vasc Interv Radiol 27:1441–1450.e2. https://doi.org/10.1016/j.jvir.2016.06.001

Wezgowiec J, Kulbacka J, Kotulska M (2016) Electroporation in modern oncology. Electrically Active Materials for Medical Devices 473–490

Withrow SJ, Vail DM, Thamm D, et al (2019) Withrow and Macewen's small animal clinical oncology–E-Book. Elsevier Health Sciences (2015) Cancer Statistics. In: National Cancer Institute. https://www.cancer.gov/about-cancer/understanding/statistics. Accessed 18 May 2020

New Electrodes and Treatment Planning for Deep-Seated and Intraluminal Localized Tumors

Roberta Fusco, Valeria D'Alessio, Francesco Izzo, Raffaele Palaia, and Ruggero Cadossi

Abstract

Clinical use of electroporation in combination with chemotherapeutic drugs, Electrochemotherapy (ECT), has gained acceptance and its effectiveness has been widely demonstrated in several cutaneous pathologies including metastatic melanoma, basal cell carcinoma, squamous cell carcinoma, Kaposi sarcoma, and cutaneous recurrence from breast cancer. Overall, the body of clinical evidence available provided the support needed to expand clinical investigation and application of the therapy to pathologies and tumor nodules not confined to the skin or immediate subcutaneous tissues.

Efforts to translate the application of ECT from easily accessible lesions, e.g., cutaneous metastasis of different malignancies, to the treatment of deep-seated and intraluminal localized tumors require addressing the critical importance of achieving complete electroporation of the target lesions.

The development of new electrodes and the implementation of pre-treatment planning advances the acquired experience of current ECT knowledge to treatment of deep-seated and intraluminal localized tumors. The validation of new electrodes in animal model is needed to transfer their use in clinical practice.

Keywords

Electrochemotherapy · Electroporation · Deep-seated tumors · Intraluminal tumors · Pig model · Preoperative planning

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1 Background

The clinical use of electroporation (EP) techniques has spread in the last 10 years in Europe and the USA. Electroporation and electropermeabilization are terminologies both used to indicate structural changes in the cell membrane lipid bilayer induced by local electric field pulses. EP of the cell membrane occurs when electric field pulses are applied to cells or biological tissue; EP depends on cell characteristics (shape, size, cytoskeleton structure, and membrane composition) and on electrical parameters (amplitude and duration of electric pulses, number of pulse, and repetition frequency).

At the cellular membrane level, EP occurs when an external electric field above the threshold value of the transmembrane potential (approximately 1.5 V) is applied (Cadossi et al. 2014). The optimal treatment of tumors with ablative therapy requires that the technique utilized allows treatment of a large enough zone so no viable tumor tissues remain without harming adjacent vital structures. The EP technique used alone as with Irreversible Electroporation (IRE) or in association with anticancer drugs (Electrochemotherapy or ECT) has been shown to be effective in the treatment of primary and metastatic tumors depending on the parameters of applied electrical pulses (Cadossi et al. 2014; Mali et al. 2013; Sersa et al. 2008; Spratt et al. 2014). The use of EP is possible and effective in any tissue; but is currently used predominantly to the skin and to the subcutaneous tissue where it has filled a "therapeutic vacuum" (Cadossi et al. 2014; Cornelis et al. 2019a, b; Edd et al. 2006; Garcia et al. 2014; Gehl et al. 2018; Izzo et al. 2019; Liu et al. 2018; Mali et al. 2013; Marty et al. 2006; Miklavčič et al. 2010, 2012,. 2014; Mir et al. 2006; Sersa et al. 2008; Spratt et al. 2014). Malignant cutaneous lesions are resistant to most treatment andare associated with a high risk of disease dissemination, and relapse following radiotherapy or chemotherapy. Skin metastases and their treatment seldom affect survival, but they can have a negative impact on patients' quality of life. Therefore, locally enhanced chemotherapy by ECT can offer a new, important and safe opportunity to control skin localization of the tumor. Advantages include minimal invasiveness, rapid treatment effect, and absence of severe adverse events (Benevento et al. 2012). The efficacy of ECT and IRE has already been demonstrated in human metastasis of non-superficial tumors such as liver (Cornelis et al. 2019a, b; Djokic et al. 2018; Edhemovic et al. 2011; Tarantino et al. 2017), rectal cancer (Bourke et al. 2012; Edhemovic et al. 2014; https://clinicaltrials.gov/ct2/show/NCT03040180; Scala et al. 2015), primitive pancreatic cancer (Bimonte et al. 2016; Granata et al. 2015, 2017; Martin 2nd et al. 2015; Tafuto et al. 2015), esophageal tumors (Egeland et al. 2018), and bone metastasis (Bianchi et al. 2016). Unlike hyperthermic procedures, such as Radiofrequency (RFA), Microwave (MWA), high-intensity focused ultrasound (HIFU), and laser therapy (Izzo et al. 2019) that provoke coagulative degeneration of the collagen and vascular tissue, IRE, and ECT are not thermal method and since that these do not produce a variation of temperature of exposed tissue, could also be performed near noble structures (vessels and nerves) without risk of complication. Each of these technologies has its own strengths and weaknesses (Cornelis et al. 2019a, b; Edd et al. 2006; Garcia et al.

2014; Gehl et al. 2018; Izzo et al. 2019; Liu et al. 2018; Marty et al. 2006; Mir et al. 2006). One of the main advantages of hyperthermic procedures is a greater volume of cell necrosis and less susceptibility to morphological variations of the treated area due to heat-sink effects. The main disadvantage is an increase of potential risks due to collateral injury of adjacent, not target organs (Garcia et al. 2014; Izzo et al. 2019). For these reasons, an ablative technique that can spare tissue vessels and not be influenced by blood flow would be ideal.

However, increasing the number of electric pulses, both in ECT and in IRE treatments, could increase efficacy and associated thermal damage (Cornelis et al. 2019a, b), (Garcia et al. 2014), near the sharp transition around the electrodes (Edd et al. 2006). Therefore, if either the pulse number and/or the tissue conductivity are too high, there is also potential to achieve cell kill due to thermal damage in the immediate vicinity of the electrodes. This effect is minimized in case of reversible electroporation adopting an electric protocol according to ESOPE (Gehl et al. 2018; Marty et al. 2006; Mir et al. 2006), that consists of 8 pulses of 100μs divided into two groups of four with inversion of the applied voltage and a reference electric field (1000 V/cm) less than irreversible electroporation. Correct positioning of the electrodes, respectful of critical tissue structures and treatment parameters, will guarantee the maintenance of nonthermal benefits of electroporation and prevent unnecessary damage to surrounding healthy tissue, critical vascular structures, and/or adjacent organs.

2 ECT in Preclinical Studies of Deep Tumors

Electrochemotherapy is a standard treatment for cutaneous and subcutaneous tumors of various histology in human and veterinary oncology. Electrochemotherapy, unlike IRE, will cause cancer cell death, mostly by apoptosis but both necrotic and apoptotic cell death, with slow resolution of tumors is identified, based on evidence from preclinical and some clinical histological studies (Gasljevic et al. 2017; Kodre et al. 2009). Following ECT, when cells enter mitosis, they die as a result of unrepairable DNA damage caused by chemotherapeutic activity (Cadossi et al. 2014).

Clinical studies in veterinary clinical oncology have demonstrated that ECT treatment may achieve up to 80% long-lasting objective responses in cutaneous and subcutaneous tumors in dogs (Spugnini et al. 2013), cats (Tozon et al. 2014), and horses (Tamzali et al. 2012). The treatment of tumor lesions located in areas that are not superficial or immediately reachable, can be complex and prolonged unless the laparotomy is used. Percutaneous EP and the use of EP with minimally invasive techniques using laparoscopic or endoscopic approaches is still emerging.

In vivo effects of reversible electroporation in normal pancreas in a rabbit experimental model suggested that ECT could be considered as a valid alternative for the local control of non-resectable pancreatic cancer. In fact, electroporation is a safe procedure because it does not affect normal pancreatic parenchyma, and potentiate the cytotoxicity of bleomycin in pancreatic tumor cell lines (Girelli et al.

2015). To overcome the limitation to reach the deep-seated tumor, ECT has been used successfully to treat nasal cavity tumor in dogs by single-needle electrode with an appropriate electric field and 60% of survival at 1 year and 30% at 32 months and 0% of control group was observed (Maglietti et al. 2017). Safety and effectiveness of ECT have been confirmed also in 14-dog bladder neoplasm in which the median survival time of patients was improved (Rangel and Quadros 2019).

A needle-shaped multipolar probe with telescopic electrodes for percutaneous image-guided IRE as well as ECT in solid organs already exists. It can be used as percutaneous, image-guided, minimally invasive treatment option for malignant liver tumors (Ritter et al. 2018). A novel expandable electrode has been used in experimental brain tumors in rats. This treatment method resulted highly effective with complete resolution of 69% of treated tumors after once-only treatment with treatment effects localized to the target area, and with favorable toxicity profile, with no apparent influence on rat behavior or morbidity (Agerholm-Larsen et al. 2011). A novel electrode device with optimized geometry, developed for diffusion in human intracranial tumors of antineoplastic drugs and genes, improved clinical performance and geometrical tolerance (Mahmood and Gehl 2011). The first reported preclinical study of ECT in rat model of brain tumors was published in the early 1990s, reporting an almost double survival time in the treated rats. A phase I clinical study showed that ECT is safe, effective, and with low toxicity in normal tissue (Linnert et al. 2012). Endoscopic treatment of colorectal cancer as well as for the treatment of gastric and esophageal tumors have been explored with the first endoluminal electrodes developed at Cork Cancer center, University of Cork, Ireland (Miklavčič et al. 2012). EndoVe device showed to be safe and effective in spontaneous canine colorectal cancer tumor resolution (Soden et al. 2019).

3 The Expandable and Deployable Electrodes

The technological limitations of EP are linked to the basic principles on which EP of a tissue is based. To ensure a homogenous coverage of entire volume/surface of tumor, the active conductive part of the electrodes should be inserted in the tissue at the same depth and the electrodes must be parallel between them. These prerequisites, parallelism, and penetration uniformity, are not easily obtained when tumor nodules to be treated are in locations not easily accessible such as hollow or visceral organs. The solution in the classic ablation techniques such as radiofrequency, consists of an electrode container that once inserted into the tumor nodule can dislocate or expand single electrodes causing degenerative coagulation. When parallelism is lost the electrodes are displaced and expanded as in the case of radiofrequency, and there is no guarantee of obtaining the EP of all cells within the tumoral nodule. Due to the divergence between the needles, the electric field applied to the conductive part would be extremely inhomogeneous, with the risk of a short circuit in the distal part of the electrodes, leaving some areas of the tumor not properly treated.

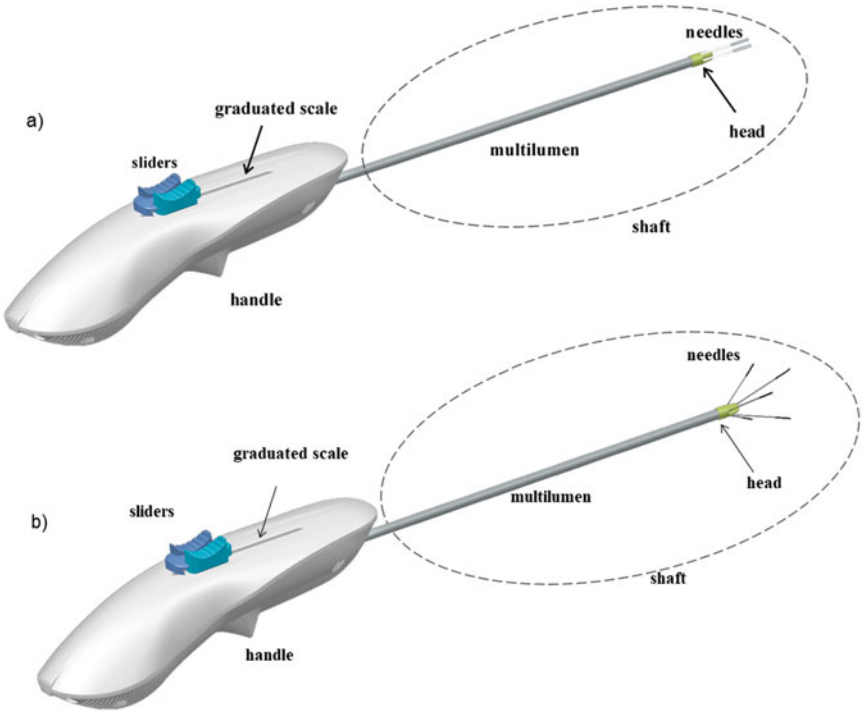

Fig. 1 Rendering representation of deployable expandable electrodes with (**a**) zero and (**b**) nonzero divergence (10° of divergence between peripheral needles and electrode shaft axis)

Segmentation treatment, in which electrodes with the conductive part varying according to the divergence within the tissue, so that EP of the tumor nodule occurs through successive steps, is a good solution. The electric field values necessary for each segment will be applied for the EP of all cell membranes. This solution allows the use of a single electrode of a small size that contains the needles. Limitation of the conductive part of the needle which reduces the electric field along the conductive part, can resolve the problem of the inhomogeneity of the total electric field.

The expandable and deployable electrodes can be extended and expanded into tumor tissue allowing an effective electric field throughout all cells to induce EP of cell membranes. With the aim to evaluate the feasibility and usability of these electrodes, EP treatment has been performed in different anatomical region on animal models with laparoscopic, open and endoscopic approaches using trans-oral and trans-anal approaches.

The electrode consists of a fixed shaft, made up of five needles (one central needle and four peripheral needles) in medical steel that extend in a multi-lumen and whose geometric configuration is determined by a head (Fig. 1, p. 8).

The configuration with five needles has been chosen to reduce the applied voltages and allow implementation of several variants with different divergences

between peripheral needles and the central one. The needles consist of the active part inserted in the target lesion, allow the delivery of the electrical impulses into the target area. The needles are adjustable in their insertion depth by means of an adjustment system implemented in the handle. The needles have an insulating polymeric sheath, which limits the conductive part (active part) exposed at 2 cm. By means of a graduated scale, present on the handle itself, it is possible to adjust the insertion depth of the needles up to 4 cm. At different steps, the distances between each needle depend on the initial distance between each needle, that is determined by the design of the shaft, and the divergence between peripheral needles and the central one. Particularly, the distance between each peripheral needle and the central one, which represents the semi-diagonal of the electrode (*d/2*), and the distance between two consecutive peripheral needles, which represent the side of the electrode (*side*). This is respectively calculated by the following formulas:

$$d/2 = (e + h_t)\tan\alpha + \frac{i}{2} - \varnothing_{needle}$$

$$side = \sqrt{2}\cdot(d/2) - \left(1 - \sqrt{2}\right)\cdot\varnothing_{needle.}$$

where the parameters h_t and i are fixed by the design of the shaft and measure, respectively, 3 mm and 3.26 mm; e is the exposition of the needles at each step; α is the divergence angle of the specific electrode variant; $\varnothing_{needle}$ is the diameter of needles, which must be subtracted in order to define the correct voltage to be applied on the inner space among each needles couple.

Consequently, the electric parameters at each step are different, based on the electrode divergence (Fig. 2, p. 10).

The central needle and the peripheral needles can be moved independently by using the dedicated cursors, in order to fix the target lesion with the central needle. The possible scenarios are:

- Insertion: First move the central needle and then advance the peripheral needles or, alternatively, move the cursor of the peripheral needles to move the entire set of needles.
- Extraction: First move the peripheral needles and then the central needle or, alternatively, move the entire set of needles back by moving the central needle cursor.

A connection cable that ends with the connector for linking to the electroporator. There are different models of electrodes that differ in the length of the shaft, for the divergence of the peripheral needles with the central needle and for maximum exposure of the needles.

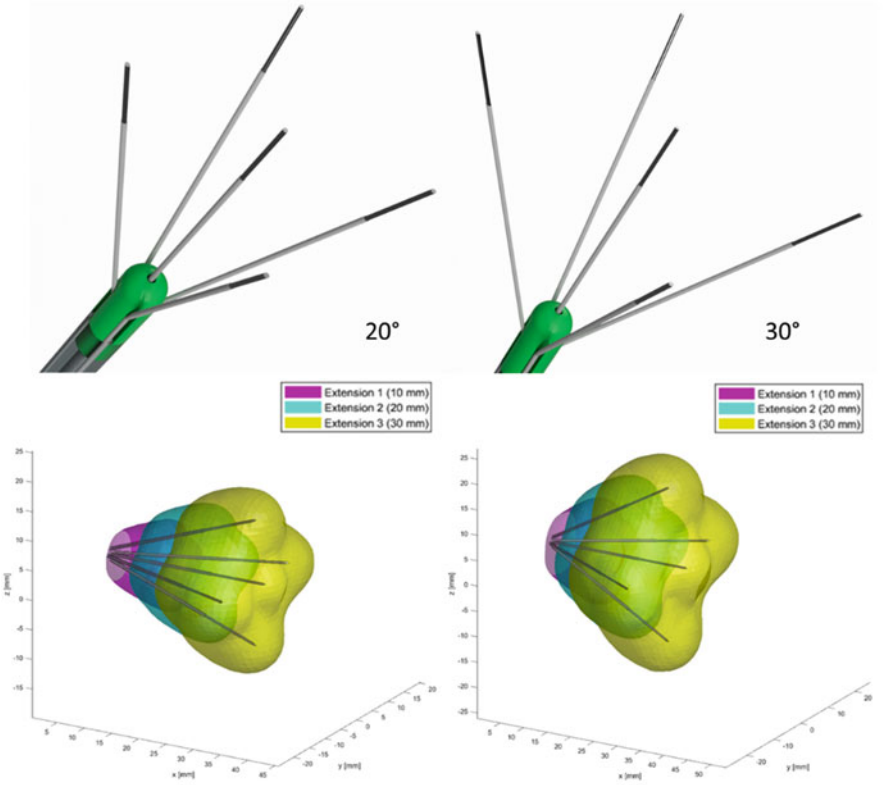

Fig. 2 Schematic representation of two expandable and divergent electrode models with of 20° and of 30° of divergence and Finite Element Method (FEM) simulations, using Comsol Multiphysics (v5.2, Stockholm, Sweden), considering three electroporation steps (10 mm, 20 mm, and 30 mm)

3.1 Our Experience on the Pig Model with Expandable and Deployable Electrodes

The expandable and deployable electrodes family have been tested on animal models with the aim to evaluate the feasibility and usability of the EP treatment performed in different districts: liver both with laparoscopic and open approach, and oral and anal anatomy by endoscopy.

The liver treatment was with ultrasound guidance using the 3-electrode type: one electrode with zero divergence and two electrode prototypes with different divergence (20° and 10°) and with 5 and 4 needles, respectively. The configuration with 4 electrodes did not have the central needle. The irreversible EP treatment was performed on liver both in laparoscopic procedure and in open surgery. The following electrical parameters with the Cliniporator Vitae (Igea SpA, Carpi, Italy) were used: 80–120 pulses; 100μs of pulse duration; 4 Hz of pulse frequency; electric field $\geq$1500 V/cm and therefore voltages based on electrode prototype variant and

deployment of needles. A Computed Tomography (CT) with iodate contrast medium injection (nonionic, water solution contrast medium—Iopamiro, Bracco, Milan, Italy) was performed 3 hours after the treatment. Then, the animals were sacrificed and liver specimens were removed for vital staining (Tetrazolium) and/or histology and immunohistochemistry evaluation.

For the oral approach, two versions of the electrode with divergence equal to zero with different rigidity of the shaft have been used. An electrode, with divergence of 10 degrees has been used in trans-oral and trans-anal approaches. The procedure of extraction of needles was with CT guided with iodate contrast medium.

The results for each anatomic region (liver, oral, and colon) showed that each electrode model, with and without divergence, tested effectively in laparoscopy/endoscopic surgical approaches (Fig. 3 p.12) as well as in open surgery Mechanical functionality (flexibility, penetrability), visibility of the electrode under radiological guidance and compatibility of the electrode with specific surgical accesses were all significant benefits of the technique. The handle is satisfactory from an ergonomic point of view and the flexibility and shape of the invasive element have proven to be suitable for satisfying the required performance. Safety (no bleeding and/or perforation) and treatment efficacy (adequate ablated volume) was also demonstrated.

The histological examination demonstrated the efficacy of the treatment, highlighting a difference between tissue hepatic treated (central part) and untreated (surrounding area). In particular, in the treated tissue, the presence of hypertrophic nuclei, blood effusion, and altered and damaged hepatic parenchyma were observed. In the untreated tissue, the presence of normal nuclei and intact hepatic parenchyma were observed (Fig. 4 p. 13).

The immunohistochemical staining also confirmed the presence of necrosis in the treated tissues, with apoptotic cells characterized by a positive antibody reaction, compared to untreated tissues where the antibody reaction was negative.

The reduction of invasiveness of the procedure, from open surgery versus a minimally invasive laparoscopic or endoscopic approach will allow a reduction in associated costs to therapy thanks to decreased hospitalization, decreased operative time, and the need for an operating theater. At the same time, it will be possible to offer therapy to a greater number of patients, those patients for whom the surgery is currently not possible as well as for those patients that are not candidates for thermal ablation.

4 Bipolar Electrodes

A new minimally invasive bipolar electrode for electroporation has been recently designed and characterized (Merola et al. 2020). The new device is different from the already marketed electrodes for its geometry, design, and utilize having anode and cathode components on a single needle. This approach allows to reduce the number of electrode insertions required simplifying the electrode placement for the procedure, minimize invasiveness, and saving time. The intended purpose of the new electrode is to reach a compromise with treatment invasiveness linked to probe

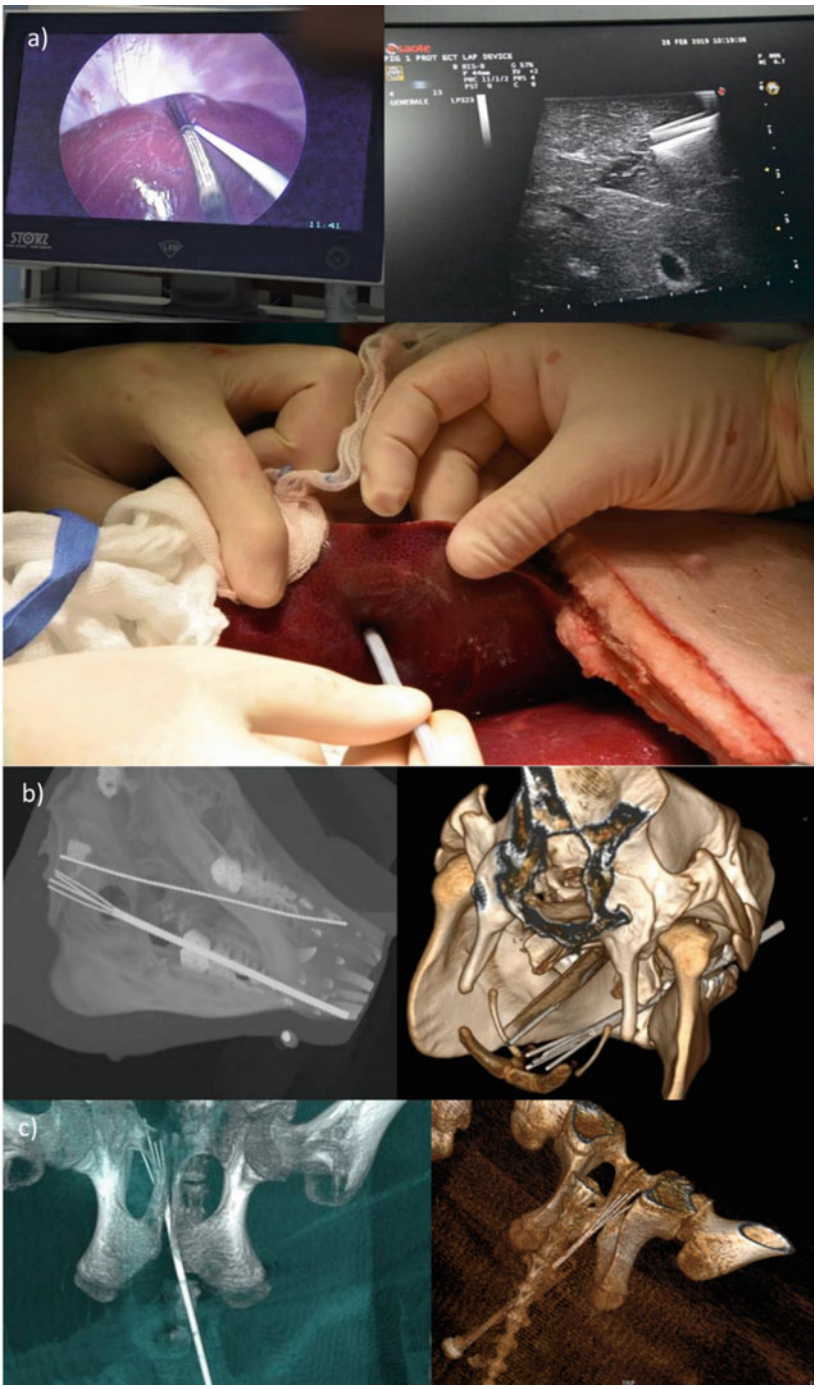

Fig. 3 (**a**) ECT treatment in laparoscopic and open access and ultrasound image of the treated area with expandable 5-needles electrode with zero divergence and with divergent expandable 4-needles electrode, respectively; (**b**) Trans-oral application for intraluminal access to upper airway with

diameter, and the electroporated area. Therefore, different geometric aspects, such as the diameter of the electrode, the length of the conductive and insulated poles, and the applied voltage were analyzed in theoretical and preclinical study ensuring an electroporated area of at least 10 mm of diameter.

To develop a minimally invasive electrode, an alternating concentric tube of insulated and conductive material, with both positive and negative poles on the same needle was constructed

Electrode prototypes have an overall diameter of 1.40 mm with a concentric structure of insulated and conductive materials characterized by minimum wall thicknesses was developed and an adequate mechanical machining was designed. The stainless steel mandrel has an outer diameter of about 0.80 mm, except for the section intended to be the proximal conductive pole (P2), whose outer diameter is of 1.40 mm. The Trocar tip was made in the end part of the mandrel and its shape helps the insertion and drilling of the target organ. Then the first insulated sheath, the stainless steel tube making the distal conductive pole (P1) and the second insulated sheath are placed (in a concentric way) on the mandrel section where the diameter is 0.80 mm. As already highlighted in the study of the material, the thickness of the insulated sheath has to guarantee the electrical insulation for all the allowed applied voltages (with a maximum functional voltage of 3000 V).

In Fig. 5 a schematic representation of bipolar electrode is shown.

4.1 Our Experience on the Pig Model with Bipolar Electrodes

Simulation findings showed that the applied voltage is the main variable to influence the electroporated volume to obtain the maximum diameter of the electric field distribution. Moreover, changing both the conductive and the insulated poles length affected the distribution of the electric field. The volume of electroporated volume is affected by conductive poles length while the electroporated volume size and shape are inversely affected by the insulated pole length. The test performed with the vegetable are in accordance with the simulations, suggesting that the voltage and the total length of the electrode are the variables that most influence the electroporated volume. Tests performed on pig preclinical model with bipolar electrode prototypes showed that a parenchymal damage could be obtained, due to irreversible electroporation protocol, in an area with a maximum diameter higher than 9 mm in all experiments. The electrode with an asymmetric geometry has determined a “drop” shaped electroporated volume, in place of the ellipsoidal shaped one determined by the electrode with a symmetric geometry.

Fig. 3 (continued) divergent expandable 5-needles electrode: Maximum Intensity projection (MIP) and volume rendering representation; (**c**) Application on rectum with Laparo-Endoscopic Single Site (LESS) procedure with divergent expandable 5-needles electrode: MIP and volume rendering representation

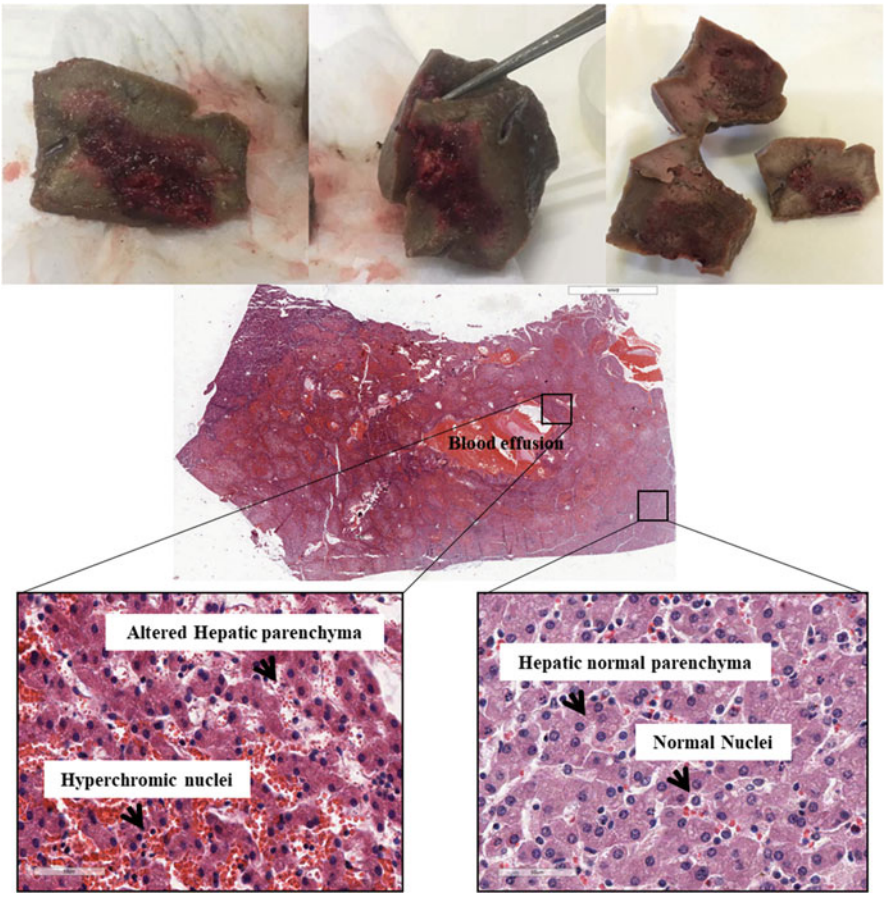

Fig. 4 Macroscopic images and histological analysis of specimens treated with electrode with zero divergence, where it is possible to observe the altered hepatic parenchyma and hyperchromic nuclei in treated area compared to normal parenchyma is not treated zone

In conclusion, using a bipolar electrode it is possible to simultaneously reduce the invasiveness of the treatment and guarantee an electroporated volume of about 10 mm in diameter. Moreover, the bipolar electrode can allow to treat metastases localized to the vertebrae with a minimally invasive percutaneous approach through the vertebral peduncle. Furthemore these electrodes allows for faster treatment compared to thermo and cryo ablation leaving intact the regenerative capacity of the bone.

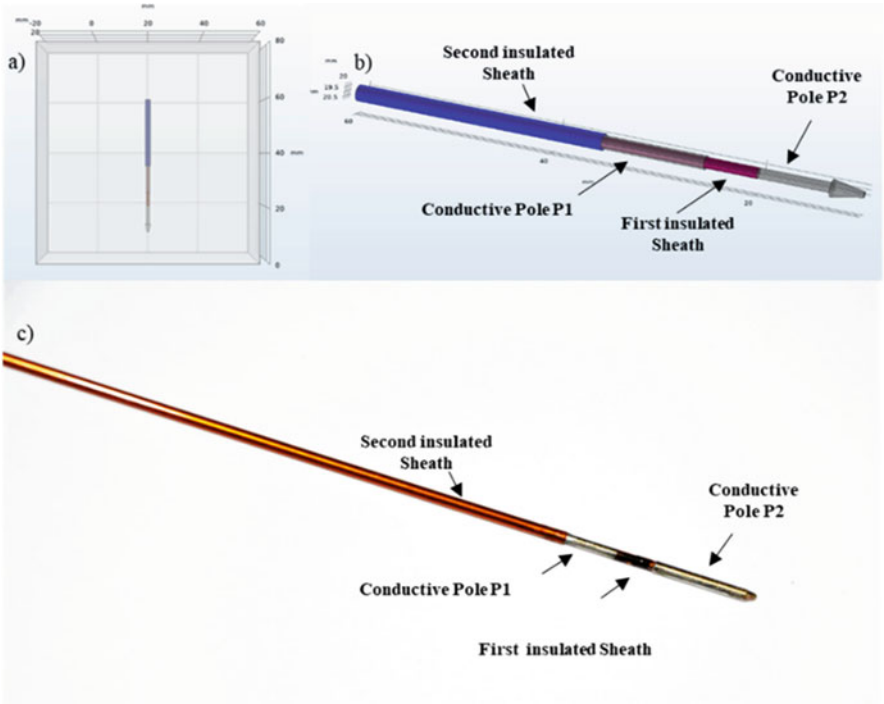

Fig. 5 Bipolar coaxial electrode. (**a**) Positioning of the device inside the simulated organ (cube). (**b**) Electrode geometry implemented in Comsol Multiphysics®: two conductive poles, a tip and two insulated sheaths. (**c**) An example of bipolar electrode prototype

5 Treatment Planning

IRE and ECT of tumors performed using multiple single long needles in a variable geometry, based on tumor morphology benefit from personalized treatment planning based on mathematical modelling along with coupling to a navigational system, providing a more accurate positioning of the electrodes and optimized electrical parameters for the treatment.

Moreover, correct spatial positioning of individual needles, requires skill and the support of intraoperative imaging: X-ray, ultrasound, or computed tomography imaging. The disadvantage of incorrect electrode positioning may be insufficient EP in the target tissue and may lead to areas where cells are not non-electroporated, thus increasing the risk of partial responses and local recurrences.

Dedicated software have been developed to guide needle insertion and positioning and the required EP pulse conditions to guarantee the complete and homogenous coverage of the tumor volume by the applied electric field (Kos et al. 2015; Pavliha et al. 2012).

To help the surgeon during the treatment preoperative planning, IGEA has d a dedicated software that would provide an approximate optimized placement of electrodes within or around a predefined area segmented by the user. The software calculates the coverage of the electric field and minimizes the number of electrodes required. The tool provides the electric field estimation in the region of interest selected by expert operators by means of approximate calculations giving indication of electrodes configuration, voltage, and distance for each couple of electrodes. The electric field coverage estimation is based on a numerical calculation with fixed electric tissue parameters and does not take under consideration different conductivities of tissues and conductivity changes due to electroporation. The estimation of the electric field is done with a reference voltage applied over a distance of 1 kV/cm considering a maximum distance between the electrodes of 3 cm, a minimum distance of electrode of 5 mm, the maximum voltage is set at 3 kV. The software allow exclusion of certain regions (forbidden zones) where the electrodes should not be placed, for anatomical reasons (e.g., nerves or vessels), within the electrodes' placement area. The software outputs all the information included in a file .xml and in a .png image. The planning file may be saved in a pen drive to be used with the electric pulse generator. An optimized preoperative planning for liver application in a pig model, showing the segmented area, the number and position of the electrodes, an estimation of the electric field coverage and the electrodes' insertion by open surgery approach is reported (Fig. 6, p. 18).

Other software proposed in literature (Marčan et al. 2015) is a web-based tool that automatically builds a 3D model of the target tissue from the medical images uploaded by the user and then uses this 3D model to optimize treatment parameters. As IGEA software, this web-based tool is intended to facilitate the treatment planning process and reduce overall treatment time, which is crucial for facilitating expansion of electroporation-based treatments in the clinic and ensuring reliable treatment for the patients.

Another tool recently developed is the EView (https://eview.upf.edu/), online platform for 3D electric field simulation in electroporation-based treatments using needle-shaped electrodes. EView provides an easy way to obtain approximate estimations of the electric field distribution for arbitrary electrode positions and orientations. EView has been developed with the aim of facilitating the understanding of how the electric field distribution depends on the geometry of the electrode setup and the applied voltage. This platform must only be used for research or educational purposes (or similar informative uses), and not for clinical use. EView offers the possibility of representation on two different sorts of background: (1) void scenario, the electrodes, and the simulation results are represented in an empty space. The background is simply black. (2) medical image scenario, a 3D medical image can be loaded as a background. This allows placing the electrodes according to anatomical landmarks and visualizing the simulated electric field distribution overlaid on the medical image. The accepted formats are Neuroimaging Informatics Technology Initiative (NIfTI), Nearly Raw Raster Data (Nrrd), and Digital Imaging and Communications in Medicine (DICOM). The easiest way to load an image is to go to the files menu and click upload. Alternatively, you can drag and drop the files from the folder to the window.

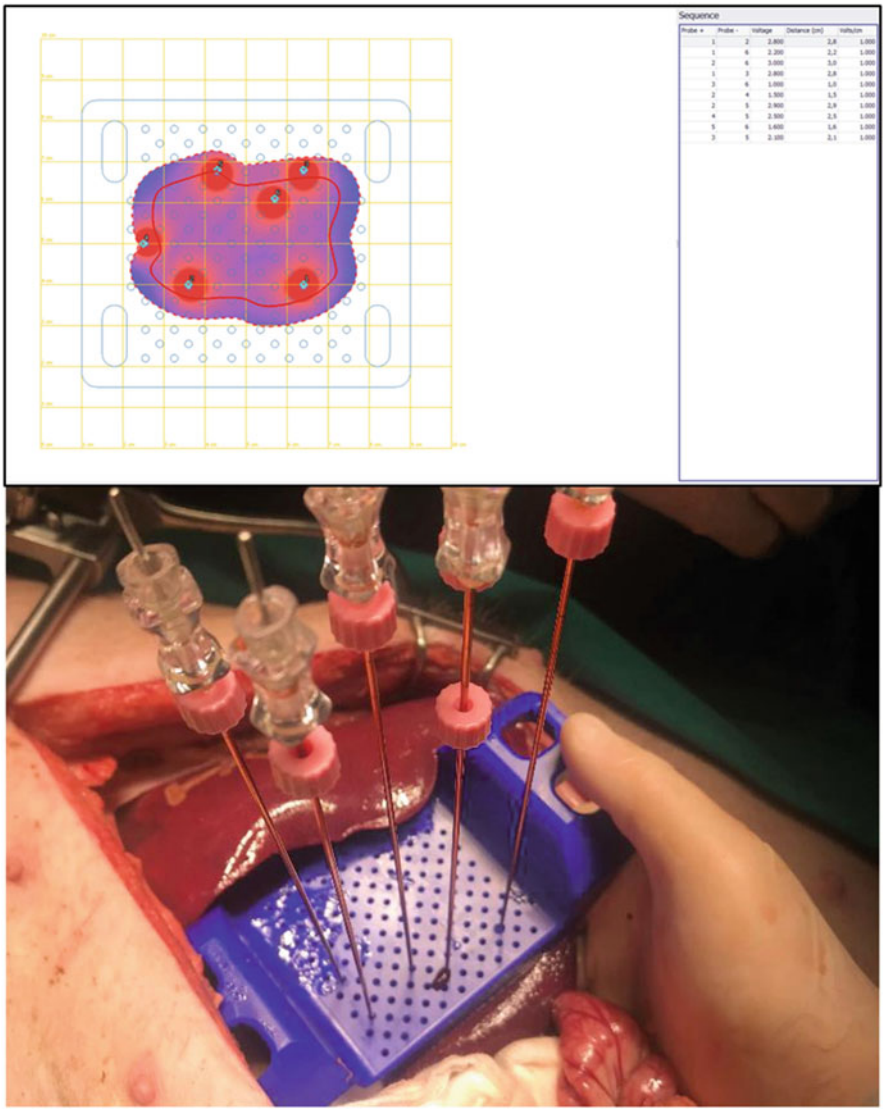

Fig. 6 Treatment planning in a liver application by open surgery approach in a pig model

6 Conclusions

The technology presented here will allow the use of EP in minimally invasive procedures such as percutaneous, laparoscopic and endoscopic approaches, greatly expanding the current use of the EP technique.

In order to effectively treat the complete tumor, electroporation, in minimizing the damage induced in critical healthy tissues or organs, should consider:

- The implementation of pre-treatment planning to obtain an optimal electrodes variable configuration 2D and/or 3D that ensure the complete target area electroporation and optimal electric field distribution minimizing the number of electrodes.
- The use of a mini-invasively surgical approach such as percutaneous and laparoscopic approach using a real-time image-guided electrode insertion to allow an accurate positioning of electrodes according to treatment plan, for either fixed or variable electrode geometries.
- The development of new minimal-invasive electrodes in the treatment of deep-seated intraluminal localized tumors.

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References

Agerholm-Larsen B, Iversen HK, Ibsen P, Moller JM, Mahmood F, Jensen KS, Gehl J (2011) Preclinical validation of electrochemotherapy as an effective treatment for brain tumors. Cancer Res 71(11):3753–3762. https://doi.org/10.1158/0008-5472.CAN-11-0451

Benevento R, Santoriello A, Perna G, Canonico S (2012) Electrochemotherapy of cutaneous metastastes from breast cancer in elderly patients: a preliminary report. BMC Surg 12(Suppl. 1):S6. https://doi.org/10.1186/1471-2482-12-S1-S6

Bianchi G, Campanacci L, Ronchetti M, Donati D (2016) Electrochemotherapy in the treatment of bone metastases: a phase II trial. World J Surg 40(12):3088–3094. https://doi.org/10.1007/s00268-016-3627-6

Bimonte S, Leongito M, Granata V, Barbieri A, Del Vecchio V, Falco M, Nasto A, Albino V, Piccirillo M, Palaia R, Amore A, Giacomo RD, Lastoria S, Setola SV, Fusco R, Petrillo A, Izzo F (2016) Electrochemotherapy in pancreatic adenocarcinoma treatment: pre-clinical and clinical studies. Radiol Oncol 50(1):14–20. https://doi.org/10.1515/raon-2016-0003

Bourke M, Salwa S, Forde P, Sadadcharam M, Larkin J, Collins C, Zeeshan S, Winter D, O'Sullivan GC, Soden D, O'Riordain M (2012) P80 endoscopically targeted electrochemotherapy for the treatment of colorectal cancer. Eur J Surg Oncol 38:1127–1128. https://doi.org/10.1016/j.ejso.2012.07.201

Cadossi R, Ronchetti M, Cadossi M (2014) Locally enhanced chemotherapy by electroporation: clinical experiences and perspective of use of electrochemotherapy. Future Oncol 10(5):877–890. https://doi.org/10.2217/fon.13.235

Cornelis FH, Cindrič H, Kos B, Fujimori M, Petre EN, Miklavčič D, Solomon SB, Srimathveeravalli G (2019b) Peri-tumoral metallic implants reduce the efficacy of irreversible electroporation for the ablation of colorectal liver metastases. Cardiovasc Intervent Radiol 32:727–736. https://doi.org/10.1007/s00270-019-02300-y

Cornelis FH, Korenbaum C, Ben Ammar M, Tavolaro S, Nouri-Neuville M, Lotz JP (2019a) Multimodal image-guided electrochemotherapy of unresectable liver metastasis from renal cell cancer. Diagn Interv Imaging 30001–4(19):S2211–S5684

Djokic M, Cemazar M, Popovic P, Kos B, Dezman R, Bosnjak M, Zakelj MN, Miklavcic D, Potrc S, Stabuc B, Tomazic A, Sersa G, Trotovsek B (2018) Electrochemotherapy as treatment option for hepatocellular carcinoma, a prospective pilot study. Eur J Surg Oncol 44(5):651–657. https://doi.org/10.1016/j.ejso.2018.01.090

Edd JF, Horowitz L, Davalos RV, Mir LM, Rubinsky B (2006) In vivo results of a new focal tissue ablation technique: irreversible electroporation. IEEE Trans Biomed Eng 53:1409–1415

Edhemovic I, Brecelj E, Gasljevic G, Marolt Music M, Gorjup V, Mali B, Jarm T, Kos B, Pavliha D, Grcar Kuzmanov B, Cemazar M, Snoj M, Miklavcic D, Gadzijev EM, Sersa G (2014) Intraoperative electrochemotherapy of colorectal liver metastases. J Surg Oncol 110 (3):320–327. https://doi.org/10.1002/jso.23625

Edhemovic I, Gadzijev EM, Brecelj E, Miklavcic D, Kos B, Zupanic A, Mali B, Jarm T, Pavliha D, Marcan M, Gasljevic G, Gorjup V, Music M, Vavpotic TP, Cemazar M, Snoj M, Sersa G (2011) Electrochemotherapy: a new technological approach in treatment of metastases in the liver. Technol Cancer Res Treat 10:475–485

Egeland C, Baeksgaard L, Johannesen HH, Löfgren J, Plaschke CC, Svendsen LB, Gehl J, Achiam MP (2018) Endoscopic electrochemotherapy for esophageal cancer: a phase I clinical study. Endosc Int Open 6(6):E727–E734. https://doi.org/10.1055/a-0590-4053

Garcia PA, Davalos RV, Miklavcic D (2014) A numerical investigation of the electric and thermal cell kill distributions in electroporation-based therapies in tissue. PLoS One 9(8):e103083

Gasljevic G, Edhemovic I, Cemazar M, Brecelj E, Gadzijev EM, Music MM (2017 Jul 7) Sersa G histopathological findings in colorectal liver metastases after electrochemotherapy. PLoS One 12(7):e0180709. https://doi.org/10.1371/journal.pone.0180709

Gehl J, Sersa G, Matthiessen LW, Muir T, Soden D, Occhini A, Quaglino P, Curatolo P, Campana LG, Kunte C, Clover AJP, Bertino G, Farricha V, Odili J, Dahlstrom K, Benazzo M (2018) Mir LM updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol 57(7):874–882. https://doi.org/10.1080/0284186x.2018.1454602

Girelli R, Prejanò S, Cataldo I, Corbo V, Martini L, Scarpa A, Claudio B (2015) Feasibility and safety of electrochemotherapy (ECT) in the pancreas: a pre-clinical investigation. Radiol Oncol 49(2):147–154. https://doi.org/10.1515/raon-2015-0013

Granata V, Fusco R, Piccirillo M, Palaia R, Petrillo A, Lastoria S, Izzo F (2015) Electrochemotherapy in locally advanced pancreatic cancer: preliminary results. Int J Surg 18:230–236. https://doi.org/10.1016/j.ijsu.2015.04.055

Granata V, Fusco R, Setola SV, Piccirillo M, Leongito M, Palaia R, Petrillo A (2017) Early radiological assessment of locally advanced pancreatic cancer treated with electrochemotherapy. World J Gastroenterol 23(26):4767–4778. https://doi.org/10.3748/wjg.v23.i26.4767

Izzo F, Granata V, Grassi R, Fusco R, Palaia R, Delrio P, Carrafiello G, Azoulay D, Petrillo A, Curley SA (2019) Radiofrequency ablation and microwave ablation in liver tumors: an update. Oncologist 24(10):e990–e1005. https://doi.org/10.1634/theoncologist.2018-0337

Kodre V, Cemazar M, Pecar J, Sersa G, Cor A, Tozon N (2009) Electrochemotherapy compared to surgery for treatment of canine mast cell tumours. In Vivo 23(1):55–62

Kos B, Voigt P, Miklavčič D, Moche M (2015) Careful treatment planning enables safe ablation of liver tumors adjacent to major blood vessels by percutaneous irreversible electroporation (IRE). Radiol Oncol 49:234–241. https://eview.upf.edu/

Linnert M, Iversen HK, Gehl J (2012) Multiple brain metastases–current management and perspectives for treatment with electrochemotherapy. Radiol Oncol 46(4):271–278. https://doi.org/10.2478/v10019-012-0042-y

Liu W, Zheng Y, He W, Zou R, Qiu J, Shen J, Yang Z, Zhang Y, Wang C, Wang Y, Zuo D, Li B, Yuan Y (2018) Microwave vs radiofrequency ablation for hepatocellular carcinoma within the Milan criteria: a propensity score analysis. Aliment Pharmacol Ther 48(6):671–681. https://doi.org/10.1111/apt.14929

Maglietti F, Tellado M, Olaiz N, Michinski S, Marshall G (2017) Minimally invasive Electrochemotherapy procedure for treating nasal duct tumors in dogs using a single needle electrode. Radiol Oncol 51(4):422–430. https://doi.org/10.1515/raon-2017-0043

Mahmood F, Gehl J (2011) Optimizing clinical performance and geometrical robustness of a new electrode device for intracranial tumor electroporation. Bioelectrochemistry 81(1):10–16. https://doi.org/10.1016/j.bioelechem.2010.12.002

Mali B, Jarm T, Snoj M, Sersa G, Miklavcic D (2013) Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis. Eur J Surg Oncol 39(1):4–16. https://doi.org/10.1016/j.ejso.2012.08.016

Marčan M, Pavliha D, Kos B, Forjanič T, Miklavčič D (2015) Web-based tool for visualization of electric field distribution in deep-seated body structures and planning of electroporation-based treatments. Biomed Eng Online 14(Suppl 3):S4

Martin RC 2nd, Kwon D, Chalikonda S, Sellers M, Kotz E, Scoggins C, McMasters KM, Watkins K (2015) Treatment of 200 locally advanced (stage III) pancreatic adenocarcinoma patients with irreversible electroporation: safety and efficacy. Ann Surg 262(3):486–494.; Discussion 492–4. https://doi.org/10.1097/SLA.0000000000001441

Marty M, Sersa G, Garbay JR, Gehl J, Collins CG, Snoj M et al (2006) Electrochemotherapy—an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: results of ESOPE (European standard operating procedures of Electrochemotherapy) study. Eur J Cancer Suppl 4:3–13

Merola G, Fusco R, Di Bernardo E, D'Alessio V, Izzo F, Granata V, Contartese D, Cadossi M, Audenino A, Perazzolo Gallo G (2020) Design and characterization of a minimally invasive bipolar electrode for electroporation. Biology 9(9):E303. https://doi.org/10.3390/biology9090303

Miklavčič D, Mali B, Kos B, Heller R, Serša G (2014) Electrochemotherapy: from the drawing board into medical practice. Biomed Eng Online 13(1):29. https://doi.org/10.1186/1475-925X-13-29

Miklavčič D, Serša G, Brecelj E, Gehl J, Soden D, Bianchi G, Ruggieri P, Rossi CR, Campana LG, Jarm T (2012) Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors. Med Biol Eng Comput 50(12):1213–1225. https://doi.org/10.1007/s11517-012-0991-8

Miklavčič D, Snoj M, Zupanic A, Kos B, Cemazar M, Kropivnik M, Bracko M, Pecnik T, Gadzijev E, Sersa G (2010) Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. Biomed Eng Online 9:10. https://doi.org/10.1186/1475-925X-9-10

Mir LM, Gehl J, Sersa G et al (2006) Standard operating procedures of the electrochemotherapy: instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or noninvasive electrodes. Eur J Cancer Suppl 4:14–25

Pavliha D, Kos B, Zupanič A, Marčan M, Serša G, Miklavčič D (2012) Patient-specific treatment planning of electrochemotherapy: procedure design and possible pitfalls. Bioelectrochemistry 87:265–273

Rangel M, Quadros PG. (2019) 3rd world congress on electroporation and Péulses electric fields in biology, medicine, and Food &Environmental technologies Toulouse, OR-134.

Ritter A, Bruners P, Isfort P, Barabasch A, Pfeffer J, Schmitz J, Pedersoli F, Baumann M (2018) Electroporation of the liver: more than 2 concurrently active, curved electrodes allow new concepts for irreversible electroporation and electrochemotherapy. Technol Cancer Res Treat 17:1533033818809994. https://doi.org/10.1177/1533033818809994

Scala D, Rega D, Ruffolo F, Pace U, Sassaroli C, Cardone E, Gromaldi AM, Caraco V, Mozzillo N, Del Rio P (2015) Electrochemotherapy for rectal cancer after neoadjuvant radiotherapy: a case report. Eur J Surg Oncol 41(1):S13–S14. https://doi.org/10.1016/j.ejso.2014.10.037

Sersa G, Miklavcic D, Cemazar M, Rudolf Z, Pucihar G, Snoj M (2008) Electrochemotherapy in treatment of tumours. Eur J Surg Oncol EJSO 34:232–240

Soden D, Forde P, Bourke M, Winter D, O'Sullivan GC, O'Riordan M, Buckley M, Endoscopic electroporation for colorectal tumours, SAGES, 2019, poster 224

Spratt DE, Gordon Spratt EA, Wu S, DeRosa A, Lee NY, Lacouture ME, Barker CA (2014) Efficacy of skin-directed therapy for cutaneous metastases from advanced cancer: a meta-analysis. J Clin Oncol 32(28):3144–3155. https://doi.org/10.1200/JCO.2014.55.4634

Spugnini EP, Di Tosto G, Salemme S, Pecchia L, Fanciulli M, Baldi A (2013) Electrochemotherapy for the treatment of recurring aponeurotic fibromatosis in a dog. Can Vet J 54(6):606–609

Tafuto S, von Arx C, De Divitiis C, Maura CT, Palaia R, Albino V, Fusco R, Membrini M, Petrillo A, Granata V, Izzo F (2015) ENETS Center of Excellence Multidisciplinary Group for Neuroendocrine Tumors in Naples (Italy). Electrochemotherapy as a new approach on pancreatic cancer and on liver metastases. Int J Surg 21(Suppl 1):S78–S82. https://doi.org/10.1016/j.ijsu.2015.04.095

Tamzali Y, Borde L, Rols MP, Golzio M, Lyazrhi F, Teissie J (2012) Successful treatment of equine sarcoids with cisplatin electrochemotherapy: a retrospective study of 48 cases. Equine Vet J 44 (2):214–220. https://doi.org/10.1111/j.2042-3306.2011.00425.x

Tarantino L, Busto G, Nasto A, Fristachi R, Cacace L, Talamo M, Accardo C, Bortone S, Gallo P, Tarantino P, Nasto RA, Di Minno MN, Ambrosino P (2017) Percutaneous electrochemotherapy in the treatment of portal vein tumor thrombosis at hepatic hilum in patients with hepatocellular carcinoma in cirrhosis: a feasibility study. World J Gastroenterol 23(5):906–918. https://doi.org/10.3748/wjg.v23.i5.906

Tozon N, Pavlin D, Sersa G, Dolinsek T, Cemazar M (2014) Electrochemotherapy with intravenous bleomycin injection: an observational study in superficial squamous cell carcinoma in cats. J Feline Med Surg 16(4):291–299

Advancing Electroporation Systems

Roberta Fusco, Elio Di Bernardo, Valeria D'Alessio, Ruggero Cadossi, and Matteo Cadossi

Abstract

In the last several decades, electroporation has continuously developed in many fields, particularly in the preclinical, clinical, veterinary, and industrial sectors. Nowadays, electroporation is used in both cancer (electrochemotherapy, irreversible electroporation ablation) and non-cancer applications. Among the most advanced techniques that use electroporation for non-cancer therapies we include gene electrotransfer, cell fusion, insertion of proteins into cell membranes, transdermal drug delivery, water treatment, food preservation, cardiac tissue ablation, electrosclerotherapy, and electroporation of cell organelles. Given the wide variety of target cells and different electric parameters required, specific generators for each approach are often necessary.

The electroporator systems differ, mainly for (1) the structure of the generator, i.e., the hardware of the power unit (particularly the signal generator); (2) the software and overall dimensions; and (3) the electrodes to be used.

New scenarios are continually explored, such as the treatment of vascular malformations, cardiac ablation, and myocardial regeneration, increasingly placing electroporation as a valid alternative to surgery and standard thermal approaches.

Keywords

Electrochemotherapy · Gene transfer · Electroporator · Genedrive · Signal generator · Cardiac ablation · Vascular malformations · Electrosclerotherapy

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1 Background

The continuous development of Electrochemotherapy (ECT) is due to its unique mechanism of action. Combining chemotherapy and the application of electric pulses, the tumor cells are dying, not from the application of physical energy, such as in the case of local ablative techniques (e.g., irreversible electroporation, microwave ablation, and cryoablation), which is the predominant difference between this therapy and these local cytodestructive options. With electrochemotherapy, the cells are dying in a more controllable way. Thanks to this proficiency, ECT has always been subject to continuous development in many fields, ranging from the pre-clinical to the clinical sector, inclusive of medical and industrial applications.

A critical milestone in the development of ECT, as more clinical centers in Europe began using Electrochemotherapy, was represented by the Standard Operating Procedures (SOPs) for ECT doctrine. Successively, ECT was included in national and European Union guidelines for the treatment of melanoma metastases, skin tumors, Kaposi's sarcoma, and liver metastases of colorectal cancer.

Since 2014 (Cemazar and Sersa 2019), preclinical studies, with the aim of applying ECT against additional tumors, have been conducted. New combinations, with other innovative techniques (cold plasma, murine melanoma, palliative cures immunomodulatory therapies) have been investigated, as well as new antitumor compounds such as calcium to achieve higher and more selective cytotoxicity. Calcium has demonstrated effective tumor necrosis and doxorubicin and sodium decahydrodecarbonate have also been evaluated. Furthermore, new electrodes, such as bipolar (Merola et al. 2020), laparoscopic/endoscopic (Izzo et al. 2020) and grid, have been designed to treat tumors that are not accessible with the current electrodes that are currently available. Newer electrodes, combined with new electroporation protocols (e.g., high-frequency pulses protocols, short bipolar pulses protocols) have also been evaluated to reduce pain (Cemazar and Sersa 2019) caused by muscle contraction, and/or skin irritation and burns, that is considered a negative association of the therapy. Advanced developments are also focused to improve ECT efficacy. Magnetic resonance electric impedance tomography, as an example, can be used for determining the electric field distribution during treatment, enabling corrective intervention during the procedure to ensure better treatment outcomes. Liquid chromatography, coupled with high-resolution mass spectrometry, could be used for determining the bleomycin pharmacokinetics, which will enable adjustments of the drug dose to a specific patient.

New developments have also affected clinical applications. In order to prevent overtreatment of the tissue, which results in irreversible electroporation, the treatment plan can be coupled to a navigation system for accurate positioning of electrodes. Furthermore, in addition to the standard chemotherapeutic drugs, calcium chloride could be used, based on very promising pre-clinical results. A double-blinded phase II study was completed comparing the effects of calcium electroporation with ECT (Cemazar and Sersa 2019). As a result, the objective response rate for the two approaches did not show significant difference. Thus, calcium electroporation is feasible and effective in cancer patients and could be used in clinical

environments where acquisition, handling, and waste disposal of chemotherapeutic drugs represents a challenge.

Additional studies in recent years have also shown interesting developments in the field of veterinary oncology. In many cases, ECT has shown to be less invasive than surgery, easy to be performed and even relatively inexpensive. The treatment of skin tumors in dogs has shown a complete response rate in 70% of treated animals (Cemazar and Sersa 2019), allowing ECT to become a standard therapy for mast cell tumors and an adjuvant treatment to surgery for soft tissue sarcomas. Electrochemotherapy is also being used for treating fibropapillomatosis in sea turtles and cutaneous squamous cell carcinoma in felines. Another application of ECT in veterinary medicine is the treatment of sarcoids in horses. ECT is emerging more often in combination with surgery, especially in the treatment of larger tumors.

A novel approach for treating oral tumors in dogs, such as non-tonsillar squamous cell carcinoma, is to combine systemic and local administration of bleomycin. Further developments in the treatment of mast cell tumors with ECT, support reduced recurrence rates and reduced time to tumor progression by combining standard ECT with gene-electrotransfer with interleukin-12.

Finally, calcium electroporation has also been evaluated recently in sarcoids in combination with surgery, supporting calcium electroporation, followed by surgical excision, can cause necrosis in over 50% of the tumor area (Cemazar and Sersa 2019).

Given the recent developments of electroporation-based therapies in the veterinary field, the demand for dedicated devices is constantly growing, putting companies in front of new challenges. Devices for veterinary applications should be compact, as they need to be easy to carry and usable in different scenarios. One of the newest veterinary devices under development is the CliniVet electroporation system (Fig. 1), marketed by IGEA S.p.A (Carpi, Italia).

ECT is not the only electroporation-based therapy. Among the most advanced techniques, that use electroporation, we include gene electro-transfer, cell fusion, insertion of proteins into cell membranes, transdermal drug delivery, water treatment, food preservation, cardiac tissue ablation, and electroporation of cell organelles.

Such a vast and heterogeneous scenario of applications determines an equally wide variety of target cells and involves different types of electrodes. Since the efficacy of electroporation depends on both physical and biological parameters, and cell parameters cannot be controlled, each electroporation application is characterized by a specific electric protocol (i.e., pulse amplitude and duration, number of pulses and pulse repetition frequency, pulse shape, and electric field direction), which provide the required electric field distribution. For example, in DNA transfection, the pulse amplitude has to be optimized to the specific cell size, while the pulse duration has to allow plasmid DNA membrane complex formation. Gene electro-transfer and cell fusion sometimes require auxiliary pulses, such as electrophoretic pulses and dielectrophoretic pulses, respectively.

Fig. 1 CliniVet electroporation system

In summary, electroporation pulses have amplitude ranging from mV to kV and frequency from Hz to GHz (Reberšek and Miklavcic 2010). Such a variety of electric parameters almost always requires dedicated generators.

2 Concepts of Electroporation Systems

An electroporation system is composed of an appropriate set of electrodes and an electroporator. Particularly, an electroporator is a pulse generator able to generate the required signal and deliver the necessary current/power. As already mentioned, in order to achieve an effective electroporation, the signal parameters have to be adequate to the specific aim and tissue target. For this reason, when designing or purchasing an electroporator, one has to know which main parameters will influence the outcome.

The main parameter to consider is the pulse amplitude. Different applications require different amplitude ranges, which can be generally referred to as high- and low-voltage pulses. Gene electrotransfer, for example, requires high-voltage pulses for reversible electroporation, while low-voltage pulses are needed for the electrophoretic drag of DNA toward the permeabilized plasma membrane. The required pulse amplitude also influences the generator design, such as the choice of switching elements: spark gaps are normally used for very high-voltage pulses, while transistors and operational amplifiers are used for high- and low-voltage pulses, respectively.

Another parameter to consider is the pulse duration. When using nanosecond pulses, for example, the rise time of the pulse is usually shorter than the length of the electrical connection between the generator and the load (the target tissue). In this

situation, if the impedance of the load does not match that of the generator, pulse reflection could occur (i.e., a pulse with an upward displacement will reflect off the end and return with a downward displacement.). On the other hand, when using microsecond pulses, it is much more important to consider that the load might require a current that is higher than the one the generator is able to deliver.

Moreover, the pulse shape also has a significant impact on the electroporation efficacy. Particularly, in the very high-voltage range, only exponential and squared wave pulses can be used, while for lower amplitudes, no specific pulse shapes are required. Dielectrophoretic force in cell fusion, for example, requires sinusoidal signals.

All these parameters determine the design of the generator, with particular reference to the signal generator circuits. More specifically, electroporators can be divided into four major groups (Reberšek and Miklavcic 2010): (i) capacitor discharge; (ii) square wave generators; (iii) modular square wave generators; and (iv) analog generators. More specialized generators can be obtained by combining two or more of these basic circuits. An electroporator for gene electrotransfer, for example, is usually made of two square wave pulse modules, while a generator for cell fusion is usually composed of a square wave pulse module for reversible electroporation and a bipolar analog generator for sinusoidal signal. Moreover, additional modules may be added in order to deliver electroporation pulses to multiple electrodes or to control the pulse polarity (e.g., for bipolar pulses).

Finally, another important aspect to consider is related to safety requirements. Safety is the primary objective throughout the development of a generator. Particularly for clinical and veterinary applications, the safety of patient and operator is crucial to prevent the risk of electrocution, which is above all related to high energy (that is accumulated on capacitors) and high electrical current generally required for the electroporation treatment (Bertacchini et al. 2007). The most significant technical standard is IEC-60601, *Medical Electrical Equipment*, which identifies a possible risk for electrical shock when a patient or operator can be exposed to a voltage exceeding 25 V_{RMS} or 60 V_{DC} (Reberšek and Miklavcic 2010). Naturally, the device's enclosure is the first protection barrier, but a galvanical separation (achieved with an insulation transformer for power signals) between the electroporator output and the ground is also necessary to minimize the leakage currents. Moreover, energy release can be controlled and limited by specific measures implemented in a software/firmware. Furthermore, requirements for electromagnetic interference and electromagnetic compatibility must also be met.

3 Generator Structure

This section explores some main aspects relating to the structure of electroporators. Particularly, since pulse shape and stability are the variables that mainly influence the electroporation efficacy, depending on the specific application, the difference between several strategies for signal generation and the choice of how to obtain

high-voltage square pulses are outlined. Finally, an example of advanced electroporator structure is analyzed.

3.1 Type of Signal Generators (Reberšek and Miklavcic 2010)

The circuit aimed to generate the pulse signal is a crucial aspect in designing the hardware part of an electroporator. It not only determines the pulse shape but also the component necessary and their required capability.

The design of the signal generator, obviously, is closely related to the applications for which the device is intended; combination of different types of signal generators, as well as additional modules, are often demanded in recently advanced therapies.

The *capacitor discharge* circuit (Fig. 2a) is the oldest concept of pulse generator. Pulse generation is obtained in two phases, namely charge and discharge. During the charge phase, the switch (S) is in position 1, allowing the high-voltage power supply (V) to charge the capacitor (C) to the preset voltage. The discharge phase starts when the switch is set in position 2 and generates an exponential pulse. The time constant is the product between the load impedance (Z_L) and the capacitance (C), thus it is related to the absolute value of the load impedance. Since the load impedance reduces during the pulse delivery, the time constant is not constant; to better define the time constant of discharge, a built-in resistance (R) is generally connected in parallel to the load. This type of signal generator can be used for gene transfection,

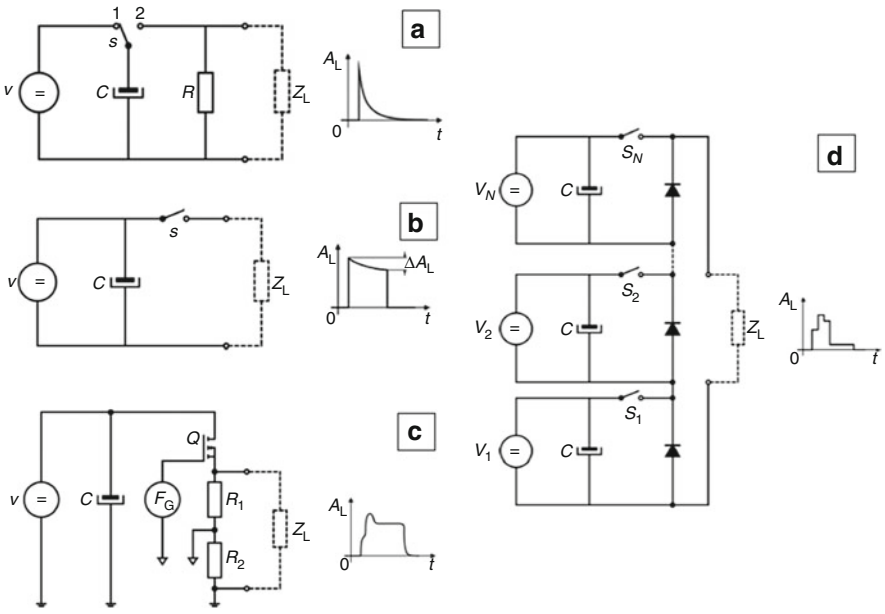

Fig. 2 Type of signal generators

since it can deliver both high- and low-voltage pulses; however, given the relatively long charge phase, the pulse frequency is highly limited.

The most frequently used signal probably is the *square wave*. The circuit (Fig. 2b) able to deliver a square wave pulse allows a better control of electric field, compared with the exponential pulse. However, a more complex control unit is required. The main differences with the capacitor discharge circuit are: (1) the voltage power supply (V) constantly charges the capacitor (C) and (2) the switch (S), usually a MOSFET or an insulated gate bipolar transistor, is capable of fast switching. Square wave pulses are usually used in both reversible and irreversible electroporation and thus are high-voltage pulses. In several applications, the required voltage is 3 kV, which is very difficult to achieve, as it requires the design of a very high-voltage amplifier. Bertacchini et al. (2007) investigated two alternative approaches for obtaining the required voltage without using an amplifier: (1) the pulse transformer and the (2) high-voltage generator. In the first case, a step-up transformer is able to transform the low voltage applied on the primary coil into the required high-voltage pulses on the secondary coil; the energy is supplied by capacitors pre-charged. When using a high-voltage generator (Fig. 2b), the capacitors (connected in series) are constantly charged by a high-voltage power supply. The pulse transformer is less efficient at low frequencies, requiring a bigger transformer. Generally, the pulse transformer does not allow pulses longer than 30μs and with a frequency higher than 81 Hz (Bertacchini et al. 2007). When using a high-voltage generator, the main advantage is a more controlled and uniform electric field but the stability of pulse shape depends on the load. If the current drawn by the load is high, this discharges the capacitors causing a voltage drop (ΔA_L) during the pulse. As a consequence, pulses are very short and require very large capacitances. For this reason, it is harder to change the amplitude between pulses, limiting the pulse generation to a few preset voltages.

The disadvantages related to the square wave pulse generator can be overcome with a *modular square wave* circuit (Fig. 2d). In this generator, N square wave pulse generators are connected in a series, each of which has a voltage source that is twice as high as the predecessor (i.e., $V_N = 2V_{N\text{-}1}$). By controlling the switch combinations, 2^N different output voltage levels can be obtained, which leads to better-defined pulses characterized by faster rise and fall times and more stable amplitude. However, the cost of the device proportionally increases with the number (N) of the square wave pulse generators required.

Analog generators (Fig. 2c) are mostly used when arbitrarily shaped and more stable electric pulses are required. A signal generator (F_G) allows the desired pulse shape and the signal generated is converted into analog; this signal is enhanced by means of a galvanically insulated amplifier with linear switching (Q). Thanks to this capability, analog generators are often designed to generate bipolar pulses. More stable pulses can be delivered by charging the capacitor with a power supply voltage higher than the maximal generated amplitude. In this way, the amplitude does not drop during pulse delivery. However, the driving stage is much more complex, and rise and fall times cannot be as fast as with a square wave pulse generator.

3.2 Design of a Generator: The Genedrive Electroporation System

Genedrive is a device for electroporation, particularly indicated for gene therapy, which is marked by IGEA S.p.A (Carpi, Italy). Thanks to its innovative structure, the Genedrive electroporation system (Fig. 3) allows the operator to design the desired pulse protocol without major constraints. The electroporator includes two-generation modules for the delivery of both high- and low-voltage pulses allowing: (a) monopolar or bipolar high-voltage pulses with a maximum voltage of 500 V (for a single wave) and lasting up to 200μs; (b) monopolar (positive or negative) low-voltage pulses with a maximum pulse amplitude of 100 V and pulse length of 400 ms.

The structure underlying this Genedrive electroporation system is shown in Fig. 4. The hardware structure is composed of a User Interface (UI) and a Power Unit (PU). The UI is the graphic interface by mean the user interacts with the device allowing the operator to set the treatment parameters and check the outcome on the *console*. The pulse generation is started using the buttons on the *handset controller*, which trigger the FPGA (Field Programmable Gate Array) in the power unit. For safety, the user must press two buttons: the first enables the FPGA to charge capacitors; the second is the trigger for the pulse generation through the *output board*.

Pulse generation is the task of the PU. Once the first button on the handset controller is pressed, the FPGA enables the charging of capacitors. Capacitors consist of two modules for accumulating energy for high- and low-voltage pulses. Capacitors charging is controlled by the *charge control system* through a specific algorithm implemented on the firmware. If the charge control system confirms that the capacitors are correctly charged, the FPGA enables the *output board* to release

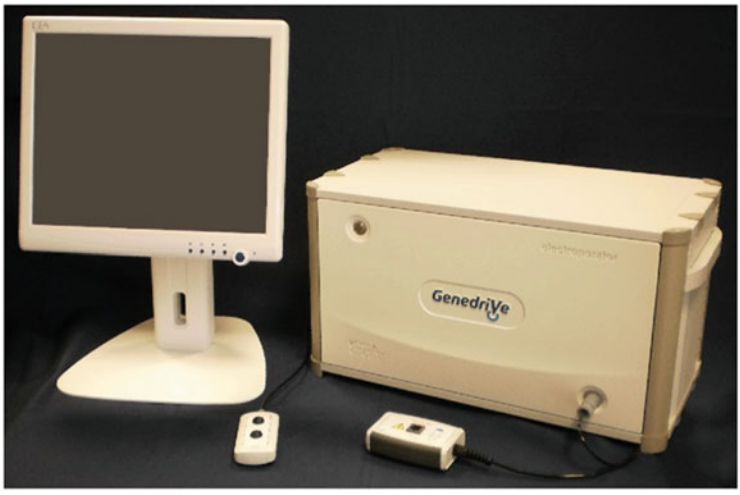

Fig. 3 Genedrive electroporation system

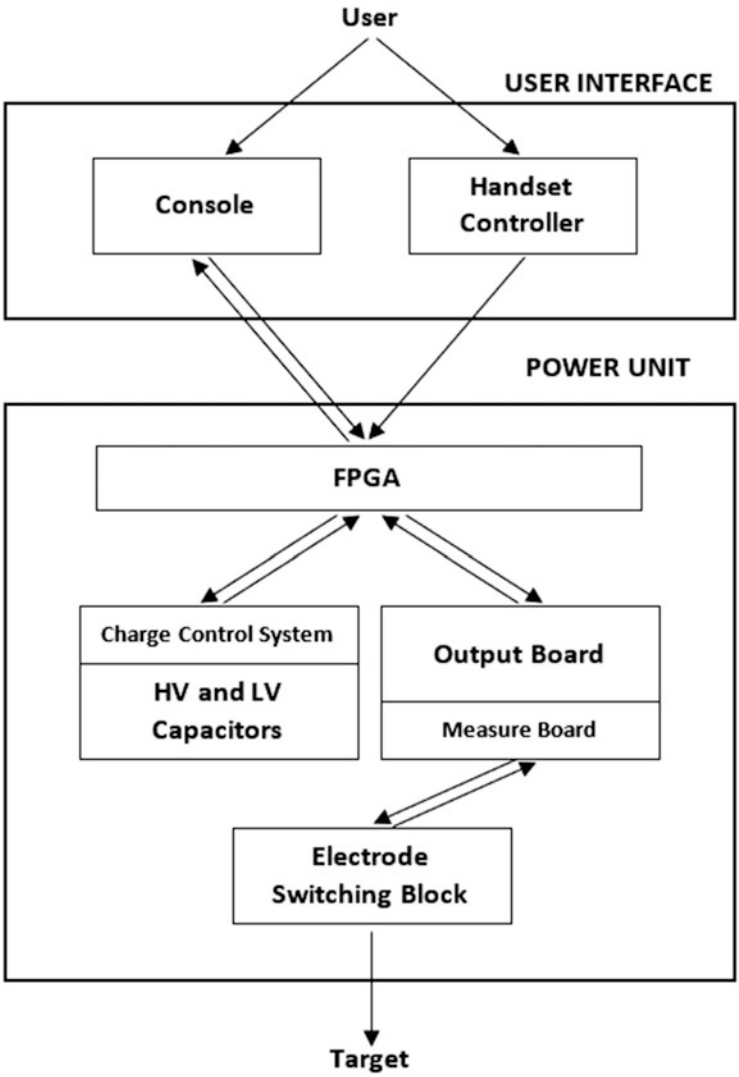

Fig. 4 Chart of main blocks composing the Genedrive electroporation system

the pulses through the two-module generator system. Pulses are delivered according to the parameters set by the user, provided that the specific algorithm implemented on the firmware, confirms the allowed operational range. Finally, a *switching board* connects each electrode in turn to the output pulse generation block (output board), while a dedicated measure system reports the voltage and current, obtained during the pulse.

4 Non-Tumor Electroporation Applications

As pointed out in the first section of this chapter, several electroporation-based techniques are developing in the most diverse sectors. Thanks to the versatility of the electroporation effect, it is possible to use controlled electric fields for purposes beyond clinical applications. Given the nonthermal nature, electroporation enables preservation of the natural quality, color, and vitamin composition of food products, and thus it can be used for food processing, cooking, and biorefinery (Mahnič-Kalamiza et al. 2014). Pulsed electric fields are also used in the food industry to improve mass transfer (Puértolas et al. 2012), thanks to their low energy consumption, short processing time, and immediate implementation ability. Even more advanced techniques, such as the real-time impedance monitoring (López-Alonso et al. 2020) and electrosclerotherapy, combine electroporation with other methods for a more enhanced efficacy of electroporation alone.

Considering the great heterogeneity of applications, we have decided to detail the clinical (and veterinary) use of electroporation in three promising sectors: cardiac ablation, electrosclerotherapy, and gene therapy.

4.1 Cardiac Ablation

In the last decade, electroporation has been investigated as a novel energy source for cardiac catheter ablation. Cardiac ablation is mostly used for the treatment of cardiac arrhythmias and has been usually performed by using thermal energy sources (e.g., radiofrequency). However, thermal ablation has been shown to cause pulmonary vein stenosis, phrenic nerve palsy or, in rare cases, paraesophageal fistula (van Es et al. 2019).

In 2007, Lavee et al. intentionally performed the first cardiac electroporation ablation and demonstrated its ability to induce transmural lesions and electrical isolation of atrial appendages. Successively, further research has been conducted to investigate the potential applications of this innovative technique. Thanks to electroporation's ability to preserve structures such as coronary arteries and the phrenic nerve, epicardial left ventricular ablation is one of the most promising advancements related to cardiac ablation. In addition, several studies suggest that atrial tissue can also successfully be ablated by electroporation (Wittkampf et al. 2018). Irreversible electroporation using various waveforms is also increasingly being investigated for the purpose of cardiac ablation.

Further studies support the development of a faster, more efficient, and safer method for catheter electroporation ablation. Wittkampf et al. (2018) designed a circular electrode catheter with the aim of determining a field distribution which linearly decreases with distance. While a single electrode determines a current density that decreases with the square of the distance, a circular catheter can induce an increased current density, thus a much greater penetration of depth and lesion size. However, further studies are required to improve electrode–tissue contact and fulfil qualification requirements.

Some limitations exist with cardiac electroporation ablation. The main limitation is related to the recognized risk of inducing ventricular arrhythmias during direct current energy delivery; moreover, myocardium may extend beyond a centimeter into the vein, and ablation for the epicardial autonomic nerves may determine the risk of stenosis developing (De Simone et al. 2014).

Electroporation leads to (skeletal) muscle contractions, which require the use of general anesthesia; in addition, current flow causes electrolysis on the electrode surface, which induces hydrogen gas production by water electrolysis at the cathode and subsequent bubble formation/coalescence (pure gold electrode) during application of the electric pulses (Mahnič-Kalamiza and Miklavčič 2020). Having entered the bloodstream, bubbles may cause damage, such as an embolism (Mahnič-Kalamiza and Miklavčič 2020). Finally, insufficient electrode–tissue contact may lead to shallow and discontinuous lesions that may cause recurrences (Wittkampf et al. 2018).

A rather interesting recent development concerns the study of alternative waveforms with the aim of reducing muscle contractions. While several authors have investigated the ability of both bipolar and high-frequency pulses in reducing muscle contractions, van Es et al. developed an asymmetric high-frequency waveform for irreversible electroporation. An asymmetric wave is a bipolar pulse composed of a short positive wave followed by a longer negative one. Given the fact that the creation of electroporation pores is proportional to the transmembrane voltage, they demonstrated that the positive phases of the asymmetrical pulses are much more effective in pore creation compared to those in the symmetrical wave. An asymmetric high-frequency pulse reaches to a deeper lesion than a symmetrical waveform of the same energy.

4.2 Electrosclerotherapy of Vascular Malformations

Vascular malformations (VM) are congenital anomalies of the vascular system defined by the International Society for the Study of Vascular Anomalies (ISSVA) as inborn errors of the vascular morphogenesis of vessels. Based on the vessels involved, VM are divided into venous malformations, lymphatic malformations, arteriovenous malformations, and capillary malformations (Horbach et al. 2020) and represent a challenge both therapeutically and diagnostically.

In the last century, many treatment modalities have been developed for treating vascular malformations. Big vessels, such as venous, have been usually treated with open resections or sclerotherapy. Particularly, the latter uses sclerosing agents (e.g., bleomycin) to occlude the pathologic vascular channels via an inflammatory reaction. On the other hand, smaller vessels, namely capillaries, are treated with pulsed-dye laser with the aim of inducing photocoagulation of the abnormal blood vessels. However, both surgery, sclerotherapy and pulsed-dye laser are effective in about half of the patients treated, with a consequently minor improvement of overall health status and frequent recurrence.

Several studies have been performed to investigate combining bleomycin sclerotherapy with electroporation. The electroporation effect, in fact, has shown to be an ideal method for localized drug delivery while avoiding systemic side effects. Furthermore, the combination of sclerotherapy and electroporation, also called electrosclerotherapy (EST), may facilitate the delivery of bleomycin to the endothelium of capillary vessels, causing a local drug effect. Finally, electrosclerotherapy utilizes a reduced dose of sclerosing agent (McMorrow et al. 2017).

In 2020, Horbach's group (Horbach et al. 2020) reported that EST can be considered as a new treatment modality for capillary malformations. They demonstrated that color and hypertrophy of all target lesion treated with EST significantly improved, based on the patient-observer scar assessment score (medians patient −11; observer −13), global assessment of change, and colorimetry (ΔE 3.4–16.5) scores.

Wohlgemuth et al. in 2020, treated 20 venous malformation in 17 patients. The EST treatment included three major steps: positioning of electrodes, intralesional bleomycin injection, and electroporation. All interventions were performed under general anesthesia. The results demonstrated that EST is effective at lesion reduction and associated with improvements in patient's quality of life despite previous unsuccessful invasive therapies. However, due to the small sample size and short follow-up period, further investigations are required.

4.3 Gene Therapy

Gene therapy is the transfer of nucleic acids into target cells or tissues to achieve therapeutic benefit. During the last decades, gene therapy has used both viral and nonviral vectors to administer plasmid DNA to the target area. However, viral vectors often cause tissue immune response and may be toxic. On the other hand, nonviral-mediated gene transfer is usually associated with a poor targeting of diseased tissues.

Naked DNA vectors are not associated with host immune and inflammatory response and thus are much safer and generally do not lose efficacy in gene delivery.

Several studies have shown that gene therapy with plasmid DNA, when combined with electroporation, has an enhanced gene transfer effect in most cells, leading to a more stable transformation or transient gene expression compared to gene transfer alone (Potter 2003). Electroporation-mediated in vivo gene delivery has provided highly effective in vaccine production, transgene expression, and enzyme replacement, when appropriate square waves or exponential pulses are applied to the target area. The levels of gene expression jump between 20- and 1000-fold (Young and Dean 2015). Although the whole mechanism by which DNA actually enters the cell is almost unclear, DNA and other nucleic acids must be present in the target area before and during the electroporation for the treatment to be effective.

Despite the large number of applications, we have chosen to focus the discussion on the use of electroporation-mediated gene transfer in the regeneration of

myocardial tissue. In fact, coronary artery disease continues to be a major cause of morbidity and mortality worldwide (Ayuni et al. 2010). Currently, the standard therapy, i.e., revascularization procedure either by percutaneous angioplasty or by coronary bypass surgery, does not reduce the risk of death. Regeneration of the infracted myocardium is the major challenge. Several studies have investigated the efficiency and the effect of electroporation-mediated gene transfer applied to the beating heart. Their results showed that this approach is feasible and safe, with a high gene expression achieved. Particularly, it was demonstrated that administering electric pulses that were synchronized to the rising phase of the R wave of the electrocardiogram resulted in enhancement of the delivered transgene, without fibrillating the heart (Hargrave et al. 2013).

Among the aspects that most influence the outcome of the procedure, apart from the electrical parameters of the impulses, we have to mention the design of the electrodes, which is the subject of continuous studies and developments.

5 Conclusions

In the last decades, numerous studies have analyzed the potential of electroporation in more detail. Starting from the first applications in the treatment of cancer. Today, electroporation is used in the most varied fields, ranging from the veterinary/medical specialty, gene therapy cardiac ablation, myocardial regeneration, wine production, and even food processing.

Given the multiplicity and diversity of applications, each sector has always investigated the optimal electrical protocol in order to optimize the effectiveness of the procedure. The subsequent results are protocols that differ in the amplitude of the pulses, in their frequency and duration, and even in their shape. As a result, a large number of electroporators are currently commercially available, pushing developers further into competition in order to improve and constantly strive for better outcomes.

The electroporator systems differ, mainly, with the structure of the generator, i.e., the hardware of the power unit (particularly the signal generator); the software and overall dimensions and the available electrodes.

Electroporation is emerging as a unique approach in a variety of different fields with many beneficial results.

References

Ayuni EL, Gazdhar A, Giraud MN, Kadner A, Gugger M, Cecchini M, Caus T, Carrel TP, Schmid RA, Tevaearai HT (2010) In vivo electroporation mediated gene delivery to the beating heart. PLoS One 5(12):e14467. https://doi.org/10.1371/journal.pone.0014467

Bertacchini C, Margotti PM, Bergamini E, Lodi A, Ronchetti M, Cadossi R (2007) Design of an irreversible electroporation system for clinical use. Technol Cancer Res Treat 6(4):313–320. https://doi.org/10.1177/153303460700600408

Cemazar M, Sersa G (2019) Bioelectricity 1:204–213. https://doi.org/10.1089/bioe.2019.0028

De Simone CV, Kapa S, Asirvatham SJ (2014) Electroporation: past and future of catheter ablation. Circ Arrhythm Electrophysiol 7(4):573–575. https://doi.org/10.1161/CIRCEP.114.001999

Hargrave B, Downey H, Strange R, Murray L, Cinnamond C, Lundberg C, Israel A, Chen YJ, Marshall W Jr, Heller R (2013) Electroporation-mediated gene transfer directly to the swine heart. Gene Ther 20:151–157. https://doi.org/10.1038/gt.2012.15

Horbach SER, Wolkerstorfer A, Jolink F, Bloemen PR, van der Horst CMAM (2020) Electrosclerotherapy as a novel treatment option for hypertrophic capillary malformations: a randomized controlled pilot trial. Dermatol Surg 46(4):491–498

Izzo F, Ionna F, Granata V, Albino V, Patrone R, Longo F, Guida A, Delrio P, Rega D, Scala D, Pezzuto R, Fusco R, Di Bernardo E, D'Alessio V, Grassi R, Contartese D, Palaia R (2020) New deployable expandable electrodes in the electroporation treatment in a pig model: a feasibility and usability preliminary study. Cancers (Basel) 12(2):515. https://doi.org/10.3390/cancers12020515

Lavee J, Onik G, Mikus P, Rubinsky B (2007) A novel nonthermal energy source for surgical epicardial atrial ablation: irreversible electroporation. Heart Surg Forum 10(2):E162–E167. https://doi.org/10.1532/HSF98.20061202

López-Alonso B, Sarnago H, Lucía O, Briz P, Burdío JM (2020) Real-time impedance monitoring during electroporation processes in vegetal tissue using a high-performance generator. Sensors (Basel) 20(11):3158. https://doi.org/10.3390/s20113158

Mahnič-Kalamiza S, Miklavčič D (2020) Scratching the electrode surface: insights into a high-voltage pulsed-field application from in vitro & in silico studies in indifferent fluid. Electrochim Acta 363:137187

Mahnič-Kalamiza S, Vorobiev E, Miklavčič D (2014) Electroporation in food processing and biorefinery. J Membr Biol 247(12):1279–1304. https://doi.org/10.1007/s00232-014-9737-x

McMorrow L, Shaikh M, Kessell G, Muir T (2017) Bleomycin electrosclerotherapy: new treatment to manage vascular malformations. Br J Oral Maxillofac Surg 55(9):977–979. https://doi.org/10.1016/j.bjoms.2017.10.002

Merola G, Fusco R, Di Bernardo E, D'Alessio V, Izzo F, Granata V, Contartese D, Cadossi M, Audenino A, Perazzolo GG (2020) Design and characterization of a minimally invasive bipolar electrode for electroporation. Biology (Basel) 9(9):303. https://doi.org/10.3390/biology9090303

Potter H (2003. Chapter 9) Transfection by electroporation. Curr Protoc Mol Biol Unit-9:3. https://doi.org/10.1002/0471142727.mb0903s62

Puértolas E, Luengo E, Álvarez I, Raso J (2012) Improving mass transfer to soften tissues by pulsed electric fields: fundamentals and applications. Annu Rev Food Sci Technol 3:263–282. https://doi.org/10.1146/annurev-food-022811-101208

Reberšek M, Miklavcic D (2010) Concepts of electroporation pulse generation and overview of electric pulse generators for cell and tissue electroporation 1st Edition Chapter 16 https://www.taylorfrancis.com/books/advanced-electroporation-techniques-biology-medicine/10.1201/EBK1439819067?refId=5a736778-efe4-459a-a072-923e2b71429a

van Es R, Konings MK, Du Pré BC, Neven K, van Wessel H, van Driel VJHM, Westra AH, Doevendans PAF, Wittkampf FHM (2019) High-frequency irreversible electroporation for cardiac ablation using an asymmetrical waveform. Biomed Eng Online 18(1):75. https://doi.org/10.1186/s12938-019-0693-7

Wittkampf FHM, van Es R, Neven K (2018 Aug) Electroporation and its relevance for cardiac catheter ablation. JACC Clin Electrophysiol 4(8):977–986. https://doi.org/10.1016/j.jacep.2018.06.005

Wohlgemuth WA, Müller-Wille R, Meyer L, Wildgruber M, Guntau M, Heydt SV, Pech M, Zanasi A, Flöther L, Brill R (2020) Bleomycin electro ScleroTherapy (BEST) in therapy-resistant venous malformations of the body. J Vasc Surg Venous Lymphat Disord S2213-333X(20):30542–30544. https://doi.org/10.1016/j.jvsv.2020.09.009

Young JL, Dean DA (2015) Electroporation-mediated gene delivery. Adv Genet 89:49–88. https://doi.org/10.1016/bs.adgen.2014.10.003