Regenerative Medicine Procedures for Aesthetic Physicians

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Editors

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Preface

Adipose tissue grafts have been the last great evolution of plastic surgery after perforator flaps. In its first applications, we observed fascinating changes, such as the improvement in the texture of the tissues, skin coloration and vascularization – changes that went beyond the simple increase in volume. Later, we would know that this is due to the regenerative properties that our own tissues can have and, properly done, can be expressed where they are applied in the pursuit of the best natural aesthetic improvement.

In this book, we wanted to integrate in a single work the current knowledge on the biological bases of the different treatments with regenerative effects, the technical foundations and the perspective of the results that can be obtained (and those that can't). And our will has been that it had the features we expect for a book for our own consultation: concise, clear and practical.

In this way, we want it to be a handbook of consultation for all those professionals interested in the fascinating world of regenerative medicine, which will surely bring many new indications as we know better their potential.

Barcelona, Spain — Joan Fontdevila

Preface

Regenerative procedures are one the most appealing and high-potential ways of treating our patients in modern medicine. The fact of being able to regenerate up to some extent a damaged structure enhances the possibilities of the physiological healing process way beyond its original duty. Any regenerating strategy implies full functional and structural restoration which is something non-acquirable by reparation.

This book presents the state of the art in regenerative procedures currently applied by aesthetic physicians, plastic surgeons and dermatologists. It is divided into two parts, the first of which provides a detailed introduction to aesthetic medicine and the aging process. The second part, in turn, addresses the current status of techniques and technologies with regard to autologous grafts, covering fat transfer, blood grafts, skin grafts and stem cells. The book examines the surgical applications of these grafts, as well as potential side effects and limitations.

With this text, we want to promote and support an evidence-based behaviour, provide the state of the art of the regenerative procedures already applied by aesthetic physicians and set the basis of a "regenerative approach" to beauty, tissue damage, cell restoration and aging.

Barcelona, Spain Hernán Pinto

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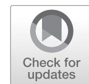
Part I

Approaches: Introduction



Regenerative Medicine Techniques: Clinical Applications in Aesthetic Procedures

Hernán Pinto

Compared to lower vertebrates, the human body has a limited regenerative capacity. To face this fact, intervening in this limited ability of tissues and organs to self-heal has been an old promise of regenerative medicine. Since first reports appeared about the possibility of expanding organ failure and tissue damage repair beyond allogenic transplantation and the use of animal-derived products, efforts have been made to deploy regenerative principles into practice [1]. At the interface between life sciences and engineering, regenerative medicine has been regarded as a therapeutic revolution [2], but being it or not, it is indeed a novel biomedical field that requires the contribution of multiple disciplines and a holistic view of human biology. It observes and enhances the properties of living cells, through the exogenous addition of growth factors, often in combination with biocompatible scaffolds, to boost the tissue's regenerative capacity [3].

Regenerative Medicine to Reverse Tissue Decline

The increasingly ageing population in first-world countries and the increasing demands to medicine for building strategies to improve elderly population's quality of life and increase their 'health span' is putting a certain amount of pressure to the old promise of regenerative medicine to succeed. Organs and tissues are also susceptible to be damaged by disease, trauma or congenital defects. Thus, virtually every medical and surgical specialty will soon be looking towards newly emerging regenerative medicine techniques. Cosmetic and aesthetic medicine will not be an exception, and in the coming years it is forecasted that the biggest advances in cosmetic medicine and plastic surgery will be derived from the application of regenerative medicine techniques [3].

The chance to reverse ageing effects on body functions is one of the reasons-to-be of aesthetic medicine. It is known that one of the major features of ageing is a decline in the regenerative *vigour* of many organs and somatic tissues. Ageing is accompanied by a progressive decline in stem cell function, resulting in less effective tissue maintenance and regeneration [4]. Thus, restoring beauty through regenerative medicine techniques will mirror a fully recovered, although limited by the idiosyncrasies of mammal species, regenerative capacity of younger tissues.

Preventing the Translational Gap

To achieve its aims, a shift in the traditional approach where clinicians work far from basic scientists becomes almost mandatory. In this

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turn, the concept of translational regenerative medicine comes into play as a key actor. It is about preventing the so-called translational gap and promoting cooperation between laboratory research and clinical care [5]. For instance, going back to the ageing problem, basic research has shown that tissue regeneration is dependent upon stem cells, and therefore, any loss in number or functionality due to ageing will likely have a profound effect on human regenerative capacity. Therefore, understanding the basic molecular pathways of age-related stem cell dysfunction in mammals and how stem cell functionality changes with age, including impaired self-renewal and aberrant differentiation potential, has significant implications for stem-cell-based therapies. Likewise, injecting adipose-derived stem cells to patients in a standardized manner requires prior research studies to define their cellular and molecular mechanisms and establish proper isolation procedures [6].

In this sense, the facts tell that the vast majority of regenerative medicine technologies and procedures have not fulfilled all steps into the clinics. There is a significant amount of basic studies looking at molecular and cellular principles of potential new therapies, but only a few clinical studies have been published to date. There is an overwhelming lack of standardization of aesthetic procedures [7]. Animal models would be desirable to help build protocols on procedures such as fat graft preparation and injection to prevent some of the well-known problems of this technique [8].

Tissue Engineering

The liver has been known to be able to regenerate itself from millennia but only recently the same is being proven about the heart [2], and yet, major advances have been made in allogenic organ transplantation, in spite of worldwide organ shortage, huge waiting lists and some transplant-derived immune problems. As an alternative to face these issues, a strong body of knowledge is beginning to be built around whole-organ bioengineering [9], 'de novo' generated cells [10] and using scaffolding materials as cellular matrices to support newly generated cell populations out of cell cultures [11], among other regenerative medicine techniques. Re-growing organs in the lab, using patients' own cells, is possible nowadays. Since Ott et al. [12] succeeded in developing a decellularized heart as a biological scaffold to 'resuscitate' using new cell populations, the field of organ bioengineering is advancing at a rapid pace. The generation of truly vascularized organ scaffolds from native solid organs has been the main trigger out of Otto's initial development, and thanks to it, the same techniques are now applied to almost every solid organ. Scaffolds serve as a sort of templates for tissue growth analogous to the extracellular matrix. Thus, a key concept in the development of tissue and organ engineering is the design and development of extracellular matrices (ECM) as scaffolds that allow surrounding cells to infiltrate. ECMs support the infiltration and further proliferation of cells and signalling molecules to promote proper tissue vascularization. Skin, as the largest organ in the body, has been since a long time an experimental battlefield in tissue engineering.

Engineered Skin for Aesthetic Regenerative Medicine

Human skin grafts have reliably reached the market and, so far, have helped patients with severe burns and other major wounds [13]. However, after 25 years of research in skin engineering, scientists, surgeons and physicians are still struggling with the creation of dermo-epidermal substitutes that can readily be transplanted in large quantities, possibly in only one surgical intervention and without significant scarring. Bioengineered skin grafts should be able to rapidly become vascularized. To speed up vascularization, matrices should ideally allow the controlled release of growth factors, which is a still in the research arena. Growth factors like fibroblast growth factor, vascular endothelial growth factor (VEGF), insulin-like growth

factor (IGF) and platelet-derived growth factor (PDGF) have a crucial role for cell migration, proliferation and differentiation in the wounded area, and thus, efforts in tissue engineering have focused on the incorporation of growth factors into matrix scaffolds. Thus, ECMs for skin engineering should be ideally nourished with a combination of cell cultures (either progenitor cells or mature keratinocytes), molecules such as lymphocytes, adhesion peptides and previously mentioned growth factors. This way, matrices acquire the ability to direct cell metabolism and cell differentiation. Former models of skin substitutes show that biomaterials were often from xenogenic origin, and bovine collagen was the main source for ECMs. Using those materials has progressively been abandoned due to the risk of prion disease transmission and host rejection issues and has been replaced by autologous pluripotent stem cells obtained from hair follicles, capable of differentiating in cells from mesodermal and ectodermal origin such as neurons, glial cells, keratinocytes, smooth muscle cells and melanocytes [14].

A Shift from Allogenic to Autologous Materials in Aesthetic Medicine

Aesthetic regenerative medicine is heading towards the exclusive use of autologous source of cells and molecules, for either skin engineering or cellular or molecular therapies. The fact that donor and host are the same person, with obviously identical genetic features, eliminates completely the risk of immune rejection and other inherent problems associated with allogenic transplants, some autologous cell- and molecular-based procedures are readily available. Aesthetic medicine prioritizes using procedures that can be performed in an ambulatory context and minimally invasive to the patient. Therefore, autologous materials obtained from a patient's blood or fat deposits and posteriorly processed are the therapeutic procedures of choice for this medical specialty.

Fat as a Source of Stem Cells

Adipose tissue is a reliable source of pluripotent stem cells. In particular, due to its mesodermal origin, precursor cells from mesenchymal stem cell lineages (MSCs) can be obtained. MSCs, whose functional decline is involved in ageing, can be easily obtained from subcutaneous fat tissue [16]. Given the ease of harvest and their abundance, fat tissue is considered a promising source of autologous stem cells known as adipose-derived stem cells (ADSCs). Those, like any MSC, are pluripotent precursor cells and have the ability to equally differentiate along multiple cell lineage pathways of mesodermal origin, such as endothelial cells and adipocytes, and are also a rich source of growth factors, cytokines and lymphocytes. Autologous fat is obtained by liposuction and lipoaspirate from easily identifiable donor subcutaneous sites and is not an invasive procedure for patients.

Regenerative medicine is turning towards researching cellular and molecular properties of adipose human tissue. In fact, fat is a complex tissue, composed by matrices of adipocytes, interspersed with collagen fibres, stromal cells, ADSCs and neurovascular structures. Adipose tissue plays a major role in ageing, metabolism and homeostasis. It provides the major contribution by volume to the connective tissue matrix, stores fat for further metabolic breakdown, regulates immunity, promotes angiogenesis and, over it, is the largest endocrine organ in the body.

New approaches in autologous fat processing for aesthetic medicine are nowadays focused in obtaining ADSCs [6]. Clinical applications of fat-derived stem cells are enormous, from tissue decline reversal to severe wound closure, as shown in experimental wound models where ADCSs accelerated wound healing by improving re-epithelization and angiogenesis [17]. Plastic surgeons are also beginning to use adipose-derived stem cells for aesthetic surgical procedures in addition to fat grafting. Their success is supported by murine models of tissue augmentation which shows that fat grafts used in combination with ADSCs improve significantly tissue

outcomes in terms of weight and appearance as opposed to fat grafting alone [18]. Cell differentiation is an important aspect of stem cell therapeutic potential, but other mechanisms such as vasculogenesis, arteriogenesis, angiogenesis, cell preservation, antiapoptosis and anti-inflammation are also a major advantage of using autologous ADSCs.

Growth Factors Obtained from Platelet-Rich Plasma

The truth is that cosmetic regenerative medicine cannot always rely on ADSCs for their procedures due to regulatory approval issues that will be briefly discussed below. Thus, autologous fat grafts are still being used as such, but recent research is showing that its efficacy increases largely when combined with a blood derivative, platelet-rich plasma (PRP), as it significantly raises fat graft survival [15]. Likewise, PRP has been shown to be a reliable supplement for adipose tissue mesenchymal stem cell expansion because it promotes these cells' proliferation without altering their phenotype, differentiation potential and chromosome stability [11]. In both scenarios, the mechanism behind PRP efficacy is based on platelet's capacity to release growth factors involved in tissue nourishing processes such as vascularization. Thus, the local joined effect of growth factors due to platelet concentrates has been termed platelet-derived growth factors (PDGF). PDGF are mitogenic for fat-derived mesenchymal cells such as fibroblasts, osteoblasts and adipocytes that stimulate the formation of collagen and structural proteins such as fibrin [19]. In addition to this, another major advance of PRP technology is that it allows the formation of a three-dimensional fibrin matrix that retains and later releases part of this bulk of growth factors and acts as a temporary resting scaffold for cells to proliferate.

Platelet-rich plasma has reached the market in cosmetic industry as gel platelet concentrates [20]. It is being used to treat highly prevalent chronic wounds in aged patients. Research has also found that PRP not only acts in wound healing but also reduces neuropathic pain associated with some injuries. Anti-ageing autologous serums based on high concentrations of growth factors and anti-inflammatory cytokines have also been used and have proven to improve cutaneous hydration and skin mechanical properties [21]. There is still an important drawback to these already omnipresent blood-derived dermo-aesthetic products: the persistent lack of standardized isolation procedures and delivery protocols. This makes clinical results difficult to compare, and thus, conclusions about their efficacy are usually not definitive.

Regulatory Approval Obstacles

Aesthetic medicine, more than other medical specialties, is often facing regulatory approval obstacles when using regenerative medicine techniques. It is usually accepted that cell-based therapies have a more arduous approval process than non-cellular approaches. This makes stem cells clinical applications difficult to implement in certain countries. In addition, plasma-derived products generate a confusion between being considered blood derivatives or drugs. Despite the adoption of process simplification and cost-effective strategies, aesthetic medicine is often facing legal regulatory decisions based on arbitrary criteria that render aesthetic regenerative advancement a slow and tough task full of restrictive permissions and regulation protocols.

Concluding Remarks

Regenerative medicine is an interdisciplinary emerging field that combines several converging technologies and requires an interdisciplinary collaboration between many scientific and medical fields. It is a novel response to the poor capac-

ity of human organism to auto-regenerate correctly and persistently throughout its life span. Cosmetic and aesthetic regenerative medicine provide a particular focus to the ageing problem and skin regeneration. Regenerative medicine is heading towards the use of autologous materials versus allogenic biological products because of their advantages in terms of host acceptance. Tissue engineering, in particular skin substitutes, is advancing at a high pace. Adipose-derived stem cells are a reliable source of cell pools necessary for tissue regeneration and many molecules such as growth factors crucial for cell proliferation and nutrient absorption. In addition, blood derivatives have reliably reached the market and are being used alone or in combination with other technologies to push tissue's regenerative full capacity and proper speed.

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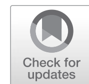

Aesthetic Medicine: Trends, Patients' Needs

Paloma Tejero

People say sometimes that Beauty is superficial.
That may be so. But at least it is not so superficial as Thought is.
To me, Beauty is the wonder of wonders.
It is only shallow people who do not judge by appearances.
The true mystery of the world is the visible, not the invisible.
(Oscar Wilde, The Picture of Dorian Gray)

Aesthetic Medicine: Reviewing Concepts

The search of the beauty and the body adornment to distinguish itself from others has been a constant in the history of humanity. The human being has always pursuit archetypes of beauty, which integrate him/her in a collective but at the same time make him/her feel different, unique. People have decorated their body with paintings, tattoos, ornaments and pendants, with a profound cultural, social or religious significance.

For the biologist, the appearance is an indicator of the quality of the genes and for that reason it plays an important role in our selection criteria. The attractiveness or beauty is something objective and mathematically measurable, which is part of the evolutionary process when considering beauty as indicative of a stronger immune system. Being beautiful in the natural world would mean being a carrier of good health. For Victor Johnston, evolutionist psychologist, there is a strong biological determinism marked by the hormones and that is part of our nature in order to perpetuate the species.

So there are no personal or cultural options. Thus, men like the faces that show fertility, that is, those whose level of testosterone is low, and women are attracted by men with a good physical aspect or good immune system which means bearer of good genes. Rosa Raich [1], on the other hand, alludes to higher cognitive processes that usually inhibit biological aspects and influence the characteristics and factor of personality and intellectuality. Today nobody discuss that the phenotype, the group of external features and characters, depends on the genes which determine it and of the pressure that the environment could have over the genetic constitution of the individual [2].

Already in 3500 BC, in the Ebers papyrus, cosmetic formulas and some tissue transplants are described. The Egyptians, a civilization advanced for their time, also reflected their medical knowledge in the papyrus of Edwin Smith (2200 BC), which describes surgical interventions and the treatment of traumatic injuries and facial alterations. The Egyptians established in an empiric way the proportions of the human figure, using segments as a unit of measurement [3].

Nowadays, medical techniques have been utilized to achieve the concept of beauty and well-being, with therapeutic and iatrogenic implications, which make it necessary for them to be indicated and controlled by medical professionals.

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At present, we know and seek the "health–beauty binomial". The waist measurement is important not only because it shows us a slender figure, but because its increase is an indicator of cardiovascular risk. The appearance in people over 70 is an indicator of vital prognosis [4]. And of course, no one doubts the "therapeutic power of the image".

Being well improves self-esteem, making our patients more "happy", improving as well their immunity. One-fifth of people with a chronic physical health problem (such as cancer, diabetes, heart disease and stroke) have depression—a rate two to three times higher than those who are in good physical health. A combination of depression and a chronic physical health problem can significantly aggravate negative outcomes for people with both conditions.

Aesthetic Medicine: Patients and Needs

Only a few decades ago aesthetic medicine was a privilege of a few; today it has become a social need and it is the patients who demand medical attention to help them be healthy and feel good. Aesthetic medicine is and has to be a preventive medicine. No medically aesthetic act can be performed without a thorough clinical history of the patient, an adequate selection of procedures, informing the patient rigorously and making him/her participate in the decision-making process. Informed consent is a key part of the doctor-patient relationship.

The prototype of healthy female patients between 40 and 60 years old that requires curbing the signs of ageing or changing some of their anatomy has changed radically.

Today in the consultations of aesthetic medicine, we face patients of all ages and conditions. In many consultations, there are three generations who come looking for that condition of "mortality", coined by Mayer, in which everyone wants to look young, healthy and beautiful [5].

The male patient has built up completely, not only requesting to maintain or recover his hair, or a muscular and attractive body, but also as a consumer of cosmetics and treatments that avoid the tired appearance of the face, or the appearance of wrinkles.

And our patients are not always healthy. We live the boom of the demand of medical-aesthetic treatments in patients over 70 years. This requires the doctor to have a broad basic training, since these patients are generally polymedicated, have comorbidities and have dermal and anatomical conditions that oblige them to modify the treatments and adapt them, sometimes even with medical-legal implications; it is sufficient to remember the contraindications for botulinum toxin use in aesthetic medicine for ages 65 and older. We must be prepared for the great challenge of the demography of ageing, which requires social sustainability, in which it is essential to promote personal autonomy in health care, as the WHO warns when it speaks of active and healthy ageing [6].

Professor Santiago Grisolia, 1990 Prince of Asturias award for technical and scientific research (at 94 years old), affirms that "Old age is not the end of the good things in life" [7]. I can boast of having in my consultation today patients over 90 years, who for more than 30 years we have been taking care of.

And what about patients with chronic diseases like diabetes and especially the great problem of autoimmune diseases, in which the conditioner is not only the altered response to many treatments, especially those that are based on the introduction of a foreign body (fillers, threads), but also in the modifications that involve the use of medicines like interferons and all biological medicines? We cannot systematically refuse to treat these patients. You have to look for the treatment and the right time. We have the experience of approaching a patient with HIV. We need solutions for these patients.

The other great challenge is the oncological patient. The number of new cases increases alarmingly, and we know that one in two men and one in three women will have cancer at some point in their lives. The good news is that survival rates increase, and we expect cancer to be for the most part a chronic disease [8, 9]. But in the oncological process, there is a "continuum",

from the diagnosis that marks and conditions the patient for his/her whole life [10]. And the cancer patient demands medical-aesthetic treatment. It not only has to face the disease, but also to change its image, skin alterations, hair loss, surgeries and their sequelae, body dysmorphisms. Aesthetic medicine, integrated in the oncological therapy, plays a fundamental role in the prevention of the disease and the sequelae of the treatments: chemotherapy, radiotherapy and surgery. New medications based on immunological treatments, as well as the molecular target drugs that affect growth factor receptors, produce a lot of cutaneous adverse symptomatology that we have an obligation to prevent and combat. And for that we need training – training and dialogue with all the actors of the disease process: oncologists, radiotherapists, dermatologists, physiotherapists and specialized aestheticians. The approach used for these patients is always multidisciplinary.

The patient who comes for a consultation for aesthetic medicine today seeks a medical professional who not only improves his/her image and attractiveness but also educates him/her in health: creation of healthy habits, food education, maintenance of ideal weight and also prevention of diseases based on these habits (photoprotection, adequate diet, exercise). The Academy of Dermatology stated that peels are a valuable weapon to prevent skin cancer [11]. Increasingly the relationship between obesity and cancer has been examined, and we know that there is a relationship between fat intake and metastases [12].

Aesthetic medicine also accompanies the patient in the great moments of life: adolescence, acne treatments, the first cosmetics, learning to eat and take care of one's self, after pregnancy, hormonal changes, menopause and andropause. Aesthetic medicine has to adapt to all these needs. That is why a new challenge has emerged, the aesthetic gynaecology field, which seeks not only to rejuvenate and beautify the "intimate zones" but also to combat the atrophy and dryness of the vagina in the menopausal woman, or who has had treatments that lead to it, or treatments in men who have had need of therapy with antiandrogens.

For all this, aesthetic medicine is in constant transformation and adaptation. It is primarily Medicine with capital letters, which requires continued training. Also the aesthetic doctor has to have extensive knowledge to be able to help his/her patient of the most appropriate treatment at any time, but that does not mean that it can be the "best among all treatments". More clearly, it becomes necessary to subspecialize within an integrative medicine. New technologies, new approaches with regenerative medicine and the new fields of treatment require an increasingly specific training in order to guarantee the quality of our treatments, which are not always free of risk and which force us to know very precisely how to address potential adverse effects and management options for potential complications of many procedures available for patients [13].

Where Are We Going: The New Challenges

Current trends in surgery and aesthetic medicine are aimed at two objectives – facial rejuvenation and body remodelling – framed in a patient with increasingly longer life expectancies. But in order to achieve these objectives, less aggressive techniques are required, with a shorter recovery time and minimal sequelae. To achieve this, it is essential to develop new technologies based on the use of different sources of energy (laser, light sources, radiofrequency), which are in continuous evolution, and knowledge of different active principles with molecules capable of preventing and repairing. But if there has been a major revolution, it is in utilizing the potentiality of our own body to repair the various processes that occur over the years [14]. Regenerative medicine is undoubtedly the protagonist of the last years and the one that will continue to advance, together with the advances in the knowledge of the genome and the individualized DNA, which will allow us to prevent different processes and to personalize not only medical treatments but also the cosmetics to be used and the medical-aesthetic treatments to be performed.

In the regenerative medicine market, it is estimated that it will bill more than USD 1 billion in 2021. Cell-based products are expected to dominate the world market, segmented to cell therapy, gene therapy, tissue engineering and immunotherapy. Immunotherapy is currently the fastest-growing segment on the world market [15].

These and other methods known to be in use or under development promise to soon bring society to surprising choices and perplexing difficulties, all in a worldwide effort to provide reliably rejuvenating stem cells and to produce immunologically adapted organs that serve to prevent, treat, cure or even one day eradicate diseases with genetic or epigenetic mechanisms.

With the era of human engineering, a major debate about regulations, procedures, prohibitions, restrictions, institutional controls and transparency rules of financing is also underway, which for many are proving ineffective in an environment where they find enormous biomedical and bioethical potential at risk and in which rights, health and heritage come into play with bioethical assumptions and formal protections that urgently need reassessment.

One of the most important challenges in the prevention and treatment of ageing of the skin is to know the characteristics of the same, so as to be able to pose the most effective treatments. If to date we had dermoanalysis equipment that allows us to know the pores' status, acne, vascular and melanin alterations, the degree of hydration, etc., we now want to have more information about the ageing process. We cannot treat what we do not know. We know that each of us, we have a genetic base, an inheritance, on which the environmental factors and our way of life will give rise to a phenotype that will condition us in our way of being, of living, of getting old, of getting sick and of dying. The use of the overall gene expression profile, also known as transcriptomic or genomic, provides a means for identifying the main affected pathways in skin ageing which may be improved with appropriate cosmetic compounds. Some of the aspects of skin ageing that can be treated include lipid synthesis, antioxidant capacity and the ability of hyperpigmentation and to respond to sunlight. The use of "gene expression profile" together with cultures of human skin cells in vitro has served to identify cosmetic compounds and understand their biological effects [16].

All this allows to perform a treatment adapted to the needs of the patient and to carry out a personalized follow-up.

The knowledge of our genes indicates our susceptibility. The new cosmetics based on genomics (genocosmetica) seeks to be a personalized cosmetics. We advance in this line, but the assets we have are still the basis of a treatment that must be continuously modified according to our physiological changes, the climate in which we find ourselves or the place where we work.

The use of stem cells, in aesthetic medicine and plastic surgery, is currently undergoing continuous development, including the best source of production, the therapeutic potential and, above all, its safe use in chronic wounds and cure of fistulae, scar management and breast reconstruction, as well as in bone and tendon repair and regeneration of the peripheral nerve [17].

Within this field, advances in regenerative medicine and hair tissue engineering in recent years have raised new hopes for the introduction of new stem-cell-based approaches to treating hair loss. It is now possible to produce hair in vitro or to manipulate the cells in their native place (live lineage reprogramming) to reconstruct the hair follicle. However, there are still problems with the functionality of cultured human hair cells, adequate selection of non-hair cell sources in cases of donor hair scarcity and the development of crop conditions. On the other hand, in the case of live lineage reprogramming, the selection of corresponding induction factors and their efficient delivery to guide resident cells in order to reconstruct functional hair requires more research for its use. We are at a crucial moment in which we highlight recent advances of the use of growth factors and stem cells obtained from both fat and skin and hair follicle structures, which must be taken into account to develop reproducible cellular treatment, safe and efficient, which is the basis for the treatment of alopecia. As several authors claim, "we are close but not yet" [18].

The objective is not to grow old: Some scientists like Juan Carlos Izpisua say that soon we will have the formula for eternal youth. As published in December 2016, in the journal Cell [19], a group of scientists led by professor Izpisua stated that through cellular reprogramming, they have succeeded in making human skin cells cultured in the laboratory rejuvenate their appearance and functioning.

They have also made mice rejuvenate, cure diseases and live longer, discovering "that the intermittent expression of genes associated with an embryonic state can reverse the signs of aging". Izpisua states that "our study shows that aging does not evolve in one direction, it has plasticity, and by properly modeling the process, aging can be reversed". As a basis for their work, they used the study of cellular reprogramming, a process in which through the expression of four genes, known as the Yamanaka factors (Nobel Prize in Medicine), scientists are able to convert any adult cell into a pluripotent stem cell (iPSC). IPSCs, like embryonic stem cells, are able to divide indefinitely and become any type of cell in our body.

The steps are very important, but we are still very far from being able to reverse the process of ageing in a safe and controlled way in humans. By improving the way we get older, we will reduce the risk of many diseases. "Our goal is not only to get us to live longer, but to live more healthy years, that the years are healthy and that we do not have to suffer the symptoms and diseases of aging", declared by Izpisúa in a recent interview with the newspaper EL MUNDO [20], in which he further states that "We alter aging by changing the epigenome, which suggests that aging is a plastic process, which can be manipulated". Epigenetic changes throughout life are the result of our interaction with the environment: what we eat, drink, exercise. Could this technique reduce the negative epigenetic marks that cause exposure to the sun or the consumption of alcohol and tobacco? Izpisúa is clear: "Because of their chemical nature, these brands are reversible and modifiable. Therefore, yes, epigenetic changes caused by sun, alcohol or tobacco could also be reversed".

Anyway, although in principle they could be reversed, we could not reverse the mutations in the DNA. Therefore, it is best to limit the consumption of these substances.

Conclusions

The current needs of patients in the area of aesthetic medicine are encompassed in a greater knowledge of the male patient who, still representing a small fraction of all cosmetic and medical-aesthetic procedures, are an emerging and rapidly growing demographic market in the field of aesthetic medicine [21, 22].

In deepening the knowledge of the process of ageing, and the possibility of reversing the changes linked to age. Recall that the number of elderly patients arriving for consultations is increasing, which also leads to the management in many cases of patients with polymedic and multiple pathologies.

Another aspect not insignificant is the demand for medical-aesthetic care of patients with cancer and other important chronic diseases, in which the challenge of survival and knowledge of the "therapeutic power" of the image make it necessary for the aesthetic doctor to have thorough knowledge about the processes of the disease and work as a team with other medical, health and aesthetic professionals.

As for trends in treatments, the goal is to achieve a beautiful, attractive, harmonious appearance—always in the frame of the binomial health–beauty.

Patients demand safe, effective, minimally invasive techniques, without sequelae and without adverse effects. We cannot offer magic or imposition of hands. All treatments can have adverse effects and it is necessary to properly inform the patient.

The new therapies based on regenerative medicine promise spectacular results, but today they are on an initial path, but still far from being applied in a generalized, reliable and safe way.

Only if we are based on a scientific study, good practice and a strict code of ethics, we will achieve quality aesthetic medicine, which satisfies the needs of our patients.

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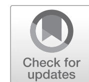

The Molecular Physiology of Ageing: New Targets for Regenerative Medicine

Salvador Macip and Mohammad Althubiti

Understanding Ageing

Finding a way to stop the damaging effects that ageing has on the human body has been a longstanding ambition for mankind. Numerous legends tell of searches for the fountains of youth and other mythical sources of immortality, but until the raise of modern biology there has been no real opportunity to interfere with the inevitable degradation that time imposes onto organisms. Over the past decades, we have acquired substantial information about the molecular physiology of ageing, but current interventions are limited at those of cosmetic nature. More research has to be conducted before the first true drug that modulates ageing reaches the market.

Despite the fact that we still lack a proper therapy that has a biological effect on the mechanisms involved in ageing, millions of dollars are currently spent annually on chemicals sold as anti-ageing drugs. This is mainly due to the fact that current laws in many countries allow compounds to be labelled as “supplements” or “cosmetics” instead of “medicines”, which would force them to undergo more severe assessments of their efficacy. This loophole has allowed this market to bloom and underscores the immense interest on these products at consumer level.

The key to designing interventions that would indeed slow down or revert the effects of ageing lies on our ability to characterize the molecular pathways involved in the changes that can be observed at the cellular level. Thus, carefully studying cell ageing (also known as senescence) is likely to provide the insights necessary to design the first strategies aimed at modifying organismal ageing. The combination of new genetic techniques and the recent advances in biochemistry are bringing us closer to understanding how the processes that contribute to the ageing phenotype are determined. The first consequence of these advances is that modulation of ageing in the lab is already possible. Fly, worms, mice and other animals that age faster than usual or that survive for more than the normal amount of time can be generated through chemical treatments and genetic manipulation. This is a proof of principle that ageing is not an irreversible and uncontrollable mechanism, as once thought, and that, like every biological process, it can be subjected to manipulation once it has been properly characterized.

Our knowledge of the ageing process in humans is still far from complete. Nevertheless, it has already been hypothesized that our lifespan and, more importantly, health span could be

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extended by a chemical intervention that interrupted the signalling pathways that determine senescence, as we will discuss later. The limits of such interventions and the impact that they may have in society are still being debated.

Ageing Is a Result of the Cellular Responses to Damage

Different theories have been proposed over the years to explain the molecular basis of cell ageing. These include the accumulation of toxic residues inside and outside the cells, the shortening of telomeres or the accumulation of damage in the mitochondria that limits the amount of energy they can produce [1]. All of them relate in one way or another to the chronic induction of damage signalling pathways that push the cell towards the process known as senescence. This, which could also enhance the progressive loss of potency of the adult stem cell niche involved in regenerative processes, could explain the impact of time on tissue physiology. Thus, ageing could be seen as an excessive accumulation of damaged cells that adopt a senescent (or "old") phenotype. It has also been proposed that the side effects of certain processes necessary for organismal survival can accelerate the processes that trigger ageing. For instance, the mechanisms that protect cells against cancer can induce senescence as well and contribute to this accumulation [2].

Within the framework of the "ageing as a result of damage" hypothesis, it is important to consider that the oxygen needed to sustain life causes important disruption to several of the components of cells, which then contributes to the progressive deterioration that will eventually lead to cell senescence. This is due to the fact that the breakdown products of oxygen, known as reactive oxygen species (ROS), produce small but measurable damage to the DNA and other macromolecules [3]. The steady accumulation of these lesions has indeed been shown to trigger cellular ageing [4]. This forms the central core of the classic oxidative theory of ageing, which is now part of a wider framework that aims to explain all the changes involved in the phenotypical changes observed in ageing [1]. Consistent with oxidation not being the sole cause of cellular senescence, it has been observed that antioxidants have only limited effects on the ageing of organisms, while they can actually increase other pathologies [5].

In the following pages, we will summarize our understanding of the main factors currently known to be involved in the molecular physiology of cellular ageing, and based on this, we will explore the interventions that could be part of the regenerative medicine tools in the future, mostly by preventing a build-up of senescent cells.

The Physiological Importance of Senescence

Ageing Cells as a Way to Prevent Cancer

Senescence is a well-known cellular mechanism with a critical role not only in ageing but also in cancer, as a tumour suppressor mechanism [6]. Senescence is usually defined as a permanent cell cycle arrest in which cells remain metabolically active and adopt characteristic phenotypic changes [7]. Senescent cells appear multinucleated, large and extended, and exhibit spindle and vacuolization features [8]. The onset of this phenotype is believed to be triggered by different types of damage, either as a result of telomere shortening after a number of cell divisions (*replicative senescence*, related to the actual age of the cell, as determined by the length of the telomeres) or as a response to a range of stress stimuli (*stress-induced premature senescence*, SIPS) [8, 9]. Expression of oncogenes, such as Ras, cyclin E, E2F3 and Raf can also trigger senescence in vitro, which underscores its tumour suppressing properties [10–12]. Indeed, the presence of senescent cells in vivo is often observed in the premalignant stages of a tumour, after which they gradually disappear.

In view of this, senescence has been considered one of the two main processes that prevent the emergence of transformed cells, together with apoptosis [13]. Since senescence stops the progression of cancer in vivo [7] and it is known

to be increased in response to many therapies [6], the presence of senescent cells in tumours could be considered an indication of a controlled or less advanced disease. Thus, the percentage of senescent cells in tumours could have a utility as a prognostic tool in cancer [14].

Other Functions of Senescence

Although the antineoplastic effects of senescence are the most well-known and studied, recently it has also been reported that it contributes to wound healing, fibrosis and embryonic development [15, 16]. Senescent fibroblasts appear and aggregate as part of normal wound healing and tissue repair [17]. Senescent myofibroblasts in mice liver are able to control fibrosis formation, while mice without senescence effectors (such as p53 and p16) suffered from extreme fibrosis and delay in wound healing [15]. Accumulation of myofibroblasts after liver injury leads to excessive extracellular matrix (ECM) secretion, liver fibrosis and, finally, cirrhosis [15]. All these observations can be explained by the ability of senescent cells to secrete proteins that degrade ECM and thus prevent fibrosis and enhance wound healing.

The involvement of senescence in normal tissue development is just beginning to emerge. For instance, it has been found that megakaryocytes undergo senescence as part of the maturation process that leads to the production of platelets [18]. In addition, senescence also was observed during the normal maturation of syncytiotrophoblasts [19]. Finally, senescent cells are found throughout the embryo, including the apical ectodermal ridge and the neural roof plate, two known signalling centres in embryonic patterning, suggesting that senescence is a mechanism essential for development [16].

The Impact of Senescence on Organismal Ageing

It has been observed that the percentage of senescent cells in tissues in vivo increases over time [20, 21]. All data obtained in rodents and primates suggest that the augment in cell senescence must play a role in age-dependent organismal changes [22–24]. Indeed, accumulation of senescent cells has actually been shown to contribute to the functional impairment of different organs [25]. This has led to the hypothesis that senescence is an antagonistically pleiotropic process, with beneficial effects in the early decades of life, mostly as a tumour suppressor, but detrimental to fitness and survival in later stages as senescent cells become more prevalent, due to its contribution to the tissue disruption that leads to age-related pathologies [26].

Because of this, senescent cells are currently thought to be at the core of the physiological changes observed in an organism during the process of ageing. Being able to prevent senescent cell accumulation, or perhaps finding a way to clear them from tissues once they become present, could be an effective strategy to regenerate tissues and maintain their functionality. Such interventions are already being considered, but they would first require a proper understanding of the molecular mechanisms that define the senescent phenotype.

The Molecular Mechanisms of Cellular Ageing

Despite the considerable knowledge accumulated in the 50 years since Leonard Hayflick first described the phenomenon of cell senescence [27], the pathways involved in this process have not been yet fully characterized [28]. One of the well-known features of both replicative senescence and SIPS is the participation of the p53-p21 and/or p16-Rb axis in triggering and maintaining the phenotype. Although in vivo suppression of p53 and/or its upstream regulator ARF is enough to prevent senescence in some models [29], other cell types rely primarily on p16 for its induction [30]. p21, a p53 target gene, has often been considered critical for establishing senescence, whereas p16 could be more involved in the maintenance of the phenotype [31]. This effect would be reinforced by an increase in intra-

cellular ROS [32, 33], thus linking senescence with the classic hypothesis of oxidative stress and ageing. Although p21 is the main cell cycle inhibitor of the p53 pathway, it can also be activated in a p53-independent manner, for example, in response to retinoic acid, IFN and TGFβ [34].

Replicative Versus Stress-Induced Senescence

The two main routes of inducing senescence (replicative or stress-induced) have many common features but diverge in the mechanisms involved in triggering the response. The main difference is that the former features a shortening of the telomeres, while the latter happens in the presence of telomeres of normal length [35–37].

Telomeres are structures located at the end of each chromosome, composed of a repeat of the TTAGGG sequence and the proteins that associate with them [38]. Consistent proliferative propagation of cells leads to shortening of telomeres [39], which causes a proliferative arrest mediated by the induction of senescence [8]. Reduction in the length of telomeres is a hallmark of tissue ageing [1]. Once telomere length reaches a limit, this triggers a DNA damage response that leads to the activation of the p53-p21 and p16-Rb pathways, similar to what is observed in SIPS [40].

Telomerase is an enzyme that adds TTAGG repeats to these sites, thus maintaining telomere length and allowing cells to continue dividing [41]. Telomerase is not expressed in most normal cells, but limited to stem cells that need to maintain their proliferative capacity. Telomerase expression can bypass senescence and this is a mechanism that many cancer cells use to avoid a permanent growth arrest [41].

The p53-p21 Pathway in Senescence

The main role of the tumour suppressor p53 is to mediate cellular responses to DNA damage [42]. p53 is a transcription factor that, among other functions, prevents the transformation of cells by triggering protective mechanisms such as cell cycle arrest, senescence or apoptosis [43, 44]. p53 is mainly regulated posttranslationally through many different modifications, including phosphorylation, methylation and acetylation [44–48]. Specifically, its N-terminal region has an important role in its stability because the E3 ligase MDM2 binds to it and ubiquitinates p53, which is then targeted for proteasomal degradation [49]. Different stresses lead to phosphorylation of residues of the N-terminal region by damage-dependent kinases such as ATM and ATR, including serine 15, which disrupts the MDM2-p53 interaction and thus increases the half-life of p53 [50, 51].

Although p53 can trigger the onset of either apoptosis [51, 52] or arrest/senescence [27, 34], the mechanisms involved in the decision between these cellular responses are not well understood. Cell type, presence of growth factors or oncogenes, the intensity of the stress signal and the cellular level of p53 have been cited as important factors in determining a specific p53-induced response [7, 12, 52, 53]. Posttranslational modifications of p53 also have been reported to influence the response observed. For example, p53 phosphorylation by different kinases in response to stress can select for arrest or apoptosis, suggesting the involvement of upstream modifiers in cell fate decisions [29]. Moreover, p53 mutants that can induce growth arrest but not apoptosis, or vice versa, have been identified [12, 49, 54], consistent with the concept that certain p53 mutations may cause selective loss of the ability to transactivate certain p53-responsive promoters [35].

Several p53 target genes have been reported to be specifically involved in apoptosis. These include KILLER/DR5 [55], Bax [39], IGF-BP3 [6], PIG3 [45], PAG608 [24], PERP [1], Noxa [43], PIDD [33], p53AIP1 [44], APAF-1 [46], FDXR [23] and PUMA [41, 56]. Some of these genes, like PIG3 and FDXR, are involved in ROS-related pathways [45]. In fact, apoptosis triggered by p53 has been reported to be dependent on an increase of ROS and the release of apoptotic factors resulting from mitochondrial damage [25]. Despite all the data accumulated in relation to the pro-apoptotic functions of p53, the

p53 target genes involved in senescence have not been properly characterized, although it is believed that p21 is its main effector.

p21 is a necessary mediator of p53-induced cell cycle arrest, as indicated by the fact that p53 cannot induce arrest after DNA damage in p21-null mice [53]. p21 is a member of a family of cell cycle inhibitors that includes p27 and p57, and it is capable of inhibiting cyclin-dependent kinases (CDKs) [57], key regulators of the cell cycle. It also acts to block DNA replication by binding to proliferating cell nuclear antigen (PCNA) [58]. p21 expression has been observed in cultured human fibroblasts after prolonged passage, during which such cells undergo senescence [55]. Moreover, p21 has been shown to be capable of inducing permanent growth arrest/senescence in a p53-independent manner [33, 56].

The p16-Rb Pathway in Senescence

Rb is a tumour suppressor protein that regulates the transition phase between G1 and S phases and can thus induce an arrest phenotype that can eventually evolve into senescence [59]. The main role of Rb is to inhibit the E2F family of transcription factors, which is crucial for DNA replication and cell cycle progression [60]. Rb can be inactivated by oncogenes that are encoded by viruses, such as SV40 and E1A, resulting in the release of E2F and senescence bypass [60]. Overexpression of cyclin-dependant kinases (CDKs), which is common in many cancer cells, can also repress Rb and suppress senescence [60]. The CDK inhibitor p16 can maintain Rbin an active state by decreasing CDK4/6 activity [28]. The p16-Rb pathway can be induced by DNA damage signals, which leads to senescence induction in association with the p53-p21 axis [60].

Other Modulators of the Senescence

There are many regulators that directly or indirectly affect the induction of senescence, mainly through their effects on the p53 and Rb pathways. For instance, PML has an essential role in tumour suppression through modulation of the activity of both p53 and Rb, by sequestering inhibitory proteins to the nuclear bodies [54]. As a result, cells that lack PML exhibit impairment in senescence induction by the p53-dependent pathway [61]. On the other hand, PML upregulates histone deacetylases that increase Rb functions [62]. PPP1CA is another effector of senescence that responds to oncogene activation. In the absence of PPP1CA, Ras is unable to induce senescence [63]. SMURF2 is an E3 ubiquitin ligase that, when activated, can induce senescence in fibroblasts independently of p21 [64]. During replicative senescence, the expression of SMURF2 is high and correlates to telomere attrition and p16 upregulation.

BTK is a non-receptor tyrosine kinase that is mutated in the inherited immunodeficiency disease X-linked agammaglobulinaemia [65]. It is expressed in myeloid and lymphoid cells but not in T cells and it is a member of the highly conserved Tec family of kinases, which play an important role in B cell receptor (BCR) signalling [66, 67]. In B cells, BTK is activated after an antigen binds to the BCR, which leads to its phosphorylation at tyrosine 551 by SRC family kinases and its autophosphorylation at tyrosine 223 [68]. Although BTK is mainly located at the cell membrane, it can also be found in the nucleus [69]. A pathological BTK upregulation has been shown in different B cell malignancies, such as chronic lymphocytic leukaemia, mantle cell lymphoma and multiple myeloma [70–72]. Because of this, several small molecule inhibitors of BTK have been developed to treat these diseases [73]. BTK was found to be induced in senescent cells and shown to be involved in the p53 pathway as a novel modulator of p53 activity through its phosphorylation [74]. In the absence of BTK, p53-induced senescence was abrogated, showing the importance of BTK in this pathway.

The Importance of Oxidation in Senescence

As we have discussed, increases in intracellular levels of ROS have been implicated at many levels

in the pathways of cellular senescence [10]. Senescent cells have higher levels of ROS than normal cells [20], and oncogenic Ras, p21 and p53 induce senescence in association with increased intracellular ROS [30, 32, 36, 75, 76]. It has also been reported that oxidative stress caused by sublethal doses of H_2O_2 [11] or hyperoxia [58] can force human fibroblasts to arrest in a senescent-like fashion [9]. Moreover, cells can be subjected to oxidative stress due to the effects of many cancer therapeutics, which could increase the presence of senescent cells in tissues [3, 77, 78].

ROS are generated by normal oxidative processes related to cell metabolism [79–81]. They are produced initially by the reduction of singlet O_2 to superoxide anion and then H_2O_2 that, if not eliminated, generates highly reactive hydroxyl free radical that causes DNA damage [3, 82]. Increased levels of ROS can be induced by inflammatory responses, certain pathological processes and exposure to agents such as ionizing radiation [83, 84]. Depending on the level of oxidative stress and the extent of the induced DNA damage, cell fate can vary from temporary arrest to death [84, 85]. For instance, exposure to H_2O_2 has been shown to induce apoptosis or necrosis depending on concentrations and cellular context [85–88], whereas low concentrations of oxidants can force normal human fibroblasts to permanently arrest in a senescent-like state [4, 86, 89–93].

When proliferating cells are subjected to oxidative stress, the cell cycle temporarily pauses either at the G_1, S or G_2 phases. Arrest at these checkpoints prevents DNA replication and mitosis in the presence of DNA damage and presumably allows time for DNA repair to occur. The proportion of cells that arrest in each phase after oxidative damage depends on cell type, growth conditions, type of damage and the checkpoints operative in the cells. The G_1 checkpoint depends on activation of the tumour suppressor p53, which through p21 induction inhibits cyclin-CDK complexes [94, 95]. Since p53 functions are lost in most neoplasias [96, 97], cancer cells often have a defective G_1 checkpoint response to oxidants. Arrest at the G_2 checkpoint results primarily from activation of the Chk1 protein kinase, which maintains mitotic cyclin B/Cdc2 complexes in an inactive state [98, 99]. Consistent with this, peroxides such as H_2O_2 or *tert*-butyl hydroperoxide (tBH) have been shown to induce both a p53-dependent G_1 checkpoint arrest, which can be attenuated by using antioxidants [100, 101], and a G_2 checkpoint response [101, 102].

The biochemical responses of normal cells to oxidative stress have been investigated in detail with respect to p53 functions. Oxidants have been shown to promote phosphorylation of p53 at serine 15, which can be blocked by antioxidants [103], and to induce an increase in p53 levels accompanied by elevation of p21 [89]. Although the activation of the p53 pathway in response to oxidative damage contributes importantly to the resulting arrest or cell death responses observed [84], there have been several studies on responses to oxidative stress in cells lacking intact p53 functions [104]. It has been proposed that genotoxic stresses can induce senescence in p53-null as well as wild type p53-containing cancer cells [105] and that this response plays a role in the suppression of tumour growth by chemo- and radiotherapy. However, other studies have indicated that cancer cell lines without functional p53 pathways do not undergo senescence in response to a variety of chemotherapeutic agents [106–108].

The fact that oxidative stress triggers a p53 response through DNA damage signals could be a common trigger of senescence and may play an important role in ageing. p53 overexpression has also been shown to cause the accumulation of ROS, presumably mediated by p53 transcriptional influence on pro-oxidant genes [32, 109]. Conversely, overexpression of antioxidant genes like superoxide dismutase or catalase causes extension of lifespan in *Drosophila* [79]. This can also be observed in cell cultures maintained in low oxygen environments [110]. All of these findings point to a strong relationship between oxidative damage, senescence and ageing.

The Senescence-Associated Secretory Phenotype

Cellular senescence results in the secretion of growth factors, chemokines and cytokines, collec-

tively known as the senescence-associated secretory phenotype (SASP). It has been found that SASP may have a positive effect on cell proliferation and angiogenesis, as well as a role in promoting ageing and tumourigenesis [111, 112]. It can also promote migration of leukocytes and tumour cells, which in turn may induce tumour metastasis [113]. Thus, the presence of SASP could explain many of the negative consequences of senescent cell accumulation, including the pro-ageing effects, and could be a target for regenerative therapies, as we will discuss in more detail later.

Common Markers of Senescent Cells

In order to prevent or stop the accumulation of senescent cells, a limiting factor is the ability to selectively detect them in vivo. Several features have been proposed as being shared by most senescent cells, although none of the currently available markers are sufficient on their own for conclusively identifying senescent cells in vivo or in vitro. This underscores the need for better characterization tools [114].

During cell cycle arrest, many genes that are involved in cell division are supressed, for example, PCNA, E2F or cyclins, and this could be used as an indication of senescence, although it is not specific. Similarly, increased expression of intracellular and/or secreted proteins, such as p21, p16, macroH2A, IL-6, phosphorylated p38 MAPK, PPP1A, SMURF2 or PGM, has been used as surrogate markers of senescence [29, 63, 64, 114–116].

Senescent cells display different modifications in the organization of chromatin that can help identify them as well. In normal cells, DNA staining reveals completely uniform colour outlines, whereas senescent cells usually show dot-like patterns, known as senescence-associated heterochromatic foci (SAHF), that appear due to intensive remodelling in the chromatin and a lower susceptibility for digestion by nucleases [117, 118]. SAHF development is not necessary for the establishment of senescence and its presence depends on cell type and the triggering stimuli [119].

Apart from these factors, the most distinctive measurable feature of senescent cells is the presence of a specific β-galactosidase enzymatic activity at pH 6.0, different from the normally observed at pH 4.0 within lysosomes [120]. This has been named senescence-associated β-galactosidase (SA-β-Gal), and it is thought to be a consequence of the enlargement in the structures of lysosome in senescent cells, and it does not have a known role in the establishment or maintenance of the phenotype [121]. Although it is currently the standard for detecting senescent cells in the laboratory, several conditions, such as high cell confluence or treatment with hydrogen peroxide, can also independently stimulate SA-β-Gal activity, leading to many false positives [122].

Recently, a series of membrane markers highly expressed in senescent cells have been identified [14]. This knowledge could contribute to define the interactions of aged cells with the microenvironment and help explain how the mechanisms of senescent cell clearance work normally and stop working with time [123, 124]. Also, specific cell membrane proteins with extracellular epitopes could be useful to rapidly detect senescent cells in vitro and in vivo [125].

Some of these membrane markers, like EBP50 and STX4, are preferentially induced by the p53-p21 pathway, while others, such as DEP1, NTAL and ARMCX3, are dependent on p16-Rb [14]. Thus, they could be used to distinguish between different triggers of senescence. Many of the new markers (such as DEP1, NTAL, ARMCX3, LANCL1, B2MG, PLD3 and VPS26A) have extracellular epitopes, which could be useful in the future to design strategies that could specifically deliver a toxic payload into senescent cells, thus providing a mechanism for clearing them. Of note, many of these proteins play a role in vesicle trafficking (including STX4, VAMP3, VPS26A and PLD3) [126–131], which underscores the importance of protein secretion in the senescent phenotype.

The Role of Cell Senescence in Age-Associated Symptoms and Illnesses

It is widely accepted that senescent cells accumulate in vivo in different tissues with time [132]. In addition, there are many age-associated diseases

in which it has been shown that accumulation of senescent cells contribute to the onset or maintenance of the symptoms, such as lung and liver fibrosis, neurodegenerative diseases or arthritis [133]. In atherosclerosis, for instance, there is evidence of a link to increased senescence of endothelial and vascular smooth muscle cells [134]. Cell senescence is also induced in myocardial ischemia and hypoxia [135]. In this context, senescent fibroblasts could be a source of fibrosis and collagen accumulation after myocardial infarction. Accumulation of senescent cells has been associated with ocular disorders as well, such as glaucoma and cataracts [123, 136].

In addition, senescence has been shown to be involved in type 2 diabetes, through a p53-dependent increase in insulin resistance in adipose tissue [137]. SA-β-gal activity and p53 and p21 levels are higher in visceral fat from diabetic patients compared to non-diabetic individuals [137]. Similarly, ageing muscle stem cells become senescent with age and the ability to delay senescence increases the potential of their regeneration [138]. In kidney transplantation, the presence of cell senescence in grafted organs associates with poor prognosis [139]. Finally, senescent chondrocytes accumulate in the articular cartilage of people with osteoarthritis [140]. These data together suggest that amelioration of all these diseases could be achieved by preventing the increase of senescent cells in tissues.

How the SASP May Define the Biological Effects of Ageing

The mechanisms by which senescent cells contribute to the symptoms related to ageing are not fully understood. A likely explanation is that impairment of organ function is due to the fact that senescent cells cannot perform their normal roles [123, 136]. However, it has recently been proposed that the paracrine impact of SASP on surrounding cells may be even more relevant for the negative effects of senescent cells, due to its ability to trigger a chronic inflammatory response and facilitate neoplastic transformation [141, 142].

Several of the changes in gene expression observed in senescence are associated with growth factors, chemokines and cytokines that, when secreted, are collectively known as SASP [111, 112]. The SASP likely evolved to create an immune response against senescent cells aimed at their clearance from tissues by phagocytosis. However, this seems to be impaired with time, for reasons that are not known. The SASP from precancerous senescent hepatocytes attract $CD4^+$ cells and are cleared by specific Th_1, showing that senescence surveillance is mediated by an adaptive immune clearance [124].

Although the SASP was first described in replicative senescent fibroblasts, it is now known that different cell types have different secretomes [143]. Secretion of inflammatory cytokines triggers proliferation and can also promote migration of leukocytes and tumour cells, which in turn may induce tumour metastasis [113]. Inhibition of the SASP could be an effective way of reducing the impact of senescent cells on tissue physiology [144, 145].

Regenerative Medicine Strategies Aimed at Preventing Ageing

A series of essential hallmarks of ageing have recently been proposed [1]. It is implied that the elimination of each of them should lead to the amelioration of the symptoms associated with ageing. Within this context, the induction of cellular senescence is the endpoint of many of the stimuli associated with ageing. As we have mentioned, the genes involved in triggering senescence belong to tumour suppressor pathways, which suggests that ageing could be, at least in part, a consequence of the natural antineoplastic defences of an organism. Thus, inactivation of these genes can result in increased risk of death from cancer at early ages. Since interfering with the induction of senescence in vivo may prove problematic, a safer approach for regenerative medicine could be to eliminate the senescent cells after they are being formed.

Identifying and Clearing Senescent Cells

The first in vivo proof that accumulation of senescent cells contributes to the deleterious effects of ageing was provided recently using two mouse models in which senescent cells were driven to apoptosis as they started expressing p16 [123, 136]. The absence of senescent cells in tissues importantly delayed the onset of age-associated changes, thus increasing lifespan and health span. These results were reproduced in both fast-ageing and normal mice and confirm that senescent cells could be a target for anti-ageing and regenerative therapies in humans. Moreover, this supports the hypothesis that senescent cell targeting could ameliorate age-related diseases such as cataracts, diabetes and atherosclerosis.

However, performing senescent cell clearance in humans is challenging. The use of anti-senescence drugs, also called *senolytics* (such as rapamycin, quercetin, dasatinib or navitoclax), could delay senescent cell accumulation in human tissue, but it might contribute to malignant transformation [114]. An alternative would be to use methods to selectively deliver apoptotic drugs to senescent cells using some of the previously described markers of senescence [14]. One possible way would be to use antibody-drug conjugates (ADCs), which have been previously proven to be effective in targeting cancer cells [146, 147]. ADCs are specific monoclonal antibodies bound to a toxic payload by a linker. Once the antibody recognizes an epitope, for instance, in the extracellular domain of a plasma membrane protein, it binds to it and is internalized. The toxin is then released inside the cell by cleavage of the linker. ADCs against markers of senescence are an alternative for designing a regenerative therapy that is currently being investigated.

Other Potential Approaches

Impairment in protein homeostasis (or proteostasis) has also been associated with ageing disorders, especially conditions such as Alzheimer's and Parkinson's diseases or muscle atrophy [148]. These are usually the result from impairment in the protein folding mechanisms and regulators of proteostasis normally act through repairing or eliminating misfolded proteins. They could potentially be used as drugs to prevent the protein damage that can contribute to the induction of senescence.

Reducing caloric intake (up to as much as 60%) has been associated with the induction of longevity and healthy life in different animal models, including non-human primates [149]. In fact, it is currently one of the most effective ways to slow down ageing in an experimental context. However, it is difficult to design a trial to assess its relevance in human unless proper markers of tissue ageing are established first. The delay in ageing phenotypes through caloric restriction is thought to be mediated by nutrient signalling mechanisms such as the growth hormone, insulin receptor, IGF-1 and mTOR pathways, and decreases in these factors have been shown to increase in lifespan in vivo [150]. Interestingly, the level of IGF-1 and growth hormone is low in old age and premature ageing syndromes [151].

Consistent with this, pharmacological inhibition of mTOR by rapamycin, a drug produced by *Streptomyces hygroscopicus*, can delay ageing in mice models [152]. However, it is also a strong immunosuppressant [153], which makes it an unlikely choice for an anti-ageing drug. Resveratrol, a compound found in grapes and other fruits, has been proposed as an alternative. Its effects on ageing seem more complex than was initially anticipated and its mechanism of action is still being discussed, although it seems to be based on the activation of the sirtuin family of deacetylases [154]. Resveratrol may not increase the lifespan of healthy lab animals [155], although it has an important effect on mice being fed a high-fat diet [156].

Finally, the other promising anti-ageing drug being studied intensively is metformin, currently being used to control mild diabetes. Due to its effects on metabolism, metformin has already demonstrated protection against age-related diseases in humans and has been shown to ameliorate ageing in diabetic populations [157]. Its effects on healthy individuals are still being characterized.

Conclusions

Ageing could be seen as a series of symptoms caused by tissues that have stop working properly. Finding a way to restore their function could not only prevent a considerable number of diseases but even prolong lifespan. There are several approaches that could achieve the regeneration of tissues needed to delay ageing and extend quality of life. Here, we have focused on potential strategies to prevent the accumulation of senescent cells, which is thought to be one of the main triggers of organismal ageing.

Anti-ageing drugs need to be highly specific while having virtually no side effects, since they would need to be taken chronically by a population of largely healthy individuals. Current clinical trials with metformin, the first putative anti-ageing drug to reach this stage, will be highly informative and will set the template for future avenues to be tested [157].

Our knowledge of the molecular and cellular physiology of ageing has allowed us for the first time to propose strategies that may have a biological effect on lifespan and health span. It is possible that we will see one or more succeed in the near future, and then regenerative medicine approaches based on chemically mediated lifespan and health span extension will finally become a reality.

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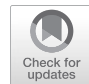
Part II

Approaches: The Process of Aging



Beauty and Aging

Eva Guisantes

Introduction

Aging affects all tissues of the body. However, it is at the facial area that more studies have focused on the aging process and on where the medical and surgical aesthetic treatments of rejuvenation are directed. The face does not age as one homogeneous object, but as a result of several dynamic components. Changes that occur with facial aging involve a complex interaction among the bone, skin, soft tissue, support ligaments, and septa. All these multiple phenomena must be considered for their proper correction.

Facial aging is a multifactorial process. Intrinsic aging includes histologic and physiologic changes resulting from cellular apoptosis and other genetically determined processes. Extrinsic aging results from long-term exposure to environmental aggressions like smoking, alcohol, UV radiation, dehydration, inadequate nutrition, extreme temperatures, traumatic damage, chemotherapy, or radiotherapy. The clinical signs of facial aging are related with changes in all structural layers (skin, fat, muscle, bone).

Proper facial analysis is the key to achieving optimal facial rejuvenation results. Undoubtedly, this analysis is based on an adequate knowledge of the underlying anatomy and the clinical implications that anatomy has for facial aging.

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Skin

The skin undergoes aging changes such as thinning of the epidermis, collagen loss, and dermal elastosis that result in fine wrinkles, skin spots, and dryness. Aging causes a fragmentation of the dermal collagen matrix. Solar elastosis is the term used to describe the histologic appearance of the photoaged dermal extracellular matrix and is characterized by an accumulation of amorphous, abnormal elastin material surrounding a decreased volume and disorganized array of wavy collagen fibrils. The loss of this extracellular collagen is responsible for the loss of the structural integrity of the matrix and the decreased mechanical tension. There is a disability of fibroblasts to produce and organize new collagen. Hence, treatments that stimulate neocollagenesis can improve the appearance of the aged skin. Both extrinsic and intrinsic aging factors affect the ability of the skin to adjust to the aging loss of underlying soft tissue volume.

Ultraviolet A (UVA) and B (UVB) radiation causes direct and indirect skin damage. UVB light is almost completely absorbed by the epidermis, and thus dermal photodamage is solely caused by UVA. UVA directly induces DNA

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changes and indirectly causes cell damage by creating free radicals, leading to an increase in oxidative stresses and a degradation of the surrounding collagen. The photoaged dermis shows a histological aspect of chronic inflammation. The epidermis undergoes characteristic histological changes with sun damage, leading to increased thickness, slower keratinocyte turnover, and decreased melanocyte counts. However, there are also regions of increased melanocyte concentration, with increased capacity for melanin production and deposition to keratinocytes, which present as solar lentigines [1, 2].

The topography of wrinkles is not arbitrary and lymphatic vessels may be the primary anatomical structures that determine the position and location of cutaneous wrinkles. Repeated skin contractions over a fixed object (vessel or nerve) may lead to a surface configurational change [3].

Treatments that stimulate the production of new collagen include laser, topical retinoic acid, deep chemical peels, hyaluronic acid, collagen, and calcium hydroxyapatite. Stimulation of collagen production may lead to stimulation of fibroblasts by a direct mechanism of fibroplasia, or indirectly through increased extracellular matrix and stretching effect. Therefore, these treatments could both replace collagen and slow its loss [4]. Laser, topical retinoic acid, and peels are useful for treating the solar lentigines. Injection of uncrosslinked hyaluronic acid improves skin hydration.

Facial Fat

The facial adipose tissue is highly compartmentalized by septa that divide the fat in superficial and deep compartments relative to the superficial musculoaponeurotic system (SMAS) or mimetic muscles forming distinct anatomical units [5]. This compartmentalization facilitates the sliding of the facial muscles between compartments during facial motion. The vessels and nerves travel through the septa that form the transition zones between fat compartments. Many of the retaining ligaments that support facial soft tissue originate within the septal barriers between these compartments [6]. In addition, these fat compartments will undergo sequential changes during aging. In a young face the transitions between them are smooth. As we age, these transitions become more marked and furrows appear.

The facial fat can be divided into two layers (superficial and deep):

Superficial Fat Compartments (Fig. 1)

- *Nasolabial fat*: it is the most medial compartment of the cheek and the least modified with age.
- *Superficial cheek fat*:
  - *Medial cheek fat*: it is lateral to the nasolabial fat.
  - *Middle cheek fat*: it is anterior and superficial to the parotid gland. It is located between the medial and lateral superficial compartments of the cheek.
  - *Lateral-temporal cheek fat*: it is the most lateral compartment of the cheek and extends from the temporal region to the beginning of the neck.
- *Forehead compartments*: there are three compartments in the forehead.
  - *Central frontal fat*: it is in the midline region of the forehead and its inferior boundary is the nasal dorsum.
  - *Middle forehead compartments* (left and right): they are located between the central frontal fat and the lateral-temporal cheek fat on each side.
- *Orbital fat compartments*: they are three, inferior, superior, and lateral. The nasolabial, the superficial medial cheek, and the infraorbital fat pads are collectively referred to as the "malar fat."
- *Jowl fat compartments*: the superior and inferior jowl fat pads.

Deep Fat Compartments (Fig. 2)

- *Deep medial cheek fat compartment (DMC)*: it has two portions, one medial and one lateral (DLC). The medial portion is located poste-

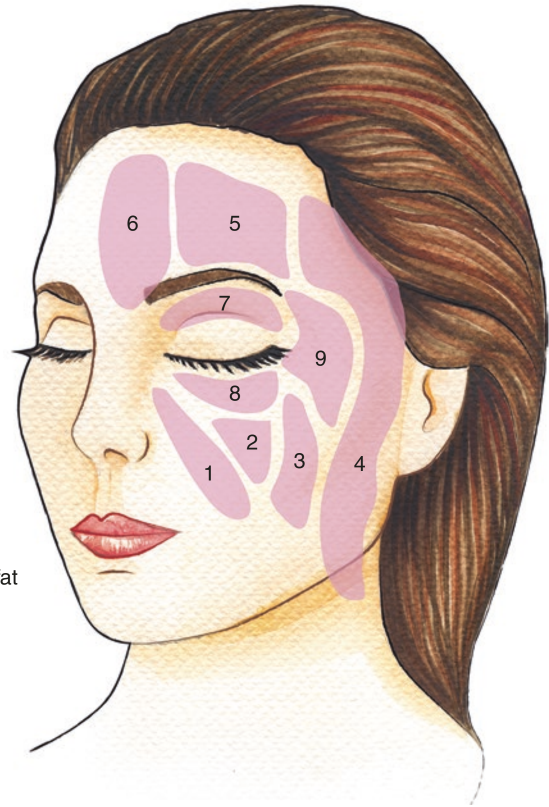

Fig. 1 Superficial facial fat compartments

rior to the nasolabial compartment and is bordered posteriorly by Ristow's space. It is one of the compartments most affected by aging.

- *Suborbicularis oculi fat (SOOF):* It is located behind the orbicularis oculi muscle and is divided into medial and lateral portions. The medial SOOF extends from the medial limbus of the iris to the lateral canthus. The lateral SOOF runs from the lateral canthus to the temporal fat compartment. The inferior boundary of the SOOF is the tear trough.
- *Retro-orbicularis oculi fat (ROOF):* it is located behind the orbicularis oculi muscle in the upper lid.
- *Intraorbital fat*: on the lower eyelid there are three fat pads, internal, middle, and external. On the upper eyelid there are two fat pads, middle and internal.
- *Buccal fat pad:* includes the Bichat pad and its superior extension that courses from the deep paramaxillary space to the superficial, subcutaneous plane inferior to the zygoma.

Aging Changes of the Fat Compartments

Classically the *gravitational theory* had been postulated as responsible for facial aging. This theory proposed that the vertical descent of the facial soft tissue was secondary to the ligamentous attenuation, leading to the sagging appearance of the aging face [7]. Repeated animation of facial mimetic muscles was also thought to contribute to this ligamentous attenuation. After the multiple studies that described the compartmentalization of facial fat, much has been

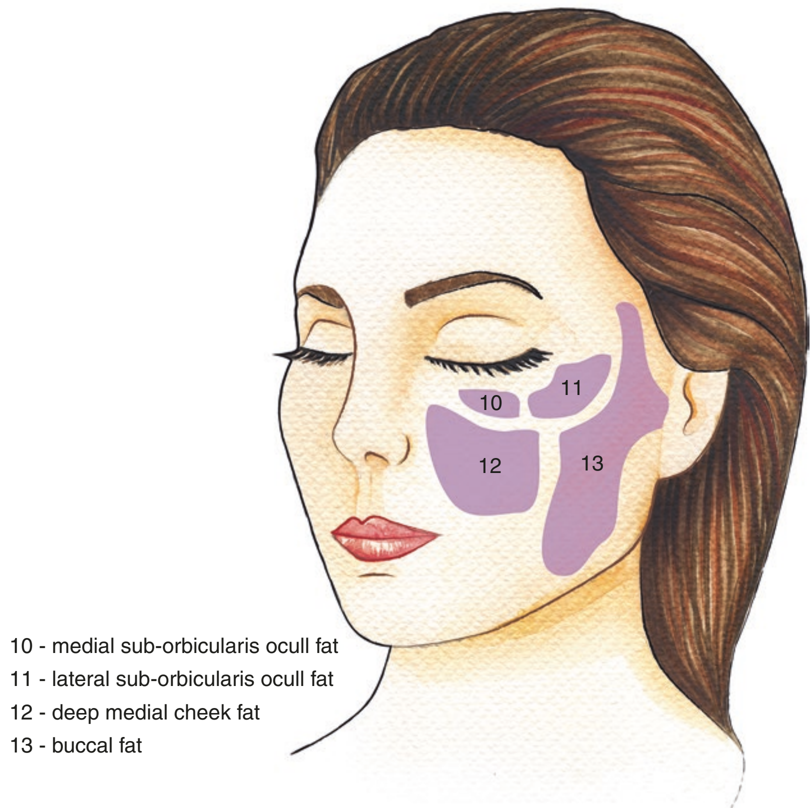

Fig. 2 Deep facial fat compartments

evolved in the theories of facial aging. At present, the most accepted theory for facial soft tissue aging is the *volumetric theory* [5, 8–14]. This theory proposes that the changing morphology of the face, especially the midface, is due to the relative deflation of certain fat pads rather than gravitational descent. Some compartments tend to deflate earlier than others (Fig. 3). These two theories are not mutually exclusive, and facial aging probably reflects a complex morphologic change that involves both elements of gravitational ptosis and volume deflation. Studies have shown that there is a relative hypertrophy of the superficial fat compartments (especially the inferior part of the nasolabial fat) and a pronounced atrophy of the deep compartments (especially DMC and buccal fat pad) with aging [15, 16]. The volumetric theory suggests that selective deflation of the deep fat pads with age leads to loss of support and descent of the overlying superficial fat, thereby contributing to the ptotic appearance of the aging face. This has led to the concept of "pseudoptosis," namely, that loss of volume in one area may lead to the development of folds in a neighboring area [4]. The cheek fat atrophy results in loss of the juvenile convexity of the middle facial third leading to a negative vector (Fig. 4). The negative vector means that the maximum projection point of the cheekbone is posterior to a tangential line to the cornea. Deflation of the deep periorbital fat contributes to the tear trough deformity and the nasojugal groove. The temporal fat compartment atrophy results in depression in the temporal region. The inferior part of the nasolabial compartment hardly suffers atrophy with aging.

But fat compartments not only suffer from deflation, midfacial fat compartments also exhibit an inferior migration and an inferior volume shift within the compartments [1, 15]

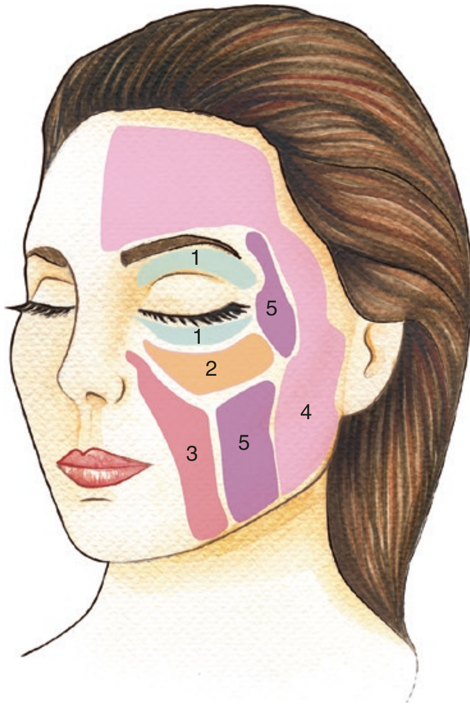

Fig. 3 Some facial fat compartments tend to deflate earlier than others with aging. Sequence of fat compartment deflation observed in facial aging

(Fig. 5). The young face is V-shaped, with a full middle facial third and a lower third with less volume. As the compartments descend, the middle third loses volume and the inferior one gains volume, which causes inversion of the facial youth V (Fig. 6). The inferior migration of fat compartments causes the transitions between compartments to become more marked. In the young face these transitions are smooth and the distances between compartments are short. In the aging face the transitions are pronounced giving rise to furrows (tear trough, nasojugal groove, etc.) and the distances between compartments are lengthened.

It has also been observed that the average adipocyte size is smaller in the deep cheek compartment than in the superficial cheek compartment [17]. Although the reasons for these variations are not clear, it seems that the mechanical environment of the two adipose layers of the midface could contribute to these adipocytes' morphological differences. The superficial compartments are adjacent to the muscles of the facial mime, while the deep compartments are in

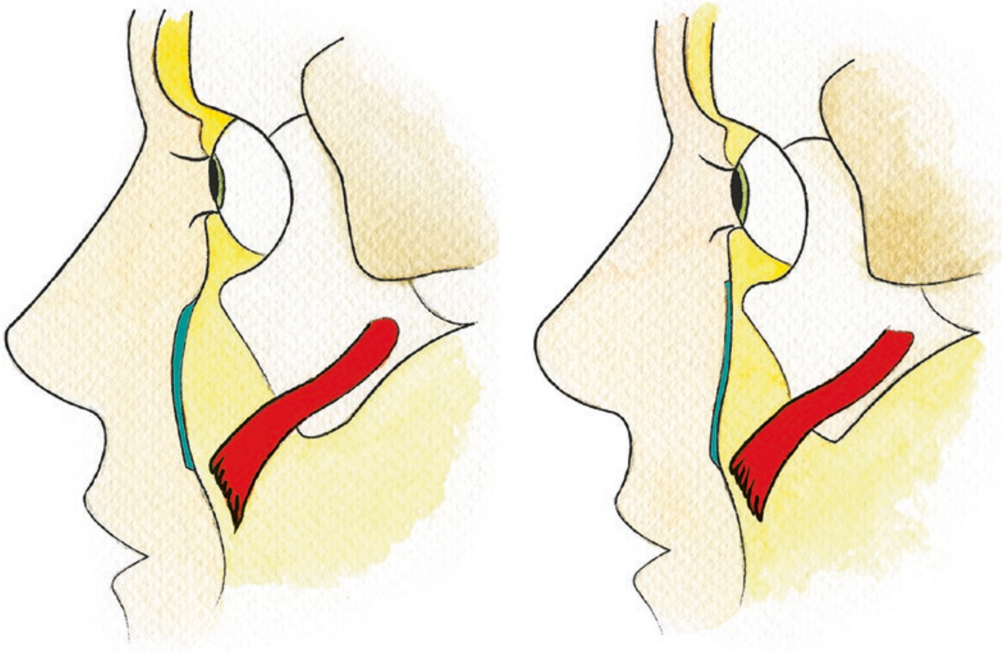

Fig. 4 The cheek fat atrophy results in loss of the juvenile convexity of the middle facial third leading to a negative vector

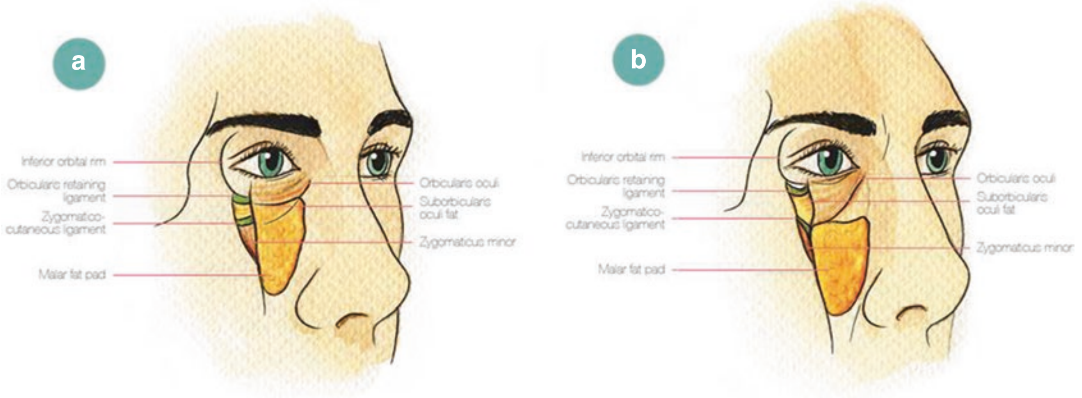

Fig. 5 Facial fat compartments not only suffer from deflation, but midfacial fat compartments also exhibit an inferior migration and an inferior volume shift within the compartments. Young face (**a**) and aged face (**b**)

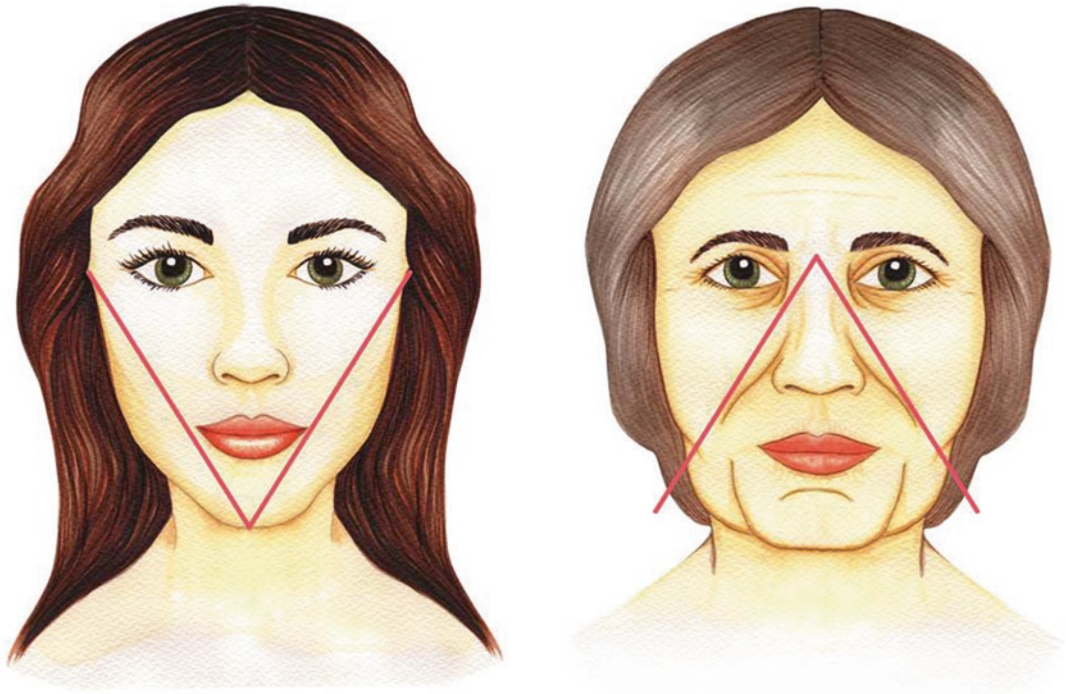

Fig. 6 The young face is V-shaped, with a full middle facial third and a lower third with less volume. As the midfacial fat compartments descend, the middle third loses volume and the inferior one gains volume, which causes inversion of the facial youth V

contact with the facial skeleton. This could be explained by the fact that the continuous compression of the deep compartments against the bone causes them to have a relatively inert role as space-filling interfaces over which the muscles of mastication slide and, hence, tend to more atrophy with time. In contrast, the superficial compartments are closer to the dynamic muscles of facial mime and this could make them more active metabolically [14].

Clinical Implications for Facial Rejuvenation

Due to the relevance that the facial fat compartments have acquired, there has been a change in thinking about facial rejuvenation, evolving from techniques aimed at lifting toward techniques aimed at filling. This evolution has allowed to rejuvenate the face in a more natural way compared to the classical lifting of skin and SMAS under the influence of gravitational theory, which leads to an unnatural appearance. The anatomical knowledge of the facial fat compartments allows us to focus facial rejuvenation techniques more precisely and directly. We can thus selectively increase the volume in the deep deflated compartments creating a more natural look rather than masking the creases of facial aging with superficial multilayering of fat or filler injection in nonspecific malar regions. This also allows economizing the product in the treatments with fillers. We can reduce successfully the nasolabial fold and restore the anterior projection of the cheek by injections into the DMC and Ristow's space [10]. The harsh transition between the lid-cheek junction and the tear trough deformity can be improved by injecting the medial SOOF and the DMC in a supraperiostal way. Augmentation of the lateral portion of the DMC can also increase the anterior cheek projection and smooth the transition between the anterior and lateral cheek. The superficial middle and lateral cheek compartment can be filled for final contour improvement [18].

The inferior migration of fat compartments and the deflation of midface fat lead to the inversion of the facial V. Hence, one should be conservative when filling the lower third (marionette lines, pre-jowls) because if the volume is increased in the lower third, the inversion of the facial V will be aggravated and we will not get an adequate rejuvenation. To restore volume in the middle facial third with fillers or autologous fat is one of the best ways to rejuvenate a face. However, in order to treat sagging of the lower third and jowls, it is preferable to perform treatments aimed at lifting and not only filling (threads, surgical facelift).

Muscle

The fat is positioned both above and below the facial mimetic muscles acting as an effective mechanical sliding plane. Over time, repeated contraction of the facial muscles contributes to changes in the facial fat distribution, expelling the underlying deep fat from beneath the muscle plane. This mechanism leads to a loss of the youthful curvilinear contour and an increase in the resting tone of the muscles. This dynamic muscular effect could explain why deep fat diminishes in favor of superficial fat overtime. With age, the facial mimetic muscles gradually straighten, changing its conformation from a broad convexity in the young face toward a rectilinear flatness in the older one. This convex contour becomes rectilinear as the underlying deep fat is expelled from behind the muscles and the superficial fat increases. The amplitude of movement of the

muscle also is greater in youth but it diminishes with aging and the face acquires a more rigid aspect [19, 20]. The contraction of the muscles of the facial mime is responsible for the appearance of expression wrinkles such as crow's feet, glabellar, and frontal wrinkles. The permanent contractures result in permanent skin wrinkling with a transformation of dynamic facial lines to static facial lines. This increased muscle resting tone with age explains why botulinum toxin treatments are so effective in facial rejuvenation. Young people usually have less strength in the depressor muscles like procerus, corrugators, and orbicularis oculi than in the levators like frontal muscle, and older people have a relatively higher strength in the depressor muscles than in the frontal muscle.

Facial Ligaments

The facial ligaments are composed of collagens, proteoglycans, glycosaminoglycans, and water. The major ligaments are robust and do not undergo significant primary aging changes in their passage from their deep origin to the SMAS. Most of the ligament change is in the multiple finer retinacular ligament branches from the SMAS through the subcutaneous layer to the dermis, which are more prone to being weakened over time by repetitive movement [18, 21, 22]. The most affected by aging are the zygomatic ligament, the orbital retaining ligament, and the mandibular retaining ligament.

The aging bony changes affect the points of origin and the firm adhesions of the ligaments to the skin, and other adjacent structures are getting affected as the position of the ligaments and thus their course are getting altered. The stability of the ligaments that serves as a hammock for the fat within each compartment (superficial or deep) show fatigue and bend along their course, promoting the appearance of sagging of the respective fat compartment and contributing to the appearance of the tear trough deformity, malar bags, and jowls.

Bone

Aging changes occur not only in the facial soft tissues but also in the underlying bony structure. Craniofacial bony remodeling is an important contributor to the facial aging process. The bony facial skeleton serves as the scaffolding for the overlying soft tissue, providing the framework on which the soft tissue envelope drapes. Selective bone resorption occurs in specific areas [9, 23–27]. The most relevant aging changes are bone resorption of the orbit, maxilla, and mandible (Fig. 7). There is an increase in orbital aperture size, more specifically, an increase in height of the superomedial and inferolateral orbital rim. The glabellar and the maxillary angles decrease with age, and there is a posterior displacement of the maxilla and a loss of its projection (Fig. 8). The pyriform aperture area increases over time causing a posterior displacement of the columella, the lateral crura, and the alar base. The anterior nasal spine also recedes, contributing to retraction of the columella, the drop of the nasal tip, and the apparent

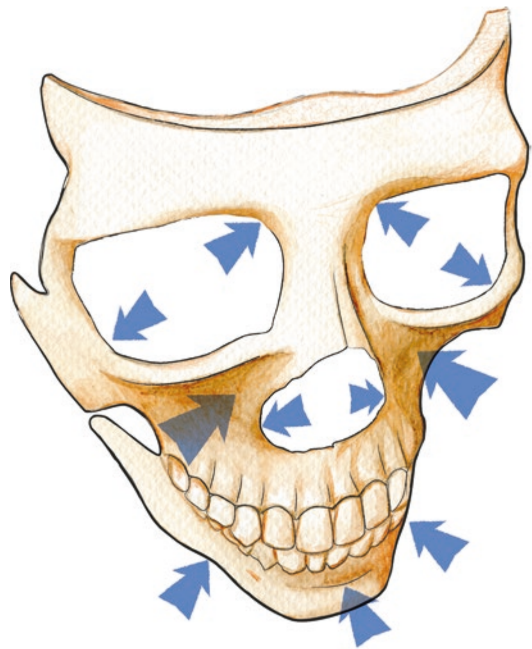

Fig. 7 One of the most relevant aging changes is bone resorption of the orbit, maxilla, and mandible

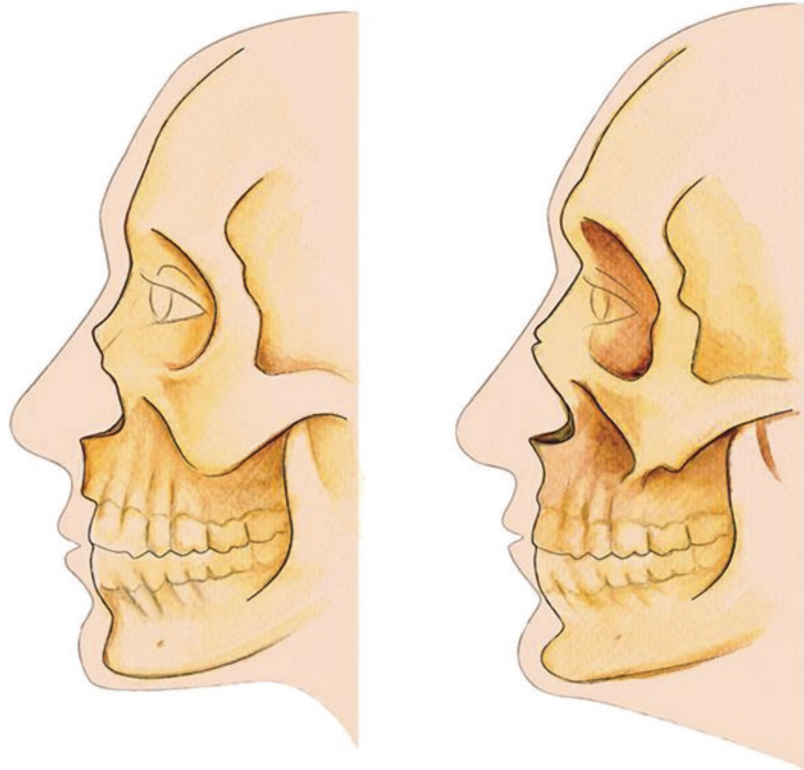

Fig. 8 Age-related bony changes (*right*): enlargement of the orbital and the pyriform apertures, posterior displacement of the maxilla, and shrinking of the mandible

lengthening of the nose. Mandibular ramus height and mandibular body height and length decrease, whereas the mandibular angle increases with age. These changes affect both sexes. Remodeling of the facial bone occurs regardless of the state of dentition, although the loss of dentition accelerates bony resorption of the maxilla and mandible.

Age-related bony remodeling causes a decrease in the space and support available for the soft tissue, especially the fat compartments, resulting in a folding of the soft tissue in a configuration that resembles an accordion [28]. The facial skeleton changes not only affect the overall facial shape but also affect the position of ligaments and septa. The expansion of the infraorbital rim causes an anterior positioning of the orbital septum and a pseudoprolapse of the intraorbital fat pads because the retaining capability of the orbital septum is reduced. The orbicularis retaining ligament loses it horizontal position toward a more inferior inclined alignment, causing loss of stability of the adjacent orbicularis oculi muscle and sagging of the ROOF and the SOOF. Superomedial orbital rim remodeling contributes to the unmasking of the medial upper lid fat, a change currently attributed to weakening of the orbital septum. The changes of the upper half of the orbit result in the soft tissues rolling into the orbit causing brow ptosis and lateral orbit hooding. The bone resorption in maxilla and pyriform area, the ligamentous fatigue, the laxity of the overlying skin, the altered muscle physiology, and gravity cause the loss of stability of the subcutaneous fat compartment superior to the nasolabial sulcus, and the fat has tendency to shift inferiorly. The zygomaticus major and buccinator muscles have strong adhesions toward the skin forming the nasolabial sulcus and border the nasolabial fat compartment inferiorly along with the terminating part of the SMAS. The fat is unable to migrate deep to the nasolabial fold inferiorly but is forced superiorly and thus a bulging of fat overlying the sulcus is clinically visible resulting in a

deep and sagged nasolabial fold. The tear trough deformity and the malar mounds are aggravated by the loss of projection of the maxilla with aging. Mandibular volume loss contributes to the laxity of the platysma and soft tissues of the neck, the loss of jawline definition, and the appearance of jowls (Fig. 9). People with poor facial skeletal support (midface hypoplasia, microgenia, and retrusive supraorbital rim) are predisposed to manifest aging changes prematurely.

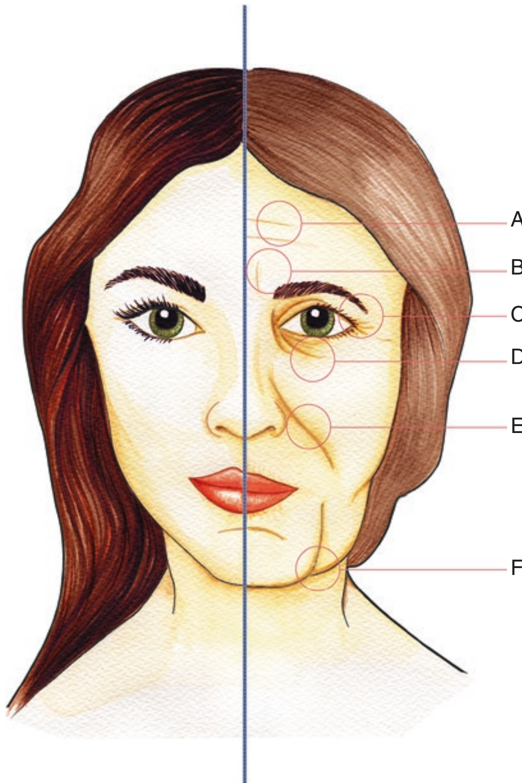

Fig. 9 The youthful (*left*) and the aged face (*right*). (**a**) Horizontal frontal wrinkles due to effect of frontalis muscle contraction. (**b**) Glabellar wrinkles due to the procerus and corrugator supercilii muscles contraction. (**c**) Periocular wrinkles due to orbicularis oculi muscle contraction. Eyebrow ptosis and sagging of the ROOF compartment due to laxity of the orbicularis oculi muscle and orbicularis retaining ligament, and bone resorption. (**d**) Tear trough deformity: aggravated by the orbital and maxillary bone resorption, the laxity of the orbital retaining and zygomatic ligaments, and changes in the SOOF compartment. (**e**) Nasolabial sulcus is formed by the overlying superficial nasolabial fat compartment and the traction of the underlying muscles of facial expression. Maxillary and pyriform bone resorption increases the sagging appearance of the nasolabial fold. (**f**) Jowl deformity. The mandibular ligament attaches the skin to the bone, and the superficial and deep fat compartments posterior to it are more loosely attached and can migrate inferiorly forming the jowls. The mandibular bone resorption contributes to the jowl deformity

Conclusions

To properly restore the youthful facial appearance, it is essential to understand the facial morphological changes over time. These changes affect facial skeleton, fat compartments, soft tissue, retaining ligaments, and skin in variable degrees depending on intrinsic and extrinsic factors. A balanced approach to facial rejuvenation between bone and fat volume augmentation and soft tissue envelope repositioning will avoid the distortions of either approach in isolation. Skeletal resorption can be improved with calcium hydroxyapatite injections or implants. The loss of volume in fat compartments can be treated with fillers or fat grafting in specific deflated soft tissue compartments. The SMAS, the retaining ligaments, and the lid structures can be modified with surgery. Botulinum toxin is useful to reduce the increased muscular resting tone present in aging. Skin rejuvenation can be performed with tretinoin, laser resurfacing, and peels. To return the characteristics of youth to the face, it is necessary to carry out an individualized and step-by-step approach.

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Reducing the Damage: Metabolism Behaviour Aesthetic Medicine

Jesus A. F. Tresguerres

Age-Related Damage

According to the free radical theory of ageing, which is one of the most widely accepted theories, the physiological decline that occurs with age is, at least in part, due to accumulative oxidative damage to cells and molecules. This oxidative damage is induced by reactive oxygen (ROS) and nitrogen (RNS) species, which are highly reactive. ROS have been implicated as major initiators of tissue damage and can upregulate enzyme activity, signal transcription and gene expression of several compounds that can exert deleterious effects on proteins, lipids and DNA which in turn are responsible for all the age-related alterations in different tissues.

It is well established that inflammation and oxidative stress are key components of the ageing process [1–3], but how early in the pathological cascade these processes are involved or which specific molecular components are key is yet to be fully elucidated. However, oxidative stress is understood as a disparity between the rates of free radical production and neutralization, which occurs when the antioxidant mechanisms are overwhelmed. Increased free radicals may in turn lead to activation of a plethora of pro-inflammatory cytokines thereby activating the cascade that leads to further inflammation [4]. Thus, inflammation and oxidative stress could be considered as the twin evils, which may act synergistically.

Cellular enzymatic antioxidant defences which are present in young persons are able by scavenging ROS, to decrease the oxidative damage that could give rise to irreversible damages of structure and functions of cellular macromolecules. Loss of these defences with age enhances oxidative damage and has been suggested to contribute importantly to the ageing process and to the pathogenesis of many age-related diseases.

Free Radicals and Oxidative Stress

Free radicals are molecules containing one or more unpaired electrons in its external orbital *(radical)* and are able to maintain an independent existence (free) although this existence has a very low duration (generally10^{-6}–10^{-9} s). The presence of unpaired electrons renders these molecules extremely reactive, since it tries to interact with other molecules in its vicinity to pair these electrons and to stabilize [5], generating in the process changes that can alter its structure and function.

Free radicals might in play a fundamental role in metabolism, the generation of mutations, the ageing process and death, which together play a role in species evolution [1].

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In nature most free radicals derive from **oxygen** and are named oxygen reactive species (**ROS**), although other free radicals derive from nitrogen (reactive nitrogen species, **RNS**), from sulphur, etc.

The mitochondrial respiratory chain is skipping 2–5% from the oxygen that we are getting and this part contributes to the generation of free radicals.

Oxygen accepts one single electron transforming it to **superoxide anion** ($^{\bullet}O_2^-$) [5]. This radical $^{\bullet}O_2^-$ is transformed to hydrogen peroxide and oxygen through a dismutation reaction, which can be catalysed or not by the enzyme *superoxide dismutase (SOD)*:

Although its reactivity is limited, $^{\cdot}O_2^-$ is able to induce tissue damage through its pro-inflammatory actions. It generates also endothelial damage, increases capillary permeability, stimulates the production of chemotactic agents increasing the recruitment of neutrophils and stimulates the autocatalytic destruction of neurotransmitters and hormones [6].

The most reactive ROS is the hydroxyl radical ($^{\cdot}OH$), mainly generated through the **Fenton reaction**, **in which** H_2O_2 interacts with reduced transition metal ions, generally Fe^{+2} and Cu^+, present as part of the prosthetic groups of multiple enzymes and proteins [5, 7]. Its half-life is in the order of 10^{-9} s and reacts with practically any biomolecule that might be located in the proximity of its place of synthesis, giving rise to chain reactions like in the case of lipid peroxidation.

NO is a relatively stable radical that does not react quickly with the majority of biomolecules [5]. However, it reacts easily with transition metals and other radicals including oxygen, peroxyl radicals and $^{\cdot}OH$ radical. The interaction with the last two plays a very important role in the damaging capacities of NO.

NO is produced by the enzyme **nitric oxide synthase (NOS).** There are three different types of NOS: **nNOS** (type I, NOS-1) that is constitutively expressed in neural tissue; **iNOS** (type II, NOS-2) that is inducible and can be expressed in a great variety of cells and tissues, especially in the pro-inflammatory-agent-stimulated macrophages; and **eNOS** (type III, NOS-3) that is present in a constitutive way fundamentally in vascular endothelial cells that play a role in vasodilatation.

There is also a mitochondrial NOS (mtNOS) in which cytochrome C release is stimulated with an increase in lipid peroxidation that could induce Ca^{2+}-dependent apoptosis [8].

Hemoxygenase

Three HO isoforms have been described, localized in the smooth endoplasmic reticulum of cells.

The inducible isoform (HO-1) is undetectable under normal conditions but can be induced by several stimuli that produce oxidative stress or through NO by means of the activation of transfer factors like NF-κB and AP-1. It modulates the response of the liver tissue to those stressors [9]. Age is also associated with an increase in the expression of HO-1, possibly due to the increase of age-associated oxidative stress. The effect could also be linked to the activation of the age-associated increase in NF-κB [10].

Actions of ROS and RNS

Physiological

Free radicals seem to play a role in some physiological processes, such as genetic regulation, cellular replication, differentiation and apoptosis, probably acting as secondary messengers in the transduction signal pathways [3]. It is also known that the reactive species are one of the elements implicated in the response to pathogens (the oxidative “explosion” of neutrophils).

Harmful Effects

Living organisms are always exposed to a certain amount of reactive species due to its oxygen-dependent metabolism, and they use several mechanisms to fight against it. Oxidative stress takes place when the balance between

pro-oxidant and antioxidant species is altered in favour of the former.

DNA damage: Reactive species and especially the $^{\cdot}OH$ radical react with the double bonds of puric and pyrimidinic bases of DNA, generating derivatives of 8-OH-deoxyguanosine, which have a very high mutagenic activity and are used frequently as a marker of oxidative damage to DNA [11].

Oxidative damage to lipids: lipid peroxidation. It is a process by which lipids and especially polyunsaturated fatty acids (PUFA) are influenced by free radicals, originating an autopropagatable chain reaction that is able to oxidize all free fatty acids of the affected systems (cellular membrane, mitochondrial membrane, etc.).

During this process several characteristic molecules are generated such as lipoperoxides, hydroxynonenals, malondialdehyde, and other byproducts, many of which can be determined by several methods [12, 13] as markers of oxidative stress.

All these alterations are especially significant in the mitochondria, whose functionality depends on the existence of an intact membrane [14].

Protein Oxidation

Oxidative stress also affects proteins, altering its structure and function. Amino acid including the appearance of proteins undergo modifications induced by the reactive species, including carbonyl groups, hydroperoxides [15] and nitrosylated derivatives [8]. These processes induce alterations in the structure and function of the affected proteins and this could influence cellular physiology [16, 17].

Sources of Free Radicals and Reactive Species

The principal source of reactive species is the mitochondrial electronic transport chain that is composed of a *group of enzymes* whose coordinated activities are able to couple metabolic substrate oxidation with the generation of ATP in a process known as "oxidative phosphorylation". Nearly 2% of the total oxygen employed by the mitochondria is not completely reduced and escapes the system in the form of $^{\cdot}O_2^-$ and H_2O_2 [18].

The enzyme *xanthine oxidase* (XO) is another important source of reactive species. This is a cytosolic enzyme that produces $^{\bullet}O_2^-$ and H_2O_2 during the oxidation of hypoxanthine to xanthine during purine metabolism.

Enzymes with an oxidoreductase activity, such as NOS; those involved in prostaglandin and leukotriene synthesis, such as cyclooxygenase and lipoxygenase; and P450 cytochromes also generate reactive species. Neutrophil, phagocyte and microsome activities are other sources of reactive species.

Endogenous Antioxidants Against ROS and RNS

The organism has developed a series of defence mechanisms, generally considered as *antioxidants*, to protect itself from the action of free radicals and act either to prevent the formation of those radicals or neutralize it or facilitate the repair of the induced damage.

These antioxidants are as follows:

Superoxide Dismutase (SOD)

This enzyme comes from a broadly present family of metalloproteins in nature that are able to catalyse dismutation of 2 $^{\cdot}O_2^-$ to H_2O_2. They are highly important since they are in the first line of cellular defence against oxidative damage that can be caused by the superoxide ion and the reactive species that derive from its presence [19].

Glutathione Peroxidase (GPx)

GPx plays a fundamental role in detoxification of hydrogen peroxide and lipoperoxides that are generated in the cells [20]. GPx catalyses also the reduction of H_2O_2 and organic hydroperoxides H_2O and alcohol, respectively, using reduced glutathione (GSH) as donors of electrons.

The reduction of oxidized glutathione (GSSG) generated in these reactions is catalysed by the enzyme glutathione reductase (GR).

Catalase (CAT)

This enzyme catalyses the breaking down of H_2O_2 to H_2O. It is localized principally in the peroxisomes, and its tissular distribution is similar to SOD.

Glutathione

It is a tiolic tripeptide present in the majority of cells from plants or animals. It is one of the most abundant antioxidants of the cell, reacting directly with free radicals or through GPx. It seems also to act in the reduction of several cellular antioxidants, like vitamin E.

Vitamin E

It belongs to a family of highly lipophilic phenolic compounds that play a fundamental role in the protective action against lipid peroxidation of cellular membranes. The organism cannot synthesize this molecule and its presence in the body is only dependent of its intake with food.

Vitamin C

Vitamin C is a hydrosoluble molecule present in the cytosolic compartment of cells and in the extracellular fluid. Although it can interact directly with radicals $^{\cdot}O_2^-$ and $^{\cdot}OH$, it seems that its principal function is to participate in the recycling of vitamin E [21].

Other Antioxidants

Carotenoids are natural dyes present in many vegetal components (tomatoes, carrots, citrus, spinach, corn) capable of neutralizing $^1O_2$ and inhibiting lipid peroxidation. Flavonoids are a group of polyphenol antioxidants present in fruits, veggies and drinks (tea, wine, beer), capable of reacting with $^{\cdot}O_2^-$, $^{\cdot}OH$ and peroxyl radicals.

Oxidative Stress and Ageing

The amount of oxidative damage present in different macromolecules of an organism increases with age and the accumulation of damage throughout life could have crucial functional consequences [2]. There are studies that demonstrate that ageing is associated with an increase of oxidative damage to DNA [11], following an increase in the rate of mutations as well as in the amount of nitrosylated or oxidized proteins in several tissues [22]. The elimination rate through proteolysis of oxidized proteins is reduced, with the consequent accumulation of defective proteins [8]. The increase in oxidative damage to lipids is also increased [23]. All these elements might influence the alterations of age-related mitochondrial functions, inducing a reduction in ATP production and uncoupling of the respiratory chain, which is associated with a further increase in free radicals and closes the evil circle [16, 17, 24].

Apoptosis and Cellular Survival During Ageing

All living organisms need to have a regulated form of cellular death that could allow to control very closely several critical aspects for the maintenance of its homeostasis like the size of the tissue, the number of cells taking part in its composition or the protection against elements that could jeopardize its integrity. Apoptosis was described for the first time by Currie in 1972 [25], is also named programmed cellular death and is really a physiological way of programmed cellular suicide.

Apoptosis takes place under normal physiological conditions in development and embryogenesis to allow for organic remodelling,

metamorphosis or normal tissue exchange, as a defence mechanism, and also in a more later part of life during ageing.

Mechanisms regulating apoptosis are not completely elucidated but the involvement of two families of proteins has been demonstrated: members of the caspase family and the proteins of the Bcl-2 family.

Caspases are cysteine proteases very well maintained through evolution from nematodes until humans. Activation of caspases is one of the principal points in the regulation of apoptosis [26].

The **Bcl-2** family is composed of a series of genes that play a critical role in the control of mitochondrial integrity. Some members of the family, like Bax, Bak and Bad, are apoptosis inducers. On the contrary the expression of other members like Bcl-2 and Ced-9 prevents apoptosis. In any case it is well known that the principal role of Bcl-2 proteins is to control mitochondrial homeostasis: under certain conditions canals or pores are formed in the outer mitochondrial membrane, allowing the exit of mitochondrial contents, being among them cytochrome C, which, in addition to playing a fundamental role in the electron chain transport, is an essential component of the activation of caspase-9 in the cytosol.

Regulatory Elements for Oxidative Stress Damage: GH

Growth hormone (GH) is the most abundant anterior pituitary hormone that accounts for 4–10% of the wet weight of the anterior pituitary in the human adult, amounting about 5–10 mg per gland [27].

The circulating levels of this hormone decline during the first weeks after birth but reaches adult levels after 2 or 3 weeks of life. A substantial increase of GH during puberty has been observed. Spontaneous episodes of GH secretion occur every 3–4 h over 24 h, being these secretory peaks more frequent and smaller in females than in males. The highest secretion of GH occurs during the two first hours of nocturnal rest in the period of slow wave sleep.

Three hypothalamic hormones are involved in GH control: somatostatin (SS), GH-releasing hormone (GHRH) and ghrelin, which is also synthetized in the stomach [28]. SS has a direct inhibitory effect on GH release in response to all known stimuli [29].

Hypothalamic GHRH binds to specific receptors in the somatotropic cells stimulating GH secretion, cell proliferation and also GH-gene transcription [30]. Each episodic secretion of GH is determined by the release of GHRH to the portal circulation together with a decrease in somatostatin. This pulsatile pattern of GH secretion seems to be more important for the peripheral hormonal effects, than the total amount of GH secreted [31].

Ghrelin is another peptide with 28 amino acids, mainly synthetized in the stomach mucosa, but it is also produced in the hypothalamus, which has been found to stimulate GH release both in vivo and in vitro [32].

Actions of GH

GH acts on tissues on a receptor (GHR) that consists in a transmembrane protein codified by a gene located in chromosome 5 [33].

GH is an anabolic protein hormone that causes cells to grow and multiply by directly increasing the rate at which amino acids are used to synthesize proteins. Due to these effects, GH induces an increase in the growth rate of long bones and skeletal muscles during childhood and teenage years. GH also stimulates lipolysis, which is the breakdown of triglycerides into fatty acids and glycerol providing substrates for the neosynthesis of glucose and thus has a sparing effect on glucose utilization. GH also promotes fat catabolism [33].

GH deficiency in the adult has been recently recognized as a specific clinical syndrome characterized by a combination of metabolic and cardiovascular features that are more evident in women than in men [34]. The syndrome includes a high prevalence of dyslipidaemia, glucose intolerance, central obesity and hypertension. Early arteriosclerosis is found in this asymptom-

atic GH deficiency. All these are important contributory factors to the increased cardiovascular risk [35]. Adults with GH deficiency [36, 37] exhibit a diminution of lean body mass and an increase of adipose tissue, which means a reduction of the muscular force capacity. Increase of force and exercise capacity has been reported in elderly people when GH therapy is instituted [38, 39, 40].

Physiological Decrease of GH Secretion with Age

Ageing is associated with several changes and alterations in metabolism, body composition and organ function. Elderly people show bone mineral density loss, lean body mass and muscular strength reduction, adipose tissue increase, insulin resistance and glucose intolerance, etc. [37, 41, 42]. The similarities detected between all the consequences of GH deficiency (GHD) in adults and the changes shown by elderly people point to a possible relationship between age-related physical impairment and the GH/IGF-1 axis decline that physiologically occurs with age [37, 42, 43]. Old age could be a physiological state of GH deficiency. The hormones of the somatotropic axis, growth-hormone-releasing hormone (GHRH), growth hormone (GH) and insulin-like growth factor-1 (IGF-1), apart from their effects on somatic growth and metabolism [27], also exert some other actions on the cardiovascular system.

Indeed, experimental evidence demonstrates that GH treatment has beneficial effects on aged animals. It improves cerebral microvasculature [44], coronary blood flow and heart capillary density [45] in ageing rats. In humans, GH treatment is able to enhance lean body mass and muscular strength, reduces body fat [38, 46, 47], improves plasma lipid profile and increases bone mineral density [47]. However, the effect of GH on vascular function and structure in aged individuals is not well established.

The central nervous system is a target for growth hormone (GH) actions [48]. GH deficiency is associated with sleep disturbances, memory loss, feeling of diminished well-being and other cognitive impairments. Memory and cognitive performances of GH-deficient patients are ameliorated by GH replacement therapy [37, 44]. In animal models, GH has been shown to protect the brain and the spinal cord from different forms of neurodegenerative stimuli and promote neuronal survival after hypoxic-ischaemic injury [49, 50, 51, 52]. These neuroprotective effects of GH suggest that decreases in the hormonal levels with age [37] may affect the brain and may contribute to the ageing-associated deterioration of brain function [53, 54].

Metabolic Effects

It has been demonstrated that GH treatment to both GHD adults and elderly people is able to improve several parameters related to body composition [47, 55], for example, reducing abdominal obesity, which is a strong predictor of cardiovascular risk [56]. The increment in lean body mass in old GH-treated rats is associated with an increase in body weight gain as compared to the weight loss observed in old untreated animals confirming the preponderance of the anabolic properties of GH in the old animals over the lipolytic effects on fat tissue [57].

It has been previously reported [58] that there is a decrease in GH and IGF-1 production with age, but in the present study reduced plasma IGF-1 levels were only seen in males, whereas hepatic IGF-1 content was significantly reduced in both sexes [59]. GH administration was able to significantly increase the hepatic content and plasma IGF-1 levels.

Vascular Effects

GH-deficient patients show a greater risk of cardiovascular alterations [35] and endothelial dysfunction, including a reduced vascular endothelial-dependent relaxation [60].

Ageing is associated with both structural and functional changes that take place in the vascular wall [61, 62]. An increase in media-intima thick-

ness as well as changes in cellular and extracellular composition of the vessel wall [62] can be seen. Ageing is also associated with an impaired endothelium-dependent vasodilatation. Similar results have been obtained in experiments carried out in humans, measured as the response to brachial artery infusion of acetylcholine by plethysmography [63]. This endothelial dysfunction seems to be parallel to the general deterioration of the animals as shown by the correlation found between the maximal relaxation to acetylcholine and body composition parameters.

A decrease in endothelial NO availability due to reduced synthesis and/or major degradation by oxidative stress has been suggested to be an important mechanism underlying the altered response to endothelium-dependent agents during ageing [61, 62], which has been confirmed by our group [64, 65]. In addition, an increase in contracting factors which can counteract the effect of relaxing ones might also be involved in this altered endothelial function. In our previous study, the administration of GH to old rats produced an expected increase in plasma levels of IGF-1 that was accompanied by an improvement of endothelial function and vessel structure [57, 66].

The mechanisms underlying the beneficial effects exerted by GH involve an increase in endothelial NO availability that has been confirmed by our group [64, 65] This enhanced NO availability could positively influence vascular function and structure. These data confirm previous studies which show that GH can exert beneficial effects on cardiovascular system in aged animals [44, 45].

Effects on the CNS

The hippocampus, a brain region involved in spatial and episodic memory [67], may significantly contribute to ageing-associated decline in cognitive abilities [54, 68]. Although in most brain areas there is no massive neuronal loss with ageing [53, 69], a significant reduction in the number of neurons has been reported in the hilus of the dentate gyrus of the hippocampal formation in aged humans [54] and in 24-month-old Fischer 344 male rats [52].

It is well known that GH exerts important effects on CNS [48] increasing psychological capacity in adults [70], memory, concentration, alertness and capacity of work. Some neurotransmitters change also under GH treatments [71]. Receptors for GH exist in CNS at different levels: neurons, glia and endothelial cells in the vessels [72]. Under GH stimulation IGF-I is produced in the cerebral tissue [73] probably playing a local trophic role [74]. Emergence of new neurons in the brain is a well-documented phenomenon, especially in young animals, but the real significance of this fact is still unknown [75].

Estimation of the total number of hilar neurons by our group revealed that 24-month-old rats that were treated with GH had more neurons in the hilus than control animals treated with vehicle. GH is a neuroprotective factor for the brain and spinal cord of young animals [50, 51] and prevents hippocampal neuronal cell loss after unilateral hypoxic-ischaemic brain injury [51, 65, 76]. The neuroprotective effect of GH was observed in both sexes.

After GH treatment a clear inhibition of the apoptosis was observed, which was accompanied by a decrease in nucleosome levels and an increase in Bcl-2 levels.

The role of GH and IGF-I on the regulation of the production of free radicals and the antioxidant defences is controversial. It has been demonstrated that the administration of both GH and IGF-I exerts a protective effect on several experimental models of free-radical-induced tissue damage. Previous data showed in our laboratory confirm these effects in the liver, pancreas, heart and brain [64, 77, 78, 79, 80]. Effects can be due to direct actions of GH or mediated by IGF-1.

However, there are also data that indicate that the hormones of the somatotropic axis could also exert a negative influence on life expectancy and antioxidant defences. Transgenic mice that overexpress GH show a reduction in life expectancy as well as a phenotypic expression of premature ageing with an excess of oxidative stress damage [81]. On the contrary dwarf Ames mice that are deficient in GH, TSH and prolactin live longer

than the normal individuals of the same strain [81, 82]. Our explanation for the apparently controversial results is that what we expect when we proceed with replacement therapies with GH in old animals is to maintain the physiological levels of GH present in young adults. Both transgenic animals and acromegaly patients show very high levels of GH starting very early in life, thus exerting at least negative effects on the whole organism, whereas compensating for the lost levels of GH in old animals should replace the positive physiological actions of GH.

Since the paper of Rudman D and colleagues [47], which has been converted in a “classical reference”, replacement therapy with GH in elderly people has been proposed, and many studies that have been carried out demonstrate beneficial effects of the treatment [83]. However, the studies have obtained positive results [37, 84]. The administration of GH to men older than 60 years of age restores normal levels of IGF-I reaching values present in young people [38], and this could be extremely beneficial.

Both in GH deficiency and in the elderly, replacement therapy with GH is able to increase lean body mass and to reduce body fat [38, 84, 85]. Replacement therapy in the elderly with GH also has beneficial effects on plasma lipid levels, since cholesterol values are reduced and also the relationship between LDL/HLD [86]. Our group has confirmed these results in old rats [64, 77, 79, 87].

There are also effects on the skin as has been described previously with an increase in cutaneous stiffness and thickness [47, 89, 90]. It has been also seen that when age-associated immunosenescence is treated with GH, a highly beneficial effect can be observed.

Melatonin

Melatonin, an indolic hormone secreted by the pineal gland, is a substance closely related to biological rhythms and has been used since decades for the induction of sleep and the treatment of jet lag. In addition to its role as a chronobiotic hormone, melatonin is a ubiquitous direct free radical scavenger and an important indirect antioxidant.

The pineal gland [91] is the link between light signals of the environment and the endocrine and nervous systems through the secretion of its hormone **melatonin**, which is regulated by the degree of environmental light.

Melatonin is synthesized from tryptophan in the pineal gland and then is secreted to the circulation, showing a circadian rhythm with maximal values present during the period of darkness. It seems that this more recently discovered action as antioxidant was actually its initial activity and appeared nearly simultaneously with the starting of life on earth as a way to reduce the oxidative damage in nearly all living beings [92].

Bright artificial light is able to reduce the amplitude of the nocturnal peak [91]. Besides these direct scavenging actions, melatonin stimulates also a host of endogenous antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRd), and inhibits the activity of nitric oxide synthase (NOS) thus making it possible to effectively fight not only against free radicals but also against inflammation, apoptosis and also several age-associated diseases.

In humans, melatonin levels start to descend from 25 to 35 years of age, and at 40–60 years the levels are about 35–50% of those present in young individuals [92, 93], and especially a reduction in the nocturnal peak has been detected [94].

In parallel with the reduction of melatonin levels, an increment in free radicals has been observed together with the reduction in antioxidant enzymes such as SOD, GRd and GPx, which are in part regulated by melatonin itself. Thus one of the reasons to have an age-related oxidative damage could be the reduction of melatonin [95].

Moreover, melatonin is a small, lipophilic and hydrophilic molecule, which allows it to easily cross biological barriers and membranes and diffuse throughout cell compartments, reaching the place where the free radicals and reactive species are generated in all tissues: the mitochondria.

Melatonin is critical for the regulation of circadian and seasonal changes in various aspects of physiology and neuroendocrine function [91,

92, 96]. In addition, melatonin is also produced at high levels in other organs (bone marrow, gastrointestinal tract) [97] and the immune system – especially lymphocytes [98]. Melatonin and its metabolites exert potent hydroxyl and peroxyl radical scavenging activity [99, 100, 101], and they increase the efficiency of the electron transport chain in the mitochondria and, as a consequence, reduce electron leakage and generation of free radicals. Melatonin also reduces the formation of 8-hydroxy-2′-deoxiguanosine, a marker of damaged DNA, more effectively than some classical antioxidants [101]. As age advances, the nocturnal production of melatonin decreases in animals of various species, including humans [93, 94]. Melatonin is an anti-apoptotic mediator. So melatonin supplementation suppresses NO-induced apoptosis by induction of Bcl-2 expression [102]. In previous publications, we found that melatonin treatment reduced NO levels and increases cytochrome C content in mitochondrial fraction of the liver of old and castrated female rats. Additionally melatonin treatment enhances hepatic antioxidant/detoxification systems, consequently reducing apoptotic rate [76, 88, 102].

Ageing is associated with a dysregulation of the immune system known as immunosenescence that is characterized by a decrease in the functional activity of NK cells, granulocytes and macrophages. Besides causing changes in innate immunity, ageing is associated with changes in cellular and humoral immunity. Reductions in CD3 and CD4 and rises of CD8 immune cells occur in elderly individuals. Changes in the expression and function of TLR (toll-like receptors) as a result of immunosenescence lead to increased secretion of pro-inflammatory cytokines and chemokines [98].

Immunosenescence

The ability of melatonin to revert age-associated thymic involution adds further support to the concept that it is a potential therapeutic agent for the correction of immunodeficiency states associated with ageing and possibly other immunocompromised situations like severe stress [98]. Immunopharmacological activity of melatonin has been demonstrated in various experimental models [79, 88]. Melatonin regulates the immune system by affecting cytokine production in immunocompetent cells [103]. Inasmuch as melatonin is able to stimulate the production of intracellular glutathione [104], its immuno-enhancing effect may be partly a result of its action on glutathione levels.

Melatonin and Diabetes Type 2

Diabetes type 2 appears in the fourth decade of life in persons with a genetic predisposition and that generally are overweight. The first indication of glucose metabolism impairment is the appearance of peripheral insulin resistance. The pancreas reacts initially with beta cell hypertrophy leading to hyperinsulinaemia, thus making it possible to maintain normoglycaemia, and later on, after some months or years, with beta cells getting exhausted or entering apoptosis with the corresponding reduction in plasma insulin levels and the appearance of hyperglycaemia. Drugs used until now allow the treatment of diabetes as a chronic disease but are not able to cure it. The elements responsible for insulin resistance in peripheral tissues are related with oxidative stress and inflammation and the same occurs in the endocrine pancreas itself. Melatonin when given both to experimental animals (1–10 mg/Kg) that show already insulin resistance and hyperinsulinemia and to humans (40–60 mg) with increased HOMA index is able to revert the situation, reducing plasma insulin levels and enhancing the islet production of insulin by reducing several inflammation- and oxidative-stress-associated factors present in both the peripheral tissues like muscle and fat tissue such as TNF-alpha, NF-kB and IL1/2. The administration of melatonin to experimental animals was able also to increase the pancreas genes of survival like SIRT1 and FoxO as well as genes of differentiation like PCNA, Pdx and Sei1 and to reduce apoptosis markers restoring its normal function [79, 80, 105].

These effects have been observed also in humans suffering from insulin resistance in which

melatonin besides of a reduction in plasma insulin levels a potentiation of the beta cell capacity to produce insulin, which reduces the risk of exhaustion of these cells and thus allowing the maintenance or restoration of normoglycaemia [105].

Oestrogens

Oestrogens have shown to exert fundamentally antioxidant effects but also under some circumstances pro-oxidant actions [106, 107], as well as anti-inflammatory actions in several experimental models [108]. They have also shown protective effects against peroxidative membrane damage [106, 109]. These hormones are able to protect hepatocytes undergoing oxidative stress [110], and they preserve hepatic integrity and function in several experimental models of liver injury in which oxidative damage is involved [111]. They have also shown to exert protective effects in other tissues, such as CNS [112, 113] and heart and skeletal muscle [114]. Moreover, it has been demonstrated that mitochondria isolated from brain and liver of female rats exhibit higher antioxidant gene expression and lower oxidative damage than males [115]. All these findings support the idea of oestrogens acting as protective agents against oxidative damage of different aetiologies, and our studies are in accordance with this, showing a protective effect of oestrogens against age-induced oxidative injuries in the liver but also in other tissues.

Both menopause and ovariectomy are known to induce deleterious effects on different organs and systems, such as cardiovascular system, plasma lipid profile and bone turnover [116, 117]. On the other hand, oestrogens have shown to exert positive effects on vascular function [117, 118], to have antioxidant properties in vitro [106, 109] and to play neuroprotective actions [112]. Epidemiological data suggest that the rate of progression of chronic hepatic disease is higher in men than in women, suggesting a possible protective effect of oestrogens on the liver [119]. Moreover, hepatic tissue in both males and females contain oestrogen receptors and respond to these hormones [120].

The degree of damage found in these parameters is, in general, lower in intact females than in ovariectomized ones. Thus, a possible protective effect of oestrogens on oxidative and inflammatory age-induced liver injury can be suggested. Moreover, when ovariectomized female rats were treated with oestrogens, they showed a clear improvement in the values of all studied functions.

On the other hand, oestrogens have also shown to exert anti-inflammatory actions in different experimental models, such as experimental arthritis [121], uveitis [122], shock [123], amyloid-b-induced inflammatory reaction [124] and carrageenan-induced pleurisy [108]. All these data are in accordance with those found in the present experiment, in which treatment with oestrogens were able to reduce NO release in cells isolated from old rats.

Several mechanisms have been proposed to be involved in these protective actions of oestrogens. They have shown to maintain the level of endogenous antioxidants, such as GHS and other antioxidant enzymes [111, 115, 125]. Oestrogens are also able to inhibit the activation of the gene regulator NF-κB in different cell cultures [112, 126], which is involved in immune and inflammatory response. They may also act as direct free radical scavengers and iron chelators, since some of their protective effects are not mediated by the interaction with the classical ER [113, 114]. And oestrogens have shown to modulate the expression of cytokines and other molecules related to inflammation [108, 125].

A naturally occurring sexual dimorphism in the immune response has been shown by both clinical and experimental data [127, 128]. Females show, at least during the reproductive period, a more vigorous cellular and humoral immune response than males [129]. Furthermore, data suggest that physiological levels of oestrogen stimulate the humoral and cellular immune response, whereas male testosterone does the opposite [130]. Recent studies on many experimental models have demonstrated that sex hormones may regulate immune reactivity, and concretely T cell response, and the subsequent release of various cytokines [125]. Moreover,

oestrogen deprivation affects several aspects of the immune function. Thus, a decrease of NK activity has been found after oestrogen withdrawal in rhesus monkeys [131]. In fact, the results of the present work show that intact female rats demonstrate higher values of the immune parameters studied than ovariectomized in which these values decrease in some functions such as lymphoproliferation.

If a good immune system is a predictor of longevity [132], the better response found in females could explain in part the longer lifespan of female rats as compared to males, which was attributed to oestrogen [133]. Many studies have examined the cytoprotective role of oestrogens on the prevention of age-related diseases, but the mechanism(s) underlying its effects on the ageing process has not been elucidated. Oestrogens have been also found to exert a protective effect against oxidative stress and mitochondrial DNA damage. This protection against free-radical-mediated damage could explain the different lifespan of both genders [134]. Oestrogens have been reported to act as antioxidants in vitro [111, 135]. Ovariectomy caused an increase of oxidative stress in mitochondria and oestrogen replacement therapy completely prevented this effect [134]. Same effects can be seen in heart and skeletal muscle [114]. Although no data are available about its effect on immune cells, a similar mechanism could be suspected for them. But the physiological levels of these sex hormones are not high enough to show a direct antioxidant action. Oestrogens are also able to increase the antioxidant defences of the organism. Thus, oestrogens increase the expression of Mn-superoxide dismutase and glutathione peroxidase, two of the major antioxidant enzymes found in the mitochondria [134].

Phytoestrogens are plant-derived substances, with molecular structures similar to those of oestrogens, that share some of the effects of these hormones. Isoflavones, which is one group of phytoestrogens, are now being widely studied, since they seem to exert beneficial effects on health [136, 137]. Isoflavones are organic plant substances found in soy, legumes, fruits and vegetables, which have been suggested to exert beneficial effects on health. These compounds seem to reduce the incidence of various cancer types [136] and coronary heart disease [138].

Isoflavones have shown to behave both as agonist and antagonist of oestrogenic action, similarly, but not identically, to the action of a SERM. In the presence of a stronger oestrogen like estradiol, an antagonist action on both cell growth and oestrogen-induced protein synthesis has been observed [139, 140]. One of the possible explanations for the dual activity of isoflavones could be its interaction with oestrogenic receptor. A more intense affinity for receptor ERβ has been described. ERβ receptors have different transcriptional activities. It has been shown that isoflavone excretion, derived from vegetal food, is much higher in Japanese than in North American women and that this excretion is negatively correlated with the incidence of climacteric symptoms and breast cancer [141, 142]. In fact, in Western countries isoflavones are becoming an alternative treatment for climacteric alterations, such as hot flushes [143]. Since isoflavones show oestrogenic effects on several tissues, it has been tested for antioxidant activity in several tissues including the immune system.

Phytosoya®, a commercial soya extract, has been shown to protect against glucose-induced oxidation of human LDL [65]. It has also shown to protect against oxidatively induced DNA damage in different cell lines [144, 145]. Our group has shown that Phytosoya® is able to improve some parameters related to oxidative stress in several tissues that are altered by ageing and ovariectomy. And we also have shown that these effects are similar to that of oestrogen. The importance of these results rests in the fact that phytoestrogens are being increasingly proposed as a safer alternative to hormone replacement therapy, mainly in women who have some contraindication for being treated with oestrogens. Several mechanisms may be involved in this effect, such as a direct free radical scavenging [146], metal ion chelator activity [147], restoration of GSH levels [146] and modulation of the activity of antioxidant enzymes [137, 146, 148]. However, there are some discrepancies among the results of different studies carried out in order

to investigate these antioxidant properties of isoflavones, and some of them have been unable to demonstrate real antioxidant effects [149, 150]. It is necessary to point that these studies are difficult to compare, since they use different experimental designs and soya extracts, and on the other hand some women lack the enzymatic activity to hydrolyze the extracts in order to obtain the active aglycones. To elucidate the antioxidant properties of phytoestrogens and their intrinsic mechanisms of action, more work needs to be done.

Resveratrol

Resveratrol, a polyphenolic compound found in appreciable amounts in grapes and red wine, is currently a widely investigated molecule for its potentially beneficial effects on health and its capability to promote longevity [151]. In animal models, from lower metazoans to vertebrates, including small mammals, resveratrol administration has the remarkable property to prolong lifespan [151, 152, 153]. Several genes have been identified to play a role in the control of lifespan, including genes implicated in insulin-like signalling and genes coding for the Sir2/SIRT1 sirtuin family of deacetylases [154, 155]. In aged individuals, intense inflammatory activities characterized by the presence of cytokines, apoptotic cells, immune cell infiltration, amyloid deposits and fibrosis may cause a reduced function or failure in pancreas and other tissues [156]. Several factors are responsible for inflammation, including elevated nuclear-factor kappa B (NF-κB) activity, increased levels of cytokines such as tumour necrosis factor-alpha (TNF-α), interleukins (ILs), resistin, leptin and free fatty acids [157, 158, 159, 160]. NF-κB is a family of transcription factor that regulates expression of genes which are involved in immunity and inflammation. Sirtuins are NAD^+-dependent deacetylase enzymes related to histones and transcription factors like p53, FoxO family and PGC-1. Sirtuins and FoxO factors could remove NF-κB signalling and, thus, delay the ageing process.

Resveratrol supplementation has been shown to exert anti-inflammatory effects in various mammalian models of ageing [151, 161]. Previous studies have established that resveratrol can exert significant cardiovascular protective effects in various models of myocardial injury [162, 163, 164], hypertension [164, 165, 166] and type 2 diabetes [167, 168, 169].

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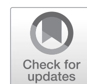

Fat Harvesting: The Latest Scientific Evidence on Cell Viability

Jesus Benito-Ruiz

Introduction

The first known historical reference for the transfer of adipose tissue corresponds to Neuber, which transplants fragments of 1 cm adipose tissue, from the forearm to the face. Lexer in 1910 used fat for facial and malar atrophy and increased malar, and Bruning in 1919 reported the first fat injection through a needle in 1919 [1]. Peer in 1950 refers to a 40–50% retention of transplanted fat per year [2]. The introduction of liposuction increased interest in lipotransfer [3, 4].

In 1987, Bircoll [5] introduced the use of adipose tissue as a breast enhancement material, but it raged with criticism as bad experiences and complications from steatonecrosis accumulated. That is why a panel of experts from the American Society for Plastic and Reconstructive Surgery issues a statement advising against its practice. Coleman is responsible for the standardization of an atraumatic procedure that allows to obtain good and reproducible results with the adipose tissue grafts and denominated Lipoestructura ™ [6]. Basically it consists of an atraumatic fat collection (with 3 mm blunt cannulas and 10 mm syringes), centrifugation at 1286 g (3000 rpm with the Coleman centrifuge) for 3 min to separate the adipose cells from the blood components and cells Broken, and transferred to the tissue by blunt cannulas of about 2–3 mm in multiple passes, using 1 cc in each pass. The importance of micrografting has already been pointed out by Bircoll and Coleman as the most important part of the procedure. In 1993 Carpaneda and Ribeiro compared the viability of various fat cylinders and found that survival is greater in those with less than 3 mm in diameter. The central part of the grafts of more than 3.5 mm was necrosed [7]. This observation has been confirmed more recently by the works of Eto et al. [8] which showed that the adipocytes of a graft begin to die on day one and that only a few adipocytes within 300 μm of the edge of the tissue survive.

Despite the pioneering effort of Coleman and the enormous advance that his standardization of the method supposed for fat grafting, one of the major criticisms and drawbacks of lipofilling is the variable retention range reported, between 20 and 80% at 1 year [9, 10]. This unpredictability has led to the search of the best method of harvesting which would ensure the best cellular viability and retention. Research has focused on the three phases of preparing the fat: harvesting, processing, and injection. However, there is insufficient scientific evidence to permit the standardization of procedures. Since 2011, there are only five clinical trials and 32 prospective comparative studies [11].

Regarding harvesting, retention could depend on the size of the fat particles. Liposuction

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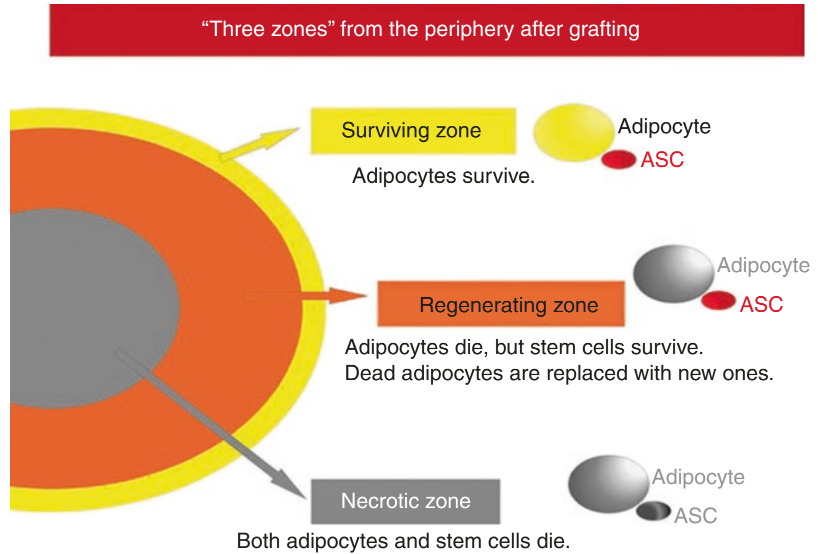

Fig. 1 "Three Zones" from the periphery after grafting

disaggregates the fat in particles of different sizes, depending on the cannula diameter. As we have read above, fat diameter is a paramount issue for survival. Eto et al. [8] in their landmark study showed that adipocytes closer to the surface of the particle were more likely to survive. They established that in the outer surviving layer (100–300 μ) all adipocytes and stem cells survive; in the middle layer (600–1200 μ), the adipocytes die but they are replaced by proliferating stem cells; and in the core of the particle, all cells die (Fig. 1).

The aim of this chapter is to review the latest data available regarding harvesting techniques for fat grafting.

Mechanical Damage

The main disadvantage of excising en bloc fat for grafting is the size of the scars. Therefore one of the main goals is to demonstrate that liposuctioned fat conserves the features of the whole fat while being less invasive in its harvesting. Moore et al. [12] showed that cells isolated from intraoperative liposuction and lipectomy samples did not differ functionally, responded similarly to insulin stimulation of glucose transport and epinephrine-stimulated lipolysis, and retained the same growth pattern in culture. Lalikos [13] did not see any difference in architecture comparing liposuctioned and whole fat. In another work, Pu et al. [14] showed no difference in the cellular architecture between the liposuctioned and the en bloc fat, although the liposuctioned cells showed less enzymatic activity of glycerol-3-phosphate dehydrogenase, a marker of cellular metabolism. For the Yoshimura group, however, the aspirated tissue is poorer in ASC and adipocytes, and therefore they defend the need to enrich the aspirate with stem cells (what they call CAL or cell-assisted lipotransfer) [15].

What Is the Best Donor Site?

There is no uniformity of opinions or results regarding the different work performed. For some the abdomen, especially the lower part of it, is the richest in stem cells (it must be borne in mind that it is assumed that the higher the concentration of ASCs, the greater the survival of the graft). For Fraser [16] the best donor site is the hip, and for Rohrich et al. [17] and Li et al. [18], there is no difference between donor sites (level of evidence 1). Another work by Small et al. in breast reconstruction concluded as well that there was no difference in longevity between fat harvested from the abdomen or from the thigh [19].

The main conclusion is that the current literature suggests that there is no significant difference

between different donor sources regarding cell viability or volume retention. On the other hand, if we consider the layers of the adipose tissue, there could be a difference. Di Taranto et al. [20] reported that the superficial adipose tissue has a higher stromal compound and higher CD105+ cells comparing to the deep tissue, which would make it a better donor tissue for fat survival.

Effect of Local Anesthesia

Local anesthesia (lidocaine) appears to adversely affect the metabolism of adipocytes, with reduced glucose transport and lipolysis, and viability and differentiation of preadipocytes (ASC) [20]. Articaine/epinephrine and 2% lidocaine are especially harmful. The time between infiltration and aspiration may be relevant in terms of the longer contact between cells and anesthetic [21]. It must be taken into account in any case that these works are done in vitro and does not take into account the actual concentration of the anesthetic in the fluid that infiltrates. They consider in their work as 30 min of exposure, and it is possible that many surgeons wait much less time to obtain the fat once the infiltration is done. Lidocaine potently inhibited glucose transport and lipolysis in adipocytes and their growth in culture [14]. That effect, however, persisted only as long as lidocaine was present; after washing, the cells were able to fully regain their function and growth regardless of whether the exposure was as short as 30 min or as long as 10 days. In fact, it seems that the inhibitory effects of lidocaine disappear when the anesthetic is removed [14].

Epinephrine at different concentrations has not deleterious effect on the number of living cells in a 100× field [22].

Finally, tumescence makes no difference regarding cell viability comparing to the dry technique [23].

Suction Pressure

There is no conclusive data to ensure that syringe harvesting is better than with liposuction. The syringe gets a pressure of 660 mmHG (0.86 at). The percentage of cells in the stromal fraction is greater when using aspiration at 350 mmHg than 700 mmHg and higher in both cases than the syringe. Obtaining a 10 cc syringe and after aspirating 2 cc of air (which is what Coleman recommended) results in a negative pressure of 0.37 at. The 50 cc syringe arrives at a vacuum pressure of 0.76 atm [12]. Ould-Ali shows that with lower vacuum pressure for harvesting, greater adipose tissue survival and less fibrosis [24]. Therefore either the 10 cc syringe is used with the plunger removed 2 cc or a liposuction device at 0.5 at.

Cheriyan et al. [25] compared high pressure (−760 mmHg) versus low pressure (−250 mmHg) for cell viability using trypan blue vital stain technique after digestion with collagenase, and they observed that aspirate collected under low pressure appeared to have a compact, homogenous fat layer without any obvious oil layer, indicative of less rupture of fat cells during low-pressure aspiration. Furthermore, the average number of adipocytes after harvest was 47 percent higher in the low-pressure simple.

Cucchiani and Corrales [26] compared fat aspirated through straight cannulae (15 cm long, 3-holed, 3 mm hole diameter) under low vacuum (220 mm Hg) or high vacuum (720 mm Hg) with a Luer-Lock Terumo™10-mL syringe with plunger set at 2 mL or with a 60-mL Luer-Lock Terumo™ syringe with plunger set at 60 mL, respectively. Vacuum pressures were determined at these plunger positions by means of a vacuometer. The adipocyte viability was studied with a MTT assay. They observed that viability is reduced with higher vacuum pressures, even though the cells are quite resistant to both positive and negative pressures.

Chen et al. [27] compared the results of two different suction pressures on the cell yield of the stromal vascular fraction and the functionality of adipose derived stem cells of the SVF. The adipose tissue was obtained from the abdomen of ten patients at −30 ± 5 or −55 ± 5 kPa (101,32 kPa = 1 at). The cell yield for the lower pressure was twofold higher than with the higher pressure as well as faster cell growth and secretion of basic fibroblast growth factor and vascular endothelial growth factor.

Charles-de-Sá et al. [28], however, comparing samples obtained with different syringes (10 mL, 20 mL, and 60 mL) and different pressures (350 mmHg and 700 mmHg) with 2-hole blunt cannulas measuring 3 mm in diameter, found no significant changes in adipocyte cell count, percentage of endothelial progenitors, viable cells, and rate of late or recent apoptosis. They concluded that the amount of negative pressure used for harvesting adipose tissue by syringes of 10 mL, 20 mL, and 60 mL and by −350 mmHg and − 700 mmHg pressure does not affect the integrity and viability of adipocytes and AMSCs.

The Cannula

Coleman has designed a series of cannulas aimed at obtaining atraumatic fat and its safe infiltration (reducing the possibility of intravascular injection). Özsoy et al. [29] compared 4, 3, and 2 mm cannulas and found higher cell viability in samples harvested with 4 mm cannula. A similar result was reported by Erdim et al. [30] who found a greater viability with large cannulas (6 mm) comparing to 4 and 2 mm liposuction cannulas, and also they did not find any difference in infiltration between cannulas of 14, 16, and 20 G (gauge). Kirkham et al. [31] harvested adipose tissue from the abdomen with 5 mm and 3 mm cannulas with negative pressure (25 mmHG) and grafted the samples in nude mice. The analysis of the graft after 6 weeks showed better results for the group obtained with the 5 mm cannula.

However there are some evidences that harvesting with microcannula (2 mm multiperforated) could be better for tissue regeneration and micrografting. Trivisonno et al. [32] observed higher number of stromal and vascular cells in samples obtained with 2 mm cannula with five round ports along the sides of its distal shaft than with 3 mm and single suction port on the side of its distal end. And Alharbi et al. [33] observed better viability and migration of isolated cells in collagen elastin matrices in the microcannula samples (2 mm multiperforated).

Liposuction techniques have evolved in the last years, and new techniques have been described for removal of unwanted adipose tissue. The physics of these devices rely on their ability to detach the adipocytes from the tissue. Therefore these devices have been tested to know if they are suitable for harvesting fat for grafting. The most popular are ultrasound-assisted liposuction (UAL), power-assisted liposuction (PAL), and water-assisted liposuction (WAL).

Several studies have been done for UAL, showing no difference in cellular damage between conventional (suction-assisted) liposuction and UAL by studying the cellular damage with glycerol-3-phosphate dehydrogenase enzyme assay [12, 34, 35]. Other studies have not shown any difference for fat outcomes comparing handheld syringes, conventional liposuction, and UAL [36–39].

Using power-assisted liposuction is as well safe for harvesting fat with no difference with manual aspiration [37]. Barzelay et al. [40] studied the difference between samples obtained by resection (en bloc) and power-assisted liposuction (PAL). They did not find any difference between samples regarding the number of nucleated cells and their viability.

Something similar happens with water-assisted liposuction [41]. Meyer et al. [42] reported a good yield of adipose stem cells by using WAL, comparable to other methods of harvesting.

Finally, a recent technology using 1470 nm radial laser that disrupts the collagen seems to avoid cell damage, with a viability of adipocytes of 95.7% [43].

Conclusions

Even though considering the heterogeneity of the published works, the conclusions that can be drawn from the literature are:

- Donor site is not an important factor.
- The presence of lidocaine is deleterious for the cell, so the fat should be washed.
- Aspiration pressure should be around 0.5 at.

- The diameter of the cannula does not seem an important issue although bigger cannulas are linked to better cell viability.
- The technology for harvesting has not influence (WAL, PAL, UAL).

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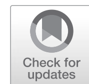
Part III

Approaches: Strategies to Reduce the Clinical Impact of Aging



Graft Processing and Enrichment Strategies

Jordi Descarrega and Juan Cruz

Autologous fat graft is known to be the ideal filler since it is inexpensive; it can be easily harvested as needed and is biocompatible without risk of allergic reaction or rejection. On the last decades, fat grafting has gained a significant interest both in the cosmetic field and for the treatment of a variety of pathologies such as reconstructive breast surgery, pathologic scars, vocal cord pathologies, and facial lipoatrophy, among others [1].

The autologous fat transfer was first described more than a century ago. However the interest in grafting the autologous fat tissue increased significantly with the expansion of the liposuction procedure. On the decade of the 1980s and 1990s, the complete absence of consensus concerning the fat grafting technique leads to significant variety of results in the long-term viability of the fat grafted. The work of Dr. Coleman contributed to establish a specific and reliable method for the fat grafting. However, it is considered that his major contribution to the fat grafting procedure is not the technique itself, but the highlight of the importance of detailing accurately each step and the exact instruments used in the fat grafting procedure to ensure specific results. This strictness in the method enables reproducible techniques, reliable results, and studies to be comparable and understandable [2, 3].

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The diversity of processing methods is considered to play an important role in the lack of uniform results of fat grafting (the survival rate of grafted fat has been reported to range from 40 to 80%). On the last decades, many studies have been published focusing on this issue. Although much knowledge had been acquired, still no definitive consensus is found concerning the gold standard technique for the fat graft processing. When it comes to compare different processing and enrichment strategies, very little strong evidence is found based on clinical results. The lack of consensus and evidence is especially true for the enrichment strategies of the fat graft. When analyzing bibliography, it is important to highlight the difference between biological or histological variables of the fat graft and clinical in vivo relevant results for patients [4].

Fat Graft Processing

Processing includes all the techniques or procedures performed on the fat graft since it is obtained until it is injected again. Each step on the processing phase pursues the aim of obtaining the fat tissue on the best condition for each receptor area and guarantees the highest graft survival. To achieve this major goal, it is essential to eliminate contaminants from the fat tissue obtained, such as cellular debris, the tumescent solution infiltrated, free oil, and hematogenous

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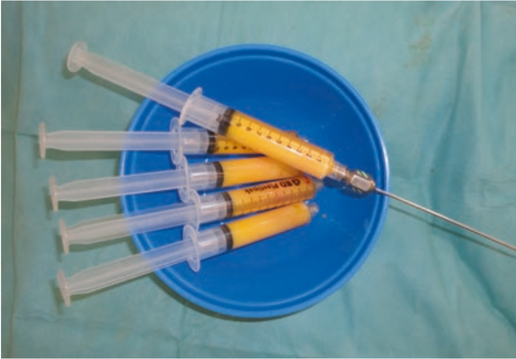

Fig. 1 Adipose tissue ready to graft after processing according to the Coleman's technique

cells, among other nonviable components. Contaminants elimination reduces the false volume of the fat graft and decreases the proinflammatory reaction that they may produce (Fig. 1).

Many studies have compared different processing methods; however some of these studies focus excessively on the cell viability with each processing technique and do not pay enough attention to the contaminant elimination and the false volume they allow. It is believed that the ideal processing technique is the one which lets to the greatest adipocytes cell viability in the fat graft with less contaminants and false volume, which may enable the greatest graft retention (in vivo clinical survival of the fat graft) [5].

Fat Graft Processing Techniques

Many different processing techniques and minor modifications of previously described techniques have been mentioned in bibliography. Some techniques incorporate a significant variability in the method and are not easily reproducible on the exact same way. Moreover some authors advocate multimodal methods, which combine different techniques. Therefore definitive consensus and evidence will persist complicated to achieve.

The different methods have been grouped under five techniques titles. The evidence found for each technique and their advantages and disadvantages will be analyzed.

Centrifugation

Centrifugation is probably the most widely used technique for fat grafting processing, and it was considered the gold standard system. Its popularity initially came from the fact it is the processing method in the Coleman's fat grafting technique, which proved reliable clinical results. Moreover, centrifugation is a precise system, with less intersurgeon variability in the method that makes it more reproducible than the other classic techniques.

According to original Coleman's technique, the tissue obtained by aspiration is placed in 10 mL Luer-Lock syringes, which are centrifuged at $1200 \times g$ (approximately 3000 rpm for a 12 cm rotor) for 3 min. Three layers are obtained: the aqueous inferior layer, the middle layer with the adipose tissue, and the small top layer of oil coming from damaged adipocytes. Only the middle layer is preserved and prepared to inject in adequate syringes for each receptor area, while the other two layers are discarded [6] (Fig. 2).

Several investigations have proved a relation between the centrifugation force and the cellular

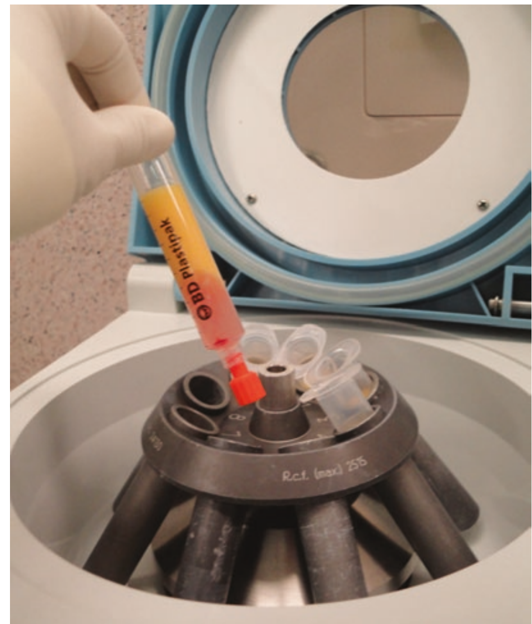

Fig. 2 After centrifugation, three layers are obtained: the aqueous inferior layer, the middle layer with the adipose tissue, and the small top layer of oil

damage. However no major evidence is found according the ideal force and time of centrifugation. It's generally accepted that forces above 1200 × *g* are not recommended. Some investigators advocate soft centrifugation and shorten the centrifugation time to 1 or 2 min since it may preserve a higher number of adipose-derived mesenchymal stem cells and cause less damage in the adipocytes [7]. It has also been suggested that other techniques, such as washing or filtration, might be superior to standard force centrifugation. Therefore, lower forces and times to standard Coleman's centrifugation might gain popularity in the next years. For those used to centrifugation at 1200 × *g* for 3 min, it is essential to take notice of the greater overcorrection produced when fat tissue is obtained with softer centrifugation, since more false volume is contained in the prepared fat graft [8].

Decantation

Decantation consists in simple separation, by difference of density, of the harvested adipose tissue in two layers: the aqueous inferior layer and the upper layer containing the fat tissue to be preserved and grafted. There is no agreement on the time that one should wait for components to separate adequately and decantation process lasts until components get visually separated. Therefore it is considered an imprecise method (Fig. 3).

Fig. 3 Decantation method showing the two layers obtained (in the left of the picture). Then, the superior fat tissue layer is frequently centrifuged in a multimodal processing technique

Decantation should not be contemplated as a single method for fat graft processing. Although it could be acceptable as an initial step in a multimodal processing technique, there is enough evidence to ensure its inferiority as a single method. Simple decantation permits a great amount of contaminants to be included in the graft that harm the fat tissue through the proinflammatory reaction they produce. It has been proved that simple decantation, as an isolated method, reduces de graft survival and causes severe cystic changes in comparison with the other techniques [9].

When used as the first step in a multimodal processing technique, decantation should be considered as a gross separation method of the aqueous component from the lipoaspiration. Therefore, decantation should only be considered as an initial handling that effectively reduces the amount of tissue to process [7].

Filtration

Filtration consists in isolation of fat tissue from the lipoaspirate by a mechanical separation. It requires a sieve or a filter that allows contaminants to pass through it but retains the fat tissue. Many different methods have been described for filtration: cotton gauze simple filtration, cotton gauze rolling, metal sieve concentration, or washing over cotton gauze or metal sieve. Moreover, there is no consensus among the amount of tissue to process depending on the surface of the sieve or the time that it has to be kept in the sieve. Thus it is considered a laborious method with a great impact of the individual manipulation of the tissue, which incorporates an artistic component in the technique. It is not a suitable method when great amount of tissue is required. When the sieve method used has not been previously validated, it is also possible that waste coming from the sieve (i.e., gauze) contaminates the fat graft obtained.

There is no sufficient data to compare the long-term survival of the fat graft obtained by filtration in relation to the other processing methods. However, it has been published equivalent or higher number of viable cells and higher percentage of isolated adipose-derived mesenchymal

stem cells when a specific filtration method (rolling on a specific referenced cotton gauze) was compared with standard centrifugation [10–12].

Some of the new commercially available technologies, like the Puregraft system, are based on the filtration technique. With these new applications, the filtration method eliminates the variability and permits greater amounts of tissue to be processed.

Washing

Washing consists in cleaning the lipoaspirate with a physiologic solution. There is no agreement on which solution is more appropriate and for how long it has to be washed. According to the washing solution, it was theorize that the lactated Ringer's solution may cause less harmful acid environment for the fat graft than normal saline solution. However there is no sufficient data supporting lactated Ringer's solution above saline 0.9% solution as the ideal washing solution. It is also important to mention that washing can be performed as a previous step to other methods, like centrifugation, in a multimodal processing technique.

It has been proved that washing effectively achieves a gross separation of contaminants from the lipoaspirate preserving adipocyte viability [10, 13, 14]. As a matter of fact, there is one study supporting superior histological viability and graft retention in an animal model of the single-step washing technique relative to centrifugation method [9]. Washing is certainly strongly recommended as a first step in a multimodal processing technique, particularly when a very hematic lipoaspirate is obtained.

New Proprietary Systems

In recent years some new commercially available technologies for fat graft processing have raised. They are semiautomatic, precise, and reproducible methods, which can process larger amounts of tissue [15, 16]. However, their price can make them inadvisable when small amount of fat graft is required. These new methods usually combine some of the previous classic processing techniques. They boast of being closed system with less probability of external contamination. Although this feature seems to be positive, it has not proved to be a major advantage, and one should not concern about processing with standard "open" systems when working in adequate sterile conditions.

These methods may be a wonderful choice for surgeons with little experience in fat grafting since they allow rigorous and constant processing, reducing the surgeon experience factor.

Some of the best-known systems are Puregraft (Cytori Therapeutics, Inc., Bridgewater, NJ), Revolve (LifeCell Corp., Bridgewater, NJ), Tissu-Trans Filtron (Shippert Medical Technologies, Inc., Centennial, CO), or Lipivage (Genesis Biosystems, Inc., Laguna Hills, CA).

Puregraft® System is probably the most popular of the new commercially available technologies. It is a closed-membrane semiautomatic filtration system, combining washing and dialyzation through a proprietary membrane technology. It favors predictable results since it is a precise method, reducing human factor variability. The membrane is contained in a sterile bag with three different accesses. The first is used to add the lipoaspirated and to obtain the filtered adipose tissue, the second permits to add the washing solution, and the third is used to separate out contaminants and part of the false volume. There is consensus supporting that the fat graft survival obtained with Puregraft® System is comparable to the standard centrifugation method. Even more, some studies support higher graft retention with this new method than with the classic techniques (Fig. 4).

Other devices, such as Tissu-Trans Filtron®, Revolve®, Lipivage®, Aquavage (MD Resources, Livermore, CA), and Lipo Collector 3 (Human Med AG, Schwerin, Germany), connect the processing method to the aspiration system, in an effort to achieve an inline course, leading to an automatic process and reducing the manipulation of the fat tissue. Similar to Puregraft® system, Tissu-Trans Filtron®, Revolve®, and Lipivage® are based on the filtration processing through a

Fig. 4 Puregraft system consists in a closed technology combining washing and filtration of the adipose tissue

membrane. In the first technology, the membrane is contained in a sterile basket (five different volume baskets are available ranging from 140 cc to 2000 cc) that is connected to the aspiration system and allows to process variable amounts of fat tissue. Revolve® system consists in a 200 micron mesh filter combined with a propeller for active washing and filtration inside a sterile canister. It allows relatively high fat volume processing in less time than other conventional methods. The filtration system of Lipivage® is contained in a sterile hand-sized syringe, which makes it preferable when smaller volumes of fat tissue are required. Although the plunger has to remain completely retracted during the aspiration, the vacuum level remains low inside the Lipivage® syringe, which reduces the traumatization of the fat tissue. Although more studies are required, Tissu-Trans Filtron®, Revolve®, and Lipivage® appear to show equivalent fat graft survival rates to the reference methods. Aquavage® and Lipo Collector 3® are based on the decantation method, easily allowing a gross separation of the fluid and the adipose tissue in the same recipient where the lipoaspirate is obtained. Lipo Collector 3® automatically starts separation of the discarded fluid when the lipoaspirate reaches a certain level in the receptacle. Similar to other previously described devices, different volume containers are available for these systems. The same limitations as previously described for the decantation method have to be considered for these new proprietary systems based on simple separation by density difference.

Above all, it is important to highlight the importance of the familiarity of the surgeon with the method he is using. The new proprietary systems have confirmed that there is not a unique reliable method for fat grafting processing and that filtration through high technological membranes or meshes may allow equivalent or even better results than standard centrifugation. Probably in the following years new rigorous evaluated technologies will appear. Therefore it is advisable that every surgeon remains faithful with the same method for enough time to guarantee his ability to self-evaluate his results. Conversely, continuous changing of the processing technique may lead to unpredictable results for the same surgeon (Fig. 5).

Fat Graft Enrichment

Enrichment is a concept that includes all strategies, different to the regular processing methods, performed with the intention of increasing the fat graft survival rate.

Some of the strategies that have been described are stem cell enrichment, PRP enrichment, vacuum-based external tissue expander system in the receptor area, and experimental enrichment with other substances with proangiogenic, anti-apoptotic, and antioxidative capacities. However, there is still no consensus supporting the systematic use of any of these strategies to enhance fat graft survival.

Fat Graft Enrichment Techniques

In recent years some researchers have focused on increasing the amount of adipose-derived mesenchymal stem cells (ASC) in the transplanted tissue. This school of thought is based on the

TECHNIQUE	DESCRIPTION	ADVANTAGES	DISADVANTAGES	MAJOR EVIDENCE
CENTRIFUGATION	- According to Coleman's technique: 1200g for 3 min - Was considered the gold standar	- Precise method	- Variable forces and times recommended by different authors	Cellular damage with strong centrifugation (above 1200g)
DECANTATION	- Simple separation by different density	- Simple - Initial step for other methods	- Imprecise	- Shouldn't be used as a single method - Inferior graft survival
FILTRATION	- Mechanical separation through a sieve - Frequently combined with washing	- Inexpensive	- Imprecise - Labor intensive - Possible waste from the gauze - Not suitable for great amount of tissue	- Equivalent to centrifugation when cellular results are analyzed - No conclusive data when comparing fat survival in vivo
WASHING	- Cleaning with physiological solution - Frequently combined with filtration	- Simple and inexpensive - Initial step for other methods	- Imprecise	- No conclusive data when comparing fat survival in vivo
NEW TECHNLOGIES	- **Puregraft:** Washing + Filtration - **Revolve:** Washing + gentle shake - **Tissue-Trans Filtron:** Inine filtration method - **Lipivage:** Inline filtration method	- Closed systems - Semiautomatic and precise systems - Ideal for great amount of tissue	- Price - Inadequate for small amounts	- Promising results in biological and histological results - Few evidence in vivo studies

Fig. 5 Summary comparison of the different processing methods grouped under five technique titles

demonstration that ASC are highly proliferative, can promote angiogenesis, and have antiapoptotic and antioxidative proprieties that could increase the fat graft survival rate [17]. The adipose-derived mesenchymal stem cells are found in the stromal vascular fraction (SVF); therefore, the first step for stem cell enrichment is isolating the SVF of the aspirated adipose tissue. SVF not only contains mesenchymal progenitor cells, but also other cell populations such as preadipocytes, endothelial cells, pericytes, T cells, and macrophages.

Different commercially available methods have been described to achieve isolation of the SVF from the lipoaspirate. However, still no standard protocol or method has obtained agreement among researchers. Some of the methods that have been compared in bibliography are the manual processing MultiStation (PNC International, Gyeonggi-do, Republic of Korea); the semiautomated Cha-Station (CHA Biotech, Kang-namgu, Republic of Korea); the closed, manual processing Lipokit with MaxStem (Medi-Khan, West Hollywood, CA); and the closed, fully automated Celution 800/CRS System (Cytori). According to the biological results obtained, the Celution® System reaches the highest amount of adipose-derived stem progenitor cells compared with other methods [18]. However there is no study comparing in vivo fat graft retention with the different SVF isolation methods.

The Celution® System is a reliable and reproducible method to isolate ASC from the lipoaspirate. It achieves ASC isolation and concentration by proteolytic digestion with a proprietary enzyme reagent specifically optimized for separation of cells from aspirated adipose tissue. The entire process since the lipoaspirated is introduced until the ASC are isolated approximately lasts 1.5 h (Fig. 6).

ASC-enriched fat graft has proved to achieve graft retention with no side effects reported until date. When comparing the survival of ASC-enriched fat graft with regular processed fat tissue, some studies are showing higher graft retention with the enrichment method. Even more, the quality of the graft has reported to be superior with the enriched fat graft. The capillary density of the transplanted adipose tissue has

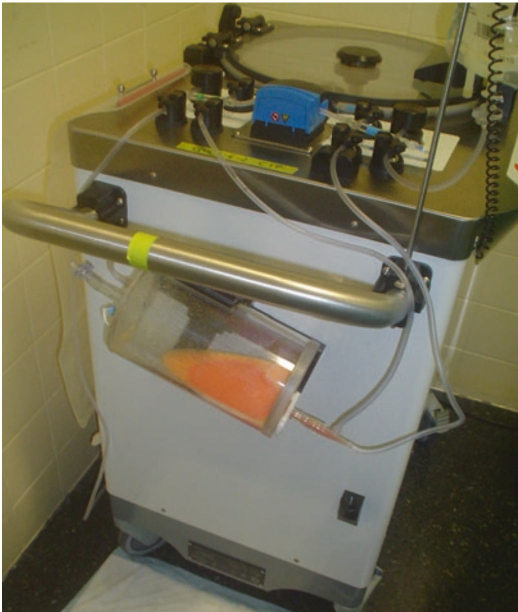

Fig. 6 Celution® 800/CRS System

been found to be higher in enriched grafts, which is thought to come from the ASC capacity to promote angiogenesis. However, the proportion of fat graft retention achieved with ASC-enriched adipose tissue in comparison with regular processed grafts remains uncertain and variable between investigators [19–22]. Moreover, other studies are not showing statistical differences when comparing the graft survival of ASC-enriched adipose tissue and the regular processed one [23]. In the same way, the cost of the SVF isolation methods remains a major issue when analyzing the disadvantages of this enrichment strategy.

In conclusion, there is no enough data until date, to reach agreement about the role of ASC enrichment in fat grafting. However, studies are showing promising results that may turn this strategy into a great clinical tool in the future.

Another studied strategy for fat graft enrichment consists in platelet-rich plasma (PRP), also called plasma rich in growth factors (PRGF), addition to the prepared adipose tissue. PRP contains a reservoir of growth factors that promote cellular regeneration and repair. Based on this capacity, PRP was thought to facilitate fat graft survival.

Platelet-rich plasma contains a concentration of autologous human platelets three to five times higher than baseline platelet count. This type of cells incorporate granules containing the nine main growth factors known to be actively secreted in the wound-healing process. PRP also contains other significant substances that act as a scaffold for cellular processes or are necessary for cell adhesion (Fig. 7).

To obtain the PRP that is added to the fat graft, a small volume of peripheral vein blood is needed. PRP will represent approximately 10% of the volume of blood obtained. The blood is procured in a tube containing sodium citrate, a substance with anticoagulant capacity. After centrifugation, 90% of platelets are obtained in the upper layer. Specifically the lower portion of the upper layer contains the higher number of platelets and is the PRP or PRGF.

In vitro and animal model studies have shown PRP stimulate angiogenesis and proliferation of adipose-derived stem cells [24]. However, when it comes to clinical assays no conclusive data can be obtained. No side effects have been reported until date. Thereby PRP enrichment of the fat graft is considered a safe procedure. According to fat graft survival, some studies are showing better retention with PRP enrichment [25–27], while others are showing no statistical differences with the regular fat graft [28, 29]. Therefore, it is not yet stated that PRP enrichment of the adipose tissue offers any improvement to the conventional

Growth factors	Transforming growth factor	TGF-ß1
		TGF-ß2
	Platelet-derived growth factor	PDGF-AA
		PDGF-AB
		PDGF-BB
	Vascular endothelial growth factor	VEGF A
		VEGF C
	Insulin growth factor	IGF-1
	Epidermal growth factor	EGF
Other substances		Fibronectin
		Vitronectin
		Fibrinogen
		Osteocalcin
		Osteonectin

Fig. 7 The nine growth factors and other substances contained in PRP

technique. More clinical studies are needed to determine the role of PRP enrichment in fat grafting.

The perioperative use of a vacuum-based external tissue expander system (i.e., BRAVA tissue expansion system) should be also considered an enrichment method although it is not applied to the fat graft but on the receptor area. This method is known to permit greater fat grafting volumes in the surgical procedure since it expands the scaffold that will harbor the adipose tissue [30]. However it is not clear to enhance the survival of the grafted fat. Although the negative pressure could stimulate vascularization of the receptor area and promote better bedding for the adipose tissue, the retention of the graft has not been reported to be superior. Other disadvantages that have to be taken into account are the inconvenience caused to the patient, the price of the device, and possible skin damages caused by the negative pressure when the system is not correctly applied.

Other enrichment strategies have been described and tested in vitro or in animal models. Most of them are substances that have been experimentally added to the fat graft. Some of them are insulin, beta-blockers, growth media, and N-acetyl cysteine. Also the exposure of the adipose tissue to hyperbaric oxygen has been experimentally tested. These strategies have their theoretical basis in the proangiogenic, adipogenic, antiapoptotic, or antioxidative capacities that they promote, hypothetically enhancing the graft survival rate.

Insulin in vitro cell culture has shown to promote the proliferation and differentiation of preadipocytes to mature adipocytes. In adipose tissue enriched with insulin, fat shows certain hypertrophy that may be attributed to induction of acetyl-CoA carboxylase enzyme by insulin. In addition to the adipogenic activity, insulin can promote the proliferation of vascular endothelial cells and boost microvessel formation, enhancing the revascularization of the grafted adipose tissue. However, the long-term effect of insulin enrichment on the survival rate of the fat graft in the clinical setting remains controversial.

Selective beta-1-blocker can inhibit adenylate cyclase in fat cell membranes, which may prevent lipolysis, and block cyclic-AMP that can enhance adipogenic activity. Very promising results in the fat graft survival rate were obtained in rat models. However, similar to insulin enrichment, there is no evidence obtained up to date in terms of significant clinical outcomes that supports a prevalent use of this enrichment method.

N-acetyl cysteine is a harmless and widely available antioxidant. It has been recently tested as an addition substance to the tumescent solution in rat models, showing an increase in the adipose-derived stem cell proliferation and improved graft retention at 3 months. These findings provide proof of principle for the addition of this substance to the tumescent solution that will need to be studied in the clinical setting.

In conclusion, wide clinical validation has not been achieved for any of these mentioned enrichment methods. Therefore these strategies should not be performed routinely in patients, except in the case of clinical trials under a validated protocol. Certainly, more clinical assays are needed to achieve broad evidence about any of these promising strategies.

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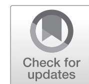

Variants of Fat Grafting: From Structural Fat Grafting to Microfat, Sharp-Needle Intradermal Fat (SNIF), Nanofat, Emulsion, SNIE, FAMI, and SEEFI

José M. Serra-Mestre and José M. Serra-Renom

The wide acceptance of fat grafting in recent years has ushered in a large number of new clinical applications. Specifically in the facial region, in addition to its standard role in correcting volume loss in facial fat compartments [1–4], fat grafting has aroused a great deal of interest as a possible regenerator of tissues and as a means of improving skin quality [5–7].

The expansion of fat grafting and its applications in areas of the face such as the eyelids, where the skin is very thin and where any irregularities are likely to be visible or palpable, has sparked interest in the development and study of new ways of fat processing. Today, ever thinner and smaller grafts are being obtained and injected, without compromising their cellular viability [8–11].

Traditionally, fat grafting has achieved good results as a filler. However, it has not become widely accepted in finer contouring, where conventional fillers such as hyaluronic acid continue to be preferred. At our practice, we initially performed fat injections only via cannulas, and in cases of marked folds and a deep, long-standing central wrinkle, we found that the wrinkle reappeared even though the surgery had been successful. In these cases, attempts at overfilling did not produce satisfactory results. Much the same occurred with facial fine lines. Today, the development of needle injection techniques [7, 12–14] has allowed surgeons not only to be more precise but also to perform the injections in a more superficial plane and thus to use fat grafts as fine fillers.

Thanks to improvements in our understanding of the composition of the grafts that are being injected, new types of grafts have been proposed which aim not to add volume but to improve skin quality.

Given the growth in the number of techniques now available for fat injection, in this chapter, we describe the methods for obtaining, preparing, and injecting these grafts and outline their main indications.

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Fat Grafting: Types and Preparation

Microfat Grafting

Unlike Coleman's structural fat grafting [15] and similar variants which used a 2–3-mm diameter suction cannula, the fat is obtained through low-pressure liposuction (0.5 atm) with a 2.4-mm microport harvester cannulas with barbed and beveled 1-mm ports (Tulip Medical Products, San Diego, California, USA).

Once obtained, the fat needs to be isolated from the blood, debris, water, the components of the solution used for the tumescence, and the oil

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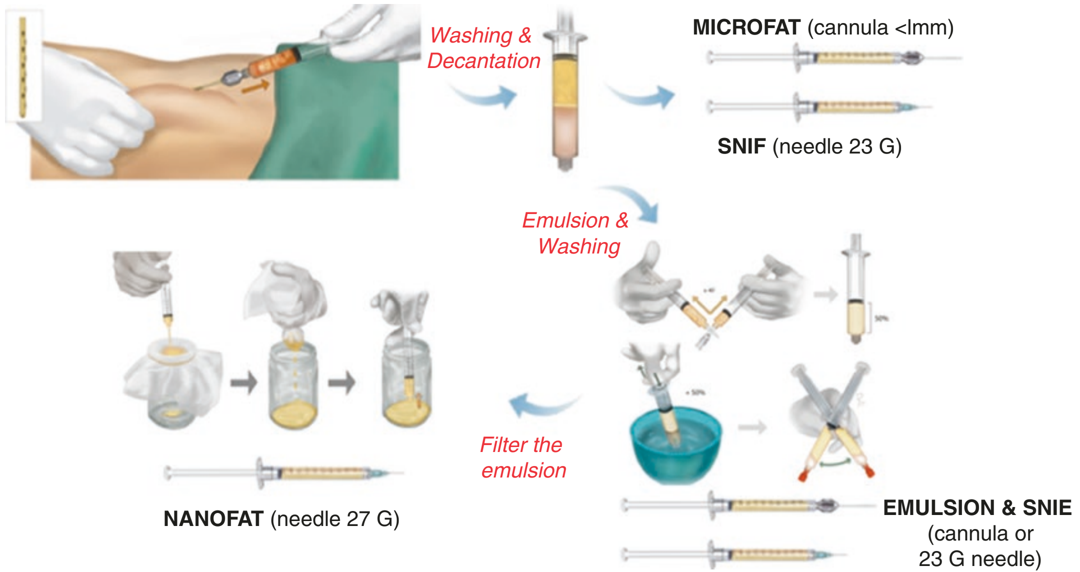

Fig. 1 Preparation of the main variants of fat grafting: microfat grafting, sharp-needle intradermal fat grafting (SNIF), emulsion, sharp-needle intradermal emulsion (SNIE), and nanofat grafting

resulting from the breakdown of the fatty acids during aspiration. To do this, either centrifugation, decantation, or washing can be used. In the case of injection via a cannula, any of these three methods is appropriate; however, if part of the fat is to be injected via a needle, it is advisable not to use centrifugation since the fat obtained is more compacted and can easily obstruct the needle.

Microfat is injected via 0.7–0.9-mm cannulas (always less than 1 mm in diameter) into the subcutaneous plane [8–11], in contrast to structural fat grafting (Fig. 1).

Sharp-Needle Intradermal Fat (SNIF)

Needle injection has been carried out for a number of years and several authors have already described its use [12]. However, it was Tonnard's group [13] who coined the term sharp-needle intradermal fat (SNIF) for the injection of microfat via a needle.

With SNIF, the surgeon can work in either a superficial subdermal plane or a deep intradermal plane using 23-gauge sharp needles. This technique minimizes the appearance of fine lines and wrinkles and allows greater precision.

Emulsion and Sharp-Needle Intradermal Emulsion (SNIE)

After obtaining the microfat, a mechanical emulsion is created by passing the fat 30 times between two 10-cc syringes connected by a Luer-Lock connector. As the emulsion is created, it acquires a lighter yellowish color. It is then decanted and washed with saline solution before being transferred to 1-cc syringes for injection [16].

A recent study [17] of the mechanical procedure of shuffling lipoaspirated fat found that it does not alter the tissue viability or its microscopic structure, nor does it affect the stromal vascular fraction (SVF).

The injection can be performed with cannulas, but also with 27-gauge needles (SNIE) in cases of fine lines, injecting the fat in a superficial plane.

Nanofat Grafting

Nanofat grafting was recently described by Tonnard et al. [6], not for adding volume but for injecting SVF cells. Although the evidence is very limited, Tonnard et al. saw that by creating a

mechanical emulsion of a sample of a microfat graft and filtering it through a nylon membrane, they were able to preserve a significant proportion of the stromal vascular component with the same proliferation and differentiation capacity of the stem cells and without any viable adipocytes.

Unlike the commonly accepted methods for obtaining SVF cells such as separation by the use of collagenases or other digestive enzymes, mechanical emulsion can be created by transferring the fat 30 times through two syringes and filtering the emulsion through non-absorbent nylon membranes in order to separate the cells from the remnants of connective tissue.

The nanofat is injected with 27-gauge needles in a superficial dermal plane in order to improve skin quality. After the injection, the color of the skin becomes a little lighter.

Superficial Enhanced Fluid Fat Injection (SEFFI)

Superficial enhanced fluid fat is obtained by liposuction using a 2-mm diameter cannula with a side-port size of 0.5–0.8 mm. After rinsing and centrifugation for 1 min at 2000 rpm, the fat is enhanced with platelet-rich plasma (PRP). The concentrated PRP is mixed with the fat to obtain a final concentration of 10% of the total fat harvested [7, 14].

Superficial enhanced fluid fat is usually injected using 20–23-gauge needles in a superficial plane and is useful for treating the periorbital area and the lips, among other sites.

Fat Autograft Muscle Injection (FAMI)

FAMI [18, 19] differs from the above techniques mainly in terms of the site where the fat tissue is deposited: directly inside the muscles and beneath the periosteum, via the injection of 1–3 cc of fat in a retrograde fashion from the muscular insertion to its origin. Engrafting the muscles of facial expression may improve graft retention and therefore its predictability and symmetry.

FAMI is harvested via syringe aspiration, refined with centrifugation, and injected with specific curved cannulas to the muscles of facial expression.

Clinical Applications: Our Approach to Facial Rejuvenation

One of the clearest indications for these fat grafting techniques is the restoration of volume in specific sites as a complement to classical facial rejuvenation surgeries such as blepharoplasty and face lift.

Practically all elderly patients present a negative facial vector [1–3], with loss of volume in the malar region. To restore the facial projection and contour in this area, we inject microfat grafting through two entry points—one in the proximal third of the zygomatic arch and the other at the height of the nasolabial groove—dividing the fat compartments into “deep malar,” “deep medial malar,” and “high lateral deep malar.” A more superficial injection is also performed until the desired volume and contour are achieved (Fig. 2a–d).

Later, in order to obtain a more natural brow-palpebral-malar transition, we usually complement the blepharoplasty with a volumetric rejuvenation of the periorbital rim in an attempt to create a supportive frame for the periorbita [16]. Above the upper eyelid, fat is injected in the glabellar region and in the superomedial angle of the orbital rim, followed by an enhancement of the tail of the eyebrow from the midpupillary line in order to avoid damage to the supraorbital neurovascular bundle. The microfat adds volume, and great care is taken to perform the injection above the orbital rim.

In the lateral part of the rim, we inject variable amounts of microfat and try to improve the static periorbital wrinkles using SNIE. In the lower eyelid, both the tear trough and the tear valley are injected with emulsion via a cannula. Microfat is also injected in the brow-palpebral-malar transition. This area frequently presents irregularities, and so great care is needed to use the correct quantity and to ensure a homogeneous distribution of the grafts.

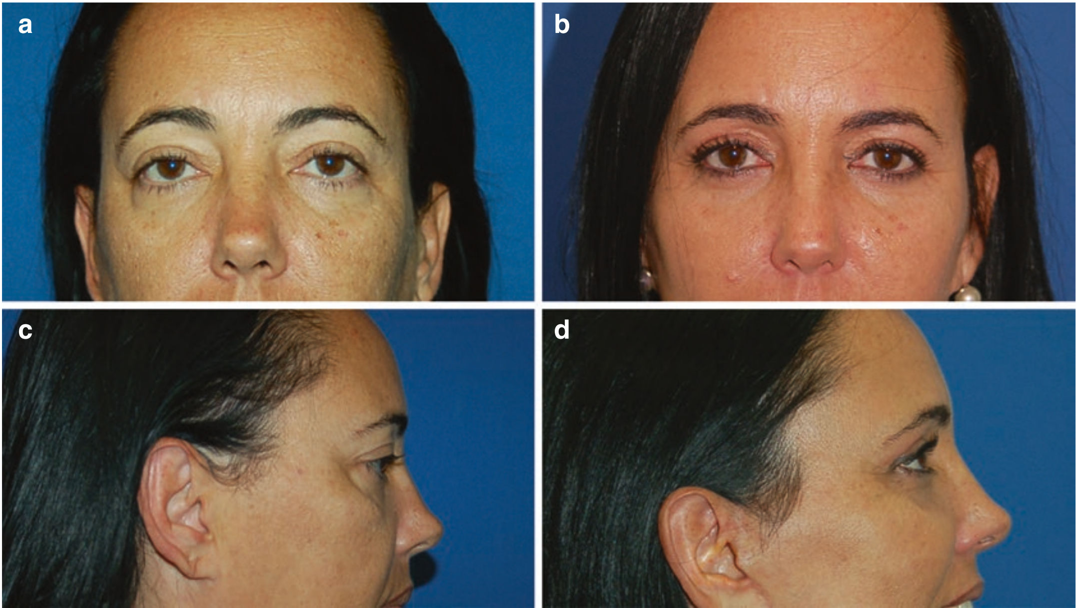

Fig. 2 Clinical case of a minimally invasive facelift, blepharoplasty and lipofilling. Fat grafting with microfat was performed in the malar region, the eyebrow, and the temporal region. Emulsion was used in the lower eyelid, in the tear trough, and for correction of crow's feet and the outer edge of the orbital rim. Also a mesotherapy with emulsion and PRP was performed to improve skin quality. (**a**) Preoperative frontal view; (**b**) final result, frontal view; (**c**) preoperative lateral view; and (**d**) final result, lateral view

In the lower facial third [16], both the nasolabial and the melomental grooves can benefit from a deep injection of microfat. If the central wrinkle persists, we perform SNIF injections perpendicularly to the wrinkle in a superficial subdermal plane over a distance of approximately 1 cm in each injection. The remaining volumetric applications in this area, such as chin augmentation in cases of rhinoplasty or homogenization of the mandibular ridge, are performed with microfat grafting in order to restore the lost volume.

In older, poor quality skin, we may also perform mesoplasties with emulsion or nanofat [6] throughout the facial region. Nanofat can also be useful in patients who have dark circles around the eyes or in the tear trough. When nanofat is injected in the periorbital area, the surgeon should warn the patient that the bruising may remain for between 3 and 4 weeks.

After the treatment, the area is washed with alcohol and an antibiotic cream and cold packs are applied for the first 2 h. All patients are seen the day after the intervention and weekly during the first month, and then at three-, six-, and twelve-month intervals. At 6 months the need for further treatment is assessed.

Summary

The development of new ways to process and inject fat, reducing the graft size and performing the injection via needles, has given rise to a range of new applications for fat grafting, above all in the facial region. Combinations of deep and superficial injections of autologous fat grafts are an efficient complement to classical surgical techniques for achieving the most natural results. To a large extent they resolve volumetric deficits when working at deep levels and can also be used for finer remodeling.

Further research is needed to assess the impact on clinical practice of these new methods for processing fat grafts as well as to compare their efficacy with respect to conventional fillers.

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Fat Graft Application

Mauricio Raigosa and Tai-Sik Yoon

Introduction

Autologous fat grafting has become a great tool in all fields of plastic surgery. Fat grafting involves transfer of nonvascularized but viable fat cells from one location to another within the same individual. Although fat grafting is generally successful in many circumstances, the results can be unpredictable in terms of volume maintenance. It does transfer fat and stroma in a single setting, although it usually requires multiple stages to achieve satisfactory clinical results, especially when larger contour defects are treated.

Since Coleman formalized the technique in 1997 [1], many authors have focused their effort to improve graft viability in terms of processing of the harvested fat and graft transfer or injection technique [2, 3].

Autologous fat grafting possesses many of the most ideal properties desirable to work as a filler throughout the body. Unfortunately, transplanted graft survival can be highly inconsistent [1, 4, 5]. Atraumatic harvesting, handling, and transfer are key points to maximize fat cell viability during fat grafting. However, the most decisive step in the success of fat grafting procedure is the last step: the injection of the fat once processed into the target area.

As many efforts have been focused on harvested fat processing in order to improve graft survival, little evidence exists about the role of fat injection.

Instruments and Materials

Instruments for fat grafting must be efficient and cause minimal trauma to the grafted tissue during injection.

Cannulas

A blunt 17-gauge cannula with one distal aperture just proximal to the tip is the most commonly used cannula for fat injection in our practice. The injection cannulas vary in length and shape. The most useful lengths are from 7 to 9 cm for facial procedures and from 9 to 15 for body contouring procedures. Cannula tips also come in various sizes and shapes for individualized treatment (Fig. 1). The proximal end of the cannula has a hub that is connected to a Luer-Lock syringe.

Coleman [6] developed three different types of blunt-tip cannulas:

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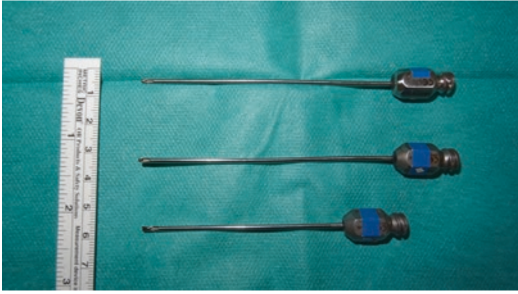

Fig. 1 17-gauge injection cannulas developed by Coleman, type I completely capped (above), type II partially capped, and type III with flat end

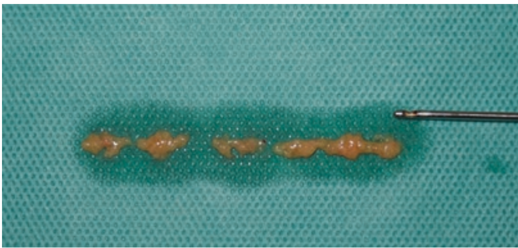

Fig. 2 Small aliquots of fat graft are delivered through the injection cannula to maximize graft contact with surrounding tissues and its survival

- Type I is completely capped on the tip with a lip that extends 180° over the distal aperture.
- Type II is similar to type I but it is not completely capped and has a lip that extends over the distal aperture only about 130°–150°.
- Type III is flat at the end to allow dissection of the tissues in specific situations. It makes easier to push through scars or fibrotic tissues.

Coleman introduced the principle of "microdroplets" [6] (Fig. 2). It is generally accepted nowadays since Carpaneda [7] that a "tiny" fat graft surrounded by vascularized tissue enhances graft survival through revascularization. Mashiko and Yoshimura [8] recommended the diameter of fat graft particles to be as small as 2 mm. Khouri developed the concept of "microribbons" [9] as small units of fat that can survive acute transfer to subcutaneous plane. According to this theory, if we set a conservative limit of 0.1 cm^2 for the area of the base of a cylinder, the maximum volume of fat delivered by a 10-cm-long injection should be 1 cc. Khouri et al. stated fat injections larger than 0.16 cm in radius will have an area of central necrosis through a mathematical model [10]. Del Vecchio and Rohrich stressed the importance of placing fat within 2 mm of an arterial supply to survive, but fat placed beyond that distance will undergo necrosis [2]. We believe following the microdroplet principle is mandatory to achieve a significant fat graft survival rate, and thus a good clinical result. In our opinion, one condition necessary to guarantee microdroplet principle is that grafted fat tissue must flow easily through the injection cannula without clogging to avoid "huge" fat lobules deposits. To do so, we agree with Del Vecchio and Rohrich that hole size of the injection cannula matters and should match closely the hole size of the aspiration cannula [2] (Fig. 3).

Although shear stress has proven to be harmful to fat [11, 12] and flow rates on the order of 0.5–1 cc of fat graft/s have been recommended to optimize fat viability during injection [12], no significant differences have been reported on following injection with 14-, 16-, and 20-gauge needles [13, 14]. Technique described by Coleman uses a 17-gauge cannula (1.20 mm external diameter) but does not enable direct reinjection at a subdermal level nor in inextensible tissue such as fibrotic dermis. The smallest transfer cannula used in the Coleman procedure is a 22-gauge blunt cannula [6], which clogs very often during reinjection. Nguyen et al. presented micro-fat injection with a 20-, 23-, and 25-gauge cannula after harvesting fat with a multiperfo-

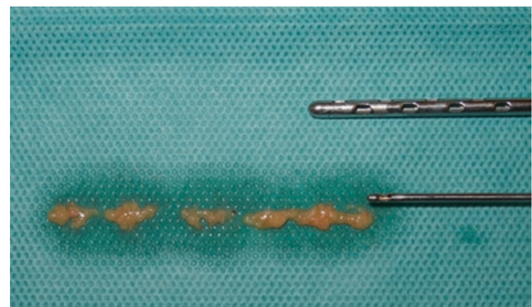

Fig. 3 Size of the holes of liposuction cannula should be very similar to the size of the hole of injection cannula to provide smooth delivery of fat avoiding clogging and bolus injection

rated cannula with holes of 1 mm in diameter [15]. In our daily practice, 17-gauge cannula is used. We strongly believe this cannula caliber works really well in a great variety of receptor tissues like breast, buttock, and even face. We agree when dealing with periorbital fat grafting, especially upper eyelid and lower eyelid tear-trough deformity, a thinner cannula is advisable to avoid complications [16].

Smith suggests using cannulas to inject the fat is less harmful to fat than using needles [17]. For some authors, blunt-tip cannulas cannot be manipulated easily inside the tissue, and the cannula must be pushed with high pressure in order to reach a target point [18]. On the other hand, sharper-tipped cannulas have higher risk of penetrating blood vessels causing vascular damage and hematoma, being described blindness due to retinal artery injury or embolism, stroke, and skin necrosis [19]. Yazar et al. presented a pointed-tipped cannulas, which are blunted to a certain degree that can be applied easily through the tissues avoiding such complications [18]. Based on our experience, blunt-tip cannulas are our first choice. They are safe, with low risk of blood vessel penetration and vascular damage. A severe hematoma during postop, even when dealing with highly vascularized areas such as the face, is extremely rare. It is true that blunt-tip cannula manipulation can be sometimes difficult inside the tissue, especially in irradiated or scar tissues. But with the help of the opposite hand bounding the tip and exerting a controlled pressure on the cannula, this problem can be overcome easily, avoiding sharp tips.

When fat grafting is indicated to treat scared or fibrotic tissues, these should be released prior to fat injection. "Rigottomy" [20, 21] or three-dimensional ligamentous band release consists in "meshing" the scar tissue using a needle or a pointed or even a blunt cannula. Meshing the scar makes it spread. Fat grafting after the release acts as a filler and a spacer, filling the gaps in the "mesh" avoiding tissue collapse and further scarring [9, 10].

Syringes

Basically two types of syringes are used in our clinical practice. We use 1 cc syringes when applying fat grafting in the face and 10 cc syringes for the rest of the body contouring (Fig. 4).

Fat grafting in the face needs to be very precise and for that reason extreme control over the amount of fat that is delivered is mandatory. To do so, 1 cc syringes offer the best control and the exact amount of desired volume can be delivered [1, 16]. When facing body contouring, we still have to be precise but higher fat volume is delivered. For that reason 10 cc syringes are preferred. However, for those starting in fat grafting tech-

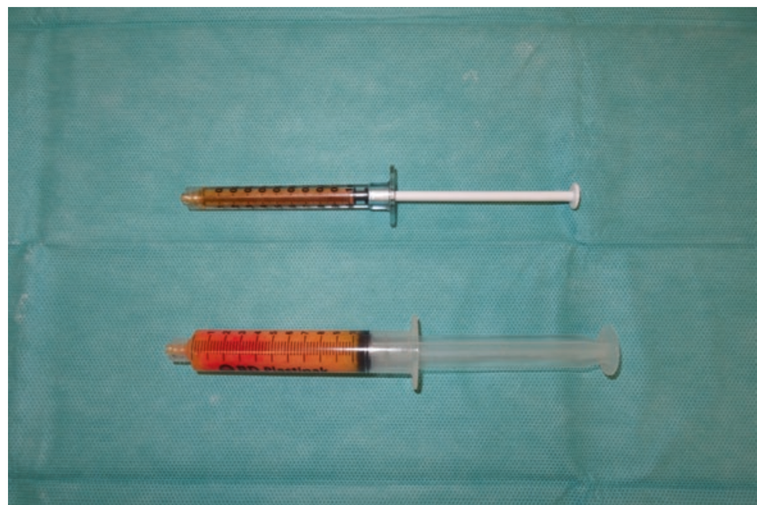

Fig. 4 1 cc syringes with Luer-Lock hub are used in face contouring and 10 cc syringes with Luer-Lock hub are used in body contouring

niques, even out of the facial area, we recommend to start with lower volume syringes (3 or 5 cc) until they become familiar with the technique.

All syringes must have a Luer-Lock hub at the distal end to be connected to different type of cannulas. This kind of tight connection is very convenient to avoid leaks and sudden cannula unplugging.

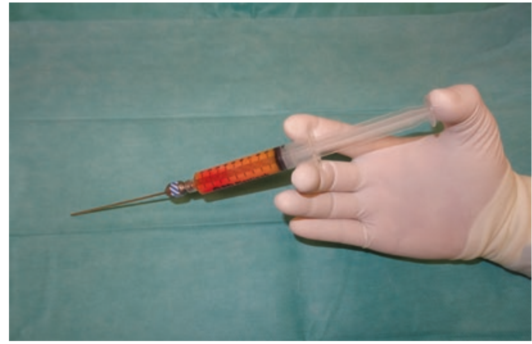

Fig. 6 Holding the end of the plunger with the thumb, we lose fine control over speed of fat delivery through the syringe

Injection Technique

Incisions

Small stab incisions are made in the skin at the previously designed entering points with a 16-gauge needle. These incisions are so small that do not need to be sutured and become inconspicuous after healing takes place.

Holding the Syringe

There are different ways of holding the syringe [16]. If the end of the plunger is held with the thumb, we lose fine control over speed of fat delivery through the syringe, but if the plunger is held with the palm, the grade of control over the injection speed increases greatly (Figs. 5 and 6). Not only the speed but holding the syringe in that way gives more control over the direction of the cannula toward the target area and the plane in which the surgeon wants to inject.

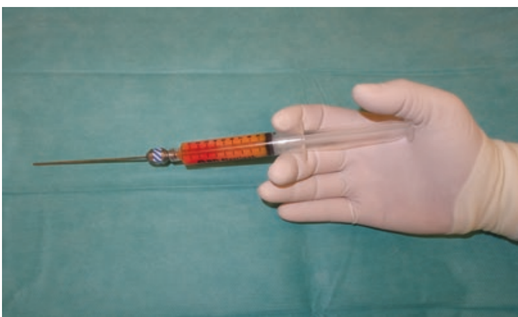

Fig. 5 Holding the end of the plunger with the palm provides greater control over the injection speed and the volume you want to inject and at the same time allows better control over the direction and the plane you want to inject in

Cannula Movements

There are currently two established methods of fat grafting: mapping technique and the reverse liposuction technique [9, 10, 16, 22]. Reverse liposuction refers to a constant motion of the injection cannula back-and-forth while fat is being injected. Generally, soft pliable non-scarred tissues without underlying implants can be treated more efficiently with reverse liposuction technique. In the mapping technique, fat is injected during axial withdrawal of the cannula only in a retrograde manner. This technique is preferred when grafting scarred, or irradiated beds, or over implants, when a more precise and cautious fat deposition is required.

When performing fat grafting, it is important to place fat in a fan-shape mode from a given injecting point and with a crosshatched pattern using long radial passes from multiple entering points. This helps to avoid placing an excess of fat in a single place or line [9, 10, 16].

Plane of Placement

When injecting fat into a target area, we should keep in mind the principle that the key to successful application of adipose tissue is to maximize

the contact area between the grafts and the recipient vascularized tissue [9]. The grafts should be small enough to increase this contact area while maintaining the original architecture of the fat. According to that principle, fat should be placed with multiple passes developing a single layer of fat and avoiding bolus injection. Each injection will be made into a new tunnel, creating multiple levels in a three-dimensional manner. Usually the fat graft is placed just under the dermis, but all available vascularized tissues should be grafted in order to gain volume. If a bolus injection occurs, it can be flatten with digital manipulation, but this can lead to fat necrosis so the best is to avoid bolus injection.

Speed of Placement

How fast we inject and how much pressure we apply onto the syringe are important issues. Advancement and withdrawal of the injection cannula is made slowly by beginners or when dealing delicate areas (periorbital, periprosthesis, etc.). When experience is gained, these movements are made quicker and steady. According to Marten [16], with rapid and constant movement, intravascular injury is less likely to happen and fat is infiltrated in a more uniform manner. The pressure over the plunger should be gentle and constant, which will indicate the fat delivery is homogeneous and uniform. If higher pressure is needed, the cannula may be blocked. In that case it is better to remove and check the cannula. Exerting higher pressure will end up in bolus injection.

Assisted Manual Injection

Manual injection is the most popular injection technique among surgeons performing fat grafting. As mentioned before, a learning curve is necessary to deliver the grafts uniformly and in tiny droplets in order to maximize graft survival.

Several devices are available in the market in order to guarantee the procedure is performed in the correct way and make it easier.

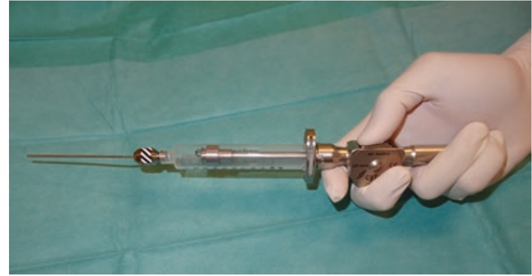

Fig. 7 Stainless steel device for precise delivery of microdroplets. A simple brush of the thumb permits accurate control over the content of the syringe, delivering a small volume of fat with every movement

- Lipografter TM [23]. It is a sterile, single-use disposable kit that is used in harvesting, and transferring of autologous fat. It provides minimal manipulation of the fat, and its patented atraumatic tissue valve allows for harvesting, processing, and reinjection in a closed system. The graft is delivered using a 1 cc syringe.
- Celbrush TM [24]. It is a stainless steel device for precise delivery of microdroplets. A simple brush of the thumb permits accurate control over the content of the syringe, delivering a small volume of fat with every movement. The "10 mL Celbrush is designed to deliver approximately 0.50 mL of tissue for each full brush of the operator's thumb" (Fig. 7). Another advantage of this system is that minimizes clogging and overfilling.

Volume of Graft

When to finish injection is a question all beginners ask when performing this technique. It is important to feel some resistance during advancement of the cannula [16]. It indicates the new tunnel is surrounded by non-touched tissue, and the fat infiltrated will have maximum exposure to vascularized tissue. Once you feel large open space with each pass, probably it is good to stop. Sometimes, especially for beginners, it is difficult to decide when to stop injecting. Overcorrection, especially in face contouring, must be avoided. Blanching or stiffness of the treated areas due to high pressure after injection must be avoided as

stated by Khouri [9, 10]. We recommend injecting until contour deformity is corrected (completely or partially) keeping the injected tissue soft and turgid.

Complications

The two most significant complications are intravascular injection [17, 19] and overgrafting [1, 4, 5]. Fortunately, these phenomena are very rare. Using blunt cannula, low-pressure injection, moving constantly the cannula, and placing epinephrine to achieve vessel contraction, vascular injury can be avoided.

On the contrary, overgrafting is becoming an increasing problem due to large volume injection, as practitioners become more familiar and confident with fat grafting techniques. This is seen more frequently in younger patients who have had superficially placed injections. Unfortunately, weight gain causes all fat grafts to enlarge, which, in the face, can result in significant contour distortion. The treatment of overgrafting requires microliposuction with only limited improvements and risks of excessive scar formation. Therefore, even for experienced surgeons, it is recommended to use small to moderate amount of grafts in the face with minimal overfilling.

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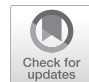
Part IV

Regenerative Medicine Procedures for Aesthetic Physicians—State of the Art: Fat Transfer, Lipo-Filling



Lipofilling in Reconstructive Surgery: Indications, Outcomes, and Complications

Joan Fontdevila

Lipofilling has different potential applications both in aesthetic surgery and in procedures intended to treat pathologies [1]. Indications are increasing every day since fat grafting has been recognized as a useful and reliable technique and has become a technique used by many specialties in many different areas and pathologies. Fat grafting can work by two different ways: by volume addition (e.g., breast volume restoration in breast reconstruction) or by side regenerative phenomena (e.g., scar release in retracted breast lumpectomy scars).

As volumizer, lipofilling has an unlimited potential, only conditioned by the availability of enough fatty tissue in the donor areas, and an appropriate strategy of procedures sequencing to assure the maximal tissue uptake and the minimal lost in form of reabsorption or necrosis. This volumizing effect is usually used in spaces between the skin and the underneath bone structures, and in some anatomical spaces amenable to be grafted by the common fat grafting techniques, as the vocal cord, or the spaces surrounding the vagina.

As a regenerative agent, the fat graft can release the fibrosis of any scar, can improve the tissue elasticity, increase the vascularization, and induce the reversal of the aging and toxic agents damage to the tissues [2].

Indications can expand in the future if the technology provides solutions to improve the ability of the graft to overcome ischemia stress and the lack of nutrients, and if we can achieve the best conditions to integrate the graft in the receiving tissue. Furthermore, improvements and new technology in regenerative cells procurement, growth factors, and biological scaffolds can contribute to achieve these goals [3].

We have divided the indications in treatments by the kind of pathology. In some indications, we will suggest the volume to use, but as was exposed in the previous chapter about injection technique, this is the most experience dependent technical tip. Nevertheless, the area where we are going to apply the grafts has more features to control before deciding the volume, as the skin thickness, the age of the patient, the skin laxity, asymmetries, previous treatments with permanent or resorbable materials, and many others. Our advice is to be cautious with the former patients, avoiding overcorrection, and visit experienced surgeons to see how they decide the volume to use in each case.

Lipofilling is a relatively simple technique with a low complication rate if performed correctly. The surgeon should keep in mind that the first is do no harm, so the first common step of every lipofilling procedure, the harvesting of fat,

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must be performed with accuracy to avoid sequels at this level. Since fat transference techniques have increased its popularity in recent times, infrequent complications have been seen in clinical practice, and these will be discussed for every area.

Fibrosis, Scars, and Radiodermatitis

Any area with fibrosis or a scar is suitable to be treated by means of fat grafting, especially those retracted or depressed, improving the contour, hardness, and color, and also providing improvement of the symptoms as pain or itching [4, 5].

The graft can be of help to release the fibrosis, which is anchoring the scar to the deep planes, by means of its antifibrotic properties and also by the tunneling of the scar. The tunnels will be stuffed with the fatty tissue, providing foundation to the scar to sit on, and limiting the following fibrosis and relapse of the retraction (Fig. 1).

Scars from burns can also benefit of this treatment, smoothing and softening the burned area, decreasing inflammation, alleviating the pain, and improving the function [6].

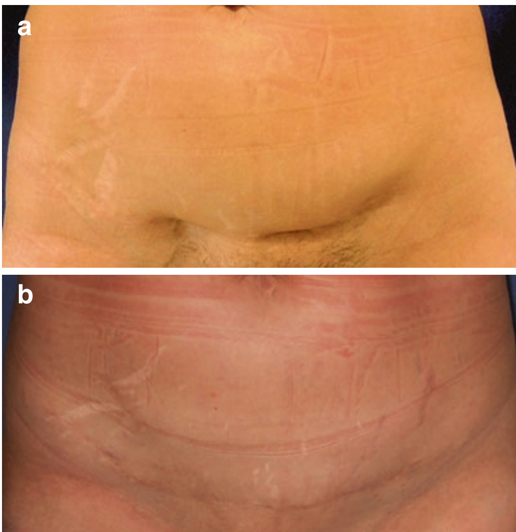

Fig. 1 (**a**) Suprapubic incision retracted and adherent after necrotizing infection. (**b**) After two procedures of fat grafting without any new incision, the scar now is flat and non-adherent

For a successful technique, tunneling and subcision of the scar is mandatory. Sharp cannulas or beveled needles can be used to ease this work, but bleeding, swelling, and posterior bruising are higher than using a blunt cannula. Multiple punctures on the skin have been proposed as a method to release the fibrosis before the graft placement. We do not recommend the use of sharp instruments neither multiple punctures of the skin because the same outcomes can be achieved by means of a 16G spatulate blunt cannula, with less trauma to the tissue and without the risk of multiple small white scars where the skin was punctured.

The skin that has been under the effect of the radiotherapy can develop fibrosis in the skin and in the underlying soft tissues as a chronic sequel, among skin color changes and telangiectasia. In most severe cases, the ischemia in the tissue induced by the radiation damage can lead to the skin breaking and ulceration. Ulcerations of the irradiated skin do not usually respond to conventional topical treatments, requiring of surgeries able to remove all the irradiated skin and direct closure with healthy tissue, or covering them with flaps obtained from non-irradiated areas. Some authors have achieved good results using lipofilling, with or without enrichment with stromal vascular fraction or growth factors [7, 8]. The healthy fatty tissue of the graft is able to promote neoangiogenesis in these ischemic tissues, improving the vascularization and also helping the granulation and epithelialization of the ulceration [7, 9–11].

Acne

Fat grafting provides a new approach to the treatment of acne scars, which complements the traditional dermabrasion, chemical peeling, or laser resurfacing. In fact, fat atrophy is a subdermal damage produced by the severe acne that contributes with the scarring to the irregularities on the skin surface. As a kind of fibrosis and scar, sequels of acne can be improved by means of fat grating, and this can also provide volume of fat were this was damaged by the disease [1, 12, 13].

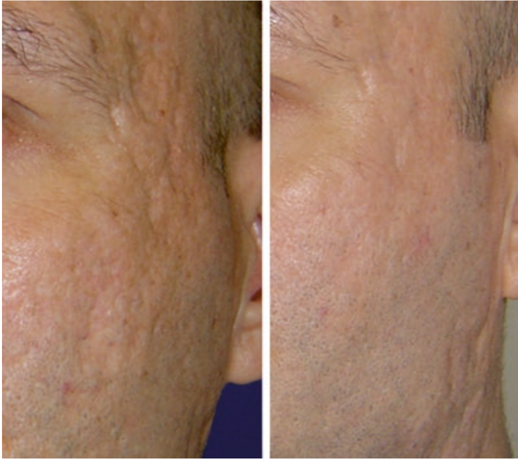

Fig. 2 Acne scars treatment with lipofilling. Left: before, Right: after. Amounts used are discrete, the most important is to release every scar with subcision and fill the space created with fat

Subcision of the scar is necessary to create the space to place the fat under the acne scar. Many authors suggest the use of a beveled needle but, for us, sharp instruments will produce more swelling, ecchymosis, and bleeding, which will interfere with the graft uptake. We rather use spatulate blunt cannula, but as difference with the general treatment of the scars, we will choose a narrower diameter of cannula (18G–21G) (Fig. 2).

HIV Lipoatrophy

Antiretroviral treatments used at the end of the 90s showed an exceptional effectiveness to control the disease but had a devastating side effect, the HIV-related lipodystrophy. This implies changes in the body fat distribution, with lipoatrophy in the limbs and the face, and fat accumulation in the trunk (especially intra-abdominally and in the upper back as a hump) and the neck. These changes make noticeable that the patient, otherwise healthy, is infected by the HIV [14, 15].

The features are a face with more or less sunken cheeks, depending on the degree, with a skeletonized look in the most severe cases. In the limbs, buttocks flattening and very noticeable muscles in the legs, thighs, and the arms are the main signs of the disease, concerning more to the women because they use to have more fat than men in these locations.

The options to treat the more concerning feature of HIV lipoatrophy, the facial atrophy, comprise synthetic fillers injection or lipofilling. Given that the atrophy does not improve over the time and does not have any etiological treatment, the use of reabsorbable fillers is not the first option, being preferable a permanent one. Synthetic permanent materials are sometimes related with serious local problems, which can be difficult to treat. Lipofilling is a good option because is permanent and absolutely biocompatible, and its effectiveness and durability in these patients have been demonstrated, so should be the first to be considered in the treatment's algorithm in those patients with enough fat in the potential donor areas, usually abdomen or hump [14, 15] (Fig. 3).

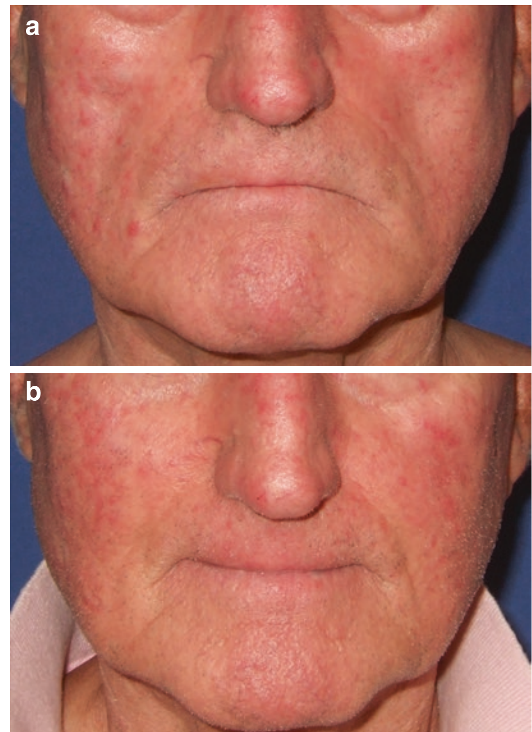

Fig. 3 Patient with facial HIV lipoatrophy. (**a**) (up) Before the treatment with lipofilling. A noticeable lack of fat in the cheek, with the skin sunk under the malar bone it the severe grade of atrophy. (**b**) (down) 1 year after grafting 9 cc of centrifuged fat, a natural correction of the atrophy is observed

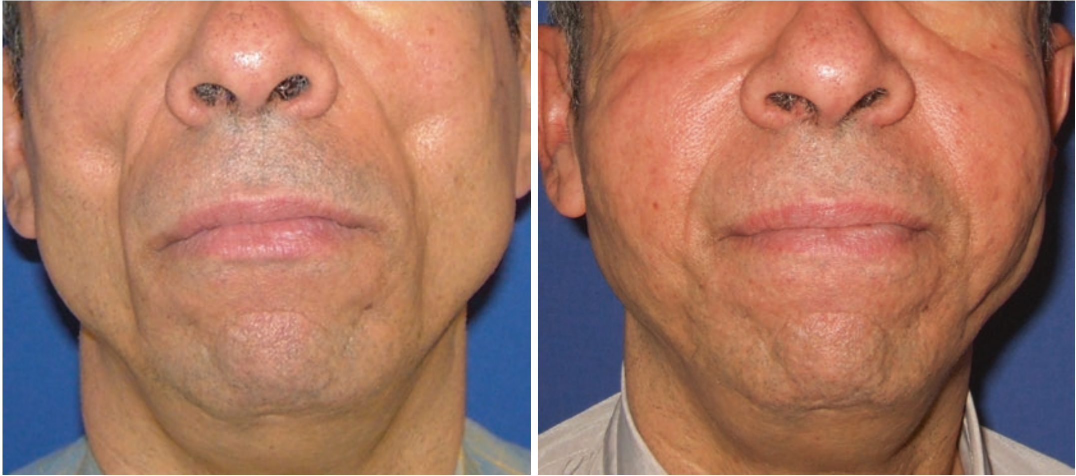

Fig. 4 Patient with facial lipoatrophy, left, before lipofilling treatment, and right, 1 year after, showing hypertrophy of grafted fat (Hamster syndrome)

Lipofilling can also be used for the buttocks atrophy, but many patients have a severe atrophy of the buttocks with a limited amount of fat available. The amount of fat required to enhance the gluteal area is high compared with the face (around 500 cc each side), so many of them requesting a treatment for this area require buttocks implants additionally to the lipofilling, which can be performed simultaneously [16].

A special warning should be given about complications of fat grafting in these patients, because an excessive overcorrection can lead to an excessive full-face appearance that has been called "Hamster syndrome (Fig. 4) ". This complication is not frequently reported out of the HIV lipoatrophy context; probably in these patients the particular changes in fat metabolism pose an especial risk for it. This can be prevented obtaining the fat from volume stable donor areas in patients with well-established and stable lipodystrophy and avoiding overcorrection [14].

Parry–Romberg Syndrome, Hemifacial Atrophy

The features of the Parry–Romberg disease, hemifacial atrophy and some other entities in the maxillofacial area featured by atrophy of the facial subcutaneous tissue (among other changes in the underlying bones and muscles) are pretty similar to those of the HIV lipoatrophy. Lipofilling is the most convenient option for those changes in the facial soft tissues, while for the underlying skeletal anomalies, osteotomies or hard implants are the preferred treatment.

For those with Parry–Romberg disease, lipofilling use to be enough to achieve good symmetry and long-lasting results [17, 18]. For the other maxillofacial syndromes as Treacher Collins or Goldenhar syndrome, lipofilling is necessary as the main step in their treatment or as a secondary procedure in the following reconstructive times.

The results are quite satisfactory for the cheek area, but curiously in these cases the results in the chin are not satisfactory in terms of symmetry, being very difficult to achieve a good expansion of the thick skin of this area.

Complications can appear as those described for HIV facial lipoatrophy and we should take the same cautions, especially overcorrection avoidance given this condition is difficult to treat [19] (Fig. 5).

Breast Reconstruction

In the last decade, indications of fat grafting for breast reconstruction have become the last significant advance in breast reconstructive surgery

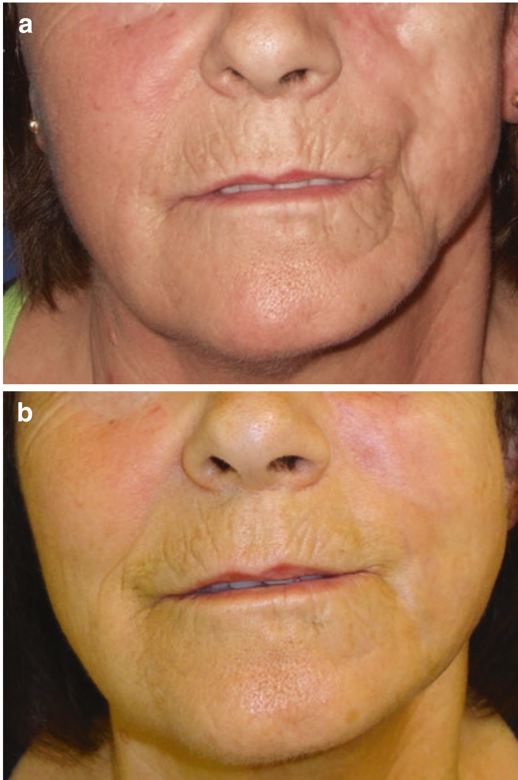

Fig 5 Patient with Parry–Romberg syndrome: facial atrophy of the left cheek (**a**). (up) Before the surgery (**b**). (down) After two procedures of lipofilling

after the boom of the perforator flaps in the early days of this millennium. Concerns about its safety and effectiveness kept the fat grafting in the backstage of the surgical options in breast surgery both for aesthetic and for reconstructive purposes.

Based on the improvements in the fat grafting technique, currently the controversy of the effectiveness and durability of fat grafts is not an issue, but some controversy remains about its safety in the breast. This controversy refers to the potential risk of neoplastic promotion mediated by the adipose tissue stem cells (ASC) and the possible interference in breast imaging. Despite that, current evidence from the clinical series indicates that this procedure is safe in patients who have suffered a breast cancer [20–23].

Fat grafting in breast reconstruction provides a versatile option to treat many of the breast defects resulting from an oncologic surgical approach, which has offered a new approach to treat these defects in a more effective and conservative way than the options offered by the implants and flaps. Nevertheless, flaps and implants still remain the main option for mastectomy reconstruction, but even with these the fat can play an important role as an ancillary procedure to improve the results that can be obtained with the basic technique [24–26].

Fat grafts in the breast after breast cancer surgery can also give additional advantages aside of volume as an improvement of the Postmastectomy Pain Syndrome improvement. Chronic pain in the breast and surrounding areas affects up to the 60% of the patients after surgery of breast cancer, boosted by some factors, being the radiotherapy one of them [27].

The most usual complications are common with those appearing in its use in aesthetic surgery and will be presented there, as like some breast defects as tuberous breast and breast asymmetry, which can be considered both reconstructive and aesthetic problems.

Partial Defects

Breast-conserving surgery (BCS) of breast cancer can lead up to a 35% of bad cosmetic results due to the shape distortion and asymmetry from the surgery and the adjuvant radiotherapy [28]. Common features of the breast after a BCS are contour deformities, pigmentation and hard touch due to the fibrosis and the radiotherapy. Some of the morphological changes can be prevented by an oncoplastic approach, remodeling the breast at the time of the tumor removal, avoiding dead spaces that will lead to a skin retraction and distortion of the breast contour. Another option to prevent secondary defects is the use of flaps from the thoracic wall (intercostal perforator or latissimus dorsi flaps) but the use of these mean deep planes dissection, even muscle sacrifice, and new scars in an area without any disease, with a more visible sequel that the incision needed to treat the cancer. When the defects appear, fat grafting can provide a more convenient way to treat it, providing volume and contour restoration, with fewer

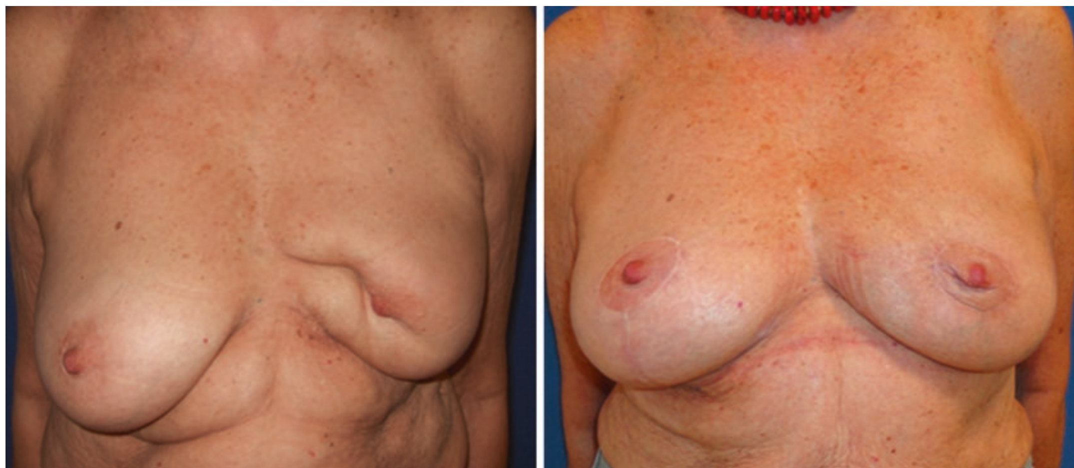

Fig. 6 Left: Skin retraction after breast-conserving surgery of breast cancer and radiotherapy. Right: After two procedures of fat grafting and periareolar mastopexy the shape of the breast has been restored

scars, without functional compromise and with a regenerative parallel effect of fibrosis release that can soften the hardened breast (Fig. 6). This approach has also been proposed to be applied at the same time of the lumpectomy, but with the aim of adding volume to the whole breast to avoid retraction of the exceeding envelop [29].

The cannula in a breast lipofilling procedure will cross the breast repeatedly, and this maneuver can spread a cancer in the breast, so we must be sure that the breast does not have any lesion suspicious of malignancy, given the risk of relapse or a second cancer, so a mammogram of less than 3 months before the surgery is advisable.

As we induce some changes in the breast architecture, those patients with a breast difficult to assess by imaging will be excluded of lipofilling treatment to avoid interferences in the disease control.

Total Reconstruction

Total breast reconstruction after a mastectomy is usually performed by means of implants or flaps. Both present limitations as any other technique but they are the more convenient way to do it because they can provide enough volume in a single procedure. Fat grafting in these case has many limitations as the high volume of fat needed, the inelastic tight skin which hampers the graft placement, and the need of many grafting surgeries to achieve the same volume that the healthy side. But in some cases fat grafting can be an option to provide a scarless reconstruction: patients with a single scar, with some excess of skin in the lower and inner quadrants to achieve a good cleavage definition, and without a big breast in the contralateral side (Fig. 7).

Expanding the skin previously can be of help to perform a total breast reconstruction by means of fat grafting. Two different strategies can be used in this way: internal expansion and progressive deflation with simultaneous grafting, or external expansion using an external vacuum device and grafting [30–33]. These need two or more procedures to achieve the final result.

Ancillary Procedure

As in the case of the partial defects, fat grating has revolutionized the breast reconstruction surgery being nowadays a step more in the reconstructive process, being used as an ancillary procedure, before or after the main reconstructive procedure [34].

Fat grafting can be applied before the implant based reconstruction to increase the thickness of

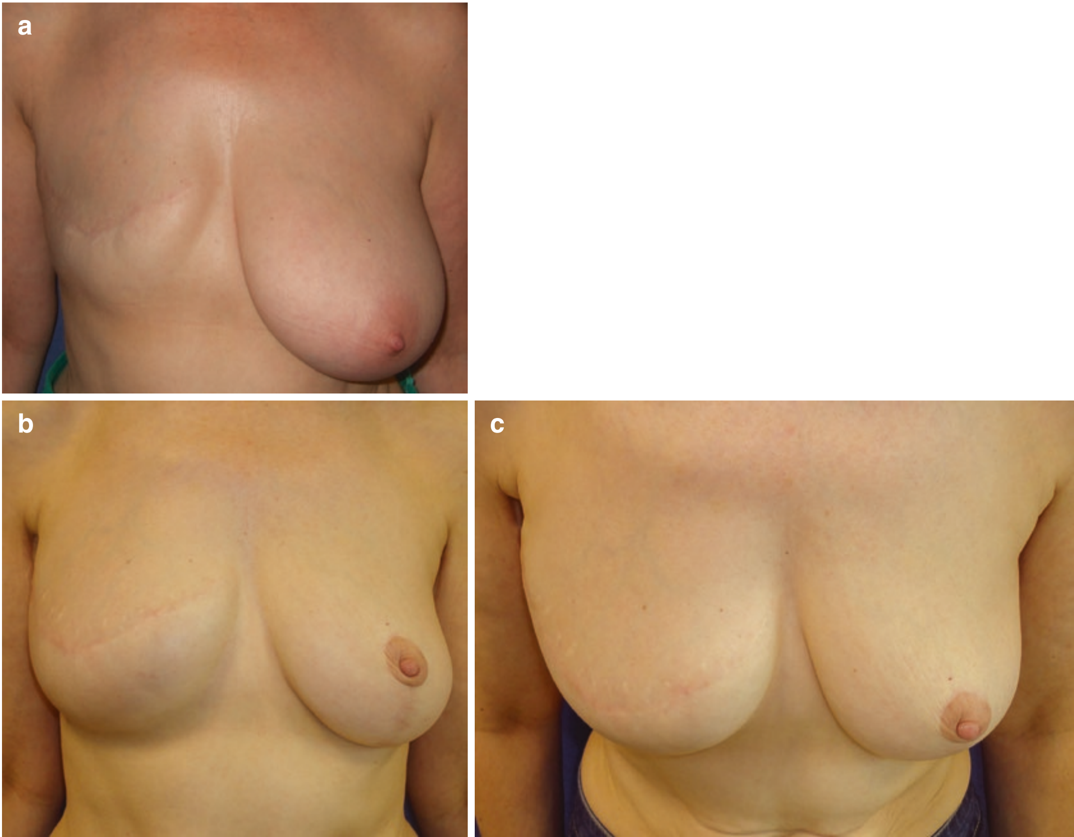

Fig. 7 (**a**, up) Right mastectomy, with enough remaining skin in the lower and inner quadrants (**b**, left) that can help to achieve a natural shape of the reconstructed breast after two fat grafting procedures (**c**, right) including a good cleavage definition

the skin with the objective of avoiding implant extrusion. If the breast has been irradiated, fat grafting seems to provide regeneration of this area that will limit the problems of the implants applied in previously irradiated area, as contracture, upper displacement, and extrusion [35–37].

After the reconstruction, fat grafts can help to provide a smoother transition between the clavicle and the upper pole of the implant or flap, a better defined cleavage when applied in the inner quadrants, and can also supplement the volume of the flaps and even the implants, improving the satisfaction of the patient with her reconstruction [26]. Moreover, fat grafting enhanced latissimus dorsi flap, provides us the same results in terms of volume and shape than those obtained by the DIEP flap (the contemporary "gold standard" of breast reconstruction) in a more predictable surgery and with less limitations than the microsurgical procedure (Fig. 8).

Poland's Syndrome

Fat grafting is an option of treatment for Poland's syndrome among the implants and the transference of the latissimus dorsi [38–40]. Like in the case of the breast reconstruction, fat grafting can be used as a primary treatment or as an ancillary treatment, supporting implants and flaps. As primary treatment can be successful in those cases with a mild deformity, but in most severe cases, with severe chest wall compromise (sometimes involving also the ribs) could be insufficient to provide a good result. In these cases, the use of implants or flaps is mandatory,

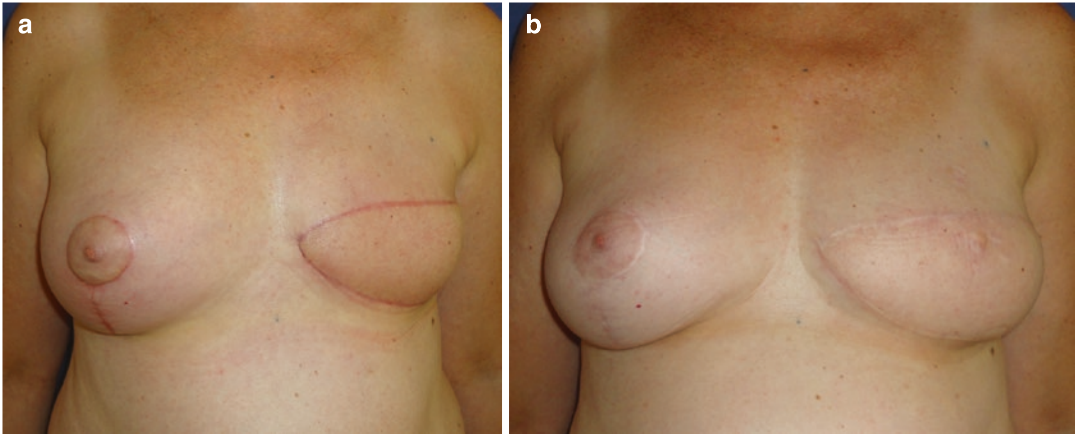

Fig. 8 (**a**, left) Left mastectomy reconstructed with latissimus dorsi pedicled flap without implant. (**b**, right) After two procedures of fat grafting, the flap has gained the volume of the contralateral side, with a permanent and more natural shape and touch than what is achieved when using implants

and fat grafting can help to achieve a more natural result, improving the contour and adding volume where needed [40].

Pectus Excavatum

Nowadays, the standard technique for the correction of pectus excavatum is the minimally invasive repair of pectus excavatum (MIRPE), which implies the use of a metal bar, which pushes out the sternum and corrects the deformity. Custom-made silicone implants placed in a subcutaneous pocket are another surgical option. The invasive techniques for pectus correction may not eliminate the need for ancillary methods of extrathoracic remodeling and aesthetic refinement of minor or remnant defects, and this is the main role of fat grafting in this pathology [41, 42].

Lipofilling have not succeed as a primary treatment of pectus excavatum because there are some important factors limiting its effectiveness: the funnel deformity in the moderate and severe cases require of a thickness increase to be corrected that is hardly attainable in only one procedure, the firmly adherent skin of the presternal area to the deep plane makes difficult the placement of the graft, and these patients usually are young and slim being difficult to harvest enough transferable fat.

Autoimmune Diseases

Many of the autoimmune diseases have changes in the subcutaneous fat, skin and fibrosis, which can be managed with the use of lipofilling. Diseases as rheumatoid arthritis have features like hands and feet fat atrophy that give them an aged look, with noticeable tendons and veins, and in the feet can lead to pain in the sole due to the lack of fat cushion. Increasing the fat thickness there can help to hide the skin underlying structures, and makes the patient feel more comfortable when steps on.

Lupus and scleroderma can show facial depression deformities caused by scars along with skin and subcutaneous fat atrophy. In “coup de sabre” scleroderma of facial skin, changes can mimic those of Parry–Romberg disease and the differential diagnosis should be established. Fat grafting can reduce the aesthetic and functional impact of the disease increasing the facial volume and improving the quality of the skin, and even the mouth opening in the scleroderma [43, 44].

The use of fatty tissue and stromal vascular fraction (SVF) has been proposed to treat the symptoms in the hand of the systemic scleroderma: Raynaud phenomenon, finger retraction, and skin ulceration. Fat is injected at the hand palm and in the dorsum, SVF is injected under

the skin in the fingers, and they reduce the fibrosis and the vessel hyper reactivity, reducing the risk of amputation [45, 46].

Dupuytren Disease

Dupuytren disease has as a main feature a fibrotic cord retracting the fingers (mostly the 5th and the 4th). The classic approach so far has been the surgical cord resection, with or without skin plasties, with a high rate of relapse. A new treatment using collagenase is used nowadays with the advantage of being no invasive, but the relapse can also appear. Lipofilling is another option, breaking the cord by means of needle subcision and grafting directly the fibrotic area [47].

Due to the antifibrotic properties of the stem cells contained in the graft (which inhibit the proliferation of the contractile myofibroblast) and the placement of the fatty tissue right inside the cord, relapses are limited being a less invasive treatment than the open resection option, especially in a disease that have no effective option to avoid the relapse [48].

Urinary and Bowel Incontinence

Even though lipofilling has been used in these indications, references about this use are week, probably because it is not easy working in these delicate spaces with cannulas, with risk of urethral or bowel perforation, and other strange complications reported as pseudolipoma, with results not equal to those that can be achieved with other techniques [49–51].

Vocal Fold Paralysis

In the event of vocal fold palsy, the vocal fold is unable to medialize, changing the features of the voice. Any procedure that increases the volume of the paralyzed cord can help improve the quality of the voice. Synthetic materials as calcium hydroxyapatite are used with this purpose, but lipofilling can also be used with the advantages of being softer and permanent. The fat is injected under direct microlaryngoscopy into the defective vocal fold [52].

Eye Enucleation

Loss of fat volume in the eye socket can occur after enucleation. This limits the ability to wear ocular prosthesis because the orbit is not able to retain the implant. Adding more fat by means of lipofilling can help to increase the volume of soft tissue and help to retain it there, improving the general aesthetics of this zone [53, 54].

Vulvovaginal Atrophy/Lichen Sclerosus

Vulvovaginal involutive diseases, as senile atrophy and lichen, painful episiotomies scars, and others, which use to have the common features of skin stiffness, dryness, irritation, soreness, and pain (dyspareunia) with urinary frequency and urgency are tributary of treatment using lipofilling as volumizing agent and with the benefit of the immunomodulation of the stem cells. Lipofilling can provide relief from pain, volume, softness, and humidity [55]. Alternative treatments are hyaluronic acid or platelet growth factors (PRP) injections, being reported good results.

Foot

Loss of foot sole fat cushion can have many etiologies, as autoimmune and rheumatic diseases, diabetic foot, ischemia, previous local trauma, surgeries or radiotherapy, age related among many others. Most of these patients have used custom made insoles to reduce the pain and discomfort in walking, but if these are unsuccessful fat thickness restoration is sometimes the only one available option for them [56, 57]. This indication is not well reported, but by the authors experience in some patients the improvement worth the surgery.

Lipofilling is applied in the sole from the toes web spaces and can also be applied in the heel from the instep [58]. Volumes used are very different to one patient to another due to the different features of the atrophy in each one. The volume used is limited by the stiffness of the sole skin, and we should avoid an excessive volume infiltration because a compartmental syndrome is one of the overpressure risks in the limbs. We recommend to use only the volume enough that do not bleach the skin, keeping in mind that this volume is less than the expected volume if we compare with face, breast, or buttocks procedures.

In diabetic foot, fat grafting is a promising option to provide cushion to insensitive areas exposed to pressure ulceration [59], and is also an option for painful scarring impairing walking [60].

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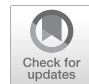

Lipofilling in Aesthetic Surgery: Indications, Outcomes, and Complications

Joan Fontdevila and Ariel Marshall

The indications of the lipofilling in aesthetic surgery have bloomed in the last years, embracing areas far from the early applications in the face [1]. The good results obtained in one area boost the interest of the surgeons to apply it in other. Usually, the first intention of use of lipofilling is to add volume, but its regenerative properties have expanded its indications to secondary surgeries, when we need a gentle procedure with the tissues, able to provide new healthy tissue and improvement of the fibrosis of the previous surgeries [2].

Volume increase maybe is the main goal we look for in aesthetics applications of fat grafting, and this is limited by the biological properties of any graft. In order to have satisfied patients, we should know the results desired by the patients and plan carefully the lipofilling procedure, in order to determine the amount of fat available for harvesting, how many grafting procedures we will perform, and how we will perform it. As with the reconstructive procedures, improvements and new technology in regenerative cell procurement, growth factors, and biological scaffolds can contribute to expand the indications [3].

Many of the principles stated in the reconstructive indications chapter apply to the aesthetics, but unlike the reconstructive surgery, aesthetic surgery is a field where the use of synthetic materials has been the state of the art for many indications until the breakthrough of the lipofilling and the beginning of this century. So, depending on the material chosen, indications, outcomes, and potential complications may be different. It is important to inform the patients and make them aware about the differences of using one or other kind of treatment.

Face

The face is one of the main targets of lipofilling for aesthetic purposes because nice and natural results can be achieved, even in lean patients with limited volume donor areas, and because it does not require a huge amount of fat. Moreover, a regenerating effect of the grafts provides additional aesthetic improvement with dermal thickness and vascularization increase, and also a color and pigmentation improvement [4]. Even patients with facial bone anomalies can benefit a simpler approach to harmonize their face [5].

But the results differ quite depending on the unit of the face treated, being the cheek and the chin where the integration of the graft is best and

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the temple, nose, lip and frontal areas where integration is low [6]. Patient should be informed before of the limitations with fat grafting in some areas and consider the use of synthetic materials if with the lipofilling is not possible to achieve the expected result.

Regarding the synthetic materials, every patient requesting a lipofilling procedure should be questioned about previous synthetic filling materials use. If the patient has implanted a permanent filler (methacrylate, silicon, etc.), it should be rejected. The surgical procedure can stimulate a foreign body reaction against the allogenic material, with possible devastating consequences. If the material used was reabsorbable, then we should advise the patient to wait until 1 year after the treatment to enable us to assess the volume with accuracy.

Complications of lipofilling in the face are rare. The mild ones are the most common, such as edema, ecchymosis, pain, skin tattoos, nodules, asymmetries, reabsorption, displacement, hypocorrection, hypercorrection, or hypertrophy, while more serious complications may occur secondary to infection, injury of anatomical structures, and intravascular injection.

Despite they are more pronounced than those seen with synthetic injectable materials, edema, ecchymosis, and pain will be managed as usually is done in any other facial surgery: local cold, lymphatic drainage massages and soft pain killers.

If the injection is very superficial, it can result in visible or palpable nodules. This is especially relevant in the eyelids, where the skin is very thin and a superficial infiltration would generate an evident irregularity. In this zone the correct technique would be to make a deeper infiltration between the orbicular muscle and the periosteum. In case of immediate appearance, a massage of the area should be performed to remove the excess tissue. Nodules can appear immediately (due to superficial or excess infiltration), but may also appear months or years after infiltration as steatonecrosis, suggesting a technical deficiency: traumatic harvesting or processing altering the viability of the graft, or an indiscriminate infiltration of fat, which is prevented by properly infiltrating in the form of small drops of graft. If its center shows liquefaction producing an oily cyst, puncture and drainage under local anesthesia could be an appropriate treatment.

Asymmetry can be very visible in the face since this is a very exposed area and slight differences are easily detected. We must identify asymmetries prior to surgery and make it note to the patient. Most of them are there before the surgery and can be difficult to correct if the problem is in the bone structures. A useful way to identify them is through photographs on every possible plane to compare the silhouettes from different angles. A usual origin of asymmetries is the lack teeth pieces, creating a depression that can hardly be fixed only with fat. The correct procedure would be to first apply dental implants and then perform fat infiltration.

Hypocorrection is generally masked by local inflammation and becomes visible once it disappears. To prevent it, it is for us important to use a very dense graft, using centrifuged fat rather than only decanted or filtered. Using high dense fat, the amount of injected fat and the retained fat will be as close as the 1:1 ratio, like happens with the synthetic materials. Hypocorrection usually requires one or more corrective infiltrations. It is recommended to wait at least 6 months to let the first graft stabilizes and avoid hypercorrection, with better uptake due to less fibrosis.

Fat hypertrophy may appear after more than 10 years and does not disappear spontaneously. It manifests as an increase in volume in the infiltrated area, usually after a rapid weight gain and more likely in patients treated in the youth. Hypertrophy can appear as a nodulation but, unlike steatonecrosis and oily cysts shows a normal consistency of fat. Its etiology is unknown, although it is believed that adipocytes that survive after the graft increase its content due to the weight gain, so patients should maintain a stable weight after surgery to avoid it.

There are no studies that demonstrate which fat donor area is the best, but in most cases of hypertrophy described, the donor area has been the abdomen, so to infiltrate very visible areas such as the face, it is preferred to obtain fat from other areas; for example, the inner part of the

knees, which do not fluctuate too much with the weight gain. Even though it's not necessary to perform imaging studies for its diagnosis, if an MRI is performed, the fibrous tracts in the infiltrated area can be seen increased; therefore, the correction through liposuction is harder than usual. Even supposing it can be corrected by liposuction, it can recur, and that's why the most effective treatment is surgical excision.

The most worrisome complications are those generated by the intravascular injection, ranging from cutaneous necrosis, blindness, paralysis, or death. The manifestations may appear within the first 24 h, although they usually emerge during infiltration. For this complication to occur, no large amounts of fat are necessary since cases have been described after infiltration of 0.5 mL. The highly vascularized periocular areas are frequently involved; for example, glabella, nasal root, nasolabial fold, and frontal and temporal regions.

Fat injected in small arteries of areas mentioned above can travel oppositely to blood flow through the arteries in the injection area to the ophthalmic artery and internal carotid artery due to the high pressure of infiltration. The ophthalmic artery occlusion causes a painful blindness and ocular ptosis. Meanwhile if it travels further reaching the internal carotid artery, it may occlude the anterior or middle cerebral artery, presenting with neurological manifestations that can be as severe as death.

Superficial lesions as cutaneous necrosis may improve after local treatment; therefore, if a change in coloration of the skin is observed during infiltration, it is recommended to stop immediately, apply topical nitroglycerine, and occlude the area. In addition, local infiltrations with sodium heparin have been reported.

In cases of cerebral involvement, supportive treatment, anticoagulant, and intravenous corticosteroids are advised, while in cases of blindness it is recommended to perform eye massage, pharmacological intraocular pressure reduction, and intravenous vasodilators. However, in most cases blindness is usually irreversible.

To avoid intravascular injection, several precautions must be taken, including the use of blunt cannula, avoiding infiltrating deep planes with needles or pointed cannulas, always aspirate before infiltrating, withdrawal of infiltration and with low-pressure syringes, limiting the size of the syringe to 1 mL and the volume of fat to 0.1 mL in each pass, and using vasoconstrictors in the area of injection. Avoid treating areas with previous trauma, chronic inflammation, or scarring.

Skin marking with permanent pens can leave tattoos in the cannula entry orifice since the ink can infiltrate the dermis and remain in. The most suitable way to solve would be by laser. A different kind of pigmentation to avoid is that caused by sun exposure for a while as it can cause pigmentation of the skin that is then difficult to remove, requiring application of laser or chemical pilling. Ecchymosis can also be camouflaged with specific makeup.

Lids and Periorbital

Some signs of aging of the eyelids such as the tear trough depression, upper palpebral sulcus hollowing, and a marked eyelid to cheek transition are suitable to be treated using lipofilling [7–11].

Treatment of tear trough deformity has focused the interest of the surgery of this area in the last years, being suggested the use of hyaluronic acid and other synthetic fillers, orbital fat repositioning, and fat grafting [9, 12]. Fat grafting is becoming very popular, as a common graft or as the emulsion of the fat, known as "nanofat" [13], the latter being used more like a skin regenerating agent of this area rather than as a filling material.

This area is not easy to treat because the periorbital skin is very thin and soft and continuously exposed to the other's sight. These special features make this area sensitive to noticeable complications if the grafted lid shows irregularities, usually as a thread of small lumps in the orbital rim or in the upper eyelid (Fig. 1). To avoid this, graft should be very thin, harvested with cannulas with holes less than 1 mm of diameter, and injected in a small amount (from 1 cc to 2 cc for

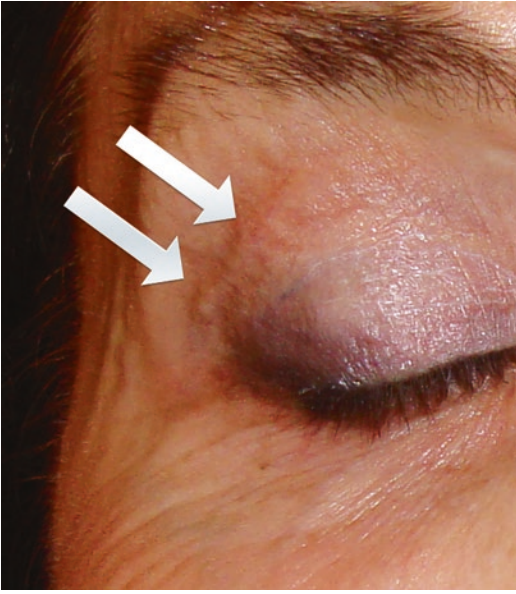

Fig. 1 Small lumps of grafted fat, noticeable in the upper eyelid after attempting the correction of sunken upper palpebral fold with fat. Meticulous technique should be used in this zone to avoid complications: small size of the graft (<1 mm), 21G cannula, and deep injection close to the periosteum

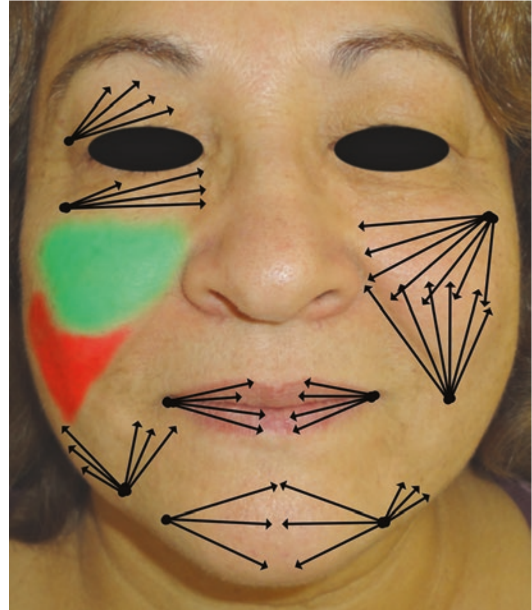

Fig. 2 Injection points recommended for the main areas tributary on fat grafting in the face. Over the malar bone (green area), the graft provides a lift effect that enhances the results obtained with the lipofilling. But in the lower cheek (red area), the extra weight of the graft can make it to sag, so moderate volumes should be used there

each unit of the periorbital area) deeply close to the periosteum using 21G cannula.

As previously commented, this area is more prone to intravascular injection and its complications, and a cautious injection should be performed [11, 14].

Cheek

The best area to observe the power of this technique in the face is the cheek. As the other mammals, humans loose fat from the peripheral areas of the body with aging, redistributing to central location. This atrophy is located mostly in the limbs and can also be evident in the cheek. So, in a different extent, with the time, everybody is eligible to improve his face with lipofilling. Due to the thick dermis in the upper cheek, we can use fatty tissue obtained with cannulas with holes wider than 1 mm and inject them with 16G. But the skin of the cheek gets thinner inferiorly, and any thick graft or excess of volume in the lower cheek can be easily noticeable. We recommend applying the same principles of thin graft and few volume used for periorbital injection when injecting the cheek down from the level of the anterior nasal spine (Fig. 2).

The support of the malar bone provides a lift effect in the middle third of the face that contributes to the nice results obtained when applied in the upper cheek. But the lower cheek does not have the support of the bone and an excessive volume of fat will add weight in a bad supported area and this will sag easily, especially in older patients because of the flaccidity. In this case, it would be necessary to reconsider performing a repositioning technique of the facial soft tissues (face lift) (Fig. 3).

The entry point of the cannula in this area will be the upper lateral area of the malar bone and lower limit of the nasolabial fold. The plane of infiltration is from the deep dermis deep to the periosteum, including the facial muscles. Special attention should be paid to the emergency point

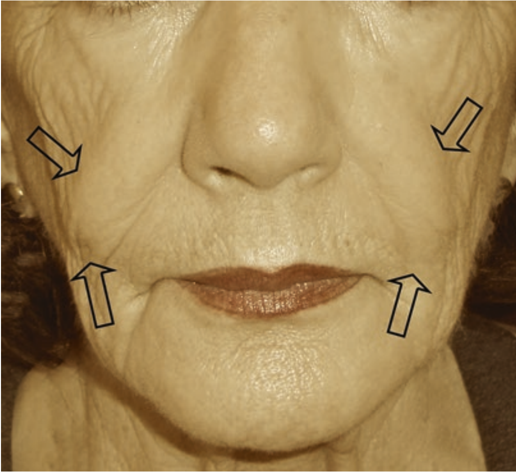

Fig. 3 In older patients we must consider first a face-lift surgery, because the skin flaccidity is unable to support the weight of the graft. In this case, an 80-year-old, 1 year after fat grating in the malar area, the skin of the cheek has displaced downward. A face-lift procedure with a simultaneous fat grafting could provide more predictable results

of the infraorbital nerve to avoid numbness, pain, or paresthesias of its innervation area.

Swelling and bruising, which can last up to 2 weeks after the treatment, are more evident than with the use of synthetic fillers, so we should warn the patient that this is not a rapid recovery treatment.

Combination of face-lift procedure and lipofilling has become a standard procedure nowadays in the facial rejuvenation surgery, based in the knowledge of this relative lack of fatty tissue in the face, which will not be improved by the face-lift techniques. Lipofilling is an easy maneuver that leads to better results in these surgeries [9, 15].

Nose

Lipofilling in the nose is indicated to treat secondary defects of rhinoplasty or to avoid a rhinoplasty if it is applied in strategic points as an augmentation technique to hide the hump [16, 17]. This area is not easy to treat if a previous surgery has created a fibrosis under such thin skin, so we do not recommend this indication to be used by those surgeons without huge experience in fat grafting. Moreover, retrograde fat embolism of the ophthalmic artery is a possible complication described in the nose lipofilling [16].

The technique here is like that used for the periorbital lipofilling, entering the cannula by a small stab incision in the nose tip and auxiliary entries at both sides of the upper dorsum can be also used.

Lips

The gold standard in lips enhancement is the hyaluronic acid. With this product the volume and profile of the lips can be improved by means of small-needle injection with precision and immediate results. The main limitation is the reabsorption over several months [5]. Lipofilling can provide an option for more lasting results but with the inconveniences of more swelling, bruising, and less control of the outcomes compared with the hyaluronic acid due to the unpredictability of the final volume or different rates of graft survival in each side which can hamper the symmetry [18]. Moreover, there is not agreement about the survival rates of the fat in the lips, and some authors think that this is not the most convenient indication of the lipofilling while we have other options as hyaluronic acid [6]. The plane of infiltration is also controversial, because it is not clear that a muscle with a constant movement like the orbicularis oris muscle is able to allow the graft integration. We suggest applying it only at subcutaneous and submucosal level.

The strategy used for the lips is harvesting of fat with cannulas with holes of 1.5 mm and 1 mm. The 1.5 mm fat will be used to increase the volume injecting under the vermillion, and the 1 mm fat will be used to improve the profile injecting under the white roll, and to improve the bar code, we will inject this under the lines of the skin of the lip. SNIF and nanofat can also be used to improve the skin of the lips.

Chin

Lipofilling can provide volume and projection to the chin [9, 18]. Chin implants are another option: they are easy to place and provide good projection

and volume, but, as any other synthetic material, can have undesired complications as displacement, palpability, mental nerve damage, and infection, especially whether the implant is placed by intraoral approach. Another option is the use of reabsorbable injectable materials such as hyaluronic acid or calcium hydroxyapatite, but the amount needed is high and the effect will not last far than 1 year.

Like for the malar area, here the bone supports the soft tissues, this guarantees a good projection, and the technique used is alike which is used there: fatty tissue obtained with cannulas with holes wider than 1 mm and injected with 16G cannula. Infiltration is performed from the lateral of the chin area, and only one incision for each side is needed. The graft can be placed both subcutaneously and intramuscularly.

Some older patients show a very deep wrinkle between the chin and the lower lip. This wrinkle can improve dramatically if we place fat underneath, and additionally performing inside the wrinkle a SNIF technique, because the surrounding dermis here is very thick and SNIF will help us to restore its thickness.

Perimandibular

In selected cases, adding volume to the skin surrounding the mandible can provide a youthful look [18]. Before deciding to perform lipofilling in the tissues surrounding the mandible, we should think if the patient actually needs to increase the volume in this area or in fact needs a skin-tightening procedure. In the case that the skin over the mandible is sagging, we should consider to perform a facelift because the lipofilling can add an extra weight that can worsen the sagging. We do not recommend this technique for those without experience in face rejuvenation surgery and with lipofilling.

The goals in this area are to hide the transition between the jowls and the mandible and to smooth the mandible contour with the neck [9, 19].

Even with a good skin quality, we will avoid to graft this area with high volumes and thick grafts, using a thin graft harvested with less than 1-mm-holes cannula and injecting with a cannula of 18G. The infiltration level is only at the subcutaneous tissue.

Temple

There are patients who are worried about the hollow aspect of this area, requesting to fill it. Some authors have noticed that the results are not as good as in other locations because reabsorption rates here are high [6]. This is anatomically a singular area with factors hampering the potential of fat grafting as volumizer: the skin is thin, with the temporal vessels just underneath, and the soft tissue contains the temporal deep fascia, which is a hard and non-expandable structure. So we can only place the graft under a thin space where overcorrection is difficult to compensate high reabsorption rates. Moreover, given that this is a flat area with thin skin, irregularities in the distribution of the fat will be easily noticeable.

The technique will be the same than that used for areas with thin skin as the periorbital fat grafting. Entry points are placed in the infero-anterior angle of the temporal fossa and in the implantation line of the hair.

Breasts

As was discussed in the breast reconstruction indications, concerns about the safety of fat grafting in the breast make many surgeons to avoid its indication also in patients without health disease. Nowadays there is no clinical evidence of this risk to discourage the surgeons to apply it [20, 21].

The main benefit as a breast volumizing method in front of the implants are the long-lasting results avoiding implant limitations as capsular contracture, displacement, rupture, and implant replacement over the years, along with the versatility to reshape the breast with a material that should not be placed in a specific plane of the breast. The fat will follow the breast in its aging without secondary surgeries over time to update the breast position regarding the implant. Given this natural shape and touch provided by this technique, patients should be warned that the results are pretty different from those obtained with the implants: some patients prefer a round tight shape and hard touch of the breast. The main drawback of the use of fat as a method of breast enlargement is that it is not able to fill the

upper poles as the implants do, and we must advice this to those patients looking specifically for a corrective surgery of these breast areas, mostly women with atrophy after pregnancies.

Because we cross the breast tissue blindly with cannulas to perform the lipofilling, it is important to check the breast thoroughly before the surgery to discard any disease that can contraindicate the technique [22]. Breast should be palpated in search of any lump, and mammogram or ultrasonography should be performed close to the date of the surgery (we recommend less than 3 months). If any benign nodule is detected, we should consider removing it in the same surgery before performing the lipofilling. If a suspected malignant lesion appears, then we will not perform the lipofilling and patient will be studied to establish the diagnosis. Those patients with a difficult radiological evaluation of the breast, who previously need biopsies to discard malignancy, or having a gene mutation increasing the risk of breast cancer, should better opt for a surgery with implants because lipofilling will create changes in the breast that can compromise the imaging follow-up. Women with very dense breast can be also difficult kind of patients for lipofilling because the radiological assessment is not easy and the hard breast tissue hinders the fat infiltration.

The recommendation to not overcorrect in excess because this will create oily cysts and fat necrosis (Fig. 4) applies especially for all the indications of breast lipofilling because this kind of benign complications can hamper the cancer screening of the breast, can alter the shape of the breast, and make the patient feel pain or make them hypersensitive [23].

Negative pressure to expand the breast tissues before grafting has been proposed as a way to increase the volume, vascularization, and softening of the receiving tissue [24]. This can help to perform higher volume grafting reducing the complication rates [25]. The drawback is wearing an uncomfortable vacuum device for some weeks.

We recommend performing a mammogram after 1 year of breast lipofilling. This will be the baseline record of how is her breast after the treatment. In the future, if the patient has any suspicious change in the mammogram, this can be compared with the basal mammogram to know if the changes existed before or are new [22]. Patients should make their radiologists aware about the previous lipofilling procedures in the breast, because they can see changes difficult to explain not knowing that fat grafting has been performed before.

As for most of lipofilling procedures, serious complications are rare. The most common include the previously commented oily cyst and steatonecrosis (fat necrosis) that should be identified and treated [26]. In fact, both of them are

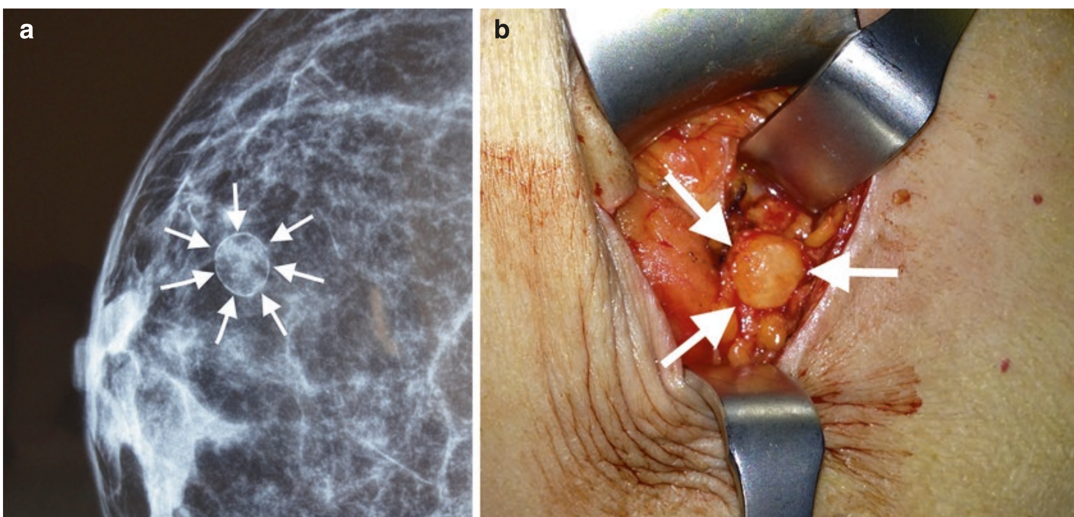

Fig. 4 Oily cyst. (**a**) (left) In the mammogram. (**b**) (right) Intraoperative image of the oily cyst

benign conditions and small lesions are usually painless, so they can be untreated but patient should be advised that these can appear in the mammogram. Bigger lesions are palpable and cause discomfort to the patient and can also hamper the radiological evaluation of the breast. These can be treated by direct excision or in the case of the oily cyst can be punctured to empty them and reduce their volume.

Augmentation

Usually patients demanding a breast augmentation procedure are young lean patients who need a considerable increase of the initial volume and do not have much fat to harvest. For this reason, breast implants still are nowadays the gold standard in breast augmentation. The outcomes are different to those achieved using implants, and we should warn the patients that the breast will not be firmer and round as with an implant. But being a noninvasive surgery without incisions in the breast, sensibility and breastfeeding will be preserved, and the recovery will be faster, painless, and with fewer restrictions regarding movements and weight lifting because there is no risk of displacement like with the prostheses.

If the patient considers achieving the same volume than with a breast implant, then she should plan two procedures of fat grafting separated as less by 6 months. We will advise the patient to harvest only the fat necessary for the first procedure in order to keep fat sources for the second procedure.

The injection of the fat will be done from a stab incision by sharp needle of 16G in every quadrant in the color-changing area of the areola and another entry point in the middle of the newly designed inframammary fold. Another one can be done at the outer limit of the new inframammary fold, in the inferior-lateral quadrant. The inframammary fold approach lets us graft the subglandular plane easily. Injection from the parasternal area must be avoided due to the risk of hypertrophic scars (Fig. 5).

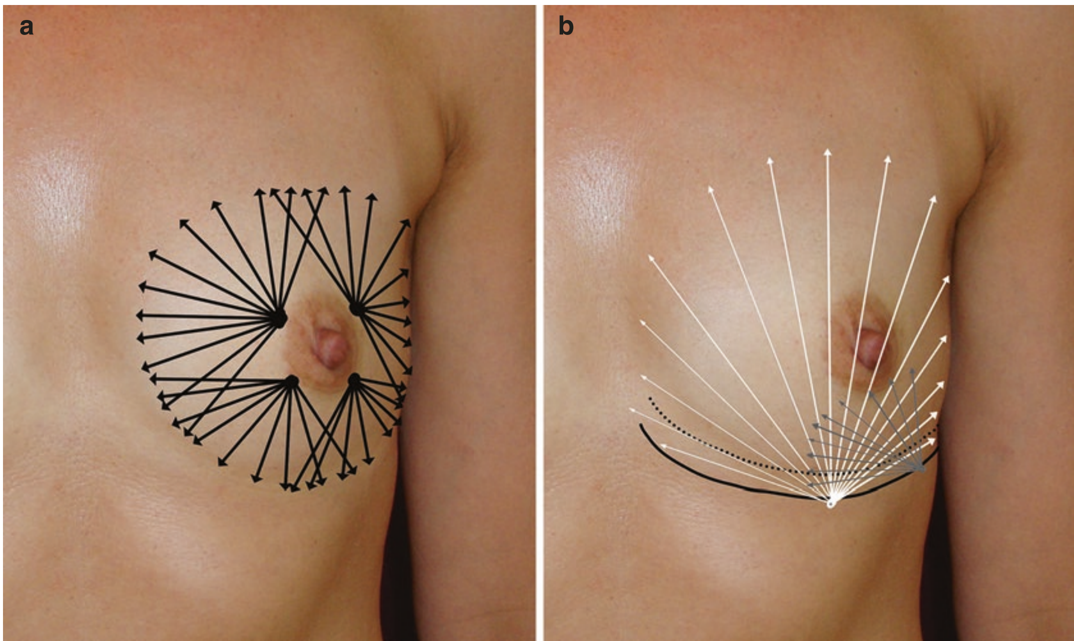

Fig. 5 Entry points for breast lipofilling. (**a**) (left) Four points in each quadrant of the outer rim of the areola. (**b**) (right) White arrows show another access for submammarian plane infiltration in the new inframammary fold (black line, dotted line shows existing inframammary fold). If needed, as for very wide breasts, another one in the lateral inframammary fold can be done (gray arrows)

Injection will be mostly subcutaneous and subglandular, and depending of the quality of the breast, also in the gland. Older patients with softer breast, with less glandular tissue and more fatty tissue, are more likely to receive fat in the gland. The amount of fat will be around 40–50 cc for every quadrant and 50–70 cc for the subglandular plane. Additional 30–50 cc can be placed inside and under the pectoralis muscle, remembering that only its caudal portion is of interest in breast augmentation; otherwise the breast will look excessively full in the upper quadrants.

Care should be taken to control the volume placed in every quadrant, to ensure symmetry. Not all the fat are the same and the fat origin also matters to avoid asymmetries due to the different behavior regarding different fat metabolism: fat from the same origin will be used for every quadrant (e.g., if fat from the abdomen is used for the upper quadrants in the right side, we will use the same origin for the upper quadrants of the left side).

Stitches are not used to close the entry points; we will only place an adhesive strip over them.

A soft bra will be worn for the first month, without limitations about exercises or positioning. Patient should be warned that the initial swelling will gone, so the volume that she sees the days after the surgery is not the final volume. Compared with submuscular implant placement, this procedure is almost painless. Over the months, the breast will become softer, and the volume at the third month usually is definitely.

Composite Breast Augmentation

This technique combines a lipofilling procedure and simultaneous breast augmentation with implants. The advantage is an increased thickness of soft tissue over the implant hiding the presence of this, allowing a more superficial placement (over the pectoralis muscle), reducing the volume of implant needed, and improving the cleavage definition in a smooth transition with the breast, therefore providing a more natural result [27–30]. The disadvantages are an increased surgery time and a more expensive procedure.

Lipofilling can be also used after any other breast surgery as an ancillary procedure to achieve better results or to treat some complications and as a method to hide the implant rippling, release and volumize retracted scars, add some volume, and improve the contour [31]. In these cases where the breast has an implant, a very cautious injection should be performed in order to avoid implant puncture, using a blunt cannula and rather injecting closer to the dermis than deep.

Tuberous Breast

Tuberous breast is a congenital deformity of the breast with the onset in the puberty when the female breast develops. Constriction of unknown origin of the soft tissues surrounding the breast is responsible of a limited growth especially noticeable in the lower quadrants, which creates an elevated inframammary fold and a lack of volume (hypoplasia). The breast can only grow by the areola, where the skin is always thin and elastic, resulting in a wide and protruding areola. This condition is usually bilateral and asymmetrical; therefore both breasts are treated and this treatment should be personalized considering the differences between every breast.

The classic proposed treatment is based on the use of breast implants to provide volume and try to expand the hypoplastic inferior pole, usually creating multiple incisions inside the gland with the aim of helping the implant to round the breast and adding a periareolar mastopexy to reduce and retrude the areola [32, 33]. This approach is very aggressive to the breast tissues, which can lead to complications and usually fail to provide the desired round shape to the breast because the lack of soft tissue in the lower pole creates a double-bubble deformity [34, 35].

Fat grafting can provide just what this kind of breast needs: release of the constriction and volume in the inferior pole. If the deformity is severe, two procedures separated by at least

6 months are advisable to assure the integration of the grafted tissue, resorption of the non-viable tissue, and benefit of the antifibrotic effect. Breast implant can be avoided if the patient only wants to improve her breast shape. However, if the patient desires more volume, as any other breast augmentation using lipofilling, a second procedure of fat grafting can be performed, or an implant can be placed with better results once the breast shape was normalized with the first procedure of lipofilling. We should keep in mind that especially the young women may prioritize more volume and firmness over only shape restoration [36].

The technical principles are the same than those described for breast augmentation, but in the lower pole the surgeon should be more aggressive with the cannula breaking the breast tissue to reduce the constriction and fill this space with fat [33, 34, 37]. We do not recommend performing "percutaneous fasciotomies" (the so-called rigottomies after Dr. Gino Rigotti) because these can leave a permanent white spotting in the lower pole skin. We rather perform the same maneuver internally with a spatulated cannula, feeling a "click" every time that a tight fiber of breast tissue is disrupted.

When marking the tuberous breast before the surgery, we should pay attention to how much we want to lower the inframammary fold. Descends of 2 cm are easily achievable in a single step, but if more descent is needed, a second surgery should be planned or the inferior pole will not get the desired rounded shape. Patient should wear soft bra for some months after the surgery to allow the fat grafts to provide the round shape of the lower pole (Fig. 6).

Breast Asymmetry

When the volume of both breasts is different and these are of small size, the common approach is performing a breast augmentation surgery using implants of different sizes in each side. The drawback of this approach is that we are in fact creating a new asymmetry using different implants with different diameter or different profile, which can be noticeable after some months and worsens over time. If one breast is small and the other one has a good size, the use of implant in the smaller one will mean a different behavior of both breasts with aging, with more sagging over time in the bigger side compared with the augmented side.

Lipofilling is a better option for these cases because we can correct the volume differences using a tissue with a natural behavior and evolution in the breast, and we can be more precise and individualize more with the volumes than with the implants, which have definite sizes and volumes [38]. If the patient desires a high volume in

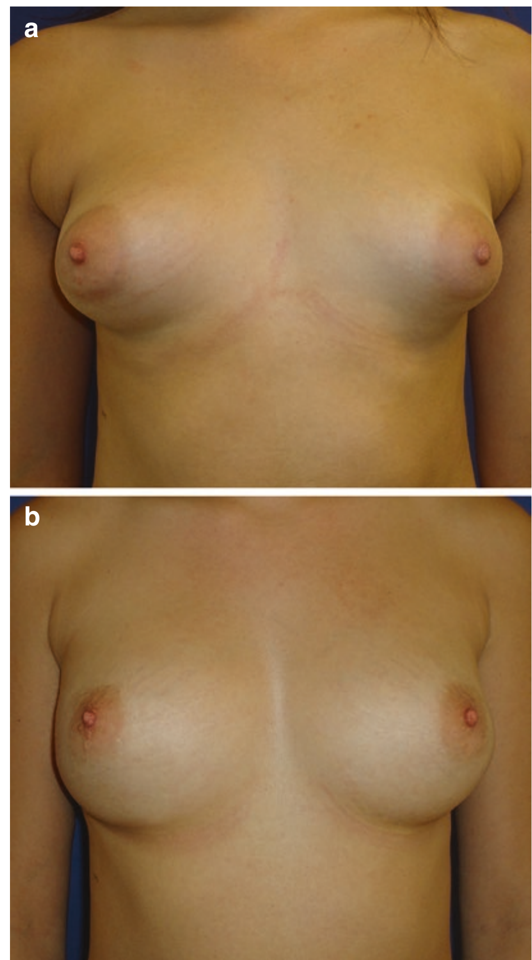

Fig. 6 (**a**) (upper) Tuberous breast with severe constriction of the lower quadrants and widened areola. (**b**) (lower) After two fat grafting procedures, the breast is rounder and the constriction release helps to relax areola protrusion

her breast, a good option is the "composite" breast augmentation, applying lipofilling to the smaller breast to compensate the lack of gland volume, using the same size of implant in each side. This strategy ensures better outcomes in terms of symmetry in the long run.

Implant Explantation and Simultaneous Fat Augmentation

Patients who have suffered of complications with implants for breast augmentation (capsular contracture, implant displacement or rotation, implant extrusion, etc.) frequently request a treatment of these complications without the use of implants but without resigning to the volume provided by these. Lipofilling has emerged as a more convenient option for these patients. An explantation with simultaneous fat grafting, with or without mastopexy, can provide the patient a good breast shape and volume with more comfort [39, 40].

The basic principles of the surgery are the same than those for breast augmentation with lipofilling, with some tricks to do the surgery easier. Fat grafting should be performed before the implant removal; this provides a tight breast, being the infiltration more comfortable than with a deflated breast. Periprosthetic capsule is removed only partially, leaving the anterior and posterior capsule in site with the purpose of placing fat grafts under the well-vascularized capsule tissue and also helping to retain the graft. Mastopexy, if needed, is performed after grafting, keeping in mind that the markings can change after the volume changes of implant removal and lipofilling.

Arms

Surgical procedures to enhance the aesthetics of the arms are not usually reported, even when the patients, commonly women, are concern about a body zone that is very exposed with short sleeve dresses or tank tops. Proposed treatments range from surgical excision of skin and fat (brachioplasty), liposuction, or a combination of both. Recently, the focus also went on fat grafting as a way to improve the results, combined with liposuction [41, 42].

The arms are evaluated in order to check the harmony of its fat distribution, removing fat from the exceeding areas as those between the muscle groups, defining more the muscle shape, and grafting this fat where the muscles use to protrude more, as the deltoid prominence [41, 43, 44]. The approach is be different in women than in men, wherein the volume of muscle infiltration in men can be higher than in women.

The technique is the same as the one previously described for the breast, but here larger cannulas, 12 cm long, 14G, and holes of 2 mm, can be used for infiltration to make it faster and more comfortable.

Hands

Technical options to rejuvenate effectively the hands are scarce, being one of the most exposed areas of the body, which can disclose the age of the person. Fat atrophy, pigmentations, noticeable dorsal veins, tendons, and bones are some of the features of hand aging. Lasers and chemical peelings can contribute to improve the superficial changes of the hands skin, but tissue thickness can only be corrected by means of synthetic fillers or lipofilling. Synthetic filler is an effective option but expensive in the long run because the volume needed is far high than what is needed in facial treatments, and furthermore, the volume fades totally in a year.

Lipofilling provides an inexpensive option for the volume restoration of the dorsum of the hands, which is durable, autologous, and with regenerative parallel effects [45–48]. Fat will be obtained with a cannula with holes of medium size (from 1 to 2 mm) and infiltrated from stab incisions in every web space and at ulnar and radial sides of the hand. The dorsum of the distal phalanxes will be also treated from these incisions (Fig. 7).

Here it is very important to use a blunt cannula, to avoid damaging the veins, tendons, and nerves that run just where we will place the fat:

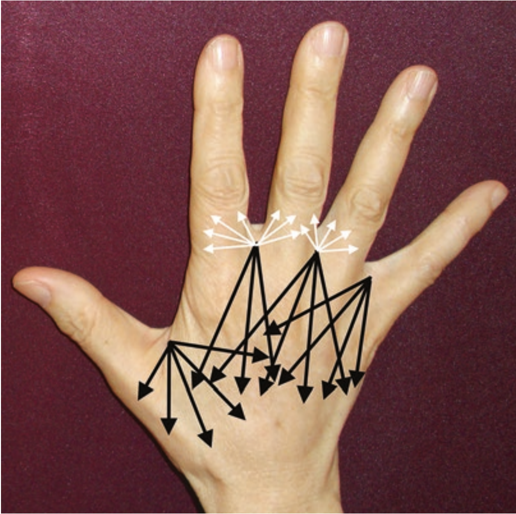

Fig. 7 Entry points for hands lipofilling. Black arrows for dorsal hand infiltration, and this access can be also used for proximal dorsal aspect of finger infiltration

the subdermal plane. Marking these anatomical structures before the surgery is useful to locate them when the swelling hinders their visibility. It is also noteworthy that patients requiring treatment of the hands will have atrophy of the skin, so injection should be done evenly avoiding clumps of fat, which will be very noticeable.

Injection will be done by means of 1 cc syringes, like the other sensitive areas of the body. Cannulas will be of 17G, with a length of 9 cm (or 7 cm for short hands). For each hand, 20–30 cc of fat will be used. At the end of the surgery, stab incisions are protected by means of adhesive strips, and the patient is advised to keep the hands in an elevated position and avoid massaging the treated area to prevent displacement of the fat.

Complications of hands lipofilling are not usual, being the prolonged edema the only issue to discuss with the patients because it takes weeks to resolve. Infections have been reported occasionally [49, 50].

Buttocks

Together with breast augmentation with lipofilling, buttocks augmentations by means of fat grafts have risen notoriously in the last years since it became one of the most popular procedures in many countries, especially in Latin America [51]. As for the breast augmentation, the standard for buttocks augmentations has been the silicone implants. But in this location, which is mechanically very demanding (sitting, walking, exercising, etc.), complications of the implants are more common and concerning than in the breast (extrusion, displacement, pain, discomfort, etc.) [52, 53]. Postoperative course is also tough because the usual intramuscular plane for the implants means many days of pain and limitations for walking.

For this reason the use of lipofilling has meant a shift in this region surgery, because it is almost painless; if there is enough donor areas, it provides more volume than the implants can (submuscular plane here has a limited capacity for solid implants); volume can be placed where the surgeons want instead of placing an implant in a specific anatomical pocket; and the combination with liposuction of the flanks and hips harmonize all the area [54].

An important limitation of buttocks lipofilling is the amount of graft necessary to obtain a noticeable change. Volumes close to 500 cc for each side are advisable for a satisfactory result. If is not expected to obtain this amount, the patient should be informed that maybe an implant is a better option. Like for the breast augmentation, the combination of implants and fat grafting leads to better results because the fat hide the contour of the implants and can be also placed in locations not indicated for the implants, e.g., the lower pole or outer quadrants.

The cannulas used here can be larger than those used in the breast and the face: 12–14 cm long, 14–12G, and holes of 2–2.5-mm are acceptable. Infiltration is performed from the peripheral limits of the buttocks, being very important to avoid high pressures of infiltration deep in the area of emergence of the gluteal vessels, because it is considered a dangerous area for intravascular fat injections and subsequent fatal fat embolism (Fig. 8).

Patient is warned to avoid long periods of sitting on the same area of the buttocks during the first month after the surgery to allow the graft to vascularize. As this surgery implies extensive liposuction to harvest a high volume of graft,

compression garment must be worn; this should be designed with a low-pressure zone in the buttocks area to ensure good blood flow to the grafted area (Fig. 9).

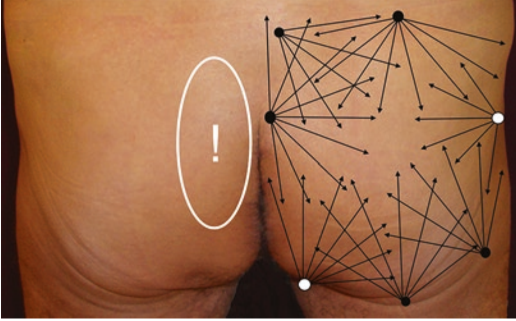

Fig. 8 Injection points for the gluteal lipofilling. As many as needed can be done at the peripheral of the buttocks, but as shown in the right side, deep injection in the inner quadrants should be avoided because of the risk of injection in the gluteal vessels

Concern about complications of buttocks lipofilling have raised recently, as this procedure has become more popular, especially fat embolism because of its potential fatal outcomes [51, 55, 56]. Common complications with other liposuction and lipofilling procedures can appear, such as fat necrosis or contour irregularities, but the most worrisome is fat embolism in the pulmonary vessels or right ventricle, which does not have a specific treatment and is usually fatal when diagnosed. These should be suspected when the patient presents a sudden cardiovascular collapse during the surgery or the first hours after the surgery. Supportive measures should be established as soon as possible. To limit this risk, avoiding deep infiltration in the gluteal veins emergence points, in the piriformis muscle area, is recommended. This problem does not seem to have a direct relationship with the volume of fat injected.

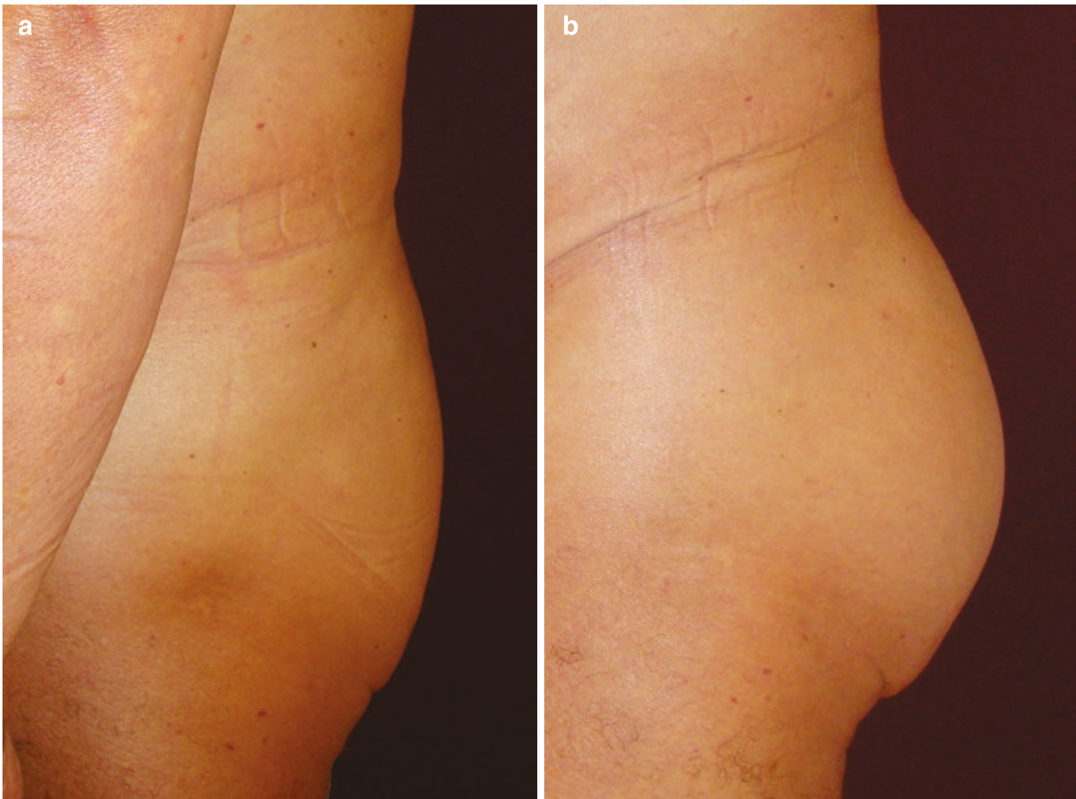

Fig. 9 Buttocks lipofilling. (**a**) (left) Before. (**b**) (right) After. If enough fat is available, nice results in terms of volume and projection can be achieved

Thighs and Calves

These areas are not the most popular for fat grafting, although calves augmentation with implants is a procedure requested from some patients. Results can be different to those obtained using implants, which usually are placed submuscularly creating an effect of hypertrophied gastrocnemius muscles. Lipofilling can provide wider calves but not defined muscle shape. Therefore, the best indications will be women with thin legs who are looking for more defined calves [57].

For the thighs, indications are more restricted to lean patients looking for a less skeletonized appearance. This applies to the HIV+ patients affected of lipoatrophy in whom the limb muscle boundaries are very noticeable.

In the limbs, fat will be injected subcutaneously, avoiding muscles because these distal areas differ from the buttocks in having important vascular and nervous structures more superficial, prone to be damaged. However, as for the buttocks, high amounts of fat are necessary for satisfactory results, up to 300 cc for each thigh or each calf. The cannulas recommended for this area are the same than for the buttocks.

Female Genitalia

In women, different signs of aging can be noticeable at the genitalia level. In those overweight women, a sagging bulky pubis can indicate the need of pubis liposuctions and skin reduction. But in lean women, sometimes the problem is a deflated and wrinkled appearance of the labia majora, with more exposed and visible labia minora.

Lipofilling in the labia majora is an easy way to provide a fleshier look, hiding the labia minora [58]. The procedure is easy and can be performed under local anesthesia and sedation, and the volumes used for each side range from 20 to 40 cc, applied in multiple layers, from deep to superficial, with a single-holed blunt-tip 16G cannula 9 cm long.

Male Genitalia

Penile girth enlargement is an indication of the lipofilling [59]. It is only a cosmetic indication, because it is not reported that sexual performance improves after this kind of procedure. Different options include dermal fat grafts, vein grafts, and acellular dermal matrix, among others. These options are more invasive and complications should be discussed thoroughly with the patients.

Due to the particular features of this area, technique is very specific. Fat is injected by means of a blunt cannula of 16G in four stab incisions around the penis at the 1, 5, 7, and 11 o'clock positions. Up to 15 cc are injected from each of these locations. The plane of injection is under the superficial fascia, in the areolar tissue over the Buck's fascia. Fat graft should be of small size, obtained with cannulas with holes of 1 mm, and due to the thin and soft tissue over the grafted plane, this area becomes at high risk of developing lumps.

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Enriched Plasmas: Concepts and Processing

Paloma Tejero, Lucía Jáñez, and Victoria Sunkel

Introduction

The increase in life expectancy in the population makes necessary to search for safe and affordable treatments, which improve the quality of life and help to slow down the aging process. Platelet-rich plasma (PRP), as a restorative and autologous treatment, achieves rejuvenating effects, with the application of minimal amounts of blood plasma into the skin, and restores normal metabolism, and cutaneous functions deteriorated over the years, by biological activation of skin cells. Among the many biological materials which are available today, the use of blood plasma has attracted a special interest. Nevertheless, as it was initially considered a minor therapeutic option against cell autotransplant from other tissues, its knowledge and development has been slow [1].

History

The first growth factor (NGF) was discovered by Rita Levi-Montalcini in 1948 (Fig. 1), opening a new era in Cell Biology. Subsequently, EGF was isolated by Stanley Cohen. Their findings have been essential for the understanding of the control mechanisms which regulate cells and tissue growth [1].

Fig. 1 Rita Levi-Montalcini won the Nobel Prize in Physiology or Medicine in 1986 for her discovery of Nerve growth factor (NGF) (Reproduced from Sandrone 2013)

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In 1954, Kingsley [2] first used the term PRP to earmark thrombocyte concentrate, during experiments related to blood coagulation. The investigations which led to the use of autologous plasma rich in growth factors began in 1965, with Marshall Urist [3], who discovered and described the importance of bone morphogenetic proteins (BMP) in tissue regeneration.

The term "fibrin glue" was introduced by Matras [4], in 1970. This biological material, made by polymerizing fibrinogen with thrombin and calcium, was found to improve healing of skin wounds in rat models. However, due to low concentration of fibrinogen in donor plasma, the quality and stability of fibrin glue was suboptimal [5]. Between 1975 and 1978, numerous research works suggested an enhanced concept for using blood extracts and designated them as "platelet–fibrinogen–thrombin mixtures" [6].

In the 1980s, autologous platelet-poor plasma (PPP) began to be used in the United States during surgery as a hemostatic element or adhesive gel, rich in fibronectin, Von Willebrand factor, vitronectin, thrombospondin, and other adhesive proteins [1]. In 1986, Knighton et al. [7] first demonstrated that platelet concentrates (PC) successfully promoted healing, and they termed it as "platelet-derived wound healing factors (PDWHF)", which was successfully tested for the management of skin ulcers.

Until the beginning of the 1990s, PRP had not been used as a tool for tissue regeneration in surgery and biostimulation [1]. In 1994, Tayapongsak et al. [8] studied autologous adhesive fibrin as an intrinsic mechanism of the cellular response applicable to tissue regeneration. In 1997, Whitman et al. [9] named their product PRP during preparation, but as the final product had a gel consistency, it was finally named "platelet gel".

The development of these techniques continued slowly until the article of Marx et al., in 1998 [10], who described three platelet growth factors: platelet-derived growth factor (PDGF), transforming growth factor β1 (TGFβ1), and transforming growth factor β2 (TGFβ2). At that moment, the interest in these techniques began to grow. However, all these products were designated as PRP, without deliberation of their content or architecture, and this paucity of terminology continued for many years. Some commercial companies, in order to achieve better visibility, started labeling their products with distinct commercial names [5]. In 1999, Anitua [11] proposed to use the term, coined by himself, plasma rich in growth factors (PRGF), and explained that these proteins had properties such as chemotaxis, proliferation, and cell differentiation, all of them key processes for tissue repair and regeneration. This was one of the popular methods advertised on large scale to prepare pure platelet-rich plasma. It was commercialized as plasma rich in growth factors (PRGF) or also called as preparation rich in growth factors (EndoRet, Biotechnology Institute BTI, Vitoria, Spain). Another widely promoted technique for P-PRP was commercialized by the name Vivostat PRF (Alleroed, Denmark). However, as the name implies, it is not a PRF, but produces a PRP product [5]. In 2000, Choukroun et al. [12] developed another form of PC in France, which was labeled as PRF, based on the strong fibrin gel polymerization found in this preparation. It was called a "second-generation" platelet concentrate, because it was obviously different from other PRP. This proved an important milestone in the evolution of terminology [5]. Everts et al. [13, 14] focused on the leukocyte component of the platelet concentrate, and the two forms, i.e., non-activated and activated. The inactivated/non-activated product was called "platelet-leukocyte rich plasma" (P-LRP) and activated gel was labeled "platelet-leukocyte-gel" (PLG). In 2009, the first classification about platelet concentrates was proposed by Dohan Ehrenfest et al. [15]. In 2012, Mishra et al. [16] proposed another classification, which was limited to PRP, and only applicable to sports medicine. At about the same time, DeLong et al. [17] made a similar classification. In 2013, Tunalı et al. [18] introduced a new product called T-PRF (Titanium-prepared PRF). One year later, Choukroun [19] introduced an advanced PRF called A-PRF (claimed to contain more monocytes). In 2015, Mourão et al. [20] provided a detailed technical note on preparation of i-PRF [5].

Concepts and Foundations

Platelet-rich plasma (PRP) or plasma rich in growth factors (PRGF) is defined as an autologous sample of blood with platelet concentration above baseline values [21] (Fig. 2). It is an autologous platelet concentrate, in a reduced plasma volume, containing trophic factors, which are released when platelets are activated by calcium chloride, thrombin, fibrinogen [22], or any other platelet activator. Theoretically, these two plasmas are not the same, because all PRP is a PRGF (with a suitable platelet activation stimulus), but not the other way around. However, in practice, both terms are used as synonyms. On the other hand, trademark protections and trade names make it difficult to unify terminology [1].

Terminology and Classification

Literature on PRP is extensive, but the published results are often contradictory, and their relevance is controversial. There are many preparation techniques, terminology, and numerous applications, which make their classification difficult. According to the international scientific literature on the subject, and the evolution of clinical trends, it is difficult to establish which products are really useful [23, 24]. This is due to several factors: (1) Many techniques are available for the production of platelet concentrates, leading to different final preparations; (2) There is no unified terminology to classify and describe the many variants of platelet concentrates; (3) The lack of precise characterization of products and techniques tested in most of the articles published gives rise to a huge literature of thousands of articles which constitutes an authentic "blind library of knowledge"[25]. Improving classification and clarifying terminology are mandatory; however, this effort is still in its beginning.

Fig. 2 PRP appearance

In 2009, Dohan Ehrenfest et al. [15] proposed the first classification of platelet concentrates. Three main sets of parameters are necessary for a clear classification of platelet concentrates: The first set of parameters (A) relates to the preparation kits and centrifuges used; the second type of parameters (B) relates to the content of the concentrate; the third set of parameters (C) relates to the fibrin network which supports the platelet and leukocyte concentrate. Based on parameters B and C four families are defined:

1. **Pure Platelet-Rich Plasma (P-PRP) or Leukocyte-Poor Platelet-Rich Plasma** are preparations with a low-density fibrin network after activation. By definition, all the products of this family can be used as liquid solutions, or as an activated gel. They can be injected, or used as a gel in wounds or sutures (as a use similar to fibrin glue). There are many methods of preparation, particularly the use of cell separators (continuous flow plasmapheresis), although its use in daily practice is not efficient. A widely known method of P-PRP production is the trade name PRGF [11] (Plasma Rich in Growth Factors or EndoRet, Biotechnology Institute BTI, Vitoria, Spain) which was tested in many clinical trials, particularly in sports medicine. Another P-PRP technique was promoted for the treatment of skin ulcers, and is known as Vivostat PRF (Fibrin rich in platelets, Vivostat A/S, Alleroed, Denmark); however, this technique is not a PRF, but clearly a product of P-PRP [26].
2. **Platelet-rich plasma and leukocytes (L-PRP)** is prepared with leukocytes, and with a low-density fibrin network after activation. All the products of this family can be

used as liquid solutions or as an activated gel [6]. The largest number of commercial and experimental systems, with many interesting results in general surgery [13], orthopedics and sports medicine [14], belong to this family. Recently, many automated protocols, which require the use of specific kits, allowing a minimum manipulation of blood samples, and a maximum standardization of preparations, have been developed, i.e. Harvest Smart-PreP, Biomet GPS III, both from USA and other kits such as Plateltex (Czech Republic) or Regen PRP (Switzerland) [25].

3. **Leukocyte-poor or pure platelet-rich fibrin (P-PRF)** concentrates have a high-density fibrin network. These products can only be found as an activated gel, and are not suitable to be injected or used like traditional fibrin glues. However, due to their fibrin matrix, they can be manipulated as a real solid material for other applications. In this category, there is only one method available, the Fibrinet PRFM kit, by Cascade Medical (New Jersey, USA), also marketed for orthopedic applications by Vertical Spine, USA. The main drawback remains the cost and relative complexity compared to the other forms of PRF available, L-PRF (fibrin rich in leukocytes and platelets) [25].
4. **Leukocyte- and platelet-rich fibrin (L-PRF)** concentrates are prepared with leukocytes and a high-density fibrin network [27]. This PRF protocol is a simple and free technique developed in France by Choukroun et al. [28]. It can be considered as a second-generation platelet concentrate, because the natural concentrate is produced without any anticoagulants or gelifying agents, and this is still a key difference with the other product families. The PRF clot forms a strong fibrin matrix, with a complex three-dimensional architecture, in which most of the platelets and leukocytes from the harvested blood are concentrated [29]. The only system approved by the FDA with certified materials is marketed under the name Intra-Spin L-PRF (USA) [17, 30].

This classification system was widely cited, defended and validated by a multidisciplinary consensus conference published in 2012 [31]. This terminology and classification are now considered as a basis of consensus in many fields, particularly in oral and maxillofacial disciplines. The POSEIDO (*Periodontology*, Oral Surgery, Esthetic and Implant Dentistry Organization) keeps it as its guideline for all its publications on the subject [16].

Other classification systems have been proposed in recent years, but they are limited to platelet-rich plasma products and sports medicine applications:

- Mishra et al. [16] proposed a classification only for sports medicine applications, based on the presence of leukocytes, and the activation of PRP: (1) Type 1 PRP: L-PRP solution; (2) Type 2 PRP: L-PRP gel; (3)Type 3 PRP: P-PRP solution; and (4)Type 4 PRP: P-PRP gel. This classification is limited only to PRP, and is less intuitive. The only new parameter of this classification is the evaluation of the platelet concentration: type A PRP is five times or more the blood platelet concentration, and type B PRP is less than five times the blood platelet concentration. This last parameter is debatable, since the concept of platelet concentration was largely abandoned in previous years for a logical reason: the concentration of platelets depends solely on the serum volume used to keep the platelets in suspension. The amount of serum varies greatly according to the protocol and the expected application, and has no impact on the expected effect. The concept of absolute platelet count would be more accurate, although most publications failed to detect a clear and reproducible impact of this parameter on clinical outcomes [32].
- Delong et al. [17] proposed the PAW classification system, which is based on three components: (1) the absolute number of Platelets, (2) the manner in which platelet Activation occurs, and (3) the presence or absence of White cells. This system is again limited to PRP families, in fact it is very similar to the one proposed by Mishra et al. [16]. Regarding the number of platelets, no publication has been able to define what would be the optimal

number of platelets, or even if the concept actually exists, in complex multi-component materials such as platelet concentrates [32]. The two previous classification proposals are interesting, but they do not rely significantly on the evidence, and do not really allow updating the current terminology and classification.

- Mautner et al. [33] considered that none of the previously published PRP classifications presented all the characteristics that might influence PRP activity and efficacy. The authors evidenced that is important to describe the platelet count (absolute number/μl), leukocyte content (positive or negative) and, if present, the percentage of neutrophils, red blood cells content (positive or negative), and activation. This classification is known as PLRA (Platelet, Leukocyte, Red blood cells and Activation).
- In 2016, Magalon et al. [34] published a new classification proposal, the DEPA, which classifies PRP based on: (1) the Dose of injected platelets; (2) the Efficiency of the production, in other words, the percentage of platelets recovered; (3), the Purity of PRP obtained; and (4) the Activation process. In this classification, it is necessary to determine the complete cell count for whole blood and PRP, as well as the volume of collected blood and injected PRP.
- In 2017, Lana et al. [35] made another attempt to standardize the PRP procedure, the MARSPILL classification, which describes PRP contents, studying cellular and molecular components. The main focus is on mononuclear cells (progenitor cells and monocytes). This classification incorporates important variables related to PRP application, such as the harvest Method, Activation, Red blood cells, number of Spins, Platelets, Image guidance, Leukocytes number and Light activation.

Basis of the Use of Platelet-Rich Plasma

Cell Repair and Regeneration

When a tissue is injured, scarring process is initiated as a result of migration, division, and cellular protein synthesis, which ends with a tissue without functional capacity called scar. Repair is the tissue restoration without retaining its original architecture or function. On the other hand, regeneration is the restoration, maintaining properties indistinguishable from the original tissue. The higher is the specialization of the affected tissue, the lower is its capacity for regeneration. Once the tissue injury occurs, a hematoma forms at the site of tissue damage, the platelets adhere to the exposed collagen creating a clot, and the inflammatory phase begins with platelet activation, resulting in its degranulation and release of growth factors. The access to the site of the wound by neutrophils and macrophages occurs within a few hours of the injury, and then phagocytosis of tissue remains begins. A few days after the injury, the proliferative phase begins, and is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction. Finally, the phase of remodeling involves the maturation of collagen, and the apoptosis of the excess cells, which can take several weeks to months after an injury, depending on the degree of damage [1].

Platelets are anucleated cell fragments originated from megakaryocytes cytoplasm [36]. They are lens-shaped, with a diameter of 2-3 μm. Their normal concentration in peripheral blood is 150 to 400 × 10^9/L, and their half-life is between 7 and 10 days [1]. Physiological activation is originated after three fundamental episodes: platelet adhesion, shape change, and platelet aggregation. In addition to its well-known role in hemostasis, with the formation of a clot in response to the injury of a vessel, platelets perform essential functions in tissue regeneration, angiogenesis, immunity, inflammation, tumor progression, and thrombosis. This activity is due to the different proteins contained in their granules, which are divided into three types: (a) alpha: they contain fibrinogen, Von Willebrand factor, platelet-derived growth factor (PDGF) and other growth factors; (b) delta (dense): they contain ADP, ATP and serotonin, which are potent agonists or platelet activators; and (c) lambda: they are lysosomes, which help to dissolve the clot once it has fulfilled its function [36].

The large amount of growth factors contained in the platelet granules; its ability to synthesize proteins; its microbicidal and inflammatory modulating activity, which favor cell proliferation and extracellular matrix synthesis, have led to propose the use of autologous PRP for the repair and regeneration of tissues [37]. Platelets are chosen as substrate, since they are relatively easy to obtain, and in addition to growth factors, they transport other proteins useful in tissue regeneration and repair. Platelet-rich plasma is a volume of autologous plasma which contains an amount of platelets four to six times higher [36] (1,000,000 platelets/μL) of the one found in normal blood (150,000 platelets/μL) [1].

Growth Factors in Platelet-Rich Plasma

In recent years, the properties of growth factors in the induction of tissue repair and regeneration have been described: (a) they attract and lead cells toward the place where they are necessary for tissue repair; (b) they take part in cell division; (c) they stimulate angiogenesis; and (d) they activate the synthesis of cell matrix [1].

Growth factors (GF) are significantly increased in PRP, compared to whole blood and platelet-poor plasma [38]. Growth factors are biological mediators which regulate essential functions in tissue regeneration/repair: chemotaxis, angiogenesis, cell proliferation, differentiation and regulation, and extracellular matrix synthesis. These growth factors are synthesized and stored in the megakaryocytes of multiple cells and tissues: fibroblasts, osteoblasts, kidney, salivary glands, lacrimal glands and platelets, among others. After activation and degranulation, the platelets will release several growth factors, of which, those in highest concentration are platelet-derived growth factor (PDGF) and transforming growth factor (TGF) [1].

Several growth factors have been found in platelet-rich plasma, including the three isoforms of platelet-derived growth factor (PDGF): PDGF AA, PDGF BB and PDGF AB; two isoforms of transforming growth factor (TGF): TGF β1 and TGF β2; vascular endothelial growth factor (VEGF); epidermal growth factor (EGF); and fibroblastic growth factor (FGF) [1].

- Platelet-derived growth factor (PDGF) is a potent growth factor for various connective tissue cells. Many other cell types also synthesize PDGF, including macrophages, endothelial cells, fibroblasts, and glial cells. It is a powerful chemo-attractant and stimulator of cell proliferation [39]. PDGF acts as a key mediator in wound healing. It was the first approved recombinant growth factor in topical application to accelerate wound closure. However, the PDGF plays a role throughout the wound healing process. At first, PDGF attracts fibroblasts, neutrophils, and monocytes to the site of injury. It is also a mitogen for fibroblasts, and promotes the production of extracellular matrix (ECM). In the proliferative phase, PDGF stimulates the differentiation of fibroblasts into myofibroblasts, promoting the contraction of the wound. During remodeling, it stimulates collagenase production in fibroblasts. PDGF AA and PDGF BB are the principal mitogens of human cells in vitro. PDGF also has a role in the activation of macrophages [1].
- TGF-β is a multifactorial regulator of cellular growth in developing systems. TGF-β molecules are one of the most important regulatory growth and differentiating factor superfamily [39]. TGFβ is an important mitogen and morphogen. TGF β1 and TGF β2 act as autocrine or paracrine growth factors, mainly affecting the fibroblasts, the stem cells of the marrow, and the preosteoblasts. TGFβ represents a mechanism for the maintenance of a long-term healing. It also has an inhibitory effect on epithelial cells migration [1].
- VEGF is involved in both vasculogenesis and angiogenesis, which paves the way for healing [40]. VEGF is also a vasodilator, increases microvascular permeability, and promotes the recruitment of pericytes to support neovascularization. It is found in the blood clots present in the wounds, and begins to act when the fibrin clot forms [1, 39].
- EGF stimulates epidermal regeneration, promotes wound healing by stimulating the proliferation of keratinocytes and dermal

fibroblasts, and enhances the production and effects of other growth factors [41]. EGF stimulates the growth of various epidermal and epithelial tissues in vivo and in vitro, and of some fibroblasts in cell cultures. It is also usually present in tears. Two other members of the EGF family, heparin-binding epidermal growth factor (HB-EGF) and TGFα, have a role in wound healing by stimulating the proliferation of keratinocytes, and consequently, re-epithelialization. In general, EGF is the growth factor that is most administered topically, but it can also be administered intravenously. It is applied in burns, ulcerations, and corneal injuries [1].

- The insulin-like growth factor (IGF) is a group of peptide hormones produced by the liver, under the stimulation of growth hormone (GH). Two different IGFs (IGF-I and IGF-II) have been described. The first isoform (IGF-I) is present throughout life, decreasing with age. The second form (IGF-II) is present only during the fetal stage. The structure of both IGFs is homologous to human pro-insulin [39]. Because of this similarity in chemical structure, the topical application of insulin has been found to accelerate wound healing in experimental models [1]. However, IGFs do not cross-react immunologically with each other. IGF is constitutively produced in many tissues, including liver, kidney, heart, lung, fat tissues, and various glandular tissues. IGF-I is also produced by chondroblasts, fibroblasts, and osteoblasts. IGF-I is chermotactic for fibroblasts, and stimulates protein synthesis. It stimulates type I collagen biosynthesis, cellular proliferation and differentiation [39]. Many tissues contain IGF-I receptors (muscle, digestive tract, skin, among others). In terms of wound healing, evidence suggests that IGF-I is mitogenic for keratinocytes and fibroblasts, inhibits apoptotic pathways, attenuates production of proinflammatory and anti-inflammatory cytokines, and stimulates the production of extracellular matrix components. IGF-I levels in the wound fluid demonstrate a positive direct correlation with the success of wound healing [1].
- Fibroblast growth factor (FGF) increases hair growth by inducing the anagen phase of hair follicles. It promotes dermal papilla cell proliferation, and has been found to increase the hair follicle size in mice. Another function is to stimulate angiogenesis [42].
- Hepatocyte growth factor (HGF) is an angiogenesis stimulator [42].

PRP also includes three proteins in the blood known to act as cell adhesion molecules: fibrin, fibronectin, and vitronectin [43].

Platelet-Rich Plasma Mechanism of Action

It has been hypothesized that platelets, and particularly platelet-derived growth factor (PDGF) contained in their alpha granules, are responsible for the biologic effects of PRP [44, 45]. The GF released from the alpha granules of platelets during thrombosis interacts with surface receptors on the target cells, activating intracellular signaling pathways, which induce the expression of genes required for regenerative processes, such as cell proliferation and extracellular matrix formation [21]. Based on the physiological processes described above, the acceleration of wound healing through the addition of platelet-rich plasma is based on diverse platelet growth factors that stimulate the different stages of the wound repair cascade. Compared to the application of individual or recombinant growth factors, which are applied in supraphysiological concentrations, PRP has the advantage of offering multiple growth factors which work synergistically at the site of the wound, and at concentrations closer to physiological conditions. In addition, the production of type III collagen, elastin and hyaluronic acid from its precursors, proline, lysine and glucosamine, respectively, and the strengthening of the fibrin mesh would allow maintaining the sustained release of bioactive molecules [1]. The active secretion of these growth factors by platelets begins within 10 min after activation, with more than 95% of the pre-synthesized growth factors secreted within 1 h [46]. These growth factors act on the target cells and, in this way, PRP initiates a larger and faster cellular response

than a normal blood clot. Fibrinogen (already converted to fibrin), in combination with growth factors present in PRP, could effectively promote wound healing at the site of the injury, and the formation of the fibrin clot could stimulate collagen synthesis. The ability of PRP to modulate cytokine release has also been demonstrated. PRP has been found to promote significant changes in monocyte-mediated proinflammatory cytokine/chemokine release. LXA(4) was increased in PRP, suggesting that PRP may suppress cytokine release, limit inflammation, and, thereby, promote tissue regeneration. Monocyte chemotactic protein-1 (MCP-1) was suppressed by PRP, whereas RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) was increased in monocyte cultures. PRP stimulated monocyte chemotaxis in a dose-dependent fashion, whereas RANTES, in part, was responsible for PRP-mediated monocyte migration [38].

The complex mix of growth factors released by the platelets acts in synergy, while protecting themselves against rapid degradation. A sudden increase in platelet concentration can cause a saturation, and negative regulation of the growth factor receptors. It has been suggested that growth factors might be more effective when they are released directly by the platelets, in a slow and pulsed way, instead of being administered as a bolus dose, to improve the physiological response to the injury. In addition, it has been observed that platelet growth factors have a dose–response relationship in fibroblast proliferation and the production of type I collagen. High platelet concentration inhibits this proliferation, while low platelet concentration does not show optimal results; therefore different concentrations of platelet growth factors can have an impact on the results which can be obtained in vivo [1].

Platelet-Rich Plasma Anti-aging Mechanism

Aging of human skin results from a combination of a gradual decline in function over time (intrinsic aging) and cumulative damage caused by environmental factors (extrinsic aging), which include smoking, exposure to chemicals, and notably, ultraviolet B (UVB) radiation. In dermis, UVB has shown to stimulate collagenase production by human dermal fibroblasts. In skin, which is continually exposed to UVB, collagen degeneration and altered deposition of elastic tissue occur. As a result of this, the structural integrity of the dermal ECM is impaired, causing wrinkles and loss of skin resilience. Dermal fibroblasts play a key role in the aging process, through their interactions with keratinocytes, adipocytes, and mast cells [47]. They are also the source of ECM proteins, glycoproteins, adhesive molecules, and various cytokines [48]. Fibroblasts contribute to the fibroblast–keratinocyte–endothelium axis that maintains skin integrity and youthfulness, by producing ECM molecules and supporting cell-to-cell interactions [47, 49]. Growth factors present in PRP may induce the synthesis of collagen and other matrix components by stimulating the activation of fibroblasts, thus regenerating and rejuvenating the skin, and therefore treating wrinkles [47].

Autologous platelet-rich plasma (PRP) has received a lot of attention recently for its healing and rejuvenating properties and has been used to treat scars, or hair loss. PRP has been investigated for its effects on the remodeling of the extracellular matrix, a process which requires activation of dermal fibroblasts, whose function is essential for rejuvenation of aged skin [50]. Topical application of growth factors stimulates the rejuvenation of photoaged facial skin, improving its clinical appearance and inducing new collagen synthesis [51]. Nevertheless, experimental studies confirming the effects of PRP on aged fibroblasts are very limited [47]. In 2010, Redaelli et al. [52] studied the in vivo effects of PRP injection on facial and neck rejuvenation, and demonstrated that injections of high concentration PRP increased levels of type I collagen and the expression of MMP-1 and MMP-2 in human dermal fibroblasts. The authors found that levels of procollagen Type 1 carboxy-terminal peptide were high in cells grown in the presence of 5% PRP. In addition, PRP increased the expression of Type I collagen, MMP-1 protein, and mRNA in human dermal fibroblasts. These findings suggest that PRP may have the potential to promote the remodeling of aged and photoaged skin [47]. The improvement

of skin aging has been attributed to the effect of platelet concentration in the proliferation of mesenchymal stem cells. Another mechanism is the acceleration of endogenous hyaluronic acid production. Hyaluronic acid absorbs water and produces redensification of the ECM, increasing the skin volume and turgor. All the processes described above, and some other unknown, contribute to PRP skin rejuvenation [1].

Platelet-Rich Plasma Processing

Currently, there is a great discussion and no consensus regarding PRP preparation. PRP is prepared through a process known as differential centrifugation, in which acceleration force is adjusted to sediment certain cellular constituents, based on different specific gravity [53].

PRP is obtained through a series of protocolized procedures that vary according to the technique to be used. PRP preparation can be carried out under two different techniques [42]:

- **Open technique**: the product is exposed to the environment and comes in contact with different materials, such as pipettes or product-collection tubes. In the blood processing to obtain PRP with the open technique, it should be guaranteed that the product is not contaminated during handling.
- **Closed technique**: it involves the use of commercially available disposable kits (including centrifuge equipment and application) in which the product is not exposed to the environment (recommended).

PRP processing depends on the type of device chosen, and should be done according to the manufacturer's instructions. There are different PRP systems, which facilitate the preparation of PRP in a reproducible manner. All of them operate on a relatively small volumes of drawn blood, and are based on the principle of centrifugation [42].

The systems to obtain PRP differ extensively in their ability to collect and concentrate platelets, depending on the method and time of its centrifugation. Each preparation may produce different concentrations of platelets and leukocytes, with different applications. It is difficult to assess which kit for PRP preparation is better [53]. There is controversy in the number of centrifugations required and their speed duration, the ideal volume of PRP to be administered, the frequency of application, the site of administration, and the most suitable techniques and devices [42].

The technique to obtain PRP is very simple, however, it is important to take into account the legislation in force in the territory where the procedure is carried out. PRP use is immediate, therefore it does not require storage or transport. Six moments can be clearly distinguished throughout the process: extraction, centrifugation, collection, concentration, activation, and application [1].

Extraction

PRP is obtained from a sample of patients' blood drawn at the time of treatment. A 30 cc venous blood draw will yield 3–5 cc of PRP, depending on the baseline platelet count, the device used, and the technique employed [53]. The whole blood is obtained by venipuncture in anticoagulated tubes (usually with acid citrate dextrose or sodium citrate solution) [42], to prevent platelet activation prior to its use [53]. The volume of blood drawn may vary depending on the treatment to be applied. The tube volume will vary depending on the manufacturer [1].

Centrifugation

The blood is centrifuged with single- or double spin centrifugation, depending on the device (Fig. 3). The settings of the centrifuge, established to obtain PRP at an adjustable concentration, are defined by the manufacturer and cannot be changed by the physician [42].

The tubes are centrifuged to compact as much as possible the cell fractions, but without breaking the platelets, obtaining in this way the maximum platelet concentration [1].

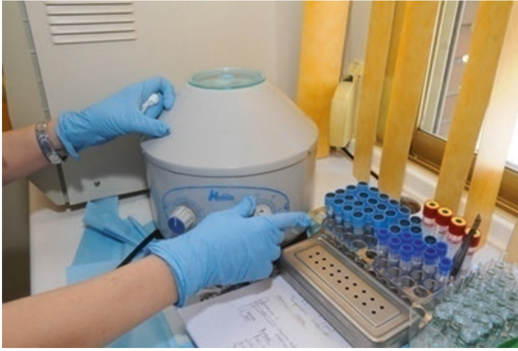

Fig. 3 Centrifugation device

Fig. 4 Platelet-rich plasma extraction

Obtention

After centrifugation, the tube shows three basic fractions: (1) at the bottom of the tube, there are red blood cells (representing almost 50% of its volume), with leukocytes deposited immediately above (a small volume difficult to visualize); (2) the middle layer corresponds to the PRP (Platelet-Rich Plasma); and (3) at the top, there is the PPP (Platelet-Poor Plasma) [42]. The platelet fraction is indistinguishable from the plasma fraction (acellular) supernatant. The lower third of the platelet plasma fraction is where there is the highest concentration of platelets, and is the most interesting from the therapeutic point of view. The upper third of the same fraction is mainly plasma, with very few formal elements (PPP). From the clinical point of view, it is important to keep in mind that centrifugation creates gradients of concentrations of each element, also within each fraction [1].

A needle is inserted to the lower third of the platelet plasma fraction (Fig. 4), almost up to the level of the leukocyte fraction, but without contacting it, and 0.5–0.8 mL of plasma are extracted. It may vary depending on the volume of the tube, and the quality of the PRP that is intended to be obtained [1].

Concentration

The previous step is repeated with all tubes, obtaining 0.5–0.8 mL of each tube, and collecting the platelet-rich plasma in a single syringe. It is very important to make clear that if 1 mL of PRP is obtained from 0.2 mL of 5 tubes, this milliliter will have more platelets, and will be of higher quality than 1 mL of PRP obtained from 0.5 mL of 2 tubes; and the latter, in turn, will be of higher quality than 1 mL of PRP obtained from only 1 tube. In general, it is advisable to take a little volume of PRP from several tubes. Therefore, the PRP vial is constituted by the sum of the lower thirds of platelet fraction of several tubes [1].

Activation

Platelets can be activated before application of the PRP, although there is no consensus on whether or not platelets must be previously activated before their application, and with which agonist [54]. Thrombin and calcium chloride, which are aggregation inducers, are used with the aim to activate platelets and stimulate degranulation, causing the release of GF [55]. A volume of 0.05 mL of 10 M calcium chloride is added for each milliliter of plasma to be injected, i.e. if there is 2 mL in the syringe, it would be activated by adding 0.1 mL of 10 M calcium chloride. This activator of the platelets makes them de-granulate, releasing their

GF. The use of other platelet activators, such as calcium gluconate, more appropriate for gelation, has been also described. Finally, there is also a current which holds that platelets should not be artificially activated [42], claiming that a natural activation occurs at the moment of injection [1].

Conclusions

- Tissue repair begins with clot formation and platelet degranulation, which release the GFs necessary for wound repair.
- GF are potent inducers of normal tissue repair.
- In addition to their tissue-forming and proliferative effects, GFs exhibit chemotactic effects that cause the migration of neutrophils and macrophages, adding an antimicrobial component to the wound site [56].
- Some evidence shows that PRP accelerates healing [1].
- Since the platelet concentration, the levels of GF, the activation method, and the time of application may affect the result, more research is needed to standardize PRP processing [1].
- The discrepancies between in vivo and in vitro studies reflect not only differences of technical protocols but also the greater complexity of healing vital tissues compared to controlled in vitro studies [56].

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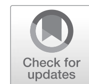

Was It Gold What Shone? Platelet-Rich Plasma Applications, Outcomes and Security Considerations

Lucía Jáñez, Paloma Tejero, and Marina Battistella

Introduction

The clinical application of PRP (platelet-rich plasma) began in the 1990s, with the use of autologous tissue adhesive (fibrinogen and thrombin), which developed as a substance with hemostatic and adhesive properties. Platelets, as a rich source of growth factors, were subsequently added [1]. PRP has been used for rapid healing and tissue regeneration in numerous and growing fields of medicine [2, 3]. Initial applications of PRP were predominantly in musculoskeletal and maxillofacial fields; however, in recent years, its application has been extended to many different fields (Table 1) [3, 4]. The benefit and safety of PRP is documented in more than 5000 studies [5]. Numerous studies are showing a greater scientific evidence in each of these applications, due to its stimulating role in the proliferation of diverse epidermal and mesodermal cell lines [3]. The local application of platelet gel has a positive impact on tissue repair in the first weeks, accelerating the process, although it is not extensible in the long term [1]. One of the applications in which there is general unanimity about the advantages of applying PRP, is in those cases where its effect is sought as a biological adhesive [6].

In 2015, Sclafani et al. [7] reviewed 61 studies (observational, in vitro, animal models, and clinical trials) noting that the vast majority of them suggested a tangible effect of both topical and injectable platelet preparations on cellular changes, facial aesthetics, and wound healing. There are many limitations to the studies included in this review. First, the possibility of a publication bias favoring positive results must be acknowledged. Secondly, the results in many of the case series and case–control studies were difficult to objectively be quantified. The inability to convincingly show dramatic results and quantify evidence to support those results may explain why platelet preparations are not more widely used in plastic surgery [7].

PRP seems to be a promising therapeutic modality but the level of evidence from the available published data is low. Well designed, larger population-based trials are required to validate PRP use and to reaffirm its efficacy and safety [8, 9].

The most representative studies of PRP application will be described below.

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Table 1 PRP applications (modified from Anitua et al)

Plastic and reconstructive surgery Lifting Rhynoplasty Mamoplasty Blepharoplasty Flaps and grafts Burns Fat grafting	**Dermatology** Non-healing wounds Decubitus ulcer Venous ulcer Diabetic ulcer Neuropathic ulcer Bites	**Traumatology** Arthroplasty Spinal fusion Bone repair Grafts Chondroarticular regeneration Intraarticular infiltrations
Aesthetic Medicine Rejuvenation Alopecia Wrinkles and folds Sagging Dyschromia Scars Striae distensae Cellulite Post-peeling/Post-laser	**Neurosurgery** Nerve reconstruction Craneotomy Dural repair CSF leak Pituitary tumors surgery Transphenoidal hypophysectomy	**General surgery** Abdominal surgery Hepatic lobectomy Pancreas surgery Hernia repair surgery Splenectomy Gastrectomy Enterocutaneous fistula
Cardiothoracic surgery Coronary bypass Valvular surgery Bronchopleural fistula Sternal and costal repairs Mediastinum sealing	**Vascular surgery** Carotid surgery Aortic aneurysms Vascular grafts Chronic ulcers	**Oral and maxillofacial surgery** Mandibular reconstruction Bone grafts Sinus repair Dental implant
Ophthalmology Epithelial regeneration Corneal abrasions and ulcers Incisions closure Macular holes	**Otorhinolaryngologic surgery** Thyroidectomy Parotidectomy Acoustic neuroma Timpanoplasty Grafts	**Urologic surgery** Prostatectomy Nephrectomy Retroperitoneal resections

PRP Applications and Outcomes in Aesthetic Medicine

In the field of Cosmetic and Aesthetic Medicine there are several ways to use PRP [6].

PRP Application Modes

Depending on the time and temperature upon activation, a liquid for infiltration or a malleable gelatinous solid, useful for sealing or compacting (platelet gel or clot), may be obtained. If only the adhesive gel without factors is needed, PPP (platelet-poor plasma) can be activated to obtain a fibrin clot [10].

Topical

PRP can be used topically for its stimulatory effects on dermal fibroblasts, as a clot or as a mask, or more frequently, by spraying it. It is usually used as topical therapy after chemical or physical exfoliations or after laser resurfacing [6].

Filler

Platelet concentrates can also be used as a clot (Fig. 1) to fill in scars, wrinkles, or folds such as tear trough [6]. The so-called *Plasma Gel* is obtained from platelet-poor plasma and can be used as a filler. For its preparation, the syringe is prefilled with ascorbic acid 100 mg/mL, 1 mL per 5 mL of blood drawn, and mixed in the syringe. Then, the blood without anticoagulant is centrifuged. The product obtained is charged into 1 mL syringes and introduced into water at 95–98 °C for 3 min, whereby the plasma is gelled by protein denaturation and the material obtained is used as a facial filler (Fig. 2). The heating can also be carried out with a commercial machine. Special attention must be paid to the denaturation

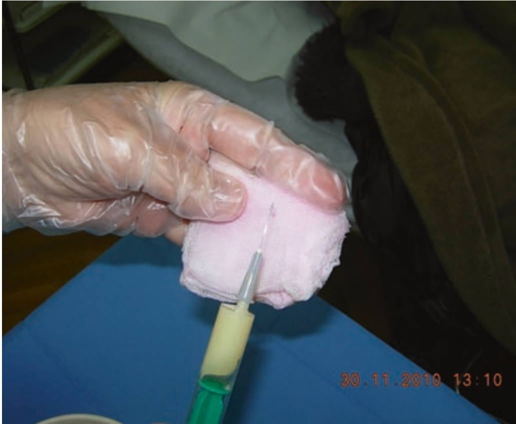

Fig. 1 *Plasma Gel* can be used as a filler

Fig. 2 Heating process to obtain *Plasma Gel*

of proteins, to achieve the formation of a gel with a viscosity that facilitates its injection. Infiltrations are usually performed with a 1 mL syringe and a 27 G needle. This procedure keeps its filling effect for 3–4 months. Once used as a filler, ozonated rich in factors PRP can be used to biostimulate. The biostimulation is repeated with PRP once a month, for 3 months, and then the patient is re-evaluated for a new filling with *Plasma Gel*, which in this case largely replaces the hyaluronic acid [10].

As advantages, this procedure offers maximum profitability and economy by being autologous, and by allowing obtaining several syringes of the product with little blood volume. A disadvantage is the unavailability of growth factors and other cytokines in the plasma, due to the denaturation process. So far, no bibliographic reference that details the effectiveness of this method or the biological effects of this filler on the fibroblast and the synthesis of collagen have been found [11].

Dermocosmetics

There are several commercial topical preparations in the dermocosmetic market, which include growth factors and soluble matrix proteins secreted by human dermal fibroblasts. Topical application of human growth factors has been shown in multiple clinical studies to reduce the signs and symptoms of skin aging, including a reduction in wrinkles and elastosis, and an increase in the synthesis of dermal collagen. At the histological level, it has been demonstrated that growth factors produce an increase of epidermal thickness, and an increase in the density of fibroblasts in the superficial dermis [6].

Intradermal

The general procedure of PRP application is intradermal infiltration. With the aim of performing a skin biostimulation, PRP can be used as a mesotherapy, for the treatment of wrinkles, elastosis, or dyschromias. The intradermal administration of PRP stimulates the production of type I collagen by fibroblasts. Intradermal injection of growth factors produces remarkable clinical changes on aged skin: restores skin vitality, increases skin thickness, recovers elastic consistency, improves vascular inflow, stimulates secretions, and improves smoothness and skin appearance. The growth factors regulate the remodeling of the epidermis and dermis, and have a great influence on skin appearance and texture [6].

Indications in Aesthetic Medicine

Bibliographical references on the use of PRP in the field of Aesthetic Medicine are increasingly proliferating, most of them focused on cutaneous rejuvenation. A limited number of publications are relative to other indications (Table 2).

Table 2 PRP indications in Aesthetic Medicine

Skin rejuvenation
Wrinkles and folds
Sagging
Dyschromia
Alopecia
Tissue repair
Scars
Striae distensae
Cellulite
Post-peeling
Post-laser

Rejuvenation

PRP is one of the most novel regenerative medical tools in anti-aging medicine, and its beneficial effects on skin rejuvenation are patent. However, the mechanism of action of growth factors is a field which needs more research [6]. PRP has rejuvenation capacity per se, and can be used to fight aging cellular involution [5, 12]. It can be used on nappage technique, on mask and as a temporary regenerative filler in combination with thrombin [5]. PRP application in skin rejuvenation improves skin aspect, elasticity, texture, and firmness [9, 13].

Histologic changes have been demonstrated after PRP treatment [14, 15]. Shin et al. [15] demonstrated that PRP thickened the dermal layer, increased the length of the dermoepidermal junction, the amount of collagen, and the number of fibroblasts and keratinocytes, thus increasing dermal elasticity [16].

Animal studies performed by Cho et al. [16] showed the effectiveness of P-PRP (pure platelet-rich plasma) in reducing wrinkles in photoaged mice. In the same line, Liu et al. [17] argued that P-PRP could potentially delay aging in an animal model, by demonstrating that monthly bone marrow injections of P-PRP could promote cell growth, increase osteogenesis, decrease adipogenesis, restore cell senescence-related markers, and resist the oxidative stress in stem cells from aged mice. The preliminary in vitro study of Anitua et al. [18] suggested that PRGF (platelet rich in growth factors) were able to prevent UVB-derived photooxidative stress, and to diminish cell damage caused by ultraviolet irradiation.

Many positive clinical results have been published. Sclafani et al. have carried out several studies to assess PRP efficacy in rejuvenating procedures. In 2010, Sclafani et al. [19] evaluated the efficacy of a single injection of autologous PRFM (platelet-rich fibrin matrix) into the dermis and immediate subdermis, below deep nasolabial folds. Over 12 weeks, treated patients had statistically significant reductions in wrinkle assessment scores. One year later, Sclafani et al. [20] reported a study of 50 patients who were treated with PRFM for aesthetic purposes with a mean follow-up of 9.9 months. Most patients were treated for deep nasolabial folds, while the volume-depleted midface region, superficial rhytids, and acne scars were other commonly treated areas. Patients underwent an average of 1.6 treatments. Complications were minimal and most patients were satisfied with the results, although one patient felt that there was limited or no improvement after two treatments. Later, Sclafani et al. injected PRFM into the deep dermis and immediate subdermis of the upper arms in human volunteers. Full-thickness skin biopsy specimens over a 10-week period supported the clinical observation of soft tissue augmentation. As early as 7 days after treatment, activated fibroblasts and new collagen deposition were noted, and continued to be evident throughout the course of the study. Development of new blood vessels was noted by 19 days; also at this time, intradermal collections of adipocytes and stimulation of subdermal adipocytes were noted. These findings became more pronounced over the duration of the study, although the fibroblastic response became much less pronounced [14].

Small observational studies have been performed looking at clinical effects of P-PRP (pure platelet-rich plasma) on facial skin [7]. Redaelli et al. [21] performed mesotherapy with P-PRP in face and neck of 23 patients, and found that after four weeks 30% reported mild improvement and 61% reported good improvement. They carried out three sessions separated one from the next one month. No control group was used in this study. Mehryan et al. [22] assessed the effect of P-PRP on infraorbital dark circles and crow's feet. Ten participants were treated in a single session with intradermal injections of 1.5 mL of P-PRP on each side. The improvement in infraor-

bital color homogeneity was statistically significant, but no statistically significant changes were observed in melanin content, stratum corneum hydration, wrinkle volume, and visibility index. Yuksel et al. [2] applied P-PRP three times at 2-week intervals in ten healthy volunteers. P-PRP was applied to the forehead, malar area, and jaw by a dermaroller, as well as injected using a 27-gauge needle into the wrinkles of crow's feet. The authors found that there was a statistically significant difference between general appearance, skin firmness–sagging and wrinkle state before and after the applications.

Moya et al. [23] studied 140 articles, concluding that facial biostimulation with platelet-rich plasma is a simple method, free of complications, since it works with autologous material, through which positive changes in the skin are achieved.

Dyschromia

The role of PRP in hyperpigmentations is controversial. PRP might increase pigmentation especially in the face region, thus some authors advise against its use for the treatment of postinflammatory hiperpigmentation [24]. Contradictory findings have been noted by other authors [13, 22, 25, 26]. The effect of EGF (epidermal growth factor) on skin whitening and as a protection against postinflammatory hyperpigmentation could be due to accelerated wound healing and decreased melanin production [26].

Scars

The usefulness of PRP in scar treatment has been evaluated mainly in acne scars, showing good results. Du Toit [27] studied the application of intradermal PRP in a black patient with facial scars, sequelae of acne. Four weeks after a session of mesotherapy, improvement of the tone, texture, and pigmentation of the skin was evidenced, as well as light filling of the dermal defects. Lee et al. [28] conducted a split-face trial and found that treatment with P-PRP after ablative CO_2 fractional resurfacing enhances recovery of laser-damaged skin, and synergistically improves the clinical appearance of acne scarring. In the same line, Gawdat et al. [29] found that intradermal P-PRP injections after fractional ablative CO_2 laser for atrophic acne scars treatment had a significantly better response, fewer side effects, and shorter downtime than laser treatment alone. They also noted that there were no significant differences in intradermal and topical PRP-treated areas, in degree of response and downtime. Yuksel et al. [2] performed skin microincisions, with subsequent application of PRP in gel form or by subcutaneous injection. The improvement in the appearance of atrophic scars would be based on a rearrangement of collagen.

Gentile et al. [30] studied ten patients with burn sequelae and post-traumatic scars. Patients were treated with autologous fat mixed with PRFM, compared to a control group treated with centrifuged fat. The authors observed a 69% maintenance of contour restoring after 1 year in the PRFM group compared to 39% in the control.

Ibrahim et al. [31] performed a histological study to compare the efficacy and tolerability of intradermal injection of PRP vs. microdermabrasion in the treatment of striae distensae. Collagen and elastic fibers were markedly increased in the dermis at the end of treatment sessions. Platelet-rich plasma alone was found to be more effective than microdermabrasion alone in the treatment of striae distensae, but the combination of both treatments improved efficacy.

Androgenetic Alopecia

Several studies have specifically looked at the effect of platelet preparations on hair loss. Most studies have been performed in androgenetic alopecia. PRP produces a thickened epithelium, proliferation of collagen fibers and fibroblasts, and increases vessels around follicles and follicular density, increases the number of newly formed follicles and accelerates hair formation [32–35]. Sclafani [36] noticed that intradermal injections of PRFM increased the hair density index in patients with androgenetic alopecia, but these results did not reach statistical significance. In 2017, Giordano et al. [37] performed a meta-analysis, including six studies involving 177 patients, in order to investigate the effectiveness of PRP local injections for androgenetic alopecia. The authors concluded that local injection of

PRP for androgenic alopecia might be associated with an increased number of hairs and some hair thickness improvement in the treated areas with minimal morbidity. The results of this meta-analysis should be interpreted with caution as it consists of pooling many small studies. Larger randomized studies could verify this perception.

After applying PRP in a cell culture of cells of the dermal papilla, an increase in proliferation, a greater expression of the antiapoptotic protein bcl-2, and an increase in the amount of FGF-7 in the culture medium were demonstrated. An increase in the activity of β-catenin, which plays a role in cell proliferation and hair growth, was also demonstrated [38, 39]. Li et al. [38] also carried out an in vivo study, and demonstrated PRP ability to accelerate the passage to anagen, after its application on the back of mice, in the telogen phase. Afterwards, Rastegar et al. [40] supported these findings, demonstrating that combination of herbal extracts and PRP plays an active role in promoting the proliferation of human dermal papilla cells.

Schiavone et al. [41] performed scalp injections of L-PRP (leukocyte- and platelet-rich plasma) on 64 patients, and observed some improvement in most patients at 6 months. Cervelli et al. [42] injected PRFM every month for 3 months into scalps with hair loss. They found an increase in a mean number of hairs, and a mean increase in total hair density. Khatu et al. [35] performed injections of P-PRP, and found an increase in hair counts at 12 weeks.

The application of PRP in humans has been studied in patients with alopecia areata in a clinical trial. PRP was found to increase hair regrowth significantly, and to decrease hair dystrophy compared with triamcinolone acetonide or placebo. Cell proliferation was significantly higher with PRP. No side effects were noted during treatment [43].

Cellulite

PRP biostimulation has been used as the first step in treating cellulite, to help connective tissue recovery and microcirculation before mesotherapy [44]. Hernández et al. [45] injected PRP mixed with autologous fat intradermally in patients with mild and moderate cellulite, obtaining satisfactory results, with smoothing of the injected area at 8 weeks post-procedure. Side effects were mild (erythema and transient bruising). The main drawback of this technique is the need to extract more than 30 mL of blood, to be able to repair the defects, due to its wide extension. In addition, the procedure is difficult to perform in a single step.

Application Techniques

Before any treatment, a cutaneous analysis must be performed (i.e., Fitzpatrick scales). The patient must have signed an informed consent. Prior to application, the area to be treated should be devoid of makeup or any cosmetic and disinfected. The physician who applies the PRP treatment must be adequately prepared to perform the procedure [11]. An intimate understanding of facial vascular anatomy during cosmetic injections is mandatory. Full awareness of injection plane to be intradermally rather than subdermally may help to reduce or eliminate vascular compromise. Aspirating before injection, applying topical vasoconstrictors, and using smaller needles (30–32 G) with slow technique and judicious use of pressure are recommended precautionary measures [46–50]. Caution should be taken when injecting fillers in the glabellar region, due to its rich vascular supply, to prevent skin necrosis or devastating visual complications. Furthermore, periocular injections must be performed by licensed practitioners who are familiar with orbital anatomy and the rich anastomosis of facial arteries [51].

To conceptualize the ideal treatment, it should be developed as close as possible to the patient, with minimal instrumentation, guaranteeing asepsis/antisepsis standards, and should be applied in a short time. Usually, 20–25 min are enough to obtain and apply PRP before coagulation of plasma proteins [11]. Whatever the method used for PRP activation, the activated mixture must be applied within 10 min to prevent the clot from retracting and sequestering secretory proteins on its surface [6].

Ideal plasma is translucent yellowish, thus it should never be infiltrated if the following appearances are noticed:

- Whitish sebaceous appearance, which can have two possible origins: (a) the patient has had an important lipid intake; (b) the patient is suffering from dyslipidemia.
- Rose appearance with red blood cells: this is due to a traumatic extraction of blood which causes a release of thromboplastin, giving rise to micro-clots retaining some erythrocytes, which could produce a tattoo if injected [10].

Regarding the application technique, PRP is injected intradermally (mesotherapy) (Fig. 3), in micropunctions of maximum 0.05 mL. The volume of each puncture and its depth depend on the physiopathology of the entity to be treated and may vary depending on therapeutic protocols. In general, there is no specific protocol on the number of sessions and the amount of PRP injected in each session. In some patients an average of 2–4 sessions are applied per year. In others, a shock program can be initially performed, before an annual maintenance phase. The decision of the protocol could thus be left to each specialist, depending on aspects such as photo-aging degree and other qualities to improve (scars, stretch marks, alopecia, etc.) [11]. Some examples of application techniques are described below.

Fig. 3 PRP intradermal injection

Gómez-Font et al. [52] perform multiple intradermal injections with 30 G ½ 0.4 × 4 mm needles, using the nappage mesotherapy technique and facial mesopuncture points. Prior to application, topical anesthesia cream EMLA (lidocaine/prilocaine 25 mg/g + 25 mg/g) has been applied for an hour. Injections are made in facial mesopuncture points (eyebrow tail, ocular, tragus, lateronasal, commissure, hyoid) and by mesotherapy in submandibular axis, mandibular axis, tragus-commissure axis, tragus-nose, upper and lower lips, expression lines, infraorbital axis, frontal lines, and interciliary area. Then, a gauze soaked in PPP (platelet-poor plasma) activated with calcium chloride is applied as a mask to treated areas, in order to obtain a soothing-healing effect. García et al. [53] propose two sessions per year (one in case of young patients with mild aging), in which intradermal infiltrations are performed on the entire face and neck. Deposits of 0.01–0.02 cm^3 are injected 1 cm apart. If necessary, at least 10–15 days before, a superficial-medium exfoliation may be performed and a session of topical therapy by spraying could be performed after epidermal removal.

Combination of PRP and Other Medical Aesthetic Treatments

Laser

Ablative and nonablative lasers have been used in combination with PRP for striae, acne scarring, rejuvenation, and hyperpigmentation with mostly favorable outcomes. PRP accelerates healing, enhances recovery, and promotes better results after laser treatment [28, 29, 54–56]. P-PRP treatment increased the length of the dermoepidermal junction, the amount of collagen, and the number of fibroblasts. Shin et al. [15] found that P-PRP combined with nonablative fractional laser increased subject satisfaction and skin elasticity, and decreased the erythema index.

Different studies of PRP treatment after CO_2 laser resurfacing have been carried out. PRP after ablative CO_2 fractional resurfacing synergistically improves the clinical appearance of acne scarring [28]. P-PRP following fractional CO_2 laser resurfacing decreases erythema index and melanin index [54]. Gawdat et al. [29] found that intradermal P-PRP injections after fractional ablative CO_2 laser had a significantly better response and fewer side effects than laser treatment alone. Kim et al. [57] injected PRFM subcutaneously after fractional ablative CO_2 laser, and noted a statistically significant decrease in edema and erythema postoperatively, but no difference in the reepithelialization rate.

Ozone

Ozone promotes platelet aggregation and release of its growth factors, and has been shown to be effective for PRP activation. Applying it by intradermal injection adds the effects of ozone: sterility, anti-inflammatory effect, and pain control [10, 58, 59].

Botulinum Toxin Type A

Bulam et al. [60] demonstrated that PRP resulted in less botulinum toxin muscle paralysis activity. When PRP was administered separately through skin mesotherapy after toxin, botulinum toxin activity failure was more severe in comparison with direct contact. The authors could not explain the exact mechanism underlying this interaction.

Microneedling

PRP may be used topically, before or after microneedling for improvement of scarring, hyperpigmentation, fine lines, wrinkles, striae, and hair loss [61]. Yuksel et al. [2] applied PRP to forehead, malar area, and jaw by a dermaroller, and injected into the wrinkles of crow's feet. Significant difference was found regarding the general appearance, skin firmness-sagging and wrinkle state, according to the grading scale of the patients, whereas there was only statistically significant difference for the skin firmness-sagging according to the assessment of the dermatologists. The authors concluded that PRP application could be considered as an effective procedure for facial skin rejuvenation. Chawla [62] performed a study comparing microneedling with PRP vs. microneedling with vitamin C. Overall results were better with microneedling and PRP, which proved to be good in treating boxcar and rolling scars, but had limited efficacy in dealing with ice pick scars.

Peelings

After chemical exfoliations, PRP can reduce erythema and accelerate healing [63]. In terms of the combination of PRP and peelings there are studies with different results, most of them favorable. Nevertheless, Aguilar et al. [64] concluded that the association of salycilic peeling and carboxytherapy with Jalupro® (Glycine, L-Proline, L-Leucine, L-Lysine and Sodium Hyaluronate) is better than with PRP.

Based on facial skin image analysis results, also considering the efficacy and safety profile, combination of skin needling, platelet-rich plasma and glycolic acid 70% chemical peeling can be the treatment of choice for atrophic acne scars among people with Fitzpatrick Skin Type IV–VI [65].

Hyaluronic Acid

Ulusal [66] demonstrated that platelet-poor and platelet-rich plasma, and hyaluronic acid injections on the face, provided clinically visible, and statistically significant improvement on general appearance, skin firmness-sagging, and skin texture. The improvements were more remarkable as the injection numbers increased.

Aguilar et al. [67] described the vulvo-vaginal rejuvenation by lipofilling and an injection of combined PRP and hyaluronic acid, with no complications and good results.

Radiofrequency

Several authors have used radiofrequency (RF) after PRP [61, 68]. Favorable results of this combination have been shown in striae treatment. Intradermal RF combined with PRP appears to be an effective treatment for striae distensae also found good results in striae distensae treatment with TriPollar RF [68, 69].

Other PRP Applications

Wound Healing

PRP significantly accelerates wound healing [70–75]. In addition, an angiogenic potential has been demonstrated [76, 77].

Oral and maxillofacial surgery

Autologous platelet gel was first used by Whitman et al. [78], in the placement of osteointegrated titanium implants. Local application of growth factors enhances bone regeneration, soft tissue healing, and is useful in dental implantology and in the treatment of periodontal disease [1, 79–82]. Other authors have found little or no effect of PRP [83, 84]. This discrepancy may be due to the lack of controlled clinical trials under standardized conditions [1].

Traumatology and orthopedics

PRP accelerates fracture repair [85]. Tajima et al. [86] showed that transplantation of the adipose-derived stem cell and PRP mixture had dramatic effects on bone regeneration overtime.

PRP has been used in epicondylitis, lesions of rotator cuff, Achilles tendon and patellar tendon, anterior cruciate ligament lesion, muscular tears, meniscopathies, fractures and their complications, intervertebral discopathies, plantar fasciitis, osteoarthritis, chronic elbow tendonitis, correction of genu varus, ankle arthroplasty, and repair of articular cartilage [87–92].

Ophthalmology

Riestra et al. [93] examined the available evidence regarding the application of plasma rich in growth factors and its variations on the ocular surface. These preparations have been used in the treatment of dry eye or persistent epithelial defects, among others, with good safety and efficacy profiles. PRP promotes faster corneal epithelialization and reduces inflammation and pain [94–96], and significantly accelerates healing of the cornea and conjunctiva in ocular burns [97]. PRP has also been used for the treatment of retinal and macular pathology [98–100].

Otorhinolaryngology

PRP has been applied topically, observing a faster closure in tympanic perforations [101]. PRP has also been used in patients undergoing thyroidectomy and parotidectomy, with good results [102, 103].

Dermatology

In chronic skin ulcers of different etiologies (diabetic, vascular, neuropathic, decubitus, post-therapy), PRP promotes the formation of granulation tissue and accelerates healing [104, 105].

PRP results have been extensively studied in diabetic ulcers, accelerating their closure, reducing pain, improving quality of life and reducing care costs, without significant side effects [106–108]. These effects have been studied with less profusion on venous and hypertensive ulcers [9]. Salazar et al. [109] observed very good results in ulcers of non-ischemic etiology. Cases of ulcer healing in lower extremities have also been described in cases of β-thalassemia intermedia [110, 111]. PRP has also been used as an adjuvant in the treatment of ulcers with grafts, with excellent results [91].

Rheumatology

Anitua et al. [112] observed an increase in hyaluronic acid and hepatocyte growth factor after intraarticular administration of platelet-derived growth factors in osteoarthritis. Sánchez et al. [113] demonstrated greater improvement of pain with PRP injections than with injections of hyaluronic acid in knees with osteoarthrosis.

Neurosurgery

PRP has a neurotrophic effect, enhancing nerve regeneration [114–116], and helping to improve remyelination [117]. So far, however, the role of PRP in the repair of peripheral nerves has not exceeded the experimental scope [118]. PRP has been used to improve the process of spinal fusion or to regenerate peripheral nerves, and in neurodegenerative pathologies such as Alzheimer's. Its applications are promising, such as regeneration of damaged tissue after cerebral ischemia [6, 119, 120].

Gynecology

PRP gels decrease postoperative pain and analgesic requirements of major gynecological surgeries [121]. On the other hand, an in vitro study confirmed the ability of PRP to seal biological membranes, an encouraging effect which should be tested in the future [118, 122].

Cardiovascular Surgery

Topical application of PRP in cardiovascular surgical wounds reduces the frequency of infection, improves hemostasis, reduces pain and the amount of drainage of wounds, and decreases the days of hospital stay [118, 123].

General Surgery

The use of platelet-rich fibrin as a glue for the placement of meshes in the correction of inguinal hernias improves tolerance, postoperative pain, and decreases the amount of suture necessary for their fixation [124]. Another study in pigs found an increase in blood flow and an increased resistance of the anastomosis after a tracheal resection, as well as faster healing with the application of PRP [118, 125].

Plastic Surgery

In the year 2013, Sommeling et al. [126] published one of the most extensive systematic reviews of the use and applications of PRP in plastic and reconstructive surgery. The PRP has been used in mammoplasties, blepharoplasties, facelifts, rhinoplasties, or as an adjuvant treatment in soft tissue reconstruction, and in other flap surgeries. It has also been used after laser resurfacing and in burns [6, 127–129].

Several animal studies have sought to examine the effect of P-PRP on survival and quality of fat grafts [5, 6, 130, 131]. Only one of these studies did not find a significant difference of P-PRP in fat graft survival in mice [132]. It has been demonstrated that PRP increases fat cells survival rate and stem cells differentiation [5, 133–136], with a lower inflammatory reaction [6]. Fat graft survival rates were found to be significantly increased in rats [5, 130, 131], and in rabbits [6].

Li et al. [137] concluded that fat grafts consisting of P-PRP and adipose-derived stem cells constituted an ideal transplant strategy, as it resulted in decreased absorption, increased volume retention, adipocyte area and capillary formation, and accelerated fat regeneration. Modarressi [5] considered that the addition of 20% PRP to fat grafts offered a better fat grafting survival, less bruising and inflammation reaction, and easier application of fat grafts, due to liquefaction effect of PRP.

The association of fat grafting and PRP has also been described for facial reconstruction [138] and for aesthetic cases, with good results [5, 134]. The group of Cervelli et al. [134] has studied the effects of PRP mixed with free fat grafts used for cutaneous rejuvenation, evidencing a longer maintenance of the contour restored when the fat graft was mixed with PRP. Willemsen et al. [139] found that the addition of P-PRP to grafted autologous fat resulted in a significant drop in the number of days needed to recover before returning to work, or to restart social activities. The aesthetic outcomes were also significantly better than without P-PRP.

Other authors have compared PRP and SFV effects on rejuvenation and breast reconstruction. Rigotti et al. [140] concluded that the use of PRP-enriched fat did not have significant advantages in skin rejuvenation over expanded adipose-derived stem cells or SVF-enriched fat. On the other hand, Gentile et al. [141] found that the use of either enhanced stromal vascular fraction, or PRP mixed

with fat grafting, produced an improvement in maintenance of breast volume. Recently, Gennai et al. [142] noticed that following PRP addition, there was a greater proliferation in the micro-superficial enhanced fluid fat injection (M-SEFFI) into the dermal region of the periocular and perioral zones.

PRP adhesive activity is used in flap and graft surgery to increase viability, accelerate healing, seal dead spaces, improve hemostasis, decrease inflammatory reaction and the need for drainage and compression dressings, and reduce edema, postoperative pain and healing time [6, 143–145]. Other authors have found that PRP had angiogenic effects that enhanced graft survival [146–148]. Marx et al. [80] found that skin donor sites treated with PRP showed a faster epithelialization, greater skin thickness, and less pain and discomfort. Chandra et al. [149] noticed that, despite an increased inflammation, no significant differences were observed in the degree of fibrosis or collagen deposition. On the other hand, Danielsen et al. [150] examined the effects of topical autologous platelet-rich fibrin on epithelialization of donor sites and meshed split-thickness skin grafts, and found no significant difference in epithelialization rates.

So far, the limited evidence does not yet allow to recommend PRP in the management of burns [151]; however, future perspectives are promising [118, 152].

PRP in Tissue Engineering

The most recent application of PRP, and the one that currently generates a greater number of research studies and articles on the subject, is its application in the last years in the field of tissue engineering. Growth factors secreted by platelets promote cell proliferation, growth, and differentiation. The applicability for tissue engineering has spread to virtually all of the above-described applications [6, 153].

Adverse Reactions, Potential Risks, and Security Considerations

Given its autologous nature, PRP is a safe product that, by definition, lacks the potential risk of disease transmission, implicit in the use of blood material from donors [63]. All products for topical use may contain a risk of irritation or dermatitis [154]. The adverse reactions produced by the injection of the product into the skin are the same as can occur in any other cosmetic treatment which uses this route of administration: local inflammation, mild edema, erythema, bruising, discomfort, pain, etc., which are transient and self-limited [3]. During blood extraction, some patients may experience dizziness or vasovagal syndrome [11]. Injectable PRP preparations which use thrombin to activate platelets have been questioned, because of the potential risks of generating cross-reactive antibodies to thrombin, factors V and XI, which could cause coagulopathy. Another potential effect would be the release of microparticles with a prothrombotic effect, such as interleukin 1 beta [1, 118].

Some authors who have clinically used PRP ensure that there are no risks of infection since it is antimicrobial and effective against most bacteria, except Klebsiella, Enterococcus and Pseudomonas; therefore, asepsis and disinfection of the skin are mandatory before the injection [11, 155]. In 2017, Kalyam et al. [51] reported the first case of unilateral blindness, after the aesthetic application of PRP for the treatment of periocular wrinkles. The good results of PRP treatments are undeniable in some cases; however, scientific evidence also shows that growth factors and their receptors appear overexpressed in tumor and dysplastic tissues [155], which suggests two possible risks: carcinogenesis and metastasis.

Carcinogenesis

In normal fibroblasts, we can find between 5000 and 10,000 normal EGFR (epidermal growth factor receptor) per cell, while about 400,000 normal receptors per cell have been observed in tumor cells. This increase is due to alterations of genes coding for the receptors, and is not a consequence of the overproduction of growth factors.

It is believed that therapeutic concentrates of growth factors could act more as promoters than as initiators of carcinogenesis, favoring the divi-

sion and promotion of cells previously mutated or initiated in carcinogenesis. However, this phenomenon may require more continuous doses in time than those applied in PRP therapy, taking into account that extracellular growth factors degrade after 7–10 days [1, 155].

Another factor to consider is the antiapoptotic capacity that has been assigned to certain growth factors such as VEGF and IGF. Taking into account this possibility, there are authors who propose to avoid the use of PRP in patients with cancer processes or in the vicinity of large vessels and precancerous processes (oral leukoplakia, erythroplasia or solar cheilitis), as well as in patients with previous exposure to carcinogens. Thus, it would not be advisable to use PRP in smokers and/or drinkers, since they are exposed to potent mutagenic, and are more likely to have cells initiated in the process of carcinogenesis. For the moment, we could only establish a possible relationship between the therapeutic application of PRP with carcinogenesis, since, so far, there is no scientific evidence to demonstrate the link between treatment with PRP and the carcinomatous transformation of normal or dysplastic tissues [1, 155–157].

Metastasis

Another phenomenon to assess would be the ability of platelets to facilitate the process of metastasis of tumor cells in the perivascular tissue. The platelets coat the tumor cells and favor their vascular permeability by means of VEGF (vascular epidermal growth factor), thus allowing the penetration of the tumor [156]. Recently, Menter et al. [158] published a review of the subject with the latest evidence, clearly demonstrating a role of the platelets in the hemostatic microenvironment, which is used by tumor cells to facilitate tumor progression, invasion, and metastasis. According to the authors, the platelets act as “first responders” in the healing process of a wound. Cancer is a non-scarring or conical wound that can be actively aided by the mitogenic properties of platelets, to stimulate cell growth. This growth ultimately exceeds the circulatory supply, which leads to angiogenesis and the passage of tumor cells into the bloodstream. The circulating tumor cells re-join platelets, which facilitates their adhesion, extravasation, and metastasis [158, 159]. Although these studies seem very strong, they are still controversial.

Contraindications and Recommendations

It is very important to obtain a careful medical history before starting treatment with PRP. During the period of pregnancy and lactation, PRP is contraindicated [160], and relatively contraindicated in patients who receive chronic platelet antiaggregants [161]. The application of this technique is not recommended in patients with severe systemic and metabolic disorders, coagulation disorders, and dermatological conditions such as systemic lupus erythematosus, porphyria, allergic dermatitis, etc. [11].

As explained above, some authors have proposed to avoid the use of PRP in patients with cancer processes, or in the vicinity of large vessels and precancerous lesions, as well as in patients with previous exposure to carcinogens, such as smokers and/or drinkers [1, 51, 155, 156].

It is recommended to stop taking nonsteroidal anti-inflammatory drugs 7–10 days before the procedure, and systemic corticosteroids 2 weeks before the injection.

Conclusions

- Nowadays, PRP is a therapy accepted throughout the world in many medical fields.
- PRP was the first revolution in the field of Regenerative Medicine applied to Aesthetic Medicine.
- PRP is already accepted as part of the range of treatments available to the aesthetic physician, and has been included in official recommendations and guidelines.
- Scientific evidence highlights the potential of enriched plasmas for aesthetic and tissue regeneration purposes, but the level of evidence from the available published data is low.

- The spread of these techniques has been held back by the lack of dramatic results and by the difficulty of quantifying them in an objective and reproducible way.
- Further large and rigorous studies, with long-term follow-up should be performed to assess the safety and efficacy of PRP.
- Facial biostimulation with platelet-rich plasma is a simple method to improve skin, with a low complication rate.
- PRP can be used as part of a rejuvenation strategy by layers, and satisfactory results can be obtained in combination with other aesthetic medical treatments.
- The result, as in other treatments, depends on adequate training and experience.

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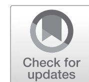

Autologous Conditioned Serum as an Antiaging Tool

Hernán Pinto

Introduction

From immemorial times, human beings have been fighting an unwinnable battle with years. Despite this, and throughout evolution, their life expectancy has increased. Based on great apes [1, 2], our common ancestor was estimated to have a life expectancy of less than 20 years. This parameter's global value has now quadrupled, being its maximum value greater than 100 years [3]. There is evidence that humans have been searching for a longer life since the beginnings of history ([4]). The yearning of not aging has now become an obsession for the modern man, overwhelmed by a way of life that they themselves have created, but for which their bodies are not prepared [5–11]. One of mankind's clear contemporaneous goals as a whole is to live longer. However, together with the desire of a longer life, a better-quality life has also surfaced, and beauty is most certainly part of this quality.

What humans consider beautiful or not is socially standardized according to each people and time. We can say that, although beauty is completely subjective, it stands on tangible pillars, which are brief but also measurable, and therefore susceptible to comparisons. These are all concepts that can be measured and objectified, and constitute beauty's parameters. For a more detailed study of these parameters, an arbitrary division can be established between two fundamental components of beauty: the quantitative and qualitative components.

Quantitative component. The "amount" of tissue is significant and often the only parameter to correct. There may be (a) wrinkles, due to tissue deficiency, which is typical in young adults; (b) cheekbones, due to the need to "reposition" tissues because of hypotrophy or ptosis, which are more prevalent with aging; and (c) localized adiposity (double chin), due to excess tissue, which may occur at any age.

Qualitative component. Tissue characteristics are essential to assess beauty. This chapter includes all sorts of concepts: firmness, distensibility, elasticity, luminosity, color, freshness, turgidity, hydration, homogeneity, smoothness, and softness.

The pass of time has the same impact on skin as on the other tissues. The difference is that this impact is more visible on skin. Loss of beauty is one of the main manifestations of skin aging, but not the only one. Loss of functionality is another essential characteristic of skin aging. However, under real conditions, this division is arbitrary and only theoretical, since they are both completely interrelated. The skin has beautiful features when the tissues that are part of it are well structured and

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work properly. And, vice versa, when the skin's structure is damaged, and tissues do not play their role normally and efficiently, then that skin will usually not be as beautiful as it can be. Furthermore, inflammation's role in aging is becoming increasingly clearer, although, due to its complexity, it is still not fully understood. The inflammatory theory of aging is similar to the free radicals theory [12] in terms of inflammatory damage being mediated by free radicals and specific inflammatory peptides [13–16]. Furthermore, this theory takes some elements from the immune theory and the fetal origins hypothesis. Oxidative and inflammatory damage accumulated by molecules and cells throughout the years (bystander damage, BD) gives cause for most aging-related dysfunctions that, in turn, increase damage and mortality in old age. Some of these dysfunctions can be found in prodromal (subclinical) inflammatory changes occurring at an early age [17].

The possibility of healing or improving conditions and pathologies using biological materials prepared with the patient's own tissues has always been a desired and highly interesting idea from every point of view. That fantasy became a reality in 1958 with the appearance of the first report on an autologous hematopoietic cell transplant attempt [18]. Two decades later, healing reports of pathologies that had been previously considered incurable started to appear ([19, 20]is; [21]). The following landmark on autologous transplant was the use of peripheral stem cells. The first successful trials on animals appeared at the beginning of the 1960s, continuing for the next 20 years [22–24]. The first attempts on humans failed [25, 26], but, during the 1980s, these treatments became established [27–29]; and, half a century after the first attempts, autologous transplants became a versatile medical resource, used for several purposes [30] and with a high frequency.

Of the multiple biological materials available today, serum has aroused a special interest. However, since it was initially considered a minor treatment option to cell autotransplantation, it had a late development and understanding. Some events turned out to be essential to drive the study and implementation of this kind of material. The first event was the partial understanding of the role played by some growth factors and other cytokines in tissue repair processes [31]. Other highly important facts were the (a) acknowledgment of many of the substances found inside platelet granules, including PDGF, TGF, EGF, and IGF [32]; (b) understanding of some of their functions—regulation of cell migration processes, vascularization, cell proliferation, and location of new cell matrix [33]—and, of course, (c) development of methods and systems that ultimately enabled the application of some of these biological materials [34] (Fig. 1).

About the use of autologous materials as tools to prevent aging, the stage has been virtually 100% dominated by one single product: platelet-rich plasma (PRP) or plasma rich in growth factors (PRGF). Commercial protections for some variants of these names make it difficult to unify the terminology. They are both used as synonyms to refer to an autologous concentrate of platelets in a reduced plasma volume containing trophic factors that are released when platelets are activated by the action of calcium chloride, thrombin, fibrinogen [35], or any other platelet activator. The alternative to PRP is serum. This is more versatile, since it is not necessary to use anticoagulants, it does not have so many temporary limitations when applying it, and it can be conditioned in different ways when

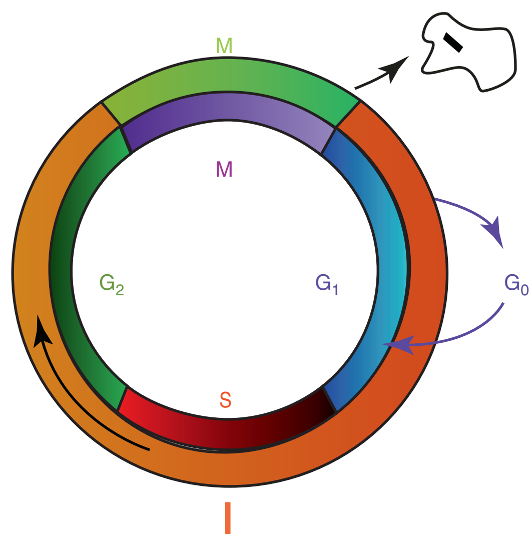

Fig. 1 Cell cycle phases

exposed to several substances. Furthermore, serum preparation systems are necessarily closed, reducing any possibility of contamination. These types of serum have been used with excellent results for other indications [36, 37], and they are also used to improve the patient's aesthetic appearance because of its great similarity with PRP. Autologous serum was developed several decades ago. The idea of being able to get certain substances with specific means and through the simple processing of our own blood has tortured scientists ever since. The application of similar conditioned serum has already been successfully tried for:

(a) Eye pathologies [38]
(b) Muscle injuries [37]
(c) Epidural and perineural injections [39]
(d) Trauma/articular pathology [40]

To date, there are many publications about the effectiveness [41, 42] of this kind of autologous biological material, although they especially make reference to the less prevalence of adverse effects [43, 44], less postsurgical recovery time necessary, and the easier resolution of complications that may occur [45]. There is also a considerable large group of publications with no statistical significance in treatment effectiveness [46–48], or which contend the impossibility to get any because they cannot compare studies that are mostly case reports or uncontrolled data sets, where very different methodologies are used to obtain PRP, and where its application varies based on the indication [49].

When studying the map of cytokines obtained from serum from patients with this conditioning [40], the idea of using it to reduce the impact of chronic inflammation and, therefore, as an antiaging tool arises logically, simply, and naturally.

Antiaging Conditioned Autologous Serum (ACAS)

The technique to obtain ACAS broadly follows the original method to obtain platelet-rich plasma (PRP) or plasma rich in growth factors (PRGF), discussed in previous chapters. There are, however, some significant variations. The main difference between serum and plasma lies in the presence or absence of proteins from the cascade of coagulation in the material to process. While tubes with anticoagulants are used to obtain plasma, with coagulation factors remaining in the plasma, to obtain serum, tubes do not contain anticoagulants, and therefore a clot is formed inside the tube, which is discarded. Inside the tube/syringe, there are small borosilicate crystal balls triggering reactions that will result in a hyper-concentration of IL-1ra without a concomitant increase of IL-1b. Autologous serum is obtained through an important induction of IL-1ra synthesis, without the need of having immunoglobulin G attached to the surface. Experimenting with different materials, including plastic polymeres, glass, and quartz, has led to the identification of borosilicate glass spheres as the most effective surface to induce *de novo* synthesis and accumulation of IL-1ra without a concomitant production of interleukin-1b in the blood [40] (Fig. 2).

The list of growth factors and interleukins present in the ACAS serum is probably higher than the one we already measured and published [50]: 11 ng/mL of IL-1Ra, 1 pg/mL of EGF, 39.5 ng/mL of TGF-B1, 108.9 ng/mL of IGF-1, 27.1 pg/mL of PDGF-AB (Fig. 3), after 24-hour incubation at 37 °C, 10 m centrifugation at 3000 × *g* and freezing at −20 °C (the numbers express measurement means obtained with

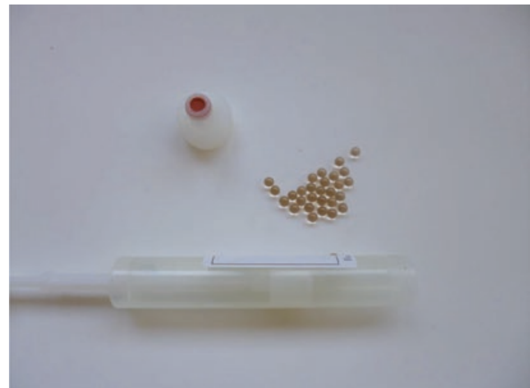

Fig. 2 Device to obtain ACAS

Factor	Blood	Post-Incubation
IL-1Ra	0.3 ng/mL	11.0 ng/mL
EGF	0.1 pg/mL	1.0 ng/mL
TGF beta 1	1.0 ng/mL	39.5 ng/mL
IGF 1	72.6 ng/mL	108.9 ng/mL
PDGF AB	5.8 pg/mL	27.1 ng/mL

Fig. 3 Comparison: growth factors blood concentrations vs. ACAS concentrations

ELISA kits from R&D Systems, Minneapolis, MN, USA) (Fig. 3).

Treatment Session

The technique to obtain ACAS is very simple and new, presenting a few differences when compared with the PRP technique previously described. Sample processing involves five consecutive steps: blood extraction, blood incubation, ACAS preparation, ACAS storage, and ACAS application.

Blood Extraction

The volume to extract will vary based on the treatment plan, since serum can be frozen and saved for future sessions. Every tube/syringe contains 8 mL, resulting in 3.5 mL of serum to apply. Fifty milliliters of blood can be extracted in six tubes to prepare the serum to inject in three sessions. The blood is directly collected into the tube/syringe by negative pressure, similarly to a standard vacuum tube. After extraction, the tube/syringe embolus is broken, and the tube is used to process the autologous serum. Tubes do not contain anticoagulants.

Blood Incubation

The tubes contain borosilicate glass spheres, which stimulate cell production of growth factors and of anti-inflammatory components without concomitantly increasing pro-inflammatory cytokines [9]. They are incubated for 24 h without shaking, at 37 °C.

ACAS Preparation

Devices are subject to a single 10-min spin centrifugation protocol at 5000 revolutions. The sample is exposed to 3000 Gs. Tubes are centrifuged to compact the clot that forms during incubation. Thus, a larger volume of serum is obtained. Unlike what happens with PRP, platelets will not rupture.

The clot and serum are perfectly separated: the clot in the bottom of the tube and the serum above it. A needle is inserted until reaching the clot, but without touching it. All the serum possible is extracted.

Then, the tubes are placed in a rack, where serum itself is individually extracted from each tube. Standard 5-mL syringes with 20 G needles × ¾ are used. Approximately 3.6 mL of AAS is obtained from each device. Each syringe containing 3.6 mL +/− 0.2 mL of AAS processed this way is called “a vial.” Four vials are used for treatment purposes, while the fifth is used for counts and measurements.

Note: Unlike what happens with plasma, there is no need to concentrate or activate serum when it is injected.

ACAS Storage

Of the 4 vials of ACAS for application, two are stored. After closing the vials with a top and properly identifying them, they are frozen at −20 °C. These vials are kept under these conditions for 15 days. Forty minutes before applying the desired injection, vials are thawed at room temperature (Fig. 4).

ACAS Application

Treatment application is performed in a mesotherapy session. Each vial is applied by multiple intradermal (ID), low-volume (0.1 mL) injections. Application is manual, with a standard mesotherapeutic technique and without leaving a papule. 30 G ½ (12.8 mm) needles are used, as

Fig. 4 Thawed vials of autologous serum

well as a 0.2 μm bacteriologic filter. Each injection is performed at a 3 mm depth. 1.8 mL +/− 0.1 mL is injected in both the right and left malar regions. Each session lasts about 15 min. Alcohol 70° is used as antiseptic.

Previous studies by our group [51, 52] have yielded encouraging results that have motivated us to continue this line of work. However, despite that all variables improved at one time or another, when analyzing results altogether, some of these variables keep their potential and confirm their beneficial action, while others yield contradictory, paradoxical, or, at least, inconsistent results.

At first, we obtained very interesting results concerning the aesthetic improvement of patients receiving applications of ACAS. These results were objectively and subjectively quantified, by the patient as well as by the treating physician. Since it was just a small set of cases, and as such, it was exclusively exploratory, we stopped focusing on results and decided to widen the scope of our study, using a larger *n*. In this study [50], the results obtained suggest a significant aesthetic improvement after ACAS application, specifically, increases of 17.08% in epidermal hydration, 10.38% in skin firmness, and 16.59% in some mechanical properties, like viscoelasticity. In this study, sessions were scheduled 15 days apart, with two vials of ACAS injected in each session. Since ACAS systemic action was still unknown, in each session one vial was intradermally applied (mesotherapy), while the second vial was intramuscularly applied.

The systemic impact (anti-inflammatory or antiaging) of ACAS observed in previous studies was even more spectacular. In the initial exploratory series of cases, some patients showed 50% reductions in plasma concentrations of interleukin 6 and PCR. This potential was confirmed by a pilot study made of eight cases, where, after intramuscular application of two vials of ACAS, reductions of up to 94.52% (maximum recorded in a patient) were observed in the plasma concentration of IL-6. Furthermore, there was a concomitant reduction of 13.27% in plasma concentration of PCR. This reduction was much less spectacular, but the sample's dispersal was lower, strengthened the data set, and gave us hope.

In both studies, volunteers' follow-up lasted 45 days. The fact that reductions in markers concentrations were observed both after intradermal application and after intramuscular injection of ACAS predicted a systemic anti-inflammatory action independently from the injection route. With this idea in mind, we decided to modify the application of intramuscular routes. In our last study, ACAS application was exclusively performed intradermally (mesotherapy) to supposedly keep the systemic effect (over the markers) but increase the local (aesthetic) effect. This would be a previous step to suggesting a dose increase. Since local, simultaneous application of two vials seemed too aggressive, it was decided to apply four vials intradermally once a week. That is, the final dose remained the same, but the dose per session was reduced, the time between sessions was shortened, sessions were increased, the intramuscular route was abandoned, and follow-up was increased to 65 days. Some results have been consistent with those previously obtained, reinforcing our thoughts and the evidence we already had. Other results, on the contrary, were not consistent, and in some cases, they were even contradictory.

Hydration increased 9.63%, which is consistent with the 17.08% previously obtained. The lower increase can be justified in several ways, but the most likely reason seemed to be the extra follow-up time the patients were subjected to. The fact of collecting the final sample at 65 days

instead of 45 days was expected to alter the results. This reduction in hydration's increase suggested that the hydrating action of this serum could reach its maximum effect about 2 months after the injection and then be reduced.

Softness was improved by 24%. These data had no precedent, since there is no evidence, and in our previous studies, friction metric data were biased. Softness is a qualitative component of beauty that can be touched and seen. As such, it is closely related with other qualitative parameters. These correlations must be studied in the future, since luminosity, freshness, softness, smoothness, and fragility should be closely related.

As for cutometry assessment, the great amount of variables involved makes its interpretation difficult. The correct assessment of the skin's mechanical behavior is complex, since there are many tissues and forces involved at the same time. The fact that 18 cutometry variables had been collected turned this into top level information, but it also made it harder to interpret. R0 and R2 yielded paradoxical values. For R0, we previously obtained an increase of 10.38%, although we observed a reduction in this study. Clinical consequences involving a similar reduction made us confident that this reduction could not be real, but biased. The case of R2 was not as marked, but it was equally unexpected. It was not contradictory, but it was inconsistent. The increase of 1.12% in R2 observed in this study is significantly different than the increase of 16.59% observed in previous studies. The opposite occurred with R5, which yielded an increase of 22.84%, double of the 11.21% yielded in previous studies. This increase suggests a much more elastic condition of the tissues. R9 was reduced 31%, suggesting a better response to mechanical repetitive stimuli and lower depletion. F0 is an area obtained from the total area under the cutometry curve. The reduction of 22.79% observed in F0 accounted for a significant improvement in skin elasticity. This increase was not consistent with the irrelevant improvement of 1.12% observed in R2, strengthening the idea that the values observed for R0 and R2 were not real. F4 is also an area and accounts for skin firmness. The reduction of 5.16% in F4 involves firmer skin and is consistent with a reduction of 10.38% obtained in previous studies. In the cutometry curve, Q0 is the maximum recovery, meaning skin firmness. The reduction of 19.36% observed in this parameter was very important, and not only did it partially support the results obtained in previous studies, but it also added to the arguments set forth about the truth of the values obtained for R0.

As for IL-6 mean plasma concentration, highly significant differences ($p = 0.002$) between pre-ACAS application values (1.150 pg/mL; SD: 0.272) and post-application values (0.063 pg/mL; SD: 0.042) were obtained in previous studies. However, values obtained in this study were different. Pretreatment mean concentration was 0.125 pg/mL (SD: 0.56), and post-treatment mean concentration was 1.17 pg/mL (SD: 2.53). The difference between them was statistically significant ($p < 0.001$), but, despite being a relatively small sample with a very important dispersal, changes occurred in an opposite direction than that expected. IL-6 mean concentrations were particularly affected by the presence of five outlayers, two of which were extreme outlayers. If we did not consider outlayer values, samples would not be significantly different, which would be neither desirable nor consistent with previously obtained values, but at least it would not be paradoxical. However, the important amount of outlayers (5) and the huge value of extreme outlayers do not allow to search a justification to discard it. Anyways, and for informational purposes, the extreme outlayer that is farther from the mean developed a cold 72 h after the application, and the second extreme outlayer farther from the mean did not develop any infectious pathology or any other condition that could justify its value.

In this protocol, ACAS was applied in weekly sessions, instead of fortnightly sessions, like in previous studies. Therefore, local damage (in injection site) caused by mesotherapeutic injections doubled. IL-6 increases are unspecific. It is highly likely that the larger amount of facial lesions found in this study cannot explain these increases on their own, and of course they cannot explain the fact that they selectively occur only in some patients. However, it is highly possible that

they played some role to increase a mean that, although without significant differences and not considering *outlayer* values, is real.

About PCR plasma concentration, in previous studies we have recorded pretreatment plasma concentrations of 0.113 mg/dl (SD: 0.005) and post-treatment plasma concentrations of 0.098 mg/dl (SD: 0.019). The difference was statistically significant ($p = 0426$). However, values obtained in this study were different. Pretreatment mean concentration was 0.1481 mg/dl (SD: 0.141), and post-treatment mean concentration was 0.42 mg/dl (SD: 0.132). The difference between them was statistically significant ($p = 0.001$), but, despite being a relatively small sample with a very important dispersal, changes occurred in an opposite direction than that expected. PCR increases are also highly unspecific, and, just like it was observed with IL-6, it is highly likely that the larger amount of facial lesions cannot explain these increases on their own. However, it is equally very likely that they played some role in the increase of its plasma concentrations.

Time between sessions is still another controversial variable. Weekly sessions do not seem to have aesthetic advantages over fortnightly sessions. The most appropriate time between sessions seems to be, up to date, 15 days. Using a constant dose throughout the different studies performed, shortening the time between sessions means fractioning the dose and, therefore, carrying more interventions. The "time between sessions" variable has not been isolated or duly studied by any protocol, and the results of measurements, with a variable time between sessions, could have been masked by other events, like, for instance, the fact that the number of sessions may alter the observable aesthetic impact or the concentrations of dosed markers.

The same thing happened when attempting to analyze the differences between measurements after intradermal or intramuscular application of ACAS. Since the puncture itself has beneficial effects, it follows that vials injected intradermally must have had a bigger local aesthetic impact than vials injected intramuscularly and remotely.

Final Considerations

Despite the inconsistent results obtained with some of the variables collected, the path that ACAS intends to follow is clearly the right one. The possibility of conditioning autologous serum to customize and fit it according to the needs of each human being is now a reality. Easy access to blood, the simplicity of conditioning, the safety of its handling, the versatility of reinjections, the huge clinical potential it holds, and low cost have turned autologous serum in an essential tool for the future treatment of multiple diseases that affect humanity today. The great challenge of this technology will be to identify the right conditioning for each serum and the right serum for each condition to be treated.

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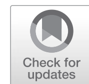
Part V

Regenerative Medicine Procedures for Aesthetic Physicians: Skin Grafts



Skin Cell Cultures and Skin Engineering

Lucía Jáñez

Introduction

Cell culture is a well-established research tool in biology and medicine [1]. The challenge of current bioengineering efforts is to generate functional organ systems from dissociated cells that have been expanded under defined tissue culture conditions [2].

Skin Histology and Physiology

Skin is formed by dermal and epidermal tissues (Fig.1). Keratinocytes are the major component of the epidermal tissue (more than 95%), with melanocytes and Langerhans cells representing a minority population [4]. Human keratinocytes derive from the epidermal stratum basale [5–9] from hair follicles [10–13] and, as recently suggested, also from eccrine sweat glands [14].

As cells divide and differentiate through the epidermal layers, their protein expression changes [15]. Cell behavior is governed by chemical messengers. Surface proteins attach to various ligands, such as growth factors, and trigger specific signaling pathways involved in stem cell differentiation. Cells are also influenced by their surrounding ECM (extracellular matrix) [16]. Each type of tissue has its own unique ECM composition. Several studies have demonstrated that ECM greatly influences cell development, migration, proliferation, differentiation, shape, and function [17–21]. ECM and surface proteins perform the so-called mechanotransduction, which transmits mechanical signals to the cell nucleus and alters gene expression [16]. It has been shown that despite the genetically programmed cell expression, phenotype can be changed by modifying the interaction with the ECM. An example of the transformation that the extracellular environment may generate is found in embryonic cells [22].

Dermal tissue comprises a dense connective tissue structure in which the major cellular components are fibroblasts. The ECM and a variety of cytokines are synthesized by fibroblasts to induce epidermal and vascular endothelial cell growth. ECM is made up of collagen, glycoproteins, and proteoglycans (chondroitin sulfate, HA, heparin, etc.). Fibroblasts produce abundant extracellular proteins (especially collagen and elastin). Collagen allows cell adhesion, growth, proliferation, and differentiation [23]. The dermis constantly renews itself via the process of degradation, rebuilding, and regeneration. Theoretically, the dermis cannot regenerate like the liver, bone, and the epidermis after being destroyed. Scarring is hard to avoid during the process of natural healing

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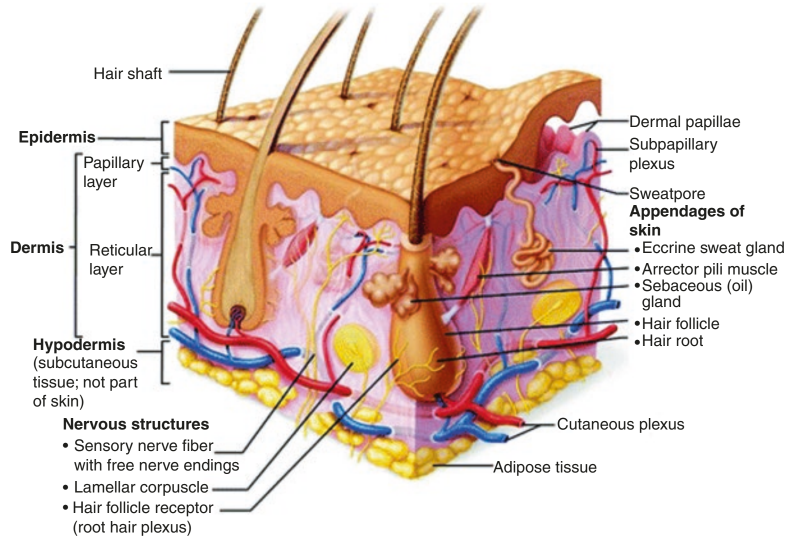


Fig. 1 Schematic representation of human skin (Reproduced from Tavakolpour et al. [3])

[24]. The scarless healing of fetal wounds is an ideal healing method [25]. Tissue formation and healing mechanisms are still unclear [26]. A lot can be learned about skin physiology and cell–ECM interactions by studying wound healing which involves cell migration, proliferation, differentiation, apoptosis, and the synthesis and remodeling of the extracellular matrix (ECM) [25]. Injuries involving the epidermis alone or the superficial layer of the dermis will re-epithelialize without surgical intervention, provided there is a sufficient number of keratinocyte stem cells in the remaining epidermis or in the residual dermis. If epidermal keratinocytes are missing, regeneration may be achieved by epithelial stem cells derived from hair follicles and/or sweat glands [26]. During wound healing the cell is fully reprogrammed. They have to de-differentiate and the genes and proteins expressed change. The Wnt pathway leads stem cell function and renewal and reprograms differentiated cells to have stem cell-like properties [15]. Growth factors can be considered the engine of wound healing, but their use as a monotherapy in clinical practice has not worked well. Human serum (a soup of factors) helps keratinocytes but is detrimental to fibroblasts [15, 27]. Aoki et al. [27] demonstrated that dermal fibroblasts, bone marrow stromal cells (BMSCs), and preadipocytes derived from subcutaneous adipose tissue promoted epidermal regeneration [27].

Fibroblasts are a heterogeneous population of mesenchymal origin that can be found in numerous tissues. Fibroblasts from different anatomical sites have their own characteristic phenotypes, synthesizing different extracellular matrix (ECM) proteins and cytokines [28]. Dermal fibroblasts release cytokines and growth factors that have autocrine and paracrine effects [29]. Autocrine activity promotes collagen synthesis and fibroblast proliferation [30]. Paracrine activity affects keratinocyte growth and differentiation [31]. Dermal fibroblasts promote the development of keratinocyte layers in addition to promoting keratinocyte proliferation [32]. Human fibroblasts regulate vascular and lymphatic endothelial cell proliferation [33].

The adult hair follicle (HF) is composed of mesenchymal cells that provide signals to regulate epithelial stem cell function during tissue regeneration [34]. The HF is accessible to experimental modulation and can be easily removed in its entirety. Moreover, the HF is the only mammalian organ that, for the entire lifespan, cyclically undergoes consecutive transformations. The HF cycles between a state of relative “quiescence” (telogen) and rapid and massive growth (anagen); and finally it cycles back toward telogen, via an apoptosis-driven organ involution (catagen) [35].

Skin Stem Cells

Stem cells (SCs) have the unique capacity to self-renew and to differentiate into the cell lineages that constitute their tissue of origin. Hair follicle and skin tissue, apart from bone marrow, are perhaps the only tissues, which hold the niche for diverse kinds of stem cells: melanocyte stem cells, keratinocyte stem cells, and mesenchymal stem cells [36].

Epidermal stem cells (Epi-SCs) reside in the basal layer of interfollicular epidermis and in the hair follicle bulge [37], which is a specialized portion of the outer root sheath epithelium defined as the insertion site of the arrector pili muscle (Fig. 2). Bulge cells contribute not only to the generation of new HFs with each hair cycle but also to the repair of the epidermis during wound healing [39]. Because Epi-SCs in the hair follicle are difficult to acquire, Wang et al. [40] investigated whether Epi-SCs in the epidermis were capable of regenerating epidermal appendages. Among the potential keratinocyte donor sites, the foreskin seems to be a promising source [41].

Adult dermal SCs have not yet been fully defined [2]. Endogenous dermal stem cells (DSCs) have been demonstrated within the adult mammalian dermis [42], which might serve to regenerate dermis or rejuvenate dermal papilla to restore follicle growth. DSCs reside in the HF mesenchyme. Endogenous DSCs can be grown in vitro as self-renewing multipotent cells named skin-derived precursors (SKPs), which can generate both mesodermal and neural derivatives [2, 43]. In addition, SKPs display all the predicted properties of multipotent dermal SCs including HF morphogenesis demonstrated in rodents but not in humans [42]. Once transplanted into skin, SKPs can generate new dermis and reconstitute the dermal papilla and connective tissue sheath [43]. It has been proved that rodent dermal papilla cells can be removed from HFs and transplanted in their intact state into recipient skin, where they induce de novo HF development and hair growth [44].

Thus, SKPs are attractive tools for regenerating the skin dermis. However, isolating SKPs from human skin requires invasive surgical procedures, and the isolated cells may have limited or variable abilities to proliferate and/or differentiate. Mesenchymal stem cells (MSCs) also have the same problems. Therefore, there have been many studies that generated MSCs from pluripotent stem cells [45].

The amelanotic melanocytes (AMMC) are considered to be melanocyte stem cell population [46].

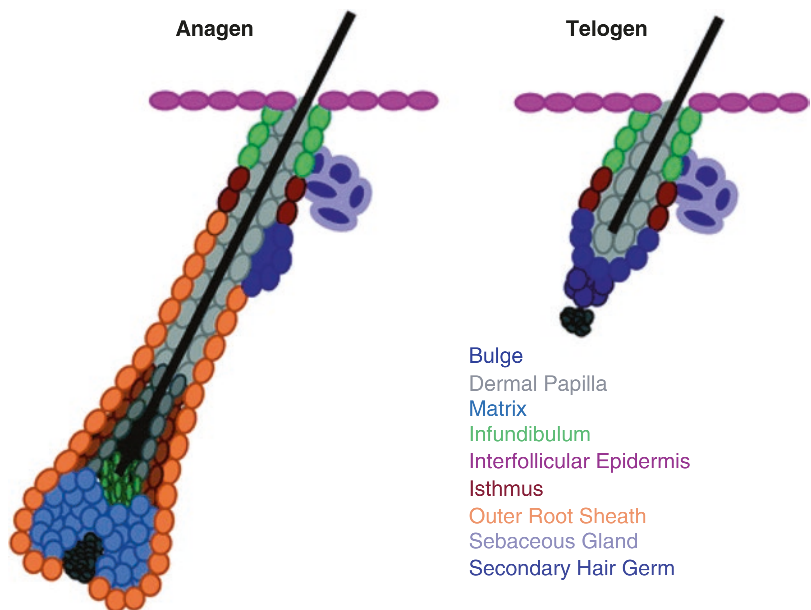


Fig. 2 Depiction of the hair follicle stem cell niche (Reproduced from Lee et al. [38])

Cell Lineages Obtained from Skin

Skin is an established tissue source for cell-based therapy. Various cell lineages may be obtained from skin. In addition, the ease of tissue harvest and the multipotent nature of skin and HF stem cells have promoted basic and clinical research in this area [47].

Some neural crest stem cells persist within crest-derived tissues. HFSCs (hair follicle stem cells) and SSCs (skin stem cells) are both originated from neural crest cells. Although both types of stem cells can differentiate into neuronal and melanocyte lineages, HFSCs are a better source for melanocyte differentiation and SSCs are more inclined to neuronal differentiation [47].

The hair follicles have been shown to harbor pluripotent neural crest stem cells [48] which can be differentiated into melanocytes, neuronal cells, adipose cells, and other lineages [47]. Bulge cells can differentiate into all types of cutaneous epithelial cells including sebaceous glands and interfollicular epidermal keratinocytes [2].

DSC and SKP are of neural crest in origin and are capable of differentiating into melanocyte and neural lineages. SKPs are closer to the neuronal cell lineage, while DSCs are closer to the melanocyte progenitors [49]. SKPs can generate both mesodermal and neural derivatives, including adipocytes, skeletogenic cell types, and Schwann cells [42, 50–54], but tend to have spontaneous differentiation toward neuronal lineage. When SKPs are transplanted to full-thickness skin wounds, they originate a variety of fibroblast phenotypes and fill the lesion with new dermal tissue. Transplanted SKPs are also able to integrate into the mesenchyme of existing HFs and initiate formation of new HFs when cotransplanted with epithelial cells [42].

Adult cells can return to the embryonic stage with the possibility of differentiating toward all the specialized cell categories [55]. Skin fibroblasts can be reprogrammed to hiPSCs (human induced pluripotent stem cells) with the potentiality of obtaining all the cellular lineages that can be derived from them. iPSC can be differentiated into specific cells with a wide spectrum of cellular phenotypes. Fibroblasts differentiated from iPSC acquired an augmented biological potency that exceeded those from their parental fibroblasts, characterized by their increased production and assembly of ECM, functional features important for application of these cells in regenerative therapies [24].

Sugiyama et al. provided an induction protocol of SKPs from human iPSCs [2]. The human iPSC-derived SKPs (hiPSC-SKPs) express several genes and proteins that have been previously reported to be expressed by human SKPs [50]. As for their differentiation potential, hiPSC-SKPs can successfully differentiate into adipocytes, osteocytes, and Schwann cells. In addition, hiPSC-SKPs were able to induce hair follicular keratinization when they were co-cultured with epidermal keratinocytes. These observations suggest that hiPSC-SKPs may facilitate the regeneration of human full-thickness skin, including skin appendages [2].

Human epidermal keratinocytes and epidermal SCs have also been developed from induced pluripotent stem cells (iPSCs). Additionally, iPSC-derived epidermal cells have the ability to reconstitute HFs with mouse dermal cells [56].

Background

The first milestones in skin research were the enzymatic separation of the epidermis and dermis [57] and the in vitro culture of human skin epithelial cells [58]. Cell culture appeared with the introduction of trypsinization by Moscona et al. [59]. In 1975, Rheinwald and Green started serial cultivation of keratinocytes (autologous epidermal cultures) and showed that the limitations of epidermal cell cultures were not intrinsic, but due to the relationship between keratinocytes and fibroblasts [5]. An epidermal graft could be expanded to more than 500 times its size within 3–4 weeks [6]. After the first clinical grafting of autologous cultured epithelium prepared from autologous epidermal cells performed by O'Connor et al. [7] in 1981, cultured epidermal autografts (CEAs) were tested in almost all leading burn centers worldwide [26].

In 1981 Bell et al. generated a dermo-epidermal substitute [60]. This technique was transformed into the product Apligraf® (human allogeneic fibroblasts and keratinocytes). On the basis of this development, dermo-epidermal skin substitutes consisting of human autologous keratinocytes and fibroblasts in bovine collagen were also transplanted in severe burn patients [61]. Researchers revealed that the cross-talk between fibroblasts and keratinocytes was essential for the establishment of a functional basement membrane [62]. In 1981, a further significant advance was the development of a bilayered "artificial skin" [63] commonly referred to as Integra® which was commercially launched in the United States in 1996. The appealing idea of combining cultured keratinocytes with Integra® generated a fascinating new field of research. However, reality has shown that simple cultured epidermal autografts do not take well on the neodermis produced by Integra® [26].

Clinical use of injectable autologous skin-derived fibroblasts was first started by Isolagen Technologies in 1995 to repair dermal and subcutaneous contour deformity. Long-term correction and no allergic adverse effects have been reported, which made autologous fibroblasts a promising alternative to the use of other foreign materials [64].

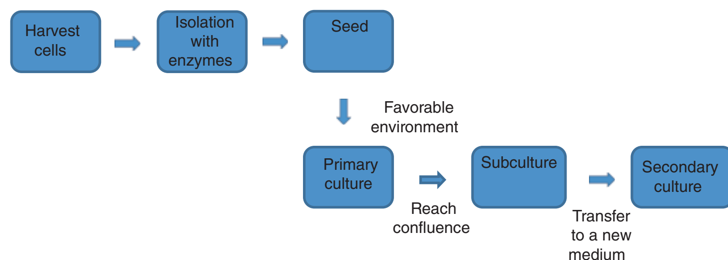
Until 1990, preclinical human hair research had been limited to histological studies or to difficult-to-perform in vivo assays with human skin transplanted onto mice [35], while human HFs could not be maintained and studied ex vivo. The main challenges were not only to maintain the HFs viable, but to keep their function. In 1990 Philpott et al. [65] developed an ex vivo model for the study of isolated human scalp HFs. Not only was the morphology and keratin synthesis of the HFs preserved up to day 4, but also more importantly, the follicles demonstrated rates of de novo hair shaft growth approximating that seen in vivo. This ability to maintain viable human HFs ex vivo constituted a methodological breakthrough in human hair research and raised the possibility of investigating the effects of a wide range of hormones, neurotransmitters, growth factors, cytokines, and drugs on human HF biology, while simultaneously promising new insights into the pathogenesis of a range of hair growth disorders [35].

Skin Cell Culture

Cell culture is the process by which cells are grown under controlled conditions in a favorable artificial environment. When primary cultures reach confluence (they fill all the substrate), the cells are then subcultured (passaged) by transferring them to a new medium (Fig. 3). The stem cells are key pieces in cell cultures. These cells can be maintained in culture for a longer period of time compared to other cell types [47].

Cultures are affected by many factors which influence cell function, proliferation, differentia-

Fig. 3 Cell culture procedure



tion, and transcriptional status. Temperature, pH, time, replicative potential, medium composition (growing factors and other cell signals), cell characteristics and origin, passage number, physical and physiological stress (i.e., shear stress), plastic plate adherence, aggregate size, agitation rate, impeller size, and volume of culture medium are decisive factors. Each cell type needs its appropriate culture medium with rigorously controlled characteristics. Stress imposed by inadequate culture conditions induces senescence [4]. Plastic culture flasks are commonly used for cell culture of single cell types [1]. An incubator is used to grow cell cultures maintaining optimal temperature, humidity, and CO2 and oxygen concentrations. The survival rate of a given type of cultured cell depends on the degree of adhesion to the plastic plate, meaning that the adherence of cells is an essential survival factor [66].

Establishment of a novel culture method can sometimes open up huge new fields of cell biology and medicine [66]. One difficulty is the large discrepancy between cell kinetics in vivo and in vitro due to the extreme difficulty to reproduce an anatomical or physiological microenvironment. Several factors, including cytokines, scaffold material, cell–cell interactions, and physical stress, constitute this artificial microenvironment [1].

A variety of studies have been developed to understand and control cell cultures, and certain assumptions have been raised. First, the presence of fetal bovine serum in cell culture medium is questionable since there is a lack of characterization and quantifying of growth factors. Furthermore, the ideal culture media should contain specific nutrients according to the cell type. Second, the in vitro cell culture environment is very different from the in vivo environment. Third, despite widespread use of proteolytic enzymes, it must be taken into account that using these enzymes in the cell passage or tissue digestion promotes the destruction of both the ECM and surface proteins and may, thus, modify signaling and mechanotransduction of signals to the nucleus [16, 17].

Unlike germline and stem cells, somatic cells have a limited lifespan. They stop dividing when cultured in vitro for a certain period of time [67]. Typical human primary keratinocytes possess an in vitro lifespan of around 15–20 population doublings in serum-free and chemically defined media [68]. When cells encounter the so-called Hayflick limit, they enter a state of permanent quiescence, often named cellular senescence [4, 69]. Continuous replication of typical primary human cells is prevented by two events: mortality stage 1 (M1) or “replicative senescence” and mortality stage 2 (M2) or “cellular crisis.” Cells entering senescence first stop responding to exogenous mitogenic stimuli and acquire increased cellular adhesion to the extracellular matrix while losing cell–cell contacts. In addition to prolonged in vitro culture of primary cells, various types of cellular stresses including telomere erosion, DNA damage, overexpression of tumor suppressor genes or oncogenes, oxidative stress, continuous mitogenic stimuli, and a variety of chemicals can also induce senescence [4]. Unrepairable severe terminal telomere shortening eventually leads to cellular crisis, a state characterized by massive cell death [70].

Cell Characterization

Each cellular type holds a particular protein expression profile (Table 1) which can be detected by different methods (i.e., immunofluorescence, flow cytometry, etc.).

Different cell type culture protocols will be summarized. Detailed description of cell culture techniques is out of scope of this chapter.

Fibroblast Culture

Techniques for culturing fibroblasts were long established prior to the discovery made in 1975 by Rheinwald and Green [5] for culturing and expanding keratinocytes, which require fibroblasts to support their proliferation. Dermal fibroblasts can be extracted from skin biopsies either through enzymatic degradation or by explant culture. The medium used for culturing fibroblasts is usually supplemented with fetal calf serum, which previously raised concerns regard-

Table 1 Cell characterization

Type of cell	Cell markers	References
Basal layer epidermal stem cells (Epi-SCs)	K15, K19, cytokeratin (CK)5, CK14, CD29 (integrin β1), and CD49f (integrin α6)	[37, 71]
Hair follicle Epi-SCs	CD34, Lgr5, or K15	[40]
SSCs and HFSCs	Cytokeratin (CK)19, CK15, and β1 integrin	[47]
Differentiated melanocytes	HMB45 (Human Melanoma Black 45) and S100	[47]
Differentiated neurons	NF (neurofilament) and TH (tyrosine hydroxylase)	[47]
Skin-derived precursors (SKPs)	Nestin, fibronectin, and BMP6	[72]
Human hair follicle stem cells	CD200	[40]
Dermal stem cells (DSCs) and their progeny	α-Smooth muscle actin (α-SMA), fibroblast-specific protein 1 (FSP1), PDGF receptor-α (PDGFRα), and dermal extracellular matrix protein collagen III	[66]
Dermal papilla (DP)	CD133, integrin-α8, and versican	[2, 43]
Hair mesenchyme DP and DSCs	SOX2	[73]
Sebocyte progenitors	Lrig1	[40]

ing transmission of bovine spongiform encephalopathy (BSE). However, the serum is obtained only from BSE-free countries [29].

Fibroblasts and adult stem cells divide through asymmetric division, which means that the replicating cell gives rise to one adult stem cell and one specialized stem cell (fibroblast), suggesting the continuity of cell division until complete differentiation of the stem cells [74].

Fibroblasts are readily cultured in the laboratory (Fig. 4) and incorporation of fibroblasts into tissue-engineered skin substitutes has produced encouraging results including symptomatic pain relief, rapid healing, less scarring, and better cosmetic results [29]. Growth parameters and the characteristics of fibroblasts in culture will be influenced by passage number, age of the donor, subtype of fibroblast (reticular or papillary dermis), and anatomical site. Older donor skin fibroblasts tend to migrate more slowly, reach cell culture senescence earlier, have a prolonged cell population doubling time, and are less responsive to growth factors. Other factors that influence fibroblast behavior in culture include vitamins, such as vitamin C, and antioxidants, including coenzyme Q10. For example, in the presence of vitamin C, fibroblasts produce twofold more collagen, a response that is independent of the age of the fibroblasts [75]. Likewise, coenzyme Q10 promotes wound healing by increasing cell proliferation and fibroblast mobility [76].

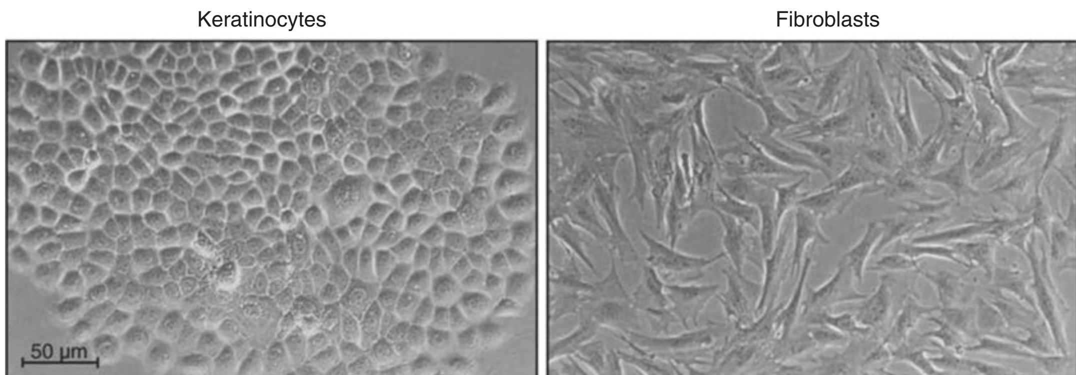


Fig. 4 Phase contrast images of minipig keratinocytes and fibroblasts grown in monolayer culture (Reproduced from Dame et al. [77])

Several protocols for culturing fibroblasts have been described. Solakoglu et al. [78] used rat biopsies which provided dermal connective tissue that was treated with collagenase B and DNAase. Fibroblast culture is usually performed in flasks at 37 °C with 5% CO2 in humidified air [74, 78, 79]. Many different culture mediums have been used:

- Eça et al. 2012 added culture medium containing L-amino acids, Earle's salts, and sodium bicarbonate, supplemented with human serum from the patients to the culture flasks containing the dermis fragments [74].
- Solakoglu et al. used DMEM (Dulbecco's modified Eagle's medium)-F12 medium and fetal calf serum [78].
- Weiss utilized Iscove's modified Dulbecco's medium (IMDM) with phenol red supplemented with antibiotics and fetal bovine serum (FBS) [79].
- Zhao et al. used DMEM supplemented with FBS, penicillin, streptomycin, and glutamine [80].
- Sugiyama et al. cultured human primary fibroblasts in DMEM including 5% FBS [2].
- Kumar et al. cultured immortalized human foreskin fibroblast (I-HFF) in IMDM supplemented with fetal bovine serum, L-glutamine, non-essential amino acids, and penicillin and streptomycin [47].

When cells reach confluence they are detached from the culture plate with trypsin solution [64, 74, 78, 79]. Solakoglu et al. cultured fibroblasts for 3 weeks by 2 or 3 passages [78]. These cultures expand rapidly resulting in a higher percentage of live cells with the human serum technique than with the use of fetal bovine serum [74]. Culturing fibroblasts at the air–liquid interface (ALI) culture system, which imitates the skin microenvironment, promotes optimal differentiation approaching that of skin in vivo [29].

Autologous fibroblasts can be cultured for their posterior injection [79, 80]. After biopsy collection, skin samples are inspected for quality and transferred to tissue culture plating. After an antibiotic wash, biopsy tissue is subjected to enzymatic dissociation in a collagenase enzyme cocktail at 37 °C. Cells are then seeded into a culture flask with IMDM with phenol red supplemented with antibiotics and fetal bovine serum (FBS) [79].

Keratinocyte Culture

Ex vivo keratinocyte short lifespan has limited many skin-related applications. In order to overcome this difficulty, many attempts to immortalize primary keratinocytes have been made with success. Different kinds of primary cells are able to become immortal through a variety of cellular events including overexpression of telomerase, epigenetic gene silencing, oxidative DNA damage, inactivation of cell cycle regulatory genes, overexpression of cellular or viral oncogenes, and inhibition of a specific host kinase. Nevertheless, immortalized keratinocyte cell lines turn out to have several undesirable genetic abnormalities. In spite of these genetic defects, immortalized keratinocytes seem to maintain some properties of normal keratinocytes, which enable them to be used as a substitute for primary keratinocytes in various skin research fields [4].

Cells that have a lifespan of 20–50 passages under in vitro culture conditions are mostly blast cells, such as fibroblasts. Cells that have a lifespan of less than 10 passages under in vitro culture conditions are typically epithelial cells, such as keratinocytes. In many epithelial cells, epidermal growth factor (EGF) has been shown to be able to increase their lifespan to 10–20 passages before senescence [4]. Human primary keratinocytes can be cultured in keratinocyte medium (J-TEC) [2].

Melanocyte Culture

Kumar et al. [47] induced differentiation of SSCs and HFSCs into melanocytes. For melanocyte differentiation, 70–80% confluent cultures of SSCs and HFSCs are incubated in molecular, cellular, and developmental biology (MCDB) 201 medium and Ham's F12 nutrient mix, supplemented with

fetal calf serum, L-glutamine, L-ascorbic acid, phorbol 12-myristate 13-acetate (PMA), cholera toxin, fibroblast growth factor, and penicillin and streptomycin. PMA is used as an inducer of melanocyte, promoting cell proliferation and helping the formation of multiple dendrites [81]. Geneticin is used to remove the contaminating fibroblasts. The protocol to differentiate the stem cells into melanocytes lasts 21 days. The culture dish becomes homogenously confluent with melanocytes in almost 25–30 days. The authors found no visible difference in the melanocytes differentiated from HFSCs and SSCs after staining with HMB-45 and S-100 antibodies for immunofluorescence. Nevertheless there were a higher percentage of cells functionally active in melanocytes derived from SSCs than in those derived from HFSCs [47].

Adipocyte Culture

Aoki et al. described a unique culture technique for floating adipocytes called "ceiling culture" [66]. Mature adipocytes are mesenchymal cells with abundant lipid droplets within their cytoplasm. As the gravity of mature adipocytes is lower than that of culture medium, mature adipocytes float in medium, and it is quite difficult for floating cells to attach to a plastic culture plate. The authors cultured adipocytes in flasks that were completely filled with medium. Under these conditions, adipocytes became attached to the ceiling of the flask. Then these cells were able to proliferate, form a cell monolayer, and exhibit accumulation of intracytoplasmic lipid droplets after reaching confluency. The authors established an adipose tissue-organotypic culture system in addition to the ceiling culture system, which was able to maintain the proliferative ability and function of mature adipocytes for more than 4 weeks [66].

Wang et al. cultured Epi-SCs derived from the epidermis of neonatal mice or adult human foreskin in CnT-07 PCT epidermal keratinocyte medium containing dexamethasone, insulin, rosiglitazone, and XAV939 for 3 days to induce sebocyte differentiation [40].

Recently, dedifferentiated fat (DFAT) has gained attention in regenerative medicine, because it contains multipotent stem cells [82]. The ceiling culture method is a fundamental technique for the fabrication of DFAT cells, which are able to differentiate into other mesenchymal cell types such as adipocytes, chondrocytes, and osteoblasts [66].

Neuronal Culture

For neuronal differentiation, 70–80% confluent cultures of stem cells are incubated in the neurobasal medium containing penicillin and streptomycin supplemented with basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), B-27 supplement, and L-glutamine. The contaminating fibroblasts are removed with geneticin. The cells start to change their morphology after 4–5 days of culture [47].

Skin-derived precursors (SKPs) are the only neural stem cells which can be isolated from an accessible tissue such as skin. Bayati et al. presented a protocol to enrich neural SKPs by monolayer adherent culture [83]. This culture method helps to increase the number of neural precursor cells. The authors found that serum-free adherent culture reinforced by growth factors was effective on proliferation of skin-derived neural precursor cells (skin-NPCs). The cells of enriched culture possessed a multipotential capacity to differentiate into neurogenic, glial, adipogenic, osteogenic, and skeletal myogenic cell lineages.

Skin Stem Cells (SSCs) and Hair Follicle Stem Cells (HFSCs)

Kumar et al. carried out in vitro expansion of skin stem cells (SSCs) and hair follicle stem cells (HFSCs) by explant culture method [47]. Skin tissue measuring approximately 2 × 2 mm and individual hair follicles were used as explants. Culture was performed according to the modified Rheinwald system [5] consisting of DMEM and Ham's F12 nutrient mix, supplemented with fetal

bovine serum, epidermal growth factor, hydrocortisone, insulin, transferrin, cholera toxin, and penicillin and streptomycin over a fibronectin-coated culture dish. HFSC could be expanded for 10 passages as compared to SSC which could be taken for up to eight passages [47].

Wang et al. demonstrated that a combination of cultured human Epi-SCs and skin-derived precursors (SKPs) was capable of reconstituting functional hair follicles and sebaceous glands (SG) in mice. The Epi-SCs formed de novo epidermis along with hair follicles, and SKPs contributed to dermal papilla in the neogenic hair follicles. Notably, a combination of culture-expanded Epi-SCs and SKPs derived from the adult human scalp could generate hair follicles and hair. In addition, Epi-SCs were able to differentiate into sebocytes and form de novo SGs, which excreted lipids [40]. Rapid attachment to plastic culture dishes has been recognized as a property of Epi-SCs [84]. Therefore Wang et al. selected the cells that rapidly attached to the dish. Epi-SCs were then cultured in the CnT-07 progenitor cell-targeted (PCT) epidermal keratinocyte medium. The dermis was digested with collagenase to isolate SKPs. Their culture was performed in Dulbecco's modified Eagle's medium/F12, supplemented with B27, epidermal growth factor, and basal fibroblast growth factor in untreated dishes [40].

Like many stem cell cultures, SKPs are typically grown in static tissue culture flasks as nonadherent, spherical colonies. Recently, Agabalyan et al. presented a new technique consisting of enhanced expansion of SKPs in computer-controlled stirred-suspension bioreactors. Rat SKPs (rSKPs) were isolated from the back skin and were grown in Dulbecco's modified Eagle's medium (DMEM) low glucose/F12 with basic fibroblast growth factor, platelet-derived growth factor (PDGF)-BB, B27 supplement, and penicillin/streptomycin. Following primary colony formation, SKPs were dissociated to single cells using collagenase and replated. SKPs were passaged three or four times in static cultures to obtain sufficient numbers of cells to introduce in the bioreactor. Then rSKPs were cultured for three passages in 500-mL computer-controlled DASGIP Parallel Bioreactor Systems (Eppendorf, Hamburg, Germany). The variable bioreactor set points were regulated at 60 rpm, 37 °C, pH 7.4, and a 21% dissolved oxygen concentration [43].

Sugiyama et al. developed a method to induce human SKPs (hSKPs) from human induced pluripotent stem cells (hiPSCs). The induction efficiency of this method is very high (over 95%) in a short period and the hiPSC-SKPs exhibit SKP characteristics. To generate SKPs from hiPSCs, the authors established a differentiation protocol in which hiPSCs were initially differentiated to the multipotent neural crest stage as precursor cells of SKPs. Human iPSCs were treated with human recombinant noggin and SB to promote highly efficient neural induction [2]. A human iPS cell line (201B7) was generated by introducing four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) into human skin fibroblasts [55]. The hiPSCs were cultured on inactivated SNL feeder cells using hiPSC medium containing DMEM/F12, knockout serum replacement, non-essential amino acids, L-glutamine, β-mercaptoethanol, bFGF, and penicillin and streptomycin. When hiPSC colonies reached 80–90% confluence, they were plated on SNL feeder cells in hiPSC medium without bFGF, including noggin and SB431542. Then, they were cultured in SKPs medium containing DMEM/F12, B27 supplement, penicillin and streptomycin, bFGF, EGF, and CHIR99021 (CHIR). When cells reached 80% confluence, they were dissociated using Accutase cell detachment solution and were subcultured in new dishes in SKPs medium without CHIR. After 5 days, a sufficient number of cells were obtained, which were termed hiPSC-SKPs[2].

Sugiyama et al. described adipogenic, osteogenic, and neurogenic (Schwann cell) differentiation from hiPSC-SKP the same as from traditional SKPs. In addition, hiPSC-SKPs can differentiate into osteogenic cells, unlike SKPs. hiPSC-SKPs can also induce follicular type keratinization. Epidermal keratinocytes and hiPSC-SKPs express trichohyalin, a hair follicle-specific protein [2].

Human Embryonic Stem Cell-Derived Endothelial Precursor Cell (hESC-EPC) Culture

To differentiate hESC effectively into endothelial cells, several approaches have been taken, including altering cytokines in the medium and co-culturing with other cells such as stromal cells [85]. The embryoid bodies (EB) formed spontaneously from hESC have frequently been used to promote differentiation of hESC into endothelial cells [86]. Culture and differentiation of hESC has been described previously [87]. Cells are cultured on human collagen-coated dishes in EGM-2/MV medium. For expansion of hESC-EPC, cells are passaged by trypsinization [85].

Stem cells have been shown to have a therapeutic effect in several ischemic animal models. We examined the wound-healing effect of secretory factors released by hESC-EPC. Conditioned medium (CM) of hESC-EPC was prepared and applied in a mouse excisional wound model. hESC-EPC CM accelerated wound healing, increased the tensile strength of wounds, and caused more rapid re-formation of granulation tissue and re-epithelialization of wounds. In vitro, hESC-EPC CM improved the proliferation and migration of dermal fibroblasts and epidermal keratinocytes. hESC-EPC CM also increased the extracellular matrix synthesis of fibroblasts. hESC-EPCs secrete many growing factors and interleukins important in angiogenesis and wound healing [85].

Human Hair Follicle Organ Culture (HFOC)

Langan et al. described HFOC culture conditions and quality control [35]. Remarkably, even after having been removed from the human body, the HF maintains some of its in vivo characteristics in HFOC. HF growth ex vivo is influenced by the stage of the microdissected follicle, its rate of growth, its intrinsic hair cycle, the rate of matrix keratinocyte proliferation, the differentiation of matrix keratinocytes into the mature hair shaft, and the HF epithelial stem cell proliferation/apoptosis. It is also important that the major stem cell component (bulge) is absent in microdissected and amputated HFs and is only present when full-length HFs are microdissected and cultured [35]. Successful growth of anagen VI terminal HFs ex vivo for up to 2 weeks has been demonstrated [88].

Despite its important role in preclinical hair research, human HFOC clearly has major limitations due to elimination of neural, vascular, and endocrine controls of human HF biology, as well as multiple factors contained in serum. Probably, the rapid HF entry into catagen ex vivo reflects that the HF is significantly stressed by the trauma of microdissection, denervation, and serum and hormone deprivation [35]. It must also be noted that anagen scalp HFs in HFOC operate in the absence of their epithelial and melanocyte stem cell populations in the bulge, even though keratin 15+ or keratin 19+ epithelial progenitor cells and amelanotic melanoblasts are still present in the proximal outer root sheath (ORS) of organ-cultured human HFs [89]. The most important limitation of current human HFOC techniques is that human anagen HFs routinely fail to reach even the telogen phase before they degenerate ex vivo [35].

The original HFOC model [65] has been adapted for a wide range of applications. Microdissected HFs can be cultured in a serum-free medium (Williams' E), supplemented with L-glutamine, hydrocortisone, insulin, penicillin, and streptomycin, and maintained at 37 °C in 5% CO2 air [35].

It is now possible to knock-down defined genes in human HFOC and to assess the gene expression profile of defined microdissected human HF in situ [12]. These recent developments have greatly enhanced the usefulness and instructiveness of HFOC for preclinical hair research [35].

Dermal Papilla Cell Culture

Human dermal papilla cells can be cultured in dermal papilla cell medium (Cell Applications) [2].

Reproducing a Physiological Microenvironment

Physiological stress is an important factor in both the morphogenesis and homeostasis of various organs. Physical stress has been implicated in the physiologic responses observed in cell behavior [90]. To replicate the tissue architecture, cell–cell interactions, and specific physical microenvironment, Aoki et al. demonstrated the effectiveness of a three-dimensional collagen gel culture system and further established two simple culture systems: air–liquid interface (ALI) and fluid flow stress (FFS) [66]. The microenvironment physical stress (forces of gas and fluid streaming) has a huge effect on the proliferation and differentiation of various cell types [66, 90]. The ALI culture system consists of three components: outer plastic dish, inner cell insert, and collagen gel scaffold. Skin is constantly exposed to air; thus the ALI system closely imitates the skin microenvironment [66].

Alternatives to Proteolytic Enzymes in Cell Culture

Proteolytic enzymes affect the balance between ECM degradation and deposition during cell passage. These enzymes not only destroy the ECM but also interfere in surface proteins; therefore profound changes in stem cell behavior may be produced [16].

Huang et al. studied the proteomic changes caused by the use of proteolytic enzymes (such as trypsin) in cell passage [91]. They found that 36 proteins were differentially expressed in the trypsin-treated cells. Proteins related to the regulation of metabolism, growth, the mitochondria electron transport, and cell adhesion showed less expression, while proteins that regulate apoptosis showed more expression. Cell detachment without proteolytic enzymes may maintain membrane proteins and preserve mesenchymal stem cell properties. Therefore, alternatives to the use of trypsin are being developed, such as cell culture in cell sheets or hydrogel 3D culture [16].

Culture in Cell Sheets

Cell sheets culture was developed to promote cell passage without the use of proteolytic enzymes [16]. Yamada et al. developed the cell sheets technology, in which the cells and their ECM are collected together, without proteolytic enzymes treatment or any tool for extracting cells [92]. The plates are coated with thermo-responsive polymers which change its cell adhesion property as the temperature changes. Several groups have been using the cell sheets technology [91, 92]. It has been demonstrated that, after three passages, cells grown in cell sheets preserve both viability and proliferation properties, and differentiation to some extent [93].

Hydrogel 3D Culture

Hydrogel can mimic the tissue-specific cellular 3D microenvironment by manipulating the ECM physicochemical properties and components, according to tissue and culture requirements. However, it is a challenge to promote appropriate oxygen, soluble factors, and the requirements of cell nutrients transport in hydrogel 3D culture. Hydrogel can be used to culture cells in bioreactors, as a 3D culture which avoids the use of proteolytic enzymes, as a mechanical vehicle to 3D cell/organ printing, and as a biocompatible material to be implanted in vivo. Despite the challenges, hydrogel 3D culture, which avoids proteolytic enzymes, is a good alternative solution for either preservation or manipulation of ECM components [16].

Three-Dimensional (3D) Cultures

Two-dimensional (2D) cell culture systems have routinely been adopted around the world for the past four decades [66]. The 2D cell culture microenvironment affects cellular function, since only one side of the cell is in contact with the ECM and the neighboring cells [16]. Creation of a 3D scaffold provides a better physiologic microenvironment for cultured cells and is expected to more closely develop the cellular function.

Several types of 3D skin culture systems have been developed. A 3D skin culture system was

introduced by Ozbun et al. [94] to grow differentiating epithelial tissues that mimicked important morphological and biochemical aspects of skin. This technique is often called an organotypic raft culture due to its apparent floating nature with growing keratinocytes on top of a collagen lattice with fibroblasts [4]. Organotypic raft culture promotes stratification and full differentiation of keratinocytes when placed at the air–liquid interface [4]. Aoki et al. demonstrated the advantages of using a collagen gel-based 3D cell culture system to analyze the effects of adipose tissue on various cell types in vitro [66]. As previously described, hydrogel 3D is an interesting culture alternative [16].

Automatic Bioprocessing

Although static tissue culture is sufficient to generate cells for experimental purposes, it is impractical for generating the large quantities of SKPs that would be required in an autologous cell therapy to repopulate the HF mesenchyme or dermis to enhance wound healing. Static tissue culture methods are time and labor intensive, and manual handling and inherent cellular variation between flasks are influential factors. Therefore, controlled cell culture processes must be developed to efficiently and safely generate sufficient stem cell numbers for clinical use. Computer-controlled stirred-suspension bioreactors can be used for this purpose and generate a large number of DSCs while maintaining their phenotype and at least some of their inherent inductive function [43].

Previous studies using cell types that included murine embryonic stem cells, human embryonic stem cells, multipotent adult progenitor cells from bone marrow, neural precursor cells, mesenchymal stem cells, and induced pluripotent stem cells have all shown that stirred-suspension bioreactors are an effective alternative for culturing stem cells. Stirred-suspension bioreactors offer several advantages over static cultures, including reduced labor and costs, higher yield, more cellular homogeneity, reduced space requirements, and increased cell density per volume. They also allow for precise monitoring and control of key process variables, such as physiochemical environment, thus providing a healthy environment for cells and often leading to increased cell proliferation [43]. The shear stresses produced in stirred-suspension bioreactors can stimulate proliferation and differentiation of stem cells [95]. Moreover, authors have shown that shear stress in stirred suspension can play a role in the expression of stem cell markers [96]. Exposure to shear force might liberate single cells from proliferating aggregates, thereby reducing the average colony size and allowing for formation of new colonies and producing an increase in viable cell number in stirred-suspension bioreactors [43].

Compared with static culture, stirred-suspension bioreactors generated fivefold greater expansion of viable SKPs which were able to reconstitute the HF mesenchyme, to induce de novo hair follicle morphogenesis, and to exhibit bipotency, reconstituting the dermal papilla and connective tissue sheath, although bioreactor-grown SKPs exhibited a significant reduction in hair-forming ability compared with static-expanded SKPs [43]. Little phenotypic differences were found in SKPs exposed to either static or automated bioreactor expansion, and most DSC markers, with the exception of SOX2, were sustained over multiple passages. In static culture, a subset of aggregates consistently adhered to the culture flask, and the frequency of adhesion appeared to increase over passages. All rSKP aggregates grown in the bioreactor remained in suspension, as the reactors were siliconized before use, thus preventing adhesion to the vessel surface [43]. Adhesion is indicative of differentiation and would largely contribute to the limited expansion observed in static culture [42].

Skin Engineering

Significant progress has been made over the past 25 years in the development of engineered substitutes that mimic human skin (Fig. 5), either to be used as grafts or for the establishment of in vitro human skin models [98]. Several methods have been described to build skin maintaining its structure and function [24].

Insights from developmental biology are already pointing to the development of "intelligent materials" that work with nature's own mechanisms of organogenesis and repair. Biologically active and appropriate matrices and factors in combination with automated (tissue printing) techniques are designed to produce a new generation of complex skin substitutes in a desired number and with a constant quality [26]. Tissue engineering is emerging as a potential solution for tissue and organ failure (Fig. 6) [40]. Tissue engineering has given tools to cover large surface wounds which has been one of the major challenges in clinical research [41]. Successful regeneration of skin with skin substitutes depends on two factors: the presence of self-renewing keratinocyte stem cells for re-epithelialization and a functional dermal substitute consisting of the appropriate cellular and acellular components, which allow no or only limited scarring of the developing skin [26]. To prevent immunological incompatibility, autologous cells may be used. However, the number of cells required for the construction of an organ or tissue is much greater than the number of cells obtained from an autologous donor cell source. In this way, expanding the number of cells in cell culture for a long period is required until the necessary amount of cells is obtained. Nevertheless, maintaining the cell characteristics throughout the expansion process is a challenge as cell processing and cell expansion protocols have not been established yet [16].

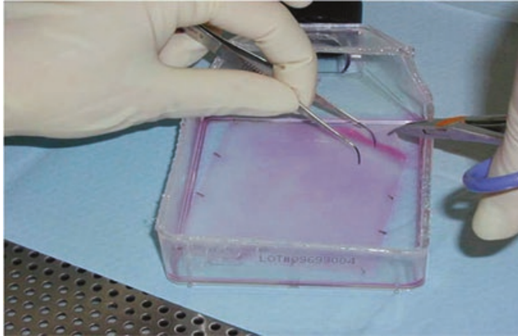


Fig. 5 Preparation of a bioengineered skin substitute (Reproduced from Hakim et al. [97])

Several "commercial" treatment modalities exist along with skin grafting. Various problems

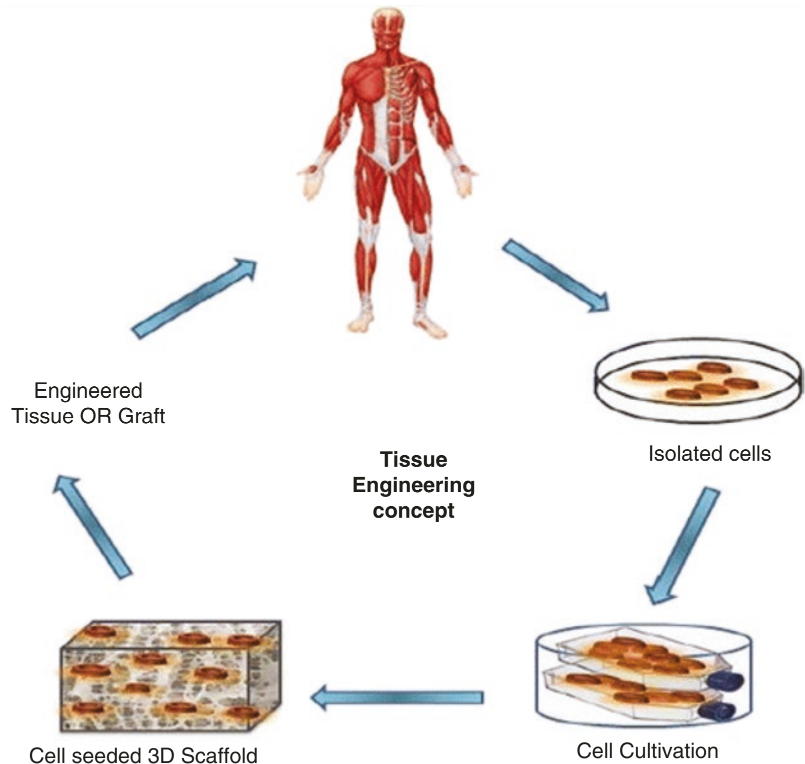


Fig. 6 Tissue engineering concept (Reproduced from Pandey et al. [99])

are still encountered. A vascularized wound bed is required for prompt graft attachment. If a dermal substitute reaches a threshold thickness [100], vascularization is too slow resulting in epidermal necrosis or graft loss. Therefore, most dermal substitutes thicker than 1 mm (Integra®, Matriderm®) are applied using a two-step approach, giving the dermal substitute sufficient time to vascularize. Transplantation of an epidermal component needs an additional operation. Features of the transplanted epidermal component may be missing elasticity, contraction, lack of pigmentation, and thereby lack of protection against UV radiation [26].

Keratinocytes and fibroblasts in tissue-engineered skin produce the correct concentration and combination of growth factors and cytokines important for efficient and effective wound repair as well as providing the necessary ECM components. In addition, one of the advantages of using skin substitutes is that they can be cryopreserved and that following thawing, the fibroblasts retain the ability to proliferate and produce appreciable amounts of VEGF, hepatocyte growth factor, basic FGF, TGF-β1, and IL-8 [29, 101].

Several commercial products have been developed during the last 30 years (Table 2). They can

Table 2 Examples of skin substitutes

Skin substitute	Structure	References
Bioseed-S	Autologous keratinocytes, fibrin glue	[61]
MySkin®	Autologous keratinocytes grown in the presence of irradiated murine fibroblasts cultured on a silicone support layer	[102, 103]
Epicel®	Cultured epidermal autograft (autologous keratinocytes grown in the presence of murine fibroblasts)	[104, 105]
Epidex®	Cultured epidermal autograft (autologous outer root sheet hair follicle cells)	[106, 107]
AlloDerm®	Acellular donated allograft human dermis	[108, 109]
Dermagraft®	Bioabsorbable polyglactin mesh scaffold seeded with human allogeneic neonatal fibroblasts	[110, 111]
Integra®	Thin polysiloxane (silicone) layer; cross-linked bovine tendon collagen type I and shark glycosaminoglycan (chondroitin-6-sulfate)	[112, 113]
Matriderm®	Bovine dermal collagen type I, III, and V and elastin	[114, 115]
Hyalograft 3D	Hyaluronic acid with autologous fibroblasts and keratinocytes	[116]
Cultured skin substitute	Autologous fibroblasts and keratinocytes in collagen	[61]
Composite skin replacement	Cultured autologous keratinocytes in acellular allogeneic dermis	[117]
Composite skin	Autologous keratinocytes and preadipocytes in Matriderm®	[118]
Autologous bioengineered composite skin	Autologous keratinocytes and fibroblasts in allogeneic plasma from blood bank	[119]
Apligraf®	Human allogeneic neonatal foreskin keratinocytes; bovine collagen type I containing human allogeneic neonatal foreskin fibroblasts	[120, 121]
OrCel®	Human allogeneic foreskin neonatal keratinocytes containing human allogeneic neonatal fibroblasts cultured onto matrix of bovine collagen	[122, 123]
PermaDerm®	Collagen sponge with autologous keratinocytes and fibroblasts	[61]
Biobrane®	Knitted nylon mesh that is bonded to a thin, silicone membrane and coated with porcine polypeptides	[124]
TransCyte®	Semipermeable silicone membrane and human newborn fibroblast cells cultured on a porcine collagen-coated nylon mesh	[125]

be permanent or temporary; autologous, allogeneic, or xenogeneic; and made of natural or synthetic materials as scaffolds for cell attachment. They can be classified into three types.

Types of Skin Substitutes

Epidermal Substitutes

Epidermal substitutes contain autologous keratinocytes, often grown in the presence of murine fibroblasts. Most products belong to the category of "cultured epidermal autografts" (Epicel®, EpidexTM, MySkinTM). Developing the final substitute from a skin biopsy takes about 3 weeks [126]. Thus, burn wounds initially need to be treated with temporary wound dressings. Several studies and multicenter trials [126, 127] show a wide range of take rates with an average value of 50% or less [128], and statements about outcomes are inconclusive due to the diversity of methods. Disadvantages are mainly their slow preparation time, variable engraftment rates, difficult handling, and their high production costs [26]. Another approach for epidermal replacement is the use of autologous keratinocytes in suspension (ReCell®) [129].

Dermal Substitutes

Dermal substitutes used as dermal regeneration templates play an important role in skin reconstruction by improving wound healing and scar formation [26]. Engineered dermal substitutes promote new tissue growth and optimize healing conditions by secretion of growth factors and deposition of dermal matrix proteins [29, 130]. There are currently a range of dermal substitutes which may be acellular or cellular. Some of them consist of acellular matrices, which are permanently incorporated into the patient's wound bed (AlloDerm®, Integra®, Matriderm®) [108, 112, 114]. The four types of commonly used natural materials are collagen, chitosan, hyaluronic acid, and carboxymethyl chitosan. Dermal substitutes need to be covered by a permanent epidermal substitute [130]. These substitutes are colonized and vascularized by the underlying cells usually 3–4 weeks after application [111]. As the autologous neodermis regenerates, the scaffold gradually disappears. Histologic evaluation of biopsies did not show any evidence of immunologic response [131]. More recent approaches are using thinner dermal layers, with the aim of transplanting the dermo-epidermal substitute in a single step [132]. Artificial three-dimensional scaffolds have been used as effective dermal regeneration templates for treating full-thickness skin defects [24].

Incorporation of stromal fibroblasts into dermal substitutes has shown great promise for their application in repairing tissues by fabricating dermal substitutes. In contrast to allogeneic cells, autologous fibroblasts carry no risk of rejection or cross-infection [29]. However, there is often a delay in obtaining sufficient autologous cells, whereas allogeneic cells are cryopreserved and therefore readily available. hiPSCs offer a novel source of autologous cells for dermal regeneration. iPSC-derived fibroblasts may improve efficacy and function for future regenerative therapies [24].

The attempt to incorporate growth factors has, in most cases, been disappointing due to their instability. Loading of functional genes into the scaffolds is a way to produce growth factors, which have drawbacks such as enzymatic degradation of the DNA and low cell transfection efficiencies [24].

Dermo-epidermal Substitutes

More than 10 years have passed since the development of cultured skin substitute (CSS), which consists of cultured autologous epidermis and dermal fibroblasts. However, the CSS does not regenerate the appendages. The incorporation of an epidermal component composed of differentiated keratinocyte layers onto a cellular dermal substrate leads to the formation of a bilayered skin substitute [40].

Few engineered, "off-the-shelf" dermo-epidermal substitutes have been produced. Human allogeneic neonatal keratinocytes and fibroblasts are combined with a scaffold to form a temporary covering (Apligraf®, OrCel®) [29, 100, 130]. Apligraf® was the first bilayered living skin equivalent produced [29]. For autolo-

gous cultured dermo-epidermal substitutes [130], keratinocytes and fibroblasts are obtained from a burned patient's biopsy and added to a collagen–glycosaminoglycan substrate [133]. In terms of graft take and scar appearance, this substitute yields results superior to conventional techniques [130, 134], but further clinical studies need to confirm these results [26]. However it has the disadvantage that it is an expensive method, which requires five weeks of preparation [134].

Keck et al. described the construction of a multilayered skin substitute with human preadipocytes from subcutaneous tissue and cultured keratinocytes seeded onto a scaffold (Matriderm®) [118].

Skin Appendages

Wang et al. identified clinically applicable stem cells for de novo regeneration of the hair follicle and sebaceous glands (SG), suggesting a great potential to develop novel bioengineered skin substitutes with appendage regeneration capacity. The authors demonstrated that a combination of culture-expanded Epi-SCs derived from adult human epidermis and culture-expanded adult human SKPs was sufficient to regenerate de novo hair follicles and hairs. In addition, they evidenced that Epi-SCs from the epidermis differentiated into sebocytes in vitro and formed functional SGs in vivo upon appropriate induction [40].

Stem Cells in Tissue Engineering

Recent research regarding the use of stem cells for skin tissue engineering has mainly concentrated on mesenchymal stem cells (MSCs), adipose-derived stem cells (ASCs), embryonic stem cells, and induced pluripotent stem cells (iPSCs). Meanwhile, a few researchers have begun to focus on dermal-derived stem cells [42, 135, 136]. These stem cells are famous for their ability to perform multi-directional differentiation [24].

MSCs in the bone marrow have the ability to differentiate into a variety of cells and tissues derived from the mesoderm and the neural ectoderm. Under certain conditions, hMSCs can differentiate into epidermal-like cells [137]. Therefore, the bone marrow MSCs are used as seed cells to construct full-thickness skin tissue [24]. MSCs have also been used in the induction of vascularization of tissue engineering scaffolds [138].

Research investigating differentiation from ASCs into epidermal cells is very minimal but may result in breakthroughs in the treatment of severe trauma and extensive burns [24]. Therefore, ASCs may be ideal seed cells for skin tissue engineering research [139].

Clinical Applications

Skin Substitutes

Many clinical indications for treatment with skin substitutes have been described:

Chronic Ulcers

Dermal equivalents and bilayered skin substitutes have been used to treat chronic nonhealing wounds, such as venous, diabetic, and pressure ulcers [29].

Burns

Burn injuries may be divided into partial-thickness burns, involving loss of epidermis and papillary dermis, and full-thickness burns where damage is deeper. Superficial partial-thickness burns may result in full regeneration by re-epithelialization without scar formation, in comparison with full-thickness burns where scarring inevitably occurs. Nevertheless, all burn injuries can lead to loss of fluid and proteins, and increase susceptibility to infection, thus requiring immediate attention. Nonbiological topical treatments and biological dressings may be used. Tissue-engineered skin substitutes as temporary biological dressings (i.e., AlloDerm®) are also effective and promote wound healing [140]. Alternatively, the use of a bilayered skin substitute such as Apligraf® or OrCel® requires just one step for skin replacement. The advantage of using Apligraf® as opposed to cadaver skin as a biolog-

ical dressing is that Apligraf® is readily available, of reproducible quality, and does not predispose the patient to infectious disease transmission. Furthermore, Apligraf® incorporates neonatal fibroblasts which have higher proliferative rates and offer the possibility of producing near normal dermis [141]. On the other hand, treatment of burns may involve two steps [29]. First a dermal template with artificial epidermis initially allows autogenous neovascularization and autologous fibroblast migration into the dermal scaffold. Second, following formation of the neodermis, the temporary epidermis is removed and replaced by an epidermal autograft. An example of this type of product is Integra®[29].

Genodermatoses and Other Dermatological Conditions

Tissue-engineered skin substitutes have been used with variable success in epidermolysis bullosa (EB), pyoderma gangrenosum, hydroxyurea-induced leg ulcers, bullous morphea ulcers, and ulcerative sarcoidosis. Fibroblasts for cell-based therapy and gene therapy have been used for the treatment of recessive dystrophic EB [29].

Cosmetic and Reconstructive Surgery

Tissue-engineered skin substitutes have also been used for the treatment of wounds following cancer excision [29]. They have the advantage of not inducing donor-site defects as well as allowing monitoring for local tumor recurrence. Dermagraft® has been successfully used for covering intraoral defects following oral squamous cell carcinoma [142]. In addition, the use of Apligraf® produced better cosmetic results in wounds following Mohs or excisional surgery [143].

Cultured Fibroblasts

Cultured fibroblasts may be utilized to promote tissue repair in a variety of conditions ranging from acute and chronic wounds through to their application in aesthetic and reconstructive surgery. For permanent engraftment, autologous fibroblasts are necessary. However, allogeneic fibroblasts may be used as a biological dressing or for preconditioning of the wound bed prior to graft application, especially when wounds are very large. In addition, using autologous fibroblasts in dermal substitutes has led to better restoration of dermal tissue and minimal scar formation compared with allogeneic dermal substitutes [29].

Skin Stem Cells

The skin stem cells (SSCs) are in clinical setup for a long period of time and have been used for the management of vitiligo, burn, and other pigmentary disorders. Hair follicle stem cells (HFSCs) have also been used for cell-based clinical needs, especially in vitiligo [47].

hiPSC-SKPs can provide an unlimited number of dermal SCs and could contribute to skin dermal regeneration that was lost due to injury or disease [2]. Enhanced expansion of SKPs in computer-controlled stirred-suspension bioreactors might provide a safe and efficient method to generate large numbers of DSCs, thereby permitting drug screening for compounds that might influence HF growth or cell-based strategies to repopulate the skin and hair follicle after injury or disease [43].

Secretory Factors

Secretory factors released from stem cells could be an important mediator of stem cell therapy in ischemic tissue diseases [85].

Clinical Applications in Aesthetic Medicine

Currently the use of cell culture techniques and tissue engineering is not widespread in aesthetic clinical practice.

Fibroblast Injections

Fibroblasts and ECM decrease during skin aging resulting in the formation of wrinkles; therefore a

therapy for rhytids consisting of autologous cell injection has been proposed. Cell injection is neither a dermal filler, nor stem cells (but may contain stem cells, which is under investigation), nor growth factors [74]. Cultured autologous fibroblasts seem to be the first successful implementation of cell therapy for the treatment of wrinkles. This treatment offers the promise of maintained growth of cells, which may persist longer than other fillers [79].

Autologous fibroblasts are the first and only autologous fibroblast cell therapy approved by the US Food and Drug Administration (FDA) for aesthetic use that is grown from patient biopsies and injected back into facial skin (Fig. 7). Fibroblast cell injections appear to be best suited for fine lines with the current on-label indication of nasolabial wrinkle correction [79]. There is evidence that autologous fibroblast injections can improve the appearance of facial wrinkles and depressed scars [145, 146]. Other indications are wounds [147] and subcutaneous atrophy [80]. Some physicians are currently using the product for off-label indications such as glabella folds, periocular rhytids, tear troughs, upper lip rhytids, marionette lines, chest wrinkles, necklace lines, and atrophic skin of the dorsal hands. Long-term results are expected, but not proved. Anecdotal evidence suggests that some patients treated in clinical trials 8 years ago still show clinical benefit for NLF [79].

Fibroblasts secrete different kinds of ECM proteins, of which collagen is the most likely involved in correcting dermal and subcutaneous defects. Type I and type III collagens are the most abundant types of collagens in the skin. In adults, type I collagen constitutes approximately 80% of dermal collagen, whereas type III collagen is abundant in healing tissue, and then it is gradually replaced by the stronger and tougher type I collagen [148]. In Zeng et al. study [64], both types of collagens were secreted by the transplanted human fibroblasts and accumulated gradually during the 3 months.

Unlike traditional fillers, cultured autologous fibroblast cells are injected more superficially and treatment may require months to show improvement. Therefore patients must be informed that this treatment does not work immediately. Autologous cells give gradual improvement after three consecutive treatments over several weeks. Autologous fibroblast cell injection may be a good alternative for patients who do not want foreign materials injected. Side effects are minimal and comparable with other injected agents. Compared with other fillers, additional costs for harvesting and culturing before injection are incurred. Autologous fibroblast treatment may be synergistic to volume fillers. Autologous fibroblasts may provide a long-term solution to the increase of dermal collagen bundles [79]. In addition cultured fibroblasts can extend the longevity of bovine collagen [78].

Clinical trials for autologous fibroblast therapy have been conducted since 2001 [79]. A major

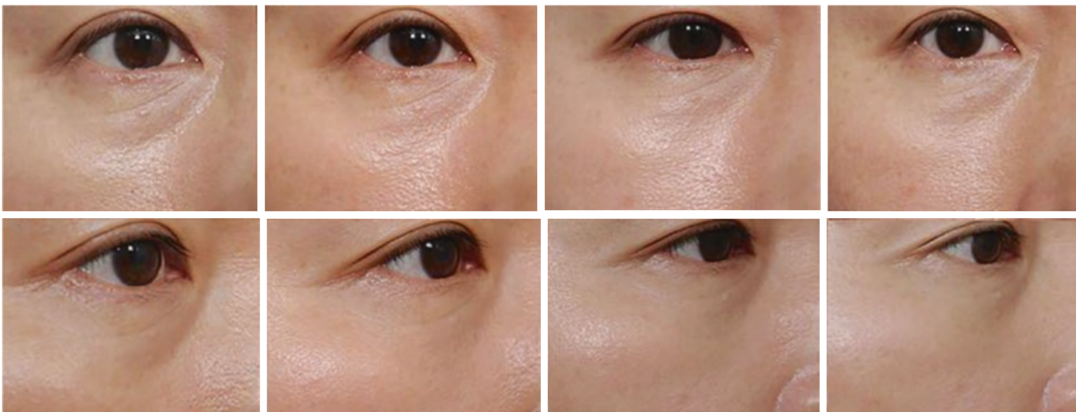


Fig. 7 Correcting nasojugal groove with autologous cultured fibroblast injection (Reproduced from Moon et al. [144])

trial ($N = 215$) with living autologous fibroblast cells for the treatment of facial contour defects was reported in 2007 [149], showing initial hopeful results. Live fibroblasts (20 million/mL) were given in three doses administered at 1-week to 2-week intervals. Efficacy evaluations were performed 1, 2, 4, 6, 9, and 12 months after the first injection. Results indicated that living fibroblasts produced greater improvements in dermal deformities and acne scars. At a 12-month follow-up, patients treated with live fibroblasts continued to show benefit from treatment. No serious adverse events were reported. This finding led to the initial conclusion that autologous fibroblast injections could safely and effectively produce improvements in rhytids, acne scars, and other dermal defects continuing for at least 12 months after injection [79].

Eça et al. performed a study to assess the safety and efficacy of the injection of autologous fibroblasts cultivated in the patient's own serum for dermal repair of skin flaccidity and wrinkles [74]. A skin biopsy was performed in the groin region. Next the dermis was mechanically separated from the epidermis and the hair follicles and then fragmented and transferred into culture flasks. After the primary culture reached 70% confluence, the cells were treated with trypsin solution, centrifuged, and resuspended in PBS (phosphate-buffered saline). Then two aliquots were separated: 1 mL for expansion and 1 mL for injection. The aliquot for expansion was cultured constituting the first cell passage (first population doubling). When confluence of 70% was reached, the cells at first population doubling were once again submitted to trypsinization, with 50% of the cells being used for injection and the remainder for expansion until completion of the second population doubling, when the entire cell content was injected. Injections into the superficial dermis in forehead wrinkles, perioral wrinkles, nasolabial fold, chin, and periorbital skin were performed using a retrograde linear threading technique. Injections were given over four sessions, with a minimal interval of 15 days between each session. The first injection was performed after the first passage (first population doubling). Injections were given every 15 days at the second, third, and fourth population doublings. The cell population increased progressively. The cells resulted in 98% viable cells at the fourth population doubling. Sixty days after completing the four intradermal injections, significant improvement was found in periorbital flaccidity in two cases, with slight improvement in surface lines in one case. No improvement was found in deeper wrinkles. Six months after completion of treatment, no further changes were found. A total of 6.4 × 106 fibroblasts/mL was injected, resulting in a good response in the periorbital region, although surface wrinkles and deeper wrinkles may require a greater number of fibroblasts, as shown in the Weiss study [149] previously described.

The current autologous fibroblast therapy product called Isolagen Therapy™ (Laviv™) is the first cell therapy cleared by the FDA for aesthetic improvement and the first to show statistically significant benefits in large blinded controlled trials [150]. A personalized biopsy kit/shipper is sent to the practice location. The biopsies are performed and processed for culture the next morning. Three 3-mm punch biopsies are performed in the retroauricular area with just enough depth to obtain cells from the dermis, but not as deep as adipose tissue, and placed in the transport media vial. After fibroblast culture, fibroblasts are harvested and cryopreserved. Before use, the cells are thawed, washed with PBS and Dulbecco's modified Eagle's medium, resuspended at a concentration of 1.0 to 2.0×10^7 cells/mL, and shipped overnight at 2–8 °C to the treatment center for administration the next day. Efficacy and safety tests are performed. Before use at the treatment center, the cell suspension is stored at 2–8 °C and then allowed to warm to room temperature for 30 min before use. Only topical anesthetic is used. The area of treatment is cleaned using alcohol with time allowed for the alcohol to evaporate. After gentle inversion of the vial to dislodge clumped cells, aspiration into a 1-mL or 3-mL syringe is performed using a 22-gauge to 25-gauge needle. The cell suspension is injected using 30-gauge needle in a retrograde threading technique or in small aliquots of 0.05–0.1 mL directly into a wrinkle. Serial puncture is most commonly used. No lidocaine

or epinephrine is added to the cell suspension before injection because it could be harmful to cells. The injection is into the superficial papillary dermis and confirmed by the appearance of blanching and wheal formation. No massage or other manipulations of the areas are performed to avoid risk of damaging the cells. Subjects should avoid the use of soaps or any other products to the face for 72 h after each injection session, although mild washing is permitted. Indirect application of ice to the treatment area is not recommended. The treatment consists of three sessions, each 5 weeks apart, with a dose of 0.1 mL of a suspension of 1.0 to 2.0×10^7 cells/mL [79]. Clinical improvement in NLF wrinkles was seen 2 months after the start of treatment, with continuous improvement in the follow-up months 2–6 after a series of three injections [150]. Unlike most dermal filler products, autologous fibroblast therapy benefit is not expected to degrade over 6 months [151, 152]. Prior studies of autologous fibroblasts showed continued benefit 1 year after treatment [149]. Zhao et al. proved that cultured autologous skin fibroblasts survive for at least 5 months after injection [80].

Cultured fibroblasts can be injected combined with hyaluronic acid (HA) to obtain longer-lasting results [78, 153]. Solakoglu et al. [78] used cross-linked HA as a biodegradable polymer scaffold for cultured human fibroblasts. Dermal fibroblasts obtained from rat skin biopsies were cultured and injected. The density of the cells in mixture was approximately 30×10^6/mL. At the end of the fourth and eighth months, the injected fibroblasts, elastin, and collagen production were found to be stable and well tolerated. Syntheses of collagen and elastin were demonstrated. HA bulks surrounded by fibroblasts suggest an interaction between HA and fibroblasts. HA also promoted vascular angiogenesis. There were no signs of apoptosis, inflammation, or necrosis, which was expected because the injected cells were autologous. Therefore, cultured human dermal fibroblasts combined with hyaluronic acid can provide a long-lasting material and should be regarded as a new method in dermal renovation [78].

Scar formation and contraction should be avoided when using fibroblast treatment. In case of correction of dermal and subcutaneous depression, where the skin remains intact, the possibility of wound contraction is very low. Thus fibrosis and scar formation are our primary concern, which may be caused by excessive growth or secretion of cultured fibroblasts after transplantation. The extracts of the dermis (extract D) could inhibit the proliferation of fibroblasts [154].

Stem Cell-Conditioned Medium

Microneedle fractional radiofrequency is a safe and effective skin rejuvenation method, and better results may be expected when combined with stem cell-conditioned medium [155]. The stem cell-conditioned medium (hESC-EPC CM) [85] is composed of a large number of growth factors and cytokines. In vitro, hESC-EPC CM significantly improved the proliferation and migration of dermal fibroblasts and epidermal keratinocytes and also increased collagen synthesis of fibroblasts. hESC-EPCs secrete cytokines and chemokines which are important in angiogenesis and wound healing [156]. Patients received three sessions at 4-week intervals. Histologic examination revealed marked increase in dermal thickness and dermal collagen content. Side effects were minimal [155].

Efficacy and Safety

Since the first transplants of adult stem cells from bone marrow in 1959 [157], there is no record in the scientific literature of any case of tumor formation resulting from the injection of these cells [74]. The technique is considered safe at an expansion of up to the fourth population doubling[158].

Immunological Impact of Allogeneic Cells

There have also been a number of studies investigating the immunological impact of allogeneic cells [29]. It has been suggested that allogeneic

cells are replaced by host cells. In addition, large trials involving grafting of allogeneic skin equivalents onto venous ulcers did not reveal evidence of rejection clinically or immunologically in the patients [159]. There was no demonstration of antibodies specific for human leukocyte class I antigens expressed on allogeneic cells and no proliferation of T cells in patients. One of the reasons for the perceived lack of acute rejection in immunocompetent hosts is that dermal fibroblasts lack major histocompatibility complex class II antigens necessary for antigen presentation [160]. It has also been proposed that during in vitro culture, the antigen-presenting cells, such as Langerhans cells, are gradually lost following serial passages [161]. One study assessed the persistence of allogeneic fibroblasts in an acute wound (porcine model) and found that after 1 week, allogeneic fibroblasts were not detectable by polymerase chain reaction [162].

Malignancies Development

It is essential that clinical safety be ensured as these cells return to patients. It is also important to investigate and understand the changes in cell culture to be certain that the cells do not carry mutations or unwanted differentiations that may cause any pathology in the medium- to long-term horizon [16].

Hayflick et al. [69] have shown that human fibroblasts could maintain their genomic stability after 40 generations, although cells at the tenth passage were too senescent for injection. Clinically, cells at passages 3–4 are most suitable for injection in terms of cell quantity and proliferative and secretory activity [64]. Eça et al. [74] ensured that no genetic alterations occurred in fibroblast expansion up to the fourth passage. Zeng et al. [64] noted that the proliferative behavior of the cultured cell population remained stable from passages 5 to 10 without overactive division or apoptosis. Cells at passages 5 and 10 maintained their normal somatic cell diploid karyotype, and no mutations or other translocations were discovered. No chromosomal abnormalities were found in in vitro expanded human fibroblasts. It was also noted that the proliferation was active at the first month and returned to normal later, indicating that the proliferation of the injected cells was under certain regulations, which prevented the cells from hyperplasia. The fact that no macrophages were found suggested that there was no abnormal apoptosis or necrosis of the injected cells. Moreover, normal cell morphology without dysplasia was observed from histological sections, and no tumors were detected from gross inspection together, suggesting that no oncogenic transformation or fibrosis formation had occurred at least 3 months after transplantation [64]. However, small segment mutations or other subtle molecular events were not completely excluded. The in vivo section of the study has limitations since the injected cells were not labeled. Second, the viability and stability of the injected cells were only demonstrated by microscope observation. Telomerase activity and carcinogenicity should be measured in a further study [64].

A theoretic risk of the enhancement of malignancies with autologous cell therapy has been raised [163]. Although one basal cell carcinoma was reported near a treatment site in the pivotal trial, it is unrelated to the autologous fibroblast injection for two reasons:

1. Given the number of subjects and duration of the trial, the incidence of cutaneous malignancy is consistent with background rates.
2. Basal cell carcinoma is not a fibroblast-derived tumor. To date, no dermatofibrosarcomas have been reported in thousands of injections [79].

Animal Disease Transmission

The use of fetal bovine serum (FBS) in fibroblast culture medium may increase the risk of infection from bovine diseases or of a reaction to foreign proteins. In addition, cell division and consequently the number of fibroblasts have been shown to be greater in autologous dermal fibroblasts cultivated in human serum than in FBS [164].

Autologous Cultured fibroblasts

The advantage of injecting a live autologous filler is obvious as it leads to longer-term correction and eliminates the problem of hypersensitivity and foreign body granulomatous reactions [29]. Many authors have studied the safety and efficacy of the injection of cultured autologous fibroblasts [29, 64, 74, 78, 79, 149]. The preclinical safety and efficacy of autologous cultured human skin fibroblasts has been studied and their proliferation and secretion activity has been demonstrated. The implanted fibroblasts can survive in vivo for more than 5 months and actively secrete new collagen [64, 80]. The use of cultured human skin-derived fibroblasts has been proven to be safe, providing a basic support for clinical use of autologous fibroblast transplantation [64].

Side effects of cultured autologous fibroblasts are minimal and temporary and comparable with other injected agents [79]. A major trial reported in 2007 [149] found no serious treatment-related adverse events. No hypersensitivity reactions have been noted, and no long-term nodules or other local problems have been seen [150]. Solakoglu et al. found no complications after injection of cultured fibroblasts combined with stabilized hyaluronic acid (HA) [78]. Furthermore, there have been no reports of hypertrophic or keloid scarring following the injection of autologous fibroblasts, suggesting that fibroblast proliferation and collagen synthesis are naturally regulated by cell–cell and cell–ECM contact and negative feedback [145]. Therefore autologous fibroblast therapy is as safe as many of the existing filler therapies [79].

Skin Substitutes

A systematic review which included 20 randomized controlled trials assessed the safety and efficacy of bioengineered skin substitutes in comparison with standard methods in the management of burns. Nevertheless the numerous subgroup analyses and the diversity of skin substitutes limited the ability to draw conclusions [41, 130]. Bioengineered skin substitutes, as Biobrane®, TransCyte®, Dermagraft®, Apligraf®, Integra®, and CEA (cultured epithelial autograft), have proved to be at least as safe as the classical skin replacements or topical agents/wound dressings [130]. Apligraf® is composed of allogeneic keratinocytes and fibroblasts which are not detectable beyond 6 weeks [165].

Present and Future of Cell Cultures

Stem Cells

The possible therapeutic use of somatic cells derived from embryonic stem cells is currently a hot topic in regenerative medicine [41]. Although skin biopsies are a regular source of keratinocytes and skin stem cells, the generation of keratinocytes from human embryonic stem cells could be a useful technique [166]. Nevertheless obtaining skin stem cells from the own patient's skin might simplify the procedure.

Work on embryonic stem cells [167] and the discovery of Yamanaka et al. [168], which demonstrated that adult cells can return to the embryonic stage with the possibility of producing all the specialized cell categories, give hope in the near future to cellular genetic therapy, which might replace tissue and organ transplants [134].

The usage of stem cells may help to overcome the limitations of current technologies (i.e., the lack of vascular networks, sensory receptors) [24]. Though using stem cells has been partially effective, the potential risks of malignant teratoma formation and long-term adverse effects of the stem cells should be taken into account, and therefore more extensive studies are required [169].

Tissue Engineering

Tissue engineering of skin is based on 25 years of research and rests on a strong background of technologies and cell and molecular biology. Despite initial unrealistic expectations, tissue-engineered skin has already delivered considerable benefits to patients with burns, accidents, infections, and chronic wounds. In this regard, "skingineering" has a huge potential that has just

begun to be realized [26]. The challenge that still remains is the generation of a complex dermo-epidermal substitute that can be safely and effectively transplanted with minimal scarring in one single-step procedure. Skin regeneration aims to achieve structural and functional reconstruction, reducing scar formation and improving the quality of wound healing [24]. Regeneration rather than scar formation is key [15].

The invention of Integra® was a major step in skin engineering. Controlled release of angiogenic factors from matrices, seeding endothelial cells directly into the matrix, and engineering the vasculature directly into the tissue would largely contribute to speeding up vascularization in these complex skin grafts [26]. The combination of tissue-engineered skin substitutes with cytokines and growth factors may in the future be used to enhance wound healing as well as the possibility of incorporating defensins for antimicrobial benefit [29]. The use of cells with its preserved matrix could exempt the need for a scaffold [16].

Stem cell biology also has to be integrated in this future concept. Human adult stem cells can be a source to generate skin in vitro [26]. iPSCs provide a novel source of bioactivity for scaffolds to promote cell proliferation and differentiation via different signal transduction pathways. However, the currently available dermal substitutes have certain deficiencies to overcome, such as inconvenient usage processes, missing hair follicles, and poor resistance to infection, and most of them require secondary transplantation in autologous skin. These include further research into the differences between fetal scarless wound healing and adult wound healing and improving vascularization of scaffold materials and seed cells, especially stem cells [24, 100].

Skin Three-Dimensional Bioprinting

In recent years, skin three-dimensional bioprinting is a potential technology which can generate stratified constructs to simulate function and morphology of the natural skin [98, 135, 169]. Until now, relatively simple skin architectures composed of keratinocytes and fibroblasts have been successfully generated through bioprinting techniques (Fig. 8). These skin architectures have

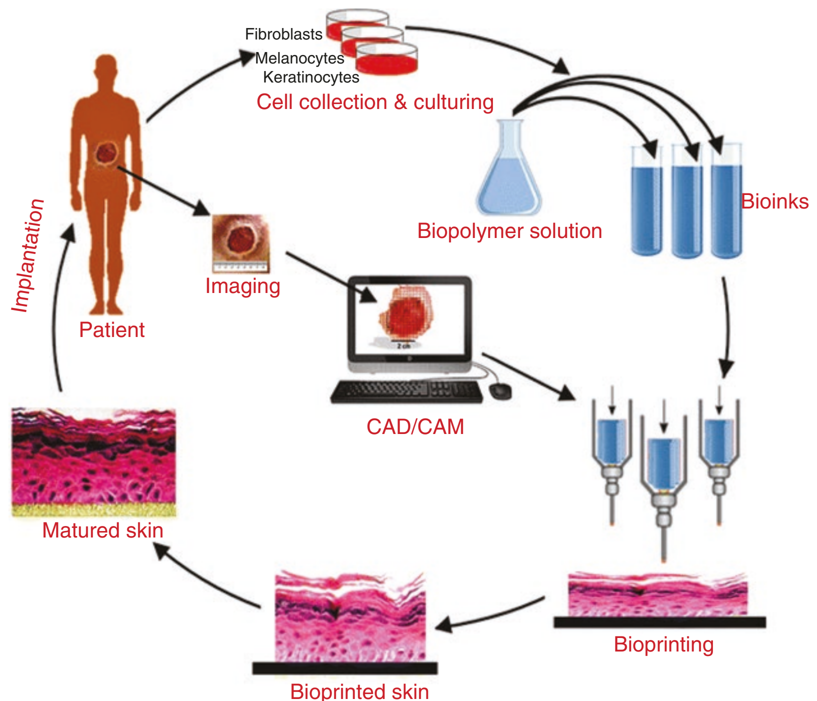


Fig. 8 Skin bioprinting (Reproduced from Augustine [170])

demonstrated similarities to native skin and have performed some skin functions in in vivo studies [24, 98, 171–173].

Cubo et al. generated a human bilayered skin using bioinks containing human plasma as well as primary human fibroblasts and keratinocytes that were obtained from skin biopsies in less than 35 min. The generated skin was very similar to human skin and indistinguishable from handmade bilayered dermo-epidermal equivalents [98]. The barrier function of the skin is closely relative to the maturation and formation of the stratum corneum. Therefore the bioprinted skin constructs cannot achieve complete function due to the absence of a fully differentiated epidermal region [171–173]. The density of ECM and cellular component can be controlled in bioprinting and the cell viability can be maintained during the whole printing process [171–174]. Furthermore, the thicknesses of printed constructs can be customized and controlled according to the wound depth [175]. Therefore 3D bioprinting is a suitable technology to generate bioengineered skin for therapeutical and industrial applications in an automatized manner [98].

Cell Cultures

The future application of fibroblasts for gene therapy also offers a huge potential in providing new strategies for treating skin genodermatoses [29]. In the future, autologous fibroblast cell therapy might replace superficial synthetic fillers, but currently that is doubtful [79].

HFOC is a versatile and accessible assay system which has only just begun being employed for research into human genodermatoses and miRNA function, chronobiology, cutaneous neuroendocrinology, HF-associated progenitor cell biology, melanocyte biology, epithelial stem cell immunopathology, and mitochondrial biology [35].

In postpartum humans, skin appendages lost in injury are not regenerated. Wang et al. demonstrated that transplantation of culture-expanded epidermal stem cells and skin-derived progenitors led to de novo regeneration of functional hair follicles and sebaceous glands. This finding could be the basis for the development of novel bioengineered skin substitutes with epidermal appendage regeneration capacity [40].

Future studies will identify the inductive signals that might explain the limited inductive capacity after bioreactor expansion. It is promising that inductive function is partially retained after extensive cell expansion [43].

Cell Reprogrammation and Immortalization

The reprogrammation of differentiated somatic cells to iPSCs offers an opportunity to generate pluripotent patient-specific cell lines [41]. These iPSC lines could help in model human diseases, drug discovery, and cellular transplantation therapies. Nevertheless, there are lots of factors regarding safety that should be resolved [24, 176–178]. Better understanding of cellular senescence will allow the immortalization of various kinds of primary cells which will be essential not only for regenerative medicine but also for the economic development of a three-dimensional skin culture system [4].

Conclusions

- Even though there is still much work to be done, the rise of cell culture and tissue engineering is providing powerful tools for regenerative medicine evolution.
- It is important to maintain not only the tissue structure but also to preserve its function.
- Efforts should be ideally directed to efficiently and safely obtain large amounts of high-quality product reducing costs, and to decrease the use of allogeneic products, thus diminishing potential risks.
- Further progress in automatic processing may improve reproducibility and reliability and may contribute to speeding up the process, reducing costs, increasing efficiency, homogenizing protocols, and decreasing manual procedures variability. Computer-controlled bioreactors and skin 3D bioprinting can contribute in this regard.

- The advance of cell therapy will possibly make it more accessible and affordable, and in the future rejuvenation treatments might not be as we know them today but would mostly consist of autologous treatments with a decrease of secondary effects.
- Further large and rigorous studies with long-term follow-up should be performed to assess the safety of cell culture and skin substitutes.
- The use of stem cells may help to overcome some of the limitations of current tissue engineering techniques (i.e., angiogenesis, sensory receptors generation).
- Great improvements have been made in this field, and now the challenge is its application to routine clinical practice.

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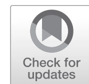

Techniques and Processing

Letizia Trovato, Riccardo D'Aquino, and Antonio Graziano

An overview of Grafts

The graft is defined as a transplant of one or more tissues from a donor site to a recipient site with an interruption of neurovascular connections between both of the sites.

From historical point of view, the first description of skin grafts dates back to 3000 BC in India where the gluteal skin segments were used for the reconstruction of the nose. In the Western world, the first skin transplant was performed in 1804 by an Italian surgeon, Baronio, on a sheep, and, in 1817, by an English Surgeon, Sir Astley Cooper, on a man [1]. In 1869, Jacques-Louis Reverdin described the first method of grafting small, full-thickness pieces of skin for wound healing based on the concept of skin islands to promote epithelialization of a wound [2]. Then, in 1872, Ollier was the first to underline the importance of dermal grafts and their component and, in 1886, Karl Thiersch [3] described another method of skin grafting, based on the application of the thin skin grafts to cover large wounds. In 1875, Wolfe described the first case of full-thickness graft used for correction of eyelid ectropion, but just in 1893, thanks to Krause, the use of full-thickness grafts is widespread in the clinical practice [1].

In 1939, Padgett introduced the dermatome [4] and, in 1964, Tanner published the technology to expand the surface of the skin grafts up to 12 times [5]. Finally, in 1975, Rheinwald and Green were the first to culture human keratinocytes in vitro [6].

The success of a graft depends on its attachment or engraftment rate on the recipient site and this process can be regulated by three principal aspects:

- Good vascularization of recipient area related to its ability to produce new vascular vessels
- Maximum adherence between the graft and recipient sites to prevent the appearance of hematomas or seromas among the surfaces
- Optimal immobilization of the graft to restore the revascularization

The other factors that can influence the success rate of a graft are the ability to control infection and fluid loss, histocompatibility, mechanical compliance and stability, absence of toxicity and antigenicity properties, and cost-effectiveness.

In general, the grafts can be divided into two main categories:

- Autologous grafts when the donor and recipient are the same individual.
- Homologous grafts when the donor and recipient are different individuals belonging to the same species.

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In turn, these categories can be subdivided into allografts or xenografts: in the first case, two individuals belong to the same species but are genetically different and usually a donor cadaver is used, while in the latter case, two individuals belong to different species, for example human and bovine or equine species.

The skin represents the largest tissue of the human body, and its main function is to create a barrier to prevent tissue damages, trauma, or infections. Due to easy access and its consistent amount, the skin represents the main tissue used for grafting procedures and tissue engineering models that are needed when the skin integrity is impaired.

The Micrografts Era

The healing of large or chronic wounds is still today a big challenge for the surgeon, despite the different approaches available such as skin grafts, allografts, xenografts, and engineered artificial skin [7]. The major part of these approaches provides a rapid but temporary wound coverage and often are extremely expensive, especially when the wound is large and the availability of donor site is limited. To overcome these limitations, the ideal graft would be immediately available, non-immunogenic, permanent, and offers low morbidity to the patient. The micrograft concept, which is based on the use of autologous tissue, overtakes these drawbacks, allowing to cover a wound by using a minimal amount of donor skin. The micrograft idea arises from the evidence that small skin colonies, cultured and expanded many times, are able to cover a wound, which is larger than the donor site [8]. In the Table 1 are summarized some of the skin expansion techniques used until now to obtain micrografts.

During the past few years, several modifications have been introduced to improve the skin expansion techniques, achieving an expansion ratio of 1:100–700 with respect to standard ratio of 1:5–9. Among these, the most common techniques are represented by dermal–epidermal grafting [9, 10] and autologous noncultured cell therapy [11]. All these techniques are based on the concept of Meek whereby smaller grafts have a greater regenerative potential due to the little distance between them [12].

Dermal-Epidermal Grafting

Dermal and epidermal grafts are constituted by a variable amount of both dermis and full-thickness skin providing a major resurfacing and stability of the wound. The methods that permit to obtain these types of graft are multiple, including the Meek-Waal dermatome, the flypaper technique, skin expansion with meshers, Xpansion® system, fractional skin harvesting, suction blister epidermal grafting, and others [13]. In 1958, Cicero Park Meek was the pioneer of skin expansion techniques using a dermatome to obtain postage stamp of 4 mm × 4 mm size, reaching a ten-fold skin expansion. Subsequently, in 1993, Kreis et al. have modified the Meek's technique using a dermatome on compressed air. This modification combined with the use of cultured grafts or allografts permitted them to improve the treatment of severe burns reaching a percentage of coverage wound of 75% [14].

The flypaper technique was developed by Lee et al. and it was based on the combination of grafts with 5 mm × 5 mm size over gauze impregnated of petroleum jelly and in this way, a wound epithelialization similarly to Meek's techniques can be obtained while reducing the costs [15].

Despite the efforts to improve the expansion ratio and therefore the wound epithelialization, reducing costs and time, all the above-mentioned techniques continued to be labor-intensive and did not provide increased expansion when compared with the original Meek technique. Cultured epithelial autografts have also been studied as an alternative but, although they could provide an expansion ratio up to 1:1000, these are fragile with poor graft take, lack a dermal component, and are extremely susceptible to mechanical shear [16].

To improve dermal–epidermal grafting, new tools were developed such us the Xpansion® Micrografting System and the fractional skin harvesting. The Xpansion® Micrografting System

Table 1 Skin expansion techniques

Comparison of Techniques				
Micrograft type	Graft size	Expansion ratio	Advantages	Disadvantages
Pinch graft	2–5 mm^2	6–7:1	Easy to cut, resists infection well, resists pressure better than split-skin graft, can be performed as an outpatient procedure, inexpensive	Donor site cannot be used for future grafts, poor cosmetic result, not useful for wounds >4.6 cm^2, tedious procedure for large wounds
Patch/postage stamp graft	1.27 mm^2–various	6–9:1	Easy to cut, resists infection well, can be performed as an outpatient procedure, inexpensive	Poor cosmetic result, tedious procedure, unpredictable expansion ratio
Meek microdermagraft	1.58 mm^2	9:1	Quicker graft preparation	Need custom dermatome
Chinese intermingled technique	0.9–2.5 mm^2	7–10:1	Less contracture formation, use of allograft protective layer	Tedious procedure, possibility of rejection
Microskin graft	<1 mm^2	7–100:1	Easy to prepare, cost efficient, resists infection well, tolerates trauma well	Orientation of grafts may be nonuniform, increased scar contracture formation
Microscopic split-skin ("diced") graft	40–200 µm^2	20–26:1	Easy to prepare, can be prepared as an outpatient procedure, comparative healing rates to meshed skin	Random orientation of grafts
Modified meek technique	9 mm^2	10:1	Good for poor quality wounds, uniform distribution of skin pieces, orientation is nonrandom, true expansion rate	More demanding technique, higher costs of materials
Modified postage stamp/fly paper graft	25 mm^2	9:1	Less expensive, larger graft size easier to handle and orient, minimal materials needed, true expansion ratios obtained	Tedious procedure
Autologous skin suspension	0.4 mm^2	10:1	Easy to prepare, fast healing rates	Viability of skin particles questionable, poor cosmetic outcome, excessive scar contracture
Microskin spray	0.04–0.25 mm^2	110–150:1	Easy to use, good distribution of grafts, shorter operating time, less donor skin needed	Custom preparation needed

In the table are summarized and compared the most common techniques of skin expansion. From Biswas et al., J Diabetes Sci Technol Vol 4, Issue 4, July 2010

(Applied Tissue Technologies, Newton, Mass.) contains 24 parallel rotating cutting disks 0.8 mm apart designed to cut the grafts twice in a perpendicular direction, resulting in 0.8 mm × 0.8 mm sized micrografts (Fig. 1a). The advantage of this system is that by providing smaller grafts, the border length increases and thereby enhances the regenerative capacity of the grafts. Further, the orientation of the skin grafts (dermal side up or down) is irrelevant in a wet or moist environment, thus simplifying the procedure [17].

In the fractional skin harvesting, a large number of microscopic full-thickness skin graft columns are harvested, ensuring a good-quality skin and faster healing of donor sites with minimal scarring. The concept evolved from the photothermolysis technique where, when the skin was subjected to laser microbeams of 300 µm

Fig. 1 New tools commercially available for dermal and epidermal grafting. (**a**) Xpansion® Micrografting System (Applied Tissue Technologies, Newton, Mass.); (**b**) CelluTome™ Epidermal Harvesting Device (Kinetic Concepts, Inc. [KCI], San Antonio, Texas); (**c**) ReCell® (Avita Medical Europe Ltd., UK). All the figures were taken from respective websites

diameter to create a microthermal zones, the epidermal closure occurred within 24 h followed by dermal healing within 2 weeks [18]. The fractional skin grafts are full-thickness grafts, collected using customized hypodermic needles with double cutting edge of 700 μm diameters. These columns of tissue are extracted by suction into a collection basket, and the micrografts are then randomly spread over the wound without dermal orientation.

For both these latter techniques, clinical results showed a wound healing comparable with the split-skin graft technique [10, 19].

To summarize, the techniques for dermal and epidermal grafting provide very promising results, even if more studies will be needed to further validate their advantage with respect to conventional split-thickness skin grafting.

Epidermal Grafting

Grafting containing both dermis and epidermis are certainly more effective in the wound healing due to reduction of wound contraction and the presence of fibroblast that induce the release of growth factors, promoting the proliferative phase of wound-healing process. However, other techniques were developed to obtain only epidermal grafts, which if on one hand are poorly stable and resistant, on the other hand do not stimulate dermal pain receptors and can be obtained in an outpatient setting [20]. The most used techniques to obtain epidermal grafts are the epidermal suction blisters grafting and autologous non-cultured cell therapy.

The epidermal suction blisters grafting was described for the first timed by Falabella, and involves harvesting of ultrathin*** skin grafts consisting of only epidermis by inducing physiological split at the dermo–epidermal junction applying a negative pressure of 200–500 mm of Hg. The graft erupts as a blister and the time required for the formation of suction blisters is inversely related to the skin temperature [21]. This method has previously showed to be an effective treatment for vitiligo [22–24] and closure of hard-to-heal wounds [25–28]; however, its use has been limited in clinical practice due to the lack of a reliable and automated method for harvesting patient epidermal skin. Additionally, it causes discomfort and it is time consuming.

In the last years, it was reported in the literature an automated epidermal harvesting tool, commercially available, based on both heat and suction to the normal skin to form epidermal micrografts. This tool is marketed as CelluTome™ Epidermal Harvesting Device (Kinetic Concepts, Inc. [KCI], San Antonio, Texas) (Fig. 1b) and creates micrografts on all inner thigh donor sites.

Briefly, after the removal of body hair and cleaning the donor site with 70% isopropyl alcohol wipes, a sterile harvester is secured around the subjects inner thigh, and the vacuum head was snapped onto the harvester. The automated suction blister epidermal harvesting system starts, vacuum and heat were applied to donor sites until epidermal micrografts were formed (time of execution is 30–45 min). At the time of harvest, the vacuum head was detached from the harvester, and immediately, a transparent film dressing is placed on top of the micrografts and then harvested [29]. For further information about this methodology, please visit the website www.cellutome.com.

Recent papers in the literature reported that the use of this automated, minimally invasive harvesting system provided a simple, low-cost method of producing uniformly viable autologous epidermal micrografts with minimal patient discomfort and superficial donor-site wound healing within 2 weeks [30]. Furthermore, epidermal micrografts retained their original keratinocyte structure, which is pivotal for potential re-epithelialization and repigmentation of a wound environment [31].

From a clinical point of view, a recent study showed that in a nonhealing ulcer secondary to pyoderma gangrenosum, not responsive to conservative treatment, this approach promotes complete re-epithelialization in 7 weeks [32].

To summarize, epidermal blister grafts act like a bioengineered skin promoting wound healing through release of autologous keratinocytes and growth factors.

Autologous noncultured cell therapy is another emerging technique to obtain epidermal grafts and is indicated for treatment of various skin wounds, especially burns. This technique consists of the isolation of cells from the donor tissue and immediate autologous replantation to the patient's wound, overcoming the timing of cell culture of 2–3 weeks related to cell culture of epithelial cells [33]. A product commercially available based on this technique is represented by ReCell® (Avita Medical Europe Ltd., UK) (Fig. 1c), where following the harvest of skin graft, cells are digested with trypsin solution and the sample mechanically agitated to separate the cells. Finally, the cells are suspended in a lactate solution and sprayed over the wound, similarly to spray techniques [34].

Wood et al. showed that, using this system, the average yield of viable cells per cubic centimeter of donor split-thickness skin graft was 1.7 million and that 86% of the original cells were viable after 24 h storage at 4 °C. On in vitro analyses by fluorescence-activated cell sorting, the same authors reported that the suspension contains primarily keratinocytes (65%) and fibroblasts (30%), with a small population of melanocytes (3.5%) that provide skin pigmentation [35].

The advantages of this technique are surely the quick application and the possibility to treat large wounds without need of a scaffold achieving an expansion ratio of 1:80 similar to sprayed cultured epithelial cell autograft [36]. On the other hand, the use of this technique is limited in the clinical practice due to high costs and due to the trypsin process that does not permit one to inoculate the suspension.

The Rigenera® Technology

In the context of new and innovative techniques commercially available, new technology named Rigenera is now popular, which permits one to obtain autologous micrografts in a easy, fast, safe, and no invasive manner, and without cell manipulation.

Rigenera® technology is a promising clinical protocol for the human tissue regeneration that can be performed in one surgical time; thus, donor and acceptor of micrografts are the same individual. From a practical point of view, this protocol is based on the use of a CE- and FDA-certified medical disposable called Rigeneracons and the Rigenera machine (Human Brain Wave srl, Turin, Italy) (Fig. 2a). The disposable Rigeneracons, through a mechanical disaggregation, permits one to obtain autologous micrografts including progenitor cells able to differentiate in several cell types and then repair or regenerate a tissue injury when applied on a lesion and to restore the form and function of

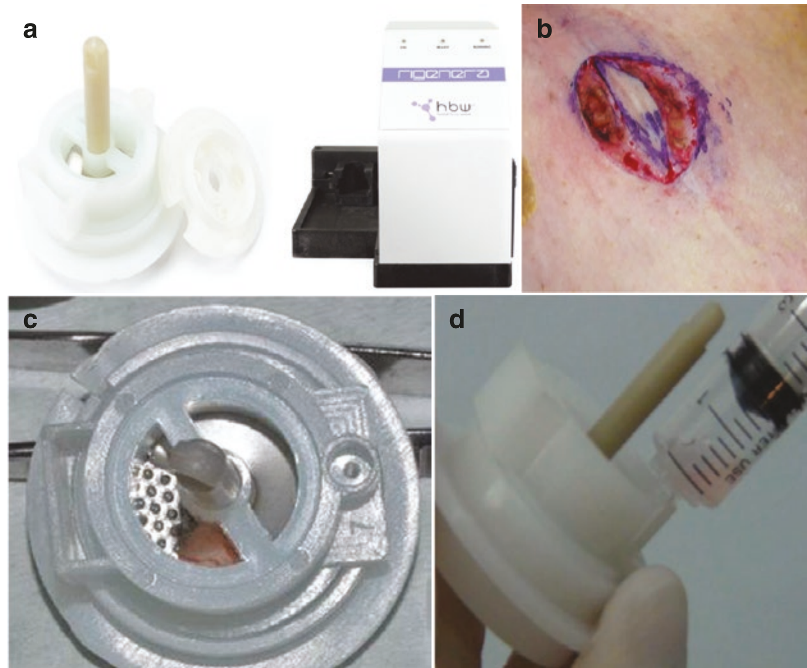

Fig. 2 Rigenera technology to obtain autologous and calibrated micrografts. (**a**) Rigeneracons and Rigenera machine (Human Brain wave srl, Turin, Italy); (**b**) collection of a small piece of skin; (**c**) disaggregation of skin by Rigeneracons adding 1.5 mL of sterile saline solution; (**d**) collection of autologous micrografts obtained after the disaggregation is performed by the use of a syringe without needle

several types of tissue, including skin, bone, and cartilage. These progenitor cells in association with growth factors derived by starting tissue form autologous micrografts ready to use, which can be used alone or in combination with different biological scaffold such as collagen, Poly Lactic-co-Glycolic Acid (PLGA), and others.

The Rigenera® technology is based on the assumption that not only in fetal, but also in adult human tissues are present specific niches where the side population is located. This is a cell subpopulation exhibiting different biological characteristics compared to the main population, such as stem cell-like characteristics [37–40]. This technology was born from scientific studies conducted by an Italian group of researchers that have initially showed a side population in the human dental pulp isolating a subpopulation of dental pulp stem cells (DPSCs) called Stromal Bone Producing DPSCs (SBP/DPSCs) able to differentiate into osteoblast producing, in vitro, a living autologous fibrous bone (LAB) tissue [41]. The following studies on the human dental pulp have confirmed the regenerative role of DPSCs [42–44] until the demonstration of a new method for their isolation. In fact, to improve the collection time and the cell viability of these cells, the enzymatic digestion was replaced with a mechanical disaggregation developing in this way the Rigeneracons device [45]. The Rigeneracons is a biological disruptor of human tissues constituted by a grid provided with 100 hexagonal blades able to disrupt and filter a cell population with a size of 50–70 µm.

In vitro tests have demonstrated that micrografts originated with this system and derived from human dental pulp or periosteum samples are able to maintain a high cell viability and express a high percentage of positivity to mesenchymal stem cells markers, suggesting an intrinsic regenerative potential. The same results were also observed in cardiac atrial appendage biopsy and lateral rectus muscle of eyeball [46].

The micrografts technology based on the Rigenera protocol is actually used in different clinical contexts, including dentistry, aesthetic medicine and wound care, and different clinical and academic groups published several scientific papers on their efficacy. In fact, it was reported that these micrografts promote the wound healing of complex postoperative and posttraumatic wounds [47, 48], wound dehiscences [49, 50], chronic ulcers [51, 52], and pathological scars

[53]. More recently, the ability of micrografts to induce cartilage repair in patients affected by nasal deformity or alar nasal cartilage defects was also reported [54, 55].

Regarding the application in dentistry, it was reported that human dental pulp or periosteum derived– micrografts are able to promote the periodontal regeneration [45] , the bone regeneration in the atrophic maxilla [56], and to preserve the alveolar socket after tooth extraction both by reducing bone resorption and increasing new bone formation [57].

The Rigenera protocol is very easy to perform, does not need any particular expertise by the operators, and can be divided into four steps:

1. Collection of a small piece of skin, approximately 1–2 mm and up to 10 mm size from a distant donor site from the recipient site: this step can be performed using a biopsy punch or a scalpel as indicated in the Fig. 2b. It is very important to discharge the adipose and epithelial component from the collected tissue to not compromise the efficiency of disaggregation.
2. Disaggregation of skin by Rigeneracons adding 1.2 mL of sterile saline solution: the addition of a sterile saline solution is important both for the final collection of micrografts and preservation of micrografts integrity and viability (Fig. 2c). The Rigeneracons device is inserted into the Rigenera machine to start its rotation and then mechanical tissue disaggregation.

   Ccollection of autologous micrografts obtained after the disaggregation: the collection of micrografts is performed by the use of a syringe without needle slightly reclining the disposable as showed in the Fig. 2d.
3. Injection of micrografts in the injured site: the micrografts obtained are ready to use, does not need further manipulation or expansion and can be applied directly on the lesion or used in combination with biological scaffold such as collagen, hyaluronic acid, and PLGA according to the tissue to regenerate.

The advantages of Rigenera protocol are multiple, the first is the contemporaneity, in fact this protocol can be performed in one surgical time where the patient is the donor and the acceptor of calibrated micrografts; this procedure leads to an enrichment of progenitor cells in the acceptor site that need to be regenerated; the procedure is not invasive and the morbidity of the donor site is extremely reduced in spite of small pieces of sample taken.

Tissue Engineering

The Grafting procedures illustrated until now have provided a strong contribution to regenerative medicine and offer to clinicians a wide choice of treatments depending on the type of wound and patient's history.

Together with grafting procedures, over the years, there has been gradually developing the tissue engineering, the purpose of which is to repair or regenerate tissues through the combined use of scaffolds and biologic mediators, such as, for example, stem cells and growth factors to improve the wound healing process, providing a new tool in the field of regenerative medicine. As reported above, over the past several years, various grafts of dermal, epidermal, or dermo–epidermal origin have been reported and have been used commercially with the aim to restore the structure of the skin tissue by repairing the wound [58–60]. Bioengineered skin substitutes not only repair the wounds, but also have various supplements, such as growth factors, antibiotics, and anti-inflammatory drugs, which eventually fasten the wound-healing process. To engineer these substitutes, various scaffold matrices have been developed to promote cell growth in 3D structure. Scaffolds are defined as the best materials for restoring, maintaining, and improving tissue function and are key players in repair and more importantly regeneration of tissues permitting essential supply of various factors associated with survival, proliferation, and differentiation of cells [61]. Such scaffolds are highly biocompatible with skin tissue and biodegradable in nature, acting as suitable dressing material for wound healing and can be made up of synthetic or absorbable, naturally occurring, biological, degradable, or nondegradable polymeric. In the

Table 2 Advantages and disadvantages of most common synthetic scaffolds. Adapted from Chaudhari AA et al., Int J Mol Sci. 2016 Nov 25;17(12). pii: E1974

Synthetic Scaffold Types	Advantages	Disadvantages
Porous scaffolds	High porosity provides a suitable environment for extracellular matrix (ECM) secretion and nutrient supplies to the cells.	Porous nature limits the homogenous distribution of the cells. Different pore sizes are required for the specific cell types and are therefore time consuming.
Fibrous scaffolds	Highly microporous structure is best suitable for cell adhesion, proliferation and differentiation. Low inflammatory response upon implantation.	Surface functionalization is required to create the nanofibers of these scaffolds.
Hydrogel scaffolds	Highly biocompatible and controlled biodegradation rate.	Limited mechanical strength due to soft structures.
Microsphere scaffolds	Provides enhanced cell attachment and migration properties.	Microsphere sintering methods are sometimes not compatible to the cells and reduces the cell viability.
Composite scaffolds	Highly biodegradable and offer mechanical strength. Greater absorbability.	Acidic byproducts are generated upon degradation. Poor cell affinity.
Acellular scaffolds	Native ECM is retained and thus normal anatomical features are maintained. Less inflammatory and immune response with higher mechanical strength.	Incomplete decellularization is required to avoid immune responses.

Tables 2 and 3 are summarized the principal synthetic and natural scaffolds today available and mostly used.

The products obtained by tissue engineering are commonly named skin substitutes, dermal or epidermal substitutes, and can be divided into natural or artificial. Natural dermal substitutes or acellular dermal matrices are produced from allogeneic or heterologous skin, fibroblasts, and keratinocytes, where these cells are incorporated inside the scaffolds [58]. Their tissue composition is the closest to autologous skin and by this approach, it is possible to promote better growth and alleviate the patient's pain once it regulates and initiates wound healing. Furthermore, natural dermal substitutes have an excellent biocompatibility being able to replicate the three-dimensional structure of collagen. The product most representative is Alloderm™ (LifeCell Corporation, Branchburg, NJ) that is derived from skin taken from donated cadavers by repeated cycles of freezing and thawing to remove cells. Alloderm™ has been reported in a wide range of applications including rhinoplasty, abdominal wall reconstruction, alloplastic breast reconstruction, radial forearm free-flap donor site coverage, and vaginal repair showing promising results [62].

Artificial dermal substitutes can be divided into two categories, natural or synthetic, according to their composition. A representative product of the first category is Integra™ (Integra Life Science Corporation, Plainsboro, NJ) that is composed of collagen glycosaminoglycan and silicone, and is successfully used to treat large-area burns or third degree burns. On the contrary, artificial synthetic dermal substitutes are mainly composed of synthetic polymer materials including polylactic acid, polyglycolic acid, and polyurethane. A representative product of artificial synthetic dermal substitute is Dermagraft™ composed of Polyglactin-910 or Polyglycolic acid seeded with neonatal fibroblasts (Advanced Tissue Science Inc., La Jolla, CA, USA) that can be used for burn wounds and chronic skin ulcers, especially for the ulcers of diabetic foot [63].

To summarize this paragraph, different bioengineered skin substitutes are available in the market and all are well distinguished by their biocompatibility, applicability, and safety characteristics in order to permit to clinicians the better option for patient wellness.

Table 3 Advantages and disadvantages of most common natural scaffolds. Adapted from Chaudhari AA et al., Int J Mol Sci. 2016 Nov 25;17(12). pii: E1974

Biomaterials Types	Advantages	Disadvantages
Natural biomaterials such as collagen, gelatin, silk and fibrinogen	Are all highly biocompatible, collagen offers tensile strength, gelatin enhances cell adhesion, silk provides great permeability for nutrients, Fibrinogen is converted into fibrin and exhibit anti-inflammatory properties	Gelatin is advantageous over collagen as it is less immunogenic.
Biomaterials of animal origin such as keratin and bovine serum albumin (BSA)	Keratin is used in various dressing materials to either release antibiotics or growth factors and BSA nanofibers are used for wound closure suturing	
Polysaccharide biomaterials such as chitosan, hyaluronic acid and alginate	Chitosan has hemostatic, antimicrobial and antifungal properties, hyaluronic acid induces a fast wound healing without scar formation, enhances mitotic division of epithelial cells, and regulates phagocytosis mechanisms	high viscosity and surface tension of HA leads to enhanced water capacity, limiting its use in scaffold designing due to poor electrospinning
Synthetic biomaterials such as Polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone (PCL)	High mechanical strength, flexible properties, easy processability, and non-toxic degradation	Less mechanical strength and biocompatibility with respect to natural or animal origin biomaterials
Composite biomaterials made up of different natural or synthetic polymers or a combination of both	Highly biocompatible and biodegradable, have potential application in drug delivery and wound dressing	

Conclusions

In this chapter, the authors tried to provide an overview on numerous techniques of grafting used in the past and in present to obtain skin substitutes. These latter are very useful in the regenerative medicine to improve the wound healing or replace injured tissues, restoring the tissue functionality and improving in this way the quality of life of patients.

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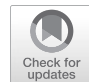

Injection/Application of Micrografts

Letizia Trovato, Antonio Graziano, and Riccardo D'Aquino

The Rigenera® Technology

In a previous chapter, the authors described the Rigenera® protocol to obtain ready to use autologous micrografts, evidencing the possibility to use them both alone or in combination with the most common scaffolds such as collagen and polylactic-co-glycolic acid (PLGA). As already reported, the micrografts are composed of progenitors cells derived from starting tissue, which express an high positivity for mesenchymal stem cell markers, suggesting an optimal regenerative potential [1]. Furthermore, the autologous micrografts obtained by this protocol are not in need of the expansion culture.

The micrografts obtained by Rigenera® protocol actually are used in different clinical contexts, including dentistry, wound care, and aesthetic medicine. For this reason, in this chapter, the authors will provide evidences based on their experience in the clinical application of this kind of micrografts.

Application of Micrografts in Dentistry

The clinical application of autologous micrografts obtained using Rigenera protocol started some years ago in the dentistry field where it was reported that human dental pulp or periosteum-derived micrografts are able to promote the periodontal regeneration [2], the bone regeneration in the atrophic maxilla [3], and to preserve the alveolar socket after tooth extraction both by reducing bone resorption and increasing new bone formation [4]. The first evidence of regenerative properties of human dental pulp-derived micrografts was reported in the paper of d'Aquino et al. where the authors showed the ability of these micrografts combined with collagen sponge to completely restore human mandible bone defects in patients requiring extraction of their third molars [5]. A further confirmation of micrografts regenerative action was provided by Graziano et al. showing that stem cells derived from dental pulp poured onto collagen sponge is a useful method for bone regeneration in atrophic maxilla [2]. According to these results, evidences, it was reported that human dental pulp-derived micrografts combined with collagen sponge also increased the efficacy of the treatment of non-contained intrabony defects in patients affected by chronic periodontitis [6, 7]. In all these studies, the micrografts were obtained from human dental pulp that represents a site easily accessible during the routine clinical practice, providing a readily available source of stem cells for clinical regenerative therapy [8]. Human dental pulp stem cells (DPSCs) have been previously isolated and characterized as a population of

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multipotent stem cells capable of differentiating in vitro in dental-pulp-like structures, osteoblasts, and endotheliocytes [9, 10].

Usually, DPSCs can be cultured by enzyme-digestion method and explant outgrowth method. In the first method, pulp tissue is collected under sterile conditions and digested with the appropriate enzyme, and the resulting cell suspensions are seeded in culture dishes until confluence and subsequent cell differentiation is achieved [11]. In the explant outgrowth method, the extracted pulp tissues are cut, anchored via microcarriers onto suitable substrates, and directly incubated in culture dishes for at least two weeks before reaching sufficient amount of cells [12]. From a clinical point of view, both these methods are not suitable for therapeutic and clinical applications due to dental pulp manipulation. The Rigenera® protocol overcomes this limitation, avoiding cell culture and manipulations. Below will be indicated a typical protocol to obtain micrografts from human dental pulp permitting its use in the clinical practice.

The dental pulp is gently collected under sterile conditions with a Gracey curette and dissociated using the Rigeneracons sterile filters, adding 1.5 mL of sterile physiologic solution. This system allows for the simultaneous mechanical dissociation of the dental pulp and filtering of the solution through a 50-μm strainer. After 60 s of agitation, the cellular suspension is collected and endorsed onto a collagen sponge scaffold, which can be gently placed to completely fill the defect that needs to be repaired. All the studies cited above report clinical benefits on average after 4–6 months from micrografts application. To confirm the regenerative properties of dental pulp stem cells after mechanical disaggregation using Rigenera® technology, a recent in vitro study showed that these cells are able to differentiate in adipocytes, osteocytes, and chondrocytes. Furthermore, in the same study, the clinical grafting of these cells in six patients showed a well-differentiated bone with Haversian system formation in the site treated with a biocomplex composed by DPSCs and collagen, while poor tissue formation was observed in the site treated with collagen alone [13].

In addition to dental pulp, the periosteum is a surprising source of stem cells, and it has been reported in the literature that after bone fracture in animal models, periosteal progenitor cells undergo an impressive expansion and differentiation into osteoblasts and chondrocytes [14]. This remarkable property of the periosteum has prompted extensive research into the use of periosteum-derived cells for regenerative approaches, and today periosteum-derived progenitor cells (PDPCs) are widely used in several clinical applications such as cartilage regeneration, bone healing, and oral-maxillofacial tissue engineering [15, 16]. In this regard, a recent paper showed that a biocomplex composed of periosteum-derived autologous micrografts and collagen is effective in the alveolar ridge preservation with respect to collagen alone, reducing the bone resorption and increasing the new bone formation after 4 months from micrografts application [4]. Other studies, not still published, showed that periosteum-derived autologous micrografts can be used in the sinus lift procedure using other scaffolds such as PLGA and PLGA/hydroxy-apatite (data unpublished). The protocol to obtain periosteum-derived autologous micrografts is similar to one of human dental pulp. Briefly, a small piece of periosteum (1–2 mm up to 10 mm) is inserted into the Rigeneracons device with 1.5 mL of physiological solution and disaggregated by rotation in the Rigenera machine (75 r/min and 15 Ncm). After 2 min the micrografts suspension is collected with a syringe using the dedicated hole and used to soak a collagen sponge for 10 minutes in order to build a biocomplex that is directly grafted on the alveolar socket.

In summary, in this paragraph has been reported the efficacy of micrografts derived by human dental pulp and periosteum in the treatment of oro-maxillo facial defects such as intrabony defects, atrophic maxilla, and alveolar socket preservation. Furthermore, it has been showed that the ability of micrografts to interact with different scaffolds such as the collagen or PLGA to better achieve the clinical benefits for the patients.

Application of Micrografts in the Wound Care

Chronic wounds remain a significant challenge for wound care specialists. While acute wounds proceed through the normal stages of healing, chronic wounds generally do not follow an orderly process of regeneration and repair, showing a lengthy healing process and requiring frequent office visits and dressing change. The use of skin substitutes provides a valid alternative therapy, showing superior efficacy and, in some cases, similar cost-effectiveness compared to traditional treatments [17].

It was reported in the last years that autologous micrografts obtained by Rigenera protocol promote the wound healing of complex postoperative and posttraumatic wounds [18, 19], of postsurgical dehiscences [20, 21] and chronic ulcers [22, 23].

In particular, it has been reported an improvement of wound healing on average after 3–4 weeks from micrografts application and as for the application in dentistry, the micrografts were mainly used in combination with collagen sponges, without the support of any other medications. The first evidence of the efficacy of micrografts in the wound healing has been reported in the paper by Giaccone et al. where the authors described two clinical cases of complex postoperative wounds. In the first case a female patient developed a necrosis at the ends of the flaps after abdominoplastic surgery and despite the treatment with VAC therapy, after two months the margins were still undermined and the area was not along the axis with respect to the skin surface. On this wound were injected autologous skin micrografts directly into the granulation tissue, showing a gradual improvement of the wound with the disappearance of undermined area and leveling of the wound to the skin surface [18]. In the second case, a man with several comorbidities such as diabetes and hiatal hernia was subjected to numerous and different surgical interventions due to the presence of adhesions on the colon, ascites, entero-cutaneous fistulas, and other complications. The wound was first treated with VAC therapy and then with skin autologous micrografts, reporting a progressive improvement in terms of good surface and size reduction of the wound despite the general and local factors of the patient, including the colonization of the wound by *Pseudomonas aeruginosa* [18].

The micrografts were also used to form a biocomplex with a collagen sponge to treat a post-traumatic lesion after two radical debridement and negative pressure therapy, showing an improvement of both re-epithelialization process and softness of the lesion. In the same study, in vitro experiments confirmed the high cell viability of micrografts and their positivity to mesenchymal stem cells markers [19].

The autologous skin micrografts were also successfully applied for the management of post-surgical dehiscences in different group of patients. In a case report was described the case of an oncological and immune-compromised patient who underwent decompressive spinal laminectomy and vertebral fixation, after which occurred a dehiscence. One month after this intervention, the patient was treated with advanced dressings without benefit, starting also the chemotherapy. Subsequently, the patient was treated with negative wound pressure therapy (NWPT) for 2 months observing a reduction of the diameter and depth of wound dehiscence but no complete re-epithelialization that was instead achieved after 70 days from micrografts application [20]. A similar result was reached in elderly patients subjected to different orthopedic surgical interventions. In all patients the micrografts were obtained by trochanteric region and injected alone into the site of injury by perilesional infiltrations or in combination with scaffolds embedded with micrografts. For all patients, the authors reported a good remission of wound dehiscence on average after 1 month from micrografts application, with a range between 15 and 60 days, depending on the wound [21].

As known, the nonhealing ulcers, especially those venous in the lower extremity, represents still today a medical challenge for all operators engaged in their management. In addition, this condition represents an economic and social issue due to decreased quality of life, reduced mobility, and social isolation for the affected sub-

jects. The management treatment of chronic venous ulcer includes compression therapy, debridement of the ulcer when necessary, and wound care but in the most of cases these treatment are not completely solving [24].

In two recent papers, the ability of micrografts to promote the healing of chronic leg ulcers to different etiology, including venous, diabetic, and posttraumatic ulcers, was described. For all treated ulcers, the micrografts were obtained by a small piece of skin as already described. In all cases, the authors reported the presence of granulation tissue after three weeks, and after four weeks a new tissue covering the area around the lesion was evidenced. The authors also reported no signs of inflammation in the skin around the wound and a reduction of pain after micrografts application [22]. To confirm the role of micrografts in the treatment of nonhealing ulcers, similar results were also observed in another independent study where was evaluated the ability of dermal micrografts to improve the healing of venous, diabetic, pressure, and posttraumatic ulcers showing a reduction of wound size, an increased granulation, and reduced exudation. In addition, in the same study, in vitro experiments showed that cultured micrografts exhibit a fibroblasts-like morphology and confirmed the expression of mesenchymal stem cell markers. Furthermore, assuming that topical delivery of micrografts on collagen sponge can improve wound healing of treated ulcers, in vitro results showed that, when combined, these form a viable and proliferative biocomplex, confirming their regenerative potential [23].

Another approach to the treatment of chronic wounds is represented by the use of minced micrografts technique consisting of spreading upon the wound bed an autologous skin sample (2 cm^2 in size) finely minced, using two surgical blades and included in a sterile hydrogel [25]. Using this technique, the authors reported the first signs of re-epithelialization after 6 days and the complete wound repair occurred within few weeks.

The success of epithelial micrografts in the treatment of chronic wounds was also confirmed by other studies, showing a more stable and complete wound healing and an increased pliability and softness of the treated areas [26, 27].

In summary, several evidences about the role of autologous micrografts combined with a scaffold in the wound healing process of different type of wounds were reported, suggesting an optimal and biological interaction or synergy when micrografts and scaffold are combined to form a regenerative biocomplex.

Application of Micrografts in Aesthetic Medicine

One of the major clinical applications of micrografts in the aesthetic medicine is represented by hair transplantation, where for micrografts the use of one or two hair follicular unit grafts applied in large numbers (>1000 grafts) in a single session for the treatment of male pattern baldness is intended. The use of micrografts (one or two hair follicular unit grafts) and minigrafts (three or four hair follicular unit grafts) has revolutionized hair restoration in aesthetic and reconstructive cases and has rapidly become the elective technique in most cases [28].

This method is also used in the androgenetic or burn alopecia to obtain the maximum survival and growth rate possible [29, 30] and in the aesthetic reconstruction of the face and scalp [31].

Micrografts and minigrafts grow anywhere on the face and thus are useful for restoring sideburns, temporal hairline, eyebrows, mustache, beard, etc. These techniques can be used in different cases, including, for example, a revision of unfavorable results from previous hair transplantation procedures, posttraumatic injuries, correction of hair loss burn injuries or congenital conditions, such hair loss due to excision of congenital hairy nevus, arterio-venous malformations, or after involution of strawberry hemangiomas [28].

Micrografting of groups of 1–2 hairs is prepared by isolating each follicle as a whole to maintain a significant amount of tissue around the entire length of the follicle [29]. Micrografting technique has been also used both for hair restoration of the scalp and the face after burn injuries

[32] and particularly for eyebrow reconstruction due to the fine control obtainable with this technique and the natural-looking results [33].

Another approach commonly used for hair transplantation is the injection of autologous platelet-rich plasma (PRP), which is able to reduce swelling and pain and to increase hair density [34]. In line with these studies, autologous micrografts obtained by Rigenera protocol were tested in the hair transplantation, showing their efficacy both promoting a continuous growth of the transplanted hair even two months after the procedure with a shortening of the dormant phase and a faster healing of the micro-wounds [35]. In this case, the autologous micrografts were obtained from hypoderma and adipose tissue of occipital region after scalp cutting and injected or dropped on the microincisions made for the engraftments of hair. The suspension was applied before and after the hair insertion [35].

The micrografts can be also used for other applications in aesthetic medicine as showed by recent papers where the authors described the capacity of autologous skin micrografts to ameliorate pathological and hypertrophic scars [36] and to induce cartilage repair in patients affected by nasal deformity or alar nasal cartilage defects [37, 38]. Regarding pathological scars, a significant improvement in terms of appearance and texture of the exaggerated scars after 4 months of autologous micrografts treatment without the use of a scaffold was shown. The authors also confirmed that micrografts are composed of mesenchymal stem cells and are able to restore the structural layers immediately below the epidermis, promoting the horizontal realignment of collagen fibers in the papillary dermis [36].

For dermal regeneration it has been proposed a protocol mainly used in the treatment of acne scars or after derma lesions caused by rejuvenation treatment performed, for example, with automatic dermaroller or dermal mechanical abrasion. In both cases, the diagnosis of the patient is extremely important, the rejuvenation treatment is based on Fitzpatrick classification, while for acne scars, it is necessary to perform a correct diagnosis of the kind of scar and it is suggested to treat only scars not Icepick.

To perform the procedure it is necessary to:

- Perform a correct diagnosis.
- Perform an accurate skin cleansing with an antiseptic (clorexidine, alcohol 70°).
- Place a topical anesthetic on the skin for at least 20 min.
- After this, remove the anesthetic and clean the skin surface.
- Start the procedure with an automatic dermaroller or any other procedure of mechanical dermal abrasion, performing circular movements, and gently flowing on the skin to obtain a level of bleeding similarly to hemorrhagic punctuation.
- After this, perform a 2.5 mm punch in a neck area without hairs.
- Collect a small piece of tissue in a sterile condition and disaggregate with Rigeneracons device for at least 1 min, having previously added in the device 1.5 mL of sterile saline solution or any other solution (such as not reticulate hyaluronic acid) to vehiculate the obtained cell suspension without reducing the micrografts cell viability.
- To ensure the efficacy of Rigenera method, the cell suspension must be visibly cloudy and opaque.
- The cell suspension can be diluted in 5 or 6 mL of serum to ensure a more consistent infiltration in the area that needs to be treated.
- Once ready, apply the micrografts on the damaged area surface within 30 min, if possible, in order to preserve the cell viability. It is very important to cover the entire affected area with a sufficient amount of cell suspension to obtain the maximum effectiveness.
- After the treatment, place on the skin a refreshing mask exerting an anti-inflammatory effect and plan with patient the application of a reparative cream or emulsion.

In the aesthetic medicine, besides derma regeneration is also important in the cartilage repair/regeneration; in fact, cartilage grafts may be used to reposition, augment, or reconstitute

Table 1 Summary of micrografts injection and clinical applications

Source of micrografts	*Injection of micrografts*	*Applications in dentistry*
Dental pulp and ligament, periosteum	Micrografts were mainly soaked on different scaffolds such as collagen and PLGA.	Micrografts injection promotes periodontal regeneration, bone regeneration in the atrophic maxilla, preserves the alveolar socket after tooth extraction reducing bone resorption and increasing new bone formation, and restores human mandible bone defects and non-contained intrabony defects.
Source of micrografts	*Injection of micrografts*	*Applications in the wound care*
Skin	Micrografts were injected by micropumps directly around the wound or soaked on the collagen scaffold.	Micrografts injection promotes the wound healing of complex postoperative and posttraumatic wounds, postsurgical dehiscences, and chronic ulcers inducing re-epithelialization and resurfacing of the injured areas.
Source of micrografts	*Injection of micrografts*	*Applications in the aesthetic medicine*
Hair follicular unit, hypoderma, adipose tissue, dermis, nasal septum	Micrografts were injected by micropumps directly on the area that needs to be repaired.	Micrografts injection can be successfully used for hair transplantation, treatment of pathological scars, including hypertrophic and burns scars, and for cartilage regeneration.

The table reports the tissues from which the micrografts can be obtained, the micrografts injection, and the main clinical applications of micrografts

nasal structure after cartilaginous resection and recontouring [39]. Until now, in vitro and in vivo studies have demonstrated that different scaffold material types, stem cells, or growth factors offer promising results in the restoration of cartilage tissue [40]. In this regard, the efficacy of micrografts was also tested on cartilage reconstruction and in two recent studies, the combined use of autologous chondrocytes-derived micrografts and PRP to reconstruct alar nasal cartilage defects and to promote cartilage regeneration in patients affected by external nasal valve collapse was described. The constructs of chondrocytes micrografts-PRP resulted in a persistent cartilage tissue with appropriate morphology, adequate central nutritional perfusion without central necrosis or ossification, and further augmented nasal dorsum without obvious contraction and deformation [37, 38].

Conclusions

The autologous micrografts are useful in different clinical application such as dentistry, wound care, and aesthetic medicine as summarized in Table 1 and can be injected alone or in combination with the most part of scaffolds. In particular, in this chapter, their efficacy in the bone regeneration of oro-maxillo facial defects, in the management of different wounds, such as postoperative and posttraumatic wounds, chronic ulcers, and dehiscences, was reported. In addition, their regenerative role was also demonstrated in the field of aesthetic medicine including the hair transplantation, the treatment of pathological and hypertrophic scars, and the cartilage reconstruction.

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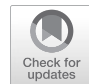

Techniques and Processing Methods to Isolate Stem Cells and Stromal Vascular Fraction Cells

Severiano Dos-Anjos and José Miguel Catalán

Introduction

The field of regenerative medicine is continuously growing and is becoming nowadays a key component in the practice of medicine. Many scientific advances in this area have been done during the last two decades. Regenerative medicine relies mainly on the use of human cells with the aim of replacing or regenerating damaged tissues or organs to restore normal function. Other disciplines like tissue engineering (using scaffolds) or the use of growth factors also belong to the wide field of regenerative medicine.

This approach of using cells has its rationale in emulating our body's physiological regenerative capabilities in adding healthy cells where there is a deficit or malfunction due to diverse causes.

A great variety of different cell types are available with this purpose, from allogeneic (from a different individual) purified cultured stem cells to the use of freshly isolated autologous (from same person) cells from different tissue sources.

Stem cells offer a tremendous potential for use in regenerative therapies to halt or reverse the effects of degenerative diseases. A stem cell is a cell that can divide to give rise to both a new copy of itself (self-renewal) and at least one specialized cell type (differentiation capacity).

We can classify the different stem cells into three main categories: embryonic stem cells, induced pluripotent stem cells, and adult stem cells [1].

Embryonic stem cells are obtained from the inner cell mass of the human blastocyst, a ball-like structure that is formed about 5 days after fertilization of the human egg. These pluripotent stem cells are grown and expanded in the laboratory and can give rise to all tissues derived from endoderm, mesoderm, and ectoderm [2].

Induced pluripotent stem cells are cells engineered in the lab by converting terminally differentiated specific cells (such as fibroblasts skin cells) into undifferentiated cells, equivalent to embryonic pluripotent stem cells. These cells are genetically reprogrammed to become a stem cell achieving pluripotency. The cells are modified in the lab introducing genes that encode four transcription factors (Oct4, Sox2, Klf-4, and c-Myc) [3].

Adult stem cells comprise a wide range of different progenitor cells that can be isolated from the great majority of all tissues in humans [4].

This group includes hematopoietic stem cells (HSCs) residing in bone marrow, mesenchymal stem cells (MSCs) from different tissues, muscle satellite cells, etc.

Adult mesenchymal stem cells (MSCs) are probably the cell type closer to the clinical reality

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due to their presence in many different adult tissues and their role in tissue regeneration and immunomodulation. The main tissue sources for MSCs isolation are bone marrow and adipose tissue, being the latter the one that provides the best yield in these cells per gram of tissue [5].

In cell-based therapies, there is still a great debate about what is the best cell type for a given clinical indication. This is caused by the development of different cellular products from biotech companies. Different cellular products or cell types have significant biological differences among them, which include: expression of cell surface markers, differentiation capacity, angiogenic potential, etc. However, the ideal cell type and/or tissue source would be the one that is autologous, abundant, easy to isolate, biologically potent, and affordable for the proposed final clinical use.

Different cell types can be obtained, either in the laboratory or fresh in the operating room, from different tissue sources and by using a huge variety of isolation methods or systems.

Certainly, the use of cells expanded in culture allows researchers to have a more homogeneous cell population (relatively pure in stem cells after several passages); however, the use of freshly isolated cells with minimal manipulation is favored in clinical practice due to reduced costs and procedural simplicity. The available preclinical literature does not show clear evidence favoring any of these approaches, and comparative studies are still necessary to clarify this matter [6].

This chapter aims to review and summarize different cell isolation techniques that can be developed in a short time intraoperatively, focusing on bone marrow and adipose tissue. A brief discussion is also included concerning some of the available clinical information and the promising future of cell-based and regenerative therapies.

Cell Isolation Techniques from Human Lipoaspirates

In the 1960s, Rodbell and collaborators developed a method to isolate cells using rat adipose tissue samples [7]. They basically extracted and minced the rat fat pads, washed several times the tissue parcels with saline solution, and then incubated the tissue pieces with collagenase to break the collagen-rich extracellular matrix, creating a dissociated tissue sample. A centrifugation step separated a yellow floating layer containing oil and adipocytes, and all other cells formed a pellet at the bottom of the sample tubes. The cellular pellet contained the stromal vascular fraction (SVF), a very heterogeneous cell population comprised of many different cell types: blood derived cells (erythrocytes, lymphocytes, monocytes, etc.), endothelial cells, fibroblasts, and other progenitor cells (including MSCs) [8]. This simple procedure allowed the separation of all mature adipocytes (tissue parenchyma) from all other supporting cells (stroma). See Fig. 1.

It was almost 40 years later when a group of scientists (led by plastic surgeons) working in Pittsburgh in 2001 demonstrated that after culturing SVF in vitro, the cells able to adhere and grow in culture were multipotent [9]. They reported that those cells (which they called PLA—processed lipoaspirate cells) had the capacity to differentiate toward the adipogenic, chondrogenic, osteogenic, and myogenic lineages. Those cells are now known as ASCs (adipose stromal/stem cells) and can be characterized by phenotypic and functional criteria: selected by adhesion to plastic (Fig. 2), proliferative potential, presence or absence of specific cell membrane markers, and the capacity to differentiate into other cell types [10]. The relative abundance of ASCs within SVF cells can be as high as 5–10% of all nucleated SVF cells obtained depending on cell isolation method used and efficiency.

During the last decade, we have seen an impressive increase in the number of publications concerning SVF focused on different features: mechanisms of action, regenerative capabilities on in vitro and in vivo models, isolation techniques, etc.

There is a huge variety of different techniques aiming to extract or isolate adipose-derived cells, which result in different cellular outputs and hence in a different biological response that affects clinical outcomes.

According to the method used to release the cells, all these techniques can be categorized into two different groups: mechanical and enzymatic

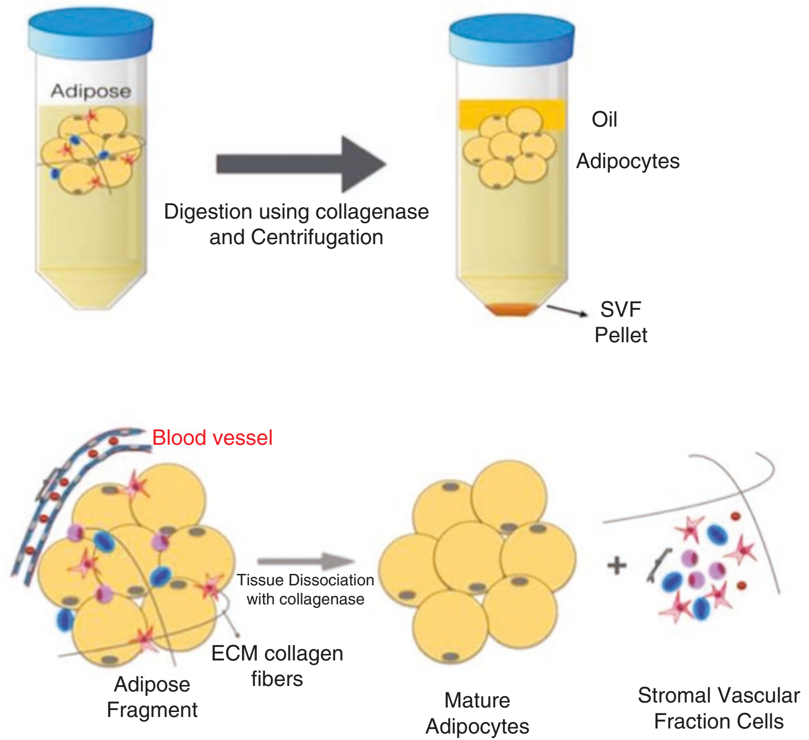

Fig. 1 Overview of SVF isolation procedure: adipose digestion in Falcon tubes showing tissue disaggregation, cell release, and concentration of SVF pellet via centrifugation

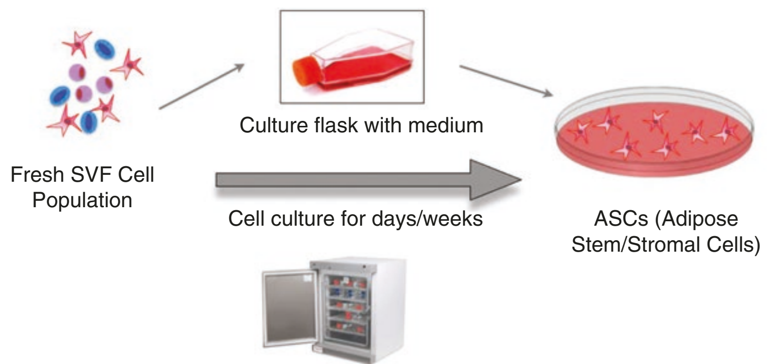

Fig. 2 Image showing ASC enrichment from SVF through cell culture expansion

methods. Nevertheless, both can be combined within the same procedure.

Mechanical methods are based on different physical methods to promote cell release from tissues. This category includes, among others, shaking, centrifugation, filtration, etc.

Enzymatic methods utilize proteolytic enzymes (proteases) to break down the tissue extracellular

matrix disaggregating the scaffold that holds the cells together. Different proteases or mixtures of them can be potentially used, but the most common and powerful one is bacterial collagenase [11].

Mechanical Cell Isolation Methods

Mechanical methods for cell isolation or concentration using lipoaspirate samples are performed using a great variety of different techniques. This has been reviewed recently by several authors [11, 12].

It is very important to point out that some of them physically extract isolated stromal vascular fraction cells, while others only decrease the relative amount of adipocytes by removing most of them in the product finally obtained.

The first group is based mainly on vortexing, vibration, or shaking followed by centrifugation to concentrate the cells that are released due to these processes [13–16].

The second group includes devices or methods that allow a relative concentration of stromal cells per volume. This is due to the removal of most adipocytes due to mechanical forces using manual techniques or point-of-care devices [17].

Some of these techniques have become very popular because of the simplicity/easiness and short-processing time, but the rationale and scientific support is still lacking or very poor [18].

Enzymatic Cell Isolation Methods

Enzymatic tissue dissociation using bacterial collagenase was the first described and is clearly the method that achieves the highest yield of isolated cells using adipose tissue samples. It is important to point out that the specific procedure determines the cell isolation efficiency and the biological characteristics of the final product. Many different factors play an important role in the cell isolation process: potency of a specific collagenase blend, concentration used, digestion time, shaking method, incubation temperature, and many more.

There are a variety of isolation systems commercially available in the market for SVF isolation, and the number is continuously increasing. It is important to highlight that their clinical use is regulated in a different way depending on each country. The regulatory framework is still not clearly defined and is still subjected to changes due to new scientific and clinical findings.

Some of these systems simplify the whole process by using specific medical devices [19] or almost fully automated systems working in a closed system [20], while others rely on a completely manual procedure using plastic disposables [21].

On last years, we are starting to see several comparative studies using some of these methods, which are helpful to the practicing physician to choose which ones would be more advantageous [22, 23].

Among the various factors that are important to compare among different systems, we would like to emphasize the following: availability of supporting scientific and clinical information, disposable cost, processing time, and user-friendliness. Regarding the scientific and clinical information, it is desirable to have as much information as possible about quality control and safety analysis of the final cellular product. This includes scientific reports about flow cytometry characterization, cell yield, collagenase residual activity, endotoxin levels, etc.

Comparison between Mechanical and Enzymatic Methods

The availability of so many possibilities and point-of-care medical devices to isolate cells from adipose tissue samples highlights the importance of analyzing critically all possibilities at hand in order to choose the best one according to the final clinical use.

In order to make an objective decision, there are different factors that should be taken into account, being the most important ones those related to the quality, safety, and potency of the final obtained cellular output. The level of automation is another factor to bear in mind, since there are manual, semi-automated, or fully automated commercial systems, which result in different processing times and simplicity of use.

Regarding the safety of a specific cellular product, it would be important to know if the processing technique is performed under strict aseptic conditions using a closed system. Moreover,

the availability of data about the absence of microbial contamination by microbiological culture, low endotoxin levels, or negligible residual collagenase activity is critical [22].

With respect to the quality parameters, the most important ones to bear in mind would be the cell yield (measured as number of nucleated cells obtained per gram of tissue processed), the cellular viability, and the phenotypical cell characterization using specific membrane markers by flow cytometry.

Biological potency assays or bioassays can provide an objective measure of biological activity by evaluating specific cellular products within a living biological system, which includes in vivo animal studies, ex vivo models, or in vitro cell culture systems [24].

These biological potency assays might give information about immunomodulatory functions, angiogenic activity, or the capacity to secrete different growth factors with regenerative properties.

There is substantial evidence that enzymatic methods yield more nucleated cells from the same amount of tissue. There is also a significant increase in the frequency of stromal/stem cells (positive for CD34 marker in vivo) with respect to the total cell population obtained. These data are summarized in Table 1.

Table 1 Comparison between mechanical and enzymatic methods for SVF isolation regarding yield, viability, and gross cell characterization. Mean cell yield values for enzymatic methods included in this table is 659.800 nucleated cells per gram, whereas for mechanical methods was only 49,571 nucleated cells per gram.

Yield (Nucleated cells per gram adipose)	Viability (%)	Method	Publication Date	First Author	Journal	CD34 + cells (%)	CD45+ cells (%)
480.000	NA	Collagenase digestion	2013	Shah	Cytotherapy [13]	81,2	27,7
25,000	NA	Mechanical (wash and centrifugation)	2013	Shah	Cytotherapy	23,7	81,7
25,000	65	Mechanical (RBC lysis and centrifugation)	2014	Markarian	Biotechnology Letters [14]	NA	NA
125,000	NA	Mechanical (shaking and centrifugation)	2014	Raposio	Plastic and Reconstructive Surgery [15]	5%	95%
1,310,000	NA	Collagenase digestion	2006	Yoshimura	Journal of Cellular Physiology [25]	20–40	20–40
719,000	83%	Collagenase digestion	2014	Dos-Anjos	Cytotherapy [19]	NA	NA
560,000	90	Collagenase Enzymatic	2016	Chaput	Plastic and Reconstructive Surgery [26]	21,45	30,59
80,000	54	Mechanical (Vortexing and Centrifugation)	2016	Chaput	Plastic and Reconstructive Surgery	5,81	41,17
50,000	45	Mechanical (Intersyringe dissociation)	2016	Chaput	Plastic and Reconstructive Surgery	38,11	19,17
230,000	80–90	Collagenase	2014	Conde-Green	Plastic and Reconstructive Surgery	NA	32
12,000	80–90	Mechanical (vortexing and centrifugation)	2014	Conde-Green	Plastic and Reconstructive Surgery	NA	70–85
30,000	More than 90	Mechanical (mystem)	2015	Gentile	PRS GO	NA	NA

Mechanical methods, in general, offer several advantages such as being less expensive and time consuming. However, enzymatic methods might be better for the clinical setting due to higher yield and the clearly superior cellular composition of the cells isolated.

Cell Isolation Using Bone Marrow Aspirate

The use of bone marrow and cancellous bone in an intraoperative clinical setting is very common, especially among orthopedic surgeons for bone healing applications.

Bone formation, remodeling, and healing depend on the recruitment of endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and their supporting accessory cells to the injured site [27].

The bone marrow is found within the central cavities of axial and long bones. It consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinuses distributed within a mesh of trabecular bone [28]. The bone marrow is the major hematopoietic organ in humans, responsible for the production of all blood cells (leucocytes, erythrocytes, and platelets) and participates in bone turnover and remodeling.

Bone grafting is widely used in hospitals to repair injured, aged, or diseased skeletal tissue. However, bone autograft material for bone regeneration is limited in quantity, and its harvesting requires an additional surgical intervention with associated morbidity, pain, and secondary complications [29].

The use of autologous bone marrow mononuclear cells (containing MSCs) and the relatively simple and noninvasive method to harvest them by aspiration is becoming very popular. The idea of extracting and concentrating bone marrow aspirate was pioneered by Hernigou in 2002 [30]. This procedure can be performed rapidly during the same surgical act using point of care medical devices [31, 32].

At present, one of the most straightforward bioregenerative strategies, specifically for those clinical conditions that cannot be addressed by standard of care treatments, is the use of autologous bone marrow aspirate concentrate (BMAC). This approach, based on the concentration of bone marrow mononucleated cells using CE-marked kits or commercially available devices, is performed in the operating room during surgery. This procedure does not involve any substantial cellular manipulation and when the cells are injected within the same histological environment (intraosseus) also would comply with a homologous use. Thus, this therapy could avoid the classification and specific regulations associated with advanced therapy medicinal products (ATMPs), regulated in Europe by European Medicines Agency (EMA). Moreover, the safety of these procedures, as well as significant clinical evidence, has been confirmed by many authors previously [33–35].

The use of bone marrow aspirate concentrate is among the different bioregenerative therapies in the field of musculoskeletal injuries, being specially used by orthopedic surgeons.

The final result of centrifuging a bone marrow aspirate is a concentrate of mononucleated cells, which includes MSCs at low frequency (0.001–0.01% of all nucleated cells). The BMAC also includes platelets, which might also be relevant in the clinical response observed.

The scientific evidence have demonstrated that its use as a single or complementary regenerative therapy enhances the physiological bone repair capacity, allowing a better and faster patient recovery.

This strategy has been used for several clinical indications, such as bone fractures, pseudoarthrosis (non-unions), avascular necrosis of the femoral head (AVN), or osteochondral lesions.

How Is BMAC Obtained?

The aspiration of bone marrow concentrate is usually performed under sedation and local anesthesia in the operating room. A percutaneous aspiration with a 13 G trocar, placed at the anterior part of the iliac crest, is performed on the ipsilateral side of the lesion to be treated.

After perforating the iliac crest, the trocar is introduced about 5 cm in depth. The trocar posi-

tion must be changed continuously (in depth and direction), aspirating a maximum of 5 cc at a time. The aspiration technique is critical in order to obtain the highest number of progenitor cells and avoid the contamination with peripheral blood.

This step is crucial, because aspirating from a single point only using high volumes might contaminate the aspirate with peripheral blood, and we would not be getting the progenitor cells that are stuck in the bone or around the walls of the blood vessels (osteoprogenitor cells and MSCs) [36]. Sometimes, several aspiration points are used on the iliac crest to improve the aspiration technique.

Following the aspiration procedure, the aspirate is filtered in order to discard blood clots or bone chips. Usually around 60–120 ml of bone marrow aspirate are obtained, which are then centrifuged and resuspended to get 8–16 cc of BMAC. The BMAC is aspirated at the lower plasma phase including the buffy coat, avoiding most of the red blood cells at the bottom.

Finally, the injection of BMAC is performed by minimally invasive techniques, either directly intraosseous through a trocar or as an adjunct to other surgical procedures such as arthroscopic subchondral bone microfractures with or without a scaffold [37].

Advantages and Disadvantages Over Other Therapeutic Procedures

Comparing the use of BMC with PRP (platelet rich plasma), for the same therapeutic indications, this procedure allows the inclusion of progenitor cells residing in bone marrow. There are several commercially available point-of-care devices for extracting and concentrating bone marrow aspirates [31].

Regarding the cell obtention from other tissues such as adipose, the main advantage is that it is easier and more convenient to perform an aspiration of the iliac crest by an orthopedic surgeon than to perform a liposuction.

Comparing the use of BMAC with conventional surgical procedures such as prosthetic surgery or osteosynthesis in pseudoarthrosis, this procedure allows the physician to obtain satisfactory clinical results (pain improvement and functional recovery) without adding aggressiveness to the surgery.

About the disadvantages, it is well described on the scientific literature that the number MSCs obtained per gram of adipose tissue is much higher compared to one cc bone marrow aspirate [5]. Adipose stromal vascular fraction also contains higher amounts of other cell types with angiogenic potential, such as endothelial progenitors or pericytes. Some authors suggest that adipose might be a better source for MSCs due to the superior phenotype and functional capacities of isolated cells (i.e., osteogenic differentiation). However, this debate is still controversial since conflicting results have been reported [38].

Another hurdle that requires careful analysis is the "contamination" of bone marrow aspirate with peripheral blood, which is called peripheral blood admixture. This happens when the volume of bone marrow aspirate increases or when most volume is obtained from the same location. This could lead to a significant increase in the percentage of nucleated cells coming from blood in the bone marrow aspirates [36].

Clinical Use of Bone Marrow Concentrate

There are several clinical reports that support the use of bone marrow concentrate for hip avascular necrosis (AVN) treatment after decompression of the femoral head, with promising results, especially in the earliest stages of the disease (grades I-II of AVN) [33].

The summarized procedure for AVN is the following: the patient is placed on the traction table, and using a fluoroscope, the guiding needle is percutaneously placed in the center of the necrotic lesion in the anteroposterior and axial hip planes. Then a tunnel is created with a 4 mm drill to inject the BMAC into the affected area of the femoral head.

The patient is discharged on the same day with partially loaded crutches, and heparin prophylaxis for 10 days [39].

Moreover, several studies have reported its use for pseudoarthrosis and non-union, where the fractures with best results were those that had a greater number of progenitor cells [40]. Pseudoarthrosis (non-union) is defined as an abnormal union formed by fibrous tissue after a bone fracture that has bone healing problems. In these cases, the non-healing lesion is perforated using drills, traversing the proximal and distal fracture sites. Through the channel created at the non-union site, the cannula is introduced and the BMAC injected, in the focus, proximally and distally, without using any type of osteosynthesis material [41].

Promising results are also being reported for knee osteoarthritis (OA) in early–moderate stages (I-II) [42]. This response could be dose dependent as supported by some authors according to the final cell dose used [43].

The pathogenesis of osteoarthritis is very complex and not fully understood. As proposed by several authors, it seems that the subchondral bone plays an important role and would affect the articular cartilage degeneration. Thus, the approach of injecting the BMAC or any other biological product intraosseus at the subchondral affected bone is advantageous. This infiltration could be more efficacious even for more severe OA, promoting the inhibition of cartilage-degrading cytokines, stimulating chondrogenesis or the production of hyaluronic acid and lubricine by chondrocytes [44].

Human Clinical Studies Using Adipose Freshly Isolated Cells (SVF)

There is substantial and encouraging preclinical and clinical information that supports the use of freshly isolated autologous cells from adipose tissue samples [45, 46]. Adipose SVF cells are currently being used in different clinical settings. As of April 2016, a total of 75 clinical studies are registered in clinicaltrials.gov, being USA and Europe the most active regions worldwide (Fig. 3). Forty-two of the total number were registered as active studies recruiting patients.

The main and more studied targets for intervention are soft tissues (radiation wounds, diabetic ulcers, etc.), musculoskeletal tissues (bone defects, tendinopathies, osteoarthritis, etc.), ischemic injuries, and immune disorders.

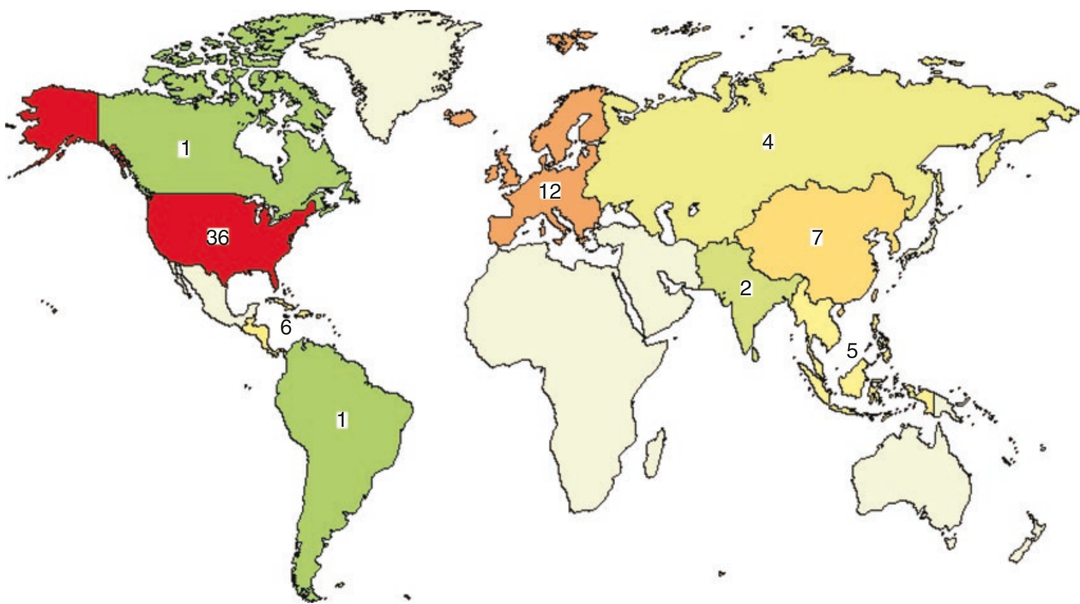

Fig. 3 Clinical studies using SVF colored by number including locations around the world. Source:https://ClinicalTrials.gov

The field of orthopedics is being most active for clinical SVF application. In this regard, many studies have reported significant clinical improvements for osteoarthritis, particularly in the knee and hip joints [47–51]. Recent clinical reports also suggest a beneficial effect of SVF cells for Achilles tendinopathy [52] and bone regeneration [53, 54]. The SVF application has been also successfully performed in patients with burn wounds [55] and other chronic wounds associated with peripheral vascular disease or diabetes [56].

Safety and feasibility is clearly demonstrated in all these studies. Patient clinical efficacy is also frequently reported. However, well-designed randomized clinical trials including controls are still needed to confirm this initial but compelling evidence. Moreover, any clinical use of cells must comply with applicable regulations.

Concluding Remarks

The field of regenerative medicine and biological therapies is evolving very rapidly and constantly changing. Many findings (preclinical and clinical) on different medical fields support this new paradigm of using cell-based therapies for treating patients. This is especially important when current traditional approaches do not provide satisfactory clinical results.

However, there is still no agreement on which tissue source or cellular product is the best for each clinical indication. Furthermore, many different devices or methods are available for the same purposes, creating more confusion. Any clinical decision based on biological or cellular products must be based only on scientific and clinical evidence. Different approaches will yield very different final products. The physician is responsible for choosing the best cost-effective method for a given clinical indication based on disease severity focusing on patient safety and all scientific information available to provide the best possible patient care.

There is currently a great opportunity to continue the scientific progress by addressing these questions developing comparative studies using different cell isolation methods or approaches for specific medical problems. The field of plastic and aesthetic medicine can be on the front of these advances. All assessments must be based on well-designed cell quality and potency assays while keeping patient safety at the highest levels. Both basic science and clinical research should complement each other in this fascinating endeavor.

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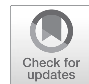
Part VI

Regenerative Medicine Procedures for Aesthetic Physicians: Stem Cells



Regenerative Medicine Procedures for Aesthetic Physicians

Martinez-Redondo Diana, Gartzia Itxaso, and Castro Begoña

The SVF is a highly heterogeneous pool of cells including mesenchymal stem cells, adipocytes, blood cells (erythrocytes, leukocytes …), endothelial cells and epithelial cells among others [1]. To purify ASC from this complex mixture we take advantage of their capacity to adhere to cell culture plastic surfaces to get rid of the non-adherent cells, and are in vitro cultured using specific conditions and culture medium that promotes ASC growth.

Once SVF cell pellet is isolated, to obtain a primary culture of cells, a first estimation of mononuclear cell number is performed by using a hemocytometer (Neubauer or Burker counting chamber), following trypan blue cell viability exclusion protocol. At this point, it should be considered that the first mononuclear cell estimation includes other cell types in addition to ASC that have the same appearance in suspension. Thus, all nucleated yellow bright cells (in the picture cells marked as orange and green) are counted knowing full well that not all of them will be ASC. Endothelial, hematopoietic, and pericytic lineages represent 10–20%, 25–45%, and 3–5%, respectively, of the total nucleated cells [1]. However, such cell counting is important to estimate total number of nucleated cells to seed them at an optimal cell density for primary in vitro cell culture (Fig. 1).

In the bibliography, cell yields for enzymatic procedures are reported from 100,000 nucleated cells/mL of lipoaspirate to 1,310,000 cells/mL [2, 3]. Cell yield in SVF not only depends on aspects related directly to the sample such as donor age, type, and location of the adipose tissue and surgical procedure [4], but also in the isolation protocol and laboratory operator. From our experience, considering 43 adipose tissue extractions in healthy donors and also in patients with different pathologies, no differences in SVF or ASC cell yields were observed with regard to age donor or extraction area (location of adipose tissue), but more cells both in the SVF and ASC at initial cell passage are obtained in women when compared with men (Table 1).

For primary in vitro cell culturing, nucleated cells are seeded at a density of 30,000–60,000 cells per cm^2 and cultured in DMEM Glutamax with 10% fetal bovine serum (complete medium) at 37 °C and 5% CO_2 in a cell incubator; this culture corresponds to passage 0 (P0). After a first overnight culture, only adherent cells attach to the plastic flask, and nonadherent cells such as erythrocytes and other cells with slower adherence capacity will be removed in following washing steps. At this point, ASCs are quite small and present a rounded morphology (Fig. 2). At least two or three washing steps with sterile PBS or culture

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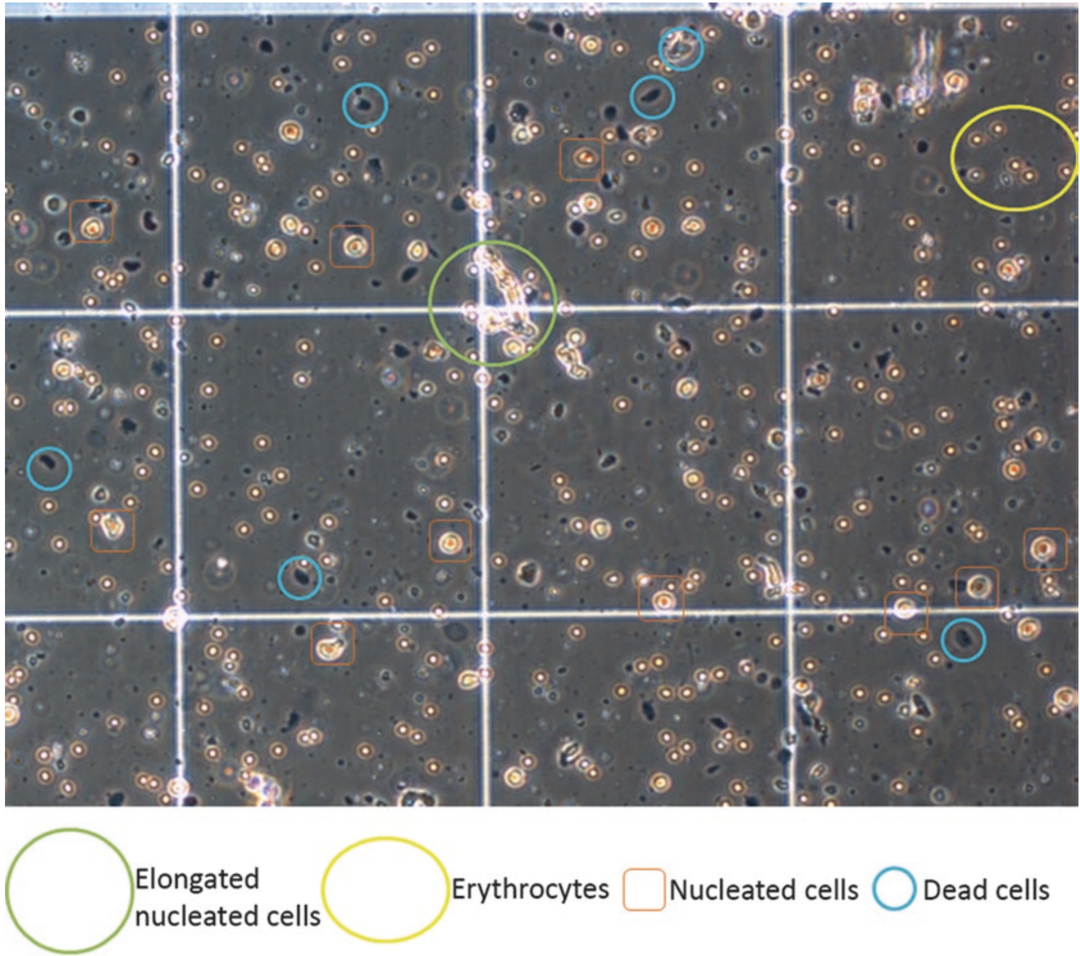

Fig. 1 Inverted microscope image of the different cell types that are present in the SVF, in a Burker counting chamber after following trypan blue exclusion staining (10x amplification)

Table 1 Donor profile: age, gender, and cell yield per milliliter of liposuction tissue

Lot no.	Gender	Age (years)	Body area	Adipose tissue vol. (mL)	SVF (cells)	SVF yield (cells/mL)	ASC P0 (cells)	ASC yield (cells/mL)
1	Man	19	A	100	9,600,000	96,000	3,960,000	39,600
2	Man	21	A	100	14,400,000	144,000	12,852,000	128,520
3	Man	21	A	200	27,600,000	138,000	11,100,000	55,500
4	Man	36	T	80	9,600,000	120,000	480,000	6,000
5	Man	38	T	30	4,800,000	160,000	560,000	18,667
6	Man	39	A	100	13,200,000	132,000	8,800,000	88,000
7	Man	39	A	100	13,200,000	132,000	11,200,000	112,000
8	Man	45	A	70	9,072,000	129,600	3,328,000	47,543
9	Man	47	A	100	10,800,000	108,000	3,000,000	30,000
10	Man	47	A and F	100	10,800,000	108,000	4,080,000	40,800
11	Man	49	A and T	100	8,400,000	84,000	2,880,000	28,800

Table 1 (continued)

Lot no.	Gender	Age (years)	Body area	Adipose tissue vol. (mL)	SVF (cells)	SVF yield (cells/mL)	ASC P0 (cells)	ASC yield (cells/mL)
12	Man	56	A	150	18,000,000	120,000	4,080,000	27,200
13	Man	57	A	200	25,200,000	126,000	17,200,000	86,000
14	Man	58	T	60	7,200,000	120,000	1,200,000	20,000
15	Man	60	T	40	3,960,000	99,000	1,220,000	30,500
16	Man	61	T	60	6,739,200	112,320	2,000,000	33,333
17	Man	63	T	100	19,200,000	192,000	12,600,000	126,000
18	Man	68	T	150	22,880,000	152,533	5,600,000	37,333
19	Man	72	T and K	75	12,000,000	160,000	12,524,000	166,987
20	Man	89	T	100	14,800,000	148,000	5,580,000	55,800
21	Woman	19	F and Tr	500	91,600,000	183,200	39,305,000	78,610
22	Woman	22	A	300	63,920,000	213,067	5,660,000	18,867
23	Woman	29	T	250	50,000,000	200,000	28,640,000	114,560
24	Woman	32	T	140	41,000,000	292,857	4,315,000	30,821
25	Woman	33	A and F	350	38,660,000	110,457	3,000,000	8,571
26	Woman	34	A and F	300	86,400,000	288,000	10,130,000	33,767
27	Woman	34	A and F	450	38,400,000	85,333	9,170,000	20,378
28	Woman	38	H and K	500	46,200,000	92,400	41,200,000	82,400
29	Woman	38	F	100	13,600,000	136,000	4,660,000	46,600
30	Woman	38	F	100	18,133,000	181,330	7,500,000	75,000
31	Woman	41	A	400	51,580,000	128,950	20,410,000	51,025
32	Woman	41	T	100	13,200,000	132,000	17,200,000	172,000
33	Woman	47	A	100	33,600,000	336,000	41,500,000	415,000
34	Woman	47	A, F and H	450	116,000,000	257,778	76,290,000	169,533
35	Woman	48	A	200	28,600,000	143,000	6,780,000	33,900
36	Woman	48	L	150	48,000,000	320,000	6,680,000	44,533
37	Woman	49	A and F	300	36,800,000	122,667	39,600,000	132,000
38	Woman	51	A and T	100	14,400,000	144,000	13,920,000	139,200
39	Woman	56	F	230	58,800,000	255,652	3,460,000	15,043
40	Woman	58	A	150	32,800,000	218,667	22,800,000	152,000
41	Woman	60	A	150	27,600,000	184,000	8,000,000	53,333
42	Woman	60	A	300	35,700,000	119,000	23,000,000	76,667
43	Woman	79	A	100	13,760,000	137,600	5,332,000	53,320

ASC P0 stands for obtained adipose tissue mesenchymal stem cells after passage 0 (4–5 days in vitro culture after SVF isolation)
A Abdominal, *F* Flanks, *H* Hips, *K* Knee, *L* Lumbar, *T* Thigh, *Tr* Trocant

medium every 24 h are necessary, followed by replacement with fresh complete medium, to leave a clean surface for ASC to grow, eliminating the toxicity that intracellular content of dead cells and tissue debris could exert. At this first passage (P0), cells that remain in the flask are mainly ASC, but also other adherent secondary populations are present (lymphocytes and endothelial cells among others), that will disappear along cell culture in subsequent passages (Fig. 3).

Approximately after 3–5 days in culture, ASC becomes confluent and is time to proceed to passage 1. For that purpose, 5 min incubation at 37 °C of cell culture in a solution of Trypsin-EDTA is commonly used for cell detachment, following a centrifugation step at 500 × *g* for 5 min, to obtain the cell pellet. In this way, cell expansion could be done by seeding the cells at a rate of 2000–3000 cells per cm^2. For a correct cell culture growth of ASC, media renewal must be performed every 48 h. Under these conditions, duplication time of ASC is between 2 and 4 days depending on cell line (donor age, type and location of adipose tissue, culturing conditions, plating density, and media formulations [5]), and ASC cell culture becomes confluent after 5–7 days when either a new cell expansion to P2 (Fig. 3) or generation of a cryopreserved cell bank can be achieved.

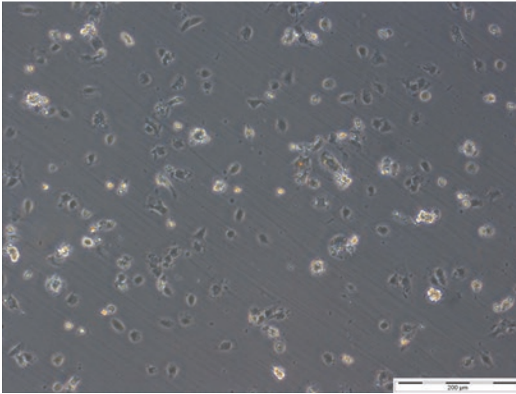

Fig. 2 Inverted microscope image of a primary in vitro culture of ASC, after overnight culture of seeded SVF. At this stage, the cell population consists mainly of ASC but other adherent cells like lymphocytes or endothelial cells are also still present (10x amplification)

Although ASC potency and characteristics seems to be maintained till P9, normally cells that are administrated to patients do not exceed passage 5–6 in order to avoid senescence state and assure genetic stability. For example, Roemeling-van Rhijn et al. [6] demonstrated that the percentage of aneuploidy remained stable in passage numbers 0–4. Moreover, at the moment, in vitro expanded stem cell clinical products must not exceed 12 generations, which means that a single cell can undergo 12

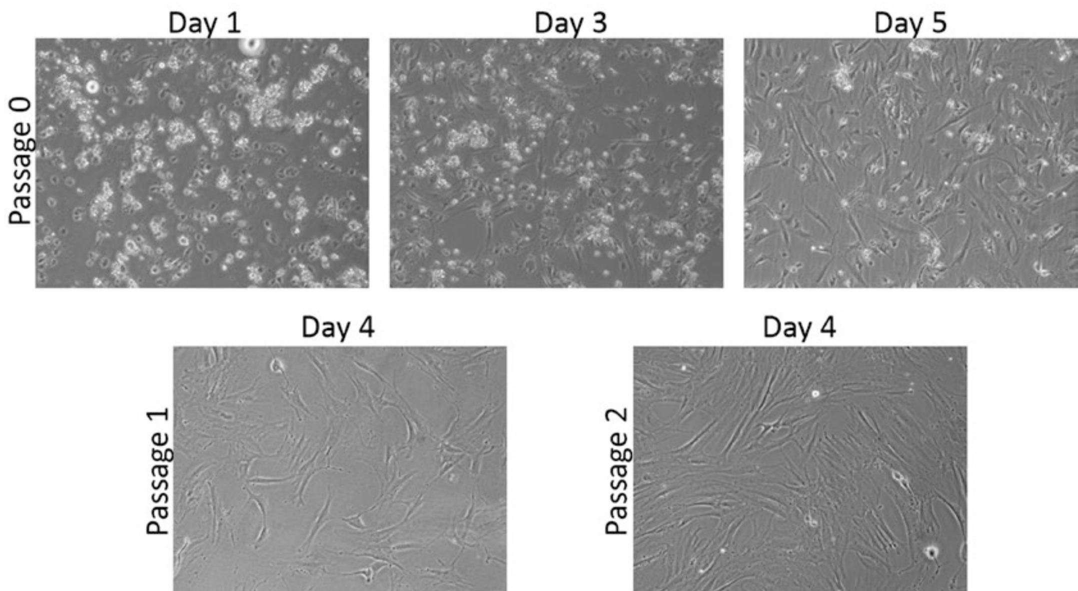

Fig. 3 Inverted microscope images of the evolution of ASC primary in vitro cell culture. Throughout P0, contaminating cells and debris are eliminated with washing steps and ASC acquires a spindle-shaped fibroblast-like morphology. This morphology is then maintained in subsequent cell passages as seen in P1 and P4 (10x amplification)

duplication cycles, thus obtaining from one single cell a total of 4.096 cell daughters with the same regeneration potency. Hence, if ASC cell yield is 75,000 cells per mL of adipose tissue (Table 1), considering 100 mL of ASC, after in vitro cell expansion a total of 30.7×10^9 ASC can be obtained to generate a therapeutic clinical product. However, in order to achieve 12 generations, there is no need to undergo cell expansion till passage 5–6 because ASC proliferation can be stimulated by several exogenous supplements to obtain the maximum amount of ASC in less time [5, 7] or by using bioreactors.

Fetal Bovine Serum (FBS) is commonly used as a supplement to cell culture media, providing high concentrations of growth stimulatory factors, macromolecules, carrier proteins for lipids, trace elements, attachment and spreading factors, low-molecular-weight nutrients, hormones, and low concentrations of immunoglobulins. However, the global serum supply reduction is an important limiting factor in serum FBS use [8].

Taking this into account, various blood products derived from mature organism have been investigated as suitable substitutes for FBS to support the expansion of human ASC, including antagonist-activated platelet-rich plasma and human platelet lysate (hPL) containing essential elements for cell expansion. Reported advantages of using hPL in place of FBS in media include a faster growth of ASC as a result of shorter doubling times and a strengthening of the inhibitory effects of ASC on lymphocyte proliferation, reaching a pure ASC cell culture in less time [9]. However, the use of human-derived components present other potential risks as routine screening of blood may be insufficient to provide the required level of safety. Additional concerns include security of supply and batch-to-batch variations, which could impact the adoption of hPL in clinical-scale production [10–12].

Increasing concern in the use of FBS and human-sourced supplements, like hPL, suggests the need of developing chemically defined media for the culture of stem cells for therapeutic applications. One of the major complications in formulating cell culture media without the use of animal- or human-derived products is the replacement of serum albumin, which carries a multitude of molecules that support and/or stimulate stem cell growth. These growth factors contained in serum albumin are perhaps the most critical components to balance properly cell culture media for therapeutic medicine applications. Not only they provide necessary mitogenic input to nonimmortalized cells, but also a correct balance of them is required to maintain the specific desired cellular phenotype. Thus, development of an optimized growth factor supplementation strategy for stem cells in vitro expansion is still pending, and remains as a major challenge to stem cell–based therapy [12, 13].

In addition, another approach to generate the required number of cells to make its application practical and cost-effective is the use of cell culture bioreactors. A variety of bioreactor vessels have been used with microcarriers, which provide a high surface-to-volume ratio, to increase stem cell production for cell therapy, including stirred-systems such as spinner flasks and stirred-tank bioreactors, and nonstirred systems such as oscillating and multiplate bioreactors. All these systems improve yield of cell expansion procedures and these cells retain their basic defining characteristics of cell surface marker expression and differentiation potential [12].

Purity of expanded ASC is another significant challenge confronting their clinical implementation. In 2006, the International Society for Cellular Therapy (ISCT) proposed minimal criteria for stromal cells obtained from bone marrow mononucleated fraction (MSC) that have also been use to define ASC: (1) plastic adhesion; (2) phenotypic expression of CD73, CD90, and CD105, and lack of CD11b or CD14, CD19, CD45, and HLA-DR expression [14]. However, as the use of ASC for therapeutic applications has grown substantially and has sparked the growth of a new research field and industry worldwide, in 2013, the ISCT updated such criteria to describe stromal cells from the SVF of the adipose tissue and ASC [1].

Expanded ASCs obtained from the SVF meet all proposed criteria to be considered a candidate

resource for therapeutic applications: be able to be collected in abundant quantities, with a minimally invasive procedure, able to differentiate into a variety of tissue types in a controlled and reproducible manner, able to be transplanted to either an autologous or allogenic host in a safe and effective manner, and manufactured in accordance with current Good Manufacturing Practice guidelines [5, 15, 16].

In contrast to SVF cells, beyond passage 2, ASCs lose their expression of HLA-DR and CD86, which serve as recognition markers for T cells. While SVF cells elicit a robust mixed lymphocyte reaction from allogeneic peripheral blood monocytes, in vitro expanded ASCs are relatively nonreactive. Furthermore, the presence of ASCs suppresses ongoing mixed lymphocyte reactions between allogeneic peripheral blood monocytes [17, 18]. The immunosuppressive properties of ASCs are due, in part, to their production of prostaglandin E_2 and indoleamine 2,3-dioxygenase [19, 20]. These unique immunomodulatory features suggest that both allogeneic and autologous ASCs will engraft successfully following application for tissue regeneration purposes [17]. On the one hand, for an autologous therapy, the individual donor is also the recipient of the treatment termed a “one-to-one” therapy and may require or not expansion of cells prior to administration; thus, the scaling of these technologies is considered “scale-out” where cells from patient may be processed in parallel, and at least one month is needed to apply the cell product in the implantation area. On the other hand, in the allogeneic approach, a single donor provides primary cells used to produce the therapeutic cells that will be administered to many recipients. For allogenic therapies, cell expansion is required in order to generate ready-to-use doses for multiple patients; these are considered “scale-up” applications, calling for larger volumes to meet huge size needs [12].

Finally, another advantage of in vitro expanded ASC and their allogenic use for therapeutic applications is the possibility to generate cryopreserved cell banks to be ready to use when needed by the clinician. In this way, the challenge becomes the optimization of freezing protocols to ensure that cell retain the characteristics of their freshly isolated counterparts. At the moment, mesenchymal stem cells for clinical use are most commonly frozen in 5% or 10% Me_2SO in an electrolyte solution with an added protein (e.g., human serum albumin). Using such cryopreservation solutions, it has been demonstrated that ASCs are stable without hampering their biological functionality for at least 6–12 months [21, 22], but probably, cell banks can be cryopreserved for longer periods of time maintaining their characteristics and function and a postthawing viability over 70%. Hence, the ability to preserve cells permits completion of quality and safety testing before use as well as transportation of the cells between the sites of collection, processing, and clinical administration. The ability to preserve the cells after production of the therapy facilitates coordination of therapy with a patient care regime and reduces staffing requirement of clinical cell production facilities [23].

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Stem Cell Research in Aesthetic Medicine

Pablo Sutelman

Introduction

Stem cells are highly specialized cells that become the foundation of every tissue and organ within our body.

Our body contains over 200 different types of cells, all of which can be traced back to the early embryo [1].

During our development, as well as throughout life, stem cells give rise to the differentiated cells that ultimately performs every distinctive function of the organism.

Furthermore, stem cells can help replace and regenerate damaged cells that are lost due to injuries or aging.

Humanity's interest in regeneration dates back as early as ancient Greece, with the myth of Prometheus (Fig. 1), who received a punishment from Jupiter after having stolen the fire, the symbol of civilization. He was chained to the Carpathian Mountains, where an Eagle would pick part of his liver every day, only to be regenerated overnight [2]. Aristotelian thesis also acknowledged that undifferentiated matter can give rise to all sorts of life [3].

Accordingly, mankind has always been fascinated with regenerative faculties, but it was not until the early 1900s that researchers recognized that different types of blood cells originate from a unique stem cell. The first successful human cell treatment was the bone marrow transplant performed in 1956 by Dr. Donnall Thomas [4].

Fig. 1 Prometheus myth [3]

Moreover, investigations leaded by Alexis Carrel, in collaboration with aviator Charles Lindbergh, involved transplantation procedures and organ repair techniques, which provided the foundation for the development of modern regenerative medicine [5].

Embryonic stem cells were first extracted from mice in the 1980s, and in 1998 a team of scientists from the University of Wisconsin was the first group to adequately isolate human embryonic stem cells in a laboratory [6].

Current therapies regarding treatment of advance organ failure ultimately requires the

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replacement of damaged tissues by organ transplant, but it is a process that is becoming increasingly complex due to limited organ donor supply and severe adverse effects related to chronic immunosuppressive therapy.

Regenerative medicine implies a revolution into a new medical paradigm, by replacement of human cells, tissues, or organs through regeneration, to restore its normal properties and physiology [7].

This novel field of research includes several strategies, from the employment of directed cell therapy to promote regeneration, to the clinical use of 3D printed biomaterials and scaffolds, which mimic the native extracellular matrix of tissues and organs, enabling structural support and function of the new tissue [8]. Simultaneously, stem cell research can modulate the immune system and enhance the normal healing capabilities of the human body to improve its functionality and, at last, unravel the hidden mechanisms of cell biology.

The future of stem cell research relies on the promise of personalized medicine based on target gene and cell therapies and tissue bioengineering.

Stem Cell Properties

Stem cells display three general properties:

- They are unspecialized cells, not containing any tissue-specific structures or markers.
- They are capable of dividing for long periods.
- They can give rise to specialized cell types [9].

Stem cell's ability of differentiation relies entirely on their evolution timeframe (Fig. 2). Totipotency is defined by the ability to originate all cell types available, including the entire embryo and placenta. The cells that arise from the fertilized egg are totipotent, but within several divisions, they make the transition to become pluripotent. Pluripotency is the capacity to form multiple cell types of all three germ layers (ectoderm, mesoderm, and endoderm) but not the whole organism. Embryonic stem cells are known to be pluripotent, being able to originate any cell type. However, their performance is shaded by some instability features in a partially differentiated state, and a limited knowledge regarding their specialization signaling. Meanwhile, once developed, tissue-specific stem

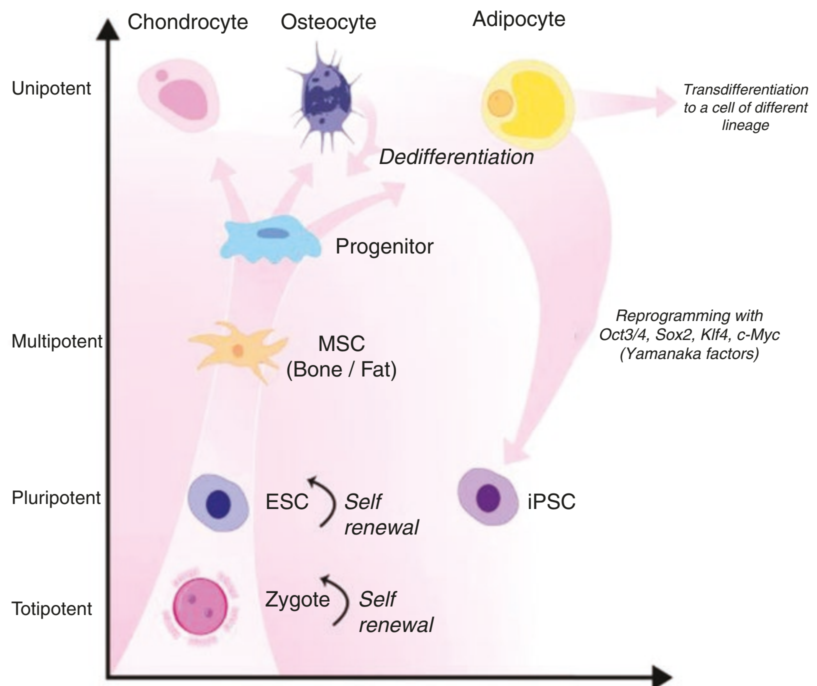

Fig. 2 Stem cell potency diagram [11]

cells are multipotent, which is the ability to give rise to a limited range of cells appropriate to their location. While restricted in their scope of cell type development, they become more suitable for a controlled differentiation [10, 11].

Differentiation. Transdifferentiation

The normal differentiation pathways used by adult stem cells are tightly controlled by each cell's genetic material included in the DNA, carrying the information necessary for every cellular function.

The external signaling involved in cell differentiation comprises chemicals and molecules present in the microenvironment, as well as physical contact with other cells. This type of interaction during differentiation causes the DNA strands to acquire epigenetic marks that then restrict DNA expression.

Stem cells are able to maintain their undifferentiated state for long periods, and later enter the mitotic cycle to give rise to specialized cells of a particular tissue. On the other hand, a varied number of experimental data endorse the existence of a distinctive process known as transdifferentiation, which is the property of certain adult stem cells to differentiate into cell types found in different tissues than the one predicted from its lineage [12].

Stem Cell Types

Embryonic Stem Cells

From early stages in stem cell research, embryonic stem cells (ESCs) from a variety of species have been employed to generate all different types of cells found in the body. Given their pluripotency ability, embryonic stem cells have been proven to differentiate into cells from all three embryonic germ layers.

ESCs were first reported by Gail Martin in 1981 [13]. They are normally obtained from the blastocyst at a very premature phase of development, between the fifth and sixth day in a preimplantation stage, consisting of approximately 200 cells [14].

Human blastocysts are derived primarily from in vitro fertilizations for assisted reproduction that are donated for research purposes through informed consent.

In contrast with adult stem cells, they are easier to identify, collect, and maintain in culture.

Another source of embryonic stem cells is through a technique known as somatic cell nuclear transfer, which consists in the removal of DNA from an unfertilized egg and replacing it with the targeted genetic information of a somatic cell. Nuclear transfer was reported by Briggs and King in 1952 [15], and it has been used to produce embryos with the purpose of explanting ESC lines with equal genetic markers as the donor cell.

Due to ethical debates raised with the use of this particular cell type, their potential use has not been fully developed. Moreover, with the possibility of tumor growth and abnormal tissue formation, their employment requires rigorous control.

Tissue-Specific Stem Cells

Tissue-specfic stem cells are undifferentiated cells with a primary function of maintaining tissue homeostasis, repairing potential injuries by differentiating into specialized cell types of the organ where they reside [16].

They have been identified in most organs, with distinctive regenerative properties, such as bone marrow, skin, skeletal muscle, brain, heart and peripheral blood, and adipose tissue, among others [17]. They reside frequently in a specific area of each organ referred as stem cell niche, which implies the physiological connections between stem cells and the extracellular matrix, intercellular contact, and specific signaling molecules [18]. They represent a very small fraction of the general population of the organ, reason why they are more difficult to isolate and grow in a laboratory setting.

Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSC) are adult somatic cells that have been genetically repro-

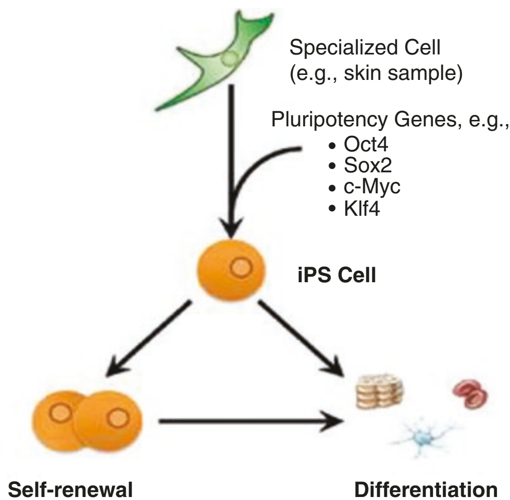

Fig. 3 iPS cells production and potential applications [18]

grammed to an embryonic stem cell-like state (Fig. 3), resulting in pluripotency capability [19].

Human iPSCs were first described by Sir John Gurdon and Shinya Yamanaka, for which they later received the 2012 Nobel Prize in Physiology and Medicine.

The original iPSCs were originated introducing viruses to insert extra copies of determined genes responsible for the embryonic stem cell undifferentiated state, although there are currently nonviral delivery strategies being employed.

Therefore, iPSCs become disease-specific stem cells, which are important tools to study particular pathologies and their response to specific treatment options. As they arise from the patient's own cell pool, it minimizes the risk of rejection or the need for adjuvant immunosuppression therapy.

Amniotic Fluid-Derived Stem Cells

The amniotic fluid usually contains several types of cells derived from the fetus itself, some of which display undifferentiated markers associated with the ESC lineage, with multipotency capabilities [20].

Amniotic fluid-derived stem cells expand without a feeder layer, retaining long telomeres, and have not been proved yet to form teratomas in vivo [21].

Umbilical Stem Cells

Umbilical stem cells (USCs) are located in the blood from the placenta and the umbilical cord.

They present several advantages, as cord blood represents a potentially unlimited source of stem cells. Their isolation procedure is a rather noninvasive technique, and they become easily simple to process and store, with a low risk of infection. USCs display low immunogenicity activity, mainly mediated through IL-10 and TGF-β pathways [22, 23].

Current Therapies

Wound Healing

The skin comprises the largest organ within our body. In its layers and hair follicles, niches of stem cell can be identified, becoming essential sources of skin homeostasis and injury regeneration.

In the epidermis, interfollicular stem cells are present, near the basal membrane. Within the hair follicle itself, follicular and neural crest stem cells can be isolated.

In the dermis, hypodermis, and adipose tissue, mesenchymal stem cells are derived from a mesodermal origin [24]. These stem cells are involved in regenerative processes, generating fibroblasts, producing growth factors, and recruiting host cells to coordinate an adequate tissue repair response [25].

These mechanisms contribute to appropriate skin repair after an injury, and restoration of tissue integrity. However, unsuccessful wound healing produces chronic skin damage that requires an appropriate resolution.

Presently, reconstructive surgery is the therapeutic option of choice to address this issue, with various results. Nonetheless, often extensive procedures are needed to treat even small lesions, and frequently enough skin defects are accompanied by local ischemia and muscle loss, leading to treatment failure and recurrence [26].

In this setting, cell therapy is becoming an increasingly useful therapeutic alternative to treat these lesions. Mesenchymal stem cells (MSCs)

are the most common type of stem cell used in regenerative medicine protocols to aid in wound healing processes. In the meantime, other groups of stem cells have been proposed in several studies, in particular bone marrow-derived mononuclear stem cells, umbilical cord-derived mesenchymal stem cells, peripheral blood mononuclear cells, and adipose-derived stem cells [27].

In different bone marrow preparations, Rodriguez Menocal et al. corroborated that cells from whole bone marrow (BM) displayed the most significant positive effects in wound healing, in comparison to BM-MSC [28].

Another interesting option is regenerative therapy based on adipose-derived stem cells (ADSCs). They are becoming the cell type of choice given their easiness to be harvested by liposuction and fat tissue digestion [29].

ADSCs have been shown throughout several studies to induce angiogenesis (Fig. 4), display immunomodulatory properties and have the ability to differentiate into multiple lineages. Regulating the inflammatory process by a paracrine release of specific cytokines and growth factors, ADSC therapy results in a reduction of chronic inflammation and promotes regenerative responses [30].

Kim et al. demonstrated that ADSC injected in wound sites possesses healing effects derived from collagen synthesis from dermal fibroblasts [31]. The vast majority of animal studies have used allograft or xenograft cells with positive outcomes [32].

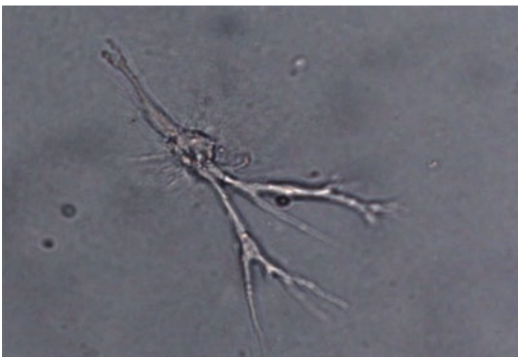

Fig. 4 Neoangiogenesis properties of ADSC, developing microtubules to form capillaries [25]

The number of repetitive sessions of cell therapy required to treat these defects depends mainly on the size and type of lesion, often requiring repeated cell injection in the target site [25].

ADSC's immunosuppressive properties can be harnessed in the treatment of aberrant scar defects. Yun et al. reported that subcutaneous ADSC therapy in a swine model of abnormal wound healing resulted in a significantly reduction in scar area and improvement flexibility and color [33].

Stem cell therapy in wound healing can be applied by three different approaches: directly through local injection, systemically by intravenous infusion, or in association with scaffolds or biomaterials.

Scaffolds can be developed with natural materials as well as synthetic or hybrid ones [34], and are seeded with particular stem cells to promote tissue regeneration and cell proliferation. 3D bioprinting and electrospinning are current techniques used in the design and implementation of skin scaffolds [35].

Rustad et al. demonstrated that MSC-seeded scaffolds promote significant tissue repair in comparison with MSCs delivered via direct injection [36]. Moreover, Lee et al. proved that tissue engineering methods applied in skin regeneration with the use of 3D bioprinting materials are successful strategies for unhealed wounds [37].

In regard to ADSC seeded biomaterials, Liu et al. compared three distinctive devices in a mice wound model, observing improved healing with the use of scaffolds, without significant differences between different types [38].

At the same time, Lin et al. evaluated the efficacy of single-layered ADSC sheets in comparison to multilayered sheets, finding better results with the latter [39].

However, there remains a lack of overall comparative studies between different biomaterials, resulting in an absence of a consensus on the delivery method of choice.

Another alternative therapy is the use of keratinocyte stem cells, which have shown to be therapeutic options in patients with great epithelial

damage, assisting in tissue repair by fibroblast proliferation and secretion of growth factors and cytokines. Autologous keratinocyte stem cells can be cultured and grown in vitro into sheets of stratified epithelium, and then be implanted into injured sites, demonstrating epidermal regeneration and angiogenesis [40].

Finally, the development of novel gene and molecular therapies as regenerative strategies in aesthetic medicine is becoming an interesting option to be taken into account in the near future. MicroRNAs (miRNAs) are noncoding RNAs that take part in the regulation of gene expression by messenger RNA repression. The advantage of MicroRNA-based therapies is that a single miRNA is able to target hundreds of genes. Therefore, miRNAs are deeply involved in many biological processes, and play a fundamental role in normal dermic development and skin homeostasis. They can also interact with distant target cells, regulating their communication with the microenvironment by being secreted into the circulation and transported via exosomes [41]. In a study, MSCs were proven to generate exosomes with specific miRNAs that assisted in wound healing processes [42, 43].

Adipose Tissue Engraftment

Adipose tissue constitutes a part of the connective tissue widely spread throughout the body, accounting for up to 4% of the adult human body mass [44]. It is responsible for regulating energy balance, and it also performs an endocrine function, producing and secreting numerous peptides, such as tumor necrosis factor alfa, leptin, and others [45].

The main advantage adipose tissue possesses is that it is a highly available source of adult stem cells, obtained through minimally invasive procedures.

Adipose-derived stem cells (ADSCs) can be easily isolated from lipoaspirated fat, and undergo further processing using filtration, mechanical or enzymatic separation, and centrifugation to obtain the stromal vascular fraction (SVF). SVF contains a mixed composition of the structural and stromal vascular cells, including adipocytes, endothelial progenitor cells, and adipose-derived stem cells [46]. Clinical application of SVF requires they be processed by a standardized methodology to obtain an adequate product.

There are several adipose processing models available presently. The Celution system is an adipose tissue processing technology that enables bedside or laboratory treatment of adipose cells using an automated sterile pathway system, generating high-quality ADSC, capable of being used in different settings [47].

Adipose-derived stem cells have a mesodermal origin, but it has been demonstrated to differentiate into cells from ectodermal, endodermal, and mesodermal lineages.

ADSCs from SVF can be cultured in the lab, generating homogeneous ADSC population, and they exhibit almost the same cell markers as bone marrow mononuclear stem cells [48].

Adipose cells were first successfully applied in humans in 1987 [49]. From then onward, much has been developed in ADSC therapy. ADSC offers potential opportunities in regenerative medicine, as they are able to differentiate into multiple lineages pathways (Fig. 5), harvested in minimally invasive procedures, and effectively implant in either autologous or allogenic hosts, without eliciting major immunogenicity response. Moreover, ADSC regulates inflammatory processes and promotes angiogenesis [50].

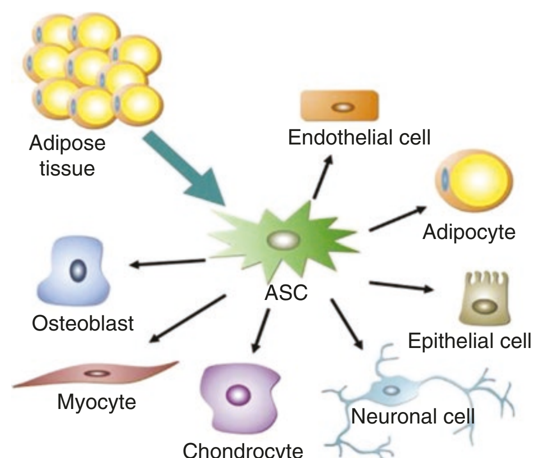

Fig. 5 Adipose stem cell differentiation lineages [49]

Human ADSC have the ability to engraft in many organs, indicating a potential to assist in tissue repair. They have been demonstrated to produce virtually all growth factors required for normal wound healing [51]. The expression of such factors increases significantly with preconditioning techniques, when ADSC are exposed to certain variables, such as hypoxia [52].

Adipose graft is an aesthetic technique employed in soft tissue filling, in the setting of various conditions as breast augmentation, reconstruction, and several lipodystrophies [53].

ADSCs are key elements in maintaining fat graft homeostasis and survival, promoting neoangiogenesis and reducing cell apoptosis [54].

In that manner, ADSC therapies have been identified to enhance fat grafting. Cell-assisted lipotransfer (CAL) is a method that combining concentrated ADSC with lipoaspirated fat produces ADSC-rich adipose grafts that enable significant improvements in tissue survival with a decreased rate in graft complications, such as fibrosis [55]. CAL has been used in different clinical settings, from breast augmentation to lipoatrophies [56, 57].

In other studies, ADSC therapy has been employed for scar formation. Kim et al. demonstrated 75% recovery in volume of scar with the use of immature ADSC [58].

A phase I clinical trial involving ADSC therapy in chronic fistulae in Crohn's disease patients showed complete healing in 75% of patients within 8 weeks of adipose cell inoculation, despite having previously failed with standard medical and surgical treatment [59].

Rodriguez et al. implanted human ADSC in dystrophin-deficient immunocompetent mice. Ninety percent of the treated animals displayed dystrophin in the myofibers at 6 months, not only in the inoculation site but in adjacent muscle, suggesting also cell migration [60].

ADSCs have been proven to possess hematopoietic functions, supporting reconstitution of endogenous hematopoiesis sources. This was demonstrated in a small animal study of lethally irradiated mice, where up to 40% of them could be salvaged with ASDC intraperitoneal injection, promoting the differentiation of hematopoietic stem cells [61].

Cartilage Formation

Cartilage defects comprise a complex therapeutic challenge, due to a lack of vascularization and limited regenerative capacity [62].

It has been reported in a number of studies the presence of stem cells from sinovial fluid collected during reconstructive surgery. They display multilineage potential differentiation, being able to give rise to osteoblasts, adipocytes, and chondrocytes, therefore regulating tissue repair [63].

Human cartilage progenitor cells show chondrogenic potential generating cartilage tissue, although its ability for cartilage development, even in vitro, is limited [64].

Vidal et al. compared bone marrow-MSCs versus adipose-MSCs evaluating their chondrogenic potential, resulting in BM-MSCs superiority in producing more suitable matrix components and better structural support [65].

Koga et al. demonstrated that MSC injection generated better outcomes with an adequate safety profile [66].

Stem cell therapy in this disease model has two potential routes of administration: percutaneous injections or through surgical arthroscopic placement.

Meanwhile, magnetic resonance imaging (MRI) has been extensively used to assess treatment effectiveness after cell implantation.

Centeno et al. reported 339 cases where MRI demonstrated cartilage and meniscus regenerative changes after MSC injection, with a long-term follow-up of 3 years with a suitable safety feature, without evidence of tumor formation [67].

In surgical implantation of culture-expanded bone marrow-derived MSCs, Wakitani et al. showed in 45 patients with a long-term follow-up period of 11 years adequate safety profile with improvement in tissue functionality [68].

Haleem and colleagues studied cultured BM-MSCs implanted in cartilage defect sites, displaying healing cartilage tissue at 1-year follow-up [69].

In 2013, Peeters et al. published a meta-analysis of 8 studies with 844 patients treated

with MSCs injection in peripheral joints, demonstrating better outcomes than single hyaluronic acid injections [70].

In addition to cell implantation, several biomaterials and 3D constructs have been evaluated to treat cartilage degeneration. These scaffolds are engineered either from natural protein models or synthetic polymers, such as polyethylene glycol.

Shafiee et al. evaluate MSC therapy with synthetic nanomaterials, showing adequate MSC support and differentiation into chondrogenic lineages, with overall improvement in healed cartilage [71].

Bone Reconstruction

Bone tissue displays a natural physiological regenerative capacity. However, large bone defects and 5–10% of all bone fractures are associated with impaired healing and require bone grafting [72].

After bone injury has been established, inflammation factors activate regenerative cells, in particular bone marrow mesenchymal stem cells, skeletal stem cells, and endothelial progenitor cells, which coordinate specific mechanisms that ultimate lead to bone regeneration [73].

Stem cell therapy has rapidly evolved from basic research into preclinical and clinical studies, with the primary focus on bone regeneration. Tissue engineering techniques combine the association of stem cells and biomaterials to generate constructs implanted in vivo in the injured site.

Current techniques have displayed positive outcomes applied to craniofacial lesions. ADSC have been employed in bone regenerative therapy, observing complete ossification of calvarian defects at three months [74].

Regarding mandible defects, mesenchymal cell therapy has been employed in different study protocols, obtaining significant improvement in functional and aesthetic results [75–77].

Antiaging Therapy

During normal skin aging, an increased activity of metalloproteinase enzymes can be observed in order to degrade extracellular matrix components. At the same time, it is associated with a decline in collagen synthesis [78]. Histological studies have shown that fibroblasts are key cells in maintaining skin homeostasis, producing several cytokines and extracellular matrix peptides that restore tissue integrity [79, 80].

As the first visible signs of skin aging appear, usually near the third decade of life, fibroblast activity tends to decrease along with collagen and elastin synthesis. There can be found a reduction in appropriate vascularization as a result of abnormal vascular integrity, and melanin production declines significantly [81].

There are numerous external factors that can account for accelerated skin aging besides inherited genetic markers, such as the exposure to free radicals and UVA radiation, both presulting in DNA mutation and alteration of mitochondrial genome, lipid peroxidation and cell-membrane degradation [82].

Cell therapy in this condition has been established in order to stop aging mechanisms and promote regenerative effects in damaged skin.

In animal studies, ADSC implant in aging skin has been shown to increase dermal thickness and collagen production, reducing radiation-induced wrinkles (Fig. 6). An enhancement of angiogenesis and fibroblast activation has been proposed as the main physiological events behind the decrease in aging signs [31].

A different approach employed in antiaging therapies has been the use of platelet-rich plasma (PRP). PRP is defined as an autologous concentration of platelets in a small volume of plasma [83].

After platelet activation by coagulation factors, numerous growth factors are secreted, including transforming growth factor, vascular endothelial growth factor, and platelet-derived growth factor [84]. PRP has been shown to induce anti-inflammatory effects and analgesic properties [85]. Following growth factors activation, an

improvement in cell proliferation and differentiation can be observed, in association with extracellular matrix upregulation [86].

Furthermore, hyaluronic acid production is enhanced by PRP, leading to overall skin regeneration [87].

In clinical trials, PRP was reported to induce collagen type I synthesis and fibroblast proliferation. At the same time, collagenases activity is improved, thus removing damaged collagen fibers and stimulating new fibers' turnover [88].

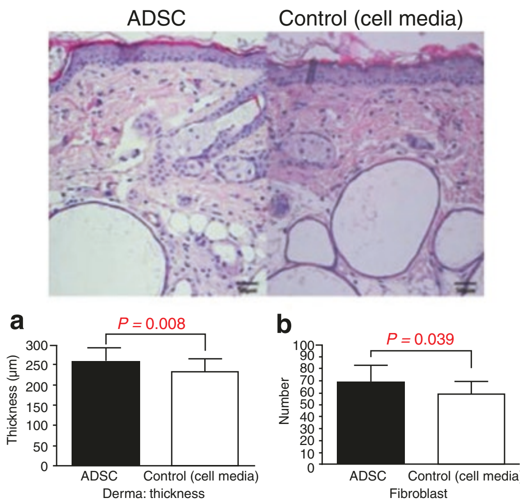

Fig. 6 Adipose-derived stem cell compared with control group in terms of dermal thickness and fibroblast proliferation [82]

Inflammatory Diseases

Inflammatory skin diseases have increased significantly their prevalence in the past decades due to modern living conditions.

They display a wide variety of symptoms that mainly affect patients' quality of life and disrupt normal skin barrier, leading to a higher incidence of dermal infections.

Current treatment alternatives for patients refractory to steroid therapy are limited. Biological agents have recently arised as an adequate strategy in patients with severe symptoms, but high costs and safety concerns have also restricted their use in clinical practice.

Regenerative therapy through stem cell administration is a new field of therapeutic possibilities, given their known anti-inflammatory properties (Fig. 7).

Mesenchymal stem cells are the primary source of cells employed in clinical protocols in inflammatory diseases, proving a suitable safety profile [89]. Intravenous injection of allogenic MSC has been shown to exert positive results in

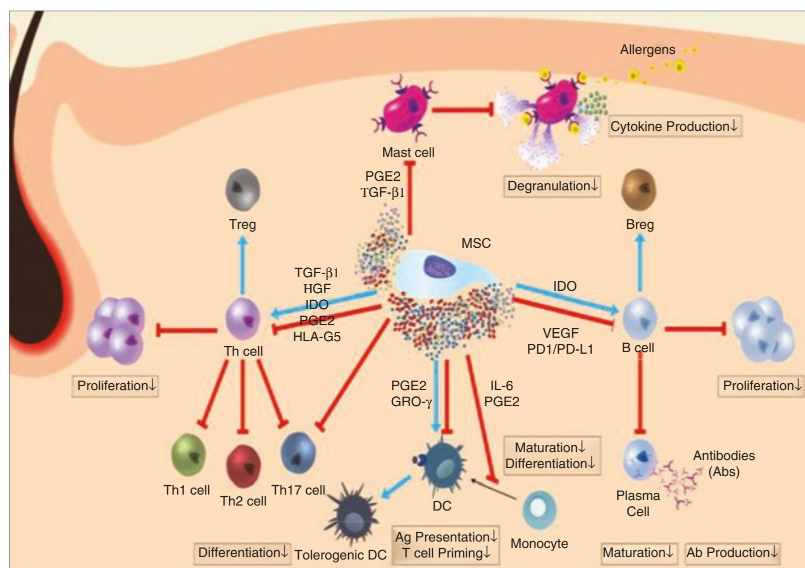

Fig. 7 MSC immunomodulating pathways [90]. Red line: inhibitory effect. Blue line: stimulatory effect

skin symptoms in the setting of several inflammatory conditions [90, 91].

Graft versus host disease (GvHD) is a devastating condition following hematopoietic stem cell transplantation, associated with poor clinical outcomes with standard therapy. MSC administration in GvHD animal models has been proven to inhibit T lymphocytes differentiation, delaying symptoms onset and improving graft survival [92]. A phase II clinical trial in 55 patients with severe acute GvHD demonstrated significant improvement after MSC injection [89]. In the chronic case of GvHD, allogenic MSC transplantation has shown recovery in dermatological symptoms by restoring Th1/Th2 balance [93].

In SLE, MSC therapy in addition to current treatment has displayed significant clinical improvement in both renal and skin involvement [94].

Psoriasis is becoming one of the most prevalent chronic inflammatory diseases, with severe cases often refractory to medical and biological agents. MSC immunomodulatory effects exhibit beneficial results in such patients [95], although further studies are required in order to properly evaluate their impact.

Finally, therapeutic properties of MSC therapy in severe atopic dermatitis patients have shown positive results, in a dose-dependent manner [96].

Conclusions

The exponential growth in the development of stem cell-based therapies has led to an increasing number of experimental and clinical breakthroughs, becoming a promising therapeutic modality in aesthetic medicine.

Despite the success observed in specific areas of regenerative medicine, there still remain several challenges that need to be properly addressed before the potential reach of this field can be broadened into new horizons.

There is still much debate over the potential physiological mechanisms behind cell therapy and tissue enhancement.

A rigorous control of cell differentiation is required to ensure the appropriate generation of a specific cell type, not only for its efficacy but also for its safety profile, avoiding abnormal cell proliferation and tumor growth.

The identification of an abundant and attainable source of stem cell is essential for maintaining a suitable cell supply. Moreover, the design of an adequate microenvironment by paracrine function or through tissue engineering will likely promote optimal regenerative responses.

Stem cell therapy has been proven to display positive results in several pathologies in aesthetic medicine, from skin regeneration to wound healing, adipose graft, and tissue reconstruction. Importantly, the trials evaluating stem cell efficacy have shown minimal complications with this therapy, although further research is necessary in order to advance into the translational field.

The discovery of iPS cells has enabled the production of patient-specific pluripotent stem cell lines that can be used without major immunosuppression and rejection concerns, but its clinical competence is yet to be achieved. Human tissue culture models of disease will allow pharmaceutical and treatment testing with a more suitable response than current animal models.

Additionally, an appropriate cell delivery system will be required in order to improve and guarantee the correct cell and molecule supply into target zones.

As the field of regenerative medicine goes forward into the future, more complex structures and biomaterials will emerge, for which new technological approaches must be adopted to ensure acceptable tissue response, sustainability, and, ultimately, appropriate application into the clinical setting.

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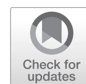
Part VII

Regulation and Conclusions



Regulation

Herrero Jone and Castro Begoña

Advanced therapy medicinal products (ATMP) are new medical products based on genes (gene therapy), cells (cell therapy), and tissues (tissue engineering) and include products with autologous, allogenic, and xenogenic origin. They offer groundbreaking new opportunities for the treatment of disease and injury. Diseases like cancer, spinal cord injury, stroke, critical limb ischemia, and multiple sclerosis, among others, are being therapeutic targets in clinical trials with advance therapy medicines. From a regulatory point of view it is necessary to address the specific aspects of these medicines regarding manufacturing, controls, clinical research, assessment, and authorization.

In Europe, these products must complain the Regulation (EC) No 1394/2007 of the Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004.

EU pharmaceutical legislation covers the whole life cycle of medicinal products, from manufacture to clinical trials, marketing authorization, pharmacovigilance, and patient information through a comprehensive and complex set of rules.

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Somatic cell therapy medicinal products are considered biological medicinal products that contain or consist of cells or tissues that have been subjected to *substantial manipulation* so that biological characteristics, physiological functions, or structural properties, relevant for the intended clinical use, have been altered, or of cells or tissues that are *not intended to be used for the same essential function(s) in the recipient and the donor*. They are presented as possessing properties to treat, prevent, or diagnose a disease through the pharmacological, immunological, or metabolic action of its cells or tissues.

Considering Annex I of the Regulation (EC) No 1394/2007, the following processes are NOT substantial manipulations: cutting, grinding, shaping, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, cell separation, concentration or purification, filtering, lyophilization, freezing, cryopreservation, and vitrification. On the other hand, cell expansion, cell culture, cell activation, or the combination of cells with biomaterials is considered as substantial manipulations, and products that include some of these processes are considered a medicinal product.

Products that are not considered ATMP must also comply with the European regulation of tissues and cells. The Tissues and Cells Directives are set out to establish a harmonized approach to the regulation of tissues and cells across Europe. The Directives set a benchmark for the standards

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that must be met when carrying out any activity involving tissues and cells for human application (patient treatment). The Directives also require the establishment of systems to ensure that all tissues and cells used in human application are traceable from donor to recipient. The regulation is made up of three Directives, the parent Directive (2004/23/EC) which provides the framework legislation and two technical directives (2006/17/EC and 2006/86/EC), which provide the detailed requirements for tissue and cell products. Some of the aspects of these Directives are also applicable to ATMP.

Development of a new advance therapy medicinal product is a long and costly process with the aim to demonstrate that the new medicine complies with quality, safety, and efficacy requirements for its commercialization. It includes several phases from basic research and preclinical development to clinical development and registry that are mandatory for medicines' authorization and commercialization.

Basic research and preclinical study phases must provide enough data about product toxicity, safety, and biological activity. With respect to clinical phases, in case of ATMPs, they can directly start at phase I/II to demonstrate safety and first endpoints for efficacy determination. Depending on clinical indication, clinical phases of this type of products do not necessarily include as many patients as conventional medicinal products. Recruitments of 10–20 patients are common in Phase I/II of many ATMP clinical trials as it can be verified in *clinicaltrials.gov* database.

Before starting the clinical phase, a clinical trial application (CTA) must be performed by the product sponsor. Each European country has its own regulatory authority that evaluates the submitted dossier. For clinical trials that are conducted in more than one European country, a harmonized voluntary procedure exists that allows submitting a unique application to the authorities of the relevant countries. In addition, to start the clinical trial, it is necessary to obtain the specific authorization by the country regulatory agency, the favorable opinion from the ethical committee of the implicated hospitals and the accordance with clinical centers direction.

The CTA consist of a medicinal product dossier that must be presented to the country regulatory authority as it is indicated in the Annex I of Regulation 536/2014. This product dossier must include the following documents:

- Cover letter.
- EU application form.
- Protocol: document that shall describe the objective, design, methodology, statistical considerations, purpose, and organization of the clinical trial.
- Investigator's Brochure (IB): The purpose of this document is to provide the investigators and others involved in the clinical trial with information to facilitate their understanding of the rationale for and their compliance with key features of the protocol such as the dose, dose frequency/interval, method of administration, and safety monitoring procedures.
- Documentation relating to compliance with good manufacturing practice (GMP) for the investigational medicinal product.
- Investigational Medicinal Product Dossier (IMPD): The IMPD is the document that gives information on the quality of any investigational medicinal product, the manufacture and control of the investigational medicinal product, and data from nonclinical studies and from its clinical use.
- Content of the labeling of the investigational medicinal products.
- Recruitment arrangements.
- Subject information, informed consent form, and informed consent procedure.
- Suitability of the investigator and facilities.
- Proof of insurance cover or indemnification.
- Financial and other arrangements.
- Proof of payment of fee.
- Proof that data will be processed in compliance with union law in data protection.

The legal framework also requires ATMP to be manufactured in facilities that comply with good manufacture practices (GMP) certification and that has been previously authorized by regulatory agency.

The European Medicines Agency (EMA) gives **scientific support** to developers concerning the ATMPs Regulation on Advanced Therapies. The Committee for Advanced Therapies (CAT) is composed of experts in the field of Advanced Therapy Medicinal Products (ATMPs). In this way, the CAT plays a key role in early contacts with developers of ATMPs and is responsible for preparing a draft opinion on the quality, safety, and efficacy of each ATMP for final approval by the CHMP. It provides the expertise that is needed to evaluate advanced therapy medicinal products. Based on the CAT opinion, the CHMP adopts an opinion recommending or not the authorization of the medicine by the European Commission. The European Commission makes its final decision on the basis of the CHMP opinion.

Once the investigational phases of the medicinal product are over, before the product is manufactured, it is necessary to apply for authorization. All advanced therapy medicinal products are **authorized through the centralized procedure following an application to the EMA. The centralized procedure is laid down in Regulation (EC) No 726/2004 which introduces a single scientific assessment procedure of the highest standard for the medicinal products falling within scope. The centralized procedure results in a single marketing authorization that is valid in all Member States and offers the benefit of direct access to the EU market.**

Under the centralized procedure, the company submits its application directly to the EMA. After evaluation, the committee gives a recommendation on whether the medicinal product should be authorized or not. This opinion is then forwarded to the European Commission, which has the final say in the granting of marketing authorizations in the EU. After considering the opinion, the Commission can issue a legally binding EU-wide marketing authorization. Once it is granted, the marketing authorization holder can begin to market the medicine in the EU.

Finally, all medicinal products in the EU are subjected to a strict testing and assessment of their quality, efficacy, and safety before being authorized. Once placed on the market, they continue to be monitored to assure that any aspect which could impact the safety profile of a medicine is detected and assessed and that necessary measures are taken. This monitoring is called pharmacovigilance and is considered a key public health function. Pharmacovigilance is the process and science of monitoring the safety of medicines and taking action to reduce the risks and increase the benefits of medicines. The legal framework of pharmacovigilance for medicines marketed within the EU is provided for in Regulation (EC) No 726/2004 with respect to centrally authorized medicinal products as ATMPs.

Further Reading

Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use.

Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating in the implantation of good clinical practice in the conduct of clinical trials on medicinal products for human use.

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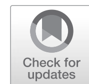

General Conclusions: The Current Status of the Regenerative Medicine in the Aesthetics Applications

Joan Fontdevila and Hernán Pinto

The goal of aesthetic medicine and surgery is to restore the features of the youth with natural appearance and texture, and long lasting with the minimal risk of secondary effects. In the search of methods and materials to achieve these objectives, have been great advances as the application of hyaluronic acid to treat facial folds and wrinkles, or great disappointments as the limited biocompatibility of the permanent injectable fillers. Overall, autologous or materials alike those naturally contained in the human body (collagen, hyaluronic acid, hydroxyapatite, etc.) are what have provided the best results. With the blooming of new biomedical technologies based on the regenerative properties of cells (stem cells or stromal vascular fraction) and humoral factors (cytokines, growth factors, or platelet-rich plasma), and focused on providing new ways of treatment for pathologies without effective options (e.g., multiple sclerosis, Crohn disease, myocardial infarction, Parkinson), expanding the indications to the aesthetic procedures seems very attractive both for the physicians and for the patients [1–3]. This is the point where the controversy starts: indications for diseases are still experimental and most of them are restricted to trials, but for aesthetics the offer of them is growing with a doubtful scientific base and with an unknown effectiveness [1, 2, 4] usually under the promise of faster recovery, safety, and improved results regarding conventional treatments.

The cost of the treatments based in cells is highly expensive because sophisticated technology must be used to obtain cellular products. This has made many doctors around the world, with the aim to overprice their treatments, to claim that they perform "cell-based" aesthetic procedures, when in fact they only use fatty tissue naturally provided with cells with regenerative properties [3, 5–7]. The excessive hype of many doctors about the properties of these new kinds of treatments, with the attribution of "magic" properties to the cells and growth factors, can lead to patient disappointment when they do not achieve much more different results than those provided by well-known conventional treatments [7].

PRP and products derived from autologous platelets and fibrin are cheaper to obtain than cells, and this has made to grow quickly the offer of treatments, but also there are concerns about its effectiveness and whether it is an advantage or not to use it [8, 9].

On the other hand, fat grafting has been accepted by the medical community as a reliable and effective treatment, which can provide in

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some degree the regenerative effects attributed to the more sophisticated treatments mentioned before. Again, enrichment of the fatty tissue with one of these regenerative agents, cells, and/or growth factors gives a role to these in the lipofilling procedures of unknown relevance in the final result. Even if we can find many reports about the success of the search of new technologies that provide solutions to improve the regenerative effects of the fat grafts [10, 11], to increase the graft's ability to overcome ischemia stress and the lack of nutrients, or to achieve the best conditions to integrate the graft in the receiving tissue, clinical evidence of their effectiveness is still very weak [1, 12].

Regenerative treatments available nowadays are based on elements of autologous sources, which reduce the concerns about compatibility and adverse events [13, 14]. However, in the search of patients for premium expensive treatments, and with the aid of loose regulation in stem cell use in some countries, allogenic cells or autologous cells are being used in unproven indications with the risk of significant adverse events [7, 15]. Even in countries with leading regulatory agencies, some professionals make use of imaginative tricks to bypass regulations and advertise treatments with cells or apply them in unproven indications, sometimes assuming risks of unknown adverse effects [2, 5].

Those practicing regularly cosmetic medical procedures may be tempted to use these new technologies to treat serious degenerative illnesses, like those previously mentioned. Given that these pathologies do not have a curative treatment, some may believe that these can expand indications from less sensible targets as dermis or subcutaneous tissue to pathologies in which they are unfamiliar. We do not recommend doing this, despite some desperate patients can call your office door because they have heard about the availability of this technology in many cosmetic medicine facilities.

Tissue scaffolds are another promising technology able to provide regenerative solutions in the field of aesthetics. Scaffolds in the field of biology and medicine are structures made of materials (biological or synthetic) able to host cells providing structural and biochemical support, like what the extracellular matrix does. Scaffolds can be "seeded" with stem cells, and these are driven to proliferation by signaling molecules. Their use is increasing in many fields, especially in bone [16] and skin regeneration [17], but nowadays there are no scaffolds developed to be used with aesthetic purposes.

Stem cells, growth factors, and tissue scaffolds have shown in experimental environment that they can be the future main actors of the regenerative effects useful for aesthetic purposes [18, 19]. However, at the present time in our hospitals and offices, out of the laboratories, they still have very limited applications, and this issue has to be exposed honestly to the patients to decide freely if they would opt for them or otherwise prefer a traditional one [3, 5, 20].

Although advances in the knowledge of the potential of regenerative therapies make us imagine the future role of them in aesthetic medicine, standardized protocols for preparation and application are yet to be established. We should be watchful in the following years for the advances in this promising field.

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