

# Important parameters in plasma jets for the production of RONS in liquids for plasma medicine: A brief review

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**Abstract** Reactive oxygen and nitrogen species (RONS) are among the key factors in plasma medicine. They are generated by atmospheric plasmas in biological fluids, living tissues and in a variety of liquids. This ability of plasmas to create a delicate mix of RONS in liquids has been used to design remote or indirect treatments for oncological therapy by treating biological fluids by plasmas and putting them in contact with the tumour. Documented effects include selective cancer cell toxicity, even though the exact mechanisms involved are still under investigation. However, the “right” dose for suitable therapeutical activity is crucial and still under debate. The wide variety of plasma sources hampers comparisons. This review focuses on atmospheric pressure plasma jets as the most studied plasma devices in plasma medicine and compiles the conditions employed to generate RONS in relevant liquids and the concentration ranges obtained. The concentrations of  $\text{H}_2\text{O}_2$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and short-lived oxygen species are compared critically to provide a useful overview for the reader.

**Keywords** atmospheric plasma jets, liquids, ROS, RNS, plasma-dose

## 1 Introduction

The interaction of cold atmospheric pressure plasmas with liquids has gained increasing attention due to the importance of biological effects of plasmas themselves as well as plasma treated liquids. The main kinds of

atmospheric plasmas employed in the treatment of liquids for biological/medical applications without direct contact with liquids are atmospheric pressure plasma jets (APPJ), followed in the distance by dielectric barrier discharges. This paper will focus only on APPJ, which will be described succinctly in the following paragraphs.

Due to the emergence of the plasma medical field, and especially of plasma oncology, many recent reviews have lately focused on the biological action of plasmas. These have in general gone over the lethal and selective effects of plasmas on cancer cells, and on the wide range of cancers studied up to now. Reactive oxygen and nitrogen species (RONS) have been highlighted as essential players in the biological effects of plasmas [1,2]. A large number of works studying plasma activated media (PAM) or plasma-activated liquids have put forward the effects of indirect treatment of liquids for oncological therapies instead of direct treatment of the biological tissues by APPJ. One of the key issues under debate is the right “dose” of RONS for the therapeutical action of plasmas. However, comparison among the different works dealing with the biological action of plasmas is hard due to the absence of standard protocols, with differences in plasma source configuration, working conditions and experimental settings such as liquid volume, surface area or even liquid temperature (which is often disregarded or not mentioned in the works), which then alter the production of RONS.

The aim of present work is to provide a summary of the main reactive species produced by plasmas in close vicinity to liquids with biological relevance and to critically review on the efficacy of different plasma sources and the different reactivity of the liquid media employed in this field. The final purpose is to provide the readers with tools for the selection of plasma source and liquid media for their final application, depending on the desired dosage.

## 2 Types of plasma devices without direct electrical contact with liquids

The main atmospheric plasma devices employed in medical applications are APPJ [3,4]. Their key advantages are easy (possibly hand-held) application for medical treatments, local delivery of plasma plume and active species to the treated tissue/organ and no need of a counter-electrode. APPJ are commonly operated as dielectric barrier discharges with a central needle electrode and one outer ring electrode, or single electrode with capacitive coupling [5,6] (Fig. 1). Another usual configuration comprises two cylindrical electrodes about the same diameter as the dielectric tube that are inserted in the tube with a certain separation and are each composed of a thin copper ring attached to a centrally perforated dielectric disc. To ignite the plasma, a high voltage in the 1–10 kV range can be used in the form of repetitive, microseconds wide pulses and applied between the two electrodes. Typically, the generated plasma flows through a dielectric tube with diameters from 0.8 to 4 mm. Its flow rate can be varied from 0.05 to 8 L · min<sup>-1</sup>. The plasma jet source can generate stable atmospheric plasma in various gases such as helium, argon, and their mixtures with oxygen, nitrogen, carbon dioxide, air, etc. When the discharge is ignited, it launches a plasma plume that can reach up to 5 cm into the ambient room air.

Another group of APPJs use radio or microwave frequency discharges. A particular kind of APPJ which deserves particular attention is the commercial kINPen developed at INP Greifswald. kINPen is a radio frequency (1 MHz) atmospheric pressure argon plasma jet (Fig. 1(a)) [7]. This plasma jet is a pin type dielectric barrier plasma jet with an outer, grounded ring electrode. The diameter of the nozzle exit is 1.6 mm. The plasma jet is operated with argon. The operating frequency is in the order of 1 MHz and voltage amplitude is in the range of 2–6 kV.

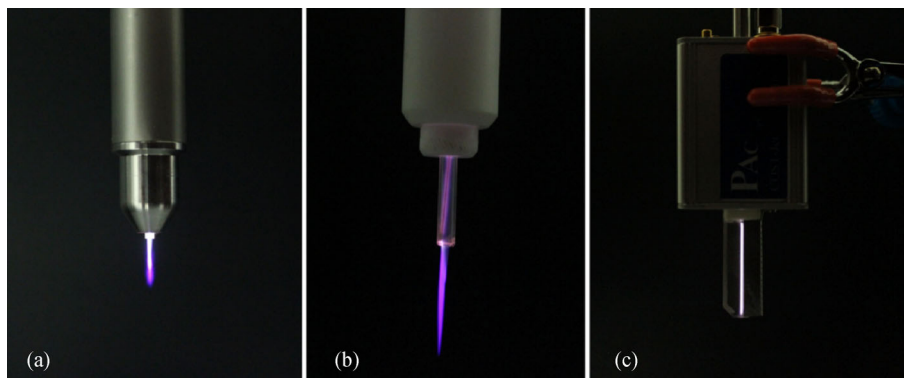
In the last years a new device was developed within COST action MP1101 with the aim of providing a reference plasma source for biomedical applications. This

is known as the COST reference microplasma jet (Fig. 1(c)). The micro plasma jet is capacitively coupled at 13.56 MHz RF-discharge with symmetric, co-planar, stainless steel electrodes enclosed by two quartz panes and a discharge volume of 1 mm × 1 mm × 30 mm [8]. The plasma gas phase of this jet generated in helium, has been extensively characterized in [9].

## 3 Chemical effects of plasma in liquids

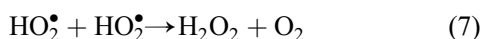
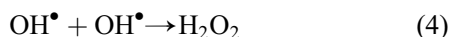
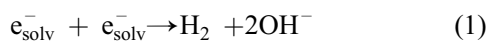
Plasma-liquid interactions have gained tremendous attention in the last few years [10]. The understanding of plasma-liquid interactions and, in particular, the transfer of reactivity from the gas to the liquid phase has been highlighted as being of prime importance for biological effects. Such interaction is a combination of physical (UV, heat and electromagnetic field) and chemical factors (which include a wide number of reactive species that are generated in the gas phase of cold plasma and will be discussed further here).

Biological liquids (i.e., blood plasma, cell culture media) are essential components in plasma treatment of biological entities (cells, wounds, tumours). Moreover, the creation of PAM has been an important new development that has shown similar biological effects to direct plasma treatment, so it has interesting therapeutic potential, and opens a versatile approach for many therapies [11,12]. It has been shown that the depth of penetration of the cold plasma can be limited to 60 μm (referred to induction of apoptosis in tumor cells) [13], so direct treatment of tissues with plasmas requires access to the site of action (i.e., surgery to expose a tumor to the action of plasma, or even use of plasma after resection of the tumor to reach remaining tumor cells). Contrarily, indirect treatment through PAM has the great advantage of allowing to target different regions of the body/tumor by injection of the PAM within the therapeutical site. This is thus a minimally invasive approach which can have many advantages for the patients. Moreover, it is technically easy to treat liquids with plasmas to obtain PAM.

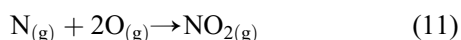
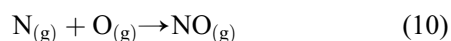
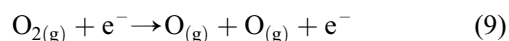
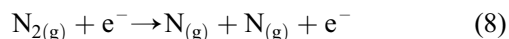


**Fig. 1** Representative picture of three examples of plasma jets of interest in plasma medicine: (a) atmospheric pressure argon plasma jet (kINPen), (b) plasma needle, and (c) microplasma COST

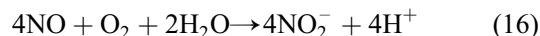
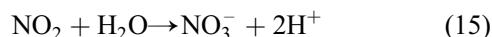
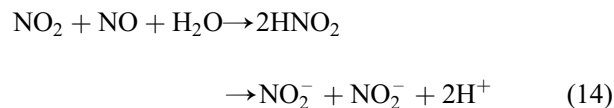
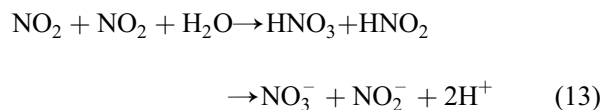
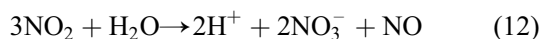
RONs are known to act in a wide variety of intracellular and intercellular processes. RONs are involved in key signaling processes within the cell and hydrogen peroxide is thought to be one of the most important key players in the biological effects of plasma generated RONs. Also, nitric oxide, which is a key signaling molecule in biological processes and its presence can lead to important biochemical modifications [14]. It has been shown that RONs can be created in liquids by atmospheric plasma treatment. In fact, plasma treatment of aqueous solutions leads to their activation and non-equilibrium dissociation of water molecules. This results in the formation of short-lived species (around a few nanoseconds), such as  $H^\bullet$  atoms,  $OH^\bullet$  radicals, and hydrated (solvated) electrons ( $e_{\text{solv}}^-$ ). Very quick reactions between these species lead to the formation of transitory and more stable species (lifetimes longer than 1 s) such as  $O_3$ ,  $H_2$ ,  $O_2$ ,  $H_2O_2$  etc. Some of the main reactions [15], mainly proceeding from water molecule dissociation, are described as follows (Eqs. (1–7)):



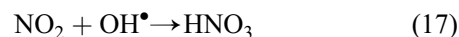
In the presence of air, reactive nitrogen species (RNS) ( $NO^\bullet$ ,  $NO_2^-$ ,  $NO_3^-$ ,  $ONOO^\bullet$ ) are also formed in liquid medium. Nitrogen oxides can be formed from well-known gas phase (g) reactions of dissociated nitrogen and oxygen (Eqs. (8–11)):



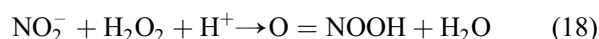
These nitrogen oxides subsequently react in water forming acids (Eqs. (12–16)), which affect the conductivity and pH of the plasma-treated liquids and based on pH dissociate to nitrite ( $NO_2^-$ ) and nitrate ( $NO_3^-$ ) ions:



The NO can be oxidized to  $NO_2$  in the case that air is used as working gas or mixed with the plasma effluent which might depend on the gas flow, thereby increasing nitrate concentration. Reactions of hydroxyl radicals with  $NO_2$  lead to the formation of nitric acid in solutions (Eq. (17)):



Subsequently, reactions between the different species formed in solution may lead to the formation of peroxyntitrous acid (in acidified conditions in non-buffering solutions, Eq. (18),  $pK_a = 6.8$ ) or peroxyntitrite ions (in neutral or basic conditions, e.g., phosphate buffered saline (PBS), Eq. (19)) [16]. Peroxyntitrites/ peroxyntitrous acid is another very important species in cell biology:



With views on biomedical applications, various water-based liquids (aqueous solutions) are of interest. For *in vitro* biological testing (assays with cells or tissues), the most commonly employed liquids are cell culture media. Cell culture media are aqueous solutions which contain a number of salts, proteins, glucose, amino acids, vitamins, etc., with up to 30 components. Depending on the cell lines studied, recipes for cell culture media can vary in composition (i.e., glucose concentration, growth factors, and other nutrients), which poses a challenge for plasma medicine researchers dealing with the reactivity of liquids. Plasma may react with the components of the medium (via reactive species) transforming them into their oxidized state [14].

For *in vivo* therapies (with animals and aimed at humans in the last instance) most usually either water or saline solutions such as phosphate buffer saline, Ringer's saline or Ringer's lactate, are employed. The effects of atmospheric plasmas on the reactivity of these species have been evaluated and will be discussed further in this work. Given the interest of these saline solutions for *in vitro* studies, they are often put in contact with cells following plasma treatment, and either diluted by adding them to cell culture

media or put in contact with the cell cultures for a brief period of time (up to usually 3 h maximum) and then replaced by the cell culture media.

### 3.1 Detection of reactive species in liquid media

A major challenge in plasma medicine remains the control of plasma-induced liquid-phase chemistry. Plasma generated reactive species transferred into the liquid or formed consequently in the liquid have been measured with an emphasis on long-lived species for obvious experimental difficulties to measure short-lived ones. Currently, there are different methods for determination of reactive species in liquids, and Bruggeman et al. [17] have reviewed them in detail. For the purpose of this work we can distinguish between “*in situ*” measurements of RONS and “*ex situ*” or indirect methods for the detection of RONS in liquids. The “*in situ*” measurements of short-lived species are based on the direct detection of ultraviolet absorption spectra. For instance, Kim and coworkers investigated hydroxyl radical density in cell culture media—Dulbecco’s Modified Eagle’s Medium (DMEM) and in PBS by ultraviolet absorption spectroscopy [18]. Rumbach et al. utilized total internal reflection absorption spectroscopy for a direct detection of the plasma injected electrons in solvation process (the solution was used as an anode because the plasma was formed with a stainless steel capillary suspended 1–2 mm above the liquid surface. A glowlike discharge was initiated at the solution surface by applying high voltage between the suspended capillary and the solution) [19]. There are also “*ex situ*” methods of short-lived reactive species detection which employ scavengers of reactive oxygen species (ROS).

Indirect methods are often easier to implement and do not require of very sophisticated equipment, so they have been widely employed in literature. To detect reactive species by using indirect methods, it is necessary to use a scavenger/chemical probe to entrap the reactive species of interest. The main techniques employed are absorbance spectroscopy, high performance liquid chromatography, ion chromatography, kit assays based on strips (with much lower precision), and fluorescence spectroscopy for detection of the products of fast reaction with reactive species. Electron paramagnetic resonance spin-trapping has been recognized as a technique capable to monitor radicals with high sensitivity and low detection limits [20,21], which allows to use one compound for detection of  $H^{\bullet}$  atoms and  $OH^{\bullet}$  and oxygen radicals.

UV-vis spectrophotometry is an appropriate method for detection of RONS in aqueous solutions. For example, the amount of hydrogen peroxide is usually quantified by a colorimetric method using titanium oxysulfate or less often sodium orthovanadate as chemical probes [22,23]. For detection of nitrite/nitrate ions a colorimetric method using Griess reagent and nitrite reductase is often employed [24].

More details on specific methods for measuring RONS can be found in the review of Bruggeman et al. [17].

As mentioned earlier, RONS have been described as some of the key players for the therapeutical action of plasma treated liquids. In particular, the cell-selective effects of plasmas and plasma-treated liquids have been attributed to them in different works [25,26]. One issue of debate in the scientific community is about the therapeutical “dose” of these RONS.

The issue is not easy to deal with since the proportion of the different RONS varies in the cocktail generated by plasma treatment as function of the different treatment conditions described. As shown in [27] the plasma gaseous products—and therefore the species generated in liquids—strongly depend on the discharge regime, its deposited power and gas flow conditions. The gaseous products then determine the chemical properties of the PAW and the dominant aqueous RONS.

Having an indication of the amount of RONS generated by plasmas in liquids could be a useful tool for researchers investigating this therapeutical “dose”. Figure 2 summarizes the main RONS generated in liquid phases by different APPJ devices. As compiled in the figure, a wide variety of liquid media have been studied, together with different detection methods for RONS quantification.

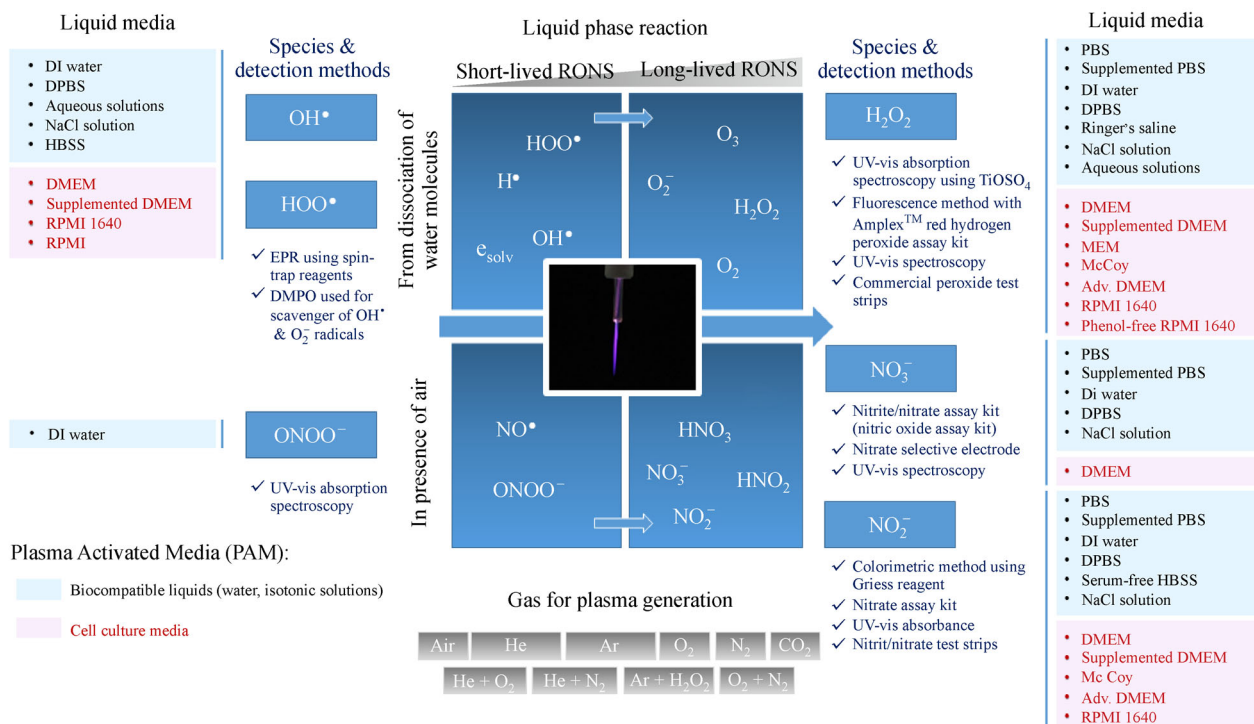
In the following sections some of the main RONS generated by atmospheric plasmas in liquids of interest in plasma medicine are compiled in different tables (Tables 1–4). Particular attention is put on the concentrations generated as a function of the specific liquid and the conditions of the plasma treatment.

#### 3.1.1 Long-lived RONS

##### 3.1.1.1 Hydrogen peroxide ( $H_2O_2$ )

Hydrogen peroxide is a very relevant species in plasma medicine, as it can be generated by APPJ in liquids (Eqs. (4,7)), and it is also present already in cells. As such  $H_2O_2$  is one of the species which has been mostly characterized in APPJ treated liquids. Table 1 summarizes the concentrations described in the literature with detail on the conditions of the plasma treatment and the particular liquid employed. The technique for measuring each RONS is mentioned as it can be of influence on the values obtained.

In the case of kINPen, as it is a commercial device with CE marking, the conditions such as plasma gas, applied voltage, frequency and in most cases, gas flow rate are fixed. Ar was used as working gas with a flow rate of  $3 L \cdot min^{-1}$  (in most cases), applied voltage of 2–6 kV and frequency 1 MHz. As shown in Table 1, the amount of  $H_2O_2$  detected in the different media displays a wide range of concentrations. It is difficult to compare the measured concentration of hydrogen peroxide due to the many different types of plasma jets and wide range of solutions



**Fig. 2** Overview of the short- and long-lived RONS generated in liquid media by APPJ with detail of the liquid media in which each species has been quantified in literature and the corresponding detection methods employed therein

of biological interest that are used. Many parameters in the experimental setup (for example, the nature of the gas, the gas flow, the applied voltage, the distance between the plasma and the solution, the composition of the solution, the volume of the solution, etc.) play a role in the amount of plasma-induced RONS in solution. Oh et al. [43] found that H<sub>2</sub>O<sub>2</sub> is the major RONS formed at distances of up to 30 mm in water. At such close treatment distances, the rate of water evaporation from the liquid is high, creating a more humid environment. Other authors have reported increasing H<sub>2</sub>O<sub>2</sub> with increasing specific energy of the plasma deposited in the liquid, which supports H<sub>2</sub>O evaporation, dissociation and OH• formation and its subsequent recombination to H<sub>2</sub>O<sub>2</sub> [33,51,52]. In addition to diffusion and solvation of species from the plasma phase, OH• can also be formed directly in water through UV photolysis. It was assumed that the contributions of humidity and UV photolysis decreased as the treatment distance increased. At longer treatment distances, there will be more mixing and reactions of the plasma effluent with ambient air before the solvation of reactive species in the water and thus in principle lower H<sub>2</sub>O<sub>2</sub> concentration.

As mentioned earlier, the plasma devices of utmost importance in plasma medicine are currently plasma jets. As reflected in Table 1, other devices, such as the COST microplasma jet device have been very well characterized on their plasma phase [50], but their interaction with liquids has not been widely investigated. As could be

expected given their configuration/design, the amount of ROS—H<sub>2</sub>O<sub>2</sub> in this case—produced by the COST microplasma jet is much lower than the other jets (Table 1). This source is therefore of limited interest for medical applications, but rather conceived as reference source to correlate the gas phase species with RONS generated in liquids, especially the COST source. In general terms, other types of APPJs produce higher H<sub>2</sub>O<sub>2</sub> concentrations than the COST microplasma jet.

Huge increase in the concentration of peroxides is recorded in PBS when doping gases such as O<sub>2</sub> or N<sub>2</sub> are added to the main Ar gas flow [39]. Another critical parameter is treatment distance as increasing of distance in kINPen from 10 to 30 mm was related to a decrease of more than one order of magnitude in the concentration of H<sub>2</sub>O<sub>2</sub> [49]. In general, treatment time linearly increases the amount of H<sub>2</sub>O<sub>2</sub> in PBS [28].

### 3.1.1.2 Nitrite ions (NO<sub>2</sub><sup>-</sup>)

Nitrite ions in liquid media are formed by dissolution of nitrogen oxides, which are formed in the gas phase of plasma jet. Concentration levels of NO<sub>2</sub><sup>-</sup> described in literature in different plasma-activated biological liquids are reported in Table 2, as function of the corresponding plasma settings and experimental conditions.

Formation of nitrites in liquids following atmospheric

plasma treatment leads to the decrease of pH. Experiments comparing DI water with buffered solutions showed that higher concentrations of  $\text{NO}_2^-$  are obtained in buffered solutions. This is most likely due to the depletion of  $\text{NO}_2^-$  by the reaction with  $\text{H}_2\text{O}_2$  leading to ONOOH that occurs

in acidic conditions in non-buffered plasma treated water solutions (Eq. (18)) [16,54]. This reaction runs slower in the solutions buffered at neutral pH which better preserves  $\text{NO}_2^-$  produced by plasma-created  $\text{NO}_x$ . Cell culture media are buffered and in addition contain many organic

**Table 1** Concentration of hydrogen peroxide after treatment by APPJ from different authors and configurations (working conditions and measurement technique are specified for each device)

Gas	Flow rate/ ( $\text{L} \cdot \text{min}^{-1}$ )	Conditions	t/s	V/ $\mu\text{L}$	Liquid	$[\text{H}_2\text{O}_2]$ /( $\mu\text{mol} \cdot \text{L}^{-1}$ )	Ref.
He	0.05	$U = 8 \text{ kV}, f = 20 \text{ kHz},$ $d = 10 \text{ mm}$	240	500	PBS ( $\text{Ca}^{2+}/\text{Mg}^{2+}$ )	1300 <sup>a)</sup>	[28]
	0.1	$U = 8 \text{ kV}, f = 15 \text{ kHz},$ $P = 5 \text{ W}$	120	100	DMEM + FBS	14 <sup>b)</sup>	[29]
					DI water	26 <sup>b)</sup>	
	1	$U = 7 \text{ kV}, f = 10 \text{ kHz}$	300	4000	DI water	18 <sup>b)</sup>	[30]
	1.67	$U = 7.5 \text{ kV}, f = 10 \text{ kHz},$ $d = 2 \text{ mm},$ shielding gas $\text{O}_2/\text{N}_2$	600	2000	PBS	265 <sup>b)</sup>	[31]
					2	$U = 18 \text{ kV}, f = 24.9 \text{ kHz},$ $d = 10 \text{ mm}$	60
	3	$U = 10 \text{ kV}, f = 9.69 \text{ kHz}$	150	100	DI water	1750 <sup>a)</sup>	[33]
					DMEM	1600 <sup>a)</sup>	
					DMEM + 10% FCS	1600 <sup>a)</sup>	
	4.7	$U = 3.16 \text{ kV}, d = 30 \text{ mm}$	60 or 120	1000	DMEM	35 <sup>c)</sup>	[34]
PBS					42 <sup>c)</sup>		
5	$U = 5-9 \text{ kV}, f = 5 \text{ kHz},$ $d = 25 \text{ mm}$	60	500	MEM	30 <sup>c)</sup>	[35]	
				5	$U = 2 \text{ kV}, P = 6 \text{ W},$ $d = 20 \text{ mm}$	1800	4000
advDMEM	2117.6 <sup>d)</sup>						
He + O <sub>2</sub>	2	$U = 15 \text{ kV}, f = 1 \text{ kHz},$ $d = 5 \text{ mm}$	300	360	PBS	174 <sup>c)</sup>	[37]
			600			391 <sup>c)</sup>	
			900			1250 <sup>c)</sup>	
	8	$U = 28 \text{ kV}, f = 2 \text{ kHz}$	60	300	Phenol-free RPMI 1640	6 <sup>c)</sup>	[38]
Ar	1	$U = 9 \text{ kV}, f = 16 \text{ kHz},$ $d = 9 \text{ mm}$	60	100	PBS	88.2 <sup>c)</sup>	[39]
	2	$U = 7 \text{ kV}, f = 60 \text{ Hz},$ $d = 13 \text{ mm}$	300	3000	DMEM + P/S + FBS	110 <sup>c)</sup>	[40]
					DMEM	92 <sup>c)</sup>	
	2	$U = 10 \text{ kV}, f = 60 \text{ Hz},$ $d = 3 \text{ mm}$	120	8000	Ringer's saline	8 <sup>c)</sup>	[41]
	3	$U = 0.7 \text{ kV}, I = 3 \text{ mA},$ $f = 16 \text{ kHz}, P = 0.2 \text{ W}$	1800		DI water	800 <sup>a)</sup>	[42]
3	$U = 7 \text{ kV}, f = 10 \text{ kHz},$ $d = 15, 25 \text{ and } 80 \text{ mm}$	300	4000	DI water	$d = 15 \text{ mm}, 16^{\text{b)}$ $d = 11 \text{ mm}, 80^{\text{b)}$ $d = 25 \text{ mm}, 3.7^{\text{b)}$	[43]	
Other gases	1, O <sub>2</sub>	$U = 9 \text{ kV}, f = 16 \text{ kHz},$ $d = 9 \text{ mm}$	60	100	PBS	1559 <sup>c)</sup>	[39]
	1, N <sub>2</sub>					735 <sup>c)</sup>	
	1, CO <sub>2</sub>					1265 <sup>c)</sup>	
	1, air					58.8 <sup>c)</sup>	
Ar, RF plasma jet	1.5	$F = 13.7 \text{ MHz},$ $d = 5-13 \text{ mm}$	60	3000	PBS ( $\text{Ca}^{2+}/\text{Mg}^{2+}$ )+ 1 g · L <sup>-1</sup> D-glucose	15–250 <sup>c)</sup>	[44]

(Continued)

Gas	Flow rate/ (L · min <sup>-1</sup> )	Conditions	t/s	V/ $\mu$ L	Liquid	[H <sub>2</sub> O <sub>2</sub> ]/( $\mu$ mol · L <sup>-1</sup> )	Ref.
Ar, kINPen	3	$U = 2-6$ kV, $f = 1.1$ MHz, $d = 5$ mm	60	1000	Phenol-free RPMI 1640	60 <sup>e)</sup>	[45]
	3	$U = 2-6$ kV, $f = 1$ MHz, $d = 9$ mm	100	5000	RPMI 1640 + 8% FCS + 1% P/S	45–210 <sup>d)</sup>	[46]
	3	$U = 2-6$ kV, $f = 1$ MHz	180	5000	Phenol-free RPMI 1640	100 <sup>e)</sup>	[47]
	3	$U = 2-6$ kV, $f = 1$ MHz	180		NaCl	78 <sup>d)</sup>	[48]
	3				DPBS	60 <sup>d)</sup>	
	3				RPMI 1640	88 <sup>d)</sup>	
	3	$U = 2-6$ kV, $f = 1$ MHz, $d = 10$ mm	300	2000	PBS	1300 <sup>a)</sup>	[49]
	3	$U = 2-6$ kV, $f = 1$ MHz, $d = 2-18$ mm	60	3000	PBS (Ca <sup>2+</sup> /Mg <sup>2+</sup> ) + 1 g · L <sup>-1</sup> D-glucose	19–20 <sup>d)</sup>	[44]
COST micro-plasma jet	1.4	He		250	RPMI1640	75 <sup>e)</sup>	[50]
		He + O <sub>2</sub>		250		5 <sup>e)</sup>	
		He + H <sub>2</sub> O		250		25 <sup>e)</sup>	
		He + H <sub>2</sub> O + O <sub>2</sub>		250		20 <sup>e)</sup>	

For detection of H<sub>2</sub>O<sub>2</sub> different methods were used: a) UV-vis absorption spectroscopy using TiOSO<sub>4</sub>; b) UV-vis spectroscopy; c) fluorescence method with Amplex<sup>TM</sup> Red hydrogen peroxide assay kit; d) commercial peroxide test stripes.  $d$ : distance between nozzle and surface of liquid;  $f$ : frequency of discharge;  $t$ : time;  $U$ : applied voltage; DPBS: Dulbecco's phosphate-buffered saline; FBS: fetal bovine serum; FCS: fetal calf serum; MEM: minimum essential medium; P/S: penicillin/streptomycin; RPMI: Roswell Park Memorial Institute

components, which results in higher NO<sub>2</sub><sup>-</sup> concentrations possibly due to the interaction of plasma with amino acids to form additional nitrite ions. On the other hand, nitrate/nitrite anions can be the targets of short lifetime ROS such as hydroxyl radical leading to the formation of peroxynitrites (ONOO<sup>-</sup>) according to reaction (Eq. (19)). In complex liquids such as cell culture media, this reaction will compete with the oxidation of biomolecules by ROS [29,33].

The amount of NO<sub>2</sub><sup>-</sup> decreases at increasing gas flow rate in the case of APPJ using He in experiments with DI water [28,29,33], as also discussed for hydrogen peroxides. This trend might result from the fact that less air is admixed to the plasma jet channel when the gas flow is higher and, thus, less RONS are produced in the gas phase [28]. The surrounding atmosphere, especially air, has a greater impact on the formation of RNS than in the formation of H<sub>2</sub>O<sub>2</sub> in solutions [45]. Higher concentration of nitrite observed in DMEM with FCS than in DMEM can be explained by the presence of copper-containing proteins such as cytochrome C in FCS that can contribute to the oxidation of nitric oxide into nitrite [33]. The maximum value of nitrite ions recorded in PBS is referred to APPJ in air [39].

In the case of kINPen plasma using argon, the concentration of nitrite ions is higher in phenol-free RPMI medium than in the presence of phenol even at short time of plasma treatment [48]. This suggests a

possible reaction of phenol red with NO<sub>2</sub><sup>-</sup> during plasma treatment (acidification process).

### 3.1.1.3 Nitrate ions (NO<sub>3</sub><sup>-</sup>)

Nitrates are also obtained from the reaction of nitrogen oxides formed by plasmas in liquids. The concentrations recorded in representative works in literature are detailed in Table 3.

The highest concentration of nitrate ions in DI water was obtained by Chauvin et al. [33] after 150 s of treatment by APPJ in helium. In general, shorter treatment times up to maximum of 1 min which could be of interest in direct treatment of tissues, have been evaluated and provided larger concentrations of nitrate, up to 2500  $\mu$ mol · L<sup>-1</sup> in PBS with air plasma jet [39]. This is related to the fact that air plasma can produce RNS such as NO and NO<sub>2</sub> in the gas phase, which react with water forming nitrite/nitrate ions as described in section 3 (Eqs. (12–16)).

For all species discussed up to now (Tables 1–3), the effect of liquid volume on the concentration of RONS is critical, as in the same treatment conditions it has been observed that smaller volumes allow generating higher concentrations (both parameters are inversely related). However, although this is not straightforward, it seems that decreases of one order of magnitude in volume may increase the concentration of RONS in one order of magnitude.

**Table 2** Concentration of nitrite ions in different liquids following APPJ or kINPen treatment

Gas	Flow rate/ (L · min <sup>-1</sup> )	Conditions	t/s	V/ $\mu$ L	Liquid	[NO <sub>2</sub> <sup>-</sup> ]/( $\mu$ mol · L <sup>-1</sup> )	Ref.
He	0.05	$U = 8$ kV, $f = 20$ kHz, $d = 10$ mm	240	500	PBS (Ca <sup>2+</sup> /Mg <sup>2+</sup> )	1600 <sup>a)</sup>	[28]
	0.05	$U = 8$ kV, $f = 20$ kHz, $d = 10$ mm	60	500	PBS (Ca <sup>2+</sup> /Mg <sup>2+</sup> )	400 <sup>a)</sup>	[28]
	0.1	$U = 8$ kV, $f = 15$ kHz, $P = 5$ W	120	100	DMEM + FBS	340 <sup>b)</sup>	[29]
					DI water	137 <sup>b)</sup>	
	1	$U = 7$ kV, $f = 10$ kHz	300	4000	DI water	18 <sup>c)</sup>	[30]
	1.67	$U = 7.5$ kV, $f = 10$ kHz, $d = 2$ mm, shielding gas O <sub>2</sub> /N <sub>2</sub>	600	2000	PBS	620 <sup>c)</sup>	[31]
	2	$U = 1.8$ kV, $f = 35$ kHz	180		Serum-free HBSS	125–138 <sup>b)</sup>	[53]
	2	$U = 15$ kV, $f = 1$ kHz, $d = 5$ mm	300	360	PBS	5 <sup>a)</sup>	[37]
	2	$U = 15$ kV, $f = 1$ kHz, $d = 5$ mm	600	360	PBS	10 <sup>a)</sup>	[37]
	2	$U = 15$ kV, $f = 1$ kHz, $d = 5$ mm	900	360	PBS	19.5 <sup>a)</sup>	[37]
	3	$U = 10$ kV, $f = 9.69$ kHz	150	100	DI water	25 <sup>a)</sup>	[33]
					DMEM	200 <sup>a)</sup>	
					DMEM + 10% FCS	500 <sup>a)</sup>	
5	$U = 2$ kV, $P = 6$ W, $d = 20$ mm	1800	4000	McCoy	435 <sup>d)</sup>	[36]	
				advDMEM	174 <sup>d)</sup>		
He + N <sub>2</sub>	2	$U = 7.5$ kV, $f = 10$ kHz, $d = 15$ mm	600	2000	PBS	590 <sup>c)</sup>	[54]
Ar	1	$U = 9$ kV, $f = 16$ kHz, $d = 9$ mm	60	100	PBS	8.8 <sup>a)</sup>	[39]
	2	$U = 7$ kV, $f = 60$ Hz, $d = 20$ mm	300	3000	DMEM + P/S + FBS	3350 <sup>a)</sup>	[40]
					DMEM	2750 <sup>a)</sup>	
	3	$U = 0.7$ kV, $I = 3$ mA, $f = 16$ kHz, $P = 0.2$ W	1800		DI water	10 <sup>b)</sup>	[42]
	3	$U = 7$ kV, $f = 10$ kHz, $d = 15, 25$ and $80$ mm	300	4000	DI water	$d = 15$ mm, 11 <sup>c)</sup> $d = 25$ mm, 16 <sup>c)</sup> $d = 80$ mm, 3.7 <sup>c)</sup>	[43]
	1, O <sub>2</sub>	$U = 9$ kV, $f = 16$ kHz, $d = 9$ mm	60	100	PBS	< 2.5 <sup>a)</sup>	[39]
	1, N <sub>2</sub>				PBS	< 2.5 <sup>a)</sup>	
	1, CO <sub>2</sub>				PBS	< 2.5 <sup>a)</sup>	
1, air	PBS				1782 <sup>a)</sup>		
Ar, RF plasma jet	1.5	$F = 13.7$ MHz, $d = 5$ – $13$ mm	60	3000	PBS (Ca <sup>2+</sup> /Mg <sup>2+</sup> ) + 1 g · L <sup>-1</sup> D-glucose	250 <sup>b)</sup>	[44]
Ar, kINPen	3	$U = 2$ – $6$ kV, $f = 1$ MHz	180		NaCl	10 <sup>a)</sup>	[48]
	3			DPBS	6.5 <sup>a)</sup>		
	3			Phenol free RPMI 1640	19.5 <sup>a)</sup>		
	3			$d = 10$ mm	300	2000	PBS

For detection of NO<sub>2</sub><sup>-</sup> ions different methods were used: a) Nitrite assay kit (based on Griess reagent); b) colorimetric method using Griess reagent; c) UV-vis absorbance; d) Nitrite/Nitrate test strips. HBSS: Hank's balanced salt solution



**Table 3** Concentration of nitrate ions in solutions after APPJ treatment.

Gas	Flow rate/ (L · min <sup>-1</sup> )	Conditions	t/s	V/μL	Liquid	[NO <sub>3</sub> <sup>-</sup> ]/(μmol · L <sup>-1</sup> )	Ref.
He	0.05	$U = 8 \text{ kV}, f = 20 \text{ kHz}, d = 10 \text{ mm}$	240	500	PBS (Ca <sup>2+</sup> /Mg <sup>2+</sup> )	500 <sup>a)</sup>	[28]
	1	$U = 7 \text{ kV}, f = 10 \text{ kHz}, d = 12 \text{ mm}$	900	100	DI water	2.4 <sup>b)</sup>	[55]
	1	$U = 7 \text{ kV}, f = 10 \text{ kHz}$	300	4000	DI water	0.47 <sup>c)</sup>	[30]
	1.67	$U = 7.5 \text{ kV}, f = 10 \text{ kHz}, d = 2 \text{ mm},$ shielding gas O <sub>2</sub> /N <sub>2</sub>	600	2000	PBS	260 <sup>c)</sup>	[31]
	3	$U = 10 \text{ kV}, f = 9.69 \text{ kHz}$	150	100	DI water DMEM	250 <sup>a)</sup> 400 <sup>a)</sup>	[33]
He + N <sub>2</sub>	2	$U = 7.5 \text{ kV}, f = 10 \text{ kHz}$	600	2000	PBS	460 <sup>b)</sup>	[54]
Ar	3	$U = 0.7 \text{ kV}, I = 3 \text{ mA}, f = 16 \text{ kHz},$ $P = 0.2 \text{ W}$	1800		DI water	20 <sup>c)</sup>	[42]
	3	$U = 7 \text{ kV}, f = 10 \text{ kHz}$	300	4000	DI water	$d = 15 \text{ mm}, 0.9^{\text{b)}$ $d = 25 \text{ mm}, 1.5^{\text{b)}$ $d = 80 \text{ mm}, 0.2^{\text{b)}$	[43]
Air	1	$U = 9 \text{ kV}, f = 16 \text{ kHz}, d = 9 \text{ mm}$	60	100	PBS	2580 <sup>a)</sup>	[39]
Ar, kINPen	3	$U = 2\text{--}6 \text{ kV}, f = 1 \text{ MHz},$ shielding gas O <sub>2</sub> + N <sub>2</sub> at 5 L · min <sup>-1</sup>	180		NaCl	7 <sup>a)</sup>	[48]
	3	$U = 2\text{--}6 \text{ kV}, f = 1 \text{ MHz},$ shielding gas O <sub>2</sub> + N <sub>2</sub> at 5 L · min <sup>-1</sup>	180		DPBS	5 <sup>a)</sup>	[48]

Methods of detection were: a) Nitrite/Nitrate assay kit (Nitric oxide assay kit); b) UV-vis spectroscopy; c) Nitrate selective electrode

### 3.1.2 Short-lived reactive species

In this section, the concentration of short-lived reactive species in PAM is discussed. Due to their lifetime and low stability, these species are harder to measure and require special equipment or adapted experimental protocols, so there is less abundance of relevant results in the literature.

#### 3.1.2.1 Hydroxyl (OH<sup>•</sup>), peroxy- (HOO<sup>•</sup>) and oxygen radicals

Nowadays the most popular method for detection of short-lived reactive species is electron paramagnetic resonance spectroscopy (EPR) using spin-trap reagents. For instance, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is used as scavenger for OH<sup>•</sup> and superoxide O<sub>2</sub><sup>-</sup> radicals [20,21,56].

Analysis of the data in Table 4 suggests that higher amounts of short-lived species generated by APPJ can be obtained in water or saline solutions such as PBS rather than in cell culture media. This may be ascribed to the fact that the complex composition of cell culture media with many components such as amino acids, vitamins and proteins can trap short-lived ROS. This trend has been confirmed by Chauvin et al. [33] by treating DI water, DMEM and supplemented DMEM under the same plasma setting conditions and comparing the amount of the short life radicals generated in the different media. On the other hand, literature data for kINPen plasma jet, operating with

Ar, are contradicting this tendency (Table 4) [48]. The smallest amount of oxygen radicals was formed in sodium chloride solution, followed by RPMI while DPBS had the highest amount of plasma-generated oxygen radicals. Authors suggested that in RPMI and DPBS, the additional compounds (aminoacids, proteins, etc) are further sources for oxygen radicals [48].

#### 3.1.2.2 Peroxynitrites (ONOO<sup>-</sup>)

It has been remarked that usually low concentrations of hydrogen peroxide are accompanied by high amounts of nitrate/nitrite ions [28,30,33,43]. This may be explained by reactions (Eqs. (18,19)). Reaction (Eq. (18)) leads to the formation of peroxynitrous acid, which is not stable in acidic solution and decays to OH<sup>•</sup> and NO<sub>2</sub> radicals or transforms to the more stable nitrate ion. Peroxynitrite has a half-lifetime about 1 s at pH 7.4 which allows its diffusion in solution. Machala et al. [58] indirectly estimated the concentration of this ion to be of tens of μmol · L<sup>-1</sup> by fluorescence spectroscopy (after transient spark discharge ignited above distilled water). Girard et al. [54] identified the formation of peroxynitrite anion in PBS (at pH 12) under He APPJ treatment by using UV-visible absorption spectroscopy. The maximal concentration detected is of 47 μmol · L<sup>-1</sup> after 10 min of treatment. The presence of shielding gas O<sub>2</sub>/N<sub>2</sub> in the ratio 20%/80% decreased formation of ONOO<sup>-</sup> to 23 μmol · L<sup>-1</sup>. For 100%

**Table 4** Concentration of short-lived ROS detected by spin-trap after APPJ treatment, using different gases for plasma generation

Gas	Flow rate/ (L·min <sup>-1</sup> )	Conditions	t/s	V/ $\mu$ L	Liquid	[ROS]/( $\mu$ mol·L <sup>-1</sup> )	Ref.
He	1	$U = 9$ kV, $f = 16$ kHz, $d = 9$ mm	60	100	DI water	OH $\cdot$ , 68	[57]
	2	$U = 18$ kV, $f = 24.9$ kHz, $d = 10$ mm	60	100	Aqueous solutions	OH $\cdot$ , 23.5 HOO $\cdot$ , 17 O <sub>3</sub> /O <sub>2</sub> /O, 170	[32]
	3	$U = 10$ kV, $f = 9.69$ kHz	150	100	DI water DMEM DMEM + 10% FCS	OH $\cdot$ , 4.12 OH $\cdot$ , 3 OH $\cdot$ , 0.4	[33]
N <sub>2</sub>	1	$U = 9$ kV, $f = 16$ kHz, $d = 9$ mm	60	100	DI water	OH $\cdot$ , 130	[57]
Ar	1					OH $\cdot$ , 84	
Ar + H <sub>2</sub> O	1					OH $\cdot$ , 210	
O <sub>2</sub>	1					OH $\cdot$ , 32	
CO <sub>2</sub>	1					OH $\cdot$ , 28	
Air	1					OH $\cdot$ , < 10	
Ar, kINPen	3	Shielding gas O <sub>2</sub> , O <sub>2</sub> + N <sub>2</sub> , flow rate 5 L·min <sup>-1</sup>	180		NaCl	OH $\cdot$ + O <sub>2</sub> <sup>-</sup> , 1.9	[48]
					DPBS	OH $\cdot$ + O <sub>2</sub> <sup>-</sup> , 5.6	
					RPMI 1640	OH $\cdot$ + O <sub>2</sub> <sup>-</sup> , 3.6	

N<sub>2</sub> and 100% O<sub>2</sub> as shielding gas composition, concentrations of peroxy nitrite reached about 10  $\mu$ mol·L<sup>-1</sup> [31]. In these conditions (with shielding gas) the intensities of NO $\cdot$  and OH $\cdot$  bands were decreasing drastically. Thus, lower concentrations of the gas-phase precursors for the species generated in liquids NO<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> may decrease the amount of ONOO<sup>-</sup> in solution.

Analysis of the data presented in Tables 1–3 shows that the largest amount of hydrogen peroxides is obtained by plasma jet treatment with He while the largest concentration of nitrite ions is produced by plasma jets working with Ar. The difference between the species generated by Ar and He plasmas can be attributed to the reactive species formed in gas phase. As it is known, emission spectra of plasma jets for both gases contain molecular bands assigned to hydroxyl radicals (306–310 nm), neutral nitrogen molecules (315–380 nm and 399–405 nm), nitrogen molecular ion (N<sub>2</sub><sup>+</sup>, 391–470 nm) and atomic oxygen lines at 777 nm and 844 nm. These species are originated from impurities or diffusing surrounding species. In the case of He plasma jet the atomic lines of helium are registered between 501 and 728 nm. In the case of Ar plasma jet the visible region from 400 to around 700 nm is mostly free of emission features and atomic lines of argon are detected starting from 697 to 923 nm. In some works, [59,60] the vacuum ultraviolet region (105–300 nm) was also registered for Ar and He plasma jets. It was established that the atomic oxygen

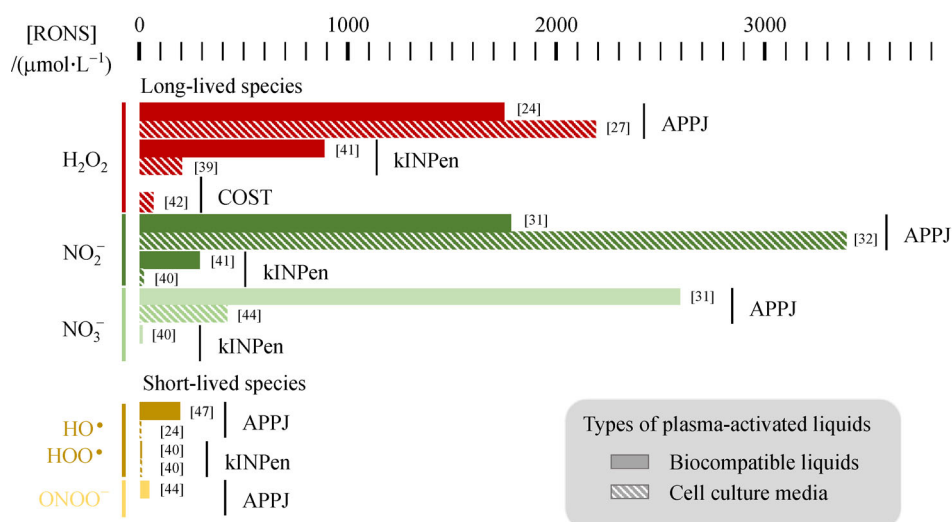
density in this region is higher for Ar plasma jets than for comparable conditions in plasma jets operating with helium. Thus, more nitrous oxides are formed in the gas phase with Ar plasma jet, thereby increasing gaseous NO<sub>x</sub> concentrations (Eqs. (10,11)), and also the amount of NO<sub>2</sub><sup>-</sup> in liquid media.

To summarize the aforementioned findings, Fig. 3 presents the range of concentrations of long-lived (H<sub>2</sub>O<sub>2</sub>, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>) and short-lived (HO $\cdot$ , HOO $\cdot$  and ONOO<sup>-</sup>) species described in literature and detailed in the previous tables. It can be highlighted that APPJ, as they include a wide range of device configurations, yield high concentration of RONS either for long-lived or short-lived species.

#### 4 Correlation between RONS and their biological effects

In the last years, different authors have studied the antitumor effects of PAM on cell viability of some cancer cell lines, and excellent reviews have analyzed this subject in detail [3,61–64]. In this section we aim to briefly correlate some of the works which determined the amount of RONS to the cell viability they obtained to highlight the importance of these species in antitumor action.

From the literature cited in the previous sections, the comparison between cell viability data and concentration of RONS achieved by indirect plasma treatment of the



**Fig. 3** Concentration ranges of RONS in PAM described in literature with emphasis on the nature of the liquid media

media is reported in Table 5, with emphasis on the maximum amount of long-lived RONS generated and its possible relation with cytotoxicity. With regards to the cytotoxicity effects on cancer cells, this increase of plasma-generated H<sub>2</sub>O<sub>2</sub> concentration is presented as predominant with respect to the increase in concentration of nitrite ions. Many different cancer cell lines have been studied but, as an example, in the case of glioblastoma cells U87 [29,34,49], it can be noticed that increasing concentration of hydrogen peroxide results in a decrease cell viability from 50% to 95% depending mainly on the time of incubation with the cell culture medium and the plasma treatment time. However as discussed in other works [24,36], the cytotoxicity is not only related to concentration of H<sub>2</sub>O<sub>2</sub>, suggesting that it can be synergistic action of hydrogen peroxide, NO<sub>2</sub><sup>-</sup> and other species (i.e., formation of ONOO<sup>-</sup> in liquid media). It is also interesting to highlight that the studies presenting the highest NO<sub>2</sub><sup>-</sup> concentration generated by atmospheric plasma treatment, from Kurake et al. [40], and the highest H<sub>2</sub>O<sub>2</sub> concentration in PBS, from Van Boxem et al. [49], present both very high cytotoxicity on the different cancer cell lines studied, with 95%–98% cell death for 24-hour cell culture experiments. In addition to presenting the most lethal effects on cancer cells, different works [36,40,63] also demonstrated the selectivity of the plasma treatment between cancer cell and healthy cells. For example, Kurake et al. [40] used human mammary epithelial cell line as healthy cells, showing a healthy cell viability around 90% in the same plasma setting conditions used on glioblastoma cancer cells with a cell viability ≤ 10%. This is pointing out a key feature of plasma jets in term of their relevance to be used in cancer therapy not only for their

efficiency but also for their potential selectivity. A lot of further research is however needed to confirm and understand these breakthroughs but so far relatively scarce results. For more details on other cancer cell lines studied in contact with APPJ or PAM, please refer to other review works [62,64].

## 5 Concluding remarks

APPJs have gained relevance in plasma medicine as they effectively produce RONS in different liquid/aqueous media, including biological fluids, and are able to deliver them locally to the treated tissue/organ. Many parameters affect the generation of RONS in solutions, the differences in chemical composition being critical to that aim. The nature of the gas and conditions of the plasma discharge also have a very important effect on the interactions with liquids.

Long-lived species such as H<sub>2</sub>O<sub>2</sub> and NO<sub>2</sub><sup>-</sup> are by far the species mostly characterized, followed by NO<sub>3</sub><sup>-</sup>. The methodology/equipment for detection of short-lived species such as hydroxyl (OH•), peroxy- (HOO•), O<sub>2</sub><sup>-</sup>, or peroxynitrite (ONOO<sup>-</sup>) radicals is more challenging so it is less often measured in literature.

Although H<sub>2</sub>O<sub>2</sub> is one of the key species with anticancer action, it has been shown that it is not the sole responsible player and that the interplay of other reactive species is crucial.

The increasing importance of plasma activated liquids in plasma oncology allows to foresee close interactions between the field of liquid diagnostics and biology. Especially, determination of the right “dose” for suitable

**Table 5** Correlation between RONS concentration generated by atmospheric plasma treatment in cell culture media by indirect treatment and the corresponding cell viability

PAM	$t_{\text{plasma}}/s$	$[\text{H}_2\text{O}_2]_{\text{max}}/(\mu\text{mol}\cdot\text{L}^{-1})$	$[\text{NO}_2^-]_{\text{max}}/(\mu\text{mol}\cdot\text{L}^{-1})$	Cell viability ( $C_v$ ) /%	Cell lines	Ref.
PBS( $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) (1 h exposure followed by DMEM addition)	120	1300	1600	$C_v^{24h} \approx 4\%$	Normal human skin fibroblasts (NHSF)	[28]
	480			$C_v^{24h} \approx 1\%$		
	120			$C_v^{24h} \approx 10\%$	MRC5V <sub>i</sub> cell line (derived from normal human lung fibroblasts MRC5)	
	480			$C_v^{24h} \approx 1\%$		
	120			$C_v^{24h} \approx 45\%$	Human colon cancer cells (HCT116)	
	480			$C_v^{24h} \approx 1\%$		
	120			$C_v^{24h} \approx 30\%$	Melanoma cell line (Lu1205)	
	480			$C_v^{24h} \approx 1\%$		
DMEM + FBS	120	14	340	$C_v^{24h} \approx 60.8\%$ $C_v^{48h} \approx 45\text{--}49\%$	Glioblastoma (U87MG)	[29]
DMEM/modified DMEM	240	35–42	–	$C_v^{72h} \approx 40\%$	Human breast cancer (MDA-MB-231) cells	[34]
	240			$C_v^{72h} \approx 15\%$	Human glioblastoma (U87MG) cells	
	240			$C_v^{72h} \approx 20\%$	Pancreatic cancer (PA-TU-8988T) cells	
MEM + 10% FBS	120	30	–	$C_v^{12h} \approx 50\%$ $C_v^{48h} \approx 50\%$	ScaBER (from human bladder cancer)	[35]
	240			$C_v^{12h} \approx 10\%$ $C_v^{48h} \leq 5\%$		
RPMI 1640	20–300	6	–	$C_v^{24h} \leq 10\%$	Human hepatocellular carcinoma Bel7402	[38]
	20			$C_v^{24h} \approx 92\%$	5-FU-resistant Bel7402/5FU cells	
	40			$C_v^{24h} \approx 80\%$		
	60			$C_v^{24h} \approx 60\%$		
	120			$C_v^{24h} \approx 40\%$		
	300			$C_v^{24h} \approx 10\%$		
DMEM w/wo 10% FBS	180	110	3350	$C_v^{24h} \approx 10\text{--}12\%$	Human glioblastoma cell line (U251SP)	[40]
	180	64 <sup>a)</sup>	1740 <sup>a)</sup>	$C_v^{24h} \approx 95\text{--}98\%$	Human mammary epithelial cell Line (MCF10A)	
PBS	300 & 540	1300	500	$C_v^{24h} \approx 2\text{--}5\%$	Glioblastoma human GBM cell lines (U87, U251 and LN229)	[49]

<sup>a)</sup> Concentration of RONS generated in cell culture media in the conditions of the cell viability assay presented in the table

biological action is in the limelight and not straightforward to determine given the great number of physical and chemical parameters influencing the generation of RONS in liquids and their subsequent biological response.

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