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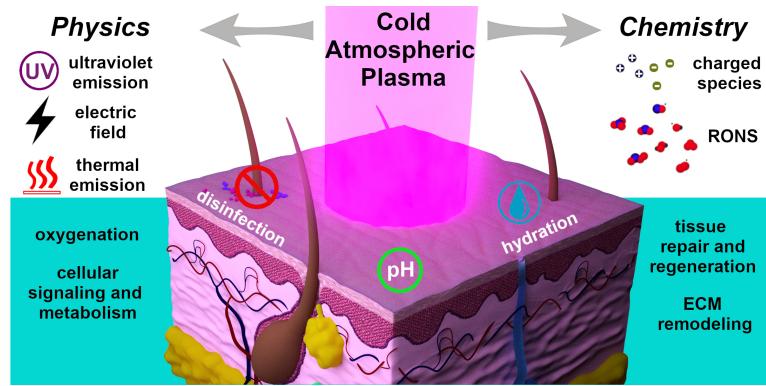
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1 The emerging potential of cold atmospheric plasma in skin
2 biology

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8

9 **ABSTRACT**

10 The maintenance of skin integrity is crucial to ensure the physiological barrier
11 against exogenous compounds, microorganisms and dehydration but also to
12 fulfill social and aesthetic purposes. Besides the development of new actives
13 intended to enter a formulation, innovative technologies based on physical
14 principles have been proposed in the last years. Among them, Cold
15 Atmospheric Plasma (CAP) technology, which already showed interesting
16 results in dermatology, is currently being studied for its potential in skin
17 treatments and cares. CAP bio-medical studies gather several different
18 expertise ranging from physics to biology through chemistry and biochemistry,
19 making this topic hard to pin. In this review we provide a broad survey of the
20 interactions between CAP and skin. In the first sections, we tried to give some
21 fundamentals on skin structure and physiology, related to its essential functions,
22 together with the main bases on cold plasma and its physicochemical
23 properties. In the following parts we dissected and analyzed each CAP
24 parameter to highlight the already known and the possible effects they can play
25 on skin. This overview aims to get an idea of the potential of cold atmospheric
26 plasma technology in skin biology for the future developments of dermo-
27 cosmetic treatments, for example in aging prevention.

28

29

30 Keywords

31

32 Skin, Cold Atmospheric Plasma, RONS, Aging, Dermatology, Cosmetic.

1
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1

2

3 **1. Introduction.**

4

5 The skin is the largest organ of the human body and ensures several distinct
6 functions because of its particular position, in connection between the outside
7 and the inside of the body. This keratinized tegument envelops the whole body
8 and protects it from environmental aggressions and from massive water loss [1].
9 Although the physical barrier against environmental and pathogen insults is the
10 main role of this organ, the skin possesses several other functions such as
11 vitamin D production [2], humidity, temperature and mechanical sensing [3-5],
12 temperature regulation [6], molecule absorption [7], excretion and secretion [8-
13 11] and some immunological functions [12]. In view of the above, daily cares of
14 this organ are necessary to preserve its integrity and functions. Skin care
15 practices are not only needed for the whole body health but also for social and
16 aesthetic purposes. Cosmetic skin care was practiced from the dawn of time by
17 the ancient civilizations. In the 21st century, with the increase of span life,
18 people from all walks of life ask to live healthy and look younger. Consequently,
19 the global consumer demand of cosmetic products is rapidly expanding today.
20 In 2018, the value of the global cosmetics market was 508 billion U.S. dollars.
21 The market is projected to value at about 758 billion U.S. dollars by 2025 [13].
22 Modern skin treatment-offer ranges from chemical product application to
23 physical treatments. Among the treatments, cream, sera and oils are commonly
24 used as at-home beauty treatments while skin peeling treatments are often
25 administered by professional beauticians. Physical treatments are also
26 administered in beauty centers although today some devices can be bought for
27 domestic use. Among them, LED light and lasers are often used for
28 rejuvenation purposes. These light sources stimulate skin renewal by physically
29 removing the external layers of the skin (resurfacing) thus activating skin cell
30 metabolism [14, 15]. For a deeper skin rejuvenation, more invasive and
31 expensive techniques such as aesthetic surgery are required. Currently, a
32 physicochemical approach, based on ionized gases, is joining the skin non-

surgical treatments. This technology, named Cold Atmospheric Plasma (CAP), was already used in dermatology to promote wound healing. Today, CAP is entering into the cosmetic field, thus providing a new challenge. In reason of their unique ability to generate a complex chemical mix and thanks to their physical properties, CAPs could be a promising alternative in non-invasive treatment of skin. However, the scientific bases of cold plasma effects on skin and the identification of their exact mechanisms of actions, both at the cellular and at the molecular levels, are still lacking and they constitute a new active field of investigation.

In the present review, based on skin fundamental notions and on chemo-physical properties of plasmas, we described the possible benefic interactions between CAPs and skin and how they could participate in improving skin wellness and regeneration.

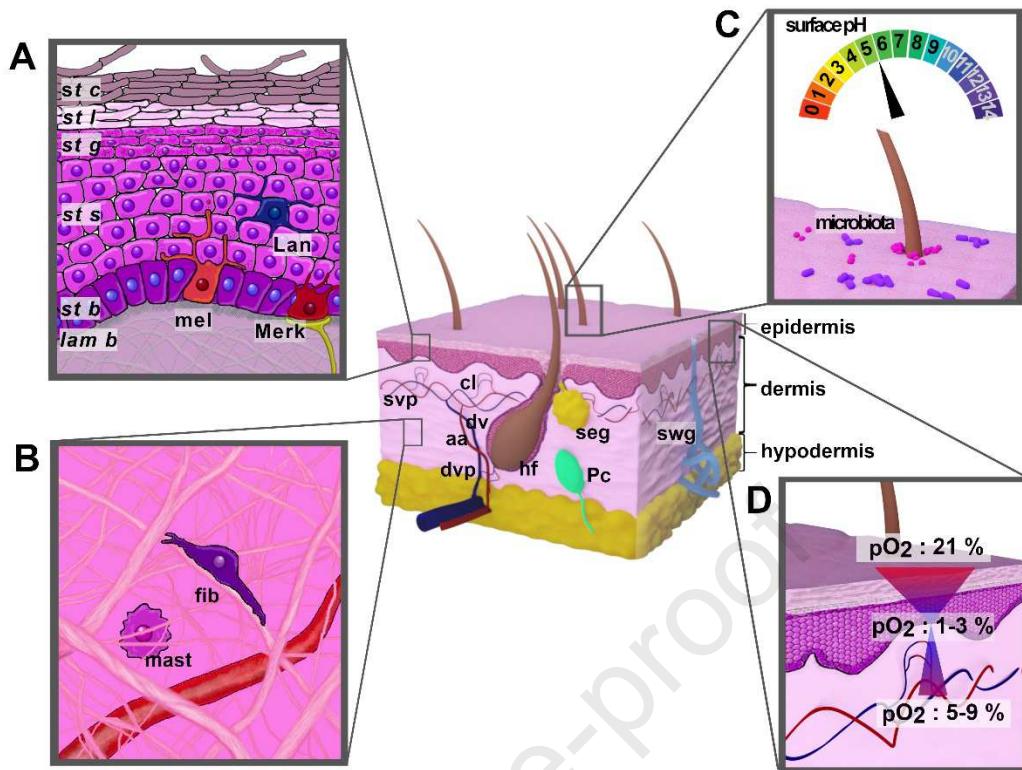
2. Fundamentals on skin and its microenvironment

To understand how CAP could be used for skin treatments, it is important to take into consideration all skin parameters contributing to skin health that could be potentially modified by plasma treatment.

2.1 Skin structure

The skin covers and isolates the whole body. In humans its thickness can vary from 0.5 to 4 mm. Skin thickness strictly correlates with the collagen content of the dermis and it is gender dependent [16]. The different cell types composing the skin form a complex structure that can be divided in three main layers: the epidermis, the dermis and the hypodermis (figure 1). The epidermis is the layer that takes contact directly with the external environment. Its thickness can change at the different body sites and it is also age, gender and phototype dependent [17, 18]. The epidermis (figure

1A) is mainly made up of keratinocytes and melanocytes, these latter confer
2 the typical color tone of the skin. Some other cells can be found in this part
3 of the skin, the immune system Langherans cells and the mechanosensors
4 Merkel cells. Human epidermis can be further divided in four or five
5 functional layers. The deeper layer of the epidermis is called *stratum basale*
6 or *germinativum* and is made of non-differentiated keratinocytes,
7 melanocytes and Merkel cells laying on the basal lamina (*lamina basalis*).
8 The presence of keratinocyte stem cells permit the epidermis turnover [19-
9 21]. Once divided, one of the keratinocyte daughter cells moves and
10 differentiates toward the upper layers of the epidermis forming, in
11 sequence, the *strata spinosum*, *granulosum*, *lucidum* (found only in the
12 palms of the hands and soles of the feet) and the *corneum*. Epidermis in
13 healthy and young humans forms digitations that penetrate the dermis
14 (papillary dermis). The epidermis is separated from the dermis thanks to a
15 thin layer of extracellular matrix called basal lamina. This latter is mainly
16 made up of collagen IV, laminins and glycosaminoglycans (GAGs) and
17 ensures the mechanical link between the basal layer of the epidermis and
18 the underlying connective tissue, the dermis. This middle layer between the
19 epidermis and the hypodermis (figure 1B), is made up of a network of
20 fibrillar proteins, collagens, elastin and an amorphous ground substance
21 rich in GAGs such as dermatan sulfate and hyaluronic acid. These
22 negatively charged macromolecules attract water molecules keeping the
23 skin hydrated. Few cell types can be found in the dermis, essentially
24 papillary and reticular fibroblasts with some mast cells. The dermis
25 possesses a sensitivity to mechanic stimuli thanks to the presence of
26 mechanosensors such as the Ruffini's, Meissner's and Pacini corpuscles.
27 The hypodermis is the third deeper layer of the skin. It is an adipose tissue
28 made up of fibroblasts, adipose cells and macrophages. The main function
29 of this tissue is to store fat for thermal insulation and as a source of energy.
30 Besides its three horizontal layers, mammalian skin possesses typical
31 appendages crossing this structure. During the embryogenesis some cells
32 of the ectoderm forming the epidermis invaginate into the dermis creating
33 cutaneous annexes like hair follicles, nails, sweat and sebaceous glands
34 (figure 1).



1

Figure 1. The skin and its microenvironment. In the middle, the 3D structure of the whole skin with its vascular system: superficial vascular plexus (svp), capillary loops (cl), ascending arteriole (aa), descending collecting venule (dv), deep vascular plexus (dvp). Typical skin appendages: hair and hair follicles (hf) with the associated sebaceous gland (seg) and a sweat gland (swg). Sensory organs such as the Pacini corpuscle (Pc) permit to feel the external stimuli. A) Keratinocytes forming the epidermis layers: *stratum corneum* (st c), *stratum lucidum* (st l) only present in the palms of the hands and soles of the feet, *stratum granulosum* (st g), *stratum spinosum* (st s) and *stratum basale* (st b). The epidermis is separated from the dermis by the *lamina basalis* (lam b). Some other cell types found in this epidermis: melanocytes (mel), Langerhans cells (Lan) and Merkel cells (Merk). B) The vascularized dermis layer made up of extracellular matrix fibers and few cell types such as: fibroblasts (fib) and mast cell (mast). C) The acidic surface of the skin and the associated microbiota living on the *stratum corneum* and inside the hair follicles. D) Skin oxygenation with the two gradients created by oxygen coming on one side from the atmosphere and on the other side from dermis blood vessels

17

18

19

1 The skin possesses a heterogeneous vascularization (figure 1). Vessels
2 coming from the hypodermis enter the dermis and form the deep vascular
3 plexus in the reticular dermis, supplying nutrients and oxygen to hair bulbs
4 and glands. The whole dermis layer is vascularized thanks to the ascending
5 arterioles and descending collecting venules coming from the deeper layers
6 of the skin. In the upper dermis, these vessels form the superficial vascular
7 plexus from which originate the capillary loops extending in dermal papilla
8 [22]. Unlike the other cutaneous layers the epidermis is not vascularized at
9 all.

10

11 *2.2 Skin oxygenation and antioxidant status*

12

13 A consequence of the typical skin vascularization is a non-uniform
14 oxygenation. Indeed oxygen diffuses inside the skin from two sources: the
15 atmosphere (21% O₂) and the blood stream circulating in the dermis (5-9%
16 O₂) [23, 24]. This results in two oxygen gradients that reach a point of
17 minimum (1-3% O₂) at the basal lamina where keratinocytes stem cell
18 reside (figure 1D). Although oxygen has a vital importance for aerobic cells,
19 during the respiratory metabolism a small part of this diatomic molecule is
20 converted in reactive oxygen species (ROS). While in low doses ROS play
21 an essential role in cell signaling and in the maintaining of cell homeostasis,
22 in case of overproduction, these free radicals can induce the so-called
23 oxidative stress [25]. Being exposed to different percentage of oxygen, the
24 different skin layers are likely to possess a different sensitivity to the
25 oxidative stress. However, to avoid ROS-induced damages, skin cells are
26 able to eliminate the excess of these reactive species keeping their
27 concentration at a physiological level. This task is performed by the
28 antioxidant system that is made up of both small molecules and enzymes
29 such as glutathione peroxidases, catalase and superoxide dismutases
30 allowing the conversion of ROS into harmless molecules [26].

1

2 2.3 Skin surface and its physiological pH

3

4 A healthy epidermis is relatively acidic. Its outer layer, the *stratum corneum*,
5 possesses an “acidic mantle” that confers it a pH ranging between 4 and 6
6 (figure 1C). Keeping an acidic pH fulfills several physiological roles [27]. At
7 birth, the skin is exposed to a non-sterile environment and it is rapidly
8 colonized by microorganism. The acidic mantle inhibits the growth of
9 pathogen microorganisms and promotes the growth of the physiological
10 microflora [28]. The acidic mantle is generated thanks to endogenous
11 factors but also to exogenous ones such as bacteria. Among the
12 endogenous factors, the relatively-low pH seems to be induced by the
13 generation of urocanic acid [29], and free fatty acids [30]. The maintaining
14 of this acidic mantle seems to be due to the activity of the sodium-proton
15 exchanger (NHE1) [31]. The low pH contributes also to preserve the skin
16 permeability barrier. Indeed, *stratum corneum* enzymes, involved in the
17 synthesis of the ceramide barrier, possess an optimal activity at acidic pH
18 [32]. In addition, a disregulated acidic mantle can have a negative impact on
19 the skin barrier [33]. Moreover, a high pH of the *stratum corneum* is
20 correlated with skin pathologies such as atopic dermatitis [34, 35]. To keep
21 the pH at physiological values, the skin possesses its own buffering system
22 permitting to avoid rapid pH variations induced by external insults [36]

23

24 2.4 The skin as a semipermeable barrier of the body

25

26 As already described, the *stratum corneum* creates a protective,
27 semipermeable barrier [37-41]. The first representation of the *stratum*
28 *corneum* was made in 1975 by Michaels and collaborators who
29 simplistically described the outer layer of the skin as a *brick and mortar like*
30 *structure* [42]. Although the skin isolates and protects the body from
31 external aggressions, this cornified organ is not a sealed barrier. Skin
32 actively communicates and interacts with the external environment. The

1 organ can expel, in a controlled way, water containing organic and inorganic
2 molecules but can also absorb exogenous compounds from the
3 environment. Molecules can penetrate the skin in three ways: through the
4 cells (intracellular way), via the inter-cellular space (intercellular way) or
5 through the skin pores, glands and hair follicles (transappendageal way)
6 [43]. In order to understand how molecules can enter the skin, several
7 models have been proposed. In 1992, Auton tried to construct a
8 mathematical model of skin penetration considering either diffusion or
9 metabolism of the compound passing through the skin layers [44]. He
10 considered two main barriers in his skin model 1) the external barrier of the
11 skin, the *stratum corneum* - this physical barrier lets lipophilic compound to
12 pass more easily than hydrophilic molecules -, 2) the second barrier made
13 up of living cells in the epidermis and the dermis where exogenous
14 molecules are subjected to enzymes such as esterase and oxygenase. In
15 2001 a more complex model, called “single gel phase” was proposed by
16 Norlén and colleagues to describe the *stratum corneum* of the skin [45].
17 This model is in accordance with the Michaels’ “brick and mortar” one.
18 Other approaches, focused on how different chemical compounds can
19 penetrate the *stratum corneum*, were developed by Trommer and Neubert
20 [46]. Mathematical models of skin permeability have been summarized in a
21 complete review by Mitragotri and collaborators [47].

22

23

2.5 The skin microbiota

24

25 Thanks to the previously described structure, the skin possesses a unique
26 and complex microenvironment. Because of the low water content of the
27 *stratum corneum*, the low physiological pH, lysozyme and RNase
28 production, skin is a poor substrate for microbial colonization. However,
29 even in healthy conditions, the skin is host of commensal or symbiotic
30 bacteria [48] at the epidermal but also at the dermal level [49]. After birth,
31 the skin is directly exposed to environmental microorganisms and rapidly
32 colonized by some of them leading to a balance that is unique to each

1 individual. In adults, considering the skin appendages such as follicles and
2 sebaceous glands, the total exposed surface is estimated to be 30 m². This
3 makes our external organ the largest epithelial surface for interacting with
4 microorganisms [50]. Different types of microorganisms can be found in
5 different sites of the skin, depending on humidity, sweat production and
6 local temperature. Commensal bacteria not only protect skin from pathogen
7 bacteria colonization, avoiding thus skin diseases, but play also a symbiotic
8 role with this organ [51, 52] that is currently under investigation [53, 54].
9

10 **2.6 Skin homeostasis and aging**

11
12 The whole skin wellness resides on the physiological equilibrium of all the
13 mentioned biochemical, bio-physical and symbiotic parameters. Although
14 the skin is a powerful organ able to rapidly regenerate, the continuous
15 biological, chemical and physical aggressions weaken its structure over
16 time.

17 Skin aging is a natural process driven by our genes (chronological aging),
18 that can be drastically accelerated by our way of life, sun and pollutant
19 exposures [55] more largely named exposome [56]. This aging process is
20 mainly mediated by the oxidative stress, an overproduction of ROS able to
21 damage biological molecules such as lipids, proteins and nucleic acids and
22 inducing the alteration of their activity. Typical signs of time in skin are the
23 loss of elasticity and hydration, the slowing down of cell metabolism and the
24 alteration of melanocyte activity. All these changes lead to a decrease of
25 the complexion radiance and the occurrence of light or dark spots and the
26 appearance of wrinkles.

27 Since the dawn of humanity, people tried to find methods to hide the signs
28 of senescence. Cosmetic treatments have always been the answer to slow
29 down skin deterioration process and ameliorate its aesthetic appearance.
30 To achieve such goal, besides cosmetic products, new physical
31 technologies such as LED- or ultrasounds-based devices have recently
32 been developed. Among these, cold atmospheric plasma may be an

1 innovative and interesting approach in this field. To know CAPs
2 characteristics and how they can interact with the skin, the following chapter
3 will provide some fundamentals about these ionized gases.
4

5

6 **3. Plasma bases and biomedical applications**

7

8 Understanding the physicochemical aspect of plasmas, and the
9 mechanisms involved in the interactions between them and biological
10 tissues, is primordial in order to use them on skin in efficient and secure
11 conditions

12

13 *3.1. Introduction to plasmas*

14

15 The stars, the sun, the lightning and the aurorae have always fascinated
16 human being. All of these wonder of the nature share a common feature,
17 they are forms of plasma: the fourth state of the matter. From the first
18 attempt to artificially generate and master these ionized gases, plasmas
19 have been exploited in different fields. The first artificial plasma was
20 generated by the German physicist Johann Heinrich Wilhelm Geissler who
21 created the ancestor of the fluorescent tube in 1857. Based on the Geissler
22 tube, William Crookes built the first cathode tube in 1879. In his high
23 vacuum tube, Crookes observed that the rarefied gas, exposed to the high
24 voltage between two electrodes, emitted a weak light at the cathode. Since
25 he did not know that the light was due to the electrons flowing from the
26 cathode, he called them “cathode rays”. Crookes understood that the
27 rarefied gas inside the tube, exposed to the high voltage, was in a particular
28 state that he described as a fourth state of the matter or “radiant matter”.
29 Gases possess almost no interatomic or intermolecular forces. Since they
30 are formed by neutral atoms or molecules, gases are perfect electric
31 insulators. In 1889, Friedrich Paschen studied the loss of the insulating

1 property of the gases exposed to a high electric field between two planar
2 electrodes. Paschen called “breakdown voltage” the minimum voltage
3 needed to generate a discharge in the gas. This voltage is proportional to
4 the gas pressure and the distance between the two electrodes [57]. Arc
5 generation is due to the ionization of the gas with consequently a loss of the
6 insulating property. It's only in 1928 that the chemist Irving Langmuir
7 described the state of a rarefied gas exposed to a high voltage between two
8 electrodes. In his paper, Langmuir wrote that *except near the electrodes,*
9 *the ionized gas was made up of an equal mix of ions and electrons* and
10 proposed to use the term of plasma for this state of the matter [58]

11

12

3.2 Hot and cold plasmas.

13

14 To generate a plasma, a gas must be supplied with enough energy to ionize
15 it. The gas could be heated at very high temperatures in order to permit the
16 external electrons to escape the atoms. The electron and the ionized atoms
17 can interact with other atoms creating a cascade reaction. Thus the
18 generated plasma is a mix of electrons, ions and neutral species. Although
19 it is formed by charged species, the plasma is neutral on its whole. As
20 described previously, a gas can be ionized and form plasma also under a
21 strong electric field generated between two electrodes. Plasmas can be
22 divided in two main categories: thermal and non-thermal plasmas. Most of
23 the natural occurring plasmas belong to the thermal category, these hot
24 plasmas possess electrons and heavy particles at the same temperature.
25 Artificial hot plasmas are produced for some applications such as hard
26 material cutting and toxic waste destruction [59, 60]. In non-thermal
27 plasmas (NTPs), free electrons possess a higher temperature than ions and
28 neutral gas molecules, these latter conferring a relatively low temperature to
29 the whole plasma. Thanks to this characteristic, NTPs are used in a
30 plethora of industrial applications: micro-electronics, lighting, surface
31 treatment and functionalization. Most NTPs conceived for industrial
32 purposes are produced using low pressure gases inside reactors. The

1 controlled atmosphere inside the reactors results in a well-controlled
2 plasma generation. NTPs, can also be generated at atmospheric pressure
3 and are called Cold Atmospheric Plasmas (CAPs).

4

5 *3.3 Cold Atmospheric Plasmas generation and characterization*

6

7 CAPs are mainly generated by means of energy coming from electric
8 alternate or direct currents, radiofrequencies or microwaves. Several
9 methods and configurations are today used to produce these ionized gases:
10 volume and surface Dielectric Barrier Discharge (DBD) [61, 62],
11 Atmospheric Pressure Plasma Jets [63], plasma needles and Plasma
12 Pencils [64] (figure 2A, B, C). These different configurations, together with
13 the choice of the feeding gas, result in a plasma having peculiar
14 characteristics. Commonly used gases are atmospheric air, pure nitrogen,
15 noble gases or custom gas mixtures of these latter. Once the gas is ionized
16 to plasma, the charged species can react with the matter (target) with which
17 they enter in contact. In the case of CAPs the first encountered medium is
18 the atmospheric air. Reacting with the nitrogen and oxygen composing the
19 air, CAPs produce the so-called Reactive Oxygen and Nitrogen Species
20 (RONS) (Figure 2 D). Plasma treatment can be performed on several types
21 of targets, either solids or liquids. When CAPs interact with a solid surface,
22 the generated reactive species can modify, charge or ablate the external
23 layers of the target. These modifications can change the physical
24 characteristics of the surface modifying for example its wettability [65].

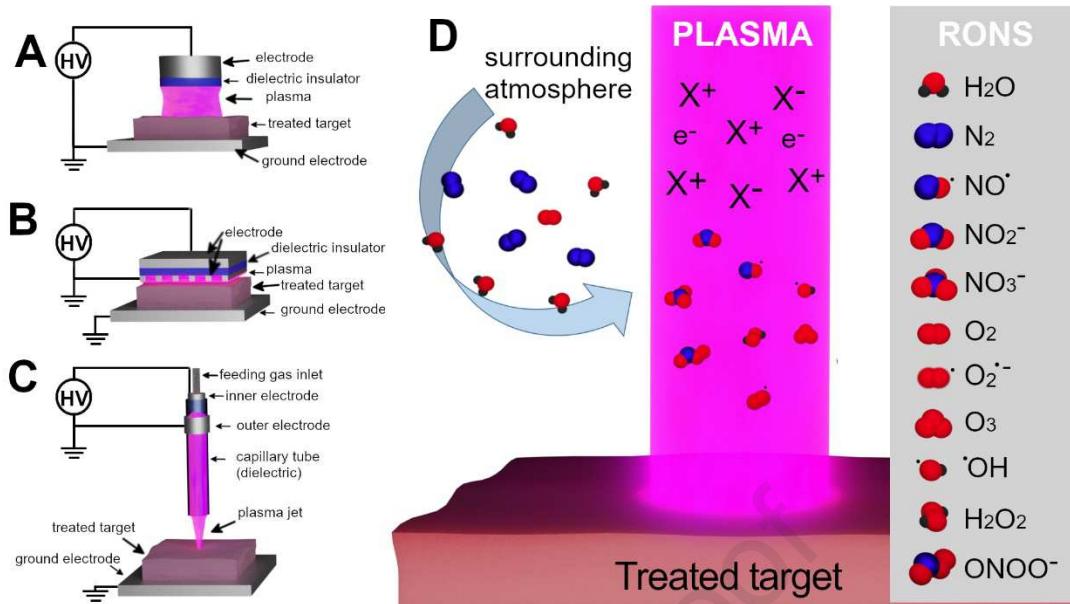
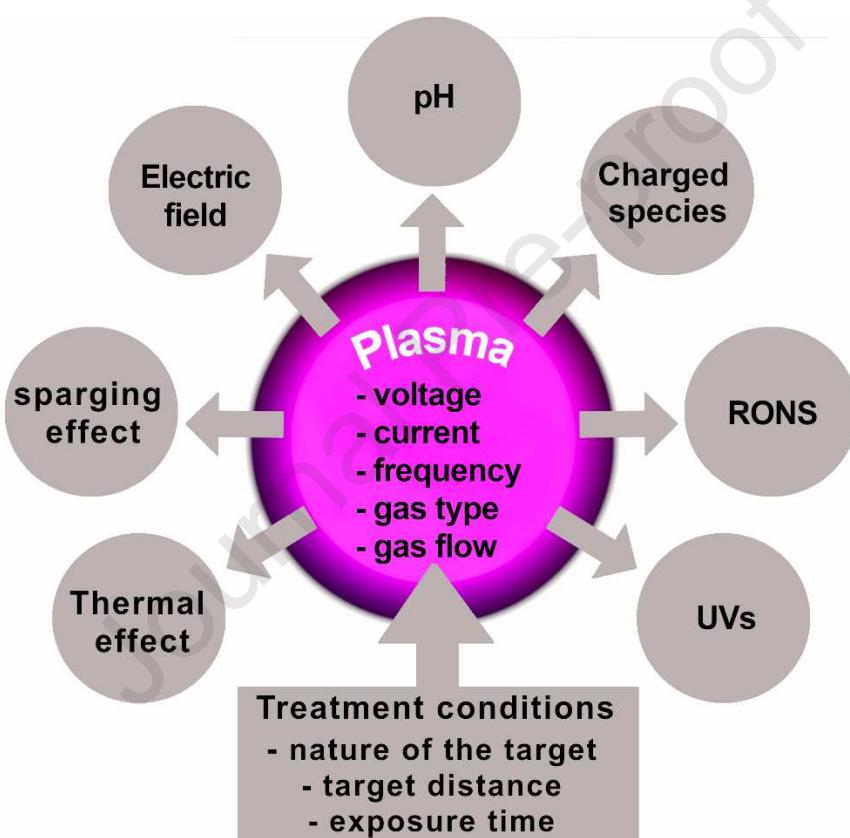


Figure 2. Cold atmospheric plasma devices and plasma chemistry. Most commonly used CAP-setup for bio-medical applications: A) Volume dielectric barrier discharge reactor; B) Surface dielectric barrier discharge reactor; C) Plasma jet reactor. (HV) High Voltage generator. D) The plasma chemistry: the ionized gas forming a plasma, with its negative and positive ions (X^+ , X^-) and free electrons (e^-), generates a mix of reactive oxygen and nitrogen species (RONS) from the atmospheric gases and water vapor.

Aqueous media exposed to a CAP are enriched of new chemical species. Once generated in the surrounding atmosphere, RONS can be carried and solubilized in the liquid medium. [66-68]. Some species such as hydrogen peroxide (H_2O_2) are produced directly from water vapor entering in contact with the plasma effluents [69]. Oxygen already dissolved in the liquid medium also participate to RONS generation [70, 71]. The amount of generated and solubilized species is proportional to the plasma working frequencies [70, 72], the voltage waveform, the CAP exposure time [72, 73] and the distance from the treated target . Gases passing to the state of plasma emit electromagnetic radiations in the visible and infra-red spectrum. Low amount of UV are commonly generated together with other visible radiations whose wavelength depends on the feeding gas used to produce the plasma. Since CAPs are generally ignited by a strong electric

1 field, once the plasma is generated, the movement of the charged species
 2 produces itself an electric field. Lastly, although these plasmas are named
 3 cold, they generate thermal energy and develop temperatures between 30°
 4 and 100°C. While temperatures higher than 40°C are not suitable for the
 5 treatment of mammalian tissues, tuning the gas flow rate and the distance
 6 of the CAP source from the target permits to avoid overheating effects. The
 7 complex interplays between CAP and the treated target are summarized on
 8 figure 3. In view of the above mentioned characteristics CAPs can be
 9 compatible with applications on biological tissues.



10
 11
 12 **Figure 3. The interplays between CAP and the treated target.** CAP characteristics
 13 intrinsically depend on the electrical setup, the feeding gas and its flow rate. The fine
 14 tuning of these parameters influences the amount of the produced charged species and
 15 RONS, the CAP-generated electric field, the acidification and de-gasification (sparging) of
 16 the target. Although marginal, the emitted UVs and the small thermal increase can play a
 17 role in the administered treatment. In addition, the treatment conditions such as the
 18 nature of the target, the plasma-target distance and the exposure duration also strongly
 19 influence the CAP behavior and performances.

1 Taking into account all the above-mentioned parameters and knowing that
2 each research laboratory developed its own CAP device, the plethora of
3 data obtained with these sources are not easy to compare. Moreover,
4 depending on the selected technical parameters, such as voltage,
5 frequency, carrier gas composition and gas flow, as well as at different
6 temperatures and humidity, a single individual device also produces very
7 different plasmas inducing different effects. Conscious of this
8 heterogeneity, in the following paragraphs we will make use of the generic
9 term of CAP to indicate different devices and settings employed to
10 generate a Cold Atmospheric Plasma.

11

12 3.4 Biomedical applications of plasmas

13

14 Plasma application in the bio-medical field is called plasma medicine [74]. In
15 the last decade this field has rapidly expanded. CAPs have shown
16 promising results in cancer therapy either *in vitro* or in clinical case studies
17 [75-80], being more sensitive to RONS than normal cells, cancer cells can
18 be selectively killed by plasma exposure [81, 82] Non-thermal plasmas for
19 biomedical applications can be used in indirect or direct configuration. In
20 indirect configuration, the long lived reactive species generated between the
21 electrodes are carried to the target thanks to the gas flow. In direct
22 configuration, the biological target is one of the two electrodes and actively
23 participates to plasma generation [83, 84]. Although CAP effluents can now
24 be brought inside the body by means of catheters for *in situ* treatments [85,
25 86], at the beginning plasma medicine was conceived for surface, non-
26 invasive applications. Most of the *in vivo* studies are performed by treating
27 directly the external tissues of the body. Besides cancer applications, CAPs
28 are also used in dermatology with promising effects on chronic wound
29 disinfection and healing [87-94]. Lately some CAP devices have been
30 developed for skin regeneration [95, 96]. These plasma sources, operating
31 with atmospheric air, nitrogen or argon, can reach temperatures higher than
32 60°C and are mostly used in skin resurfacing. Like laser resurfacing, this

1 type of cosmetic plasma treatment is mostly used to burn the outer layers of
2 the skin and force its renewal [97-100]. Although plasma resurfacing is a
3 less invasive alternative to facial plastic surgery, the high temperatures, the
4 long recovery after treatment and the limited trial data on long term side
5 effects suggest a careful use of these treatments [101]. Whereas the above
6 mentioned plasma treatments work by a mechanic ablation of the dead
7 outer skin layers, some recent evidences suggest that mild CAP treatments
8 may actually stimulate the deeper layer of the skin and play an anti-age role
9 on skin cells [102]. Hence, plasma applications in dermatology and in
10 cosmetic are, today, hot topics.

11

12 4. Cold Atmospheric Plasma activity on skin

13

14 As previously mentioned, CAP feature is under the dependency of several
15 parameters of the device such as the voltage and the pulsed frequency.
16 The characteristics of the generated plasma are modified by the
17 environment and the gas composition, the nature of the target, the distance
18 from the target and the treatment time (figure 3). This results in differences
19 in terms of RONS nature and amount, in the generated electric field and so
20 in a different effect on the treated biological tissue. This is why it is
21 important to understand how plasma chemistry and physics can interact
22 with the skin and how the different parameters can be adapted to achieve
23 the desired effect.

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1 *4.1 Effects of CAP-generated RONS on biological components*2
3 *4.1.1 On biomolecules*4
5 Liquid physiological media treated by plasma are enriched in RONS
6 species and have been shown to carry out interesting biological activities
7 on cells and tissues [103, 104]8 Among the CAP-produced RONS, nitrites (NO_2^-), nitrates (NO_3^-), nitric
9 oxide (NO^\bullet), hydroxyl radical ($\cdot\text{OH}$), superoxide anion ($\text{O}_2^{\bullet-}$), singlet
10 oxygen (${}^1\text{O}_2$), hydrogen peroxide (H_2O_2) and ozone (O_3) are of great
11 interest in biology [105, 106].12 Biomolecules, in contact with the plasma-generated RONS, can be
13 chemically changed, inactivated or irreversibly damaged [107, 108]. Takai
14 and collaborators analyzed the chemical structure of water-dissolved
15 amino acids after CAP exposure and demonstrated that most of the
16 treated amino-acids can be oxidized and acquire new chemical groups by
17 hydroxylation or nitration [109]. The amino acid cysteine, involved in the
18 redox activities of enzymes and abundant in the keratin, has been shown
19 to be oxidized by CAP exposure [110]. Since amino acid can be modified
20 by cold plasma treatment, the whole proteins exposed to CAP can be
21 altered either at their primary amino-acid sequence or at their secondary
22 structure. These modifications can change the activity or induce a loss of
23 function of the protein [111-114].24 Lipids have also been shown to be oxidized by CAP exposure [115-117].
25 Phospholipids composing cell membranes can directly be peroxidized by a
26 non-thermal plasma treatment [118]. This oxidation can temporarily
27 change the permeability of the cell membrane facilitating the penetration of
28 exogenous molecules [119]. Lipid oxidation is a phenomenon that
29 naturally occurs during oxidative stress and chronic inflammation [120-
30 122].31 CAP-induced modifications and damages were observed equally in nucleic
32 acids [123, 124] although strong DNA damages have been observed after

1 long plasma exposure [125]. Taking together, these CAP-induced effects
2 on biological molecules underpin the toxic impact that plasma can have on
3 living matter. Although cold plasmas are a source of potentially toxic
4 reactive species, CAPs delivery can induce a hormesis effect [126] since a
5 proper use of these ionized-gas source, with adapted and controlled
6 conditions, can bring beneficial effects on the treated biological tissue.

7

8

9 *4.1.2 On cell metabolism.*

10

11 As already mentioned, a proper CAP treatment can, for example,
12 accelerate wound healing or selectively kill cancer cells with little or no
13 impact on normal cells [81, 82, 127, 128]. CAPs have been largely used to
14 treat easily accessible tissues and organs and therefore they are suitable
15 to treat the skin, the external organ of our body. Being exposed to the
16 external environment, the skin is continuously stressed by physical and
17 chemical insults. Every day the epidermis is exposed to UV radiations,
18 ozone, cigarette smoke, pollutants, organic solvents or alcohols that
19 directly generate or induce the formation of large amount of ROS [129].
20 Beside these exogenous sources, cellular metabolism also generates in a
21 lesser extend endogenous free radicals [130]. Human and animal tissues
22 produce reactive oxygen species such as O_2^- , 1O_2 , H_2O_2 and O_3 [131].
23 These ROS are mainly produced in mitochondria during cell respiration
24 [132]. Although these highly reactive species can be harmful, the cells are
25 able to neutralize free radicals in excess using their anti-oxidant system.
26 Moreover our body can adapt to chronic exposure of toxic compounds.
27 Human adaptation to lethal poisons by ingestion of sub-lethal doses was
28 known thousands of years ago thanks to Mithridates and his Antidotum
29 Mithridaticum [133]. In mitochondria the adaptation to chronic exposure of
30 small doses of ROS is a phenomenon called mitohormesis [134, 135].

31

1 *4.1.3 On skin cellular signaling*

2
3 Besides their damaging role, well known as oxidative stress when in large
4 amount, ROS are required molecules for cell viability and activity. In fact,
5 right amounts of ROS play some physiological effects as they can act as
6 second messengers [136-138], stimulators for stem cells proliferation [138,
7 139], and as a booster for the immune system [140, 141]. In injured skin,
8 the immune system cells entering in contact with pathogens, start to
9 produce large amounts of RONS. This phenomenon called “respiratory
10 burst” helps wound disinfection. Correct wound healing depends on the
11 equilibrium between ROS generation and the activity of the antioxidant
12 system [142]. Among the reactive nitrogen species (RNS), the small
13 gaseous molecule NO[•] possesses several biological activities [143] acting
14 as a signaling molecule [144], a vasodilator [145], an angiogenesis
15 modulator [146], an immune system stimulator [147] and a melanogenesis
16 enhancer [148]. In skin NO[•] is also involved in hair growth, in the
17 proliferation and differentiation of epidermal cells and in wound healing
18 [149, 150]. Nitrates present in sweat can be converted in nitrites and then
19 in NO[•] thanks to the skin bacterial microflora [28] or directly from the
20 photo-decomposition of nitrites [151, 152]. NO[•] derived from photo-
21 decomposition of nitrites has been shown to protect human skin cells from
22 lipid peroxidation and thus apoptosis induced by UVA exposure [152, 153].
23 However an over production or a high exposure to these reactive species,
24 can be harmful [154, 155]. Indeed high doses of ROS can contribute to
25 the development of several pathologies such as psoriasis [156-158].
26 Moreover the oxidative stress in skin can accelerate the natural process of
27 ageing [159, 160]. As cold plasmas generate a heterogeneous mix of
28 RONS and considering the “Janus effect” of most of the above mentioned
29 reactive species, the final effect on the treated tissues will depend on the
30 amount of the released reactive species. CAP treatment has been shown
31 to induce an oxidative stress in human keratinocytes [161] but the same
32 cell line exposed for short periods to a helium-fed CAP with a low flow rate
33 increases its viability [126]. Moreover, Schmidt and collaborators observed

1 a hormesis-like increase of the antioxidant system in human keratinocytes
2 cells exposed to an argon fed CAP [162]. In light of the biphasic effect of
3 CAP treatment on skin, mastering the delivery of CAP-produced RONS is
4 a challenge in dermatology and in cosmetic where CAP could be a
5 powerful tool.

6

7

8 4.2 CAP influence on skin proliferation and motility

9
10 Cutaneous cell proliferation is important for epidermis turnover and to
11 ensure the healing process in case of skin injuries. When correctly
12 administered, CAP treatment can stimulate these processes. Short
13 exposures to a helium-fed CAP were shown to stimulate HaCaT human
14 keratinocytes proliferation and motility [126, 163]. A beneficial effect of
15 plasma treatment on HaCaT cells was also observed with the use of
16 argon-DBD. Choi et al. showed that CAP treatment inhibits the E-caderin-
17 mediated intercellular junctions and activates a β -catenin-mediated
18 proliferative signal. This plasma-activated proliferative pathway
19 accelerates *in-vivo* re-epithelialization in mice wound [164]. Furthermore,
20 short treatments (1-3 min) with argon-based plasma jet were shown to
21 increase the proliferation of the *stratum basale* keratinocytes in human,
22 intact skin explants [165]. On dermal fibroblasts very short treatments
23 were shown to increase the proliferation [163, 166] while treatments of few
24 minutes exert a toxic effect [166, 167]. The higher sensitivity of fibroblasts
25 to CAP treatment, when compared to keratinocytes, can be explained by
26 the fact that these cells reside in a deep layer of the skin. Being protected
27 in the deep dermis, fibroblasts are less equipped to endure an external
28 oxidative stress. *In vivo*, skin fibroblast can be directly exposed to CAP
29 only in case of wounds. Moreover, in wound treatments, the CAP anti-
30 proliferative effect can be advantageous to avoid the anti-aesthetic side
31 effects of the healing process such as excessive scarring [168].

1

2 *4.3 CAP influence on skin oxygenation*

3

4 CAPs have been shown to influence the oxygen content in the treated
5 target. In plasma jets, the gas flow rate exerts the so-called “sparging
6 effect”, de-oxygenating the treated liquid [70, 169] while *in vivo* CAP
7 applied on mouse skin has been shown to enhance underneath tissues
8 oxygenation [170]. An increase of the post capillary oxygen saturation
9 after plasma treatment has also been observed in human skin [84, 171].
10 The mechanism on how the plasma source increases *in vivo* skin
11 oxygenation is still unclear. As previously mentioned CAPs are not really
12 cold sources and the devices used in biology develop temperatures
13 between 30° and 40°C. The application of this relatively warm sources on
14 the skin could induce a local vasodilation and so the increase of tissue
15 oxygenation. Furthermore, since CAP directly or indirectly induces NO[•]
16 formation, the vasodilator molecule could be also responsible of the
17 observed phenomenon. A correct oxygenation is fundamental for a proper
18 cellular metabolism. Some skin cosmetic therapies are based on the
19 increase of tissue oxygenation. Hyperbaric Oxygen Therapy, already used
20 for decompression sickness, is currently used in skin-rejuvenation. The
21 administration of pressurized oxygen seems to protect the skin from UVB-
22 induced photoaging [172]. However skin oxygenation needs to be carefully
23 controlled. Oxygen has a double face and its excess can generate an
24 oxidative stress accelerating the ageing process. Moreover skin stem cells
25 need a very low oxygen partial pressure to keep their stemness [23] and
26 thus ensure the renewal of the epidermis.

27

28 *4.4 CAP effect on skin vascularization and extracellular matrix*

29

1 Skin oxygenation is directly related to the vascularization of the organ. A
2 healthy skin possesses a well-organized vasculature. With the age, skin
3 microcirculation reactivity such as vasodilation and vasoconstriction as
4 well as vascular density are impaired [173]. The inability to handle ROS
5 generation and the endothelial oxidative stress seem to be one of the
6 reason of the loss of this age-related vessels functionality [174]. Although
7 ROS are down regulators of vascular endothelial functions, CAP
8 treatments, in right conditions, seem to play a stimulating action. Human
9 Umbilical Vein Endothelial Cells (HUVEC) were shown to release pro-
10 angiogenic factors and increase *in vitro* angiogenesis when exposed to an
11 argon-fed CAP for 30s [175]. An increase of proliferation was also shown
12 in porcine endothelial cells where a 30s treatment with an air-DBD induced
13 the release of the fibroblast growth factor-2 (FGF2) [176]. The CAP-
14 angiogenesis stimulatory effect was recently showed by Dzimitrowicz et al
15 who used a He-fed CAP on human endothelial cells for very short
16 treatments (10s) [163]. *In vivo*, since endothelial cells are not directly
17 affected by plasma treatment, the increase of angiogenesis could be
18 induced via a paracrine mechanism mediated by keratinocytes exposed to
19 plasma treatment [175, 177]. The small molecule NO[•] also plays a role in
20 angiogenesis. NO[•] can acts as a pro- or anti-angiogenic factor depending
21 on its amount [146, 178, 179]. Moreover NO[•] can increase the synthesis of
22 collagen IV and activate endothelial cells adhesion [180] . Duchesne et al
23 demonstrated that CAP treatment stimulates endogenous NO[•] synthesis in
24 *in vitro* and *in vivo* models. Indeed, in a murine burn wound model,
25 plasma-generated RONS was shown to increase the expression of the
26 endothelial nitric oxide synthase (eNOS). The increase of the enzyme
27 producing endogenous NO[•], together with proangiogenic factors, speeds
28 up the healing process [181]. In wounds, the extracellular matrix (ECM) of
29 the dermis is directly exposed to CAP treatment and the improvement of
30 the healing process could depend on a direct modification of the ECM
31 properties. Ring and collaborators showed that plasma-treated collagen-
32 elastin matrix scaffolds implanted under mice skin induce an enhancement
33 of neovascularization [182]. In addition, a well-organized ECM is also
34 essential to keep a youthful looking skin. Murine fibroblasts directly

1 exposed for 15s to an argon-CAP were shown to increase the expression
2 of collagen I and III while slightly longer treatments exert an opposite effect
3 [166]. Unlike wounds, in intact skin CAP cannot directly influence the
4 extracellular matrix metabolism. However some *in vivo* studies suggested
5 that CAP can indirectly induce dermal remodeling. An increase of dermal
6 collagen content was observed in intact mouse skin exposed to an argon-
7 DBD plasma [183]. Type I collagen is the major component of the dermis.
8 While the neo-synthesis and a well-organized network of the fibrillary
9 protein is a sign of a young and healthy skin, its overproduction is
10 sometimes the expression of skin damages and diseases. Interestingly
11 CAP is able to stimulate physiological collagenogenesis while inhibiting the
12 pathological synthesis of the protein. *In vitro* and *in vivo* studies have
13 shown this selective effect in dermo-aesthetic disorders such as scars and
14 keloids [184, 185].

15

16 5. Cold atmospheric plasma effect on skin surface

17

18 5.1 CAP-induced pH modification

19

20 In addition to their stimulating/oxidizing effects, CAP-generated RONS
21 were shown to induce an acidification of the treated target. A decrease of
22 the initial pH is commonly observed in non-buffered or weakly buffered
23 treated liquids [186, 187] and in hydrated 3D matrices [188]. The pH-drop
24 can be mainly attributed to acidic species originating from the precursor
25 NO[•] that generates nitric (HNO₃) and nitrous (HNO₂) acids in solution
26 [187]. The induced acidification is proportional to the plasma exposure
27 time. It has been shown that the pH of alkaline or neutral non-buffered
28 media drops rapidly after few minutes of CAP treatment. The quick pH-
29 decrease tends to stabilize at pH values between 3.5 and 2.5 thanks the
30 transient formation of HONO/ONO⁻ buffer and to the generation of nitrous
31 acid (pKa 3.3) [187-189]. In light of the above, CAP exposure can lower
32 the pH of biological tissues.

Pork skin sebum and human lipids can be rapidly acidified when exposed to an air-fed CAP [190]. The CAP-induced acidification was also confirmed by clinical trials on intact human skin [84, 150]. Thanks to these acidifying properties, CAP treatment can contribute to keep the skin in healthy conditions. Indeed, by lowering the pH, cold plasmas can stimulate and accelerate skin renewing. In acute skin wound, a physiological acidification was shown to enhance the proteases activity and stimulate fibroblast proliferation [191]. While skin pH higher than the physiological values can lead to pathologies, very acidic pH can burn the external tissues of the organ. In order to avoid chemical burns, skin exposure to CAP should be carefully controlled [150]. However, in cosmetic, chemical peel or chemexfoliation is used to gently ablate the external layer of the epidermis and force skin renewal. This approach uses often organic acids to lower the pH and peel off the epidermis layers [192]. A well administered plasma treatment could exert a similar noninvasive exfoliating effect. Moreover, as the pH of the acidic mantle increases with the aging process leading to a weakening of this barrier [193], CAP treatments could be beneficial to re-establish the physiological pH barrier and stimulate the regeneration in mature skin.

5.2 CAP effect on bacterial decontamination and skin disinfection

Cold atmospheric plasmas possess a well-known bactericidal activity [194-197]. In CAP-treated solutions such as plasma-activated water (PAW), short and long lived species react with each other and create a powerful anti-microbial mix. Some authors reported that the biocidal properties mostly derives from a combination of oxidative and nitrosative effects induced by the synergistic activity of H_2O_2 and NO^* [198]. Zhou et al. demonstrated that the biocidal effect is achieved by the combination of H_2O_2 and NO_2^- . The two molecules alone possess a very weak anti-bacterial activity while reacting together they can form peroxynitrite ($ONOO^-$), an unstable isomer of NO_3^- . Peroxynitrite was described as the key species in PAW-induced bacterial damages [199]. Moreover in PAW

the protonated form of peroxy nitrite, the peroxy nitrous acid (ONOOH), can be further oxidized by H₂O₂ and produce the peroxy nitric acid (O₂NOOH), a stronger bactericidal molecule [200]. Within the ROS, O₃, mainly produced by air-fed CAPs, also contributes to the plasma-induced biocidal effect [201]. While bacteria directly exposed to plasma can be quite easily killed by the chemical attack of short and long lived RONS, in real life most of these microorganisms are protected by biofilms. Biofilm is a complex consortium of various microorganisms growing on a substrate. Bacteria are embedded in a sort of clammy extracellular matrix made up of extracellular polymeric substances such as polysaccharides, lipids, protein and DNA that are meant to protect bacteria from dehydration and external environment insults. Thanks to this physical barrier, bacteria are more resistant to antibiotic treatments. *In vitro* tests have shown that CAP is able to destroy biofilms of skin pathogens such as the *candida albicans* yeast [202] and the *staphylococcus aureus* [203]. In direct CAP treatment, biofilms are not only exposed to the highly reactive species but also to an intense electric field that can destabilizes the physicochemical structure of the microorganisms [204]. Interestingly, the CAP ability to destroy biofilms has been demonstrated also *in vivo* in wounds that are not any more responsive to common antibiotics [93, 205, 206]. Although CAP treatment can be efficiently used to deteriorate biofilms, some bacterial strains could adapt and tolerate plasma treatment stress. Tailoring of CAP devices and treatment conditions are to date a big challenge for a safe and efficient biofilm decontamination [207]. It was already mentioned that skin microbiota plays a physiological role on the intact organ. However when the symbiotic equilibrium between the skin and the microorganisms is disrupted, these latter can become pathogenic [208]. The previously mentioned acidic mantle controls this equilibrium by inhibiting the growth of pathogen organisms and by promoting commensal skin bacteria proliferation. Low pH is unfavorable to the growth of several pathogens, and the already discussed CAP-induced acidification enhances the antimicrobial activity of these sources.[187, 209-211]. Thanks to their anti-microbial activities, CAP devices can be used as an alternative and efficient method to sanitize intact skin [92, 212]. CAPs can inhibit the

1 growth of the anaerobic pathogen *Cutibacterium (Propionibacterium) acnes* involved in the oily skin inflammation known as acne vulgaris and in
2 other more severe pathologies [213]. A CAP-patented device was shown
3 to inhibit, *in vitro*, the growth of *Malassezia restricta* and *Malassezia globosa*, yeasts responsible for dandruff [214]. Onychomycosis caused by
4 the bacterium *Escherichia coli* and by the fungus *Trichophyton rubrum* was
5 successfully treated by a helium CAP [215]. In most cases pathogens like
6 the above proliferate in the deep skin appendages. The eradication of
7 these microorganism with topic antibiotic applications is often difficult since
8 the drugs fail to penetrate inside the deep structure of the skin. As will be
9 explained later, CAP treatment can overcome this issue.

12

13 *5.3 Plasma and skin hydration*

14

15 A healthy and functional skin needs the right amount of water. In the
16 dermis the hydration is guaranteed by the high hygroscopic GAGs such as
17 hyaluronic acid. In the epidermis, water content varies between 70% in the
18 viable part and 15-30% in the external layers [216]. The *stratum corneum*
19 is able to sense the environmental humidity and adapt its biochemical
20 metabolism [217]. Corneocytes, the dead cells composing the *stratum corneum*,
21 can keep water thanks to hygroscopic molecules composing the Natural
22 Moisturizing Factor and keratins [218, 219]. Corneocytes adhere
23 tightly each other avoiding massive water loss. Furthermore, intercellular
24 lipids such as ceramides create a hydrophobic barrier against desiccation
25 [220]. Cold plasma treatment can exert a double effect on skin hydration.
26 CAP can at first destabilize the skin barrier and desiccate the external
27 layer of the epidermis. A small, temporary water loss was observed *in vivo*
28 in human *stratum corneum* after plasma exposure [221]. Epidermis
29 desiccation is a desired effect in plasma skin resurfacing where the non-
30 ablated dried epidermis protects the thermally damaged layers during the
31 recovery process [222]. Paradoxically since CAPs can deposit charges on

the treated surface, it is likely that after a plasma treatment skin could attract more water molecules. Human *stratum corneum* wettability rapidly increases in the first seconds of plasma treatment [223]. The increased hydrophilicity was also demonstrated in finger nails where plasma treatment ameliorates the adhesion of cosmetic nail varnish [224].

5.4 RONS skin penetration and CAP-mediated percutaneous absorption

During CAP treatment, RONS can directly influence the skin cell physiology or mediate the activation of downstream responses. One of the hot topic in dermocosmetic plasma therapies is to understand the ability of these reactive species to penetrate and diffuse inside the skin layers. While short lived species rapidly react and deactivate in contact with the *stratum corneum*, long lived molecules such as H_2O_2 , NO_3^- , NO_2^- and NO^\cdot can diffuse deeply. Knowing how these molecules penetrate inside the skin during CAP treatment is of vital importance in treatment planning. Whether in dermatological therapies the deep penetration of these species is allowed, in pure cosmetic treatments the effect of RONS must be superficial and affects only the epidermis. Simple organic skin mimics such as agarose gel and gelatin have been used to quantify and measure the diffusion kinetics of the reactive species after CAP treatment [225]. These models permit to have an idea on the ability of RONS to cross the mesh of semi-solid gel but they overestimate the ability of these molecules to diffuse into the biological tissues. In real world, the physicochemical barriers of the skin can slow down the diffusion and neutralize most of the reactive species. Even using real skin, the exact quantification of RONS passing through the tissues can be distorted by the nature of the liquid medium used to collect and analyze them [226]. As already mentioned, molecules can cross the skin either through the cells, the intercellular space or via the external pores. Small species such as NO^\cdot , can passively diffuse through cell membrane, whereas some others can pass via channel proteins. Indeed, the water channel aquaporins [227, 228], were

1 shown to facilitate the cellular diffusion of H₂O₂ produced upon CAP
2 treatment [229, 230]. In addition, as previously evoked, thanks to lipid
3 peroxidation RONS can generate transient pores on the membrane and so
4 facilitate their own penetration. Furthermore, by destabilizing the cellular
5 membrane, the electric field generated during the plasma treatment
6 participates to the pore formation [231, 232].

7 Beside the direct role of CAP in skin treatment, cold plasma sources could
8 be also used to ameliorate the effect of topic dermocosmetic cares.
9 Thanks to their ability to destabilize the skin barrier, CAP were shown to
10 facilitate the transdermal delivery of other molecules with a mechanism
11 similar to electroporation [233-236]. This temporary loss of the epidermis
12 barrier could permit the intercellular diffusion of drugs or cosmetic actives.
13 Lastly, RONS and ointments can easily penetrate through the
14 appendageal way whose density depends on the body zone.

16 6. CAP delivery and skin electrical parameters

17 CAPs can behave differently depending on the electrical nature of the
18 target. [237-240] . Human organs possess different values of electrical
19 conductivity and this latter depends on the biochemical and biophysical
20 structure of the organ, on its water content and on its extracellular
21 electrolyte composition. In skin, the conductivity varies greatly in the three
22 different layers composing the organ. The *stratum corneum*, with its low
23 water content and its lipid composition, acts as a real dielectric barrier,
24 insulating the whole body from electric shocks. However as already
25 mentioned in the previous paragraph, CAP can temporarily increase the
26 conductivity of the epidermis promoting transdermal penetration of
27 molecules. The electroporation induced by high-voltage pulses lets also
28 the current flow deeper into the tissues. CAP-transported electric current,
29 penetrating the skin layers, could have beneficial effects. High frequency
30 electrotherapy was used since the early 20th century to treat several
31
32

1 dermatological conditions and other diseases [241]. It is known that a
2 certain electrical stimulation can speed up the wound healing process by
3 promoting dermal fibroblast motility [242, 243]. In addition, direct and
4 pulsed currents were shown to stimulate keratinocytes differentiation,
5 epidermis proliferation, vascularization and new collagen deposition [244,
6 245].

7 The interaction between CAPs and the skin is bidirectional, CAP can
8 modify the initial physico-chemical parameters of the targeted tissue while
9 the characteristics of this latter can influence the plasma performance
10 (figure 3). The knowledge of the body electrical parameters is crucial for
11 planning a safe, controlled and effective CAP treatment [240, 246]. We
12 already mentioned that the thickness and the structure of the skin change
13 along the body which is why the same plasma treatment can produce a
14 different effect depending on the treated zone. Moreover, the conductivity
15 of the skin itself can vary over the time according to its hydration and to
16 the temporary psychological and hormonal state of the treated person
17 [247, 248]. To overcome these variations, CAP delivery should
18 dynamically adapt to the electrical changes of the skin [249]. The applied
19 electric field has to be carefully chosen and controlled and will depend on
20 the desired effect on skin, either a stronger for dermatological use or a
21 lighter for cosmetic applications.

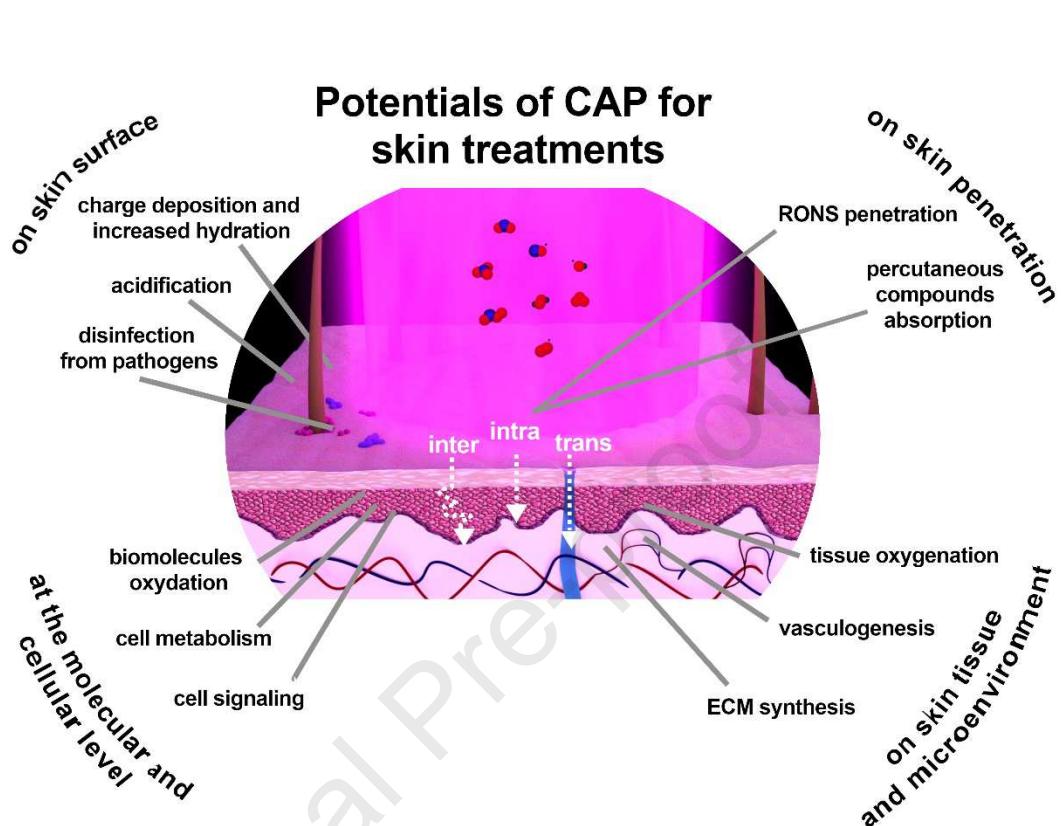
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23 7. Towards a potential role of CAP in skin biology and ageing prevention

24

25 In this review we tried to summarize the interactions between cold
26 atmospheric plasmas and the skin, based on the current knowledge of
27 their own properties and characteristics, highlighting the already known
28 and some other possible effects of these sources on the integumentary
29 system biology (figure 4). We described how the multiple physical
30 parameters involved in cold plasma can individually or together impact the
31 cutaneous microenvironment and skin cell activities. The coaction of all
32 these effects tends towards a beneficial role of CAPs on skin biology and

1 brings relevant arguments in favor of the use of cold plasma for restoring
 2 skin functional barrier and thus improving skin health and appearance.



5 **Figure 4. Potentials of CAP in skin biology.** CAP performs its activity at various levels
 6 of the skin. At a superficial level it promotes the hydration, acidification and
 7 decontamination of the stratum corneum. CAP-generated RONS can penetrate inside the
 8 skin via the intercellular way (**inter**), the intracellular way (**intra**) or via the
 9 transappendageal way (**trans**). By loosening the cutaneous barrier, CAP also promotes
 10 the absorption of other molecules such as drugs. At a molecular level, once penetrated
 11 into the skin, RONS can have a direct effect on skin biomolecule oxidation or activate cell
 12 metabolism and signaling. At the tissue level, CAP treatment lead to an increase in skin
 13 oxygenation, stimulates the vasculogenesis and the ECM remodeling or *de-novo*
 14 synthesis.

15
 16 In the light of these findings, one could ask: can CAPs be considered as a
 17 fountain of youth for the skin? Answering this question is not simple.

18 From a technical point of view, the interesting *in vitro* and *in vivo* data
 19 found in the literature come often from different cold-plasma devices.

Indeed, worldwide researchers have developed devices with different designs, gases and characteristics for plasma generation. This makes the biological results hardly comparable. Whether one specific device is better for one desired biological effect remains to be determined. Moreover, for a single device, parameters such as voltage, frequency and distance from the target also strongly influence plasma behavior and composition. In one hand, while these technical variables add a new level of complexity, in the other hand they also bring the possibility to modulate the plasma production. Moreover CAP physics and chemistry also depend on the operating conditions, such as ambient temperature and humidity. Thus the device should automatically adapt to the environmental conditions in order to deliver the same performance at every use. Hence, for each treatment, a systematic diagnosis of the device plasma composition should bring a new tool for the control and modulation of RONS generation. The development of means to finely analyze and control plasma delivery in interaction with skin with the support of modern plasma medicine studies should allow to determine the optimal conditions of use and contribute to the valuation of this technology for skin wellbeing. Finally this CAP “plasticity” can become a strong point for specific applications.

From a biochemical point of view, the mechanisms involved in plasma effect on cells and tissues still remains to be determined. Progress in this field are currently ongoing by making a precise diagnosis of plasma composition with the characterization of RONS produced in liquid or in tissues. The next step is to understand how these species are able to induce a biological effect either alone or in synergy [250]. The last challenging step is to master all the other CAP components (charged particles, UV, electric field, thermal emission) that cooperate to the final biological effect. Such rational approaches should strengthen plasma effects understanding and bring new insight in plasma applications for skin but also more generally in plasma medicine domain [251].

From a biological point of view and according to the results reported till now in the literature and presented here, CAPs should be a promising technology to stimulate and/or regenerate skin. However, as noted in this

1 work, relatively little is known about the molecular and cellular
2 mechanisms involved in the plasma-induced biological effects and about
3 the consequences of these treatments in the long term. Data obtained
4 from *in vitro* experiments are essential to understand the cellular signaling
5 activated by the CAP exposure. However, the biggest limit in the use of
6 cell cultures in plasma treatments is that most of the time the cells are not
7 directly exposed to the CAP. Cells are either treated through the
8 physiological medium covering them or put in contact with plasma-
9 pretreated medium. Thus, cells in culture receive only long-lived species
10 diffusing from the surface of the medium or dissolved in the plasma-
11 activated medium. Conversely, *in vivo*, the skin will be impacted by all the
12 CAP-produced species since the *stratum corneum* of the epidermis is
13 directly exposed to the atmosphere. To overcome this issue, the use of
14 skin explants or more complex skin models such as Reconstructed Human
15 Epidermis or Reconstructed Whole Skin could strengthen the
16 understanding of the CAP-activated processes *in vitro* and allow to
17 modulate the effects, define and correct the limits of this technology.
18 Finally, once clear cause-and-effect mechanisms are defined in these
19 studies, real life treatment must be adapted to each user as the skin
20 structure depends on the phototype but also on the age, sex and ethnicity.
21 Indeed dermo-cosmetic CAP-based treatments should be considered as
22 adapted and personalized therapies which are currently increasingly in
23 demand. Thus a new generation of plasma-based treatments could
24 emerge in the field of cosmetic and dermatology to improve skin aspect
25 and health.

26 Taking into account the flexibility of plasma generation, thanks to its many
27 parameters, CAPs could become new and promising treatments for skin
28 care and regeneration. This will go through an optimization of devices, in
29 terms of efficacy, control and safety for use as well as through the
30 deciphering of mechanisms involved in plasma-induced effects on the
31 skin. This innovative technology opens new areas of research at the
32 interface between plasma physics and skin biology and should lead
33 towards new applications in the dermo-cosmetic field.

1

2 Competing interests

3 The authors declare that they have no competing interests.

4

5

6

7 Abbreviations

8 CAP (Cold atmospheric Plasma), ROS (Reactive Oxygen Species), RNS
9 (Reactive Nitrogen Species), RONS (Reactive Oxygen and Nitrogen
10 Species), DBD (Dielectric Barrier Discharge), ECM (Extracellular Matrix)

11

12

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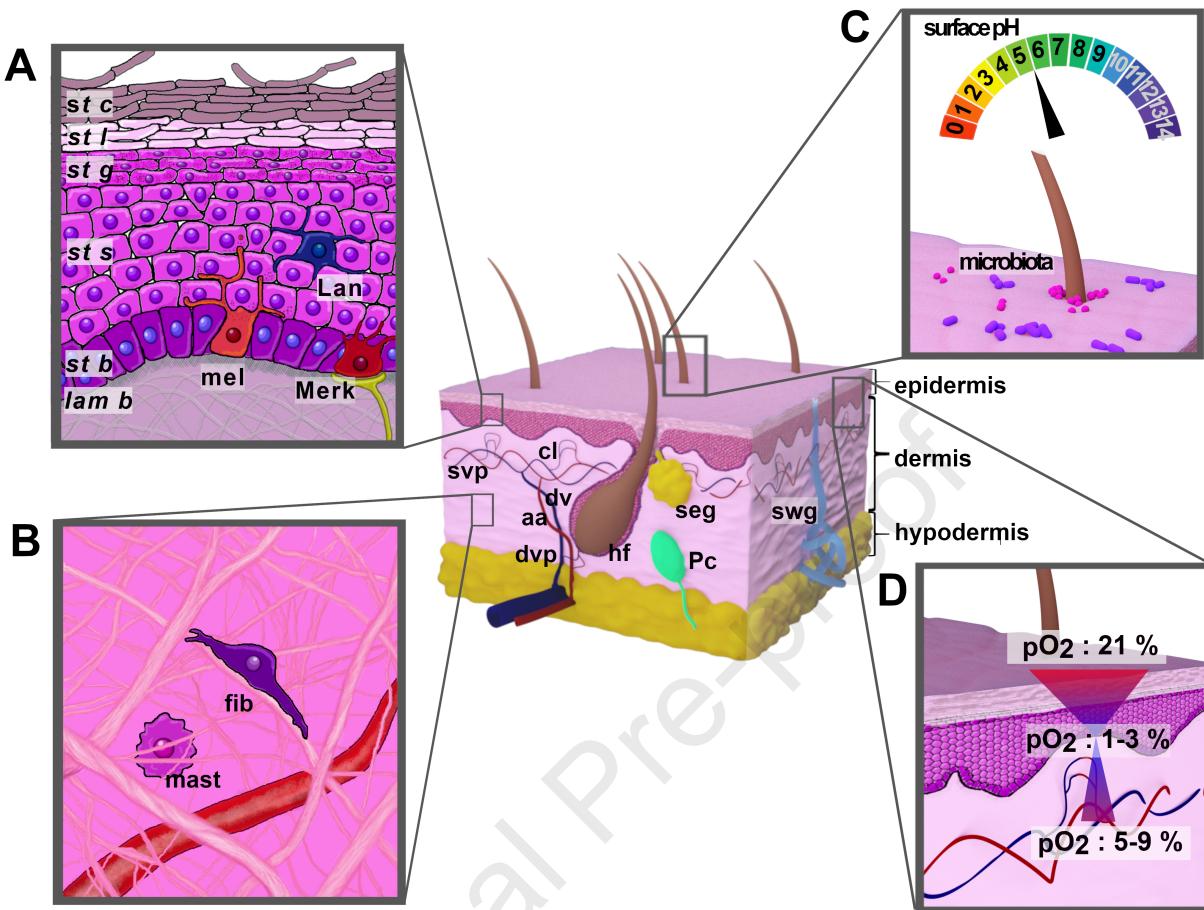
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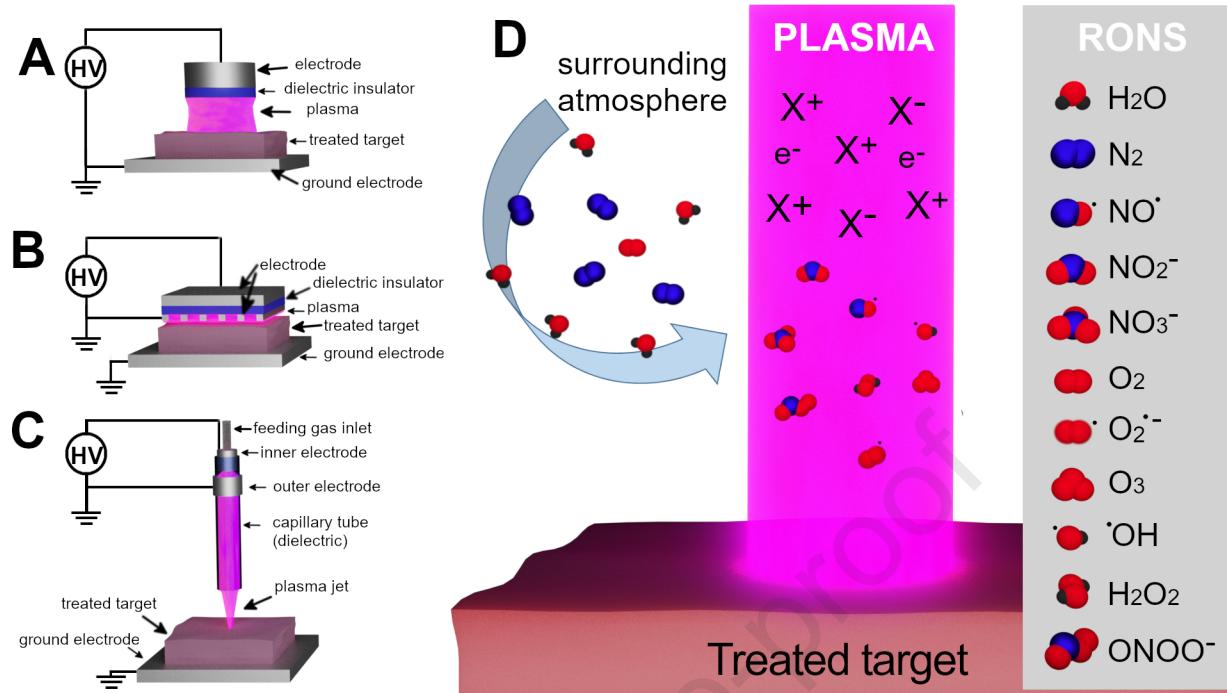
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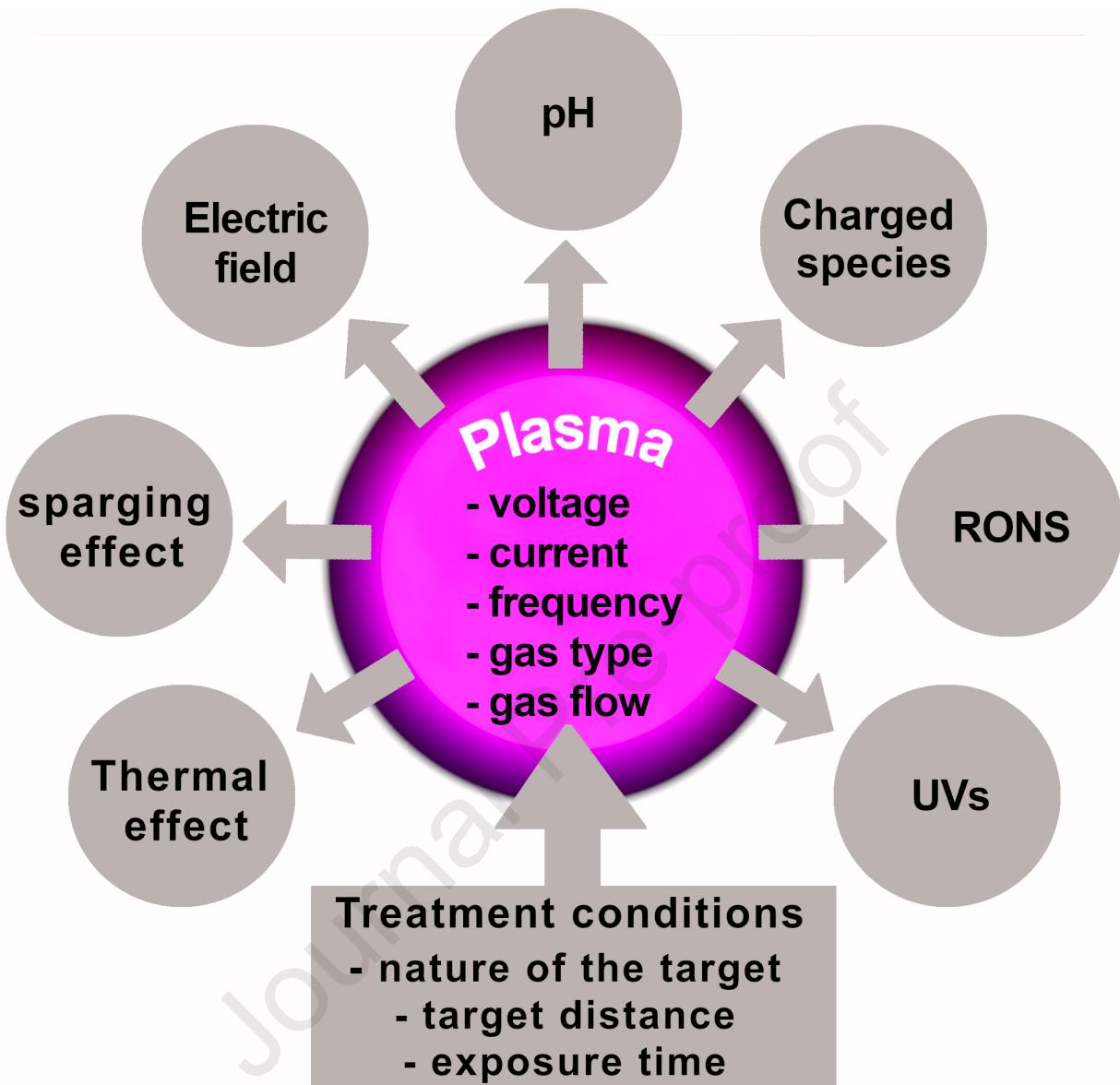
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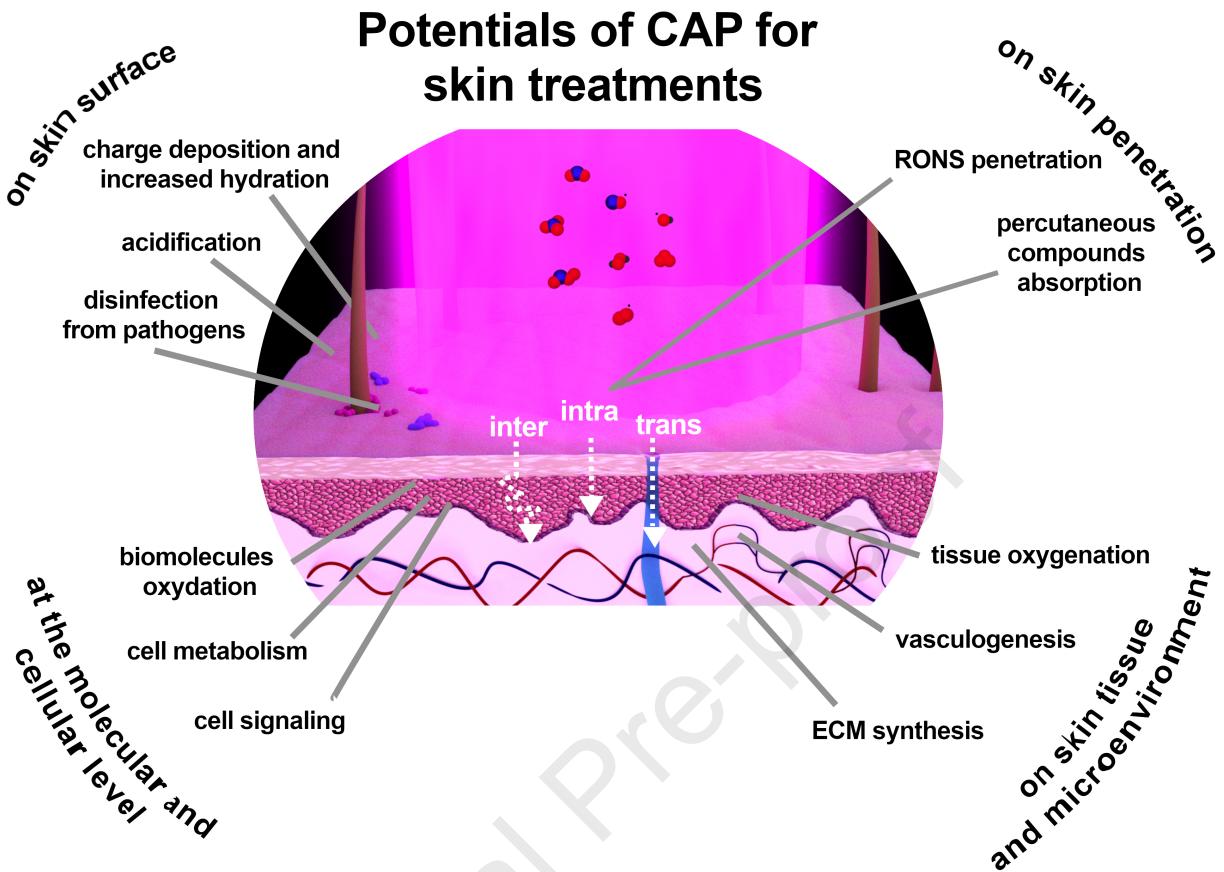
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The emerging potential of cold atmospheric plasma in skin biology

Highlights

- Taking care of the skin is essential to preserve its physiological and social functions.
- Cold Atmospheric Plasmas are already used in skin disinfection and wound healing
- Reactive Oxygen and Nitrogen Species play a key role in plasma biomedical applications
- Plasma tailoring and control are essential to achieve a benefic biological effect
- Cold Atmospheric Plasma is a promising technology for dermo-cosmetic treatments such as aging prevention