Wound healing improvement in large animals using an indirect helium plasma treatment

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ABSTRACT

Background: To optimize wound healing, the use of low-temperature atmospheric pressure plasma (ionized gas) has been proposed as an innovative therapeutic method for treating extensive and chronic wound. The aim of this study was to evaluate the specific effects of an indirect helium plasma treatment in a sheep surgical wound model based on clinical, histopathological and molecular analyses. This study was part of a larger study aimed at assessing five different wound healing improvement methods.

Methods: Six sheep were used in this study. Six square wounds were performed on the back of each sheep. Five of the lesions were used to analyze the effect of five different treatments, one of which was the plasma treatment. The sixth lesions was used as control. A 2-min plasma treatment was daily performed, using a radiofrequency helium plasma source for indirect treatments, until complete wound healing was obtained. Biopsies were performed at two and six weeks. The plasma effects were tested through clinical evaluation, histopathological and immunohistological evaluation, real time PCR analysis, bacteriological evaluation and intracellular ROS evaluation.

Results: Wound closure time for the plasma-treated wounds was quicker than that of the control group. Plasma treatment drastically reduced the bacterial load in the wounds. A strong increase in intracellular ROS was observed. After six weeks, the inflammatory process was significantly reduced in the plasma-treated case with respect to the control. The plasma treatment was found to lead to an anticipated induction of blood vessel formation, as detected through mRNA expression of VEGF. The high Ki67-positivity at two weeks indicated a strong stimulation of cell proliferation induced by the plasma treatment. The expression of the hair keratine (hKER) at six weeks indicated that the plasma was promoting hair regrowth. The plasma treatment induced an increased rate of reformation of cutaneous adnexa at six weeks.

Conclusions: The obtained results suggest that the plasma action, at the used dose, induces an increase in cell proliferation, a reduction of inflammation, a reduction of the bacterial load, a stimulation of blood vessel formation, and an improvement in the formation of cutaneous adnexa with a positive consequence on hair regrowth.

KEYWORDS

Plasma medicine, wound healing, large animals, skin, plasma treatments, cutaneous adnexa

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1. INTRODUCTION

The skin represents the largest organ system of the body, consisting of a superficial epidermis derived from the primary germ embryonic layer (ectoderm), an underlying dermis, derived from the mesoderm, and a hypodermis subcutaneous layer, derived also from the mesoderm [1]. There are differences of thicknesses in the epidermis and dermis in various regions of the body, between species and within them. However, usually the skin is thicker over the dorsal surface and on the lateral surfaces of the limbs and is thinner on the ventral surface of the body and the medial surface of the limbs [2]. The skin represents a complex system with many functions such as a mechanical protection, a barrier against pathogens, a water barrier, the ability to regulate the body temperature, synthesis of vitamin D, a sensory organ and also functions in sexual signaling [1].

Normal intact skin is a natural defense for the whole body, because it prevents the entry of most microorganisms [3], while cutaneous adnexa produce lipids and fatty acids that regulate the pH and select the composition of the normal microbiota of the skin [4]. Dry skin, exposure to heat or cold, disruption of skin integrity and other types of traumas and wounds make microorganisms like *Staphylococcus intermedius*, *Streptococcus sp.*, *Corynebacterium pseudotuberculosis*, *Pasteurella sp.*, *Proteus sp.*, *Pseudomomas sp.* and *Escherichia coli* enter the body [3]. Mites or bacteria can inhibit hair follicles and reach the body when the wall of the follicle is ruptured, causing empting of the follicle contents into the dermis. The entrance of the microorganisms then stimulates a robust host immune response and can spread to the blood stream and reach regional and distant lymph nodes via the lymph flow [3].

A wound is an injury to the body that results in disruption of the continuity of the body's structure. Wound healing is a term used to describe the host mechanisms involved in restoring the continuity of the tissue [5]. It is a highly organized and coordinated series of processes that result in the restoration of tissue integrity and function. An interruption in the normal wound healing process can lead to the development of non-healing chronic wounds. A number of factors that can cause a delay in wound healing are: venous or arterial insufficiency, renal diseases, diabetes, trauma, advanced age and local pressure effects. Nevertheless, local factors like tissue hypoxia, ischemia, foreign bodies, maceration of tissue, exudate, infection, disruption of regulation of inflammatory processes, malnutrition and immune deficiencies can all influence the healing processes [6]. Wound healing proceeds through three phases: hemostasis and inflammation, proliferation, and remodeling. These phases are regulated by various cells, cytokines, and growth factors [3,7].

Wound healing re-establishes the skin's tensile strength and natural barrier function [8]. Dysfunctional healing can lead to lifelong disability and, in veterinary medicine, an economic impact on breeding [9,10]. To optimize wound healing, the use of low-temperature atmospheric pressure plasma (ionized gas) has been proposed as an innovative therapeutic method for treating extensive and chronic wounds [11-22]. The plasma action is understood to be mediated by an intracellular Reactive Oxygen Species (ROS), which stimulate cell proliferation and migration [23-25]. Furthermore, the well-known antibacterial effect of low temperature plasmas is exploited to prevent infections.

The aim of this study was to evaluate the specific effects of an indirect helium plasma treatment in a sheep surgical wound model based on clinical, histopathological and molecular analyses. The increase of intracellular ROS was also assessed, as well as the reduction of bacterial load on the treated wound. This study was part of a larger study aimed at assessing the effectiveness of five different wound healing improvement methods. In this paper only the plasma effect will be described and compared with the control case. The other methods were the use of allogeneic mesenchymal stem cells [26], and three more conventional methods (i.e. application of hyaluronic acid, Manuka honey and acemannan) the results of which will be described in a forthcoming publication.

The paper is organized as follows: in Section 2 the methods used in the study are described; in Section 3 the experiment results are exposed; in Section 4 a discussion of the results is given; finally, in Section 5 conclusions are drawn.

2. METHODS

2.1 Animal model

Six female Bergamasca sheep, homogeneous for size and age, were used in this study. The sheep were acclimatized to a shed of the MAPS Department, University of Padua, Italy, two weeks prior to the beginning of the experimental study. Parasitological and biochemistry examinations were carried out to ensure the good health of the subjects. The experiment was approved by the Body for the Protection of Animals (OPBA), ministerial decree n° 51/2015-PR released by the Health Department of Italy. In this study, sheep were chosen because they are less neurologically developed in comparison to carnivores/equines and have sufficient superficial space on their back for multiple experimental lesions. Moreover, sheep is also considered as a possible animal model for human medicine. The number of sheep was chosen based on statistical models and on the "The 3Rs principles replacement-reduction-refinement" [27]. On the basis of these principles, it is possible i) to make the replacement of animals with other experimental methods when possible (replacement), ii) the reduction of the number of employed subjects (reduction) and iii) the improvement of the techniques and procedures in order to eliminate or minimize stress and suffering of the animals (refinement) [27]. At the end of project, the animals have not been sacrificed, but transferred to a didactical farm. The performed experiments comply with EU Directive 2010/63/EU for animal experiments.

2.2 Experimental design

Six full-thickness square wounds (4×4 cm) were performed under general anesthesia and analgesia on the back of each sheep, using a scalpel and a sterilized square guide model. The distance between each lesion did not influence the result of trials. Five of the lesions were used to analyze the effect of five different treatments. The sixth lesions was used as control, in which only phosphate saline buffer (PBS) treatment was administered. In this study, the plasma treatment is compared to the control. At 15 and 42 days after the induction of the lesions, samples for histology, immunohistochemistry (IHC) and molecular analyses were collected by means of a 6-mm punch biopsy with appropriate sedation and analgesic drug administration to the sheep. Once a week the bacterial load on the wounds were assessed with tampons prior and subsequently to the plasma treatment.

2.3 Plasma treatment

The plasma source used for this experiment is an elaboration of a RF plasma source originally developed for the treatment of corneal infections [28].

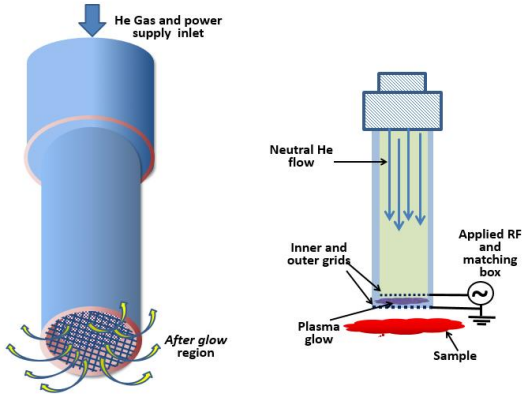

Fig.1: Schematic of a single plasma source: the neutral Helium gas inlet, and the electric scheme for gas ionization, made of the grid electrodes and of a radiofrequency power supply are indicated.

The original source is mainly constituted by two parallel planar grids, 1 mm apart. An inner one with a diameter of 10 mm, made of a brass wires (0.3 diameter, regularly spaced by 0.6 mm) is at the end of a tube

made of an insulating plastic material. The outer grid, with a diameter of 12 mm, is also made of brass wires, but with a 0.4 diameter, regularly spaced by 1.6 mm. The latter is electrically connected to one end of a grounded copper tube (12 mm outer diameter). As shown in the scheme of Fig.1, a Helium gas flow of 1.75 l/min is induced in the tube connected to the inner grid. A radiofrequency (at about 5 MHz) voltage difference ΔV is applied to the two grids with peak to peak values typically of the order of 1 kV, so that a diffuse glow discharge is established in the space between them.

Such ΔV is obtained by using a radiofrequency transmitter coupled to the source itself through a matching box, mainly a LC circuit, where the value of the capacitance is determined by the stray one due to connectors, cables and the source structure. The transmitter can be operated at a variable frequency, so that the best RF matching is obtained by continuously varying this quantity in order to minimize the reflected power. The output power from the transmitter is about 5 W, the one coupled to the plasma, as discussed more in detail later in the paper, is of the order of 0.5 W.

It is important to highlight that the adopted electrical scheme, with all the external components of the source grounded, guarantees that the electric currents sustaining the plasma are confined in the space between the two grids, thus avoiding the potentially dangerous formation of electrical arcs towards the samples to be treated. This is a safety precaution particularly relevant when dealing with extremely sensitive biological materials.

As mentioned, such simple scheme was originally designed in order to be used on the human cornea [29]. For the present study, an array of sixteen sources was built, so as to cover the whole wound area to be treated. The use of an ensemble of small sources guarantees a better control of the He flow on the various sections, and hence a more uniform plasma generation, than that produced by a single larger source. A picture of such matrix during plasma formation is shown in Fig.2a.

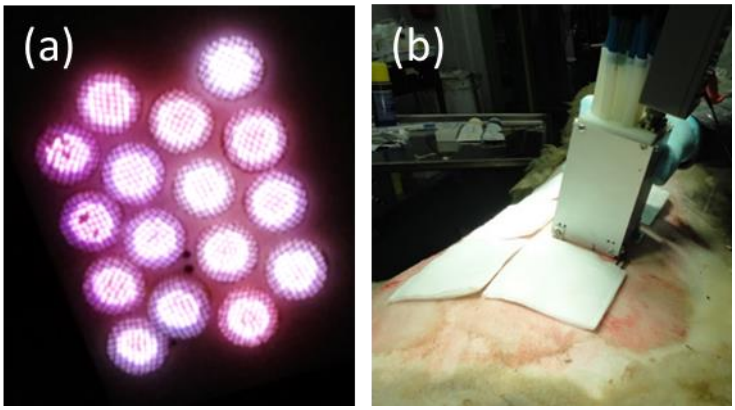

Fig.2: a) A picture of the matrix of plasma sources realized to treat the whole induced wound; b) image taken during the experimental clinical phase in the stall.

The mechanical support containing the 16 sources was made light and handy for the operator. On the bottom of the source, a system of spacer was mounted, which allows to safely and directly place the source itself on the healthy skin surrounding the wound and to maintain an average 1 mm source-lesion distance.

A 2 min plasma treatment was daily performed until complete wound healing was obtained, as will be described in the following. A picture taken during the treatment phase in the shed is shown in Fig.2b.

Prior to the plasma treatment, PBS was administered topically to the wounds. The same was done on the control lesions. After the treatment, the lesions were bandaged with sterile gauze using the “wet-to-dry” method. This method provides adequate wound protection and coverage, helps maintain a moist wound environment, and absorbs moderate amounts of wound exudates without drying up which could result in unnecessary pain during the next treatment and change of bandages. The bandaging is shown in fig.3.

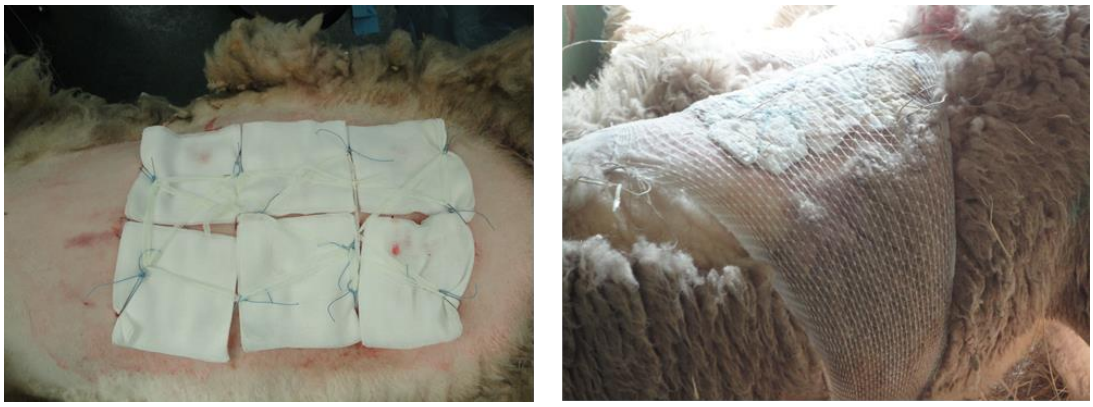

Fig.3: Bandaging adopted for wound protection and coverage in between treatments.

2.4 Clinical evaluation

The macroscopic aspect of the lesions was documented with photographs that were taken every day using a ruler for the evaluation of the process of healing of the wounds. Every week, the same operator performed a clinical evaluation without knowing the number of the subject and the type of treatment that he was judging. The evaluations obtained were catalogued using the model of Hadley [30], giving different parameters such as presence, color and character of the exudate, the aspect of the gauze after removal and the hydration of the wound. The percentages of re-epithelization and wound contraction were measured at different time periods after 14, 28 and 42 days.

2.5 Histopathological and immunohistological evaluation

All 24 biopsy samples (six plasma-treated at day 15, six plasma-treated at day 42, six control at day 15, six control at day 42) were used for histological evaluation and were glowed in OCT (Kaltek) and frozen in isopentane and liquid nitrogen. Samples were cut with cryostat into 5µm slices, mounted on slides and stained with Hematoxylin and Eosin (H&E). All samples were examined at different depths (six chosen points) for a full thickness examination. The presence of dermal and subcutaneous infiltrates, (immature) granulation tissue, undifferentiated mesenchymal tissue, and the development of adnexa were evaluated using a 0 to 4 scale (0 absence, 1 presence, 2 small amount, 3 moderate amount, 4 abundant amount).

Sections of the same biopsy samples were cut and immunostained with polyclonal rabbit anti-human CD3 (1:100, Dako), polyclonal rabbit anti-human CD20 (1:100, Biocare Medical), monoclonal mouse anti-human MHCII (1:40, clone CR3/43, Dako), monoclonal mouse anti-human Ki67 (1:10, clone MIB-1, Dako) and polyclonal rabbit anti-human vWF (1:3200, Dako) (Table 1). Immunolabeling was achieved using a highly sensitive horseradish peroxidase (PO) mouse or rabbit diamonobenzidine kit, with a blocking of endogenous PO (Envision DAB+ kit, Dako), in an auto-immunostainer (Cytomation S/N S38-7410-01, Dako). In order to block hydrophobic interactions, an antibody diluent (Dako) with background-reducing components was used. All skin samples (including negative and positive controls) were stained to the same immunohistochemistry staining procedure. Positive staining was confirmed on microscopy, and area percetanges of three random fields (at 20× magnification) from each slice were calculated with the LAS V4.0 Software (Leica Microsystems).

	Clone	Reactivity	Host	Clonality	Catalog #	Reference
CD3	N/A	Multiple species	Rabbit	Polyclonal	A0452	[31-34]
CD20	N/A	Multiple species	Rabbit	Polyclonal	3004	[26]
MHCII	CR3/43	Multiple species	Mouse	Monoclonal	M0775	[26]
Ki67	MIB-1	Multiple species	Mouse	Monoclonal	M7240	[35-38]
vWF	N/A	Multiple species	Rabbit	Polyclonal	A0082	[39]

Table 1: List of antibodies used in the study.

2.6 Real time PCR analysis

All 24 biopsy samples were used for molecular biology. Total RNA extraction was performed using TRIzol reagent (Life Technologies) and quantified using a Nanodrop spectrophotometer (Thermo Scientific). RNA (2 µg) was retrotranscribed using SuperscriptTM II Reverse Transcriptase protocol (Invitrogen) and a mixture of random hexamers were used as primer in order to synthetize the first-strand cDNA. The obtained cDNAs were used as template for Real Time PCR to study the relative gene expression.

The relative expression of genes involved in the wound healing process (Collagen 1α1, hair Keratin, Vascular Endothelial Growth Factor, Transforming Growth Factor Beta 1 and Vimentin) was assessed. RPS24 and 18S genes were used as housekeeping genes to normalize the data obtained. Primers for these genes (listed in Table 2) were designed by using Primer Express 3.0 software (Applied Biosystems) based from sheep specific gene sequences available on the GenBank database (sheep genome assembly: GCA_000298735.1). In order to check the specificity of the designed primers PCRs for the obtained cDNAs were performed. PCR products were electophoresed on a 2% agarose gel and visualized under UV light.

The designed primers specific for sheep genes were also validate with the standard curve method to assess their efficiency before the relative quantification study. In brief, standard curves were obtained by using serial dilutions of sample cDNA (1:10, 1:50, 1:100, 1:1000, 1:10000, 1:100000, 1:1000000). Standard curves, created on the basis of linear relationship between the Ct values and the logarithm of the starting amount of cDNA, showed acceptable slope values (between -3.8 and -3.3). The efficiency was calculated with ABI 7500 System SDS Software (Applied Biosystems). All samples were then tested in triplicate to study the relative expression of the genes of interest and untreated skin was used as the calibrator sample. The specific amplification of the cDNA target and the absence of nonspecific products was confirmed by the study of the melting curve (dissociation curve). The $2^{-\Delta\Delta Ct}$ method (comparative method) was used to normalize the RNA expression level of the target genes to the endogenous housekeeping genes.

GENE	ACCESSION NUMBER	SEQUENCE (5' → 3')	Amplicon size (bp)
18S	XR_003587981.1	F: AAACGGCTACCACATCCAAG R: TCCTGTATTGTTATTTTTCGTCAC	90
RPS24	XM_004021507.3	F: TTTGCCAGCACCAACGTTG R: AAGGAACGCAAGAACAGAATGAA	66
TGFβ1	NM_001009400.2	F: CGAGCCCTGGACACCAACT R: GTCAATGTAGAGCTGACGAACACA	72
VEGF	NM_001025110.1	F: GCTCTCTTGGGTGCATTGGA R: TGCAGCCTGGGACCACTT	70
VIM	XM_004014247.4	F: ACGCCATCAACACCGAGTTC R: AAGCGCACCTTGTCGATGTA	100
hKER	NM_001199070.1	F: TGGTTCTGTGAGGGCTCCTT R: GGCGCACCTTCTCCAGGTA	91
Col1α1	XM_027974707.1	F: GTACCATGACCGAGACGTGT R: AGATCACGTCATCGCACAGCA	86

Table 2: Primer sequences used for the rt-PCR analysis.

2.7 Bacteriological evaluation

The load of bacteria on the wounds was assessed weekly for the whole duration of the experiment by rubbing on the wounds sterile swabs. The assessments were performed both on the control wounds and on the plasma-treated ones, right before and right after the treatment. The swabs were subsequently placed in 10 mL of sterile medium, and 100 µL were finally inoculated in microbiological plates containing BHI (brain heart infusion) agar medium. The plates were incubated at 37°C in aerobic and anaerobic conditions After 24 hours of incubation, the evaluation of the bacteria load was performed by colony counting and the data were expressed as CFU (colony forming units)/mL.

2.8 Intracellular ROS evaluation

Biopsies collected at days 14 and 42 of the experimental period at the margins of the control lesions and of the plasma-treated lesions were examined in order to evaluate the levels of Reactive Oxygen Species (ROS). Briefly, tissue samples were preserved at 4°C in physiological solution and processed within two hours. Each sample was finely homogenized and digested for 10 minutes at 37°C in a solution containing type II collagenase (1 mg/mL) and DNAase (10 μg/mL). The cell suspension was separated from the non-digested tissue using filters with 100 μm pore diameter and incubated in permeabilization buffer (eBioscience) for 20 min at 22°C with KRT6 monoclonal antibody (clone SPM269, LifeSpan BioSciences, 2μg/ml). KRT6 was then detected using anti-mouse Alexa Fluor® 647 secondary antibody (Thermo Fisher Scientific) [40].
In order to determine the ROS level, 2×10^5 cell/mL were incubated for 20 minutes at 37°C with 2',7'-DCFDA (dichlorofluorescein diacetate, Molecular Probe), an originally non-fluorescent probe able to penetrate the cell membrane. In the cytoplasmic compartment, 2',7'-DCFDA is de-acetylated by cellular esterase resulting in 2',7'-diclorofluoresceina unable to exit the cell. The 2',7'-diclorofluoresceina is then oxidised by the intracellular ROS eventually present, originating a fluorescent compound yielding a signal directly proportional to the ROS levels. At the end of the incubation the cells were washed by centrifugation. Cells were finally washed and the fluorescent signal was analysed by a BD FACSCalibur™ flow cytometer. For each sample 10,000 cells were acquired. The data have been expressed as percentage of cells yielding fluorescence.

2.9 Statistical analysis

Data on clinical, histological, molecular, and immunohistochemical parameters were analysed using PROC MIXED, with animal as a random effect and repeated effect. The statistical linear model included the fixed effect of treatment (plasma vs. control), time and their interaction. The assumptions of the linear model were graphically inspected using residuals plots. For data that were not normally distributed (Shapiro-Wilks test < 0.90), the Mann-Whitney test was used (wound closure time, % of re-epithelialization and contraction, presence of exudate). The level of statistical significance was set at $p<0.05$.

3. RESULTS

3.1 Healing process assessment

Wound closure time for the plasma-treated wounds was quicker than that of the control group, with a statistically significant difference ($p=0.0325$): averages of wound closure time were respectively 27.4 and 31.8 days, with standard errors of 0.9 and 2.6 days. A visual comparison of the two healing processes for a representative case is shown in Fig.4. Two weeks after wound creation, the average percentage of re-epithelialization was similar for both plasma-treated and control wounds, around 31%. At day 28, the plasma-treated lesions had a higher average percentage of re-epithelialization in comparison with the control group (93% vs 87%). However, this was not a significant difference. After 42 days of treatment, all wounds had 100% re-epithelialization. After two weeks of treatment, the plasma-treated wounds showed 80% contraction compared to 78% for the control PBS group. However, this was not a significant difference. All lesions had 100% contraction after 42 days of treatment.

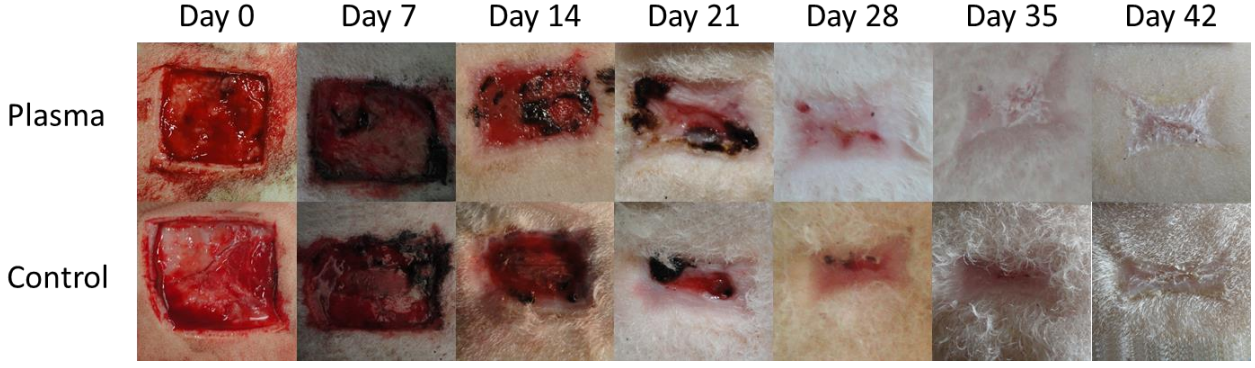

Fig.4: Comparison of the healing process of the plasma-treated wound and of the control wound in sheep n.5.

3.2 Microbiological assessment

Aside from the wound healing process, a beneficial effect of the plasma treatment is given by the antibacterial effect, described by many authors and demonstrated for our plasma source through *in vitro* and *ex vivo* studies [41-43]. As mentioned above, such capability has been evaluated *in vivo* during the present study by measuring every week the bacterial load on the wounds, before and after the treatment.

As shown in Fig.5 the untreated wounds exhibit a largely variable bacterial load, with a maximum value up to almost 1×10^6 CFU/mL. Plasma treatment drastically reduced the bacterial load in the wounds up to 25×10^4 CFU/mL. Before the treatment, the bacterial load was constant in the wounds but the antibacterial effects of plasma slightly increased over time and became even more evident starting from day 21.

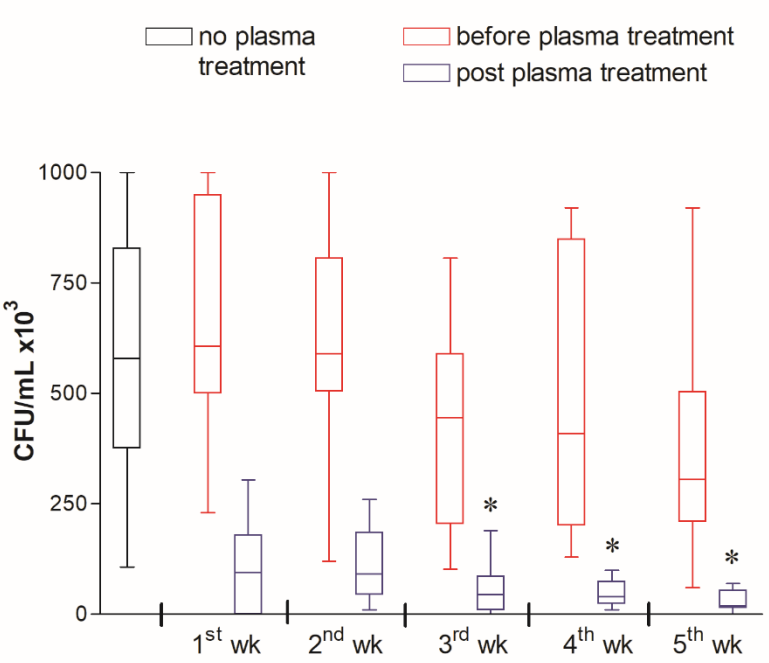

Fig.5: Bacterial load evaluation in untreated wounds, and before and after plasma treatment, at various time intervals (data are reported as CFU/mL). denotes $p<0.05$ vs. no plasma treated wounds.*

3.3 Intracellular ROS evaluation

We previously reported that plasma exposure promotes proliferation of fibroblasts in an *in vitro* model of wound healing. Moreover, plasma-induced fibroblast proliferation is ROS-dependent as cellular pre-treatment with the antioxidant agents N-acetyl L-cysteine dampened the biological effects [24]. Several studies have reported that the plasma action is mediated by generation of intracellular ROS [23] and ROS levels have a functional role in the wound healing process by stimulating cell proliferation and migration [25]. Therefore, in this study we evaluated ROS levels in cytokeratin 6A positive cells which are expressed in suprabasal cell layer in epidermis and are activated during injury of the skin [44]. As shown in Fig.6, despite a large variability within the six animals, the percentage of KRT6 positive cells exhibiting fluorescence as a consequence of the oxidation of the 2',7'-diclorofluorescein probe is sensibly larger in the samples collected after the plasma treatment with respect to that found in untreated or pre-treatment collected ones. A strong effect is found at both days 14 and 42.

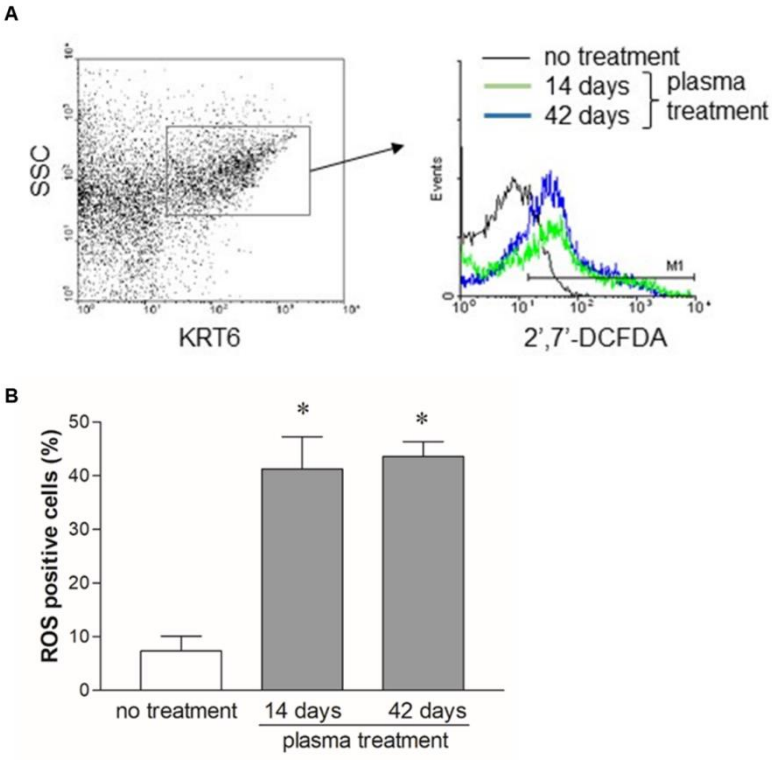

*Fig. 6: (a) 2',7'-diclorofluorescein (DCFDA) related fluorescence was detected in KRT6 positive cells. Representative image are reported. (a) Percentage of ROS positive cells in KRT6 gated cells obtained from control and plasma-treated biopsies collected at days 14 and 42 of the experimental period. * denotes $p<0.05$ vs no plasma-treated wounds.*

3.4 Analysis of inflammatory, proliferative, vascular and structural factors

The inflammatory process, as evaluated through the mRNA expression of the TGFβ1 gene (Fig. 7a), appeared to be slightly higher after two weeks in the plasma-treated wounds. After six weeks, it was significantly reduced in the plasma-treated case with respect to the control, suggesting that the plasma action induces a reduction of the inflammation/flogosis.

The mRNA expression of VEGF after two weeks (Fig. 7b) indicates that plasma treatment leads to an anticipated induction of blood vessel formation. The reduced value at six weeks, when the wound healing process has been completed, is probably due to the faster vascularization process that occurs in the treated cases with respect to the control ones.

In immunohistochemistry experiments the high Ki67-positivity (Fig. 7c) indicates a strong stimulation of cell proliferation induced by the plasma treatment, confirming results obtained *in vitro* [24]. This effect is probably related to the observed increase in intracellular ROS level. Positivity for Ki67 was especially observed in plasma-treated wound in the basal layer of the epidermis (Fig. 7d, black arrows) while in control wounds positivity was rarely observed (Fig. 7e). Proliferation is very low at six weeks both in the treated and control cases, in agreement with the fact that the wound has been completely closed and no further cell production is required.

The expression of the hair keratine (hKER) gene, which was negligible in the control case at both two and six weeks, was also negligible at two weeks for the plasma-treated case (data not shown). However, it turned out to be substantial at six weeks, indicating that the plasma is promoting hair regrowth.

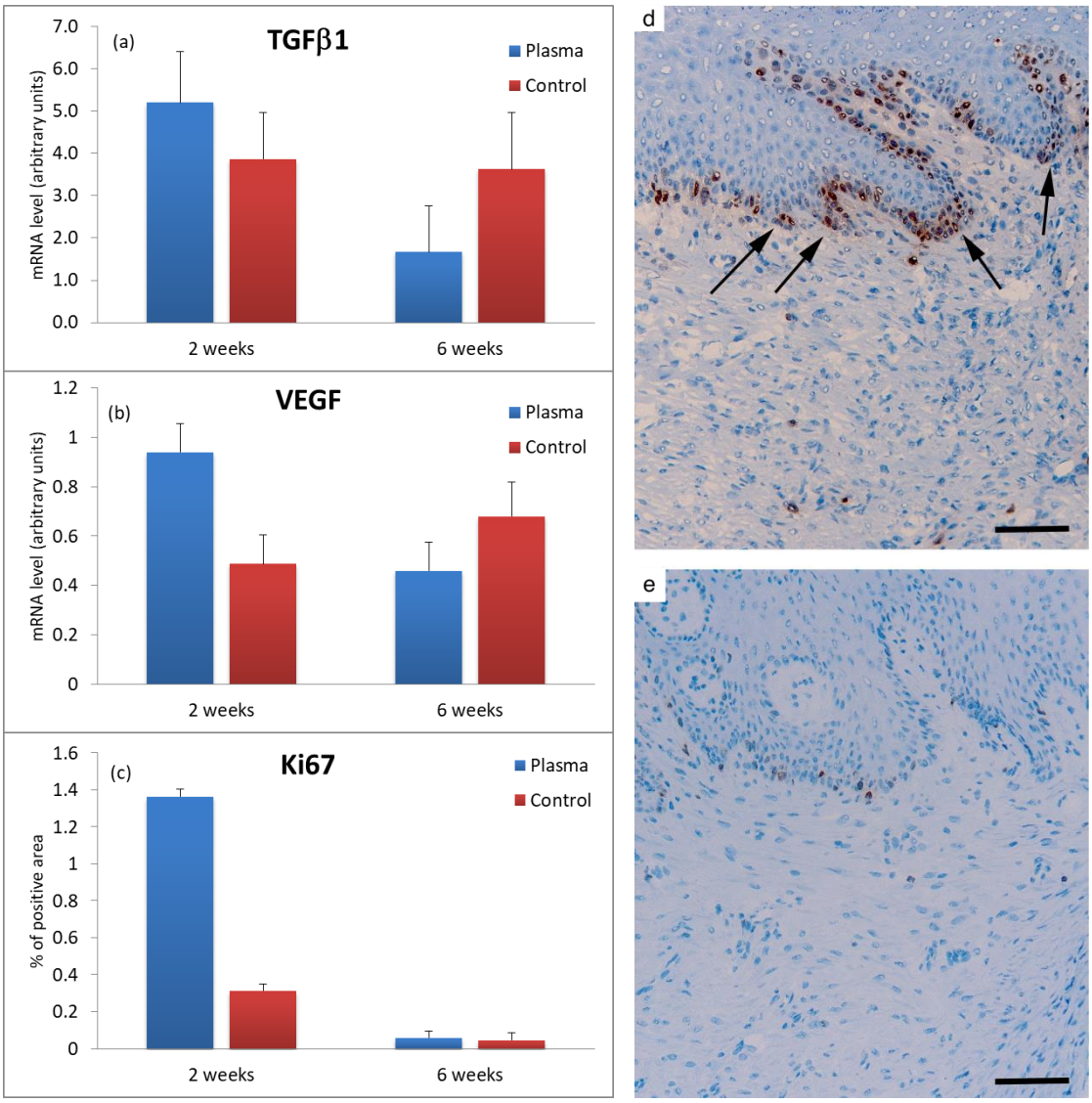

Fig.7: Comparison between the plasma-treated case and the control case for: (a) mRNA expression of the TGFβ1 protein, as obtained from real-time PCR; (b) mRNA expression of the vascular endothelial growth factor VEGF as obtained from real-time PCR; (c) Ki67 antigen, as obtained from immunohistochemistry. All quantities are evaluated at two and six weeks. Positivity for Ki67 was especially observed in plasma-treated wound (d) in the basal layer of the epidermis (black arrows) while in control wounds positivity was rarely observed (e).

Finally, the outcome of the histological analysis regarding cutaneous adnexa formation is shown in Fig. 8. Fig. 8a and 8b show histological analysis at two weeks and at six weeks respectively, for control case. Fig. 8c and 8d show the same for the plasma-treated case. Insets display the abundance of cutaneous adnexa, as evaluated from the analysis. It can be seen that, while at two weeks no cutaneous appendages formation can be seen in both cases, the plasma treatment induces an increased rate of reformation of cutaneous adnexa at six weeks. In particular, plasma samples present hair follicles, sebaceous, and apocrine glands more mature and denser compared to the control group. The promotion of the reformation of hair follicles is in agreement with the previous result concerning hKER gene expression.

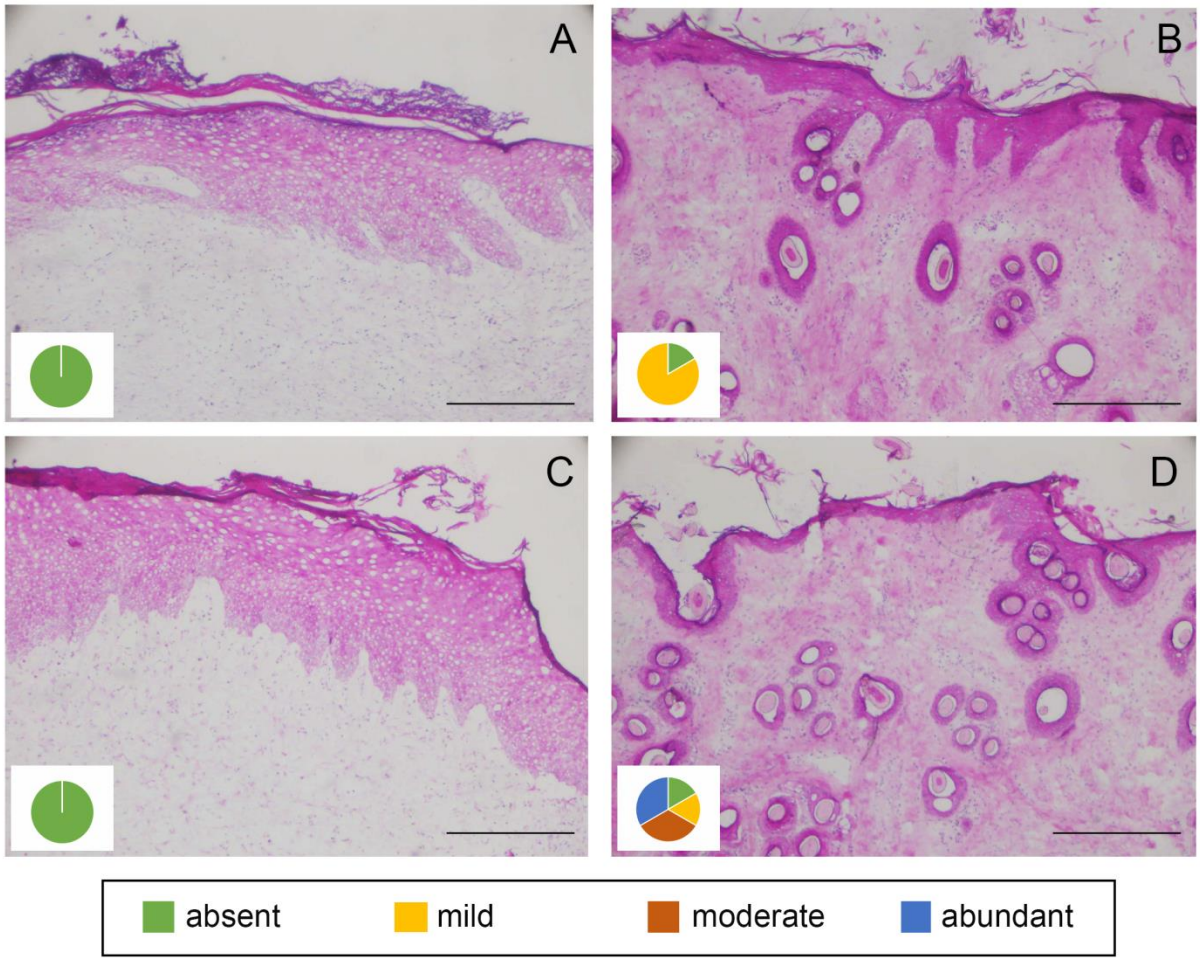

Fig.8: Cutaneous adnexa abundance, as obtained from histological evaluation: (a) control case at two weeks; (b) control case at six weeks; (c) plasma-treated case at two weeks; (d) plasma-treated case at six weeks.

The evaluation of Col1α1 and VIM gene expression and of CD3, CD20, MHCII and vWF antibodies did not show any significant difference between the plasma-treated case and the control case (data not shown).

4. DISCUSSION

The plasma treatment represents a promising approach to promote wound healing. Many research groups are working on this technique, using a variety of different plasma sources. The present study investigated a gentle approach based on an indirect plasma treatment, which is a treatment where the plasma does not get in direct contact with the wound: the healing effect is achieved by the action of the reactive chemical species produced by the plasma action present in the gas flow exiting the source. No charged species reach the treated substrate and the level of UV radiation is very low [45]. The study confirmed the effect of the plasma source action at the bacterial and mammalian cellular level already known from *ex vivo* and *in vitro* studies and mainly due to the increase in ROS level. This increase is thought to be trigger for the proliferation and migration processes responsible for accelerated healing, and evidence in this respect has been previously obtained *in vitro* [24]. Indeed, through the detection of the Ki67 antigene it was possible to confirm *in vivo* the stimulation of cell proliferation, resulting in an accelerated closure of the wound. This result is in agreement with observations performed by several authors about the ability of the plasma treatment to stimulate proliferation of fibroblasts [24,46,47]. In particular, a research group found using total proteome

profiling an overall reduction of integrin protein expression in plasma-treated fibroblasts, a condition which is often associated with loss of focal adhesion, increasing cell motility [48].
The inflammation reduction observed at a late stage of the healing process, despite not being very strong, was statistically significant. The role of cold atmospheric plasmas in reducing inflammation, either by modulating the expression of TGFβs or through other pathways, has been indeed reported by other authors [49-51]. This outcome is related to the more general topic of redox control of inflammation [52,53].
Of particular interest is the observation that the plasma treatment promotes vascularization at two weeks after lesion induction. Indeed, it is known that a moderate oxidative stress level plays a positive role in angiogenesis [54]. Chronically produced or highly concentrated ROS are detrimental for most tissues, whereas transient or low levels of ROS are able to activate signaling pathways that eventually promote regeneration and growth of blood vessels [55]. VEGF is the most potent and primary endothelial specific angiogenic growth factor, both in physiological and pathological conditions. It is to be remarked that, while our study detected angiogenesis through VEGF level, several recent studies have identified new mechanisms of ROS-activated angiogenesis that operate in a VEGF-independent manner, and which may have gone undetected in the present study [54]. The capability of plasma treatments to promote angiogenesis has been reported by several groups. In particular, a cold atmospheric plasma was found to induce pro-angiogenic and angiogenesis-related factors in skin keratinocytes, fibroblasts and endothelial cells, and to promote wound angiogenesis via autocrine and paracrine mechanisms [56]. Also, an enhancement of angiogenesis was detected through the increase of blood flow and CD31 expression in burn wounds induced in mice [57]. Angiogenesis improvement was also measured in association to a Dielectric Barrier Discharge (DBD) treatment and related to the release of Fibroblast growth factor-2 (FGF-2) [58]. Repetitive DBD treatments were also found to boost cutaneous microcirculatory effects in a prospective, cohort trial [59].
Somehow related to the angiogenesis result is the histological observation of enhanced reformation of cutaneous adnexa, and in particular of hair follicles, results in agreement with the observed expression of the hKER gene. A similar result was observed in a recent study on the effect of a helium plasma jet on pressure ulcers produced in rats [22], where the formation of new hair follicles and sebaceous glands in the epidermis and dermis layer of plasma-treated animals was observed, unlike the control animals. In the same study also enhanced angiogenesis and reduced inflammation at late stage were detected.
Overall, the results of this *in vivo* study point to an accelerated wound healing, related to the enhancement of the intracellular ROS level, which includes reduced inflammation at late stage of the healing process, a faster reformation of blood vessels and enhanced hair growth.

5. CONCLUSIONS

An indirect plasma treatment has been applied to lesions produced on the back of sheep. The obtained results suggest that the plasma action, at the used dose, induces an increase in cell proliferation, a reduction of inflammation, a reduction of the bacterial load, a stimulation of blood vessel formation, and an improvement in the formation of cutaneous adnexa with a positive consequence on hair regrowth. Overall, the results suggest that the proposed plasma treatment can be beneficial both in human and veterinary medicine. In addition, more experiments using cold plasma should be done using different dosages or adding other treatments to provide more evidence of the results obtained in this study and to confirm if the cold plasma can be used for wound healing process in veterinary medicine and the re-growing of new hair after loss.

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