World Academy of Science, Engineering and Technology
International Journal of Biomedical and Biological Engineering
Vol:6, No:4, 2012

Effect of Plasma Therapy on Epidermal Regeneration

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***Abstract*—**The purpose of our study was to compare spontaneous re-epithelisation characteristics versus assisted re-epithelisation. In order to assess re-epithelisation of the injured skin, we have imagined and designed a burn wound model on Wistar rat skin. Our aim was to create standardised, easy reproducible and quantifiable skin lesions involving entire epidermis and superficial dermis. We then have applied the above mentioned therapeutic strategies to compare regeneration of epidermis and dermis, local and systemic parameter changes in different conditions. We have enhanced the re-epithelisation process under a moist atmosphere of a polyurethane wound dress modified with helium non-thermal plasma, and with the aid of direct cold-plasma treatment respectively. We have followed systemic parameters change: hematologic and biochemical parameters, and local features: oxidative stress markers and histology of skin in the above mentioned conditions. Re-epithelisation is just a part of the skin regeneration process, which recruits cellular components, with the aid of epidermal and dermal interaction via signal molecules.

***Keywords*—**Plasma medicine, re-epithelisation and tissue regeneration

I. Introduction

WOUNDS have been dressed since prehistoric times. In the course of the ages experience accumulated as many materials were tried. The very first description of wound coverings are to be found in the Papyrus Smith (c.1700 B.C.) and the Papyrus Ebers (c. 1500 B.C.), in which oiled frog skin and greased bandages were used as wound coverings. Skin grafting was first was first reported in the ancient Hindi document Sasruta Sanhita (c.700 B.C.) for the repair of nose and ear defects. Since the previous century the increasing knowledge of skin healing has altered the abstinent attitude of the physician and encouraged him to interfere in the natural healing process [1], [2], [20].

Since the 1950's synthetic wound coverings have been appearing on the scene of clinical practice beside conventional and biological coverings [3], [4]. Such an example is the occlusive wound covering which enhances re-epithelisation under a moist atmosphere of retained exudate, rich in cytokines, growth factors and energetic substrate [9].

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In the same time, it prevents dehydration of the poorly perfused tissue in the zone of stasis of a wound, hence preventing deepening of the wound [5], [12]. An occlusive wound covering is made up mainly on a polymer matrix. Its ability to absorb, retain and concentrate wound exudate depends greatly on the polymer composition, microstructure and surface properties (i.e. hydrophily, waterability, gas permeability and surface adhesion) [6], [7]. An essential condition for the occlusive dress to work properly is to avoid applying it on infected wounds, to ensure its sterility before use [16], [17], [18]. The occlusive wound covering should not be kept on the wound surface for more than 7 days, even if it looks fine. Plasma biology and medicine are rapidly growing new areas of non-thermal plasma science and engineering [13]. Not so long ago, biomedical applications of non-thermal plasma were mostly focused on surface sterilization as well as treatment of different surfaces to control their bio-compatibility (surface functionalisation) [8]. Now, plasma is also applied in solving novel sophisticated problems of tissue engineering, sterilisation of reusable heat sensitive medical materials and devices [10], [11]. Recently, non-thermal plasma has been directly applied in medicine in treatment of living tissues, blood coagulation and sterilisation of wounds [6], [7].

The purpose of our study was to check out if the pro-regenerative properties of an occlusive wound dress can be improved by treating it with non-thermal plasma.

We tried to increase pro-regenerative properties of a Hydrocoll polyurethane wound covering by treating it with a cold plasma beam, generated by electric discharge in helium at room temperature and atmospheric pressure. The pre-treated wound covering were then applied on chemical burn wounds, made on Wistar rat skin (our own experimental model).

II. Material and Methods

In order to find some clues related to our topic we designed our own experimental model of burn wound on Wistar rat skin. We used a commercial available polyurethane occlusive wound dress (PUD), Hydrocoll, produced by Hartman. The physics laboratory of „Al. I. Cuza" University provided us with a plasma beam generator.

We have performed our experiment on Wistar rats. We have used 20 young, healthy males, 320-380g each, with a standard diet. In order to assess re-epithelisation under plasma pre-treated wound dress, we imagined and designed our own burn wound model, on Wistar rat skin. Our aim was to create a standardised, easy reproducible and quantifiable partial thickness skin lesion, involving entire epidermis and superficial dermis.

We performed chemical burns on the rat's back skin (dorsal chest and lumbar region), three lesions each side of median line. The skin burn wounds were disc shaped, with clear borders, achieved by instilling an acidic solution in a special plastic device, 5 mm in diameter (Fig.1.A) so that they have a predetermined area. We used sulphuric acid solution because of its limited action along with time (unlike alkaline solutions, whose effect might be unpredictable and more difficult to assess). We found the optimal acidic solution concentration and exposure time to achieve second degree, partial thickness skin burns. After the exposure time has passed the container and exposed skin were abundantly washed with distilled water. Immediately after burns were done, the skin looked normal (colour and texture); yet, after five to ten minutes the burnt skin becomes brown or dark red (Fig.1.B).

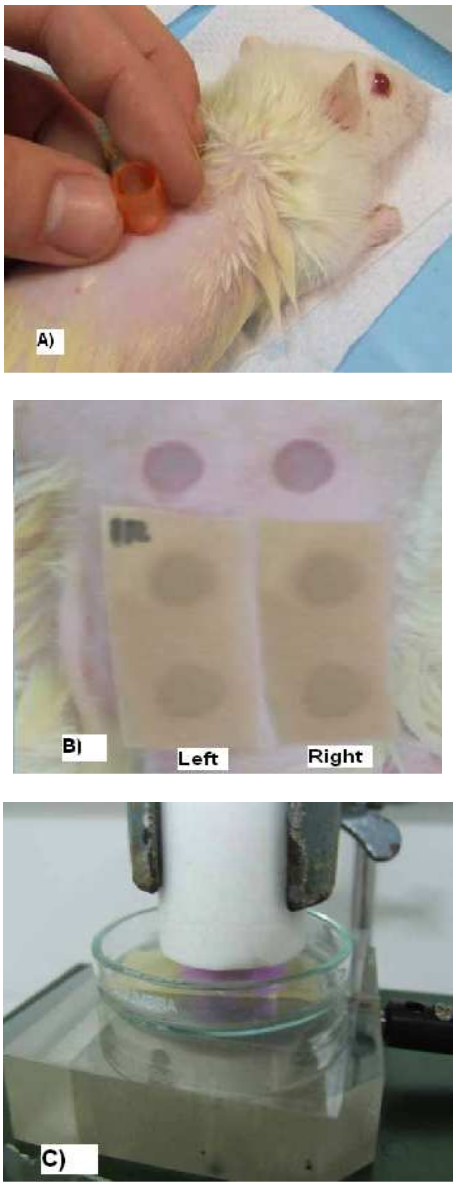

Fig. 1 A) Skin lesions achievement, B) Wound dress applied on the skin lesions, C) Cold plasma treatment of the wound dress.

The skin lesions were sterilised with etilic ether and dried out with a cotton bud before occlusive wound dress has been applied. The polyurethane wound dress pieces were applied as follows: on the left lower back side, laid down pre-treated wound dress, marked with a black coloured pen; on the right lower back side laid down untreated wound dress; the top two lesions (located cranially) were left exposed to air, to heal spontaneously (Fig.1.B). Non-thermal plasma was generated by dielectric barrier discharge in helium, at atmospheric pressure. The gas flew through a quartz cylinder connected with the power electrode. The floating (passive) electrode was the polymer being treated (Fig.1.C). The high voltage impulses had a specific geometry (Fig.2.A.), with 4 kV amplitude of voltage and 2 kHz frequency of pulses. The current vector depended on the active electrode polarity and maximal current achieved was about 4 mA which assumed no risk for the technician.

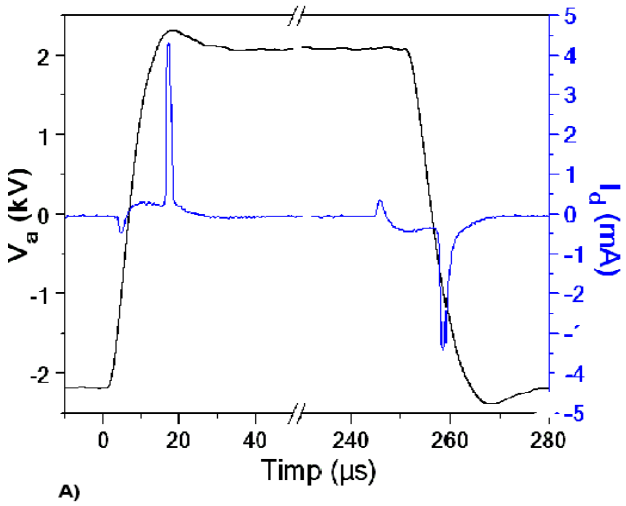

Fig. 2 Voltage geometry applied on power electrode (black) and discharge current (blue)

Maximal power applied was 0,8 watt/cm². Optical emission spectroscopy analysis showed that beside helium, our plasma atmosphere contained neutral and ionized molecular nitrogen, and active species such as atomic oxygen, singlet oxygen or hydroxyl radical. These active species interact with substrate molecules in a chain reaction, thus providing the polymer surface with many polar groups and changing its ultra structural morphology. We measured the PUD hydrophily by the contact angle method. When already unpacked, the Hydrocoll wound coverings were hydrophobic rather than hydrophilic; the contact angle measured being obtuse (Fig.3.A). After one minute of plasma treatment the polymer surface hydrophily and adhesion significantly increased as shown by the contact angle.

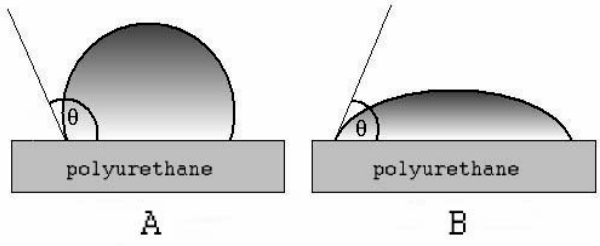

Fig. 3 Before plasma treatment polyurethane is hydrophobic (A), and becomes hydrophilic after one minute of plasma treatment (B)

At 3, 6, 9, 15 and 21 days from baseline we have assessed clinical wound appearance, exudate amount retained in the polymer matrix and the epithelisation borderline. Several pictures were taken from each wound, for computer planimetry (we have used „Image J" software to calculate the area of epithelisation versus scab surface).

III. Results

There was no statistically significant difference between the amount of retained exudate in pre-treated and non-treated polymer. At day 6 we have already noted significant difference between left side and right side wounds appearance. The difference became more obvious several days after (Fig.4 A and B).

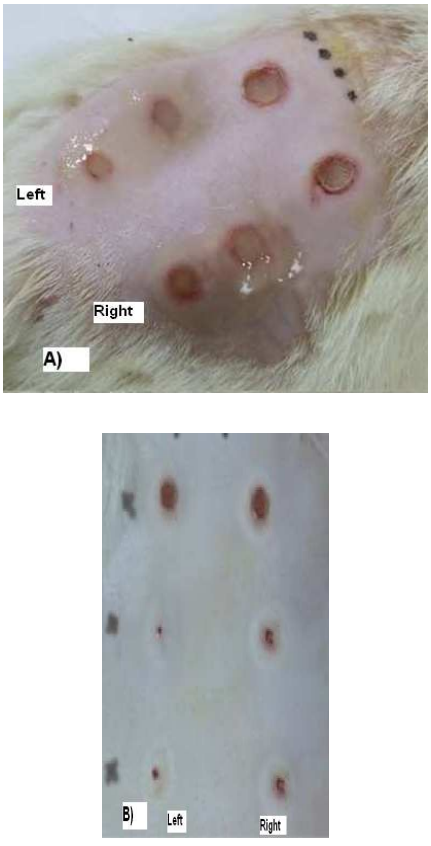

Fig. 4 A) Clinical appearance at 6 days B) Clinical appearance at 21 days

Planimetry: In order to assess the epithelisation dynamic, we took seriated pictures of each rat back skin appearance, every 3 days, after the wound dress has been removed. The figure below shows some of the most representative stages in the dynamic we followed. We have quantified the epithelisation process indirectly, by measuring the scab area which has decreased progressively all along the follow up duration. Then we have subtracted the scab area from the initial 19,6 mm² area of the already prepared wound to calculate the approximate area of the new regenerated epithelium (Fig.6)

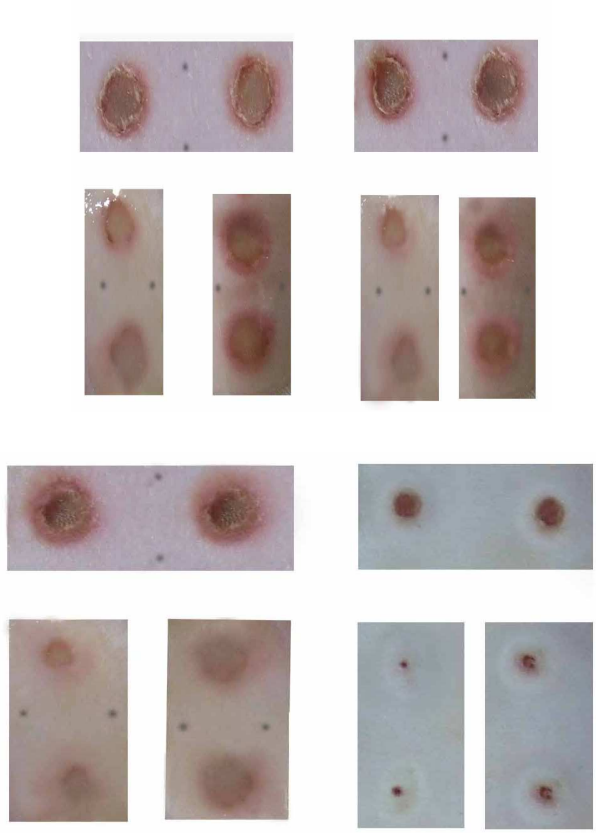

Fig. 5 Clinical appearance at day 6, 9, 15 and 21 respectively (top - plasma pre-treated polymer covered lesion; bottom – non-treated polymer covered lesion; control lesions cranially located)

We have assumed that the retraction of the dried, dehydrated scab of the non covered lesions is faster than that under the moist atmosphere of the occlusive wound dress. Yet we note that the wound scab has shrunk faster under the occlusive wound dress, perhaps because of the phagocyte activity at the border of fibrin scab, so we cannot consider it an artefact.

The planimetric examination showed significantly increase in re-epithelisation speed of the wounds covered with plasma pre-treated polyurethane (wounds of the left side) versus non-treated polymer covered wounds (on the right side) and spontaneously healed wounds (cranially located) (Fig.6).

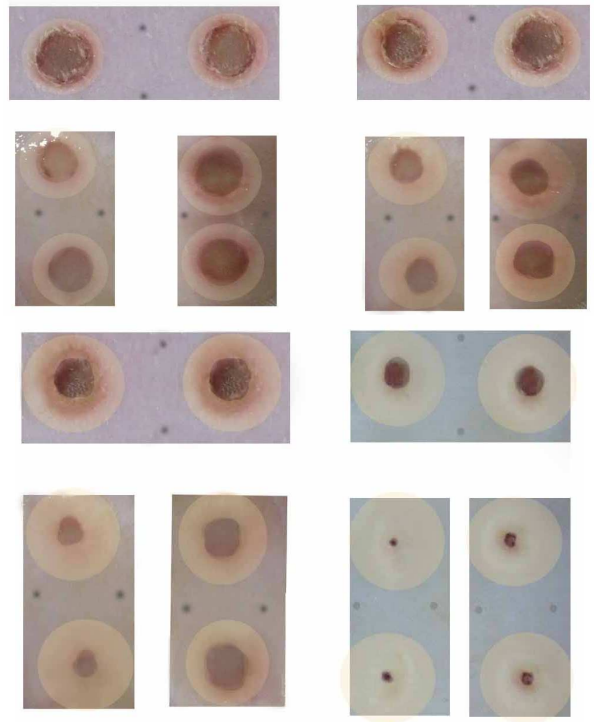

Fig. 6 Planimetry: the model of subtracting scab area from initial wound area to calculate the approximate epithelised surface

A. Histology

At day 3, 6, 9, 15 and 21, specimens were taken from the skin lesions for the histological analysis. We have used panoptic haematoxylin-eosin stain for all specimens. Beside that, for some of our samples we have used some other stain technique such as Szekely or Von Gieson (Fig.6, Fig.7, Fig.8, Fig.9). In the photos below, we discuss only some of the re-epithelisation stages, we consider more relevant (only at 3, 9 , 15 days).

B. After 3 days

- In the self healed (a) and non-treated PUD covered (b) lesions we noted epidermal necrosis, superficial and deep dermal connective tissue hyalinization (through collagen degeneration); deeply, in the surrounding dermis and beneath the burnt epidermis, there was faint perivascular inflammatory infiltrate

- In the plasma pre-treated PUD covered lesions (c) we noted the same aspect of the epidermis, but less collagen degeneration in surrounding dermis and recently vascular thrombosis.

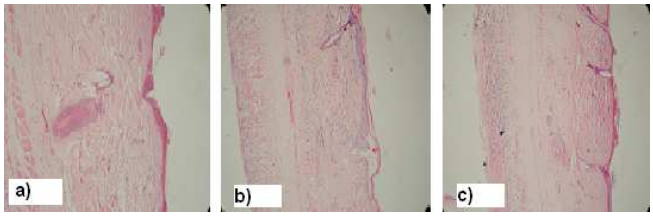

Fig. 7 Histological aspect at day 3: self healed (a), non-treated PUD covered lesions (b) and plasma pre-treated PUD covered lesions (c) (HE stain, magnification x 100)

C. After 9 days

- The self healed lesions (a) presented with a stack of fibrin and leucocytes in the superficial dermis;

- The non-treated PUD covered lesions (b) presented with an abundant stack of fibrin and polymorphonuclear neutrophils in superficial dermis and acute inflammatory infiltrate between deep dermis and muscular layer;

- The inflammatory fibrino-leukocytic detritus in plasma pre-treated PUD covered lesions (c) was poor, with less neutrophils. There was no granulation tissue in the wound bad. An abundant monocyte infiltrate at the hypodermis-muscular layer borderline was noted.

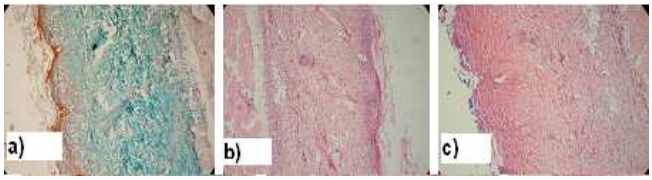

Fig. 8 Histological aspect at day 9: self healed lesions (a – Szekely stain, x100), non-treated PUD covered lesions (b) and plasma pre-treated PUD covered lesions (c) (HE stain, magnification x 100)

D. After 15 days

- In the self healed lesions (a) the epidermis was thin and the superficial dermis more homogenized, with collagen fibres horizonthalized (the homogenized area was twice as at day 9);

- The covered lesions, both with nontreated (b) and pre-treated PUD (c), presented with a heterogeneously thickened epidermis. The thickness was significantly more important than in the self healed lesions.

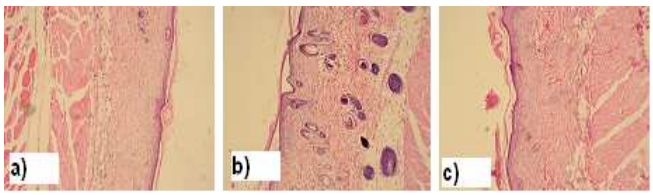

Fig. 9 Histological aspect at day 15: self healed (a), non-treated PUD covered lesions (b) and plasma pre-treated PUD covered lesions (c) (HE stain, magnification x 100).

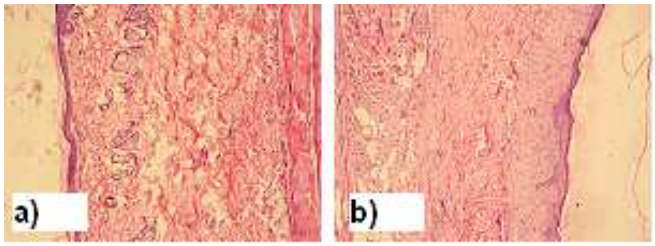

Fig. 10 At 21 days in untreated lesions noted the appearance of a thin epidermis, linear, superficial dermis with a wavy collagen fibres (restored) similar to the adjacent normal region, i.e. the lesion is similar to normal (HE staining, magnification × 100).

IV. Discussion

Epidermis always migrates in a plane between viable and nonviable tissue. With respect to the depth at which the

epidermis migrates, a donor site, which has become dehydrated, does not differ from a partial-thickness burn wound, since in both wound types scabs of necrotic dermis develop, under which re-epithelisation takes place [2]. In contrast, a donor site which remains hydrated by use of an occlusive wound covering allows epithelial migration on the top of its surface. The course of re-epithelisation can thus be advantageously diverted by using an occlusive wound covering. Winter [18] first reported that re-epithelisation takes place faster under a moist atmosphere of an occlusive wound dress. In occluded wounds epithelial resurfacing was 50% faster than in wounds left exposed to air. They calculated that epidermal cells migrate in a moist wound at a speed of about 0.5 mm per day, which is twice as fast as in desiccated wound.The occlusion concept appeared 20 years before its first use in the design of at least 14 types of commercially available synthetic wound covering. For practical use it is important to know that occlusive wound coverings must be applied within two hours after wounding and should be kept in place for at least 24 hours to accomplish a promoting effect on epidermal resurfacing.The exact mechanism by which re-epithelisation is enhanced under occlusive wound coverings is still not known in detail. Most authors think it is an increased speed of epidermal cell migration due to the absence of a scab. The reduced speed of cell migration underneath dry scabs is explained by the divergence of energy needed by collagenolytic activity or phagocyte activity of epidermal cells to create themselves a path through the collagen bundles of the dermis underneath the scab. Evidence exists that epidermal cells themselves produce collagenases. Additionally, Winter suggested that in burn wounds, an increased resistance of heated collagen against collagenase digestion might slow down re-epithelisation.Another factor explaining enhanced epithelisation under occlusion might be a decreased mitotic response, which propagates epidermal cell migration but impairs keratinisation. Another suggestion is that oxygen-permeable wound coverings increase the partial pressure of oxygen in a hypoxic wound environment, which might stimulate re-epithelisation. Other explanations for the enhanced healing under occlusion might be increased local concentration of growth factors, favourable effects of increased flora or maintenance of an electrical potential between the wound and its surrounding skin [14], [15]. We have obtained faster but nonhomogenous regeneration of epidermis under plasma pre-treated polyurethane occlusive wound dress. The planimetric analysis showed significant difference between plasma treated and non treated PUD covered epidermal regeneration with the advantage for the former. We cannot explain why the epidermis regenerates nonhomogenously under the artificial wound dress.

Ultramicroscopically, plasma may change the surface morphology, increasing its roughness, hence improving cell-polymer matrix adhesion and cell migration. Biocompatibility of most biopolymers depend greatly on the hydrophilicity of polymer surface. According to the current hypothesis of complementarity [22], in order to improve biocompatibility, it is necessary to minimize not only the average interfacial surface energy at the material-tissue interface, but also at every point on the surface. The hydrophilic and hydrophobic regions of a polymer surface that contacts with living proteins should be complementary in order for the biomaterial to be highly biocompatible. The hydrophilicity and surface energy of biomaterials can be varied over wide ranges by generating various surface polar groups with the use of treatment of the polymer surface with plasma [22]. Thereby, the protein adsorption and cell adhesion can be regulated and perhaps even more complex processes, such as those involved in cell migration and proliferation might be manipulated this way.

Yildirim et al. [25] reported the effect of non-thermal plasma pre-treatment of culture dishes upon osteoblasts in culture. Favourable cell-substrate interaction during the early stage of cell seeding is one of the most desirable features of tissue engineering. Plasma made the dish surface more hydrophilic which led to successful formation of tissue construct and cell proliferation, differentiation and new tissue ingrowths. In our experiment plasma treatment of the polyurethane wound dress increased the polar groups at the surface, hence improving keratinocytes ability to adhere, migrate and probably differentiate. The attachment phase of the anchor-dependent cells starts with formation of a cell adhesive protein layer from serum containing media on the substrate at the cell-matrix interface. The cells get attached to these absorbed proteins via cell-surface adhesion receptors like integrins and extracellular matrix proteins including fibronectin, vitrinectin, fibrinogen, and collagen that have a cell-binding domain containing the arginine-glycine-aspartate (RGD) sequence. Interactions between the proteins and the surface determine the residence time of the initial attachment, thereby influencing the cell proliferation and differentiation capacity on contact biomaterial [19], [21].

Recently, plasma-medicine supporters have speculated that polar groups at the surface of living proteins, induced by plasma treatment, might be able to activate some, and inactivate some other proteins expressed at the surface of the living cell [22], [23], [24].

V. Conclusion

Re-epithelisation is just a part of the skin regeneration process, which recruits cellular and humoral components, with the aid of epidermal and dermal interaction via signal molecules. Environmental bio-chemical factors, electro-magnetic fields and interactions may influence the genetically programmed normal course of this complex bioprocess. The bio-sciences are yet at their beginning with the knowledge about protein function and interaction. Perhaps the future data and techniques will make us able to understand many of our dilemmas today.

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