CASE REPORTS

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Therapeutic protocol using growth factors in electrocution wounds – case reports and review of the literature

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Abstract

Injuries by high voltage electrocution represent rare and very complex accidents. In order to provide support to surgical therapy, to limit the extension of injuries or to support the natural process of tissue repair, we oriented towards the use of two growth factors, insulin and platelet-enriched plasma (PRP) administered locally, on the injury site. The therapeutic protocol was applied on three cases of electrocution with promising results in reducing the duration of surgical and pharmacological treatment and of time of hospitalization. The influence of growth factors on healing wounds, the mechanism of action and recent therapeutic applications are also discussed.

Keywords: electrocution, growth factors, immune response, cytokines, insulin, PRP.

Introduction

Injuries caused by high voltage electrocution are rare and very complex accidents and most often end with the patient's death or with partial or complex disabilities. High-voltage injuries are a result of exposure to 1000 V or more. The three major mechanisms of electricity-induced injury are as follows: electrical energy causing direct tissue damage, altering cell membrane resting potential, and liciting tetany; conversion of electrical energy into thermal energy, causing massive tissue destruction and coagulation necrosis; mechanical injury with direct trauma resulting from falls or violent muscle contraction [1].

Although the treatments and therapeutic protocols have been constantly evolving, such traumas have a reserved prognostic, joining the same group as very severe burns, trauma caused by explosion, complex polytraumas with limb amputation, etc.

Solutions were sought for improving the therapeutic management, starting with a rapid and qualified taking over of the victim and continuing with various radical therapeutic protocols aiming to limit the post-electrocution ischemic areas. It has to be admitted that this is a type of progressive injury, the original injuries rapidly transforming towards deepening, surface extension, under the influence of the tissue oxygenation level. This parameter can display a negative progression, concomitantly with the decrease of hemoglobin (Hb) level, impairment of renal function, onset of a septic process and turning towards multiple system organ failure (MSOF).

Considering the particular features of electrocutions, where ischemic injuries of profound tissue are greater than those on the surface, the surgical treatment needs to be adjusted to the specific characteristics of each case. Electrocutions are always accompanied by burns, generated by the inflammation of clothes or by electrical flame. These burns need to be approached surgically, depending on their depth and the covered surface [2, 3].

Surgery needs to be targeted, aggressive in the positive sense, since it is known that stalling may worsen the injury, but, in the same time, extended incisions increase the loss of fluids and proteins, which are, anyway, considerable in such patients.

The patients in severe state constitute another group that, after the excision procedures remain with denudations of main arteries and/or nerve stems, which can be covered by local or remote means only after the efficient re-equilibration of the patient. In such situations, solutions to limit the extension of injuries or to support the natural process of tissue repair are needed. For this reason, we sought for therapeutic strategies that were successfully applied in other surgical specialties, and that can be adapted to the particular features of electrocution cases. Thus, we oriented towards the use of growth factors administered locally, on the injury site.

Patients, Methods and Results

A number of seven cases of severe electrocution with survival over 24 hours were selected. Three patients with remaining post-electrocution injuries were chosen; their healing extended over our expectations. These injuries were considered to have a slow progression, with a lack of contraction and absent or hypotrophic granulation.

The inclusion criteria in the protocol were the following: high voltage electrocution, survival of over 24 hours from the accident, post-electrocution injuries that lasted more than expected (a precise number of days could not be provided due to features arising in each particular case, protein level, hemoglobin value, associated diseases, initial depth of injuries and total surface of injuries), the presence of ischemic areas with trends of evolution in the surface and/or depth, the presence of

mobile elements (nerves, arterial trunks) in danger to remain bare and without any possibility to be rapidly covered by viable neighborhood structures.

PRP (platelet-enriched plasma) was used on three of the electrocution patients, in one patient using in parallel rapid insulin on the granulation area. The treatments were performed in a seven days interval, using a ready to inject kit produced by RegenLab. Activation was achieved with Calcium Gluconate and Thrombin. 0.9 mL of PRP was mixed with 0.1 mL Calcium Gluconate or Thrombin, in order to obtain 1 mL of activated PRP. Application was achieved by contact with the surface in two of the cases, and by mesotherapy type injection in another case, by injection papule type at equal distances of about 1 cm where the aim was to limit the extending of the area of an ischemic type injury.

Insulin was used in only one of the cases by contact application, on a surface that was equal to that treated with PRP. After the first treatment, a brutal decrease of glycemia was found; so that glycemic re-equilibration was achieved by 10% glucose endovenous perfusion (EVP). The granulation was obvious in the first 48 hours in the PRP treated area and was not evident in the insulin-treated area. For this reason, the use of insulin was interrupted from application on large areas, since its use was difficult to control. Insulin was successfully used on granular injuries of small size, in the absence of PRP, in daily applications, without finding the occurrence of decreases in glycemia, and with good results regarding the granulation of injuries, their contraction and epithelialisation. In all cases, the insulin treatment consisted in its topical application at the injury site.

The affected area was divided into areas treated with PRP and conventionally treated areas, the last one being considered as blank areas.

The study was performed with informed consent of the patients and with approval of the Committee Ethics of Bucharest Emergency Hospital, Romania, whose president is Dr. Bogdan Popa.

PRP and insulin were used on injuries compliant to the inclusion criteria (Figures 1 and 2). PRP and insulin were applied on granular injuries (Figures 3–7) as well as after the covering of affected zones with free split skin grafts (Figures 8–10).

In all cases in which PRP was used, the granulation developed rapidly, visible accelerated compared to the untreated area or the insulin treated area, as demonstrated by photographic recorded results (Figure 11). What should also be mentioned is the fact that the PRP treated area showed an obvious increase of granulation rapidly after application (48 hours), compared to control (untreated) areas. The application of PRP was also continued immediately after grafting, in the expansion mesh of the grafts. After the area was covered by specific means (free spliced skin grafts – FSSG), biopsies in the treated areas were performed. The PRP-treated area presented an intense proliferation of inflammatory elements, an improved vascularization and an agglomeration of cellular.

The results were stable and of good quality and no subsequent lesion occurred (Figures 12–14). PRP was applied either directly on the granulation area or by mesotherapy (Figures 5, 6 and 9).

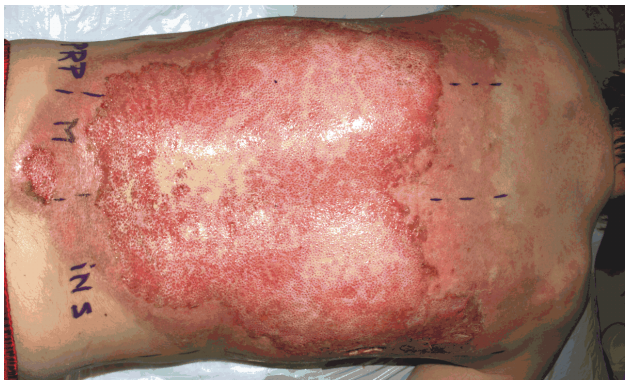

Figure 1 – ***PRP and insulin used on injuries.***

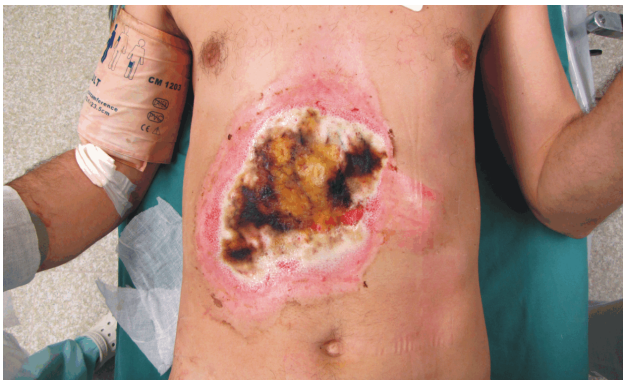

Figure 2 – ***PRP and insulin: inclusion criteria.***

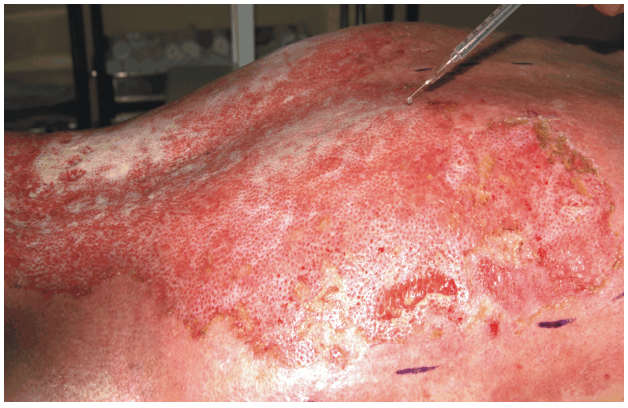

Figure 3 – ***Granular injuries on the back of the patient, treated by contact method with insulin.***

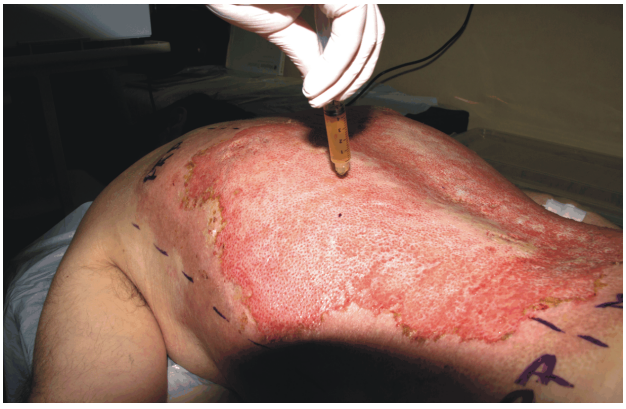

Figure 4 – ***PRP applied on granular injuries on the back of the patient.***

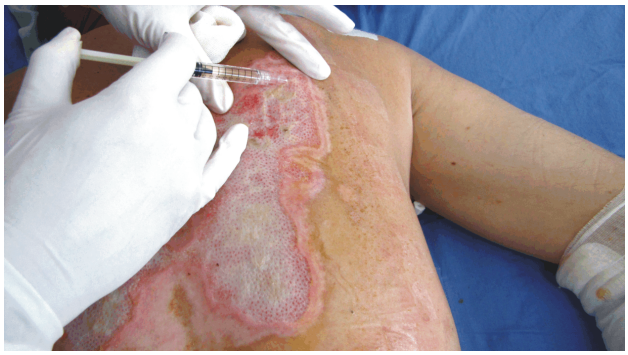

Figure 5 – ***PRP applied by mesotherapy, on granular injuries on the back of the patient.***

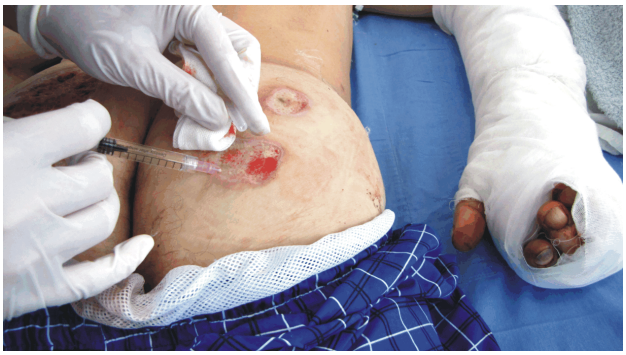

Figure 6 – ***PRP application by mesotherapy.***

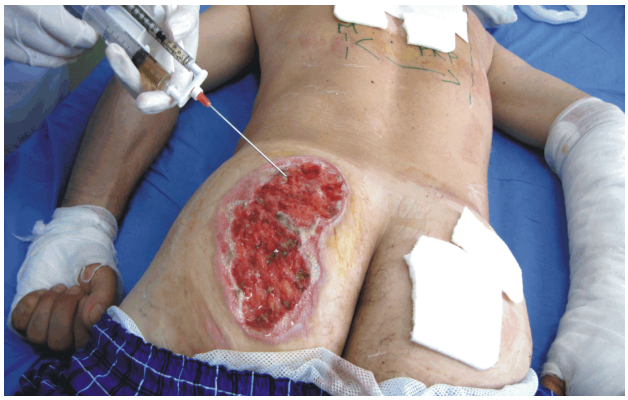

Figure 7 – ***PRP application by contact method.***

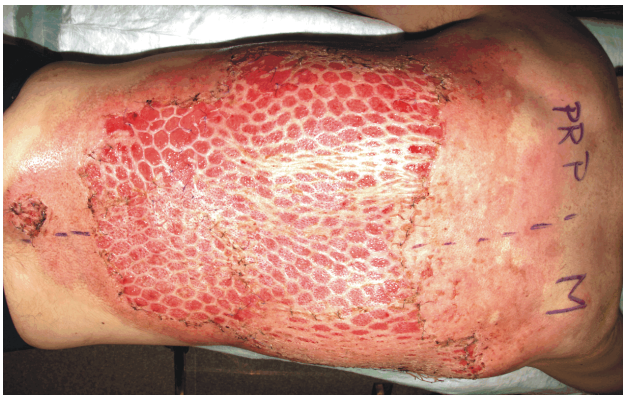

Figure 8 – ***Free split skin grafts treated with PRP and conventional method.***

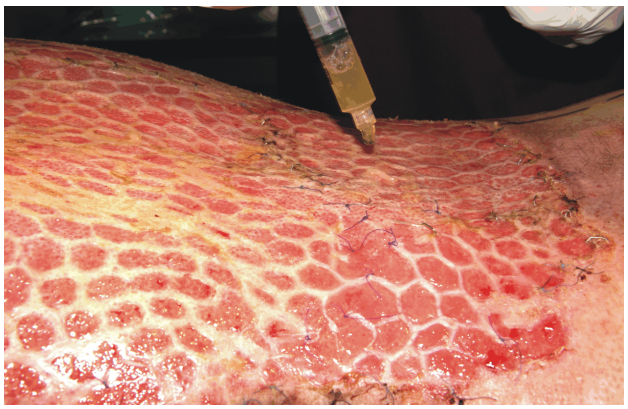

Figure 9 – ***Contact application of PRP, on skin grafted areas.***

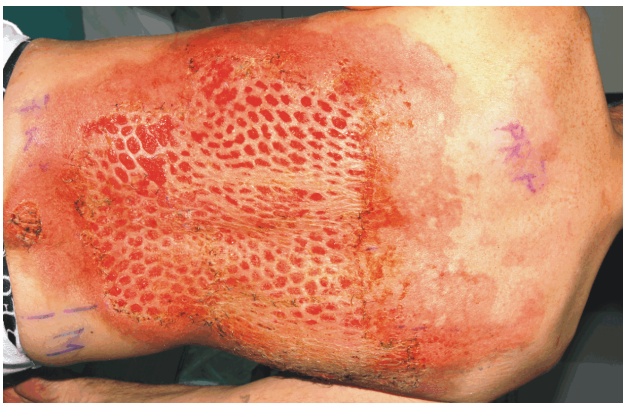

Figure 10 – ***Free skin grafted areas treated with PRP and conventional methods.***

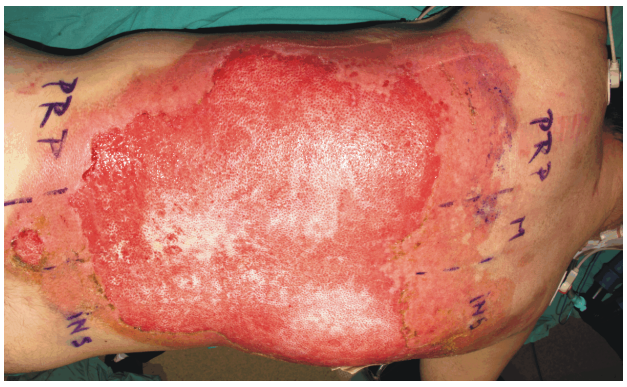

Figure 11 – ***Rapidly developed granulation on PRP marked area (48 hours after first application).***

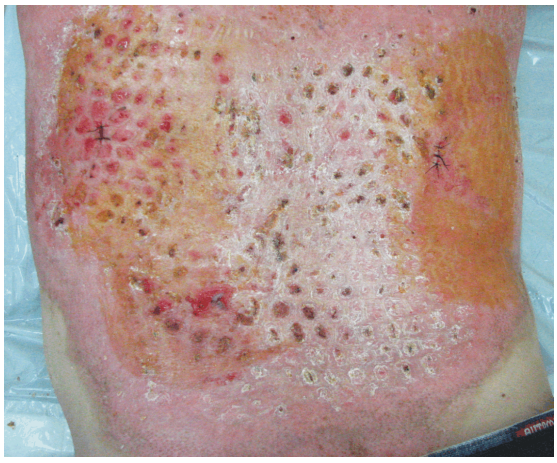

Figure 12 – ***Stable results of the treatment.***

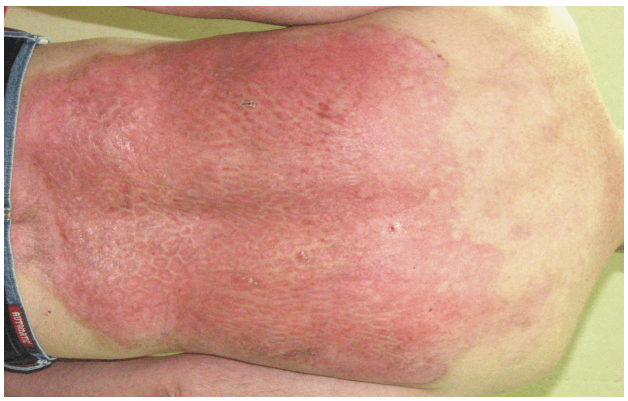

Figure 13 – ***No presence of any subsequent lesion.***

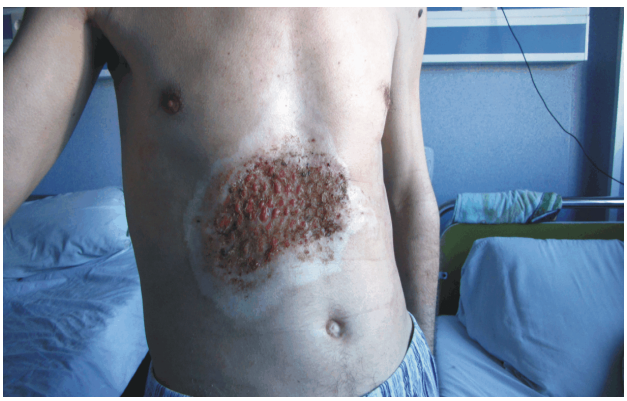

Figure 14 – ***Good quality results.***

Discussion

Therapeutic approach

We choose to use topically growth factors such as insulin and the platelet enriched plasma concentrate in three cases of sever electrocution injuries. This approach, relatively easy to use and with low costs, is new for the management of this type of injuries.

There are several experimental researches in which the two above mentioned growth factors were used for wound healing and the most relevant data are presented below, as a background for our case study:

- the granulation was obvious in the first 48 hours in the PRP treated area and was not evident in the insulin treated area;
- we consider shortening of hospitalization by about seven days.

The role of insulin in wound healing

Topical application of insulin improves wound healing. In the experimental use of insulin *in vitro*, an enhanced migration of keratinocytes is found comparatively with insulin untreated cell cultures. A large number of reticular bridges at the level of epidermis, in the papillary dermis and a well-constructed connection are suggesting that in insulin treated wounds healing is of better quality [4–8]. Insulin induces accelerated wound healing associated with diminished inflammation and increased collagen deposition [9].

The activity of macrophages was monitored by immunohistochemistry and immunofluorescence studies in insulin-treated wounds, demonstrating an intense and constant growth towards day 3, followed by a constant decrease until a minimal level. Also, high levels of mRNA expression are associated in cells from insulin-treated wounds. Insulin facilitates chemotaxis for monocytes, macrophages and phagocytes and the secretion of mediators of inflammation. Insulin promotes wound healing, normalizing the inflammatory response at their level, especially the quantity and function of macrophages.

Our previous studies strongly suggest that insulin is a potent healing accelerator (unpublished data). Regulating wound inflammatory response, especially the quantity and function of macrophages, is one of the mechanisms explaining insulin-induced accelerated wound healing [10].

Although the general role of insulin in wound healing is well-known [11], the effect of insulin on protein metabolism in skin wound has not been assessed sufficiently. To repair skin defects, new proteins have to be deposited in the wounded area to restore skin integrity. Data indicate that high doses of insulin and glucose can be safely administrated to massively burned patients to improve wound matrix formation, and, the insulin alone, stimulates protein turnover in skin wound [12].

Use of insulin in post-electrocution injuries

The use of insulin as a growth factor in granular wounds, which do not display a tendency to contraction and epithelialisation seems a cheap and easy to use alternative. Previously, we used this method on patients with trophic ulcers, atonic wounds generated by cutaneous forms of lymphomas, with good results. A limitation in the use of insulin is however noticed, the limitation generated by the lack of control on absorption in transcutaneous administration, depending largely on the area of the injury, the amount of applied insulin, the vascularization at granulation level, as well as on the level of glycemia at the time of treatment. Thus, we found out that, in non-diabetes mellitus patients, the use of insulin on granular surfaces larger than 100 cm^2 can present risks, the glycemic level decreasing rapidly (in minutes) with 20–40 mg/dL.

In such cases, the glycemia monitoring is mandatory, before and after the treatment onset, the patient being connected to a venous line for administration of 10% glucose. The fact that the treatment is achieved in a rather empirical manner, only in a hospital under careful observation and with a canulated venous line, makes the administration of insulin useful only for carefully selected cases. For the electrocution patients, who in the first stage are an intensive care patient, the use of such treatment may be aggressive, mainly by the decrease of glycemia at the cerebral level.

Based on previous experience, we consider that this treatment can be applied without major precautions on patients with granular impairments not larger than 100 cm^2, preferably diabetics. On non-diabetic patients, having the above mentioned impairment sizes, it is recommended to use carbohydrate-reach food before treatment.

The role of PRP in wound healing

An alternative treatment was the use of PRP. The use of platelet-enriched plasma was successfully applied

in other surgical specialties over time, several studies reporting the use of PRP solely or in combination with other methods. The principle is simple and it is based on the biological feature of platelets to carry growth factors and to accelerate or initiate the tissue repair processes. The large number of growth factors contained in platelet granules, the ability of *de novo* protein synthesis, and its antimicrobial activity and inflammation modulator promote cell proliferation and synthesis of extracellular matrix, promoting healing and wound repair [13].

Platelet-rich plasma (PRP) is defined as an "autologous concentration of platelets in a small volume of plasma" [14] and is considered to be a rich source of autologous growth factors. There are several other terms besides PRP, such as autologous platelet gel, platelet gel biotechnology and autologous platelet concentrate. The latter, according to Appel *et al.* [15], is the most accurate term from a hematological point of view. PRP is an otherwise normal autogenous blood clot that contains a high concentration of platelets, the only difference being, a normal blood clot, such as that found in a healing wound, contains 94% red blood cells (RBCs), 6% platelets and somewhat less than 1% white blood cells (WBCs). In contrast, a PRP blood clot contains 94% platelets, only 5% RBCs and 1% WBCs. This alteration of cellular ratios in the wound blood clot, whereby cells that do not stimulate healing (RBCs) are replaced by cells that stimulate all phases of healing (platelets), explain its ability to enhance healing [16].

Mechanism of action

The alpha granules in PRP begin degranulation within 10 minutes of clot development and secrete over 90% of their pre-packaged growth factors within one hour. These growth factors act on target cells and, thus, PRP initiates a greater and faster initial cellular response than a normal blood clot. Fibrinogen, converted to fibrin, in combination with growth factors present in PRP, might effectively promote wound healing at sites of injury, and platelet-rich plasma-derived fibrin clot formation may stimulate collagen synthesis.

PRP has also been shown to promote significant changes in monocyte-mediated cytokine release by decreasing the levels of monocyte chemoattractant protein-1 (McP-1) and increasing the levels of regulated activation normal T-cells expressed and secreted (ranTES) from the b-granules of platelets. Thus, PRP paves the way towards wound healing. Also, lipoxin a4 levels have been shown to increase, pointing towards the anti-inflammatory action of PRP [16].

Currently, individual growth factors administered for wound healing are used at much higher concentrations than those in platelet preparations in this study. The complex mixture of growth factors delivered by platelets potentially act in synergy, while also being protected against rapid degradation. In this study, the concentration of platelets in all preparations was normalized to that currently used in clinically treated wounds. Increases in platelet concentration might cause a reduction in proliferation in, due to saturation and down-regulation of the growth factor receptors. It is suggested that growth factors may be more effective when directly delivered by platelets, allowing a slow release, to enhance the physiological response to injury, rather than administered as a bolus dose [17].

Growth factors in PRP

Several growth factors have been found in PRP, including all three isoforms of Platelet Derived Growth Factor (PDGF), *i.e.*, PDGF-aa, PDGF-b band PDGF-ab; two isoforms of Transforming Growth Factor (TGF), *i.e.*, TGF-b1 and TGF-b2; Vascular Endothelial Growth Factor (VEGF); and Epithelial Growth Factor (EGF). The presence of Insulin-like Growth Factor (IGF) in PRP is still disputed [18]. These growth factors have several roles in regeneration.

All of these factors set the stage for tissue healing which involves intricate overlapping processes that are often categorized into hemostasis, inflammation, proliferation, and remodeling. Once tissue injury occurs, a hematoma forms at the site of tissue damage, platelets adhere to exposed collagen creating a clot, and the inflammatory phase begins with activation of platelets resulting in release of growth, bioactive, and hemostatic factors. Each factor plays a unique but important integrating role in the early stages of the intrinsic and extrinsic pathways of the clotting cascade. Access to the wound site by neutrophils and macrophages occurs within hours of injury thereby initiating the phagocytosis of tissue debris. Within a few days of injury, the proliferative phase begins and this is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction. Finally, the remodeling phase involves collagen maturation and apoptosis of excess cells, which can take from several weeks to months after an injury depending of the degree of damage. Based on the above-described model, acceleration of wound healing by addition of PRP is based on various platelet growth factors (PGFs) that stimulate different stages of the wound cascade. Compared to application of single, recombinant growth factors, which are applied in supra-physiological concentrations, PRP has the advantage of offering multiple, synergistically working growth factors at the wound site and in concentrations that are biologically and physiologically more pertinent [18].

PDGF (platelet-derived growth factor) is a key mediator in wound healing, and its importance is highlighted in that it was the first recombinant growth factor approved for topical application to accelerate wound closure. However, PDGF plays a role throughout the entire process of wound healing. In the beginning, PDGF serves as a chemoattractant for the migration of fibroblasts, neutrophils, and monocytes to the site of injury. Platelet-derived growth factor is also a mitogen for fibroblasts and promotes the production of new extracellular matrix components [14, 15]. Later in the proliferative phase, PDGF stimulates the differentiation of fibroblasts into myofibroblasts, promoting contraction of collagen matrices and the wound [14–16]. Platelet-derived growth factor has also been implicated in the remodeling phase through its effect on stimulating collagenase production in fibroblasts [19].

PDGF-aa and PDGF-bb are major mitogens for human PDL cells *in vitro*. PDGF also plays a role in

macrophage activation, leading to early debridement of the wound site; it is a second phase source of growth factors for continuous repair and regeneration.

TGF-b belongs to the Transforming Growth Factor superfamily; it is an important mitogen and morphogen. TGF-b1 and TGF-b2 act as paracrine growth factors, affecting mainly fibroblasts, marrow stem cells and the preosteoblasts. However, each of these target cells has the ability to synthesize and secrete its own TGF-b proteins, to act on adjacent cells in a paracrine fashion or to act on itself as an autocrine growth factor. TGF-b, therefore, represents a mechanism for sustaining a long-term healing. TGF-b also has an inhibitory effect on epithelial cell migration.

VEGF promotes capillary in growth to provide nutrients and oxygen needed. It also stimulates the basal lamina synthesis and recruitment of pericytes to support new blood vessels. VEGF is found in the blood clots present in wounds, and starts acting the moment the fibrin plug forms. VEGF is also produced by neutrophils. Injection of VEGF directly into the ischemia-injured myocardium induced the formation of new blood vessels, avoiding the need of coronary by-pass.

VEGF is used in research as a stimulant for wound healing, but more and more cardiology centers are starting to offer VEGF revascularization [20]. While VEGF appears to be beneficial in promoting burn wound healing, a caveat to this is evidence suggesting that the increased serum level of VEGF is responsible for the edema, anasarca, and edema-associated complications after burn injury [19].

EGF effects are limited to basal cells of skin and mucous membrane; it stimulates these cells to lay down a specific component of the basement membrane. It is a factor promoting the growth of epidermis, and it is found in teguments and in mucosa of the gastrointestinal tract. It is also normally present in tears. EGF stimulates the growth of various epidermal and epithelial tissues *in vivo* and *in vitro* and of some fibroblasts in cell culture.

Regarding burn wound healing, however, there is conflicting evidence about the presence of EGF in the wound fluid. Two other members of the EGF family, heparin-binding epidermal growth factor (HB-EGF) and TGF-α, have been detected at significant levels in burn wound fluid. Evidence for the roles of HBEGF and TGF-α in wound healing is seen through their effects on stimulating keratinocyte proliferation and accelerating reepithelialization [19].

The magnesiotropic hormone stimulates magnesium reabsorption in the renal distal convoluted tubule *via* engagement of EGFR and activation of the magnesium channel [21]. Increased EGF levels are correlated with cancer progression. Usually, EGF is administered topically, but it can also be administered intravenously. Applied in burns, ulcerations and cornea injuries, it acts as a promoter of healing. EGF is expensive, but accessible to medical use.

When IGF-I was discovered, it was found out that it has a structure similar to that of insulin [22]. Topical application of insulin accelerates healing, presumably because it chemically reassembles IGF-I. The Growth Hormone (GH) secreted by the pituitary, induces the production of IGF-1 in the liver, which stimulates cell growth and repair. Many types of tissue contain IGF-I receptors (muscle, digestive tract, skin, etc.). IGF-I is available for research and for clinical studies.

As for wound healing, evidence suggests that IGF-I is mitogenic for keratinocytes and fibroblasts, inhibits apoptosis pathways, attenuates pro- and anti-inflammatory cytokine production, and stimulates extracellular matrix component production. IGF-I is found in burn wound fluid as part of the normal injury response, and the expression of IGF-I and IGF-II has been shown to be significantly up regulated in epidermal cells of healing wounds that normally express minimal amounts of the protein product. In addition, the levels of IGF-I in wound fluid demonstrate a positive correlation with the success of wound healing [19].

The other components of PRP include cell adhesion molecules like fibrin, fibronectin and vitronectin. The alpha granules of platelets are rich in vitronectin [16].

The use of PRP in therapy

The use of PRP was documented in several areas. First promoted by M. Ferrari (1987) as a component of autologous transfusion after the intervention on the heart, in order to avoid the transfusion with a product with homologous blood, presently there are more than 5200 references in different areas, from orthopedics, sport medicine, dentistry, otorhinolaryngology, ophthalmology, urology, wound healing, cosmetics, cardiothoracic surgery and maxillofacial surgery [23].

There are other promising indications for PRP applications, and these include:

- accelerating the healing of bone fracture sites and/or bone grafts – particularly for maxillofacial indications;
- treating tendinopathies especially in Achilles tendon problems and rotator cuff tissue repair;
- application in aesthetic surgery as a filler and for skin rejuvenation in order to enhance the take of adipose tissue grafts;
- treating chronic non-healing wounds such as those of diabetic patients who are at risk of impaired wound healing;
- treating acute and chronic wounds, severe burns and associated skin graft donor sites which are necessary in patient management;
- taken all together, the most promising areas for using PRP use seem to be for tendinopathies [18].

The initial popularity of PRP has grown as it promises a secure and natural alternative to surgery. Proponents of PRP therapy promote its use as an organic treatment, which initiates the healing process by using natural growth factors, belonging to its own body. Recent scientific research has provided a new perspective on platelets. These studies suggest that platelets contain an abundance of growth factors and cytokines, which affect inflammation, postoperatory blood loss, infection, osteogenesis and healing of injuries. Several studies demonstrated that platelets release more bioactive proteins that are responsible for the attraction of macrophages, mesenchymal stem cells and osteoblasts, which not only promote the elimination of necrotic and degenerated tissue, but also improve the regeneration and tissue healing.

The increased use of PRP was originally based on case reports. Historically, there were just few controlled studies to assess the efficacy of PRP. In the absence of such studies, the groups were too small to allow the generalization of conclusions. Moreover, the lack of consensus regarding the technique, the number of injections, the amount of platelets compared with the basal level, the injection with or without leukocytes, the exogenous activation of injected platelets, and even the definition of patients with such indication, has led to the need of additional evaluations [22, 24]. Also, PRP was considered highly likely to have promoted the proliferation of keratinocytes [25].

PRP in burns

In recent years, PRP had been used in burn treatment, but it provoked some disputes [26].

A survey of the literature to assess the current clinical experience and the possible effects of PRP on wound healing in burn cases yields only few reports. The application of PRP is not currently standardized and the effects in wound healing are poorly understood. The use of PRP as an analog to fibrin sealant is also only seldom reported. The value of PRP application in burns remains unclear, requiring further studies [27].

To date there are very few studies that categorically demonstrate the usefulness of PRP in burn management. Experimental studies have demonstrated that the application of PRP gel to burns stimulates an intense inflammatory reaction with a significant increase in the extracellular matrix proteins and proliferation of fibroblasts, collagen and granulation tissue; however, these experiments do not report a true acceleration on wound epithelialization. On the other hand, a study in which a subconjunctival injection of PRP was administered in ten patients with ocular burns a significantly faster epithelialization of the cornea and conjunctiva was demonstrated. In another study that approached the usefulness of PRP gel in the management of diverse wounds, including friction burns, a significant improvement was demonstrated with its use. Up to now, there are no further studies to support the usefulness of PRP in the management of burns. Despite this, because of the promising results of the experimental studies there is a theoretical possibility that due to the increase reported on the inflammatory reaction, this may stimulate formation of a hypertrophic scar on superficial burns, whereas the effect may be beneficial on deeper burns. The limited evidence does not yet allow for the recommendation of the use of PRP for burn management. Some clinically relevant variables to be defined in other studies include extent of the burn, its thickness, time of application, effect on epithelialization, time and type of healing, effect on the grafts, infection rate, etc. [13].

In our case of electrocution injury, we showed that PRP increase of granulation rapidly after application (48 hours), compared to control (untreated) areas, inducing an intense proliferation of inflammatory elements, an improved vascularization and an agglomeration of cellular elements.

In the ocular area, numerous studies that confirmed the beneficial effect of PRP application. Topical autologous platelet-rich plasma therapy is safe and effective, and it promotes rapid re-epithelialization of ocular surface and can be administered along with standard medical therapy [28].

Subconjunctival infiltration of autologous platelet concentrate should be considered as a straightforward, economical and possibly effective form of treatment for traumatic accidents (burns) of the ocular surface with a shorter period of time in corneal and conjunctival healing, time on sick leave and time needed for full healing, comparing to conventional topical medications [29]. The treatment is effective both in moderate and in severe burns [30].

The pragmatic belief that PRP can promote infection is based on flawed logic that PRP is a blood clot and blood agar is used in microbiology labs to culture bacteria. In reality, PRP is identical in substrate to the blood clot that forms in every wound and, therefore, cannot support bacterial growth any more than any other blood clot. Also, PRP has a pH of about 6.5–6.7, while a mature blood clot has a pH of 7–7.2; therefore, PRP may even inhibit bacterial growth as do other acidic solutions. Moreover, platelets share structural and functional similarities with granulocytes, which are known to participate in antimicrobial host defense. It has been demonstrated that seven antimicrobial peptides from human platelets – platelet factor 4 (PF-4), ranTES, connective tissue activating peptide 3 (cTaP-3), platelet basic protein, thymosin b-4 (Tb-4), fibrinopeptide b (FP-b) and fibrinopeptide a (FP-a) – are released on activation. These peptides have shown *in vitro* antimicrobial activity against several organisms, thus suggesting a direct antimicrobial role for platelets [16].

This hypothesis is proved by a study conducted on deep second-degree burns in horses. This kind of burns involves all epidermis layers, including the basal laminae and, in general, they heal with extensive areas of scarring. PRP treatment not only accelerated repair, induced fibroses but also provided antibacterial activity (no infection was noticed although bacterial contamination was present) [31].

PRP – considerations regarding preparation

The underlying practical difficulty with designing a multiple growth factor treatment is to predict the proper time-dependent growth factor ratios for optimal healing. PRP preparations can concentrate platelets and their growth factors up to eight times the basal levels in whole blood without inducing platelet activation. The use of autologous PRP eliminates the potential for disease transmission and immunogenicity; however, preparation requires time to draw and process patient blood. Furthermore, the quality of autologous platelet preparations is inconsistent due to individual patient characteristics, many of whom have systemic diseases and are debilitated. This inconsistency may explain in part the mixed results seen in clinical studies. Healthy, allogeneic platelets may potentially circumvent this problem by allowing process standardization, but current blood-banking standards require platelets to be stored at room temperature and discarded after five days thereby limiting the availability of these cells for wound healing applications. Platelets stored for as long as 21 days retain their proliferative activity,

but are at risk for bacterial proliferation and accumulation of pyrogenic cytokines. Refrigerated preservation (4^0C) or cryopreservation methods (-80 or -196^0C) reduce the potential for bacterial proliferation, but shorten cell lifespan by inducing irreversible platelet activation, commonly known as the cold lesion. Improved understanding of the cold lesion enables preparation of platelets in such a way as to preserve their physiological properties following cold storage. One method of platelet stabilization is to directly target intracellular biochemical pathways modulated by reduced temperatures using specific second messenger effectors. Such a formulation containing Amiloride, Adenosine, and Sodium Nitroprusside has been shown to protect platelets from damage during cold and cryopreserved storage and, thereby, prevent platelet activation. Platelets cryopreserved using this biochemical stabilization method show retention of *in vitro* platelet function compared with fresh control cells, maintain circulation residence time *in vivo*, and provide clinically effective correction of platelet count following transfusion in chemotherapeutically induced thrombocytopenia. Storage modality plays a significant role in acceptance of any potential therapy. With respect to wound healing treatments, cryopreserved storage of a platelet-based product at either -80 or -196^0C would be difficult at most wound care clinics and hospitals [17].

According to the definition, PRP must contain a higher platelet concentration than the basal value; however, increasing the number of platelets represents a coarse definition and does not accurately describe the variability between different types of PRP. There are parameters to be considered, including the following: the value of platelet concentration compared to the basal level, whether leukocytes are included or not, if PRP was anti-coagulated or not, and whether exogenous activation is required or not.

PRP is produced from the blood of the patient using different methods for platelet concentration through centrifugation and cell separation. The aim is to achieve about 300% to 500% augmentation of the platelet concentration in certain plasma volume. There have been several methods used to achieve this, like plasmapheresis, blood fractionation, use of commercially available systems like the Platelet concentrate collection System® (Implant Innovations, Palm Beach, FL, USA), SmartPrep® (Harvest Technologies Corp, Plymouth, MA, USA), Curasan (Kleinostheim, Germany) or a laboratory centrifuge [16].

The number of platelets is the first variable to be considered. The absolute number of platelets is variable depending on the platelet concentration in the patients' peripheral blood. PRP can be subdivided in low PRP (2.5–3 folds higher than patient's basal level) and high PRP (5–9 fold greater than the patients' basal level). Intuitively, it is considered that a higher number of platelets contain more growth factors and could generate better clinical outcomes; however this has not been proved. Graziani *et al.* suggest that a concentration of 2.5-fold greater than the basal level is optimal, and over this level, the effect is inhibitory [2]. Variations in the platelet concentrations might influence the bone formation within the PRP-treated bone grafts. These variations in platelet concentrations may depend upon the technique of preparation and probably the method of activation [16].

When integral blood is collected, numerous PRP kits are using anti-coagulant. Many kits are using Dextrose-Citrate (ACD) as anticoagulant. ADC binds calcium and prevents coagulation proteins to initiate the clotting cascade. The addition of citrate increases the acidity over the physiological level. Since some growth factors are influenced by pH, some protocols recommend PRP buffering to the physiological pH before injection.

PRP can be activated exogenously with thrombin, calcium chloride or by mechanical trauma. PRP once activated, a fibrin network is formed, which solidifies plasma creating a fibrin clot or membrane [32]. In the case study presented, we used Calcium Gluconate and thrombin as PRP activators. Thrombin catalyzes the conversion of plasma fibrinogen into fibrin to create a platelet-rich plasma gel that has improved handling properties as compared to liquid platelet-rich plasma, but it also triggers platelet aggregation and degranulation. In the pioneering study on platelet-rich plasma by Marx *et al.*, researchers prepared the PRP gel with a mixture of 10 mL of 10% Calcium Chloride mixed with 10 000 units (U) of bovine thrombin [14]. Since then, different concentrations of thrombin have been suggested in the medical and dental literature, ranging from 1000 to 5000 U [16].

The optimal regiment to prevent un-intentional activation remains unclear. Collagen is a natural activator of PRP, therefore, when PRP is used in soft tissue, exogenous activation is not required.

Once the activation occurs at the site of injection, the release of growth factors initiates an inflammatory response that lasts for about three days. At the site of injection fibroblasts are accumulating, which marks the start of the proliferative stage of healing that lasts for several weeks. After this stage, the collagen matrix created by fibroblasts is remodeled. This remodeling stage, which leads to the formation of mature tissue, takes up to six months. The completion of all stages is required for the formation of new tissue with long-time stability [33].

The clinical efficacy of platelet concentrates depends mainly on the number of platelets and the concentration of their growth factors, which act as transmitters in most processes in tissues, particularly in healing where they are responsible for proliferation, differentiation, chemotaxis and tissue morphogenesis. They operate as part of autocrine, paracrine and endocrine mechanisms. Growth factors derived from centrifuged blood were first used in patients with chronic skin ulcers. The clinical use of PRP for a wide variety of applications has been reported mostly in oral and maxillofacial surgery, orthopedic surgery, treatment of soft tissue diseases and injuries, treatment of burns, hard-to-heal wounds, tissue engineering and implantology [34]. PRP is considered the election treatment for subacute and chronic musculoskeletal conditions. Generally, healing slows or stops 6–12 weeks from the acute injury. If the patients do not display improvements during six weeks, it is possible that the healing phase completed.

Upon over-dosing or repeated administration, the delimitation between the acute phase and the transition phase may become difficult.

The PRP contraindications are of two types, absolute and relative, each including a series of conditions. The

absolute category comprises the followings: platelet dysfunction syndrome, critical thrombocytopenia, hemodynamic instability, sepsis, infection at the injection site, patient who does not accept the risks. As regards the relative category, the following characteristics can be met: intensive use of non-steroidal anti-inflammatory drugs (NSAID) 48 hours before treatment, injection of corticosteroids at site to be treated, within one month before, systemic corticoid treatment within two weeks before, smoking, recent fever or other inflammatory conditions, cancer – especially blood or bone tumors, Hb <10 g/dL, platelets $<10^5/\mu L$ [35].

Among the patients' risks the following should be mentioned: infection: PRP is antimicrobial against moth classes of bacteria, excepting *Klebsiella*, *Enterococcus* and *Pseudomonas*, blood loss, nerve injuries, pain, lack of results, limb loss or death are rare but possible.

Conclusions

It is the intention of this report to show that a simple protocol and common laboratory materials can be adapted to standardize the preparation of PRP with the emphasis on overall preservation of platelets' physiological properties. The protocol described can be reproduced in any clinical setting, permitting a cost-effective delivery of growth factors. The purpose of the treatment in the above-mentioned cases was to limit the propagation of ischemic areas, which was difficult to achieve in the absence of correlation with other solutions of re-equilibration (increasing hemoglobin, albumin, efficient peripheral oxygenation, combating sepsis). In all situations, the patients had to be carefully selected. What should be mentioned is the fact that the PRP-treated area showed an obvious increase of granulation rapidly after application (48 hours), compared to control (untreated) areas and we consider shortening of hospitalization by about seven days. This treatment was useful in providing a good surgical background that guaranteed a good postoperatory outcome, in limiting the development of ischemic zones in the sensitive areas in which mobile elements (large vessels, nerves) could not be exposed and which could not be covered in the respective time by other procedures, in shortening the surgical and pharmacological treatment and the duration of hospitalization. In the future, to extend this therapy, there is the need of more clinical studies in order to establish a suitable therapeutic protocol for using PRP in high-voltage injuries.

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