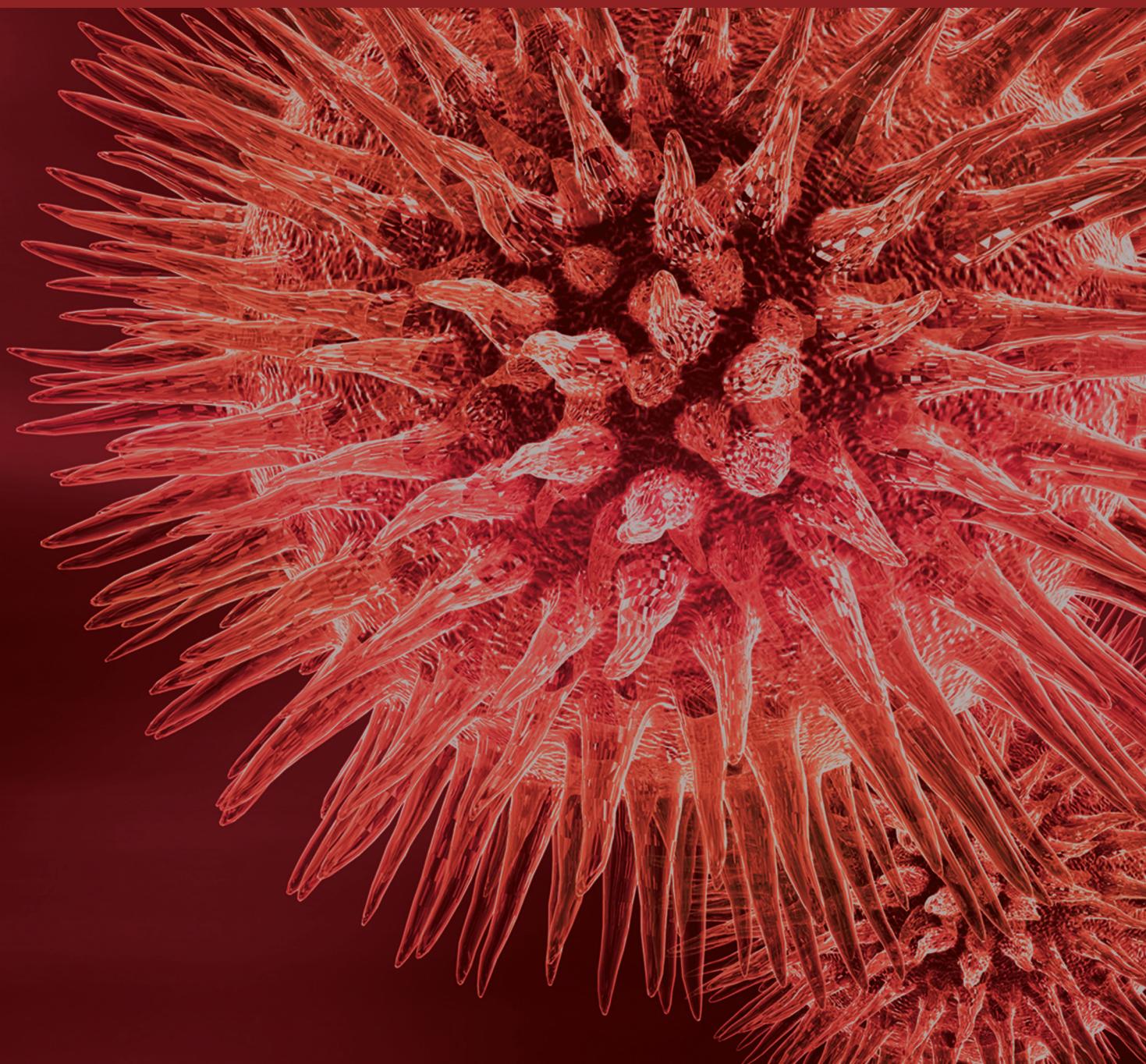


Skin Regeneration, Repair, and Reconstruction

Guest Editors: Lars-Peter Kamolz, May Griffith, Celeste Finnerty,
and Cornelia Kasper



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Contents

Skin Regeneration, Repair, and Reconstruction, Lars-Peter Kamolz, May Griffith, Celeste Finnerty, and Cornelia Kasper
Volume 2015, Article ID 892031, 1 page

The Use of Stem Cells in Burn Wound Healing: A Review, Fadi Ghieh, Rosalyn Jurjus, Amir Ibrahim, Alice Gerges Geagea, Hisham Daouk, Bassel El Baba, Sana Chams, Michel Matar, Wadih Zein, and Abdo Jurjus
Volume 2015, Article ID 684084, 9 pages

Generation of a Fibrin Based Three-Layered Skin Substitute, Johanna Kober, Alfred Gugerell, Melanie Schmid, Lars-Peter Kamolz, and Maike Keck
Volume 2015, Article ID 170427, 8 pages

Immunohistochemical Expression of Collagens in the Skin of Horses Treated with Leukocyte-Poor Platelet-Rich Plasma, Maria Verônica de Souza, Mariana Brettas Silva, José de Oliveira Pinto, Marianna Barros de Souza Lima, Júlio Crepaldi, Gabriela Francine Martins Lopes, Hélio Batista dos Santos, Rosy Iara Maciel de Azambuja Ribeiro, and Ralph Gruppi Thomé
Volume 2015, Article ID 893485, 12 pages

Diverse Roles of Heparan Sulfate and Heparin in Wound Repair, Paweł Olczyk, Łukasz Mencner, and Katarzyna Komosinska-Vassev
Volume 2015, Article ID 549417, 7 pages

GHK Peptide as a Natural Modulator of Multiple Cellular Pathways in Skin Regeneration, Loren Pickart, Jessica Michelle Vasquez-Soltero, and Anna Margolina
Volume 2015, Article ID 648108, 7 pages

Freestyle Local Perforator Flaps for Facial Reconstruction, Jun Yong Lee, Ji Min Kim, Ho Kwon, Sung-No Jung, Hyung Sup Shim, and Sang Wha Kim
Volume 2015, Article ID 707065, 5 pages

Low versus High Fluence Parameters in the Treatment of Facial Laceration Scars with a 1,550 nm Fractional Erbium-Glass Laser, Hyung-Sup Shim, Dai-Won Jun, Sang-Wha Kim, Sung-No Jung, and Ho Kwon
Volume 2015, Article ID 825309, 4 pages

Editorial

Skin Regeneration, Repair, and Reconstruction

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Skin has important functions in several life-preserving processes such as hydration, protection against chemicals and pathogens, and heat regulation.

Severe damage to the skin may therefore be life-threatening. Skin regeneration and wound healing require an orchestrated integration of complex biological and molecular events, which include inflammation, proliferation, and remodeling. Despite the current use and availability of a wide array of wound dressings, ointments, and medical devices, wound healing still remains a clinical challenge, especially in the elderly, in diabetic patients, in heavy smokers, or in burned patients, because the time-consuming conservative wound management is mainly restricted to wound repair rather than restitution of the tissue integrity.

Therefore, there is a continued search towards more efficacious wound therapies to reduce health care burden and provide patients with long-term relief and ultimately scarless wound healing, because such wounds and defects if not treated effectively eventually end up in amputations or disfiguring scars termed as hypertrophic scars and keloids, because surgical procedures such as local or free flaps go along with limited donor site availability and require stable general health condition of the patients.

Therefore, there is a need of new strategies to promote wound healing and tissue repair. When talking of wound healing, a distinction is made between regeneration and repair. Regeneration is used to refer to the complete replacement of damaged tissue with new tissue not associated with scar tissue, while repair is used to refer to the reestablishment

of tissue continuity. Regeneration can be attained by two means:

- (i) restoration, defined as “putting together what is broken,”
- (ii) reconstruction, defined as “replacing and rebuilding what is torn down.”

To grant homeostasis, most tissues undergo continuous or cyclic processes of “degeneration” and regeneration. This special issue presents and compares different aspects of regeneration, repair, and reconstruction. By discussing the common traits and the specific features of regeneration, we propose general models of regeneration and highlight various strategies adopted to cope with damage and repair.

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Review Article

The Use of Stem Cells in Burn Wound Healing: A Review

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Burn wound healing involves a series of complex processes which are subject to intensive investigations to improve the outcomes, in particular, the healing time and the quality of the scar. Burn injuries, especially severe ones, are proving to have devastating effects on the affected patients. Stem cells have been recently applied in the field to promote superior healing of the wounds. Not only have stem cells been shown to promote better and faster healing of the burn wounds, but also they have decreased the inflammation levels with less scar progression and fibrosis. This review aims to highlight the beneficial therapeutic effect of stem cells in burn wound healing and to discuss the involved pathways and signaling molecules. The review covers various types of burn wound healing like skin and corneal burns, along with the alternative recent therapies being studied in the field of burn wound healing. The current reflection of the attitudes of people regarding the use of stem cells in burn wound healing is also stated.

1. Introduction

The use of stem cell therapy is the yet to be discovered gold mine of science. A myriad of studies using stem cells are being done with promising results in various fields ranging from oncologic and hematologic diseases to organ transplants and wound healing. In the field of wound healing, the use of different types of stem cells has been reported for different types of wounds [1–3]. Burn wounds were of special interest due to the large number of cases of burns encountered nowadays, especially in the Middle Eastern Region and specifically in those areas with armed conflicts. Burn wounds have proven to be capable of having a devastating effect both functionally and cosmetically, necessitating the search for a better and more efficient cure. Being a very hot topic in the present field of research with constant studies and updates necessitated an updated review that encompasses the recent advances in stem cell therapy for burn wound healing

in addition to relevant experimental studies. The literature was searched using the key words burn, stem cells, and wound healing. CINAHL, PubMed, EMBASE, and Medline were used as search engines to broaden the resources. The studies reported were not limited neither to humans nor by language and were mostly on animals unless otherwise specified. They are mostly reported in a chronological order of their publication dates, except when found relevant to group and mentioning some related studies consecutively.

Stem cells are undifferentiated pluripotential cells that are capable of producing other types of cells, including new stem cells identical to mother cells [4]. Stem cells can be of embryonal origin or adult origin, depending on the type of tissue they are derived from [4]. Embryonal stem cells are derived from either embryonal tissue or from germ cells in adults [4]. On the other hand, adult stem cells are derived from adult tissues of different organs, especially those with a high turnover rate such as intestines and bone marrow [4].

2. Wound Healing

Stem cells have been implicated in the healing of wounds in general. However, the methods of application of the stem cells in burn wound healing are diverse, including topical application, local injection, intravenous or systemic injection, and dermal or carrier application. Several studies have shown the efficacy of stem cells in promoting faster and superior wound healing. Alexaki et al. [5] successfully used adipose derived mesenchymal stem cells in wound healing in mice and compared their effect with dermal fibroblasts. The application of stem cells in wounds promoted more efficient reepithelialization by their proliferative effect on keratinocytes [5]. Moreover, this effect of stem cells was found to be mediated by keratinocyte growth factor-1 (KGF-1) and platelet derived growth factor-BB (PDGF-BB) [5]. Amniotic fluid derived stem cells have also been used in wound healing. Skardal et al. [6] tested the effect of amniotic fluid derived stem cells in wound healing in a mouse model. Wound closure, reepithelialization, and angiogenesis were more rapid in mice treated with the stem cells in comparison to those treated with fibrin collagen gel only [6]. Additionally, stem cells did not integrate permanently in the tissue, thus, suggesting that their effect is due to released factors and not by direct interaction [6]. Additionally, bone marrow derived mesenchymal stem cells have also been used in wound healing. Leonardi et al. [7] utilized bone marrow derived stem cells in artificial dermal substitutes to promote wound healing. These stem cells were shown to increase vascular density in the wounds along with the rate of reepithelialization [7]. A study by Zhang et al. [8] examined the effect of activin signaling on the homing of stem cells to wound sites. It was also found that JNK and ERK signaling pathways were involved in activin signaling and eventually the homing of stem cells [8].

3. Physiology of Burn Wound Healing

Concerning the physiology by which stem cells enhance the process of burn wound healing, several studies have been reported. Mansilla et al. [9] found evidence of cells in the bloodstream with identical phenotypes to mesenchymal bone marrow stem cells after acute large skin burns. Hence, it was concluded that these stem cells may have a role in promoting wound healing in burns. In a similar study, Fox et al. [10] reported increased levels of bone marrow derived endothelial progenitor cells in burn patients. These levels were proportional to the extent of the burn. The study also showed increased levels of angiogenic cytokines which may be involved in the signaling pathway for promoting the release of bone marrow derived stem cells. Focusing on the role of cytokines in burn wound healing, Payne et al. [11] studied the role of amnion derived cellular cytokine solution. In the study, Payne et al. used amnion derived multipotent progenitor cells to harvest cytokines and apply them in burn wound healing. Amnion derived cellular cytokine solution showed statistically significant improvement in the epithelialization of the burn wounds and the appearance of hair growth compared to controls [11]. In addition, the results

demonstrated a faster epithelialization in burn wounds with increased frequency of application of the cytokines, further strengthening the role of stem cell derived cytokines in burn wound healing [11]. Furthermore, Foresta et al. [12] reported a positive linear correlation between endothelial progenitor cell blood levels and the total body surface area burnt. There was an increased level of endothelial progenitor cells in the bloodstream after escharectomy, posing a possible role of escharectomy in burn wound healing [12]. Additionally, stem cells could work by the release of bioactive peptides as proposed by Cabrera et al. [13] in their study where they showed that stem cells have an active role in burn wound healing by producing bioactive peptides, such as thymosin 4 and others.

More recent studies have also highlighted the role of stem cells in the process of wound healing in general and burn wound healing in specific. Koenen et al. [14] isolated acute wound fluids and chronic wound fluids and compared their effects on adipose tissue derived stem cell function in wounds. They came to the conclusion that acute wound fluids had a positive effect on the proliferation of adipose derived stem cells in wounds [14] while chronic wound fluids had a negative effect; the mentioned findings might explain the insufficient and slow healing process in chronic wounds due to a stem cell deficiency [14]. Furthermore, stem cells have been shown to decrease dermal fibrosis development in burn wound healing in mice [15]. Wu et al. performed a series of experiments which showed that bone marrow derived mesenchymal stem cells stimulate the formation of a basket weave organization of collagen in bleomycin treated skin, similar to normal skin [15]. Additionally, stem cell treatment of the skin decreased markers of myofibroblasts and downregulated type I collagen, leading to a decrease in the fibrosis that could have occurred to the skin [15]. Consequently, the role of stem cells in decreasing bleomycin induced fibrosis may be extrapolated to decrease fibrosis in burn wounds and improve their healing with less scar formation. Moreover, Lough et al. [16] performed a study in mice which showed a role for intestine derived human alpha defensin 5 in enhancing wound healing and decreasing its bacterial load. It induces leucine-rich repeat-containing G-protein-coupled receptors which are markers of adult epithelial stem cells both in skin and intestine [16]. Also implicated in the role of stem cells in burn wound healing is the role of SDF-1/CXCR4 signaling; Ding et al. [17] used interferon $\alpha 2b$ in patients with burn wounds to suppress SDF-1/CXCR4 signaling. They found out that the decreased levels of signaling lead to better remodeling of hypertrophic scarring in the wounds [17]. Additional studies on the CXCR4 signaling pathway were done by Yang et al. [18] on irradiated mice. The mice having an overexpression of CXCR4, a receptor involved in the homing and migration of several stem cell types, showed an accelerated wound healing time [18]. Furthermore, Hu et al. [19] injected bone marrow derived mesenchymal stem cells into mice and studied the effect of blocking CXCR4 receptors. They found out that blocking the CXCL12/CXCR4 pathway, leading to activation of CXCR4, caused delayed wound closure in inflicted burn wounds. Moreover, CXCL12 levels were elevated in the burn wound one week after injury

[19]. Hence, stem cells seem to be attracted to and attach to the burnt injury site by the CXCL12/CXCR4 pathway involving the CXCR4 receptors [18, 19]. The role of the ligand for the CXCR4 receptors, stromal cell derived factor-1 alpha (SDF-1 α), has also been studied. Lü et al. [20] performed a study on the role of SDF-1 α and its relation to the expression of miR-27b. It was found that SDF-1 α expression was suppressed by direct binding of the miRNA to its 39UTR site [20]. As expected, miRNA expression was suppressed in wounds hence allowing better SDF-1 α signaling and more homing of stem cells to the burn wounds [20]. In particular, miR-27b was found to be involved in the burn margins of wounds and in the mobilization of stem cells to the epidermis [20]. Chen et al. [21] performed experiments using porcine acellular dermal matrix on rats with inflicted 2nd degree burns. It stimulated collagen synthesis and stem cell proliferation and differentiation; porcine acellular matrix treated rats had a better and faster healing of the wounds.

Thus, in brief, the process of burn wound healing involves different types of growth factors, receptors, and cytokines. These factors are related to stem cell homing, differentiation, and proliferation. Additionally, when applied to burn wounds, they led to a better and faster healing process.

4. Stem Cells and Burn Wound Healing

The use of stem cells for burn wound healing, as reported in the literature, dates back to 2003 with Shumakov et al. [22]. Shumakov et al. were the first to use mesenchymal bone marrow derived stem cells (BMSC) in burn wound healing and compared them to embryonic fibroblasts [22]. The experiments were done on rats where mesenchymal bone marrow derived stem cells were applied to wounds showing decreased cell infiltration of the wound and an accelerated formation of new vessels and granulation tissue in comparison with embryonic fibroblasts and controls (burn wounds with no transplanted cells) [22]. Hence, this study marked a new era in the research of burn wound healing by being the first to test the use of stem cells in this complex process. Following this, a study by Chunmeng et al. [23] found that systemic transplantation of dermis derived multipotent cells promoted the healing of wounds in irradiated rats compared to controls with no transplantation, noting that topical transplantation of the cells had no superior effect. In 2004, Rasulov et al. [24] were the first to report using bone marrow mesenchymal stem cells in humans; a female patient with extensive skin burns (IIIB 30% of body surface area) had the stem cells applied onto the burn surface. The application of stem cells caused faster wound healing and active neoangiogenesis [24]. Another study done by Rasulov et al. on rats also showed the superiority of stem cells in burn wound healing [25]. In the rat study, the application of mesenchymal stem cells on burns reduced cell infiltration, improved neoangiogenesis, and reduced the formation of granulation tissue [25]. The aforementioned conditions created a better medium for wound healing in burns. In a similar effort, Liu et al. [26] performed experiments on pigs where they applied collagen scaffolds with seeded mesenchymal stem cells onto the surface of

inflicted burns; the latter were found to induce better burn wound healing with less contraction and better vascularization and keratinization. Moreover, in human cutaneous radiation wounds, Lataillade et al. [27, 28] reported two cases where stem cells were used to aid in burn wound healing. Mesenchymal stem cells were applied, in addition to surgical excision, flaps, and grafts, to burn wounds of cutaneous radiation patients. In these patients, the application of the mesenchymal stem cells decreased the levels of inflammation and promoted a better healing [27, 28]. Further on the role of stem cells in irradiated skin were the studies conducted by Dong et al. [29, 30], where they additionally inserted a vector of human beta defensin 2 into the stem cells. The mentioned studies showed a positive role for stem cells transfected with beta defensin 2 in burn wound healing by exhibiting antibacterial properties in infected burn wounds [29, 30]. In a similar experiment, Ha et al. [31] transfected mesenchymal stem cells with vectors of hepatocyte growth factor. The experiment, done on rats, compared the wound healing of a partial thickness burn treated with stem cells alone or stem cells transfected with hepatocyte growth factor [31]. The group treated with the transfected stem cells showed a significantly larger range of reepidermalization starting the first week, along with a thicker epidermis and lower content of collagen I at 3 weeks after burn [31]. In the same year (2010), Agay et al. [32] performed experimental studies by inflicting pigs with cutaneous radiation and studying the role of stem cells in the healing of the wounds. Intradermal mesenchymal stem cell injections were given locally in the affected area. They led to the accumulation of lymphocytes in the wound with better vascularization compared to controls (pigs with no injections of mesenchymal stem cells) [32]. Later on, Riccobono et al. [33] studied, in another experiment, the role of adipose tissue derived stem cells in the treatment of cutaneous radiation. Autologous, allogeneic, and acellular (empty, control) vehicles of adipose derived stem cells were grafted onto the burn wound areas [33]. Autologous but not allogeneic adipose derived stem cells were found to promote superior burn wound healing with no necrosis and decreased pain [33].

Aside to direct stem cell application to burn wounds, Kinoshita et al. [34] inflicted cutaneous radiation wounds to pigs and used expanders with and without basic fibroblast growth factor to determine their effect on burn wound healing. The group with basic fibroblast growth factor and expander showed greater proliferation of the dermis and epidermis along with increased neoangiogenesis [34]. Thus, basic fibroblast growth factor, which is known to promote the proliferation of mesenchymal stem cells, improved burn wound healing [34, 35].

In 2010, Yan et al. [36] studied the efficacy of porcine bone marrow derived mesenchymal stem cells combined with skin derived keratinocytes, both infected with recombinant retrovirus expressing human (h) platelet derived growth factor-A, in the healing of irradiated skin. The cells were loaded onto a cultured cutaneous substitute and compared their effect on healing with a cell-free cultured cutaneous substitute [36]. The substitute with cells stimulated faster healing,

epithelialization, angiogenesis, and better granulation of the burn wound [36]. In another experiment, Collawn et al. [37] inflicted laser burn wounds to organotypic raft cultures. The burn wounds were treated with dermal grafts with and without adipose derived stromal cells [37]. The adipose derived stromal cell-containing grafts showed complete healing of the epidermis after two days, whereas the cell-free grafts still had areas of injury; hence, those stem cells had a role in promoting faster healing of the burnt areas [37]. More on cutaneous radiation treatment came from Xia et al. [38] who transfected human vascular endothelial growth factor 165 and human beta defensin 3 into bone marrow derived mesenchymal stem cells and used the cells to treat irradiated skin. The stem cell treated area, in comparison with cell-free controls, showed shorter healing times with better granulation and collagen deposition [38]. Additionally, Xue et al. [39] examined the effect of human mesenchymal stem cells in mouse models. Mice with inflicted burn wounds were injected locally, in the burn area, with the stem cells (controls injected cell-free injections) [39]. Wound healing was significantly faster when stem cells were included in the injection with an increased and denser neoangiogenesis [39]. Stem cell injections also had a role in resuming activity and regaining body weight more rapidly [39]. Similarly, Mansilla et al. [40] used mesenchymal stem cells in burn wound healing in pigs through an acellular dermal matrix embedded with anti-CD44 antibodies to promote homing and attachment of the stem cells [40]. This study concluded that the use of these dermal matrices with stem cells not only promoted better healing of the burn wound, but also stimulated the formation of hair follicles and regeneration of muscles and ribs [40].

Concerning stem cells from human umbilical cords, Liu et al. [41] studied the effect of human umbilical cord derived mesenchymal stem cells in the healing of severe burns inflicted in rats. The stem cells were intravenously injected into the affected rats [41]. Liu et al. found that the injection of the stem cells into the rats accelerated the wound healing compared to controls, decreased the count of inflammatory cells, downregulated interleukins 1 and 6, and increased the levels of interleukin 10 and TSG-6 [41]. Moreover, stem cell injected rats had increased neovascularization and VEGF levels [41]. Not only do stem cells promote faster wound healing in burns, but also they prevent the progression of burn injuries as showed by Singer et al. [42]. The latter performed an experiment while inflicting thermal burns to rats, with several rectangular burns on each rat separated by unburned interspaces [42]. Some of the rats received tail vein injections of mesenchymal stem cells, while others received saline injections [42]. After 7 days, all of the unburned spaces in the controls were necrotic [42]. However, 20% of the unburned spaces in rats with stem cells injections did not necrose [42]. Consequently, stem cells were also shown to play a possible role in the prevention of progression of burn injuries. Furthermore, in a study by Xu et al. [43], applying autologous bone marrow derived mesenchymal stem cells to grafted burn wounds, they demonstrated decreased contraction of the grafts.

In 2014, Yang et al. [44] attempted to integrate mesenchymal stem cells with fibrin glue into the dressing of burn wounds. They inflicted scald wounds on the back of rats and applied dressing with fibrin glue and stem cells in one group, fibrin glue only in the second, and no intervention in the third [44]. One month later, the treatment group with fibrin glue and stem cells showed significantly faster healing than the other two; moreover, this group had more proliferation of sebaceous glands and the appearance of hair follicle-like structures which were not present in the other groups [44]. In another experiment, Lough et al. [45] isolated leucine-rich repeat-containing G-protein coupled receptor 6 (LGR6+) epithelial stem cells from the adnexal compartment of the skin of mice. They injected the harvested stem cells locally into inflicted burn wounds [45]. The wounds injected with stem cells showed a better healing along with increased vascular endothelial growth factor, platelet derived growth factor, and epidermal growth factor levels [45]. Stem cell injection also promoted the formation of nascent hair follicles and better neoangiogenesis in the wounds of the affected mice [45].

On the other hand, it is very pertinent to report another study in 2014 by Loder et al. [46] where they also tested the effect of adipose derived stem cells in the treatment of burns. The mice with inflicted burns that received stem cells injections showed no significant difference in comparison to controls (received saline injections) with respect to proliferation and vascularization [46]. Nevertheless, the role of stem cells in burn wound healing is a dynamic field and still under extensive research.

5. Stem Cells and Corneal Burn Wound Healing

Another area of particular interest in the field of burn wound healing is the chemical burns of the cornea. In the year 2000, Dua and Azuara-Blanco [47] used autologous limbal stem cells for ocular surface reconstruction of the contralateral eye. It resulted in the formation of a better corneal surface with significant improvement in the vision and symptoms of the patients [47]. Several other experiments and trials using limbal stem cells showed similar results in inducing improvement of corneal healing and decreased neovascularization in both human (adults and children) and animal subjects [48–53]. In 2007, Oh et al. [54] studied the therapeutic effects of mesenchymal stem cells on corneas with chemical burns. They reported that mesenchymal stem cell media and mesenchymal stem cell culture media (without the stem cells) reduced the inflammation and promoted neovascularization of the corneas [54]. They were also found to reduce the infiltration of CD4 cells, as well as IL-6, IL-10, and TGF-B1 levels. It is to be noted that the direct application of the stem cells provided superior results in the healing process in comparison with the stem cell culture media [54]. Another study by Ye et al. [55] utilized cyclophosphamide to suppress inflammatory reactions and the release of bone marrow stem cells into circulation. In this study, rabbits were inflicted with corneal alkali injuries. It was found that

rabbits with an unsuppressed bone marrow had significantly greater reepithelialization of the corneas with clearer surfaces [55]. Thus, this study revealed the role of bone marrow cells in enhancing the healing of corneal chemical wounds. Furthermore, Sel et al. [56] inflicted alkali wounds on the corneal surfaces of mice and treated the corneas with bone marrow derived stem cells, CD117+ cells, or medium only as control. Reepithelialization of the wounds in the treatment groups was significantly faster than the control, with no difference in corneal transparency. Stem cells and CD117+ cells were absent from corneas after healing, thus suggesting that soluble factors may be responsible for the effect of the applied cells [56]. In a different study by Rama et al. [57], limbal stem cells were cultured on fibrin and used in corneal burns; not only did stem cells promote a better healing but also they had maintained a superior healing at a follow-up of 10 years later. Several other studies showed comparable results where mesenchymal derived or adipose derived stem cells promoted faster recovery of the corneal epithelium and decreased neovascularization, inflammation, and oxidative injury; moreover, stem cells stimulated the formation of clearer cornea media in some experiments [58–61]. Additionally, Basu et al. [62–64], in 2011 and 2012, reported a series of studies concerning the use of limbal stem cells in corneal burn wound healing. In the first study, Basu et al. [62] used limbal stem cells in corneal burn wound healing and followed them by penetrating keratoplasty procedures. Good results were observed but they were not compared to controls. However, in the second study, Basu et al. [63] observed that 66% of patients who failed primary procedures of corneal repair and who were subjected to a secondary limbal stem cell transplant on the affected cornea had successful improvement of the corneal surface with no neovascularization at a follow-up of two years. Later on, Sangwan et al. [64] used limbal biopsies from unaffected eyes and cultured them on amniotic membranes as substrates. Similar results to previous experiments were obtained with avascular epithelialization of the new corneal surfaces [64]. Furthermore and as demonstrated by Huang et al. [65], the use of allograft transplants of limbal stem cells in corneal burn wound healing also resulted in improved avascular corneal healing without the need for systemic immunosuppression. Pellegrini et al. [66] studied the biological factors that affected the stem cells' role in corneal burn wound healing; the accurate number of stem cells used expressing high levels of the p63 transcription factor was shown to have important influence.

6. Alternative Therapies in Burn Wound Healing

Stem cells do seem to have a very promising role in the treatment of burn wounds; however, other therapies are being developed to improve the treatment. For example, Klinger et al. [67] used fat injections in severe burn wounds as a trial to improve burn wound healing in humans. They did get results showing scar improvement and enhancement of tissue regeneration, but their study was limited to a small

population [67]. In other studies, Auxenfans et al. [68] investigated the role of keratinocytes in improving wound healing in burns. They reported that keratinocytes induced a more rapid burn wound healing [68]. On the other hand, stromal vascular fraction has been also shown to play a possible role in enhancing burn wound healing [69]. Atalay et al. used isolated stromal vascular fraction in burn wound healing. It stimulated an increase in vascular endothelial growth factor and reduced the inflammation with an improved fibroblastic activity [69]. Additionally, Hussein et al. [70] studied the effect of Botox injections on burn wounds healing and found that Botox increased fibroblasts, TGF-B, and TNF-alpha levels and decreased inflammation, thus improving burn wound healing. Another recent study by Zhang et al. [71] showed a beneficial effect of heat shock protein 90 alpha on burn wound healing. It promoted faster healing and less inflammation. In addition, several other studies have examined the effects of different factors and substances such as curcumin, mast cell chymase, and phenytoin with hypericin on burn wound healing with promising results and better wound healing [72–74].

Stem cells are commonly derived either from bone marrow, umbilical cord, adipose tissue, or skin. Natesan et al. [75] have even used debrided skin from severe burns as a source of stem cells for wound healing and regeneration. Hence, the adipose tissue that is discarded from burn wound debridement may now be of use for better wound healing. In addition, Natesan et al. [76], in another study, used isolated stem cells from debrided skin with fibrin and collagen based scaffolds. The dermal equivalents, created in the study, decreased wound contraction leading to a better matrix deposition and epithelialization [76]. Along the same line, van der Veen et al. [77] isolated mesenchymal stem cells from excised burn wound eschar. These stem cells showed similar abilities to adipose derived stem cells in differentiating into osteocytes, chondroblasts, and adipocytes [77].

A relatively recent approach by Li et al. [78] studied the role of electric fields in the migration of stem cells. They proved that epithelial stem cells migrate to the cathode in an induced electric field, knowing that endogenous electric fields exist naturally in wounds [78]. The migration of the stem cells was found to be proportional to the strength of the electric field and its duration, with the involvement of epidermal growth factor receptor and mitogen activated protein kinase-PI3K [78]. Hence, in addition to the use of stem cells in burn wounds, electric fields can be applied to the wounds to better direct their migration [78].

7. Discussion

The role of stem cells in wound healing has been shown to be performed through several pathways, such as JNK and ERK59, and with the involvement of different factors and mediators, such as KGF-1 and PDGF-BB [5, 8]. Additionally, this role could also be carried out by the released factors and not only by direct integration of the stem cells into the wound scaffold or matrix [6].

Stem cells in burn wound healing have been found to follow the same mechanisms. The increased levels of stem cells in burn wounds suggested a possible enhancing role in aiding in the healing process [9, 10, 12, 13]. However, a lack of consistency of the outcome was documented. Different experiments may have used different amounts of purified stem cells, or stem cells at different stages of replication or differentiation *in vitro*, leading to what may seem different results. In brief, this review depicted the improved healing with stem cells qualitatively rather than quantitatively. To really demonstrate the value of different stem cells in the process of burn wound healing, more studies need to be done under optimal and well controlled conditions, aiming to measure a quantifiable improvement. Additionally, the excess use of stem cells may lead to unwanted results, such as increased fibrosis and thicker healed epithelium. Whether the effect is observed as a result of direct stem cell proliferation, or other induced substances and cells, needs to be studied in the future. Moreover, the role of cytokines released by stem cells along with bioactive peptides such as thymosin 4 has been documented to mediate the beneficial effect of the stem cell application in burn wound healing. Further data refer the superior healing probably not to the direct integration of the stem cells into the wound [11, 13]. Acute wound fluids were also shown to have a role in promoting faster healing of burn wounds, similarly reinforcing the role of mediators released by stem cells. Additionally, human alpha defensin 5 and the CXCL12/CXCR4 pathway with its signal SDF-1 α were found to be inducers of stem cells in burn wounds [16, 18, 20].

In addition, stem cells have been shown to decrease cell infiltration, wound contraction, fibrosis, scar progression, and inflammation of burn wounds. Moreover, they have been found to promote faster burn wound healing and angiogenesis along with better granulation and the formation of hair follicles and sebaceous glands [15, 22–45]. The studies reviewed showed positive results in both animal experiments and human trials, both in partial and full thickness injury burns. In addition, different ways of stem cell application have been used ranging from using stem cell scaffolds to systemic and intradermal injection [23, 25, 32]. Furthermore, the sources of stem cells used are multiple. They are derived from bone marrow, dermis, adipose tissue, and umbilical cords, among others [22, 23, 33, 41]. Stem cells have proved to be efficient not only in skin burns but also in corneal chemical burns, thus increasing the multiplicity of their use [54–61].

Patients with burn wounds, especially those severely injured, tend to have lower quality of life [79]. The injury they suffer is not only physical but also psychological, affecting their jobs and relations with other people, especially their families [80, 81]. With the advance of burn wound treatment with time, patients' self-esteem and quality of life have been improving [82, 83]. The hope is that the use of stem cells will open up a new arena of possibilities to improve the wound healing in burn patients, allowing patients to have faster healing, better scars, and a higher quality of life.

8. Reflections on the Role of Stem Cells in Burn Wound Healing

Stem cells have attracted many controversial public opinions over time. Many people argue that embryonic stem cell harvesting would be done by killing embryos which would be unethical [84]. Others would argue that even if embryos are used for stem cell research, it is not wrong. However, the path that this may lead to would be wrong such as embryo "production" for research purposes [84]. The public view towards the therapeutic use of stem cells has become more tolerant over time [85]. The role of educating people about the colossal potential for the use of stem cell has thus proven beneficial. People are now more educated about the different sources of stem cells and have become supportive of their use [85]. Regarding the acceptance of stem cells as an efficient therapy for burn wound healing in specific, a study done by Clover et al. [86] showed a very positive opinion. The biggest majority of people were willing to accept autologous stem cells, though a big percentage was also welcoming the idea of using allogeneic stem cells. These percentages did not differ between the use of stem cells for burn wounds or for the treatment of other diseases such as diabetes or Parkinson's [86].

9. Conclusion

In brief, the use of stem cells in burn wound healing appears to be very promising. While most studies were performed on animals, the application to humans is yet at its start. Hence, what is needed is more studies. Additionally, the signaling pathways followed by stem cells involved in the burn wound healing along with their factors and signals constitute a very dynamic and promising research field.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] P. Jayaraman, P. Nathan, P. Vasanthan, S. Musa, and V. Govindasamy, "Stem cells conditioned medium: a new approach to skin wound healing management," *Cell Biology International*, vol. 37, no. 10, pp. 1122–1128, 2013.
- [2] Y. Nakamura, H. Ishikawa, K. Kawai, Y. Tabata, and S. Suzuki, "Enhanced wound healing by topical administration of mesenchymal stem cells transfected with stromal cell-derived factor-1," *Biomaterials*, vol. 34, no. 37, pp. 9393–9400, 2013.
- [3] M. Hu, D. Ludlow, J. S. Alexander, J. McLarty, and T. Lian, "Improved wound healing of postischemic cutaneous flaps with the use of bone marrow-derived stem cells," *Laryngoscope*, vol. 124, no. 3, pp. 642–648, 2014.
- [4] Overview of stem cells, 2013, http://www.uptodate.com/contents/overview-of-stem-cells?source=search_result&=1~150#H10490925.

- [5] V.-I. Alexaki, D. Simantiraki, M. Panayiotopoulou et al., "Adipose tissue-derived mesenchymal cells support skin reepithelialization through secretion of KGF-1 and PDGF-BB: comparison with dermal fibroblasts," *Cell Transplantation*, vol. 21, no. 11, pp. 2441–2454, 2012.
- [6] A. Skardal, D. Mack, E. Kapetanovic et al., "Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds," *Stem Cells Translational Medicine*, vol. 1, no. 11, pp. 792–802, 2012.
- [7] D. Leonardi, D. Oberdoerfer, M. C. Fernandes et al., "Mesenchymal stem cells combined with an artificial dermal substitute improve repair in full-thickness skin wounds," *Burns*, vol. 38, no. 8, pp. 1143–1150, 2012.
- [8] M. Zhang, L. Sun, X. Wang et al., "Activin B promotes BMSC-mediated cutaneous wound healing by regulating cell migration via the JNK-ERK signaling pathway," *Cell Transplantation*, vol. 23, no. 9, pp. 1061–1073, 2014.
- [9] E. Mansilla, G. H. Marín, H. Drago et al., "Bloodstream cells phenotypically identical to human mesenchymal bone marrow stem cells circulate in large amounts under the influence of acute large skin damage: new evidence for their use in regenerative medicine," *Transplantation Proceedings*, vol. 38, no. 3, pp. 967–969, 2006.
- [10] A. Fox, J. Smythe, N. Fisher et al., "Mobilization of endothelial progenitor cells into the circulation in burned patients," *British Journal of Surgery*, vol. 95, no. 2, pp. 244–251, 2008.
- [11] W. G. Payne, T. L. Wachtel, C. A. Smith, M. G. Uberti, F. Ko, and M. C. Robson, "Effect of amnion-derived cellular cytokine solution on healing of experimental partial-thickness burns," *World Journal of Surgery*, vol. 34, no. 7, pp. 1663–1668, 2010.
- [12] C. Foresta, M. Schipilliti, L. de Toni et al., "Blood levels, apoptosis, and homing of the endothelial progenitor cells after skin burns and escharectomy," *The Journal of Trauma—Injury, Infection and Critical Care*, vol. 70, no. 2, pp. 459–465, 2011.
- [13] C. Cabrera, G. Carriquiry, C. Pierinelli, N. Reinoso, J. Arias-Stella, and J. Paino, "The role of biologically active peptides in tissue repair using umbilical cord mesenchymal stem cells," *Annals of the New York Academy of Sciences*, vol. 1270, no. 1, pp. 93–97, 2012.
- [14] P. Koenen, T. A. Spanholtz, M. Maegele et al., "Acute and chronic wound fluids inversely influence adipose-derived stem cell function: molecular insights into impaired wound healing," *International Wound Journal*, vol. 12, no. 1, pp. 10–16, 2015.
- [15] Y. Wu, S. Huang, J. Enhe et al., "Bone marrow-derived mesenchymal stem cell attenuates skin fibrosis development in mice," *International Wound Journal*, vol. 11, no. 6, pp. 701–710, 2014.
- [16] D. Lough, H. Dai, M. Yang et al., "Stimulation of the follicular bulge lgr5+ and lgr6+ stem cells with the gut-derived human alpha defensin 5 results in decreased bacterial presence, enhanced wound healing, and hair growth from tissues devoid of adnexal structures," *Plastic and Reconstructive Surgery*, vol. 132, no. 5, pp. 1159–1171, 2013.
- [17] J. Ding, K. Hori, R. Zhang et al., "Stromal cell-derived factor 1 (SDF-1) and its receptor CXCR4 in the formation of postburn hypertrophic scar (HTS)," *Wound Repair and Regeneration*, vol. 19, no. 5, pp. 568–578, 2011.
- [18] D. Yang, S. Sun, Z. Wang, P. Zhu, Z. Yang, and B. Zhang, "Stromal cell-derived factor-1 receptor CXCR4-overexpressing bone marrow mesenchymal stem cells accelerate wound healing by migrating into skin injury areas," *Cellular Reprogramming*, vol. 15, no. 3, pp. 206–215, 2013.
- [19] C. Hu, X. Yong, C. Li et al., "CXCL12/CXCR4 axis promotes mesenchymal stem cell mobilization to burn wounds and contributes to wound repair," *Journal of Surgical Research*, vol. 183, no. 1, pp. 427–434, 2013.
- [20] M.-H. Lü, C.-J. Hu, L. Chen et al., "miR-27b represses migration of mouse MSCs to burned margins and prolongs wound repair through silencing SDF-1a," *PLoS ONE*, vol. 8, no. 7, Article ID e68972, 2013.
- [21] X. Chen, Y. Shi, B. Shu et al., "The effect of porcine ADM to improve the burn wound healing," *International Journal of Clinical and Experimental Pathology*, vol. 6, no. 11, pp. 2280–2291, 2013.
- [22] V. I. Shumakov, N. A. Onishchenko, M. F. Rasulov, M. E. Krasheninnikov, and V. A. Zaidenov, "Mesenchymal bone marrow stem cells more effectively stimulate regeneration of deep burn wounds than embryonic fibroblasts," *Bulletin of Experimental Biology and Medicine*, vol. 136, no. 2, pp. 192–195, 2003.
- [23] S. Chunmeng, C. Tianmin, S. Yongping et al., "Effects of dermal multipotent cell transplantation on skin wound healing," *Journal of Surgical Research*, vol. 121, no. 1, pp. 13–19, 2004.
- [24] M. F. Rasulov, A. V. Vasilchenkov, N. A. Onishchenko et al., "First experience of the use bone marrow mesenchymal stem cells for the treatment of a patient with deep skin burns," *Bulletin of Experimental Biology and Medicine*, vol. 139, no. 1, pp. 141–144, 2005.
- [25] M. F. Rasulov, V. T. Vasilenko, V. A. Zaidenov, and N. A. Onishchenko, "Cell transplantation inhibits inflammatory reaction and stimulates repair processes in burn wound," *Bulletin of Experimental Biology and Medicine*, vol. 142, no. 1, pp. 112–115, 2006.
- [26] P. Liu, Z. Deng, S. Han et al., "Tissue-engineered skin containing mesenchymal stem cells improves burn wounds," *Artificial Organs*, vol. 32, no. 12, pp. 925–931, 2008.
- [27] J. J. Lataillade, C. Doucet, E. Bey et al., "New approach to radiation burn treatment by dosimetry-guided surgery combined with autologous mesenchymal stem cell therapy," *Regenerative Medicine*, vol. 2, no. 5, pp. 785–794, 2007.
- [28] E. Bey, M. Prat, P. Duhamel et al., "Emerging therapy for improving wound repair of severe radiation burns using local bone marrow-derived stem cell administrations," *Wound Repair and Regeneration*, vol. 18, no. 1, pp. 50–58, 2010.
- [29] L. Hao, J. Wang, Z. Zou et al., "Transplantation of BMSCs expressing hPDGF-A/hBD2 promotes wound healing in rats with combined radiation-wound injury," *Gene Therapy*, vol. 16, no. 1, pp. 34–42, 2009.
- [30] Z.-W. Zong, N. Li, T.-Y. Xiao Tao-Yuan et al., "Effect of hBD2 genetically modified dermal multi potent stem cells on repair of infected irradiated wounds," *Journal of Radiation Research*, vol. 51, no. 5, pp. 573–580, 2010.
- [31] X.-Q. Ha, T.-D. Lü, L. Hui, and F. Dong, "Effects of mesenchymal stem cells transfected with human hepatocyte growth factor gene on healing of burn wounds," *Chinese Journal of Traumatology*, vol. 13, no. 6, pp. 349–355, 2010.
- [32] D. Agay, H. Scherthan, F. Forcheron et al., "Multipotent mesenchymal stem cell grafting to treat cutaneous radiation syndrome: development of a new minipig model," *Experimental Hematology*, vol. 38, no. 10, pp. 945–956, 2010.
- [33] D. Riccobono, D. Agay, H. Scherthan et al., "Application of adipocyte-derived stem cells in treatment of cutaneous radiation syndrome," *Health Physics*, vol. 103, no. 2, pp. 120–126, 2012.

- [34] N. Kinoshita, M. Tsuda, R. Hamuy et al., "The usefulness of basic fibroblast growth factor for radiation-exposed tissue," *Wound Repair and Regeneration*, vol. 20, no. 1, pp. 91–102, 2012.
- [35] U. Riekstina, R. Muceniece, I. Cakstina, I. Muiznieks, and J. Ancans, "Characterization of human skin-derived mesenchymal stem cell proliferation rate in different growth conditions," *Cytotechnology*, vol. 58, no. 3, pp. 153–162, 2008.
- [36] G. Yan, H. Sun, F. Wang et al., "Topical application of hPDGF-A-modified porcine BMSC and keratinocytes loaded on acellular HAM promotes the healing of combined radiation-wound skin injury in minipigs," *International Journal of Radiation Biology*, vol. 87, no. 6, pp. 591–600, 2011.
- [37] S. S. Collawn, N. S. Banerjee, J. de La Torre, L. Vasconez, and L. T. Chow, "Adipose-derived stromal cells accelerate wound healing in an organotypic raft culture model," *Annals of Plastic Surgery*, vol. 68, no. 5, pp. 501–504, 2012.
- [38] Z. Xia, C. Zhang, Y. Zeng, T. Wang, and G. Ai, "Transplantation of BMSCs expressing hVEGF/hBD3 promotes wound healing in rats with combined radiation-wound injury," *International Wound Journal*, vol. 11, no. 3, pp. 293–303, 2012.
- [39] L. Xue, Y.-B. Xu, J.-L. Xie et al., "Effects of human bone marrow mesenchymal stem cells on burn injury healing in a mouse model," *International Journal of Clinical and Experimental Pathology*, vol. 6, no. 7, pp. 1327–1336, 2013.
- [40] E. Mansilla, R. Spretz, G. Larsen et al., "Outstanding survival and regeneration process by the use of intelligent acellular dermal matrices and mesenchymal stem cells in a burn pig model," *Transplantation Proceedings*, vol. 42, no. 10, pp. 4275–4278, 2010.
- [41] L. Liu, Y. Yu, Y. Hou et al., "Human umbilical cord mesenchymal stem cells transplantation promotes cutaneous wound healing of severe burned rats," *PLoS ONE*, vol. 9, no. 2, Article ID e88348, 2014.
- [42] D. D. Singer, A. J. Singer, C. Gordon, and P. Brink, "The effects of rat mesenchymal stem cells on injury progression in a rat model," *Academic Emergency Medicine*, vol. 20, no. 4, pp. 398–402, 2013.
- [43] Y. Xu, S. Huang, and X. Fu, "Autologous transplantation of bone marrow-derived mesenchymal stem cells: a promising therapeutic strategy for prevention of skin-graft contraction," *Clinical and Experimental Dermatology*, vol. 37, no. 5, pp. 497–500, 2012.
- [44] Y. Yang, W. Zhang, Y. Li, G. Fang, and K. Zhang, "Scalded skin of rat treated by using fibrin glue combined with allogeneic bone marrow mesenchymal stem cells," *Annals of Dermatology*, vol. 26, no. 3, pp. 289–295, 2014.
- [45] D. M. Lough, M. Yang, A. Blum et al., "Transplantation of the LGR6⁺ epithelial stem cell into full-thickness cutaneous wounds results in enhanced healing, nascent hair follicle development, and augmentation of angiogenic analytes," *Plastic and Reconstructive Surgery*, vol. 133, no. 3, pp. 579–590, 2014.
- [46] S. Loder, J. R. Peterson, S. Agarwal et al., "Wound healing after thermal injury is improved by fat and adipose-derived stem cell isografts," *Journal of Burn Care & Research*, vol. 36, no. 1, pp. 70–76, 2015.
- [47] H. S. Dua and A. Azuara-Blanco, "Autologous limbal transplantation in patients with unilateral corneal stem cell deficiency," *British Journal of Ophthalmology*, vol. 84, no. 3, pp. 273–278, 2000.
- [48] P. Rama, S. Bonini, A. Lambiase et al., "Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency," *Transplantation*, vol. 72, no. 9, pp. 1478–1485, 2001.
- [49] R. Ivecovic, E. Tedeschi-Reiner, K. Novak-Laus, B. Andrijevic-Derk, I. Cima, and Z. Mandic, "Limbal graft and/or amniotic membrane transplantation in the treatment of ocular burns," *Ophthalmologica*, vol. 219, no. 5, pp. 297–302, 2005.
- [50] F. L. Gimeno, V. Lavigne, S. Gatto, J. O. Croxatto, L. Correa, and J. E. Gallo, "Advances in corneal stem-cell transplantation in rabbits with severe ocular alkali burns," *Journal of Cataract and Refractive Surgery*, vol. 33, no. 11, pp. 1958–1965, 2007.
- [51] M. Kawashima, T. Kawakita, Y. Satake, K. Higa, and J. Shimazaki, "Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency," *Archives of Ophthalmology*, vol. 125, no. 10, pp. 1337–1344, 2007.
- [52] G. Marchini, E. Pedrotti, M. Pedrotti et al., "Long-term effectiveness of autologous cultured limbal stem cell grafts in patients with limbal stem cell deficiency due to chemical burns," *Clinical & Experimental Ophthalmology*, vol. 40, no. 3, pp. 255–267, 2012.
- [53] K. Sejpal, M. H. Ali, S. Maddileti et al., "Cultivated limbal epithelial transplantation in children with ocular surface burns," *JAMA Ophthalmology*, vol. 131, no. 6, pp. 731–736, 2013.
- [54] J. Y. Oh, M. K. Kim, M. S. Shin et al., "The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury," *Stem Cells*, vol. 26, no. 4, pp. 1047–1055, 2008.
- [55] J. Ye, S. Y. Lee, K. H. KooK, and K. Yao, "Bone marrow-derived progenitor cells promote corneal wound healing following alkali injury," *Graefes Archive for Clinical and Experimental Ophthalmology*, vol. 246, no. 2, pp. 217–222, 2008.
- [56] S. Sel, U. M. Schilling, N. Nass et al., "Bone marrow cells and CD117-positive haematopoietic stem cells promote corneal wound healing," *Acta Ophthalmologica*, vol. 90, no. 5, pp. e367–e373, 2012.
- [57] P. Rama, S. Matuska, G. Paganoni, A. Spinelli, M. De Luca, and G. Pellegrini, "Limbal stem-cell therapy and long-term corneal regeneration," *The New England Journal of Medicine*, vol. 363, no. 2, pp. 147–155, 2010.
- [58] L. Yao, Z.-R. Li, W.-R. Su et al., "Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn," *PLoS ONE*, vol. 7, no. 2, Article ID e30842, 2012.
- [59] H.-F. Lin, Y.-C. Lai, C.-F. Tai et al., "Effects of cultured human adipose-derived stem cells transplantation on rabbit cornea regeneration after alkaline chemical burn," *The Kaohsiung Journal of Medical Sciences*, vol. 29, no. 1, pp. 14–18, 2013.
- [60] M. Zeppieri, M. L. Salvetat, A. P. Beltrami et al., "Human adipose-derived stem cells for the treatment of chemically burned rat cornea: preliminary Results," *Current Eye Research*, vol. 38, no. 4, pp. 451–463, 2013.
- [61] J. Cejkova, P. Trosan, C. Cejka et al., "Suppression of alkali-induced oxidative injury in the cornea by mesenchymal stem cells growing on nanofiber scaffolds and transferred onto the damaged corneal surface," *Experimental Eye Research*, vol. 116, pp. 312–323, 2013.
- [62] S. Basu, A. Mohamed, S. Chaurasia, K. Sejpal, G. K. Vemuganti, and V. S. Sangwan, "Clinical outcomes of penetrating keratoplasty after autologous cultivated limbal epithelial transplantation for ocular surface burns," *The American Journal of Ophthalmology*, vol. 152, no. 6, pp. 917.e1–924.e1, 2011.

- [63] S. Basu, H. Ali, and V. S. Sangwan, "Clinical outcomes of repeat autologous cultivated limbal epithelial transplantation for ocular surface burns," *The American Journal of Ophthalmology*, vol. 153, no. 4, pp. 643.e2–650.e2, 2012.
- [64] V. S. Sangwan, S. Basu, G. K. Vemuganti et al., "Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study," *British Journal of Ophthalmology*, vol. 95, no. 11, pp. 1525–1529, 2011.
- [65] T. Huang, Y. Wang, H. Zhang, N. Gao, and A. Hu, "Limbal allografting from living-related donors to treat partial limbal deficiency secondary to ocular chemical burns," *Archives of Ophthalmology*, vol. 129, no. 10, pp. 1267–1273, 2011.
- [66] G. Pellegrini, P. Rama, S. Matuska et al., "Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells," *Regenerative Medicine*, vol. 8, no. 5, pp. 553–567, 2013.
- [67] M. Klinger, M. Marazzi, D. Vigo, and M. Torre, "Fat injection for cases of severe burn outcomes: a new perspective of scar remodeling and reduction," *Aesthetic Plastic Surgery*, vol. 32, no. 3, pp. 465–469, 2008.
- [68] C. Auxenfans, V. Menet, Z. Catherine et al., "Cultured autologous keratinocytes in the treatment of large and deep burns: a retrospective study over 15 years," *Burns*, vol. 41, no. 1, pp. 71–79, 2015.
- [69] S. Atalay, A. Coruh, and K. Deniz, "Stromal vascular fraction improves deep partial thickness burn wound healing," *Burns*, vol. 40, no. 7, pp. 1375–1383, 2014.
- [70] I. A. H. Hussein, N. D. Balta, R. A. Jurjus et al., "Rat model of burn wound healing: effect of botox," *Journal of Biological Regulators and Homeostatic Agents*, vol. 26, no. 3, pp. 389–400, 2012.
- [71] Y. Zhang, X. Bai, Y. Wang et al., "Role for heat shock protein 90 α in the proliferation and migration of HaCaT cells and in the deep second-degree burn wound healing in mice," *PLoS ONE*, vol. 9, no. 8, Article ID e103723, 2014.
- [72] M. Kulac, C. Aktas, F. Tulubas et al., "The effects of topical treatment with curcumin on burn wound healing in rats," *Journal of Molecular Histology*, vol. 44, no. 1, pp. 83–90, 2013.
- [73] X. Dong, Z. Geng, Y. Zhao, J. Chen, and Y. Cen, "Involvement of mast cell chymase in burn wound healing in hamsters," *Experimental and Therapeutic Medicine*, vol. 5, no. 2, pp. 643–647, 2013.
- [74] H. Sayar, N. Gergerlioglu, N. Seringec, P. Ozturk, E. Bulbuloglu, and G. Karabay, "Comparison of efficacy of topical phenytoin with hypericin in second-degree burn wound healing: an experimental study in rats," *Medical Science Monitor Basic Research*, vol. 20, pp. 36–46, 2014.
- [75] S. Natesan, N. L. Wrice, D. G. Baer, and R. J. Christy, "Debrided skin as a source of autologous stem cells for wound repair," *Stem Cells*, vol. 29, no. 8, pp. 1219–1230, 2011.
- [76] S. Natesan, D. O. Zamora, N. L. Wrice, D. G. Baer, and R. J. Christy, "Bilayer hydrogel with autologous stem cells derived from debrided human burn skin for improved skin regeneration," *Journal of Burn Care & Research*, vol. 34, no. 1, pp. 18–30, 2013.
- [77] V. C. van der Veen, M. Vlijg, F. J. van Milligen, S. I. de Vries, E. Middelkoop, and M. M. W. Ulrich, "Stem cells in burn eschar," *Cell Transplantation*, vol. 21, no. 5, pp. 933–942, 2012.
- [78] L. Li, W. Gu, J. Du et al., "Electric fields guide migration of epidermal stem cells and promote skin wound healing," *Wound Repair and Regeneration*, vol. 20, no. 6, pp. 840–851, 2012.
- [79] B. Xie, S.-C. Xiao, S.-H. Zhu, and Z.-F. Xia, "Evaluation of long term health-related quality of life in extensive burns: a 12-year experience in a burn center," *Burns*, vol. 38, no. 3, pp. 348–355, 2012.
- [80] F. C. Chang and B. Herzog, "Burn morbidity: a followup study of physical and psychological disability," *Annals of Surgery*, vol. 183, no. 1, pp. 34–37, 1976.
- [81] N. J. Andreasen and A. S. Norris, "Long-term adjustment and adaptation mechanisms in severely burned adults," *The Journal of Nervous and Mental Disease*, vol. 154, no. 5, pp. 352–362, 1972.
- [82] E. F. Worthen, "Self-esteem of severely burned patients," *Plastic and Reconstructive Surgery*, vol. 67, no. 6, p. 821, 1981.
- [83] R. L. Sheridan, M. I. Hinson, M. H. Liang et al., "Long-term outcome of children surviving massive burns," *The Journal of the American Medical Association*, vol. 283, no. 1, pp. 69–73, 2000.
- [84] M. J. Sandel and D. Phil, "Embryo ethics—the moral logic of stem-cell research," *The New England Journal of Medicine*, vol. 351, no. 3, pp. 207–209, 2004.
- [85] E. Einsiedel, S. Premji, R. Geransar, N. C. Orton, T. Thavaratnam, and L. K. Bennett, "Diversity in public views toward stem cell sources and policies," *Stem Cell Reviews and Reports*, vol. 5, no. 2, pp. 102–107, 2009.
- [86] A. J. P. Clover, B. L. O'Neill, and A. H. S. Kumar, "Analysis of attitudes toward the source of progenitor cells in tissue-engineered products for use in burns compared with other disease states," *Wound Repair and Regeneration*, vol. 20, no. 3, pp. 311–316, 2012.

Research Article

Generation of a Fibrin Based Three-Layered Skin Substitute

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A variety of skin substitutes that restore epidermal and dermal structures are currently available on the market. However, the main focus in research and clinical application lies on dermal and epidermal substitutes whereas the development of a subcutaneous replacement (hypodermis) is often disregarded. In this study we used fibrin sealant as hydrogel scaffold to generate a three-layered skin substitute. For the hypodermal layer adipose-derived stem cells (ASCs) and mature adipocytes were embedded in the fibrin hydrogel and were combined with another fibrin clot with fibroblasts for the construction of the dermal layer. Keratinocytes were added on top of the two-layered construct to form the epidermal layer. The three-layered construct was cultivated for up to 3 weeks. Our results show that ASCs and fibroblasts were viable, proliferated normally, and showed physiological morphology in the skin substitute. ASCs were able to differentiate into mature adipocytes during the course of four weeks and showed morphological resemblance to native adipose tissue. On the surface keratinocytes formed an epithelial-like layer. For the first time we were able to generate a three-layered skin substitute based on a fibrin hydrogel not only serving as a dermal and epidermal substitute but also including the hypodermis.

1. Introduction

Soft tissue damage following trauma or tumor resection often results in an exigent need of highly sophisticated, complex tissue substitutes. Due to limited donor site for tissue reconstruction (flaps) and morbidity in large scale defect reconstruction, plastic surgeons often reach their limitations in reconstructing these defects. New biomaterials and improved scaffold processing techniques have been developed over the last years, and novel promising scaffold materials can be fabricated and offered to the patients [1]. However, the main focus in research and clinical application lies on substitutes consisting of the dermal and epidermal layer [2–4] whereas the development of a subcutaneous replacement (hypodermis) is often disregarded. Particularly in deep dermal burns such constructs would be of an enormous advantage.

The skin is composed of three layers. The hypodermis mainly consists of adipocytes, fibroblasts, and adipose-derived stem cells and protects the body from stress and

strain. Above the hypodermis lies the dermis which is mainly composed of fibroblasts and different extracellular matrix proteins such as collagen, elastin, and glycosaminoglycans. The outermost layer of the skin is called epidermis and consists of keratinocytes and only sparse extracellular matrix.

The ability to “engineer” a three-dimensional skin construct to restore a physical deformity without the need for multiple surgical steps and a painful, scarred donor site remains a primary goal of scientists and plastic surgeons. The substitute should incorporate a biocompatible scaffold that defines the appropriate three-dimensional tissue architecture of adequate size to have clinical applicability to current reconstructive problems and promotes host integration and implant vascularization [5]. Ultimately, the construct should demonstrate certain stability over time but also be biodegradable as it is replaced by healthy host soft tissue. Mechanical properties are particularly important, and, ideally, the scaffold mimics the native tissue into which it will be incorporated [6]. On a macroscopic level, this is important

to ensure that the implant has a natural feel and to minimize inflammation (reaction on artificial materials) and scar tissue formation. However, recent research has also highlighted the importance of mechanical properties on a cellular level, with the substrate stiffness dramatically influencing the differentiation response of seeded cells [7].

In this study we used fibrin sealant as hydrogel scaffold to generate the three-dimensional skin substitute. Fibrin is an established material in surgical applications and was shown to be highly biocompatible in tissue engineering and cell delivery [8–10]. It has already been used as matrix for generating skin substitutes *in vitro* [11] and *in vivo* [12] and was suggested by Peterbauer-Scherb et al. to represent a suitable scaffold material for adipose tissue formation [13].

The hypodermis mainly consists of mature adipocytes but also other cell types such as adipose-derived stem cells (ASCs). These precursor cells are located between mature adipocytes. They can serve as an ideal autologous cell source for adipose tissue engineering approaches, since they are more resistant to mechanical damage and ischemia than mature adipocytes [14]. Adipocytes and ASCs can easily be harvested during liposuction or resection of adipose tissue. ASCs have been shown to proliferate rapidly and differentiate into bone, adipogenic, and chondrogenic lineage both *in vitro* and *in vivo* and are known to migrate to injured sites [15–18].

Aim of the present study was to generate a three-layered skin substitute consisting of an epidermal, dermal, and a hypodermal tissue layer. Human ASCs and mature adipocytes were used to construct the hypodermis.

2. Materials and Methods

2.1. Patients and Tissue Harvesting. Skin and fat tissue were gained from otherwise healthy patients undergoing body contouring surgery. All subjects gave written informed consent before participating in the study which has been approved by the ethics committee of the Medical University of Vienna and the General Hospital Vienna (EK number 1949/2012).

2.2. Isolation of Human Adipose-Derived Stem Cells and Adipocytes. Adipose tissue was washed in PBS (phosphate buffered saline, PAA Laboratories GmbH, Pasching, Austria) and ASCs and mature adipocytes were isolated as described before [19]. Briefly, the adipose tissue was minced and digested with 2 mg/mL Collagenase Type IV (Sigma-Aldrich, St. Louis, MO, USA) in Hanks' buffered salt solution (HBSS, Lonza, Walkersville, MD, USA) for 1 h at 37°C with constant shaking. The stromal vascular fraction (SVF) was isolated by centrifugation and red blood cells were lysed in 2 mL Red Blood Cell Lysing Buffer (Sigma-Aldrich). Cells were centrifuged and resuspended in culture medium DMEM (Gibco, Life Technologies Ltd., Paisley, UK) supplemented with 10% fetal calf serum (Hyclone, Fisher Scientific GmbH, Schwerte, Germany), 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies Ltd., Paisley, UK). Isolated cells were counted in a Bürker-Türk counting chamber (Hecht Assistant, Sondheim, Germany) with Trypan Blue stain (0.4%,

Gibco, Life Technologies Ltd., Paisley UK) to prove viability. By cultivation of SVF, selection and expansion of ASCs were achieved. ASCs were cultured as a monolayer at 37°C in supplemented proliferation/cell culture medium in a humidified atmosphere with 5% CO₂. For all experiments ASC passages up to passage 2 were used.

Freshly isolated adipocytes were collected during the isolation process from the adipose phase after the first centrifugation step. Adipocytes were washed two times with PBS to remove collagenase and were used for experiments right away.

2.3. Isolation of Human Dermal Fibroblasts. Human donor skin was cut into small pieces, put in 6-well culture plate, and incubated for 14 days in culture medium DMEM supplemented with 10% fetal calf serum, 1% glutamine (Gibco, Life Technologies Ltd.), 100 units/mL penicillin, and 100 µg/mL streptomycin. Fibroblasts growing out onto the bottom of the 6-well culture plate were cultured upon confluence and were split for further cultivation in culture flasks.

2.4. Human Keratinocytes. Keratinocytes (Evercyte, Vienna, Austria) were cultured in DermaLife K Complete Medium (CellSystems, Troisdorf, Germany).

2.5. Construction of a Three-Layered Skin Substitute with a Fibrin Hydrogel Matrix. Fibrinogen and thrombin (both Baxter AG, Vienna, Austria) were reconstituted according to the manufacturer's protocol. In brief, lyophilized sealer protein component was reconstituted in 3,000 KIE/mL aprotinin solution; the lyophilized thrombin component was reconstituted in 40 mM CaCl₂ solution. The components were diluted in PBS to a concentration of 25 mg/mL fibrinogen and 2.5 IU/mL thrombin as end concentration in the clot. For fibrin clot formation, equal volumes of fibrinogen and thrombin components were mixed in ThinCert cell culture inserts (pore size 8 µm, Greiner bio-one, Frickenhausen, Germany) and clots were incubated at 37°C for 20 minutes for polymerization.

For the hypodermal layer 3×10^5 ASCs and 2.5×10^4 adipocytes were embedded in 100 µL fibrin clots in cell culture inserts. After 15 minutes of polymerization another clot was polymerized on top of the hypodermal layer with 3×10^5 fibroblasts embedded for the construction of the dermal layer of the skin substitute. After another 15 minutes polymerization period keratinocytes ($0.6\text{--}1 \times 10^6$ cells/clot) were added on top of the two-layered construct for the epidermal layer. The three-layered construct was cultivated for up to 3 weeks in DMEM + 10% FCS where keratinocytes were exposed to air according to the air-liquid interface cultivation model [20].

2.6. Histological Evaluation of Construct. All samples were fixed overnight in 4.5% neutrally buffered formalin (SAV LP, Flintsbach a. Inn, Austria). After embedding in paraffin, 4 µm cross sections were cut, deparaffinized, and stained. For morphology staining, fixed probes were stained with Mayer's Hemalaun solution (Merck KGaA, Darmstadt, Germany)

and Eosin 1 wt. % (Sigma-Aldrich, St. Louis, MO, USA) or with $2.5 \mu\text{M}/\text{mL}$ SYTOX Green Nucleic Acid Stain/dH₂O (Molecular Probes, Eugene, OR, USA) under light protection. Pictures were taken with an AxioImager microscope (Zeiss, Jena, Germany).

2.7. Viability and Proliferation Testing. Cells (ASCs or fibroblasts: 3×10^5) were embedded in the fibrin gel ($100 \mu\text{L}$) and cultivated in culture medium for 24 hours before medium was discarded and proliferation of cells was measured using a CellTiter96 Non-Radioactive Proliferation Assay (Promega Corporation, Madison, WI). Fibrin clots with no cells were used as negative control. Cell number was evaluated according to manufacturer's protocol: supernatants were discarded and $45 \mu\text{L}$ dye solution was added to $300 \mu\text{L}$ culture medium. After 1.5 hours of incubation at 37°C in a humidified atmosphere with 5% CO₂ stop solution was added. After one hour of incubation $100 \mu\text{L}$ of the supernatants was transferred in triplicates on a 96-well plate and absorbance was measured at 555 nm on a Wallac 1420 VICTOR2 plate reader (PerkinElmer, Waltham, MA, USA). Further proliferation assays were performed on day 7 and day 14 of the experiment. Additionally, fibrin clots with no cells were used for negative control measurement. Fibrin clots with cells from at least 4 different donors were analyzed in independent experiments.

2.8. Adipocyte Differentiation. To assess adipocyte maturation in the fibrin clot, ASCs ($3 \times 10^5/100 \mu\text{L}$) were embedded in fibrin hydrogel and $50 \mu\text{L}$ clots were formed in cloning rings (Sigma-Aldrich, St. Louis, MO, USA). For 5 days clots were incubated with Preadipocyte Differentiation Medium (PromoCell GmbH, Heidelberg, Germany) before the medium was changed to Adipocyte Nutrition Medium (PromoCell GmbH, Heidelberg, Germany) which was changed every third day for the rest of the experiment. Differentiation was evaluated by fluorescent AdipoRed staining 7, 14, 21, and 28 days after induction of differentiation. Clots were incubated with AdipoRed Assay Reagent (Lonza, Walkersville, MD, USA) according to manufacturer's protocol. Fibrin clots with differentiated ASCs were analyzed using a Nikon Microphot-FXA microscope with the NIS-Elements AR 3.0 software (Nikon). Differentiation experiments for microscopic analysis were conducted with ASCs from 11 different donors.

2.9. Glycerol Assay. For the quantitative determination of glycerol in the supernatants of three-layered skin substitute in culture, $3 \times 10^5/100 \mu\text{L}$ ASCs were embedded in $50 \mu\text{L}$ fibrin clots and were incubated with Preadipocyte Differentiation Medium for 5 days before the medium was changed to Adipocyte Nutrition Medium. On days 7, 14, 21, and 28 after induction of differentiation supernatants were analyzed using a Glycerol Assay (Randox Laboratories Ltd., Crumlin, UK) to quantify the progress of ASCs differentiation. Briefly, medium was changed 24 hours before analysis. $50 \mu\text{L}$ of the supernatant was transferred as triplets on 96-well plates and was mixed with $100 \mu\text{L}$ reagent. Absorbance was measured at 490 nm on a Wallac 1420 VICTOR2 plate reader. Experiment

was done with cells from 4 different donors. Native adipose tissue of the same weight as the $50 \mu\text{L}$ fibrin clots was used as positive tissue control. Fresh adipose tissue was put in Adipocyte Nutrition Medium for 24 hours and the glycerol concentration in the supernatant was measured as described above.

2.10. Statistics. Statistical analysis was done with the GraphPad Prism software. Data are expressed as means \pm SEM of at least 3 independent experiments. Statistical comparisons for all experimental settings were based on two-sample *t*-test with $P < 0.05$ considered as significant.

3. Results

3.1. Generation of a Three-Layered Skin Substitute with Fibrin Hydrogel Matrix. For the hypodermal layer ASCs and mature adipocytes were embedded in the fibrin hydrogel. On top, another fibrin clot with fibroblasts was placed for the construction of the dermal layer. Keratinocytes were added on top of the two-layered construct to form the epidermal layer. The three-layered construct was cultivated for up to 3 weeks in cell culture inserts with keratinocytes being exposed to air according to the air-liquid interface cultivation model [20]. Constructs measured $4\text{-}5 \text{ mm}^3$ for the following in vitro experiments (Figure 1).

Cross section probes of the generated artificial skin substitute (Figure 2(a)) were analyzed and showed similar hypodermal, dermal, and epidermal structures when compared to native skin (Figure 2(b)). Cells were distributed equally in the gel and showed normal morphology. On the surface keratinocytes formed an epithelial-like layer.

3.2. Viability of ASCs and Fibroblasts in the Three-Layered Skin Substitute. To determine if ASCs and fibroblasts are viable and proliferate at a normal rate in the fibrin construct, we incubated fibrin clots with either ASCs or fibroblasts embedded for 1 and 7 days in culture medium and analyzed the cell number (matrix w/cells). Fibrin clots without cells were used as negative control. Following incubation cells were analyzed using a cytotox assay.

The results showed that both cell types were viable and proliferated in the construct as the significant difference in cell number between day 1 and day 7 demonstrated in Figure 3 (ASCs: $P < 0.01$; fibroblasts: $P < 0.05$). ASCs and fibroblasts seemed to reach their highest level of proliferation on day 7 as we also tested the cell number on day 14 (data not shown).

Hence, the results show an increasing cell number of ASCs and fibroblasts in the three-layered artificial skin substitute during the first week.

3.3. Hypodermis Construction in Hydrogel Matrix: Differentiation Potential of ASCs. In our construct we seeded ASCs in combination with mature adipocytes to add a hypodermis to the epidermal and dermal layer of our skin substitute. However, using the construct in a clinical setting, ASCs are expected to be able to differentiate into adipocytes within the fibrin matrix to form new adipose tissue. Therefore, we tested

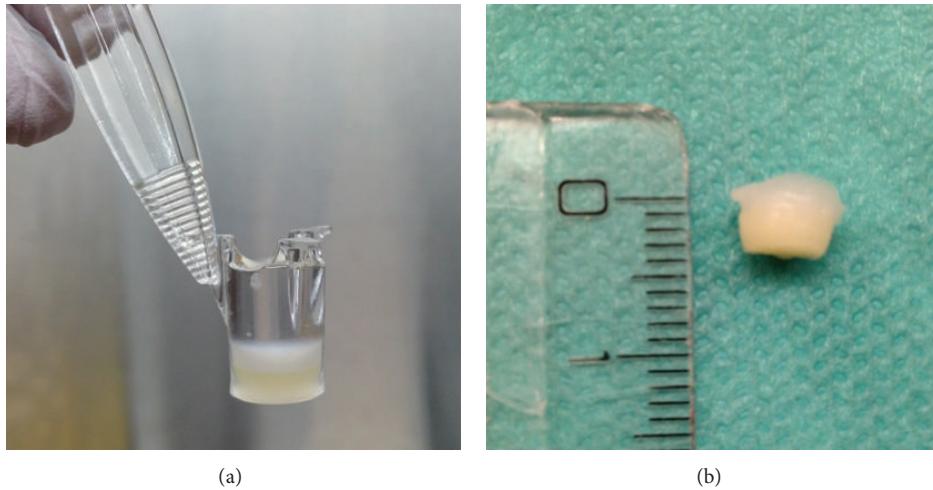


FIGURE 1: (a) Three-layered skin substitute (5 mm^3) constructed of fibrin hydrogel for in vitro analysis. (b) Lower layer (yellow) consists of fibrin clot with ASCs and mature adipocyte as hypodermal layer. Upper layer (white) consists of hydrogel mixed with fibroblasts mimicking the dermal layer. On top keratinocytes were seeded as epidermal layer.

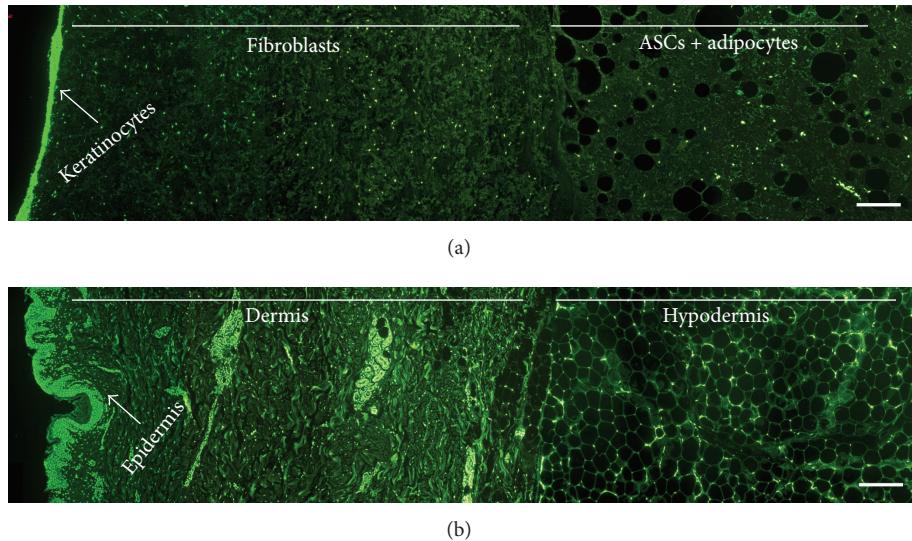


FIGURE 2: Cross sections of three-layered artificial skin show similar morphological structure to native skin. (a) Three-layered skin construct out of fibrin hydrogel stained with SYTOX Green Nucleic Acid Stain on day 4. (b) Native skin stained with SYTOX Green Nucleic Acid Stain. Bars represent $200 \mu\text{m}$.

the potential of ASCs to differentiate in the fibrin matrix and evaluated the lipid accumulation in ASCs 28 days after induction of differentiation. Cells were incubated with Preadipocyte Differentiation Medium for 5 days. For the next 24 days cells were cultured in Adipocyte Nutrition Medium.

On day 7 after induction of differentiation ASCs showed signs of lipid accumulation which increased during the following two weeks (Figures 4(a), 4(b), and 4(c)). On day 28 the majority of the cells in the fibrin hydrogel showed typical adipocyte morphology (Figure 4(d)) similar to native adipose tissue (Figure 4(e)) and filled most of the space in the matrix.

To further evaluate the differentiation potential of ASCs in the fibrin hydrogel we quantified the glycerol release of the cells within the fibrin clots in the supernatant. Undifferentiated ASCs within the fibrin hydrogel were used as control. Additionally, native adipose tissue of the same size as the fibrin matrix was used as positive tissue control.

Results showed that glycerol release increased up to three times between week 1 and week 4 of adipogenesis (Figure 5(a)). Glycerol concentration of artificial adipose tissue on day 28 was comparable to the concentration measured in the supernatant of native adipose tissue (Figure 5(b)).

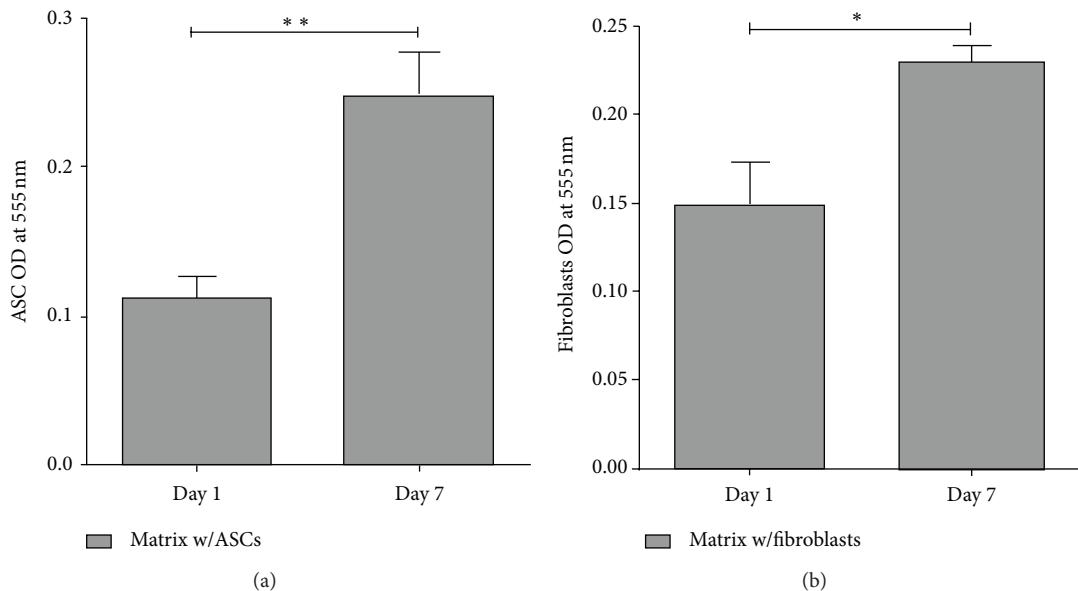


FIGURE 3: ASCs and fibroblasts are viable and proliferate in the construct. (a) ASC from six different donors ($n = 6$) and (b) fibroblasts from four donors ($n = 4$) were used in independent experiments. Data are expressed as mean \pm SEM. Significant differences are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

A certain variability was observed in terms of differentiation potential of ASCs from different donors. Still, our results demonstrate that ASCs are able to differentiate into adipocytes in a fibrin matrix and therefore are suitable for our three-layered skin substitute.

4. Discussion

In this study we were able to generate for the first time a three-layered skin substitute based on a fibrin hydrogel. By incorporating ASCs and mature adipocytes but also fibroblasts into the matrix and seeding keratinocytes on top of the construct, we were able to achieve a skin substitute not only serving as a dermal and epidermal substitute but also including the hypodermis.

In such a construct the main challenge is to generate a layer of adipose tissue replacing the hypodermis. Physical properties are of major importance to mimic the soft character of fat tissue. Mechanical properties of fibrin hydrogel resemble those of adipose tissue to a satisfying extent. In vitro it was already suggested to be suitable for adipose tissue-equivalent formation [13] and because of its biodegradability and biocompatibility we have chosen fibrin hydrogel as a scaffold for our project. Moreover, fibrin hydrogel has already been well characterized and been used successfully as a glue in clinical practice for a long time [21].

In previous studies 3D fibrin matrix of low fibrinogen concentration showed eligible results in adipogenesis of ASCs [13]. Therefore, the fibrinogen component was diluted to 25 mg/mL end concentration in the clot. Additionally, high thrombin concentrations have a negative influence on different cell types, for example, on keratinocytes [22] or endothelial cells and fibroblasts [10]. Consequently, the thrombin

component was diluted as well to an end concentration of 2.5 IU/mL in the clot.

Our results show that ASCs and fibroblast were viable and showed physiological morphology in the fibrin hydrogel. Both cell types proliferated well by showing a significant increase of cells in the construct during the first seven days. Additionally, ASCs were able to differentiate into mature adipocytes during the course of four weeks. On day 28 after induction of differentiation lipid accumulation of differentiated ASCs showed morphological resemblance to native adipose tissue. Due to high donor variability there were big differences in the adipogenic differentiation potential of our ASCs. Still, analysis of the glycerol release rate of ASCs during the differentiation period of four weeks indicated that the hypodermis of our artificial skin substitute mimics native adipose tissue.

A variety of skin substitutes that restore epidermal and dermal structures are currently available on the market. Still, there is a lack of hypodermal tissue substitutes. But especially patients with full thickness skin defects would benefit from such a replacement. Although this study showed promising results in the in vitro settings, where we used skin substitute constructs with the size of 5 mm³, the missing vascularization presents a limitation to large scale clinical approaches. Successful applications of skin substitutes are currently limited to tissue less than 2 mm in thickness [23]. Applying thicker substitutes would require a capillary network delivering oxygen and nutrients to the tissue.

A promising approach to vascularization of bioartificial skin presented a study by Sánchez-Muñoz et al. by promoting formation of capillary structures by seeding HUVECs with dermal fibroblasts and ASCs in a fibrin matrix. The extracellular matrix, produced by dermal fibroblasts and ASCs,

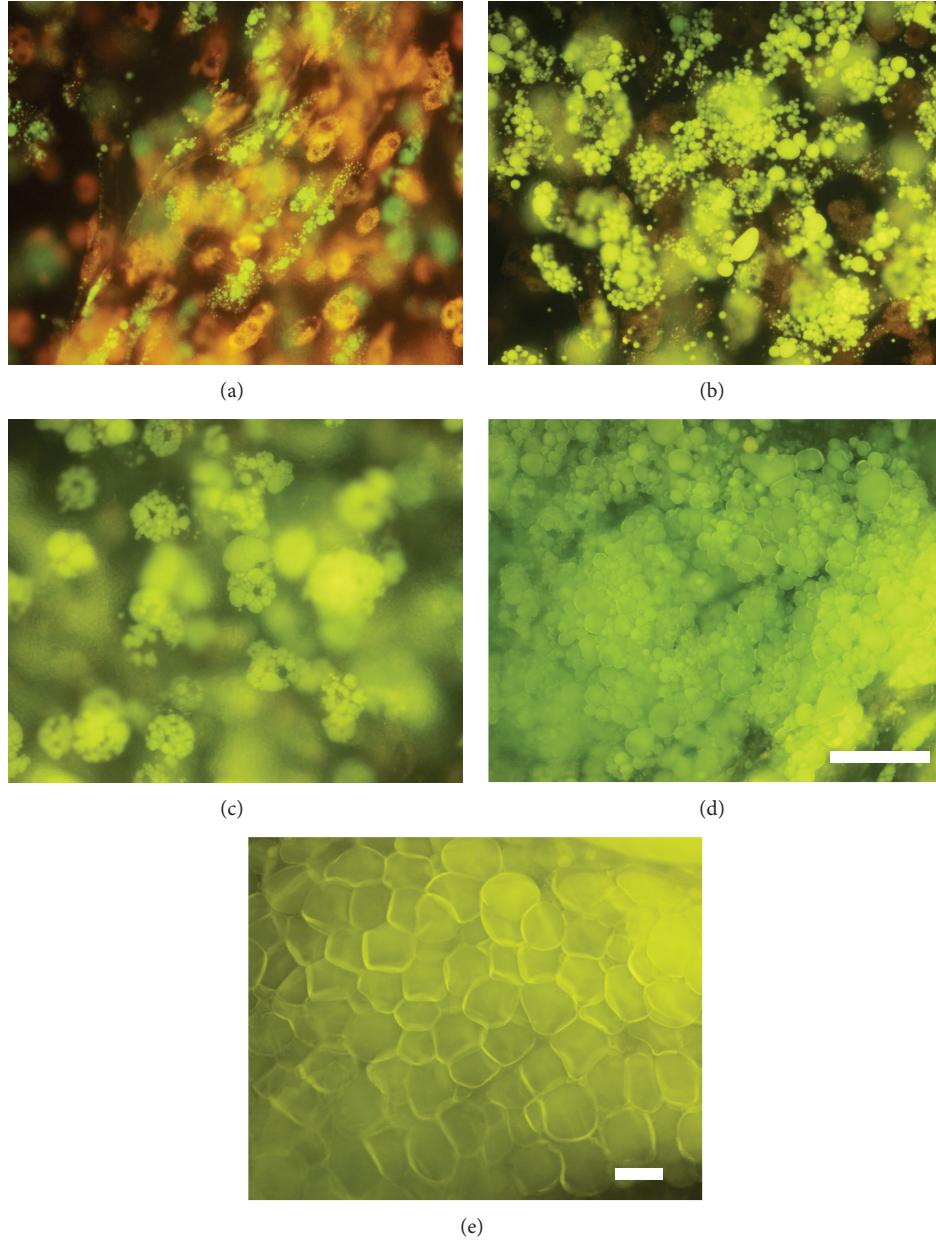


FIGURE 4: Adipose-derived stem cells differentiating into adipocytes during the time course of 28 days. Differentiation was evaluated by fluorescent AdipoRed staining (a) 7 days, (b) 14 days, (c) 21 days, and (d) 28 days after induction of differentiation. (e) AdipoRed staining of native adipose tissue. Bars represent 100 μ m. One representative out of eleven experiments is shown.

stimulates cellular growth and proliferation [11]. Further possible developments of this new multilayered model besides working with endothelial cells could be the additional use of proteins like PDGF to stimulate neovascularization [24] or incorporation of melanocytes for UV-protection [25].

5. Conclusion

In this study we were able to generate a three-layered skin substitute based on a fibrin hydrogel. ASCs, mature adipocytes, and fibroblasts were incorporated into the matrix. Keratinocytes were seeded on top of the construct and formed an

epithelial-like layer. We showed that our artificial hypodermis showed comparable characteristics to native adipose tissue in lipid accumulation as well as in glycerol release. With this work we were able to achieve a skin substitute not only serving as a dermal and epidermal substitute but also including the hypodermis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

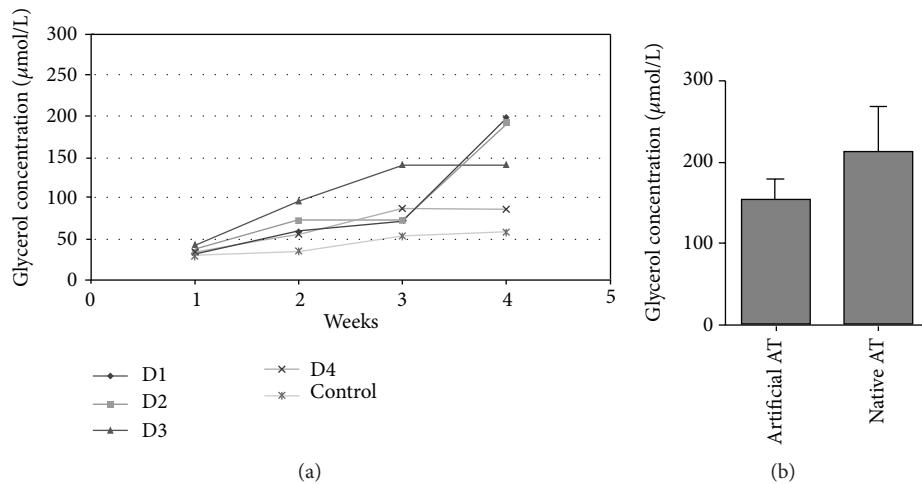


FIGURE 5: Glycerol concentration ($\mu\text{mol/L}$) in supernatant of fibrin clots with differentiating ASCs at weeks 1, 2, 3, and 4. A total of $n = 4$ donors (D1–4) are shown. Glycerol concentration of undifferentiated ASCs within the matrix is shown as control. (b) Glycerol release of artificial adipose tissue (artificial AT; $n = 4$) on day 28 compared to fresh, native adipose tissue (native AT; $n = 4$).

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References

- [1] K. Hemmrich and D. von Heimburg, "Biomaterials for adipose tissue engineering," *Expert Review of Medical Devices*, vol. 3, no. 5, pp. 635–645, 2006.
- [2] M. Kuroyanagi, A. Yamamoto, N. Shimizu et al., "Development of cultured dermal substitute composed of hyaluronic acid and collagen spongy sheet containing fibroblasts and epidermal growth factor," *Journal of Biomaterials Science, Polymer Edition*, vol. 25, no. 11, pp. 1133–1143, 2014.
- [3] C. S. Blok, L. Vink, E. M. de Boer et al., "Autologous skin substitute for hard-to-heal ulcers: retrospective analysis on safety, applicability, and efficacy in an outpatient and hospitalized setting," *Wound Repair and Regeneration*, vol. 21, no. 5, pp. 667–676, 2013.
- [4] G. Huang, S. Ji, P. Luo et al., "Evaluation of dermal substitute in a novel co-transplantation model with autologous epidermal sheet," *PLoS ONE*, vol. 7, no. 11, Article ID e49448, 2012.
- [5] E. K. Beahm, R. L. Walton, and C. W. Patrick Jr., "Progress in adipose tissue construct development," *Clinics in Plastic Surgery*, vol. 30, no. 4, pp. 547–558, 2003.
- [6] S. E. Greenwald and C. L. Berry, "Improving vascular grafts: the importance of mechanical and haemodynamic properties," *The Journal of Pathology*, vol. 190, pp. 292–299, 2000.
- [7] A. J. Engler, S. Sen, H. L. Sweeney, and D. E. Discher, "Matrix elasticity directs stem cell lineage specification," *Cell*, vol. 126, no. 4, pp. 677–689, 2006.
- [8] V. Falanga, S. Iwamoto, M. Chartier et al., "Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds," *Tissue Engineering*, vol. 13, no. 6, pp. 1299–1312, 2007.
- [9] R. A. F. Clark, "Fibrin sealant in wound repair: a systematic survey of the literature," *Expert Opinion on Investigational Drugs*, vol. 9, no. 10, pp. 2371–2392, 2000.
- [10] D. MacAsev, J. P. Diorio, A. Gugerell, A. Goppelt, H. Gulle, and M. Bittner, "Cell compatibility of fibrin sealants: in vitro study with cells involved in soft tissue repair," *Journal of Biomaterials Applications*, vol. 26, no. 2, pp. 129–149, 2011.
- [11] I. Sanchez Munoz, R. Granados, P. Holguin Holgado, J. A. García-Vela, C. Casares, and M. Casares, "The use of adipose mesenchymal stem cells and human umbilical vascular endothelial cells on a fibrin matrix for endothelialized skin substitute," *Tissue Engineering Part A*, 2014.
- [12] A. L. Mazlyzam, B. S. Aminuddin, N. H. Fuzina et al., "Reconstruction of living bilayer human skin equivalent utilizing human fibrin as a scaffold," *Burns*, vol. 33, no. 3, pp. 355–363, 2007.
- [13] A. Peterbauer-Scherb, M. Danzer, C. Gabriel, M. van Griensven, H. Redl, and S. Wolbank, "In vitro adipogenesis of adipose-derived stem cells in 3D fibrin matrix of low component concentration," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 6, no. 6, pp. 434–442, 2012.
- [14] D. V. Heimburg, K. Hemmrich, S. Zachariah, H. Staiger, and N. Pallua, "Oxygen consumption in undifferentiated versus differentiated adipogenic mesenchymal precursor cells," *Respiratory Physiology and Neurobiology*, vol. 146, no. 2–3, pp. 107–116, 2005.
- [15] J. M. Gimble, A. J. Katz, and B. A. Bunnell, "Adipose-derived stem cells for regenerative medicine," *Circulation Research*, vol. 100, no. 9, pp. 1249–1260, 2007.
- [16] P. Bourin, B. A. Bunnell, L. Casteilla et al., "Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT)," *Cyotherapy*, vol. 15, no. 6, pp. 641–648, 2013.

- [17] W.-S. Kim, B.-S. Park, and J.-H. Sung, "Protective role of adipose-derived stem cells and their soluble factors in photoaging," *Archives of Dermatological Research*, vol. 301, no. 5, pp. 329–336, 2009.
- [18] N. K. Satija, G. U. Gurudutta, S. Sharma et al., "Mesenchymal stem cells: molecular targets for tissue engineering," *Stem Cells and Development*, vol. 16, no. 1, pp. 7–23, 2007.
- [19] J. Zhong, S. A. Krawczyk, R. Chaerkady et al., "Temporal profiling of the secretome during adipogenesis in humans," *Journal of Proteome Research*, vol. 9, no. 10, pp. 5228–5238, 2010.
- [20] M. Prunieras, M. Regnier, and D. Woodley, "Methods for cultivation of keratinocytes with an air-liquid interface," *The Journal of Investigative Dermatology*, vol. 81, no. 1, pp. 28s–33s, 1983.
- [21] D. M. Albala, "Fibrin sealants in clinical practice," *Cardiovascular Surgery*, vol. 11, supplement 1, pp. 5–11, 2003.
- [22] A. Gugerell, K. Schosslleitner, S. Wolbank et al., "High thrombin concentrations in fibrin sealants induce apoptosis in human keratinocytes," *Journal of Biomedical Materials Research—Part A*, vol. 100, no. 5, pp. 1239–1247, 2012.
- [23] C. K. Griffith, C. Miller, R. C. A. Sainson et al., "Diffusion limits of an in vitro thick prevascularized tissue," *Tissue Engineering*, vol. 11, no. 1-2, pp. 257–266, 2005.
- [24] R. Mittermayr, L. Branski, M. Moritz et al., "Fibrin biomatrix-conjugated platelet-derived growth factor AB accelerates wound healing in severe thermal injury," *Journal of Tissue Engineering and Regenerative Medicine*, 2013.
- [25] S. Böttcher-Haberzeth, A. S. Klar, T. Biedermann et al., "'trooping the color': restoring the original donor skin color by addition of melanocytes to bioengineered skin analogs," *Pediatric Surgery International*, vol. 29, no. 3, pp. 239–247, 2013.

Research Article

Immunohistochemical Expression of Collagens in the Skin of Horses Treated with Leukocyte-Poor Platelet-Rich Plasma

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This study evaluated the immunohistochemical expression of type I (COL I) and III (COL III) collagens during the healing process of skin treated with leukocyte-poor platelet-rich plasma (LP-PRP). Seven healthy gelding crossbred horses aged 16 to 17 years were used. Two rectangle-shaped wounds were created surgically in the right and left gluteal regions. Twelve hours after wound induction, 0.5 mL of the LP-PRP was administered in each edge of the wounds of one of the gluteal regions. The contralateral region was used as control (CG). Three samples were obtained: after wound induction (T0), 14 days (T1) of healing process, and after complete closure of the skin (T2). The normal skin (T0) showed strong staining for type III and I collagen in papillary and reticular dermis, respectively. In the scar of the treated group, COL III showed important ($p < 0.05$) increase in immunoreaction in T2 compared with T1. The administration of a single dose of LP-PRP 12 h after induction of wound in horses does not influence formation of collagens I and III. However, the intense labeling for COL III suggests that the tissue was still weak during the macroscopic closure of the wound, demonstrating that healing was not completely finished.

1. Introduction

Large animals are considered excellent models for the study of wounds, given that they provide the proper comparison of the many physiological and immunological aspects involved in wound healing [1]. Different cell types, cytokines, and extracellular matrix molecules at the wound site interact with different systemic factors such as platelets, the coagulation cascade, and humoral cell components, which together enable the healing of wounds [2].

The most appropriate form of healing a cutaneous wound is by first intention [3, 4], but this procedure is limited to wounds located in anatomical regions that allow for the excision and adaptation of its edges [3]. Healing by second intention, on the other hand, which involves two independent processes—contraction and reepithelialization [5]—is often the only possibility to close wide and deep wounds. However, the process is slow and may result in the formation of exuberant granulation tissue. In this sense, new therapies aiming to reduce the maximum healing period of cutaneous

wounds and that result in the formation of a tissue as similar as possible to a healthy one emerge every day.

Platelet-rich plasma (PRP), a product derived from the whole blood centrifugation, is an autogenous and economic source of a variety of growth factors that participate actively in the healing process of cutaneous wounds such as the transforming growth factor beta (TGF- β), because it is associated with the stimulus to the synthesis of collagen [6–8] both type I and type III, which are the main structural components of the mature scar tissue, and it is synthesized by fibroblasts [9, 10].

Despite the numerous studies conducted with platelet-rich components in different tissues and in various species, there is still not enough evidence confirming the effectiveness of PRP in the treatment of cutaneous wounds. This is partly due to the region of the body subjected to the treatment; the form of obtaining and the composition of the PRP (considering the amount of platelets and leukocytes); its physical form (liquid/gel), route, and frequency of administration; volume; and the moment it is administered during the skin healing process; among other aspects. There are some studies and case reports on horse skin [11–16], but with contradictory results. Moreover, *in vivo* research studies evaluating the therapeutic response using highly specific and sensitive methods are rare. According to Furness et al. [17], one of the most indicated techniques to identify the collagens is immunohistochemistry, which may delineate the type present in the evaluated tissue. This methodology has been used to determine the expression of growth factors such as TGF- β 1, which is the most often studied isoform in the different species, because in addition to participating in the synthesis of collagens (as previously mentioned) it is essential for angiogenesis, chemotaxis, and cell proliferation [18].

Considering the amount of platelets and leukocytes, the platelet concentrates can be classified into different categories. There is the pure PRP (P-PRP), where the white cells are removed intentionally, and the PRP with leukocytes (L-PRP) [19]. There are also the components rich in fibrin (PRF) [20] that, unlike the other concentrated, do not require the use of anticoagulants to be obtained [21]. L-PRP should have between 5x and 8x more platelets [19, 20] and three times or more amounts of leukocytes [20] than the whole blood. However, there is no consensus of the amount of platelets that the PRP should present for it to be regarded as pure. Hutchins and Grabsch [22] report that it should contain a moderate number of platelets, which should be 1.5x to 2.5x the existing in whole blood, while Carmona et al. [20] consider that must have 1.3x to 4x the blood concentration. These authors also mention that the concentrate can contain 0.5x to 2x more leukocytes than the blood. When the concentrate rich in platelets is activated by pharmacological agents, forming a fibrin polymer, it is known as platelet gel (PG). Therefore, a PG obtained from a P-PRP is called P-PRG, and when it is originated from L-PRP, it is known as L-PRG. Finally, taking into account the basic definitions for those components rich in platelets, there is also the PRP poor in leukocytes (LP-PRP). This last component may results in lesser acute inflammatory response, lower cellularity, and vascularity when compared to PRP with a high amount in

white cells (LR-PRP), as reported by Dragoo et al. [23] five days after treatment of tendon of rats with LP-PRP or LR-PRP.

In physiological conditions, the repair process requires activation and/or suppression of several substances that may result in abnormal healing of the cutaneous wound. The immunohistochemical evaluation of proteins expressed in the different healing phases of PRP-treated skin may provide invaluable information to validate the effectiveness or ineffectiveness of the therapy. Thus, the objective of the present study was to evaluate the immunohistochemical expression of type I and III collagens during different phases of the macroscopic healing process of skin treated or untreated with LP-PRP.

2. Material and Methods

This research was approved by the Ethics Committee on Animal Use of Universidade Federal de Viçosa (UFV) (protocol number 35/2013). The procedures were conducted according to the Rules of Conduct for the Use of Animals in Teaching, Research and Extension of the Department of Veterinary (DVT/UFV), the Medical Veterinary Professional Ethics Code, the Ethical Principles for Animal Experimentation (COBEA), and the current Brazilian Legislation.

Seven healthy gelding crossbred horses aged 16 to 17 (16.37 ± 0.52) years were used. Only seemingly healthy animals with no dermatological disorders were included in the study. The animals were housed in individual 20-m² stalls fifteen days prior to the onset of the experiment, where they were fed Tifton 85 hay and chopped elephant grass (*Pennisetum purpureum*), in addition to a mash diet for horses. Mineral salt and water were provided *ad libitum*. This management was carried on over the course of the experimental trial. In the period of adaptation the horses were weighed, bathed with an acaricide solution containing deltamethrin, and dewormed orally with moxidectin paste (0.2 mg/kg). Stalls were cleaned twice daily to remove the excreta and replace the bedding.

Three rectangle-shaped skin injuries were created in the right and left gluteal regions of all animals, as described by Ferreira et al. [24]. For this purpose, the sites were previously clipped and aseptically prepared with 2% germicide chlorhexidine digluconate and 0.5% alcoholic solution. The animals were injected intravenously with 2% xylazine (0.8 mg/kg) and then subcutaneous nerve-block was performed with 2% lidocaine hydrochloride around the site to be incised, using a 21-gauge needle.

To surgically create the wounds, a scalpel and a 2.5 cm sided (6.25 m²) rectangle-shaped plastic mold were used to remove the skin fragment (epidermis, dermis, and subcutaneous tissue). Wounds were identified as A and B, from cranial to caudal on both gluteal region. The first collection was considered time zero (T0). The wounds healed by second intention and were monitored until complete closure, when the last biopsy was performed (wound B). During the postoperative period the surgical wound was dressed daily with gauze soaked in Milli-Q water. All animals received antitetanus serum on the day of the injury. Their pain was

lessened by using a single intravenous dose of butorphanol tartrate (0.08 mg/kg). No anti-inflammatory or antibiotic was used, as they could hamper or make it impossible to evaluate the treatment with LP-PRP.

The leukocyte-poor platelet-rich plasma was obtained by following the procedures described by Argüelles et al. [25]. For this purpose, 144 mL blood samples were collected from each animal, by puncturing the external jugular vein in 36 Vacutainer tubes with 3.2% sodium citrate (0.199 mol/L). The tube capacity was 5 mL : 0.5 mL for sodium citrate and 4.5 mL for whole blood. Blood samples with EDTA were also collected to quantify platelets and total leukocytes.

The samples of blood to obtain the LP-PRP were homogenized and centrifuged at 120 × g for ten minutes. After this first centrifugation, 50% of blood plasma from the surface was discarded and the rest was transferred to four polypropylene tubes with 10 mL capacity without anticoagulant. Leukocyte button and sedimented erythrocytes were discarded. Next, the plasma was centrifuged one more time at 240 × g for 10 min. After this second centrifugation, the plasma was divided into two fractions: the supernatant (platelet-poor plasma) and the remaining fraction, named platelet-rich plasma. A volume of 75% of the obtained plasma present at the surface of each tube was discarded and the PRP containing the platelet button was reserved.

The concentration of platelets and leukocytes was determined manually [26] in the LP-PRP and the blood obtained with EDTA. The count was performed in a Neubauer chamber using Türk's solution to count the leukocytes and Brecher's method for platelet count [27].

All the horses were subjected to a local treatment with LP-PRP 12 h after the injuries were performed. After clipping and sedation with 2% xylazine intravenously (0.8 mg/kg), one of the gluteal regions—chosen randomly—was prepared aseptically for the administration of the LP-PRP (treated group = TG). The wounds in the contralateral gluteal region (control group = CG) did not receive any infiltration, but only local cleaning with Milli-Q water, as was done in the treated wounds.

The platelet-rich plasma was prepared immediately before its administration, which was performed using a 24-gauge needle. Each edge of the two wounds (A and B) received 0.5 mL of the LP-PRP (Figures 1(a)-1(b)), totaling 2 mL per wound and 4 mL per side (left or right) of the gluteal region. After the treatment, the animals were kept in stalls and let out for a daily period of 2 h and monitored throughout the experiment.

Skin samples were collected to analyze the immunohistochemical expressions of type I and III collagens. Skin fragments were obtained by biopsy (full thickness) using a 6 mm diameter *Punch* with the aid of a scalpel at 14 days (T1) after wound A. In addition, a new collection was made when wound B was completely closed (T2).

For the biopsies, the animals were sedated intravenously with 2% xylazine (0.8 mg/kg) and then subcutaneous block anesthesia was performed with 2% lidocaine hydrochloride without vasoconstrictor around the site to be biopsied, using a 21-gauge needle. The last collection (T2) was performed in the center of the healed area (which was still unpigmented),

TABLE 1: Information about manufacturer, code, and dilution of the antibodies utilized in the study.

Primary antibody	Manufacturer	Code	Dilution
Anti-collagen I (mouse monoclonal antibody)	Abcam	Ab90395	1: 100
Anti-collagen III (rabbit polyclonal antibody)	Abcam	Ab7778	1: 50

whereas the second biopsy (T1) was obtained from the peripheral region of the wounds (Figure 1(c)), comprising both intact and wounded skin, including migration or reepithelialization, and granulation tissue, according to the collection time. Skin samples (Figure 1(d)) were fixed in 10% formalin, embedded in paraffin, sectioned with 5 μm thickness, and stained with hematoxylin and eosin (HE) (Erviegas Instrumental Cirúrgico Ltda., São Paulo, São Paulo, Brazil) for an overall tissue-character assessment at the different times of sample collection. This analysis was conducted by three observers blinded to origin of the biopsy specimens.

Sections were also evaluated by immunohistochemistry using the indirect immunoperoxidase technique. The primary antibodies used are listed in Table 1. For the antigen retrieval, a pretreatment was performed with citrate buffer pH 6.0 at 95°C for 30 min in water bath. After washing with PBS, a 16% milk powder solution was used to block the nonspecific reactions and tissue permeabilization, and 3% H₂O₂ (Dinâmica 1857, Blueskylab Artigos para Laboratório, Guarulhos, SP, Brazil) during 30 min to block endogenous peroxidase. The sections were incubated with primary antibody overnight at 4°C. Subsequently, the histological sections were incubated with anti-mouse and anti-rabbit universal immunoperoxidase polymer (Histofine-Simple Stain MAX PO MULTI, Biogen Comercial e Distribuidora Ltda., Sumarezinho, SP, Brazil) and the immunohistochemistry reaction was revealed with DAB (DAKO-K346811-2, Labscience de Minas, Instrumentos Científicos Ltda., Belo Horizonte, MG, Brazil). Horse and rat tendons, pig skin, and mouse and human testicles were used for the positive control. For negative control, one of the slides did not receive primary antibody. The sections were counterstained with Harris hematoxylin (Labscience de Minas Instrumentos Científicos Ltda., Belo Horizonte, MG, Brazil). Images of the slides were acquired using an optical microscope and the Axio Vision software 4.8 Rel.

The immunostained area was evaluated in the healing area where the recently formed skin showed a thick stratified epithelium and dermis without presence of annexes at the different wound evolution times. The immunohistochemically stained area (IHS_A) was calculated for collagen I and III primary antibodies as a percentage of the total area evaluated through the color segmentation analysis using software AxioVision 4.8.2. from Zeiss (<http://www.zeiss.com.br>). The brown stain was selected and a mask was subsequently applied to make a separation of permanent colors. The mean IHS_A was calculated from 30 images obtained in quadrant

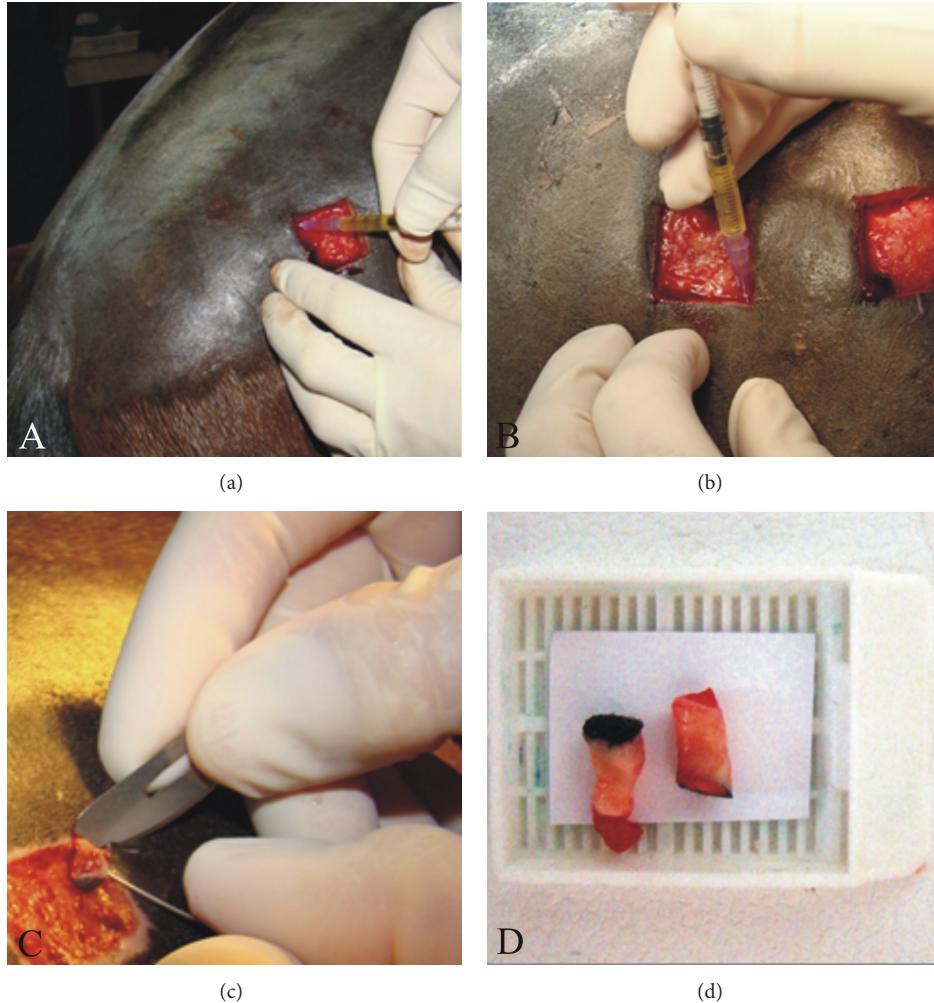


FIGURE 1: Administration of 0.5 mL leukocyte-poor platelet-rich plasma on the wound edges, using a 24-gauge needle (a, b). Skin sample collection using a scalpel and a needle, after cutting with a 6 mm diameter *Punch* (c). The obtained fragments were immediately placed on histology cassettes (d) and fixed in 10% formalin.

shape, starting from the underlying epithelial (papillary dermis) in the healing area toward the reticular dermis and then returning to the epithelium, for the time interval of treatment ($IHS_A = 100S_A/W_A$, where S_A = stained area and W_A = whole area).

The statistical analysis was conducted on GraphPad InStat version 3.05 (GraphPad Software, Inc.). The *t* test was utilized for independent samples to compare the control and treated groups at each time (T0, T1, and T2). In addition, the different times were compared with the initial situation (time 0) in both groups using the *t* test for paired data. Additionally, Student's *t*-test for independent samples was adopted to compare the mean values of platelet and leukocyte counts in the blood and LP-PRP and the mean wound-healing time in the groups. To compare the different times in both groups, an analysis of variance (ANOVA) of repeated measures was performed. All analyses were carried out at 5% significance. All data were expressed as mean \pm SEM.

3. Results and Discussion

3.1. Macroscopic Wound Closure, Platelets and Leukocyte Counts. The period for the closure of a cutaneous wound depends on the extent and anatomic location of the injury, on the presence of local infections, among other aspects. According to Theoret et al. [4], the wound healing process in horses is very similar to that of humans. The skin surface is reepithelialized, with the dermis healing by stromal granulation, migration of myofibroblasts, and wound contraction [28]. The skin healing process is classically divided into four well-defined overlapping phases, including haemostasis, inflammation, proliferation, and lastly remodeling [5, 29]. Schultz et al. [30] subdivide the third phase into migration and proliferation and the fourth into contraction and remodeling.

In the present study the maximum time necessary to heal the wounds was 47 days in both groups of animals, averaging 36.85 ± 7.45 days in the wounds that were not

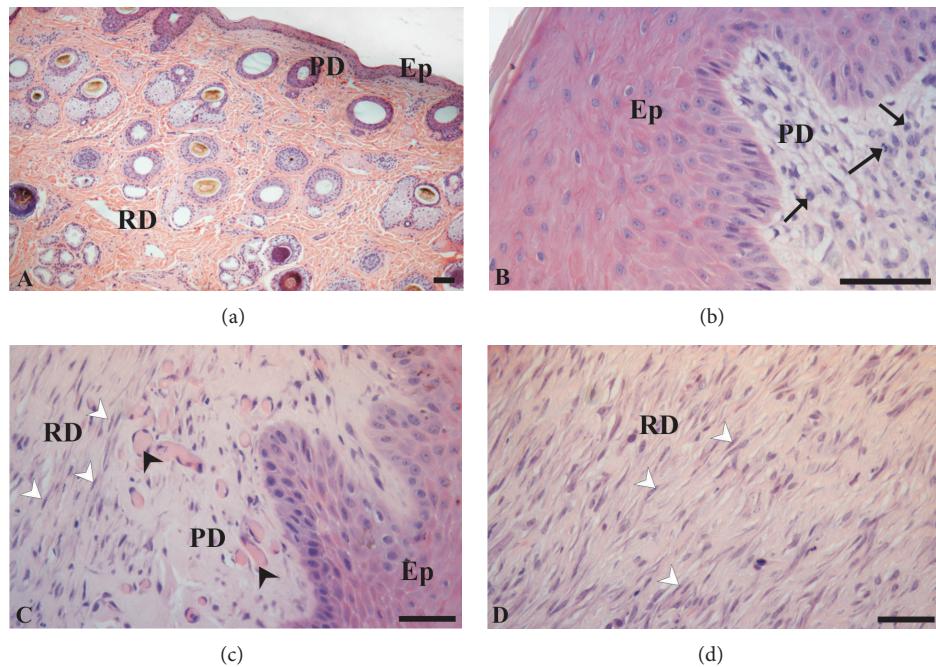


FIGURE 2: Photomicrography of skin before (a) and during the healing process (b–d), where (b) is after two weeks, and (c) and (d) after complete wound closure. Stratified squamous epithelial tissue (Ep), papillary dermis (PD), reticular dermis (RD), dilated capillaries (black arrowhead), fibroblasts (white arrowhead), and immune cells (arrows). HE stain. Bars = 50 μ m.

treated with LP-PRP, and 38.85 ± 6.46 days in the treated wounds; however, there was no difference ($p = 0.59$) between groups. Therefore, 37 days was considered as the mean time necessary for wound closure. This average healing time is close to that found in uncontaminated wounds located in the thoracic region, which is approximately four weeks [7], and of wounds performed in the same manner and in the same region as that described in the present study by Ferreira et al. [24] (38.5 ± 3.9 days). The obtained results are similar to those described by Monteiro et al. [13], who also observed that the maximum closure period of PRP-treated wounds was longer than that of untreated wounds, which suggests that the therapy has no positive effect on the maximum macroscopic healing time of the skin wound.

In the whole blood and LP-PRP the platelet count varied from 100,000 to 150,000 platelets/ μ L and from 320,000 to 390,000 platelets/ μ L, respectively. The leukocyte (white blood cells) values, however, varied from 5,600 to 10,900 cells/ μ L and 50 to 900 cells/ μ L in the whole blood and LP-PRP, respectively. The average number of platelets found in the blood was lower ($p = 0.000$) than in LP-PRP. The opposite ($p = 0.000$) occurred with the average leukocyte count.

The concentration of platelets in the LP-PRP was considered adequate, because it was above the number considered appropriate by Anitua et al. [31] and similar to that obtained in studies conducted with healthy crossbred horses [32–34]. Additionally, an *in vitro* study carried out by Graziani et al. [35] demonstrated that PRP preparations have a dose-specific effect on the proliferation of fibroblasts and osteoblasts. Better effects (considered optimal) were observed when the platelet count was 2.5x higher than that present in the whole blood.

In contrast, higher amounts (5.5x) resulted in undesirable effects such as reduction of the fibroblastic proliferation and of the osteoblast function. According to the authors, different concentrations of platelets in the PRP can also lead to different results in *in vivo* studies.

The number of leukocytes (50–900 cells/ μ L) present in the LP-PRP was also considered adequate, because it was lower than that obtained by Vendruscolo et al. [33] ($2,460 \pm 763$ cells/ μ L), who utilized a relative centrifugal force (g) and the same time used in the present study. The positive effect of leukocytes on inflammation, immunity, and cell signaling is unquestionable [36], just as on the release of growth factors. On the contrary, undesirable effects are also attributed to the leukocytes, inasmuch as in their signaling proinflammatory cells they may cause tissue catabolism [37]. Moreover, there are reports that the application of platelet concentrate with leukocyte for the treatment of patients with tendinopathy may increase the local pain [29]. Therefore, both positive and negative effects are associated with the presence of these cells in the PRP. The use of a PRP with a low number of leukocytes, as was the case of the current study, is considered appropriate when aiming to promote a rather anabolic than catabolic action, given that the wounds were not contaminated.

3.2. Histological Evaluation of the Tissue Character of the Healing Process. The images obtained in the histological analysis of the HE-stained skin are shown in Figure 2. At time zero (making of the wound) it was revealed that the skin had a normal character, that is, an epidermis with poorly keratinized, stratified squamous epithelial tissue, and dermis with two well-defined layers: papillary (superficial) and

reticular (deep). Annexes hair follicle, hair erector muscle, sebaceous gland, and sweat glands were observed in these layers (Figure 2(a)).

The papillary dermis showed loose connective tissue with numerous capillary loops in contact with the basal layer of the epidermis. In addition, nuclei of connective tissue cells such as fibroblasts and fibrocytes were observed in this layer. The extracellular matrix has an amorphous ground substance in moderate quantity and thin but abundant collagen fibers. The reticular dermis was characterized by the presence of connective tissue cells, thick acidophilus collagen fibers arranged in various directions, and scarce amorphous ground substance. The adenomes and ducts of glands were more present in this layer.

The analysis of the animals' skin at two weeks showed that the surface of the wound was coated by dehydrated serocellular crust. Granulation tissue exhibited reduction in the number of vessels mainly in the deeper layers of the wound. Extracellular matrix showed predominance of thick collagen fibers, but still with poor organization. Fibroblasts showed round nuclei and acquired appearance of myofibroblasts in the deep dermis. Reepithelialization reached two thirds of the wound surface. There was a reduction in the inflammatory process (Figure 2(c)), but with a greater number of eosinophils in the wound in the LP-PRP-treated group.

Regardless of the group, the microscopic analysis performed at macroscopic wound closure, which as previously mentioned occurred approximately at 37 days, showed complete reepithelialization of the wound with hypertrophy and hyperplasia of the covering epithelium and remodeling of scar tissue. Fibroblasts show spindle-shaped nuclei and are organized into parallel bundles, acquiring a myofibroblast aspect. Areas of inflammation characterized by dilated capillaries (Figure 2(b)), recruitment of leukocytes, and particularly neutrophils were observed in the papillary layer. In addition, plasmocytes were also identified in the same layer. Better organization of fibroblasts with less deposition of collagen, and increased numbers of eosinophils were identified in the treated group compared with the control group. The reticular dermis in the wound healing area had a connective tissue arrangement similar to the dense ordered with fibroblasts aligned in parallel (Figure 2(d)).

The histopathological findings in the present study are compatible with the healing process described by Schwartz et al. [7], although they may vary depending on the region under evaluation. The authors studied the healing process using subjective histological evaluation of second-intention healing process of wounds from the thoracic region and the distal aspect of horse forelimbs by the Picrosirius Red Histochemical technique. The injuries located in the limbs had, at time zero, normal tissue with hair follicles, blood vessels, collagen, and few red cells. The inflammation was evident in the four weeks of assessment. In the second week, the predominant cells were particularly macrophages, and there were few spindle-shaped fibroblasts, and in the fourth week there was an increase in the collagen content and reduction of inflammatory cells. Wounds located in the thoracic region had dense collagen in all layers. After 14 days,

superficial collagen deposition was observed, and the density of the collagen was increased throughout the depth of the tissues, and the fourth week showed collagen organized in parallel fibrils in all tissue layers. On the other hand, in the wounds located in the forelimbs, collagen was abundant in all tissue layers at time zero and increased gradually from week 1 to 4. However, the collagen fibrils were not aligned in an organized pattern compared with the samples obtained from the thoracic wounds. With respect to myofibroblasts, these cells were more organized in the wounds of the thoracic as compared with those of the limb in the third and fourth weeks of the healing process. In contrast, in the first two weeks there was no difference between the wounds.

According to Deschene et al. [38] skin biopsy samples should contain the skin in all its thickness, since keratinocytes should predominate in the epidermis of intact skin, whereas fibroblasts should reflect the depth of the dermis. During the early phase of skin-wound healing, inflammatory cells must prevail, with a subsequent reduction in the later stage. At this stage endothelial cells and fibroblasts must be present and the number of keratinocytes should increase for reepithelialization to take place. In the present study, although skin closure was earlier ($p > 0.05$) in the control group, the overall histological assessment revealed that fragments obtained in the final stage of the healing process of LP-PRP-treated wounds show a better organization of the collagen fiber bundles microscopically as compared with untreated wounds. Therefore, the macroscopic closure of the wound does not necessarily reflect the quality of the scar tissue.

This faster closure may be related to the process of physiologic repair of skin wounds, which classically occurs in four well-defined overlapping phases [5, 29, 30]. Although the amount of platelets present in the hematoma formed after a wound is about 4%, the remainder being erythrocytes (94%) and leukocytes (less than 1%) [39], growth factors such as TGF- β and platelet-derived growth-factor (PDGF) are released by the platelet α -granules at the site where there is tissue injury [40]. According to Marx [41], growth factors are actively secreted during the first 10 minutes after clotting.

Myofibroblasts, contractile cells that originate from the differentiation of fibroblasts, observed during the last phase of the skin healing process, are essential for wound contraction, enabling faster reduction of the wound area [42]. The process occurs faster than reepithelialization itself [43]. However, its persistence suggests that wound healing is still in progress [7]. In the present study the cells were observed in the evaluation of the already clinically healed skin.

3.3. Immunohistochemistry for Collagen Types I and III.

Figures 3 and 4 show images obtained from the samples evaluated by immunohistochemistry. For COL III, staining in healthy skin (T0) showed reticular fibers located in the papillary dermis (Figure 3(a)). In contrast, for COL I, staining was evident in the reticular dermis (Figure 4(a)). The tissue of the edge of the scar had the same staining pattern as T0 in the papillary and reticular layers, with intense staining for COL III (Figure 3(b)) and I (Figure 4(b)), respectively. As previously mentioned, the immunostained area for COL

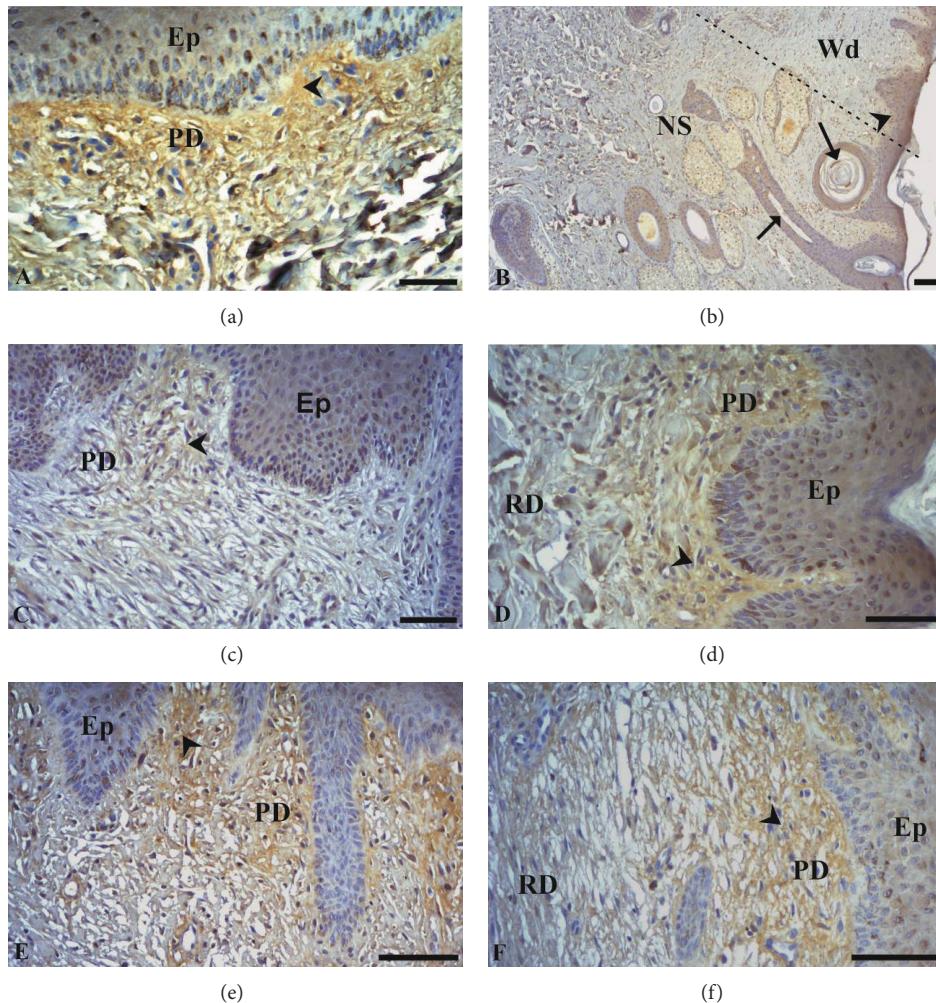


FIGURE 3: Immunohistochemistry for type III collagen in skin of horses treated or untreated with LP-PRP. Immunostaining was visible in the papillary dermis at all times and in all groups: normal skin (a), border tissue and scar area (b). Image obtained at 14 and 37 days of skin healing process from treated wounds (c and e, resp.) and controls (d and f, resp.). Stratified squamous epithelial tissue (Ep), papillary dermis (PD), reticular dermis (RD), immunostaining (arrowhead), hair follicle (arrows), scar edge skin (NS), and scar area (Wd). Bars = 50 μ m.

III (Figures 3(c)–3(f)) and COL I (Figures 4(c)–4(f)) was evaluated in the healing area where the newly formed skin had thick stratified epithelium and dermis without annexes for the different wound-healing periods.

The immunostained area of COL III in the intact skin (T0) was $3.92 \pm 0.61\%$. At 14 days of wound creation the COL III of the treated group showed a smaller immunostained area ($1.96 \pm 0.32\%$) as compared with T0 ($p < 0.05$). However, at 37 days, immunostaining of COL III ($3.00 \pm 0.58\%$) was not statistically different in wounds that received LP-PRP relative to T0. Wounds not treated with LP-PRP at 14 days ($2.97 \pm 0.27\%$) and 37 days ($2.64 \pm 0.61\%$) did not differ from T0. In T1, the area immunostained for COL III in the treated group was smaller ($p < 0.05$) than control. In T2, the values achieved in the immunostained area of the treated animals were higher than in control (Figure 5(a)).

Regarding COL I, the skin of the animals at T0 had an immunostained area of $7.29 \pm 0.87\%$. There was a difference ($p > 0.05$) in staining for COL I at T0 as compared with

the other times in animals from the treated group and control. At 14 days after surgical lesion, the immunostained area in control wounds ($1.16 \pm 0.36\%$) showed lower values ($p > 0.05$) than the treated group ($1.38 \pm 0.44\%$). Nevertheless, at 37 days the treated group had lower values ($0.43 \pm 0.15\%$) ($p > 0.05$) than control ($0.59 \pm 0.15\%$) (Figure 5(b)). Overall evaluation showed that COL III displayed more intense staining than COL I at all evaluated times.

The extracellular matrix is a complex network of cross-links of proteins and other macromolecules; among them, collagen is the main structural component, essential for the resistance and integrity of all tissues and with a vital role in the healing of wounds [5]. The synthesis of collagen is considered to start from at least the third day after a cutaneous injury. It is known that wounds acquire approximately 20% of their mechanical tensile strength by the end of three weeks from the healing process, which is the period when fibrillar collagens accumulate relatively rapidly. After this period the improvement in resistance slows down, mainly due to

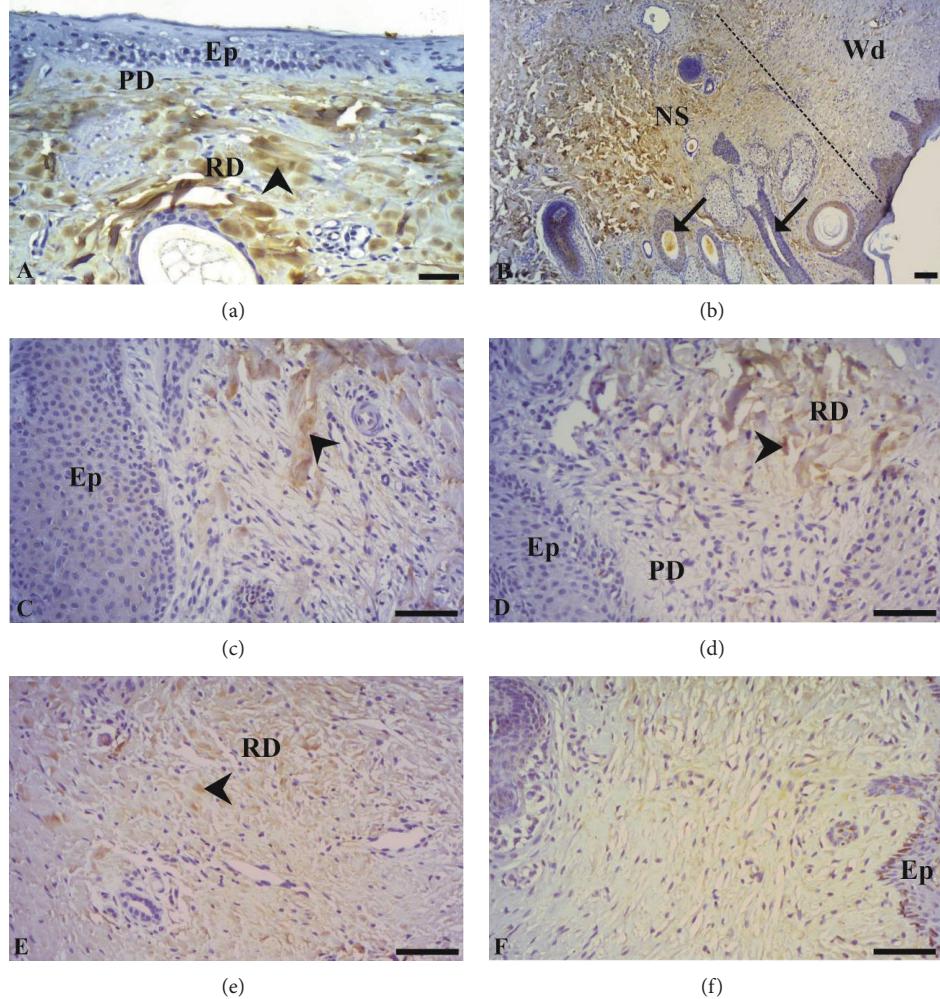


FIGURE 4: Immunohistochemistry for type I collagen in the skin of horses treated or untreated with LP-PRP. Immunostaining was visible in the reticular dermis at all times and in all groups: normal skin (a), border tissue and scar area (b). Image obtained at 14 and 37 days of skin healing process from treated wounds (c and e, resp.) and controls (d and f, resp.). Stratified squamous epithelial tissue (Ep), papillary dermis (PD), reticular dermis (RD), immunostaining (arrowhead), hair follicle (arrows), scar edge skin (NS), and scar area (Wd). Bars = 50 μ m.

the onset of the remodeling of collagens, which start to form large fiber bundles, and also due to the increase in the number of intermolecular crosslink [44], which begins during the remodeling phase.

Lefebvre-Lavoie et al. [45] utilized suppression subtractive hybridization and semiquantitative RT-PCR to identify the gene expression of type I collagen in both healthy skin and at the margin of induced skin wounds in the hemithorax of horses. The evaluation was performed one week after the wound was created, and there was a significant increase in the expression of type I collagen in the samples obtained from the wounds as compared with intact skin in both techniques. Thus, in both physiological conditions and in LP-PRP-treated wounds there is an increase in the expression of type I collagen after one week already, which, according to Schultz et al. [46], corresponds to 80 to 85% of the dermal extracellular matrix.

The results obtained in this study are consistent with those observed by Chamberlain et al. [47], who demonstrated

the spatial and temporal location of type I and III collagen immunohistochemically during the healing of wounds induced surgically in horses, healed by second intention. According to the authors, the technique was suitable for the location of the collagens during the assessment period, which was 28 days. The type I collagen remained stable at the beginning of the healing process but reduced dramatically on the 7th day, remaining low until the 28th day. Type III collagen however remained intact in the first hours after the injury, though there was an important increase in its amount 24 h after the surgical procedure, remaining high throughout the entire evaluation period. This collagen accounts for 8 to 11% of the dermal extracellular matrix [46] and should be produced at a larger amount during the final stage of the formation of the extracellular matrix. Subsequently it should be gradually degraded and substituted for type I collagen, which is essential to the increase in the strength of the healing tissue [9, 48], which is reinforced by the collagen crosslink [9].

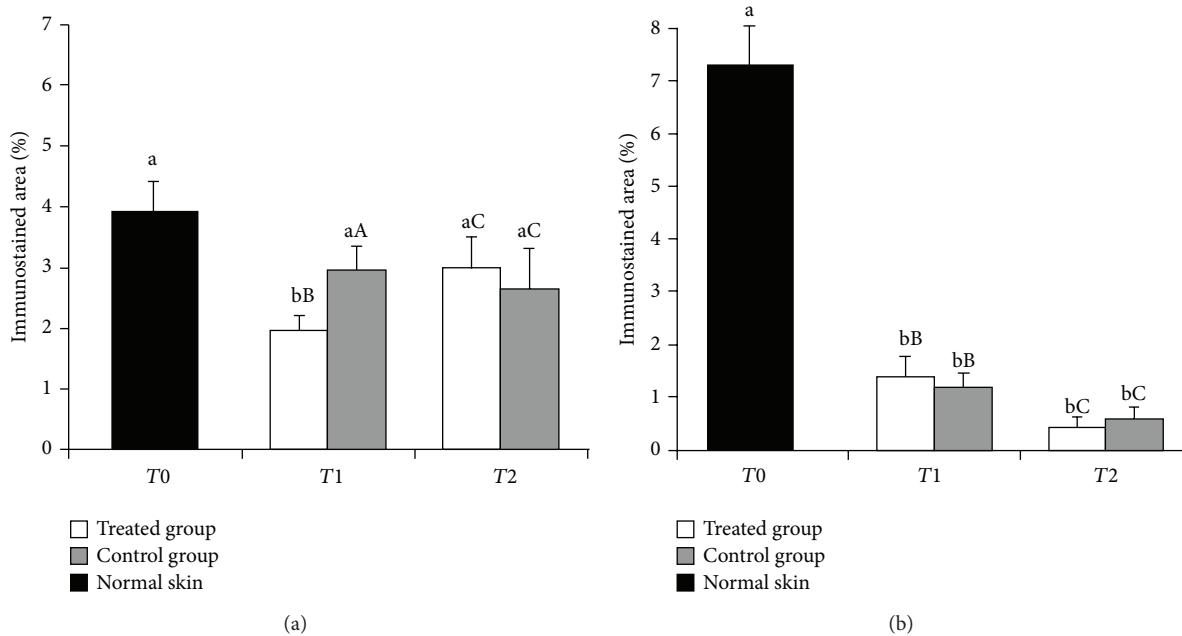


FIGURE 5: Percentage of immunostained area for collagen types III (a) and I (b) in normal skin, as well as in the treated and control groups as a function of time: on the day of wound making (T0), at 14 days (T1) of the healing process, and after complete wound closure (T2). Means followed by different lowercase and uppercase letters denote by *t*-test, respectively, differences in each group evaluated at T1 and T2 relative to T0, and between groups within each time (T1 and T2).

The intense staining of the papillary and reticular dermis for collagen types I and III, respectively, was expected. The papillary dermis is the loose connective tissue that lies just beneath the epithelium. It typically consists of type III collagen, which forms part of the basal membrane. In contrast, the reticular dermis, which is deeper, is formed by dense connective tissue and is therefore rich in type I collagen, as it provides resistance to the skin [49]. According to Schwartz et al. [7], the increase in type I collagen is progressive, continuing for at least four weeks, which was the maximum period of time in which the authors evaluated this collagen utilizing the Picosirius Red histochemical technique in wounds of horses located in the thorax and distal region of forelimbs, healed by second intention. In fact, Mignatti et al. [50] mentioned that the elevated collagen synthesis rate within the wound may take from six to 12 months to return to physiological levels, which means that regardless of whether or not the cutaneous injury is treated with PRP, the amount of type I collagen may still be elevated at the moment of wound closure.

The high presence of type III collagen in both groups suggests that the wound was still in the phase characterized by Schultz et al. [30] as contraction and remodeling, although they appeared to be healed macroscopically. This phase is characterized by the reduction in the number of fibroblasts and the balance between production and lysis of collagen. The collagen production must remain high, but with predominance of the functionally oriented collagen fibers over the nonfunctional, and without an increase in the size of the scar [51]. Contrastingly, according to Schultz et al. [46], 40 days after the injury the collagen fibers are not yet organized

enough. In this context, the macroscopic examination does not necessarily correspond to the microscopy or the expression of genes of collagen, because the nature of the wound matrix components is modified over time. As a matter of fact, the organization of the collagen in the tissue under repair is changed over the period of several months, and this will slowly increase the tissue tensile strength, which will reach approximately 80% of that corresponding to a normal tissue [5, 29, 46].

Yamauchi and Mechanic [52] define this stage of the healing process after clinical closure as intermediary. According to those authors, although the wound appears to be healed, chemical and structural changes are still in progress. In this stage, the collagen fibrils are grouped and stabilized by the formation of inter- and intramolecular crosslinks. Therefore, this elevated expression of the collagen gene even in this intermediary stage may be because the collagen synthesis still exceeds its extracellular degradation, such that it keeps increasing during scar formation [48]. More specifically, Young and McNaught [29] cite up to two years for the maturation of the scar tissue so that a wound can acquire an epithelium as close as possible to a healthy tissue. In the results presented herein it can be observed that there is a trend towards stabilization in the expression of collagens in the LP-PRP-treated group, which is appropriate. To validate this finding, further research will be necessary to evaluate the gene expression of the collagen at moments (times) subsequent to the clinical healing. Additionally, as mentioned previously, the presence of myofibroblasts at the last evaluation time (T2) used in this study may indicate, as commented by Schwartz et al. [7], that wound healing is still in progress.

Comparing the results obtained in the present study with others conducted with horse skin is practically impossible, because no studies evaluating the expression of the collagens in PRP-treated skin utilizing the immunohistochemical technique have been published. Comparison with studies utilizing other methodologies to evaluate the behavior of the collagens during the healing process is also difficult, because controlled and *in vivo* studies are scarce, and in general they aim to evaluate very precisely a specific time in the different stages of the skin healing process. In addition, most of the experiments have been conducted with cultures of tendon or ligament cells.

To the present date, no research reported results for the immunohistochemical evaluation of collagen types I and III equine cutaneous wounds treated with PRP. Recently, part of the authors who make up the team of the present research study published data on collagen gene expression during different phases of the healing process of PRP-treated skin. However, the technique used for the determination of collagen was qRT-PCR [15]. As observed in the present study, the expression of type III collagen was quite high in the apparently healed wound, whether they were treated or not. Furthermore, regression analysis showed a trend of stabilization in the expression of this collagen in the PRP-treated group, which according to the authors may be a positive aspect of the therapy. Monteiro et al. [13] also evaluated the gene expression of collagen types I and III by PCR in horse's wounds treated with PRP, but in the forelimbs. However this evaluation occurred only after complete healing of the wounds. The authors compared the mean ratio of collagen type I to collagen type III mRNA expression, though they found no difference ($p > 0.05$) between treated and untreated groups in the mean values, which were 0.502 ± 0.155 and 0.542 ± 0.118 , respectively.

Although the obtained results cannot be compared with other studies, the presented data are original and valuable for veterinary medicine. Evaluating alterations in the extracellular matrix components is an appropriate form of monitoring tissue repair. Because collagen is the most abundant component of the connective tissue, it is essential during the restoration of the skin function and reflects the quality of the matrix. The experimental trial conducted in distinct moments made it possible to monitor the dynamics of the healing process. The adopted technique, immunohistochemistry, is sensitive, reliable and easily performed, so it is suitable for research on gene expression in different biological components. Moreover, even though few studies have valuable data on density, quantification and/or gene expression of the collagens in the skin of horses, the profile of this expression in the PRP-treated wound should still be better characterized. Obtaining the skin samples for evaluation of extracellular matrix components from different periods after macroscopic healing of the wound may provide new information and verify the efficacy of or inefficacy of the therapy. This corroborates Baksh et al. [53], who mentioned that the benefits of the use of PRP in the "clinical setting remain unclear." According to Brossi et al. [54], despite the fact that the treatment of different tissues has demonstrated "beneficial effects" of platelet-rich component, "clinical evidence of its efficacy remains lacking,"

although several researchers are conducting quality studies to determine differences between treated and untreated tissues. Furthermore, Romagnoli et al. [55] observed "mild negative correlation between platelet concentration and time to return to sport activity" in spontaneous suspensory ligament injury in their study considering concentrations of platelets in the PRP. Finally, to date, the use of "the therapy in ankle surgery as an orthobiologic does not have an absolute indication" as mentioned by Grambart [56].

Because the main reason for the use of the platelet-rich component is growth factors present in platelets, it is possible that individual variations influence the effectiveness of the therapy. The body region of the horse, the method of obtaining and the composition of PRP (considering the amount of platelets and leukocytes), the presentation form (liquid, gel), route and frequency of application, and the volume and time in which it is administered during the skin healing process are already mentioned as aspects related to the lack of sufficient evidence to confirm the effectiveness of PRP in the treatment of skin wounds. Using the ELISA immunoenzymatic technique, Giraldo et al. [57] have recently reported a significant difference ($p < 0.001$) in the PDGF-BB concentration in PRP gel (P-PRG; activated with 10% calcium gluconate) from Colombian Creole females and young horses (up to 5 years of age) compared with Argentine Creole males and horses over 10.1 years old. Textor et al. [58], however, did not observe differences in the amount of growth factors TGF- β 1 and PDGF-BB in PRP of males and females. On the other hand, those authors only commented the data obtained for sex evaluation, in precisely four lines at the end of result section, without any other mention in the discussion section.

According to Minimas [59], one of the main features of senile skin is decreased synthesis of collagen. It is possible that the age of the animals used in this study, which was between 16 and 17 years, led to a delay in skin remodeling and thus in the substitution of type III for type I collagen. On the other hand, the similarity in the age of the horses included in the research is a positive aspect, given that several previously published studies used animals of different ages in a way that contributes nothing to the elucidation of the effectiveness or ineffectiveness of PRP. Nevertheless, the contradictory results between studies reinforce the need for further research *in vivo* using homogeneous samples, even considering the age and sex of animals. However, one should be careful in extrapolating and comparing the data obtained *in vitro* with those *in vivo*.

4. Conclusions

A single local administration of LP-PRP 12 h after surgical induction of cutaneous injury in the gluteal region of horses results in an important immunohistochemical expression of collagens, particularly COL III, which remains high even during the macroscopic closure of wounds. This greater expression does not result in faster closure of the surgical wound, although microscopically the tissue treated with LP-PRP shows better tissue quality. Additionally, this high COL

III staining in both the treated wound and the control wound suggests that the tissue was still weakened, despite being clinically closed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] C. L. Theoret and J. M. Wilmink, "Aberrant wound healing in the horse: naturally occurring conditions reminiscent of those observed in man," *Wound Repair and Regeneration*, vol. 21, no. 3, pp. 365–371, 2013.
- [2] C. Soo, D. N. Sayah, X. Zhang et al., "The identification of novel wound-healing genes through differential display," *Plastic and Reconstructive Surgery*, vol. 110, no. 3, pp. 787–797, 2002.
- [3] Z. Ruszczak, "Effect of collagen matrices on dermal wound healing," *Advanced Drug Delivery Reviews*, vol. 55, no. 12, pp. 1595–1611, 2003.
- [4] C. L. Theoret, O. O. Olutoye, L. K. S. Parnell, and J. Hicks, "Equine exuberant granulation tissue and human keloids: a comparative histopathologic study," *Veterinary Surgery*, vol. 42, no. 7, pp. 783–789, 2013.
- [5] S. Enoch and D. J. Leaper, "Basic science of wound healing," *Surgery*, vol. 23, no. 2, pp. 37–42, 2005.
- [6] G. L. Barnes, P. J. Kostenuik, L. C. Gerstenfeld, and T. A. Einhorn, "Growth factor regulation of fracture repair," *Journal of Bone and Mineral Research*, vol. 14, no. 11, pp. 1805–1815, 1999.
- [7] A. J. Schwartz, D. A. Wilson, K. G. Keegan et al., "Factors regulating collagen synthesis and degradation during second-intention healing of wounds in the thoracic region and the distal aspect of the forelimb of horses," *The American Journal of Veterinary Research*, vol. 63, no. 11, pp. 1564–1570, 2002.
- [8] D. Marsolais and J. Frenette, "Inflammation and tendon healing," *Medecine/Sciences*, vol. 21, no. 2, pp. 181–186, 2005.
- [9] M. P. Czubryt, "Common threads in cardiac fibrosis, infarct scar formation, and wound healing," *Fibrogenesis & Tissue Repair*, vol. 5, no. 1, article 19, pp. 1–11, 2012.
- [10] L. Yang, T. M. Witten, and R. M. Pidaparti, "A biomechanical model of wound contraction and scar formation," *Journal of Theoretical Biology*, vol. 332, pp. 228–248, 2013.
- [11] C. A. Carter, D. G. Jolly, C. E. Worden Sr., D. G. Hendren, and C. J. M. Kane, "Platelet-rich plasma gel promotes differentiation and regeneration during equine wound healing," *Experimental and Molecular Pathology*, vol. 74, no. 3, pp. 244–255, 2003.
- [12] R. DeRossi, A. C. A. D. O. Coelho, G. S. de Mello et al., "Effects of platelet-rich plasma gel on skin healing in surgical wound in horses," *Acta Cirúrgica Brasileira*, vol. 24, no. 4, pp. 276–281, 2009.
- [13] S. O. Monteiro, O. M. Lepage, and C. L. Theoret, "Effects of platelet-rich plasma on the repair of wounds on the distal aspect of the forelimb in horses," *The American Journal of Veterinary Research*, vol. 70, no. 2, pp. 277–282, 2009.
- [14] F. B. Maciel, R. Derossi, T. J. C. Módolo, R. C. Pagliosa, C. R. J. Leal, and A. A. S. T. Delben, "Scanning electron microscopy and microbiological evaluation of equine burn wound repair after platelet-rich plasma gel treatment," *Burns*, vol. 38, no. 7, pp. 1058–1065, 2012.
- [15] M. V. Souza, J. O. Pinto, M. B. Costa et al., "Expressão gênica do colágeno em ferida cutânea de equinos tratada com plasma rico em plaquetas," *Pesquisa Veterinária Brasileira*, vol. 34, no. 3, pp. 233–240, 2014.
- [16] M. V. Souza, J. d. Pinto, M. M. Costa, E. C. Santos, S. L. Garcia, and L. L. Oliveira, "Quantificação de fatores de crescimento na pele de equinos tratada com plasma rico em plaquetas," *Pesquisa Veterinária Brasileira*, vol. 34, no. 6, pp. 599–612, 2014.
- [17] M. C. Furness, D. Bienzle, J. L. Caswell, J. DeLay, and L. Viel, "Immunohistochemical identification of collagen in the equine lung," *Veterinary Pathology*, vol. 47, no. 5, pp. 982–990, 2010.
- [18] E. Anitua, M. Sánchez, A. T. Nurden, P. Nurden, G. Orive, and I. Andía, "New insights into and novel applications for platelet-rich fibrin therapies," *Trends in Biotechnology*, vol. 24, no. 5, pp. 227–234, 2006.
- [19] D. M. D. Ehrenfest, L. Rasmusson, and T. Albrektsson, "Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF)," *Trends in Biotechnology*, vol. 27, no. 3, pp. 158–167, 2009.
- [20] J. U. Carmona, C. López, and J. A. Sandoval, "Review of the currently available systems to obtain platelet related products to treat equine musculoskeletal injuries," *Recent Patents on Regenerative Medicine*, vol. 3, no. 2, pp. 148–159, 2013.
- [21] D. M. D. Ehrenfest, T. Bielecki, A. Mishra et al., "In search of a consensus terminology in the field of platelet concentrates for surgical use: platelet-rich plasma (PRP), platelet-rich fibrin (PRF), fibrin gel polymerization and leukocytes," *Current Pharmaceutical Biotechnology*, vol. 13, no. 7, pp. 1131–1137, 2012.
- [22] G. G. A. Hutchins and H. I. Grabsch, "Molecular pathology—the future?" *The Surgeon*, vol. 7, no. 6, pp. 366–377, 2009.
- [23] J. L. Dragoo, H. J. Braun, J. L. Durham et al., "Comparison of the acute inflammatory response of two commercial platelet-rich plasma systems in healthy rabbit tendons," *The American Journal of Sports Medicine*, vol. 40, no. 6, pp. 1274–1281, 2012.
- [24] J. C. Ferreira, M. V. Souza, M. I. V. Vilória, E. F. Fonseca, M. W. S. Vianna, and J. C. M. Costa, "Efeitos da monofenilbutazona na cicatrização por segunda intenção em equinos," *Ceres*, vol. 54, no. 313, pp. 263–270, 2007.
- [25] D. Argüelles, J. U. Carmona, J. Pastor et al., "Evaluation of single and double centrifugation tube methods for concentrating equine platelets," *Research in Veterinary Science*, vol. 81, no. 2, pp. 237–245, 2006.
- [26] H. M. Rees and E. E. Ecker, "An improved method for counting blood platelets," *The Journal of the American Medical Association*, vol. 80, no. 9, pp. 621–622, 1923.
- [27] M. A. Thrall, "Diagnóstico dos distúrbios hemorrágicos," in *Hematologia e Bioquímica Clínica Veterinária*, M. A. Thrall, Ed., pp. 170–187, Roca, São Paulo, Brazil, 2007.
- [28] J. E. Greenwood, "Function of the panniculus carnosus—a hypothesis," *The Veterinary Record*, vol. 167, no. 19, p. 760, 2010.
- [29] A. Young and C.-E. McNaught, "The physiology of wound healing," *Surgery*, vol. 29, no. 10, pp. 475–479, 2011.

- [30] G. S. Schultz, J. M. Davidson, R. S. Kirsner, P. Bornstein, and I. M. Herman, "Dynamic reciprocity in the wound microenvironment," *Wound Repair and Regeneration*, vol. 19, no. 2, pp. 134–148, 2011.
- [31] E. Anitua, I. Andia, B. Ardanza, P. Nurden, and A. T. Nurden, "Autologous platelets as a source of proteins for healing and tissue regeneration," *Thrombosis and Haemostasis*, vol. 91, no. 1, pp. 4–15, 2004.
- [32] L. Maia, M. V. de Souza, J. I. Ribeiro Júnior et al., "Platelet-rich plasma in the treatment of induced tendinopathy in horses: histologic evaluation," *Journal of Equine Veterinary Science*, vol. 29, no. 8, pp. 618–626, 2009.
- [33] C. P. Vendruscolo, A. D. M. Carvalho, L. F. Moraes et al., "Avaliação da eficácia de diferentes protocolos de preparo do plasma rico em plaquetas para uso em medicina equina," *Pesquisa Veterinária Brasileira*, vol. 32, no. 2, pp. 106–110, 2012.
- [34] B. M. Zandim, M. V. de Souza, L. N. Frassy et al., "Immunohistochemistry of factor VIII, histology and morphometry in equine tendon treated with platelet-rich plasma," *Revista Brasileira de Medicina Veterinaria*, vol. 35, no. 2, pp. 169–184, 2013.
- [35] F. Graziani, S. Ivanovski, S. Cei, F. Ducci, M. Tonetti, and M. Gabriele, "The *in vitro* effect of different PRP concentrations on osteoblasts and fibroblasts," *Clinical Oral Implants Research*, vol. 17, no. 2, pp. 212–219, 2006.
- [36] S. G. Boswell, L. V. Schnabel, H. O. Mohammed, E. A. Sundman, T. Minas, and L. A. Fortier, "Increasing platelet concentrations in leukocyte-reduced platelet-rich plasma decrease collagen gene synthesis in tendons," *The American Journal of Sports Medicine*, vol. 42, no. 1, pp. 42–49, 2014.
- [37] L. C. Jacobsen, O. E. Sørensen, J. B. Cowland, N. Borregaard, and K. Theilgaard-Mönch, "The secretory leukocyte protease inhibitor (SLPI) and the secondary granule protein lactoferrin are synthesized in myelocytes, colocalize in subcellular fractions of neutrophils, and are coreleased by activated neutrophils," *Journal of Leukocyte Biology*, vol. 83, no. 5, pp. 1155–1164, 2008.
- [38] K. Deschene, C. Céleste, D. Boerboom, and C. L. Theoret, "Constitutive expression of hypoxia-inducible factor-1 α in keratinocytes during the repair of skin wounds in horses," *Wound Repair and Regeneration*, vol. 19, no. 2, pp. 250–259, 2011.
- [39] O. Mei-Dan, G. Lippi, M. Sánchez, I. Andia, and N. Maffulli, "Autologous platelet-rich plasma: a revolution in soft tissue sports injury management?" *The Physician and Sportsmedicine*, vol. 38, no. 4, pp. 127–135, 2010.
- [40] R. K. Assoian, A. Komoriya, C. A. Meyers, D. M. Miller, and M. B. Sporn, "Transforming growth factor- β in human platelets. Identification of a major storage site, purification, and characterization," *The Journal of Biological Chemistry*, vol. 258, no. 11, pp. 7155–7160, 1983.
- [41] R. E. Marx, "Platelet-rich plasma: evidence to support its use," *Journal of Oral and Maxillofacial Surgery*, vol. 62, no. 4, pp. 489–496, 2004.
- [42] R. A. F. Clark, "Biology of dermal wound repair," *Dermatologic Clinics*, vol. 11, no. 4, pp. 647–666, 1993.
- [43] J. M. Wilmink, P. W. T. Stolk, P. R. van Weeren, and A. Barnneveld, "Differences in second-intention wound healing between horses and ponies: macroscopic aspects," *Equine Veterinary Journal*, vol. 31, no. 1, pp. 53–60, 1999.
- [44] A. J. Bailey, S. Bazin, T. J. Sims, M. le Lous, C. Nicoletis, and A. Delaunay, "Characterization of the collagen of human hypertrophic and normal scars," *Biochimica et Biophysica Acta*, vol. 405, no. 2, pp. 412–421, 1975.
- [45] J. Lefebvre-Lavoie, J. G. Lussier, and C. L. Theoret, "Profiling of differentially expressed genes in wound margin biopsies of horses using suppression subtractive hybridization," *Physiological Genomics*, vol. 22, no. 2, pp. 157–170, 2005.
- [46] G. S. Schultz, G. Ladwig, and A. Wysocki, Extracellular matrix: review of its roles in acute and chronic wounds, *World Wide Wounds*, 2013, <http://www.worldwidewounds.com/2005/august/Schultz/Extracellular-Matrix-Acute-Chronic-Wounds.html>.
- [47] C. S. Chamberlain, E. M. Crowley, H. Kobayashi, K. W. Eliceiri, and R. Vanderby, "Quantification of collagen organization and extracellular matrix factors within the healing ligament," *Microscopy and Microanalysis*, vol. 17, no. 5, pp. 779–787, 2011.
- [48] S. E. Mutsaers, J. E. Bishop, G. McGrouther, and G. J. Laurent, "Mechanisms of tissue repair: from wound healing to fibrosis," *The International Journal of Biochemistry and Cell Biology*, vol. 29, no. 1, pp. 5–17, 1997.
- [49] M. H. Ross and W. Pawlina, *Histology: A Text and Atlas, with Correlated Cell and Molecular Biology*, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 6th edition, 2011.
- [50] P. Mignatti, D. R. Rifkin, H. G. Welgus, and W. C. Parks, "Proteinases and tissue remodeling," in *The Molecular Biology of Wound Repair*, R. A. F. Clark, Ed., pp. 427–474, Plenum Press, New York, NY, USA, 2nd edition, 1996.
- [51] C. L. Theoret, "Wound healing," in *Equine Wound Management*, T. D. Stashak and C. L. Theoret, Eds., pp. 5–27, Blackwell Publishing, Ames, Iowa, USA, 2nd edition, 2008.
- [52] M. Yamauchi and G. L. Mechanic, "Cross-linking of collagen," in *Collagen*, M. E. Nimni, Ed., vol. 1, pp. 35–92, CRC Press, Boca Raton, Fla, USA, 1988.
- [53] N. Baksh, C. P. Hannon, C. D. Murawski, N. A. Smyth, and J. G. Kennedy, "Platelet-rich plasma in tendon models: a systematic review of basic science literature," *Arthroscopy*, vol. 29, no. 3, pp. 596–607, 2013.
- [54] P. M. Brossi, J. J. Moreira, T. S. Machado, and R. Y. Baccarin, "Platelet-rich plasma in orthopedic therapy: a comparative systematic review of clinical and experimental data in equine and human musculoskeletal lesions," *BMC Veterinary Research*, vol. 11, no. 1, pp. 98–148, 2015.
- [55] N. Romagnoli, R. Rinnovati, G. Ricciardi, C. Lambertini, G. Spinella, and A. Spadari, "Clinical evaluation of intralesional injection of platelet-rich plasma for the treatment of proximal suspensory ligament desmitis in horses," *Journal of Equine Veterinary Science*, vol. 35, no. 2, pp. 141–146, 2015.
- [56] S. T. Grambart, "Sports medicine and platelet-rich plasma: nonsurgical therapy," *Clinics in Podiatric Medicine and Surgery*, vol. 32, no. 1, pp. 99–107, 2015.
- [57] C. E. Giraldo, C. López, M. E. Álvarez, I. J. Samudio, M. Prades, and J. U. Carmona, "Effects of the breed, sex and age on cellular content and growth factor release from equine pure-platelet rich plasma and pure-platelet rich gel," *BMC Veterinary Research*, vol. 9, article 29, 10 pages, 2013.
- [58] J. A. Textor, J. W. Norris, and F. Tablin, "Effects of preparation method, shear force, and exposure to collagen on release of growth factors from equine platelet-rich plasma," *American Journal of Veterinary Research*, vol. 72, no. 2, pp. 271–278, 2011.
- [59] D. A. Minimas, "Ageing and its influence on wound healing," *Wounds*, vol. 3, no. 1, pp. 42–50, 2007.

Review Article

Diverse Roles of Heparan Sulfate and Heparin in Wound Repair

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Heparan sulfate (HS) and heparin (Hp) are linear polysaccharide chains composed of repeating ($1 \rightarrow 4$) linked pyrosulfuric acid and 2-amino-2-deoxy glucopyranose (glucosamine) residue. Mentioned glycosaminoglycans chains are covalently O-linked to serine residues within the core proteins creating heparan sulfate/heparin proteoglycans (HSPG). The latter ones participate in many physiological and pathological phenomena impacting both the plethora of ligands such as cytokines, growth factors, and adhesion molecules and the variety of the ECM constituents. Moreover, HS/Hp determine the effective wound healing process. Initial growth of HS and Hp amount is pivotal during the early phase of tissue repair; however heparan sulfate and heparin also participate in further stages of tissue regeneration.

1. Introduction

Wound healing, physiological body response to injury, is a complex series of events leading to the repair of damaged tissues and reestablishment of cellular homeostasis. These dynamic biochemical pathways involve four overlapping but well-defined phases: haemostasis, inflammation, proliferation, and remodeling [1, 2]. The restoration of tissue integrity requires precise cooperation of many cells such as neutrophils, macrophages, fibroblasts, and epithelial and endothelial cells, interacting with one another and with the components of the extracellular matrix (ECM) through their integrin receptors and adhesion molecules. In addition to various cellular interactions, wound repair process is tightly regulated by different cytokines, growth factors, and proteolytic enzymes which create balanced wound molecular environment necessary for continuing effective healing [3–6]. Matrix molecules including glycosaminoglycans (GAG) play an essential role in wound repair activity through all phases of the healing process. The GAG family consists of sulfated glycosaminoglycans, that is, chondroitin/dermatan sulfate (CS/DS), heparan sulfate/heparin (HS/Hp), and keratan sulfate (KS), as well as unsulfated hyaluronic acid (HA).

The first three types of molecules are covalently connected to core protein forming proteoglycans (PGs). Hyaluronic acid does not form covalent links with proteins but instead interacts noncovalently with proteoglycans via hyaluronan-binding motifs [7, 8]. GAGs influence wound healing process by providing both a scaffold support and a signaling role. ECM components create a temporary matrix in the repairing process [6, 9]. Signal transduction role is being fulfilled by stimulation of cellular adhesion, migration, differentiation, and proliferation as well as regulation of ECM organization and metabolism. Mentioned functions are connected with GAGs and PGs ability to bind with high affinity to a range of cytokines, growth factors, and members of chemokine superfamily. These interactions additionally can serve as a reservoir of regulatory factors that can be liberated by selective degradation of GAG chains [10, 11]. PGs may also have other roles in wound healing including a direct influence on inflammation [2]. Moreover, extensive changes in ECM components in the course of repair process may be reflected in reepithelialization and regeneration of the basement membrane but also may influence intercellular communication [12]. Heparan sulfates have diverse functions with respect to skin wound healing. A large chemical diversity of HS

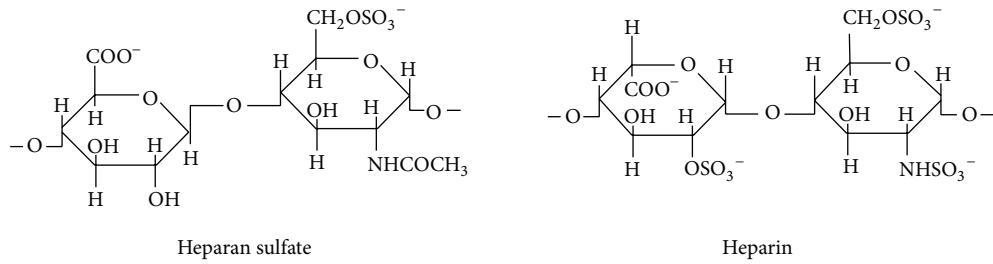


FIGURE 1: Structure of heparan sulfate/heparin disaccharides [13].

chains and capacity of these glycosaminoglycans to interact with proteins and diverse binding ligands through the varied arrangements of sulfate groups and glucuronic acid/iduronic acid residues determine their contribution to effective tissue repair. Understanding the complex mechanisms by which these ECM components influence wound repair activity promises the implementation of new therapeutic strategies.

2. Structure and Properties of Heparan Sulfate and Heparin

Heparan sulfate (HS) and heparin (Hp) are the glycosaminoglycans (GAGs) of the most complex structure among all GAGs. They are made of recurring, disaccharide units consisting of glucuronic acid and N-acetylglucosamine residues of a schematic structure [$\rightarrow 4\text{GlcA}\beta 1 \rightarrow \text{GlcNAc}\alpha 1 \rightarrow$], in which the glycosidic bond between the hexuronic acid and N-acetylglucosamine assumes the configuration of $\beta 1 \rightarrow 4$ instead of $\beta 1 \rightarrow 3$, while the bond between N-acetylglucosamine and hexuronic acid assumes the configuration $\alpha 1 \rightarrow 4$ instead of $\beta 1 \rightarrow 4$, as it takes place in chondroitin-dermatan glycosaminoglycans [11, 13–16]. HS/Hp structures are presented in Figure 1.

Despite the fact that heparin is often considered an analogue of heparan sulfate, which is caused by the fact that both GAGs are made of the same, monomeric subunits, postsynthetic modifications, the range of which is significantly different in both glycosaminoglycans, definitely differentiate these biopolymers [17]. Namely, monomeric heparin subunits are sulfated to a greater degree than the subunits of HS [13]. On average, one disaccharide unit of heparin contains 3 sulfate groups, while one disaccharide unit of heparan sulfate contains only one sulphate group [18]. The negative charge density, which is displayed by heparin, is the highest among biologic macromolecules and is responsible for the fact that this GAG is the most acidic macromolecule of human body [7, 19, 20].

Iduronic acid dominates in heparin structure constituting 90% of all acid residues, while in the heparan sulfate, glucuronic acid, being the C5 epimer of the iduronic acid [18, 21], occurs in greater amounts.

The molecular mass of the heparin molecule on average equals 15 kDa, while in the case of heparin sulfate about 30 kDa. Moreover, the chains of the latter one are longer than in the case of heparin [14].

The molecules of heparin sulfate are characterized by a greater heterogeneity of the structure compared with the structure of heparin. HS contains bigger amounts of the acetylated glucosamine residues than N-sulfated GlcN, greater content of GlcA than IdoA, but fewer O-bound sulfate groups. Moreover, HS displays the domain structure comprising highly sulfated, heparin-like sequences, poorly sulfated sequences, and unmodified regions: [GlcA-GlcNac] [14, 18, 19, 22, 23].

3. Heparan Sulfate and Heparin Biosynthesis, Postsynthetic Modification, and Degradation

The two kinds of GAGs also differ with regard to the tissue location, core proteins, to which, in the process of biosynthesis, glycans are linked, and the number of glycan chains connected with the protein [13]. Heparin is synthesized in the mast cells and basophils, in the form of side, glycosaminoglycan chains of the proteoglycan, serglycin [18, 24]. This proteoglycan, which contains numerous glycan chains of uneven length, is secreted from the mentioned cells in the process of their degranulation, after which the enzymatic degradation takes place with a subsequent release of heparin [18]. Heparan sulfate is also synthesized as a proteoglycan component, which is a constituent of many PGs occurring on the cell membrane or located in the extracellular matrix [18, 25]. Syndecans and glypcans are the two main families of HSPG which are located on the cell surface [26–28]. Moreover, perlecan, agrin, and collagen type XVIII also belong to this HSPG family and, furthermore, the isoform CD44, betaglycan, and testican [17, 29] which constitute not more than over 5% of all heparan sulfate PGs. On average, HSPG consists of only a few HS chains [17, 18].

The initial biosynthesis stages of heparan sulfate proteoglycans are not different from the initial biosynthesis stages of CS/DSPG. The linking tetrasaccharide region, connected with the seryl residue of the core protein, initiates the elongation of the HS/Hp chain [14]. In this process, the monosaccharide subunits, the N-acetylglucosamine and glucuronic acid, are alternately linked to the nonreducible end of the growing glycan chain by the glycosyltransferases, the so-called exostosin I (EXT1), and exostosin II (EXT2) [30]. During the polymerization, the glycan

chain is subject to many modifications [14]. These modifications concern N-deacetylation and N-sulfation of glucosamine, epimerization of GlcA to IdoA, 2-O-sulfating of hexuronic acid (usually IdoA), 6-O-sulfating, and 3-O-sulfating of glucosamine. They start from the removal of N-acetyl groups, after which they are replaced by the sulfate groups. The latter process is catalyzed by the enzymatic complex of N-deacetylase/N-sulfotransferase (NDST) [29]. N-deacetylation and N-sulfation are the initial condition of all next, enzymatic modifications [30]. Next to them is the transformation of glucuronic acid into iduronic acid, catalysed by C-5 epimerase, which is specific for both HS and Hp [14].

Similarly, the activity of 2-O-sulfotransferase (HS2ST) displays the specificity towards HS and Hp. This enzyme catalyses the sulfating reaction of hexuronic acid. It is thought that both last enzymes create a complex or enter the interaction in order to reverse epimerization, already sulfated IdoA back into GlcA [30]. Another enzyme in the modification process of the increasing HS/Hp chain 6-O-sulfotransferase heparin sulfate (HS6ST) transfers 6-O-sulfate group onto N-sulfated glucosamine, while heparan sulfate 3-O-sulfotransferase transfers 3-O-sulfate group also onto the residue of the mentioned hexosamine.

The modifications of heparan sulfate proteoglycans also comprise, besides the ones connected with the biosynthesis of the glycan chain, the ones concerning the transformation of the HSPG core protein [30].

HSPG comprise the syndecan family, transmembrane PGs created from 4 members- syndecan-1, -2, -3, -4, sometimes “enriched” in CS chains; glycan family PGs bound with the cell membrane by glycosylphosphatidylinositol having six members (glycan-1,-2,-3,-4,-5,-6); betaglycan PG of the cell surface, also named the type III receptor for TGF- β ; testican (chondroitin-heparan sulfate PG of extracellular matrix); perlecan; and agrin (PGs of basement membranes) [23, 25, 29, 31–34]. Perlecan may also occur outside the basement membranes, that is, in the extracellular matrix of tissues deprived of these structures, as in the case of the matrix of cartilage, sinusoid vessels of liver, spleen, or in the lymph nodes [35].

It is worth mentioning that proteins may also undergo a modification by binding HS chains which, however, usually does not occur in this kind of proteins. Such examples are isoform CD44 or collagen type XVIII [30].

Another postsynthetic modification of HSPG is the release of GAG chains from their proteoglycans (the so-called “shedding”), which leads to the transformation of insoluble glycans, connected with this protein, into free, soluble form of these compounds. These soluble GAGs may undergo further, enzymatic transformations, which are connected with the change of the length of glycan chain or with “revealing” specific domains (masked up to this moment). A special role in the mentioned enzymatic transformations is played by heparanase (endoglucuronidase), degrading the HS chains [30, 36, 37].

The degradation of heparan sulfate proteoglycans initially takes place in the extracellular space, and then is continued in lysosomes. Heparanase (endo-beta-glucuronidase) is

endoglycosidase, specific for heparan sulfates, degrades the abovementioned glycans to smaller fragments (in the place of glucuronide bonds) [30, 38].

4. Heparan Sulfate and Heparin Physiological Functions

Biological functions of heparan sulfate glycosaminoglycans have been well recognized. Heparan sulfate plays a significant role in regulating the interactions between cells and between cells and extracellular matrix. HS stimulates the adhesion of cells to ECM by binding themselves to matrix macromolecules such as fibronectin or laminin [18, 39, 40]. HS and Hp function as endogenous receptors for numerous extracellular ligands, growth factors, and chemokines which all regulate the process of cell proliferation, differentiation, and angiogenesis, processes of leukocytic migration and degranulation, and processes of carcinogenesis [14, 26, 27, 35, 41–45]. The role of HSPG in metastases depends on the type of the tissue, pathophysiologic condition of the cancer and on the HSPG tissue location. Generally, although not always, the HSPG of the cell surface prevent metastases, while the core proteins and HS chains themselves modulate the mentioned process [37]. Heparin, synthesized in basophils and mast cells of lungs, intestines, and liver, plays a role in the body immune defenses. It demonstrates antioxidant, anti-inflammatory and vasodilating properties [19]. Together with heparan sulfate, it demonstrates anticoagulant activity; however, *in vivo* the function of Hp in the regulation of the coagulation process seems to be very unlikely [24, 46, 47]. The HSPG of cell surface regulate the metabolism of lipoproteins [33]. The participation of HSPG in the processes of neurogenesis and repair of tissue damage is known [11, 46–50].

5. The Role of Heparan Sulfate and Heparin in the Process Wound Healing

Initial growth of HS/Hp amount is pivotal during early stages of tissue repair. It is known that HS/Hp play a key role in chemical signaling between cells through binding and regulating the activities of heparin-binding growth factors, proteolytic enzymes, and protease inhibitors [51, 52].

Heparin impacts hemostatic phase of wound healing by binding of various molecules. Mentioned glycan interacts with antithrombin participating in serpin’s coagulation cascade, proteinase nexin-1 functioning as an inhibitor of trypsin-like serine proteases, protein C inhibitor, which plays a procoagulant role, and factors (IIA, IXa, and Xa) taking part in coagulation cascade of serine proteases [53]. Moreover, heparin acts as a potent anti-inflammatory agent that inhibits enzymes and cytotoxic mediators, released from proinflammatory cells, responsible for augmentation of inflammation, such as elastase, cathepsin G, eosinophil peroxidase, eosinophil cationic protein, major basic protein, interleukin-8, and stromal-derived factor-1 [53, 54]. On the other hand, heparan sulphate enhances the recruitment of inflammatory cells, since endothelial surface HS decreases

neutrophil rolling rapidity via L-selectin mediated cell adhesion. Moreover, HS-mediated Mac-1-CD44v3 interaction stimulates binding of leukocytes to the endothelial surface to drive the cells extravasation [29]. Last but not least, HS can be recognized as a sensor of tissue injury, thanks to the interaction with TLR-4 on leukocytes. This action regulates the release of proinflammatory cytokines by macrophages and significantly enhances the maturation of dendritic cells. Mentioned phenomenon is confirmed by the upregulation of MHC-II, CD40, ICAM-1, CD80, CD86, and reduced antigen uptake [29]. HS/Hp are recognized as pivotal players in angiogenesis, cell growth, migration, and differentiation [51, 55, 56].

HS/Hp, abundant in acute wound fluid 24–72 h after injury, bind heparin binding growth factor (Hb-EGF), which can act as a mitogenic agent for fibroblasts, smooth muscle cells, and epithelial cells [57]. Moreover, after skin damage, heparan sulfate proteoglycan, syndecan-4, is upregulated within the granulation tissue on fibroblasts and endothelial cells, which may suggest that syndecan-4 regulates wound healing and related angiogenesis [58].

HS/Hp interact with hepatocyte growth factor, which regulates cell growth, motility, and morphogenesis of epithelial or endothelial cells and stimulates epithelial repair and neovascularization [46, 53, 59]. HS/Hp also influence fibroblast growth factor responsible for cell proliferation, differentiation, signal transduction, and angiogenesis [46, 53].

The presence of heparin at high concentrations reduces the activity of FGF-7, [60] which is responsible for enhancement of keratinocytes migration and proliferation and plays a key role in reepithelialization process [61]. The mentioned heparin “conditions” do not inhibit the action of another important factor [60], that is, FGF-1, which regulates the proliferation of fibroblasts, endothelial, and epithelial cells and influences angiogenesis via effect on the activity of endothelial cells [62]. Special attention should be paid to the fact that heparin can enhance the stability of FGF1 and may determine the formation of FGF1-FGFR (fibroblast growth factor receptor) active complex [63]. The heparin’s small fraction presents high affinity to FGF-7, particularly supporting the FGF7/FGFRIIb signaling [60]. Furthermore, HS, which builds the heparan sulfate proteoglycan, that is, syndecan-1, binds FGF-7 and its receptor, promotes the FGF-7 signaling and influences organization of granulation tissue. However, the overexpression of syndecan-1 may modify the HS effect, from stimulatory into inhibitory one, on FGF-7 signaling [60].

Last but not least, it should be noted that heparan sulfate may be responsible for accurate regulation of wound angiogenesis through binding and modulation of various paracrine agents, such as VEGF, FGF, TGF- β , PDGF- β , SDF-1, and MCP-1, functioning in orchestrated and interactive mode [58]. VEGF-A is a master regulator of angiogenesis influencing various aspects of the mentioned phenomenon, including endothelial cells differentiation, assembly, proliferation, or migration [58]. FGF-1, -2 promotes endothelial cell proliferation and the physical organization of endothelial cells into tube-like arrangements [62]. TGF- β may participate

in vessel stabilization and quiescence, since the components of the TGF- β signaling pathway, including TGF- β receptors, interact and cocluster directly with VE-cadherin at EC-EC junctions [58]. PDGF- β signaling is crucial for mural cells recruitment, vascular maturation, and stability [58]. The chemokine stromal-cell-derived factor-1 (SDF-1) inhibits human microvascular endothelial cells apoptosis and enhances cell proliferation and capillary tube formation [64]. Monocyte chemoattractant protein-1 (MCP-1) regulates the angiogenic effect of TGF- β by recruiting vascular smooth muscle cells and mesenchymal cells toward endothelial cells [65].

Moreover, the morphology of syndecan-1-null wounds was reported to be more changeable, but the reepithelialized epidermis was organized in a lesser extent and was thinner than in the case of the control ones indicating a possible role for mentioned HSPG in the signaling mediation or in remodelling the recently laid dermis [60].

HS/Hp, which interact with TGF- β 1 and potentiate its activity, are indispensable for adhesive and contractile signaling, that results in myofibroblast formation and wound closure [51, 66, 67].

In our previous experimental studies, we proved that glycosaminoglycans, including heparan sulfate/heparin, chondroitin/dermatan sulfates, and hyaluronic acid, turned out to be better effectors of natural therapeutic agent such as propolis than silver sulfadiazine (agent of choice in local burn management) in animal burn wound healing model [51, 68]. Moreover, our studies have shown the beneficial effect of propolis on the other extracellular matrix constituents, that is, collagens, fibronectin, laminin, and vitronectin, remodeling in burnt skin. Propolis, as a factor modulating the expression of the collagens, noncollagenous proteins, and glycosaminoglycans, speeds up the healing process and contributes to scar-less healing of the burnt skin [51, 69, 70]. The strong positive effect of propolis on decreasing the amount of free radicals, the factors playing an important role in the postsynthetic modification of the ECM components, was proved in our earlier study concerning burn wound healing [71].

In conclusion, understanding biochemical changes of the ECM constituents proceeding with healing process may be of great importance in the implementation of the new alternative therapeutic strategies, in the course of thermally damaged tissues repair.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] H. Sinno and S. Prakash, “Complements and the wound healing cascade: an updated review,” *Plastic Surgery International*, vol. 2013, Article ID 146764, 7 pages, 2013.
- [2] S. Guo and L. A. DiPietro, “Critical review in oral biology & medicine: factors affecting wound healing,” *Journal of Dental Research*, vol. 89, no. 3, pp. 219–229, 2010.

- [3] K. S. Midwood and G. Orend, "The role of tenascin-C in tissue injury and tumorigenesis," *Journal of Cell Communication and Signaling*, vol. 3, no. 3-4, pp. 287–310, 2009.
- [4] F. Klingberg, B. Hinz, and E. S. White, "The myofibroblast matrix: implications for tissue repair and fibrosis," *The Journal of Pathology*, vol. 229, no. 2, pp. 298–309, 2013.
- [5] G. S. Schultz and A. Wysocki, "Interactions between extracellular matrix and growth factors in wound healing," *Wound Repair and Regeneration*, vol. 17, no. 2, pp. 153–162, 2009.
- [6] R. F. Diegelmann and M. C. Evans, "Wound healing: an overview of acute, fibrotic and delayed healing," *Frontiers in Bioscience*, vol. 9, pp. 283–289, 2004.
- [7] N. Afratis, C. Gialeli, D. Nikitovic et al., "Glycosaminoglycans: key players in cancer cell biology and treatment," *The FEBS Journal*, vol. 279, no. 7, pp. 1177–1197, 2012.
- [8] A. Im and Y. S. Kim, "Role of glycosaminoglycans in wound healing," *Archives of Pharmaceutical Sciences and Research*, vol. 1, no. 2, pp. 106–114, 2009.
- [9] M. S. Ågren and M. Werthén, "The extracellular matrix in wound healing: a closer look at therapeutics for chronic wounds," *International Journal of Lower Extremity Wounds*, vol. 6, no. 2, pp. 82–97, 2007.
- [10] K. T. Tran, L. Griffith, and A. Wells, "Extracellular matrix signaling through growth factor receptors during wound healing," *Wound Repair and Regeneration*, vol. 12, no. 3, pp. 262–268, 2004.
- [11] S. Sarrazin, W. C. Lamanna, and J. D. Esko, "Heparan sulfate proteoglycans," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 7, 2011.
- [12] F. M. Watt and H. Fujiwara, "Cell-extracellular matrix interactions in normal and diseased skin," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 4, Article ID a005124, 2011.
- [13] N. Volpi, "Therapeutic applications of glycosaminoglycans," *Current Medicinal Chemistry*, vol. 13, no. 15, pp. 1799–1810, 2006.
- [14] C. Noti and P. H. Seeberger, "Chemical approaches to define the structure-activity relationship of heparin-like glycosaminoglycans," *Chemistry & Biology*, vol. 12, no. 7, pp. 731–756, 2005.
- [15] J. L. Dreyfuss, C. V. Regatieri, T. R. Jarrouge, R. P. Cavalheiro, L. O. Sampaio, and H. B. Nader, "Heparan sulfate proteoglycans: structure, protein interactions and cell signaling," *Anais da Academia Brasileira de Ciencias*, vol. 81, no. 3, pp. 409–429, 2009.
- [16] S. J. Perkins, K. W. Fung, and S. Khan, "Molecular interactions between complement factor H and its heparin and heparan sulfate ligands," *Frontiers in Immunology*, vol. 5, no. 126, pp. 1–14, 2014.
- [17] T. M. Handel, Z. Johnson, S. E. Crown, E. K. Lau, M. Sweeney, and A. E. Proudfoot, "Regulation of protein function by glycosaminoglycans—as exemplified by chemokines," *Annual Review of Biochemistry*, vol. 74, pp. 385–410, 2005.
- [18] U. Lindahl and L. Kjellén, "Pathophysiology of heparan sulphate: many diseases, few drugs," *Journal of Internal Medicine*, vol. 273, no. 6, pp. 555–571, 2013.
- [19] P. V. Peplow, "Glycosaminoglycan: a candidate to stimulate the repair of chronic wounds," *Thrombosis and Haemostasis*, vol. 94, no. 1, pp. 4–16, 2005.
- [20] R. Sasisekharan, R. Raman, and V. Prabhakar, "Glycomics approach to structure-function relationships of glycosaminoglycans," *Annual Review of Biomedical Engineering*, vol. 8, pp. 181–231, 2006.
- [21] N. Volpi and F. Maccari, "Electrophoretic approaches to the analysis of complex polysaccharides," *Journal of Chromatography B*, vol. 834, no. 1-2, pp. 1–13, 2006.
- [22] M. A. Nugent, J. Zaia, and J. L. Spencer, "Heparan sulfate-protein binding specificity," *Biochemistry*, vol. 78, no. 7, pp. 726–735, 2013.
- [23] L.-Å. Fransson, "Glycans," *International Journal of Biochemistry & Cell Biology*, vol. 35, no. 2, pp. 125–129, 2003.
- [24] E. Rönnberg, F. R. Melo, and G. Pejler, "Mast Cell Proteoglycans," *Journal of Histochemistry and Cytochemistry*, vol. 60, no. 12, pp. 950–962, 2012.
- [25] X. Xian, S. Gopal, and J. R. Couchman, "Syndecans as receptors and organizers of the extracellular matrix," *Cell and Tissue Research*, vol. 339, no. 1, pp. 31–46, 2010.
- [26] K. Catlow, J. A. Deakin, M. Delehedde et al., "Hepatocyte growth factor/scatter factor and its interaction with heparan sulphate and dermatan sulphate," *Biochemical Society Transactions*, vol. 31, no. 2, pp. 352–353, 2003.
- [27] R. V. Iozzo, J. J. Zoeller, and A. Nyström, "Basement membrane proteoglycans: modulators par excellence of cancer growth and angiogenesis," *Molecules and Cells*, vol. 27, no. 5, pp. 503–513, 2009.
- [28] U. Häcker, K. Nybakken, and N. Perrimon, "Heparan sulphate proteoglycans: the sweet side of development," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 7, pp. 530–541, 2005.
- [29] D. A. S. Davis and C. R. Parish, "Heparan sulfate: a ubiquitous glycosaminoglycan with multiple roles in immunity," *Frontiers in Immunology*, vol. 4, Article ID Article 470, pp. 1–7, 2013.
- [30] K. R. Taylor and R. L. Gallo, "Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation," *The FASEB Journal*, vol. 20, no. 1, pp. 9–22, 2006.
- [31] R. K. Okolicsanyi, L. R. Griffiths, and L. M. Haupt, "Mesenchymal stem cells, neural lineage potential, heparan sulfate proteoglycans and the matrix," *Developmental Biology*, vol. 388, no. 1, pp. 1–10, 2014.
- [32] A. Yoneda and J. R. Couchman, "Regulation of cytoskeletal organization by syndecan transmembrane proteoglycans," *Matrix Biology*, vol. 22, no. 1, pp. 25–33, 2003.
- [33] H. C. Christianson and M. Belting, "Heparan sulfate proteoglycan as a cell-surface endocytosis receptor," *Matrix Biology*, vol. 35, pp. 51–55, 2014.
- [34] A. Głowacki, E. M. Koźma, and K. Olczyk, "Biosynteza proteoglikanów keratosiarczanych, chondroitynsiarczanych i dermatanosiarczanych," *Postępy Biochemii*, vol. 50, pp. 170–181, 2004.
- [35] X. Jiang and J. R. Couchman, "Perlecan and tumor angiogenesis," *Journal of Histochemistry and Cytochemistry*, vol. 51, no. 11, pp. 1393–1410, 2003.
- [36] V. Kram, E. Zcharia, O. Yacoby-Zeevi et al., "Heparanase is expressed in osteoblastic cells and stimulates bone formation and bone mass," *Journal of Cellular Physiology*, vol. 207, no. 3, pp. 784–792, 2006.
- [37] R. D. Sanderson, "Heparan sulfate proteoglycans in invasion and metastasis," *Seminars in Cell and Developmental Biology*, vol. 12, no. 2, pp. 89–98, 2001.
- [38] L. Borsig, I. Vlodavsky, R. Ishai-Michaeli, G. Torri, and E. Vismara, "Sulfated hexasaccharides attenuate metastasis by inhibition of P-selectin and heparanase," *Neoplasia*, vol. 13, no. 5, pp. 445–452, 2011.

- [39] D. Nikitovica, M. Mytilinaoua, A. Berdiakia, N. K. Karamanos, and G. N. Tzanakakisa, "Heparan sulfate proteoglycans and heparin regulate melanoma cell functions," *Seminars in Cell and Developmental Biology*, vol. 12, no. 2, pp. 117–125, 2001.
- [40] N. S. Gandhi and R. L. Mancera, "The structure of glycosaminoglycans and their interactions with proteins," *Chemical Biology and Drug Design*, vol. 72, no. 6, pp. 455–482, 2008.
- [41] M. Belting, "Heparan sulfate proteoglycan as a plasma membrane carrier," *Trends in Biochemical Sciences*, vol. 28, no. 3, pp. 145–151, 2003.
- [42] B. Casu, A. Naggi, and G. Torri, "Heparin-derived heparan sulfate mimics to modulate heparan sulfate-protein interaction in inflammation and cancer," *Matrix Biology*, vol. 29, no. 6, pp. 442–452, 2010.
- [43] B. Mulloy and C. C. Rider, "Cytokines and proteoglycans: an introductory overview," *Biochemical Society Transactions*, vol. 34, no. 3, pp. 409–413, 2006.
- [44] R. Raman, V. Sasisekharan, and R. Sasisekharan, "Structural Insights into biological roles of protein-glycosaminoglycan interactions," *Chemistry and Biology*, vol. 12, no. 3, pp. 267–277, 2005.
- [45] X. Lin, "Functions of heparan sulfate proteoglycans in cell signaling during development," *Development*, vol. 131, no. 24, pp. 6009–6021, 2004.
- [46] J. Jia, "Structure and functions of heparan sulphate/heparin—importance of glucuronyl C5-epimerase and heparanase," in *Acta Universitas Upsaliensis*, 2009, <http://www.diva-portal.org/smash/get/diva2:228301/FULLTEXT01.pdf>.
- [47] N. W. Shworak, T. Kobayashi, A. de Agostini, and N. C. Smits, "Anticoagulant heparan sulfate: to not clot—or not?" *Progress in Molecular Biology and Translational Science*, vol. 93, pp. 153–178, 2010.
- [48] Y.-H. L. Jen, M. Musacchio, and A. D. Lander, "Glypican-1 controls brain size through regulation of fibroblast growth factor signaling in early neurogenesis," *Neural Development*, vol. 4, no. 1, article 33, 2009.
- [49] S. Q. Fan, J. L. Cai, L. Y. Qin, Z. H. Wang, Z. Z. Liu, and M. L. Sun, "Effect of heparin on production of transforming growth factor (TGF)-beta1 and TGF-beta1 mRNA expression by human normal skin and hyperplastic scar fibroblasts," *Annals of Plastic Surgery*, vol. 60, no. 3, pp. 299–305, 2008.
- [50] M. D. Bass, R. C. Williamson, R. D. Nunan et al., "A syndecan-4 hair trigger initiates wound healing through caveolin- and RhoG-regulated integrin endocytosis," *Developmental Cell*, vol. 21, no. 4, pp. 681–693, 2011.
- [51] P. Olczyk, K. Komosinska-Vassev, K. Winsz-Szczotka et al., "Propolis modulates vitronectin, laminin, and heparan sulfate/heparin expression during experimental burn healing," *Journal of Zhejiang University: Science B*, vol. 13, no. 11, pp. 932–941, 2012.
- [52] M. Tong, M. M. Zbinden, I. J. M. Hekking, M. Vermeij, D. Barritault, and J. W. van Neck, "RGTA OTR 4120, a heparan sulfate proteoglycan mimetic, increases wound breaking strength and vasodilatory capability in healing rat full-thickness excisional wounds," *Wound Repair and Regeneration*, vol. 16, no. 2, pp. 294–299, 2008.
- [53] S. M. Nelson and I. A. Greer, "The potential role of heparin in assisted conception," *Human Reproduction Update*, vol. 14, no. 6, pp. 623–645, 2008.
- [54] C. Page, "Heparin and related drugs: beyond anticoagulant activity," *ISRN Pharmacology*, vol. 2013, Article ID 910743, 13 pages, 2013.
- [55] Y. Wegrowski, A. L. Milard, G. Kotlarz, E. Toulmonde, F.-X. Maquart, and J. Bernard, "Cell surface proteoglycan expression during maturation of human monocytes-derived dendritic cells and macrophages," *Clinical and Experimental Immunology*, vol. 144, no. 3, pp. 485–493, 2006.
- [56] C. J. Malavaki, A. D. Theocharis, F. N. Lamari et al., "Heparan sulfate: biological significance, tools for biochemical analysis and structural characterization," *Biomedical Chromatography*, vol. 25, no. 1, pp. 11–20, 2011.
- [57] G. S. Schultz, J. M. Davidson, R. S. Kirsner, P. Bornstein, and I. M. Herman, "Dynamic reciprocity in the wound microenvironment," *Wound Repair and Regeneration*, vol. 19, no. 2, pp. 134–148, 2011.
- [58] M. M. Fuster and L. Wang, "Endothelial heparan sulfate in angiogenesis," *Progress in Molecular Biology and Translational Science*, vol. 93, pp. 179–212, 2010.
- [59] J.-F. Li, H.-F. Duan, C.-T. Wu et al., "HGF accelerates wound healing by promoting the dedifferentiation of epidermal cells through β_1 -Integrin/ILK pathway," *BioMed Research International*, vol. 2013, Article ID 470418, 9 pages, 2013.
- [60] N. Ojeh, K. Hiilesvuo, A. Wärri, M. Salmivirta, T. Henttinen, and A. Määttä, "Ectopic expression of syndecan-1 in basal epidermis affects keratinocyte proliferation and wound re-epithelialization," *Journal of Investigative Dermatology*, vol. 128, no. 1, pp. 26–34, 2008.
- [61] S. Barrientos, O. Stojadinovic, M. S. Golinko, H. Brem, and M. Tomic-Canic, "Growth factors and cytokines in wound healing," *Wound Repair and Regeneration*, vol. 16, no. 5, pp. 585–601, 2008.
- [62] Y. R. Yun, J. E. Won, E. Jeon et al., "Fibroblast growth factors: biology, function, and application for tissue regeneration," *Journal of Tissue Engineering*, vol. 1, no. 1, Article ID 218142, 2010.
- [63] M. Zakrzewska, A. Wiedlocha, A. Szlachcic, D. Krowarsch, J. Otlewski, and S. Olsnes, "Increased protein stability of FGF1 can compensate for its reduced affinity for heparin," *The Journal of Biological Chemistry*, vol. 284, no. 37, pp. 25388–25403, 2009.
- [64] T. K. Ho, X. Shiwen, D. Abraham, J. Tsui, and D. Baker, "Stromal-cell-derived factor-1 (SDF-1)/CXCL12 as potential target of therapeutic angiogenesis in critical leg ischaemia," *Cardiology Research and Practice*, vol. 2012, Article ID 143209, 7 pages, 2012.
- [65] J. Ma, Q. Wang, T. Fei, J.-D. J. Han, and Y.-G. Chen, "MCP-1 mediates TGF- β -induced angiogenesis by stimulating vascular smooth muscle cell migration," *Blood*, vol. 109, no. 3, pp. 987–994, 2007.
- [66] Y. Chen, X. Shi-Wen, J. van Beek et al., "Matrix contraction by dermal fibroblasts requires transforming growth factor- β /activin-linked kinase 5, heparan sulfate-containing proteoglycans, and MEK/ERK: insights into pathological scarring in chronic fibrotic disease," *The American Journal of Pathology*, vol. 167, no. 6, pp. 1699–1711, 2005.
- [67] N. Sampson, P. Berger, and C. Zenzmaier, "Therapeutic targeting of redox signaling in myofibroblast differentiation and age-related fibrotic disease," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 458276, 15 pages, 2012.
- [68] P. Olczyk, K. Komosinska-Vassev, K. Winsz-Szczotka, J. Stojko, K. Klimek, and E. M. Kozma, "Propolis induces chondroitin/dermatan sulphate and hyaluronic acid accumulation in the skin of burned wound," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 290675, 8 pages, 2013.
- [69] P. Olczyk, G. Wisowski, K. Komosinska-Vassev et al., "Propolis modifies collagen types I and III accumulation in the matrix

- of burnt tissue,” *Evidence-based Complementary and Alternative Medicine*, vol. 2013, Article ID 423809, 10 pages, 2013.
- [70] P. Olczyk, K. Komosinska-Vassev, G. Wisowski, L. Mencner, J. Stojko, and E. M. Kozma, “Propolis modulates fibronectin expression in the matrix of thermal injury,” *BioMed Research International*, vol. 2014, Article ID 748101, 10 pages, 2014.
- [71] P. Olczyk, P. Ramos, K. Komosinska-Vassev, J. Stojko, and B. Pilawa, “Positive effect of propolis on free radicals in burn wounds,” *Evidence-based Complementary and Alternative Medicine*, vol. 2013, Article ID 356737, 12 pages, 2013.

Review Article

GHK Peptide as a Natural Modulator of Multiple Cellular Pathways in Skin Regeneration

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GHK (glycyl-L-histidyl-L-lysine) is present in human plasma, saliva, and urine but declines with age. It is proposed that GHK functions as a complex with copper 2+ which accelerates wound healing and skin repair. GHK stimulates both synthesis and breakdown of collagen and glycosaminoglycans and modulates the activity of both metalloproteinases and their inhibitors. It stimulates collagen, dermatan sulfate, chondroitin sulfate, and the small proteoglycan, decorin. It also restores replicative vitality to fibroblasts after radiation therapy. The molecule attracts immune and endothelial cells to the site of an injury. It accelerates wound-healing of the skin, hair follicles, gastrointestinal tract, boney tissue, and foot pads of dogs. It also induces systemic wound healing in rats, mice, and pigs. In cosmetic products, it has been found to tighten loose skin and improve elasticity, skin density, and firmness, reduce fine lines and wrinkles, reduce photodamage, and hyperpigmentation, and increase keratinocyte proliferation. GHK has been proposed as a therapeutic agent for skin inflammation, chronic obstructive pulmonary disease, and metastatic colon cancer. It is capable of up- and downregulating at least 4,000 human genes, essentially resetting DNA to a healthier state. The present review revisits GHK's role in skin regeneration in the light of recent discoveries.

1. Introduction

GHK is a tripeptide with the amino acid sequence glycyl-histidyl-lysine. It naturally occurs in human plasma, saliva, and urine. In plasma the level of GHK is about 200 ng/mL (10^{-7} M) at age 20, but declines to 80 ng/mL by age 60. This decline in the GHK-level coincides with the noticeable decrease in regenerative capacity of an organism. The human peptide GHK-Cu was isolated in 1973 by Pickart as an activity in human albumin that caused old human liver tissue to synthesize proteins like younger tissue [1]. Subsequent studies established this activity as a tripeptide with an amino acid sequence glycyl-L-histidyl-L-lysine with a strong affinity for copper that readily formed the complex GHK-Cu. It was proposed that GHK-Cu functions as a complex with copper 2+ [2]. Pickart et al. have established that GHK-Cu accelerates wound healing and contraction, improves the take of transplanted skin, and also possesses antiinflammatory actions [3–5].

Subsequent studies directed by Borel and Maquart et al. demonstrated that GHK-Cu at a very low, nontoxic concentration (1–10 nanomolar) stimulated both synthesis and breakdown of collagen and glycosaminoglycans [6]. GHK modulated an activity of both metalloproteinases and their inhibitors (TIMP-1 and TIMP-2), acting as a main regulator of wound healing and skin remodeling processes [7, 8]. GHK-Cu stimulated collagen, dermatan sulfate, chondroitin sulfate, and a small proteoglycan, decorin [9]. In 2001 McCormack et al. established that GHK-Cu restored replicative vitality to fibroblasts from patients after anticancer radiation therapy that damages cellular DNA [10]. GHK was also found to attract immune and endothelial cells to the site of an injury [11].

Wound healing activity of GHK-Cu was confirmed in animal experiments. GHK-Cu accelerated wound healing and increased blood vessel formation and the level of antioxidant enzymes in rabbits. This molecule also induced systemic wound healing in rats, mice, and pigs. It improved the healing

of diabetic and ischemic wounds in rats, decreasing the level of TNF-alpha and stimulating collagen synthesis. It also facilitated healing of pad wounds in dogs [12–17]. Such well-documented skin regeneration activity prompted widespread use of GHK in antiaging cosmetic products [18].

Recently, GHK-Cu has been gaining publicity as a prospective therapeutic agent for chronic obstructive pulmonary disease (COPD), skin inflammation, and metastatic colon cancer [19–21]. It has been established that it is capable of up- and downregulating at least 4,000 genes in the human genome, essentially resetting DNA back to a healthier state [22]. These studies shed new light on the skin remodeling activity of the GHK-Cu peptide.

The present review revisits GHK-Cu's role in skin regeneration in the light of recent discoveries.

2. GHK Restores TGF-Beta Pathway in COPD Lungs

A collaborative study conducted by scientists from Boston University, University of Groningen, University of British Columbia, and University of Pennsylvania established that the GHK peptide reverses the gene expression signature of COPD, which is manifested by emphysema, inflammation, lung tissue destruction, and significant reduction of lung capacity.

The researchers identified 127 genes whose expression was significantly associated with emphysema severity. Among those genes, whose expression was upregulated in COPD patients, were genes involved in inflammation, while expression of genes involved in tissue remodeling and repair was markedly downregulated. Among the genes displaying decreased activity were genes involved in the TGF-beta pathway. Using the Connectivity Map, a software gene profiling tool developed by the Broad Institute, the researchers identified GHK as a compound which reversed changes in gene expression associated with emphysematous destruction. In particular, patients with COPD displayed a decreased activity of genes involved in the TGF-beta pathway. GHK reversed the gene expression pattern so it became consistent with the activation of the TGF-beta pathway.

In vitro studies confirmed that treating lung fibroblasts with GHK reversed negative changes associated with decreased TGF-beta activity. It has been established that lung fibroblasts derived from COPD patients had certain defects, which impaired their ability to contract and remodel collagen gel. When such fibroblasts were treated with either GHK or TGF-beta, the contraction and remodeling of collagen gel was restored and became comparable to fibroblasts derived from lungs of exsmokers without COPD. After GHK treatment, the lung fibroblasts derived from COPD patients were able to remodel collagen gel into fibrils. They also had an elevated expression of integrin beta 1. These findings indicate that GHK may be able to improve tissue regeneration by restoring activity of genes involved in the TGF-beta pathway [23].

It is known that skin regeneration requires the participation of multiple cytokines and growth factors. Rather than work separately, they engage in a crosstalk, which involves

interaction of different cellular pathways. For example, cellular pathways regulated by TGF-beta and integrins seem to be connected [24]. GHK's ability to restore the contraction and remodeling of collagen gel in the COPD study demonstrate that GHK is capable of activating both TGF-beta and integrin beta 1 pathways during tissue regeneration. Even though the exact mechanism of GHK's action is yet to be elucidated, it becomes apparent that the diverse and multiple effects of GHK in skin regeneration can be better understood through its ability to reset the gene pattern back to a healthier state, thereby leading to the activation or deactivation of various cellular pathways.

3. Cancer Metastasis Genes and Skin Remodeling

Hong et al. used genome-wide profiling to identify genetic biomarkers for metastasis prone colorectal cancer as well as their perturbagens, substances that modulated their expression. The search out of 1309 bioactive compounds yielded only two substances that were able to effectively downregulate expression of "metastatic" genes, GHK and the plant alkaloid, securinine. GHK suppressed RNA production in 70% of 54 human genes overexpressed in patients with an aggressive metastatic form of colon cancer. GHK produced the result at a low non-toxic 1 micromolar concentration and securinine at 18 micromolar. The authors point out that both GHK and securinine are well-known skin remodeling agents. Securinine activates macrophages and is a component of traditional African and Chinese medicines for skin injuries [25]. The 54 genes whose expression was reversed by GHK included "node molecules" YWHAB, MAP3K5, LMNA, APP, GNAQ, F3, NFATC2, and TGM2, all of which are involved in regulation of multiple biological functions through a complex molecular network [20]. The fact that GHK was able to suppress 70% of genes involved in the development of an aggressive metastatic form of colon cancer indicates that GHK is capable of the regulation of various biochemical pathways on a gene level and it seems to be resetting the gene activity back to health, which leads to the improvement of tissue repair.

In vitro studies by Matalka et al. found that when three lines of human cancer cells (SH-SY5Y neuroblastoma cells, U937 histolytic cells, breast cancer cells) were incubated in culture with 1 to 10 nanomolar GHK, the programmed cell death system (apoptosis) was reactivated and cell growth inhibited [26].

Pickart et al., using the Broad Institute data, found that GHK induces anti-cancer expression of numerous caspase, growth regulatory, and DNA repair genes. The combination of ascorbic acid and GHK-Cu strongly inhibited the growth of sarcoma-180 in mice [27].

4. Recovery of Skin Stem Cells

Skin regeneration depends on viability and proliferative potential of stem cells. Skin proliferation starts in the basal layer of keratinocytes, which are attached to the basal

membrane. When a cell leaves the basal layer, it undergoes terminal differentiation. Stem cells have unlimited self-renewal capacity; however, their proliferative potential declines with age. GHK-Cu, in concentrations of 0.1–10 micromolar, increased expression of epidermal stem cell markers such as integrins and p63 in basal keratinocytes in dermal skin equivalents, which according to the authors indicate increased stemness and proliferative potential of basal keratinocytes. Therefore, restoration of gene pattern characteristic of healthy stem cells, which leads to activation of integrins and p63 cellular pathways may be another target of GHK's gene modulatory activity relevant to skin regeneration [28, 29].

A recent study has established that pretreatment of human mesenchymal stem/stromal cells (MSC) with GHK presented in a biodegradable carrier (alginate gel) produced a dose-dependent increase in secretion of proangiogenic factors such as the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor. When pretreated with antibodies to integrins alpha 1 and beta 1, MSC failed to produce an increase of VEGF, which indicated that the effects of GHK on secretion of trophic factors by MSC involve the integrin cellular pathway [30].

5. GHK and IL-6 in Skin Repair

Wound healing and skin repair involves inflammation, cell proliferation and migration and dermal matrix remodeling. Excessive inflammation may delay healing and lead to scar formation. The copper complexes of the peptides Gly-Gly-His (GGH), Gly-His-Lys (GHK) reduced TNF-alpha induced secretion of proinflammatory cytokine IL-6, in normal human dermal fibroblasts, while *saccharomyces/copper ferment* (OligolidesA Copper) had no effect. The authors propose that GHK and GHK-Cu can be used as a topical agent in treatment of inflammatory skin conditions instead of corticosteroids [21].

6. GHK and DNA Repair

GHK was able to restore viability of irradiated fibroblasts. The researchers used cultured human fibroblasts obtained from cervical skin that was either intact or exposed to radioactive treatment (5000 rad). GHK (10^{-9} M) was added in a serum free medium directly to the cell culture. An equivalent amount of plain serum-free medium was added to control cells. Although irradiated fibroblasts survived and replicated in the cell culture, their growth dynamics were markedly different from that of intact cells. The growth of the irradiated cells was especially delayed at 24 and 48 hour measurements. However, the irradiated fibroblasts treated with GHK showed much faster growth that was similar to the normal (non-irradiated control cells). In addition, GHK-treated irradiated fibroblasts showed higher production of growth factors, which are essential for wound healing [31].

Fibroblasts are central cells in both wound healing and tissue renewal processes. They not only synthesize different components of dermal matrix, but also produce a number

of growth factors that are involved in a multitude of cellular pathways regulating cell migration and proliferation, angiogenesis, epithelialization, and so forth. Radiation damages cell DNA, thus impairing their function. Since GHK was able to restore function of irradiated fibroblasts, it has to have effects on DNA repair.

Studies using the Broad Institute's Connectivity map found that GHK significantly increased the expression of DNA repair genes with 47 genes stimulated and 5 genes suppressed (more than or equal to a 50% increase or decrease) [22].

7. Facial Studies

A number of placebo-controlled clinical studies found GHK-Cu to improve skin quality in women around the age of 50. A study of collagen production determined by studying skin biopsy samples using immunohistological techniques found that after applying creams to the thighs for one month, GHK-peptides had a significant effect on collagen production. Increases were found in 70% of the women treated with GHK-Cu, in contrast to 50% treated with the vitamin C cream, and 40% treated with retinoic acid [32].

A GHK-Cu facial cream reduced visible signs of aging after 12 weeks of application to the facial skin of 71 women with mild to advanced signs of photoaging. The cream improved skin laxity, clarity, and appearance, reduced fine lines and the depth of wrinkles, and increased skin density and thickness [33].

A GHK-Cu eye cream, tested on 41 women for twelve weeks with mild to advanced photodamage, was compared to a placebo control and an eye cream containing vitamin K. The GHK-Cu cream performed better than both controls in terms of reducing lines and wrinkles, improving overall appearance, and increasing skin density and thickness [34].

In another 12-week facial study of 67 women between 50 and 59 years with mild to advanced photodamage, a GHK-Cu cream was applied twice daily and improved skin laxity, clarity, firmness and appearance, reduced fine lines, coarse wrinkles and mottled pigmentation, and increased skin density, and thickness. The cream also strongly stimulated dermal keratinocyte proliferation as determined by the histological analysis of biopsies [35].

These placebo-controlled studies demonstrated that GHK-Cu skin creams had the following effects:

- (1) Tighten loose skin and improve elasticity.
- (2) Improve skin density and firmness.
- (3) Reduce fine lines and deep wrinkles.
- (4) Improve skin clarity.
- (5) Reduce photodamage and mottled hyper-pigmentation.
- (6) Strongly increase keratinocyte proliferation.

8. Formulation and Delivery

GHK-Cu appears to pass the skin's horny layer (stratum corneum) in quantities sufficient to activate regenerative

events. The permeability coefficients of copper complexes increase with increasing pH. It was proved that only the tripeptide GHK and its complexes with copper: GHK-Cu and (GHK)(2)-Cu are able to migrate through the membrane model of the stratum corneum [36–38]. Yet, because of its susceptibility to the actions of proteolytic enzymes it is important to ensure its sustained delivery in bioactive concentrations. Arul et al. proposed the use of biotinylated peptide incorporated collagen matrix (Boc-GHK) for dermal wound healing. They observed improved wound contraction and increased cell proliferation and a high expression of antioxidant enzymes in wounds treated with Boc-GHK compared to the control [39].

A recent study investigated formulation requirements for GHK. It has been established that the peptide was prone to hydrolytic cleavage when subjected to oxidative stressors. It was stable in water in the pH range 4.5–7.4 buffers for at least two weeks at 60°C. The distribution coefficients in octanol-phosphate buffered saline indicated the highly hydrophilic nature of GHK-Cu with log *D* values between –2.38 and –2.49 at a pH range of 4.5–7.4. GHK-Cu can be incorporated into Span 60 based niosomes. It is less stable in the presence of the negatively charged lipid diacetyl phosphate [40].

Glycyl-L-histidyl-L-lysine-Cu(II) (GHK-Cu(2+))-loaded Zn-pectinate microparticles in the form of hydroxypropyl cellulose (HPC) compression-coated tablets were developed for colon delivery of GHK. The release of GHK-Cu(2+) from Zn-pectinate microparticles was strongly affected by the cross-linking agent concentration and drug amount, but not by surfactant amount. The microparticles released 50–80% of their drug load within 4 hours. The optimum microparticle formulation (F8) coated with a relatively hydrophobic polymer HPC presented a colonic delivery system. This study indicates a possibility of including GHK into a delivery system for internal use. It should be possible to incorporate GHK into a dietary supplement with many health promoting properties and no side effects. Such formulations can be used to improve dermal healing in addition to topical delivery [41].

9. Breakdown Resistant Mixed Copper Peptide Complexes

A major problem in the treatment of human skin wounds and ulcers is dealing with infected wounds. The powerful bacteria in such wounds secrete proteases that can rapidly breakdown GHK and other types of healing growth factors.

GHK itself is formed during protein breakdown in the company of a large number of other small peptides. When we added copper 2+ to the entire mixture of small peptides formed during breakdown, we found that such a mixture had significant wound healing activity. Moreover, such peptides were resistant to further breakdown. The details of preparing these mixed copper peptide complexes and their incorporation into wound healing creams are given in the referenced US patents [42, 43].

Howard Maibach's group later tested the mixed copper peptide complexes using four human wound healing systems.

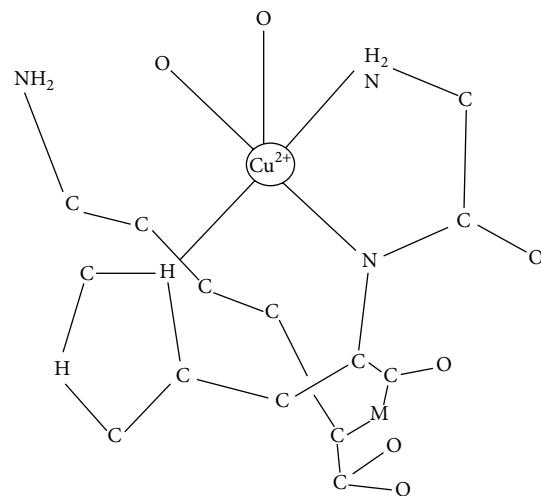


FIGURE 1: Molecular crystal structure of the tripeptide GHK-Cu. In solution, lysine carboxyl groups of neighboring complexes may participate in a complex formation.

These skin damaging methods were

- (1) removal of skin lipids with acetone,
- (2) irritation of skin with strong sodium lauryl sulfate,
- (3) irritation of skin by tape stripping,
- (4) activation of an allergic response in patients with nickel allergies.

In all four studies, there was a more rapid healing with creams containing the mixed copper peptide complexes than with the control creams without the complexes. There was also a more rapid reduction in the erythema (redness) in the nickel allergy patients [44–47].

10. Biochemistry of GHK-Cu

The molecular structure of the GHK copper complex (GHK-Cu) has been extensively studied using X-ray crystallography, EPR spectroscopy, X-ray absorption spectroscopy, and PMR spectroscopy as well as other methods such as titration. In the GHK-Cu complex, the Cu (II) ion is coordinated by the nitrogen from the imidazole side chain of the histidine, another nitrogen from the alpha-amino group of glycine, and the deprotonated amide nitrogen of the glycine-histidine peptide bond (Figure 1).

Lau and Sarkar found that at the physiological pH, GHK-Cu complexes can form binary and ternary structures which may involve the amino acid histidine and/or the copper binding region of the albumin molecule. They also observed that GHK can easily obtain copper 2+ bound to the high affinity copper transport site on plasma albumin (albumin's binding constant of $\log_{10} = 16.2$ versus GHK's binding constant of $\log_{10} = 16.44$). It has been established that the copper (II) redox activity is silenced when copper ions that are bound to the GHK tripeptide, which allows the delivery of nontoxic copper into the cell [48–50].

The most distinctive feature of GHK is its ability to form complexes with copper (II) [51]. This is very important since copper is required for more than a dozen vital enzymes in the human body and skin including those that participate in connective tissue formation, antioxidant defense, and cellular respiration. Copper also exhibits signaling function and can influence cell behavior and metabolism. For example, sufficient copper is required for stem cells to start proliferating and repairing tissues. GHK also helps to reduce the level of free ionic copper thus preventing the possibility of oxidative damage.

Apart from being able to bind with copper, GHK can also quench some toxins, in particular those that are generated during lipid peroxidation [52]. This makes GHK a quite efficient antioxidant.

Finally, GHK has been shown to be able to serve as a cell adhesion molecule, which means that it helps cells to attach themselves to the extracellular matrix. This facilitates migration, proliferation, and differentiation of repair cells in the skin [53, 54].

11. GHK: A Built-In Natural Regulator of Dermal Repair

The wound healing process in the skin goes through the following phases: hemostasis (blood clotting), inflammation, granulation, and scar remodeling. Every stage requires well-coordinated cell interaction and therefore is precisely orchestrated by a plethora of biological active molecules coming from different sources.

For example, immediately after injury degranulating platelets release growth factors (such as TGF-beta) that mobilize immune cells and attract them to the site of the injury.

Keratinocytes and fibroblasts also produce a multitude of growth factors. Neutrophils, macrophages, and other immune cells that get recruited to the site of injury produce their share of growth factors and cytokines, as well.

GHK is a rare human sequence in proteins; however, it is more common in proteins of the extracellular matrix (ECM). GHK triplet is present in the alpha 2(I) chain of type I collagen and can be liberated by proteases at the site of a wound [55]. One of the most notable GHK-containing proteins that is always present in sites of remodeling is glycoprotein SPARC. Its proteolytic breakdown after injury generates GHK-Cu [56]. It has long been established that proteolytic breakdown of some proteins and proteoglycans of ECM results in the release of important regulatory molecules, matrikines [57]. These molecules activate and regulate dermal repair processes. The ability of GHK to reset the genome to a healthier gene pattern which leads to a better regulation of various cellular pathways can explain its diverse dermal repair actions. Since GHK is present as an amino acid sequence in proteins of ECM and is released after an injury, it appears to serve as a natural built-in modulator of dermal repair.

12. Conclusion

It has long been accepted that the human copper-binding peptide GHK-Cu enhances healing of dermal wounds and

stimulates skin renewal exhibiting a wide range of effects. Cellular pathways involved in dermal repair and skin regeneration form an intricate and finely orchestrated biochemical network, where various regulatory molecules are involved in a cross-talk. When such an interaction is disrupted, the healing is delayed and may result in excessive inflammation and scarring. It appears that GHK is able to restore healthy functioning of essential cellular pathways in dermal repair through resetting the gene pattern to a healthier state.

GHK, abundantly available at low cost in bulk quantities, is a potential treatment for a variety of disease conditions. The molecule is very safe and no issues have ever arisen during its use as a skin cosmetic or in human wound healing studies.

Based on our studies where GHK was injected in a distant part of a body, such as thigh to induce systemic healing, and also on studies where GHK was injected intraperitoneally once daily to induce systemic wound healing throughout the body, we estimate that about 100–200 mgs of GHK will produce therapeutic actions in humans. But even this may overestimate the necessary effective dosage of the molecule [14].

Studies where GHK was used for the healing of bone fractures in rats used a mixture of small molecules (Gly-His-Lys (0.5 µg/kg), dalargin (1.2 µg/kg) (an opioid-like synthetic drug), and the biological peptide thymogen (0.5 µg/kg) (L-glutamyl-L-tryptophan)) to heal bones. The total peptide dosage is about 2.2 micrograms per kilogram or if scaled for the human body, about 140 micrograms per injection with treatments for 10 days [58, 59]. GHK can be incorporated into topical gels, used in dermal patches, and collagen membranes, as well as being administered orally in liposomes and other carriers. Future research is needed to establish the effective dosage in humans and the best ways of delivery.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] L. Pickart, *A Tripeptide from Human Serum Which Enhances the Growth of Neoplastic Hepatocytes and the Survival of Normal Hepatocytes*, University of California, San Francisco, Calif, USA, 1973.
- [2] L. Pickart, J. H. Freedman, W. J. Loker et al., "Growth-modulating plasma tripeptide may function by facilitating copper uptake into cells," *Nature*, vol. 288, no. 5792, pp. 715–717, 1980.
- [3] L. Pickart, "Use of GHL-Cu as a wound-healing and anti-inflammatory agent," 1988, <http://www.freepatentsonline.com/4760051.html>.
- [4] D. Downey, W. F. Larrabee Jr., V. Voci, and L. Pickart, "Acceleration of wound healing using glycyl-histidyl-lysine copper (II)," *Surgical Forum*, vol. 25, pp. 573–575, 1985.
- [5] L. Pickart, "Iamin: a human growth factor with multiple wound-healing properties," in *Biology of Copper Complexes*, J. Sorenson, Ed., pp. 273–285, Humana Press, Clifton, NJ, USA, 1987.

- [6] Y. Wegrowski, F. X. Maquart, and J. P. Borel, "Stimulation of sulfated glycosaminoglycan synthesis by the tripeptide-copper complex Glycyl-L-histidyl-L-lysine-Cu²⁺," *Life Sciences*, vol. 51, no. 13, pp. 1049–1056, 1992.
- [7] A. Siméon, H. Emond, W. Hornebeck, and F.-X. Maquart, "The tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu²⁺ stimulates matrix metalloproteinase-2 expression by fibroblast cultures," *Life Sciences*, vol. 67, no. 18, pp. 2257–2265, 2000.
- [8] H. Ehrlich, "Stimulation of skin healing in immunosuppressed rats," in *Proceedings of the Symposium on Collagen and Skin Repair*, Reims, France, September 1991.
- [9] A. Siméon, Y. Wegrowski, Y. Bontemps, and F.-X. Maquart, "Expression of glycosaminoglycans and small proteoglycans in wounds: modulation by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu²⁺," *Journal of Investigative Dermatology*, vol. 115, no. 6, pp. 962–968, 2000.
- [10] M. C. McCormack, K. C. Nowak, and R. J. Koch, "The effect of copper tripeptide and tretinoin on growth factor production in a serum-free fibroblast model," *Archives of Facial Plastic Surgery*, vol. 3, no. 1, pp. 28–32, 2001.
- [11] F. Buffoni, R. Pino, and A. Dal Pozzo, "Effect of tripeptide-copper complexes on the process of skin wound healing and on cultured fibroblasts," *Archives Internationales de Pharmacodynamie et de Therapie*, vol. 330, no. 3, pp. 345–360, 1995.
- [12] N. Y. Gul, A. Topal, I. T. Cangul, and K. Yanik, "The effects of topical tripeptide copper complex and helium-neon laser on wound healing in rabbits," *Veterinary Dermatology*, vol. 19, no. 1, pp. 7–14, 2008.
- [13] I. T. Cangul, N. Y. Gul, A. Topal, and R. Yilmaz, "Evaluation of the effects of topical tripeptide-copper complex and zinc oxide on open-wound healing in rabbits," *Veterinary Dermatology*, vol. 17, no. 6, pp. 417–423, 2006.
- [14] L. Pickart, "Method of using copper(II) containing compounds to accelerate wound healing," 1992, <http://www.freepatentsonline.com/5164367.html>.
- [15] V. Arul, R. Kartha, and R. Jayakumar, "A therapeutic approach for diabetic wound healing using biotinylated GHK incorporated collagen matrices," *Life Sciences*, vol. 80, no. 4, pp. 275–284, 2007.
- [16] S. O. Canapp Jr., J. P. Farese, G. S. Schultz et al., "The effect of topical tripeptide-copper complex on healing of ischemic open wounds," *Veterinary Surgery*, vol. 32, no. 6, pp. 515–523, 2003.
- [17] S. F. Swaim, D. M. Vaughn, S. A. Kincaid et al., "Effect of locally injected medications on healing of pad wounds in dogs," *American Journal of Veterinary Research*, vol. 57, no. 3, pp. 394–399, 1996.
- [18] L. Pickart, "The human tri-peptide GHK and tissue remodeling," *Journal of Biomaterials Science, Polymer Edition*, vol. 19, no. 8, pp. 969–988, 2008.
- [19] S. Meiners and O. Eickelberg, "Next-generation personalized drug discovery: the tripeptide GHK hits center stage in chronic obstructive pulmonary disease," *Genome Medicine*, vol. 4, no. 8, article 70, 2012.
- [20] Y. Hong, T. Downey, K. W. Eu, P. K. Koh, and P. Y. Cheah, "A 'metastasis-prone' signature for early-stage mismatch-repair proficient sporadic colorectal cancer patients and its implications for possible therapeutics," *Clinical and Experimental Metastasis*, vol. 27, no. 2, pp. 83–90, 2010.
- [21] A. Gruchlik, M. Jurzak, E. W. A. Chodurek, and Z. Dzierzewicz, "Effect of Gly-Gly-His, Gly-His-Lys and their copper complexes on TNF- α -dependent IL-6 secretion in normal human dermal fibroblasts," *Acta Poloniae Pharmaceutica*, vol. 69, no. 6, pp. 1303–1306, 2012.
- [22] L. Pickart, J. M. Vasquez-Soltero, and A. Margolina, "GHK and DNA: resetting the human genome to health," *BioMed Research International*, vol. 2014, Article ID 151479, 10 pages, 2014.
- [23] J. D. Campbell, J. E. McDonough, J. E. Zeskind et al., "A gene expression signature of emphysema-related lung destruction and its reversal by the tripeptide GHK," *Genome Medicine*, vol. 4, article 67, 2012.
- [24] S. Boo and L. Dagnino, "Integrins as modulators of transforming growth factor beta signaling in dermal fibroblasts during skin regeneration after injury," *Advances in Wound Care*, vol. 2, no. 5, pp. 238–246, 2013.
- [25] K. Lubick, M. Radke, and M. Jutila, "Securinine, a GABA receptor antagonist, enhances macrophage clearance of phase II *C. burnetii*: comparison with TLR agonists," *Journal of Leukocyte Biology*, vol. 82, no. 5, pp. 1062–1069, 2007.
- [26] L. E. Matalka, A. Ford, and M. T. Unlap, "The tripeptide, GHK, induces programmed cell death in SH-SY5Y neuroblastoma cells," *Journal of Biotechnology & Biomaterials*, vol. 2, article 144, 2012.
- [27] L. Pickart, J. M. Vasquez-Soltero, F. D. Pickart, and J. Majnarich, "GHK, the human skin remodeling peptide, induces anti-cancer expression of numerous caspase, growth regulatory, and DNA repair genes," *Journal of Analytical Oncology*, vol. 3, no. 2, pp. 79–87, 2014.
- [28] Y. Kang, H. Choi, J. Na et al., "Copper-GHK increases integrin expression and p63 positivity by keratinocytes," *Archives of Dermatological Research*, vol. 301, no. 4, pp. 301–306, 2009.
- [29] H.-R. Choi, Y.-A. Kang, S.-J. Ryoo et al., "Stem cell recovering effect of copper-free GHK in skin," *Journal of Peptide Science*, vol. 18, no. 11, pp. 685–690, 2012.
- [30] S. Jose, M. L. Hughbanks, B. Y. K. Binder, G. C. Ingavle, and J. K. Leach, "Enhanced trophic factor secretion by mesenchymal stem/stromal cells with Glycine-Histidine-Lysine (GHK)-modified alginate hydrogels," *Acta Biomaterialia*, vol. 10, no. 5, pp. 1955–1964, 2014.
- [31] J. D. Pollard, S. Quan, T. Kang, and R. J. Koch, "Effects of copper tripeptide on the growth and expression of growth factors by normal and irradiated fibroblasts," *Archives of Facial Plastic Surgery*, vol. 7, no. 1, pp. 27–31, 2005.
- [32] A. A. Abdulghani, A. Sherr, S. Shirin et al., "Effects of topical creams containing vitamin C, a copper-binding peptide cream and melatonin compared with tretinoin on the ultrastructure of normal skin—a pilot clinical, histologic, and ultrastructural study," *Disease Management and Clinical Outcomes*, vol. 1, no. 4, pp. 136–141, 1998.
- [33] J. Leyden, T. Stephens, M. Finkey, Y. Appa, and S. Barkovic, "Skin care benefits of copper peptide containing facial cream," in *Proceedings of the American Academy of Dermatology Meeting*, New York, NY, USA, February 2002.
- [34] J. Leyden, T. Stephens, M. Finkey, and S. Barkovic, *Skin Care Benefits of Copper Peptide Containing Eye Creams*, University of Pennsylvania, 2002.
- [35] M. Finkley, Y. Appa, and S. Bhandarkar, "Copper peptide and skin," in *Cosmeceuticals and Active Cosmetics: Drugs vs. Cosmetics*, P. Elsner and H. Maibach, Eds., pp. 549–563, Marcel Dekker, New York, NY, USA, 2005.
- [36] L. Mazurowska and M. Mojski, "Biological activities of selected peptides: skin penetration ability of copper complexes with

- peptides," *Journal of Cosmetic Science*, vol. 59, no. 1, pp. 59–69, 2008.
- [37] J. J. Hostynék, F. Dreher, and H. I. Maibach, "Human skin retention and penetration of a copper tripeptide in vitro as function of skin layer towards anti-inflammatory therapy," *Inflammation Research*, vol. 59, no. 11, pp. 983–988, 2010.
- [38] J. J. Hostynék, F. Dreher, and H. I. Maibach, "Human skin penetration of a copper tripeptide in vitro as a function of skin layer," *Inflammation Research*, vol. 60, no. 1, pp. 79–86, 2011.
- [39] V. Arul, D. Gopinath, K. Gomathi, and R. Jayakumar, "Biotinylated GHK peptide incorporated collagenous matrix: a novel biomaterial for dermal wound healing in rats," *Journal of Biomedical Materials Research B: Applied Biomaterials*, vol. 73, no. 2, pp. 383–391, 2005.
- [40] T. Badenhorst, D. Svirskis, and Z. Wu, "Physicochemical characterization of native glycyl-L-histidyl-L-lysine tripeptide for wound healing and anti-aging: a preformulation study for dermal delivery," *Pharmaceutical Development and Technology*, 2014.
- [41] T. Ugurlu, M. Turkoglu, and T. Ozaydin, "In vitro evaluation of compression-coated glycyl-L-histidyl-L-lysine-Cu(II) (GHK-Cu²⁺)-loaded microparticles for colonic drug delivery," *Drug Development and Industrial Pharmacy*, vol. 37, pp. 1282–1289, 2011.
- [42] L. Pickart, "Tissue protective and regenerative compositions," 1995, <http://www.freepatentsonline.com/5382431.html>.
- [43] L. Pickart, "Tissue protective and regenerative compositions," 1996, <http://www.freepatentsonline.com/5554375.html>.
- [44] H. Zhai, Y.-H. Leow, and H. I. Maibach, "Human barrier recovery after acute acetone perturbation: an irritant dermatitis model," *Clinical and Experimental Dermatology*, vol. 23, no. 1, pp. 11–13, 1998.
- [45] H. Zhai, N. Poblete, and H. I. Maibach, "Sodium lauryl sulphate damaged skin in vivo in man: a water barrier repair model," *Skin Research and Technology*, vol. 4, no. 1, pp. 24–27, 1998.
- [46] H. Zhai, N. Poblete, and H. I. Maibach, "Stripped skin model to predict irritation potential of topical agents in vivo in humans," *International Journal of Dermatology*, vol. 37, no. 5, pp. 386–389, 1998.
- [47] H. Zhai, Y.-C. Chang, M. Singh, and H. I. Maibach, "In vivo nickel allergic contact dermatitis: human model for topical therapeutics," *Contact Dermatitis*, vol. 40, no. 4, pp. 205–208, 1999.
- [48] S. J. Lau and B. Sarkar, "The interaction of copper(II) and glycyl-L-histidyl-L-lysine, a growth-modulating tripeptide from plasma," *Biochemical Journal*, vol. 199, no. 3, pp. 649–656, 1981.
- [49] C. M. Perkins, N. J. Rose, B. Weinstein, R. E. Stenkamp, L. H. Jensen, and L. Pickart, "The structure of a copper complex of the growth factor glycyl-L-histidyl-L-lysine at 1.1 Å resolution," *Inorganica Chimica Acta*, vol. 82, no. 1, pp. 93–99, 1984.
- [50] J. H. Freedman, L. Pickart, B. Weinstein, W. B. Mims, and J. Peisach, "Structure of the glycyl-L-histidyl-L-lysine-copper(II) complex in solution," *Biochemistry*, vol. 21, no. 19, pp. 4540–4544, 1982.
- [51] L. Pickart and S. Lovejoy, "Biological activity of human plasma copper-binding growth factor glycyl-L-histidyl-L-lysine," *Methods in Enzymology*, vol. 147, pp. 314–328, 1987.
- [52] G. Beretta, E. Arlandini, R. Artali, J. M. G. Anton, and R. Maffei Facino, "Acrolein sequestering ability of the endogenous tripeptide glycyl-histidyl-lysine (GHK): characterization of conjugation products by ESI-MSn and theoretical calculations," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 47, no. 3, pp. 596–602, 2008.
- [53] D. Godet and P. J. Marie, "Effects of the tripeptide glycyl-L-histidyl-L-lysine copper complex on osteoblastic cell spreading, attachment and phenotype," *Cellular and Molecular Biology (Noisy-le-Grand, France)*, vol. 41, no. 8, pp. 1081–1091, 1995.
- [54] M. Kawase, N. Miura, N. Kurikawa et al., "Immobilization of tripeptide growth factor glycyl-L-histidyl-L-lysine on poly(vinylalcohol)-quaternized stilbazole (PVA-SbQ) and its use as a ligand for hepatocyte attachment," *Biological and Pharmaceutical Bulletin*, vol. 22, no. 9, pp. 999–1001, 1999.
- [55] F.-X. Maquart, L. Pickart, M. Laurent, P. Gillery, J.-C. Monboisse, and J.-P. Borel, "Stimulation of collagen synthesis in fibroblast cultures by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu²⁺," *FEBS Letters*, vol. 238, no. 2, pp. 343–346, 1988.
- [56] T. F. Lane, M. L. Iruela-Arispe, R. S. Johnson, and E. H. Sage, "SPARC is a source of copper-binding peptides that stimulate angiogenesis," *Journal of Cell Biology*, vol. 125, no. 4, pp. 929–943, 1994.
- [57] G. S. Schultz and A. Wysocki, "Interactions between extracellular matrix and growth factors in wound healing," *Wound Repair and Regeneration*, vol. 17, no. 2, pp. 153–162, 2009.
- [58] V. Y. Cherdakov, M. Y. Smakhtin, G. M. Dubrovin, V. T. Dudka, and I. I. Bobyntsev, "Synergetic antioxidant and reparative action of thymogen, dalargin and peptide Gly-His-Lys in tubular bone fractures," *Experimental Biology and Medicine*, vol. 4, pp. 15–20, 2010.
- [59] M. Y. Smakhtin, A. I. Konoplyà, L. A. Severyanova, A. A. Kurtseva, and V. Y. Cherdakov, "Reparative activity of different functional group peptides in hepatopathies," *Experimental Biology and Medicine*, vol. 3, pp. 11–17, 2006.

Clinical Study

Freestyle Local Perforator Flaps for Facial Reconstruction

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For the successful reconstruction of facial defects, various perforator flaps have been used in single-stage surgery, where tissues are moved to adjacent defect sites. Our group successfully performed perforator flap surgery on 17 patients with small to moderate facial defects that affected the functional and aesthetic features of their faces. Of four complicated cases, three developed venous congestion, which resolved in the subacute postoperative period, and one patient with partial necrosis underwent minor revision. We reviewed the literature on freestyle perforator flaps for facial defect reconstruction and focused on English articles published in the last five years. With the advance of knowledge regarding the vascular anatomy of pedicled perforator flaps in the face, we found that some perforator flaps can improve functional and aesthetic reconstruction for the facial defects. We suggest that freestyle facial perforator flaps can serve as alternative, safe, and versatile treatment modalities for covering small to moderate facial defects.

1. Introduction

Facial defects caused by trauma or the surgical excision of tumors can be reconstructed using skin grafts, local flaps, or free flaps. Skin grafting of the face presents limitations because of contractures, poor color, and poor texture match. Local flaps harvested from adjacent tissue are aesthetically ideal for small to moderate defects, but restrictions in mobility and limited availability of the overlying skin and soft tissue are major drawbacks of this technique. Sometimes, resolving the defects necessitates a delayed or secondary procedure [1]. The successful reconstruction of facial defects requires single-stage surgery, wherein tissue that matches the color and texture of the face is harvested [2]. To satisfy these demands, the present study used various perforator flaps for facial defects.

This paper reports our experience in using freestyle perforator flaps for facial reconstruction and discusses the advantages and disadvantages of the aforementioned method.

2. Patients and Methods

Between 2004 and 2012, 17 patients underwent facial reconstruction surgery in which perforator flaps were used to cover ear, nasal, perioral, and eyelid defects.

The sample comprised 13 men and four women aged 6 to 73 years old (median age = 63 years).

Retrospective data were obtained from patient demographics, diagnosis, defect location, flap size, source vessel, and complications. These data are shown in Table 1.

To cover the nasal defects, the flaps based on the nasolabial artery or dorsal nasal artery were used. The average flap size was 3.82 cm^2 ($SD = 1.70$). Auricular defects were covered by perforator flaps based on the posterior or superior auricular artery. The average flap size was 3.79 cm^2 ($SD = 2.16$). The mental perforator flaps for reconstruction of the lower lip defects were based on the mental perforator artery. On average, the flap size was 7.19 cm^2 ($SD = 1.22$).

TABLE 1: Patient demographics, defect location, flap size, source vessel, and complications.

Number	Age/sex	Location of defect	Source vessel	Size, cm ²	Complication	Follow-up (month)
1	47/M	Nose	Nasolabial artery	3 × 2	None	12
2	72/F	Nose	Nasolabial artery	2 × 1.5	None	9
3	52/M	Nose	Nasolabial artery	3 × 1.5	None	18
4	62/M	Nose	Nasolabial artery	3 × 2	Congestion	12
5	70/M	Nose	Nasolabial artery	2.5 × 1.5	Congestion	9
6	73/M	Nose	Dorsal nasal artery	2.5 × 1	Partial necrosis	8
7	68/M	Nose	Dorsal nasal artery	1 × 1	None	15
8	70/F	Ear	Posterior auricular artery	2 × 1.5	None	12
9	66/M	Ear	Posterior auricular artery	2 × 1.2	None	12
10	44/M	Ear	Posterior auricular artery	3 × 2.5	Congestion	10
11	63/F	Ear	Posterior auricular artery	1.5 × 1.5	None	36
12	55/F	Ear	Superior auricular artery	1.7 × 2.1	None	10
13	32/M	Ear	Superior auricular artery	2.3 × 1.6	None	18
14	56/M	Lower lip	Mental perforator	4 × 2	None	12
15	70/M	Lower lip	Mental perforator	3 × 2	None	10
16	68/M	Lower lip	Mental perforator	3.5 × 2.5	None	8
17	6/M	Eyebrow	Superficial temporal artery	4 × 1.5	None	48

3. Surgical Technique and Refinements

All the operations were performed under general anesthesia. Existing skin tumors were excised by tumor resection with wide surgical margins whose appropriateness was confirmed by performing frozen section biopsies during the operations. For skin and soft tissue defects caused by trauma, the wounds were debrided until healthy tissue with pin point bleeding could be seen.

A flap was designed adjacent to a defect based on the amount of tissue that remained for reconstruction. The skin paddle was designed slightly larger than the defect size to enable insetting with minimal tension. A Doppler probe was used to identify the perforators, which were dissected meticulously using loupe magnification. Then, the final perforator was selected by the reliability of the caliber and length among the identified perforators. The flaps were elevated and inset into the defect areas along the axis of the perforator by rotation, transposition, or advancement. If a flap needed rotation for insetting, the perforator artery was dissected more meticulously. If advancement was sufficient, perforator skeletonization was unnecessary. The donor site was closed directly in two layers, the dermis and the skin, with minimal undermining. The flap was sutured in two layers in a tension-free manner, after which a slightly compressive dressing was applied.

4. Results

There were seven cases of nasal defects, six cases of auricular defects, three cases of lower lip defects, and one case of eyebrow defect.

In nasal defects coverage, two cases showed venous congestion at the early postoperative stage.

In auricular defects coverage, two patients developed complications: venous congestion and partial flap necrosis.

There were no complications after covering lower lip defects and eyebrow defects with perforator flaps. The donor sites healed completely with direct closure in all cases.

Of four complicated cases, three were reconstructed with rotational flaps and one was treated with advancement flaps.

5. Case Presentation

5.1. Case 1. A 68-year-old male patient with squamous cell carcinoma on his right ala nasi underwent wide excision with a 3–5 mm surgical margin (Figure 1(a)). The defect was reconstructed with a nasolabial artery perforator flap that was slightly larger than the defect. The flap was carefully dissected under loupe magnification and advanced to the defect area (Figures 1(b) and 1(c)). No tumor recurrence was observed during the 3-year follow-up period, and the patient was satisfied with the results aesthetically (Figure 1(d)).

5.2. Case 2. A 65-year-old woman admitted to our department presented with basal cell carcinoma on the left ear concha (Figure 2(a)). The mass was excised with a 3–5 mm surgical margin (Figure 2(b)). To cover the defect, a posterior auricular artery perforator flap was elevated and inset in a flip-flop manner (Figures 2(c) and 2(d)). The donor site was closed directly (Figure 2(e)). The procedure produced aesthetically satisfactory results, and no complications or recurrences were observed during the 5-year follow-up (Figure 2(f)).

6. Discussion

Although some reports indicate that freestyle perforator flaps have been used to cover defects of the trunk or extremities, the use of such flaps for facial reconstruction was only recently introduced [3]. With this concept, operations were carried out on the basis of facial artery perforators, nasolabial

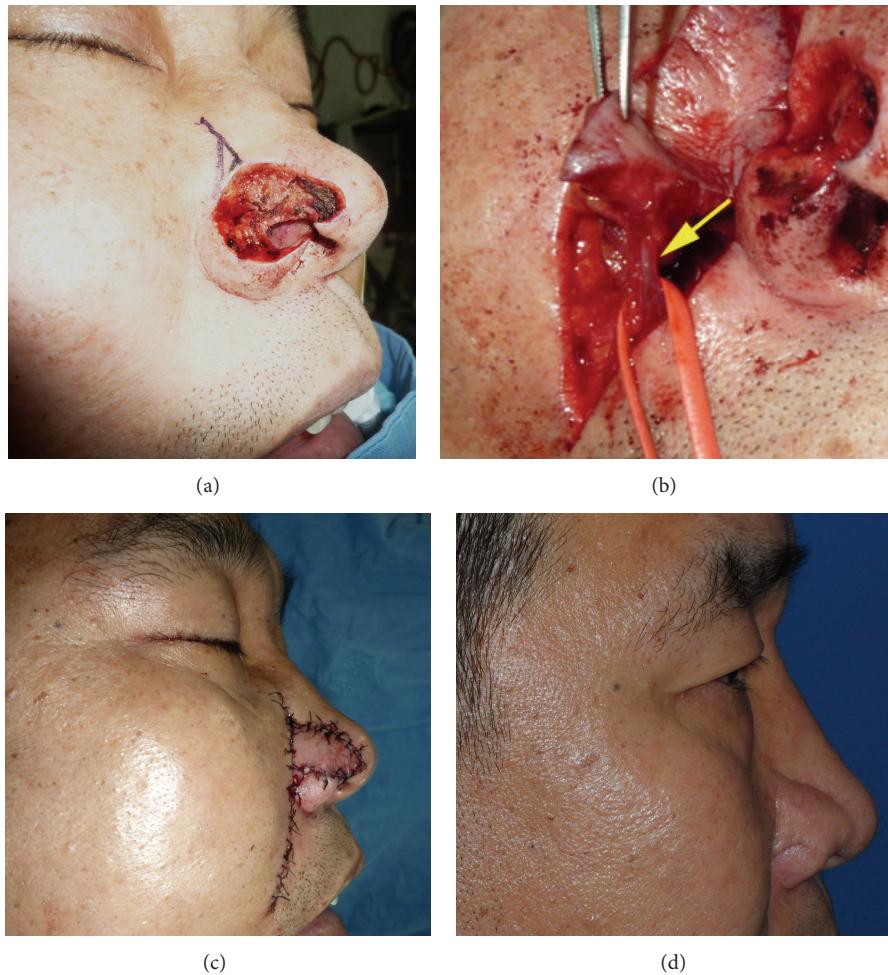


FIGURE 1: (a) Right ala nasi defect after wide excision of squamous cell carcinoma. (b) The nasolabial artery perforator flap was elevated showing its perforator (arrow). (c) Immediate postoperative photo. (d) Follow-up clinical photo 3 years after surgery.

artery perforators, postauricular artery perforators, or submental artery perforators [2].

The use of facial artery perforator flaps for the reconstruction of perioral defects was first described by Hofer et al. in 2005 [3]. Given the anatomical basis of facial artery perforators, however, the surgery was performed in a freestyle manner. In 2009, the introduction of the perforasome enabled better flap design and clinical usage [4]. Ng et al. identified a reference point where facial artery perforators were consistently found to originate in cadaveric studies [5]. The authors also classified three levels of perforator flaps based on the facial artery subsystem that are used to repair defect of the below the jawline, between the jawline and the nasal alae, and superior to the nasal alae up to the glabella [6].

Cordova et al. introduced the retroauricular island flap based on a postauricular artery perforator for the reconstruction of defects in external ear regions, such as the helix, antihelix, conchal surface, antitragus, and external auditory meatus [7].

A pedicled perforator flap on the face can increase flap mobility and provide a flap that contains only necessary tissue and presents cosmetically satisfactory results.

However, the primary closure of the donor site after flap harvest limits the size and location of the pedicled perforator flap on the face. The best areas for pedicled perforator flap harvest are the neck, nasolabial area, temporoparietal area for island flaps, and occipital area [2]. We reviewed English literature on freestyle perforator flaps for the reconstruction of facial defects (Table 2).

Submental flaps for facial and intraoral defect coverage were first described in 1993. These flaps are based on the submental artery, a branch of the facial artery, and have an anterior neck skin paddle which can be an inconspicuous donor site [8, 9].

Nasolabial flaps were the first true perforator flaps in the face used to reconstruct perioral defects. The flaps are based around the facial artery that provides several perforators between the alar base and mandibular area. It can extend to the lateral cheek and lower eyelid [10, 11].

Superficial temporal artery-based perforator flaps can be used as small hair-bearing flaps for reconstructing the eyebrows and mustache. The superficial temporal artery provides two superficial branches: the frontal and parietal branches. The parietal branches serve as the pedicles of the flap [2].

TABLE 2: Various freestyle perforator flaps used for facial reconstruction.

Originating artery	Flap	Pedicle source	Applications
Facial artery	Submental artery perforator flap	Submental artery	Cheek, perioral, intraoral defect
	Nasolabial flap	Superior and inferior labial artery	Perioral, lateral cheek, lower eyelid
	Lateral nasal artery perforator flap	Lateral nasal artery	Nasal dorsum, ala, side wall
	Angular artery perforator flap	Angular artery	Glabella, inner canthal area
	Buccinator flap	Buccal artery	Intraoral defect
Ophthalmic artery	Supratrochlear artery perforator flap	Supratrochlear artery	Nose, periorbital area
	Supraorbital artery perforator flap	Supraorbital artery	Periorbital area
Superficial temporal artery	Superficial temporal artery perforator flap	Superficial temporal artery	Forehead, periorbital area
	Retroauricular flap	Superficial temporal artery Posterior auricular artery	Ear, nose, eyelid, eyebrow, cheek, forehead
External carotid artery	Occipital artery perforator flap	Occipital artery	Chin



FIGURE 2: (a) Basal cell carcinoma on the left ear concha. (b) Skin defect after wide excision of tumor. (c) The retroauricular flap and subcutaneous tunnel between the defect and flap are shown. (d) Immediate postoperative clinical photo. (e) The donor site was closed directly. (f) At 5-year follow-up, the patient was satisfied with the level of scarring.

Furthermore, the superficial temporal artery supplies the auricle and mastoid regions along with the posterior auricular artery. The flap that contains the retroauricular area skin paddle is based on the vascular anastomoses of the superficial temporal artery and posterior auricular artery [12]. The retroauricular flap can be used to cover defects of the nose, ears, eye sockets, eyelid, eyebrow, malar area, and forehead [13–15]. A necessary procedure is to create a subcutaneous tunnel between the pedicle base and the defect area for insetting the flap into the defect.

Occipital artery-based perforator flaps are hair-bearing flaps. The occipital artery originates from the external carotid artery and has three perforator branches: the ascending, transverse, and descending branches [16]. All three perforators can function as reliable vascular pedicles for scalp and neck flaps. The donor site should be covered with a skin graft because of scalp tension. Coverage can extend to the anterior chin, but the flap is more frequently applied to posterior scalp defects.

The perforators are fixed in their locations and supply reliable skin paddles. If a surgeon detects a reliable perforator using a Doppler probe, he/she can use it as a pedicle for flaps that cover other defects on the face. This approach is limited only by the possibility of primary closure of the donor site.

7. Conclusion

With the advance of knowledge regarding the vascular anatomy of pedicled perforators in the face, several very useful perforator flaps have been developed. These flaps can improve functional and aesthetic reconstruction for the face.

We suggest that various facial perforator flaps can serve as alternative, safe, and versatile treatment modalities for covering small to moderate facial defects.

Disclosure

None of the authors have any financial interest in this research or any of the techniques or equipment used.

Conflict of Interests

The authors have no conflict of interests with regard to the publication of this paper.

References

- [1] D. J. Hodgkinson and K. Tavakoli, "The use of mimetic musculature in facial reconstruction," *Australasian Journal of Dermatology*, vol. 36, no. 2, pp. 63–74, 1995.
- [2] S. O. P. Hofer and M. A. M. Mureau, "Pedicled perforator flaps in the head and neck," *Clinics in Plastic Surgery*, vol. 37, no. 4, pp. 627–640, 2010.
- [3] S. O. P. Hofer, N. A. Posch, and X. Smit, "The facial artery perforator flap for reconstruction of perioral defects," *Plastic and Reconstructive Surgery*, vol. 115, no. 4, pp. 996–1003, 2005.
- [4] M. Saint-Cyr, C. Wong, M. Schaverien, A. Mojallal, and R. J. Rohrich, "The perforasome theory: vascular anatomy and clinical implications," *Plastic and Reconstructive Surgery*, vol. 124, no. 5, pp. 1529–1544, 2009.
- [5] Z. Y. Ng, Q. A. Fogg, and T. Shoaib, "Where to find facial artery perforators: a reference point," *Journal of Plastic, Reconstructive and Aesthetic Surgery*, vol. 63, no. 12, pp. 2046–2051, 2010.
- [6] R. Y. Kannan and B. S. Mathur, "Perforator flaps of the facial artery angiosome," *Journal of Plastic, Reconstructive and Aesthetic Surgery*, vol. 66, no. 4, pp. 483–488, 2013.
- [7] A. Cordova, R. Pirrello, S. D'Arpa, and F. Moschella, "Superior pedicle retroauricular island flap for ear and temporal region reconstruction: anatomic investigation and 52 cases series," *Annals of Plastic Surgery*, vol. 60, no. 6, pp. 652–657, 2008.
- [8] D. Martin, J. F. Pascal, J. Baudet et al., "The submental island flap: a new donor site. Anatomy and clinical applications as a free or pedicled flap," *Plastic and Reconstructive Surgery*, vol. 92, no. 5, pp. 867–873, 1993.
- [9] A. Rahpeyma and S. Khajehahmadi, "Submental artery island flap in intraoral reconstruction: a review," *Journal of Cranio-Maxillofacial Surgery*, vol. 42, no. 6, pp. 983–989, 2014.
- [10] S. D'Arpa, A. Cordova, R. Pirrello, and F. Moschella, "Free style facial artery perforator flap for one stage reconstruction of the nasal ala," *Journal of Plastic, Reconstructive and Aesthetic Surgery*, vol. 62, no. 1, pp. 36–42, 2009.
- [11] W. I. Sohn, J. Y. Choi, B. F. Seo, and S. N. Jung, "Reconstruction of nasal ala with nasolabial perforator flap after cancer removal," *Head and Neck Oncology*, vol. 4, no. 4, article 83, pp. 83–87, 2012.
- [12] R. Song, Y. Song, K. Qi, H. Jiang, and F. Pan, "The superior auricular artery and retroauricular arterial island flaps," *Plastic and Reconstructive Surgery*, vol. 98, no. 4, pp. 657–670, 1996.
- [13] D. J. Fader and T. M. Johnson, "Ear reconstruction utilizing the subcutaneous island pedicle graft (flip-flop) flap," *Dermatologic Surgery*, vol. 25, no. 2, pp. 94–96, 1999.
- [14] A. Cordova, S. D'Arpa, R. Pirrello, C. Giambona, and F. Moschella, "Retroauricular skin: a flaps bank for ear reconstruction," *Journal of Plastic, Reconstructive and Aesthetic Surgery*, vol. 61, supplement 1, pp. S44–S51, 2008.
- [15] B. Guyuron, "Retroauricular island flap for eye socket reconstruction," *Plastic and Reconstructive Surgery*, vol. 76, no. 4, pp. 527–533, 1985.
- [16] S. Ono, R. Ogawa, H. Hayashi, Y. Takami, S.-I. Kumita, and H. Hyakusoku, "Multidetector-row computed tomography (MDCT) analysis of the supra-fascial perforator directionality (SPD) of the occipital artery perforator (OAP)," *Journal of Plastic, Reconstructive and Aesthetic Surgery*, vol. 63, no. 10, pp. 1602–1607, 2010.

Clinical Study

Low versus High Fluence Parameters in the Treatment of Facial Laceration Scars with a 1,550 nm Fractional Erbium-Glass Laser

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Purpose. Early postoperative fractional laser treatment has been used to reduce scarring in many institutions, but the most effective energy parameters have not yet been established. This study sought to determine effective parameters in the treatment of facial laceration scars. **Methods.** From September 2012 to September 2013, 57 patients were enrolled according to the study. To compare the low and high fluence parameters of 1,550 nm fractional erbium-glass laser treatment, we virtually divided the scar of each individual patient in half, and each half was treated with a high and low fluence setting, respectively. A total of four treatment sessions were performed at one-month intervals and clinical photographs were taken at every visit. **Results.** Results were assessed using the Vancouver Scar Scale (VSS) and global assessment of the two portions of each individual scar. Final evaluation revealed that the portions treated with high fluence parameter showed greater difference compared to pretreatment VSS scores and global assessment values, indicating favorable cosmetic results. **Conclusion.** We compared the effects of high fluence and low fluence 1,550 nm fractional erbium-glass laser treatment for facial scarring in the early postoperative period and revealed that the high fluence parameter was more effective for scar management.

1. Introduction

Facial laceration is one of the most common trauma cases seen in the outpatient department of plastic surgery. Once the laceration is inflicted, a scar formation of some degree is inevitable no matter how meticulously the operator sutures the wound. This may be observed especially in the facial area, due to the active, constantly functioning facial musculature. To minimize the resultant laceration or surgical incision scars, various scar management and prevention methods have been introduced over recent decades, including intraleisional steroids, radiotherapy, dermabrasion, pressure therapy, cryosurgery, silicone gel sheeting or ointment massage, and surgical excision [1–5]. However, controversy regarding the most effective method remains, despite there being only a few good treatment options for facial scarring. Recently, due to increased demand for scar management, the efficacy of various methods, including laser treatment, is being evaluated [1–5]. Although various types of lasers are being used, standardized parameters for scar management have yet to be established. Thus, we sought to compare the effects of low and high

fluence parameters of 1,550 nm fractional erbium-glass laser treatment for facial scars in the early postoperative period.

2. Methods

This prospective study was designed to establish the most effective fluence parameter. After approval of the Institutional Review Board of Uijeongbu St. Mary's Hospital, a total of 57 patients with Fitzpatrick skin types III–V were enrolled [6]. All patients with facial lacerations primarily sutured in the emergency room by the same senior resident of the plastic surgery department were considered candidates. Patients ranging from 15 to 50 years of age were included. Facial laceration was defined as a laceration within a limited area bordered by the forehead hair line, the preauricular area, and the mandibular angle to the chin. Patients with a facial laceration subcutaneous in depth evenly along the length within 6 cm representing a relatively clean cut without maceration were enrolled. However, patients with acute inflammation, a history of previous trauma, and keloid tendency or those using topical agents including steroids were excluded. Laser



FIGURE 1: 5 × 10 mm handpiece tip was applied along the laceration scar.

treatment began approximately 4 weeks after primary closure. The surface was cleansed with chlorhexidine solution and EMLA cream (a lidocaine-based topical anesthetic cream, AstraZeneca AB, Södertälje, Sweden) was applied for 30 minutes prior to treatment. After removal of the anesthetic cream, the scar was treated with a 1,550 nm fractional erbium-glass laser (MOSAIC HP, Lutronic Co., Ltd., Seoul, South Korea); all patients underwent four sessions at 4-week intervals. In each session, the laceration was virtually divided in half and each half was treated with different parameters: low energy portion (L portion), fluence of 10 mJ/spot, and density of 200 spots/cm²; high energy portion (H portion), 50 mJ/spot, and 40 spots/cm². An equivalent amount of energy was delivered to each portion and the parameters and total energy for each session have been set constantly. With 5 × 10 mm handpiece tip (Figure 1), a total of three shots were delivered to each spot without overlap between the spots. The MOSAIC HP automatically starts with skin contact and stops at the end of each session after delivering a set of energy; hence the operator does not need to control the repetition rate or pulse duration.

The results were evaluated at least 6 months postoperatively utilizing two methods. Every photograph was sequentially obtained by a single photographer under identical camera settings (Nikon D600, 24.3 megapixels, Tokyo, Japan) and lighting conditions. First, assessment was performed using the Vancouver Scar Scale (VSS), which included 4 categories: pigmentation (0 = normal, 1 = hypopigmented, 2 = mixed pigmentation, and 3 = hyperpigmented), pliability (0 = normal, 1 = supple, 2 = yielding, 3 = firm, 4 = ropes, and 5 = contracture), height (0 = flat, 1 = ≤ 2 mm, 2 = 2–5 mm, and 3 = ≥ 5 mm), and vascularity (0 = normal, 1 = pink, 2 = red, and 3 = purple). This was performed by three blinded third-party physicians; each surveyor assigned points for each criterion and from this the total score was calculated. Each portion of the scar in every patient was evaluated separately and VSS score both prior to treatment and after 4 sessions of treatment (6 months postoperatively) was recorded. Second, global assessment was performed by two independent investigators,

TABLE 1: Demographic data of patients included in the study (M: male, F: female).

Patients	57 (M: 25, F: 32)
Mean age (years)	27.3 (range 15–50)
First laser session (days postoperative)	29.1 (range 27–34)
Follow-up (months after last laser session)	14.7 (range 12–23)

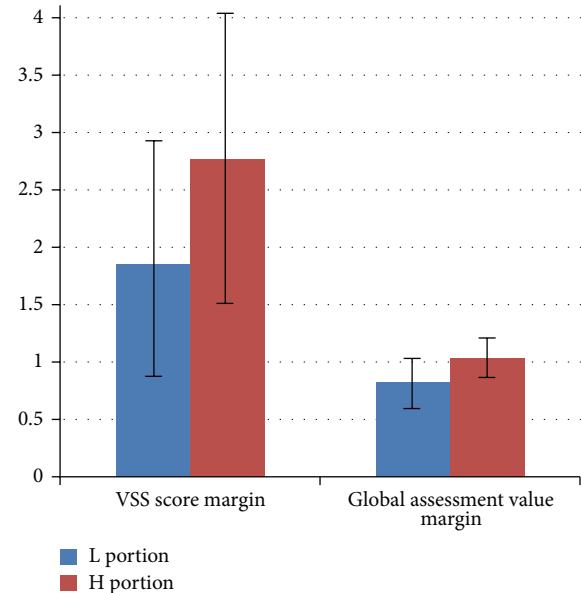


FIGURE 2: VSS score and global assessment value margins of the two scar portions in each patient. Data are expressed in mean ± standard deviation ($p < 0.05$).

who evaluated each portion of the scar before and after laser treatment and categorized the outcome as poor, fair, good, or excellent (poor = 1, fair = 2, good = 3, and excellent = 4). Also being difficult to compare the definite global assessment values, the differences of both portions in each patient were checked for comparison.

3. Results

A total of 57 patients were enrolled. Patient demographics are shown in Table 1. Every patient was evaluated two months after the last treatment session (i.e., 6 months after primary closure). Statistical analyses were conducted using SAS software version 9.3 (SAS institute, Cary, NC, USA) with an independent sample *t*-test, and $p < 0.05$ was considered significant. Because the initial status varied by individual, the difference between pre- and posttreatment scores was used rather than the absolute value for posttreatment score (Figure 2). In every patient, the change observed in the H portion (2.77 ± 1.31) was significantly greater than that seen in the L portion (1.85 ± 1.12) ($p = 0.038$), which suggests that more effective scar management was achieved in the H portion. Global assessment value revealed that the difference of the H portion (1.03 ± 0.18) was significantly greater than the difference of the L portion (0.83 ± 0.21) in every patient

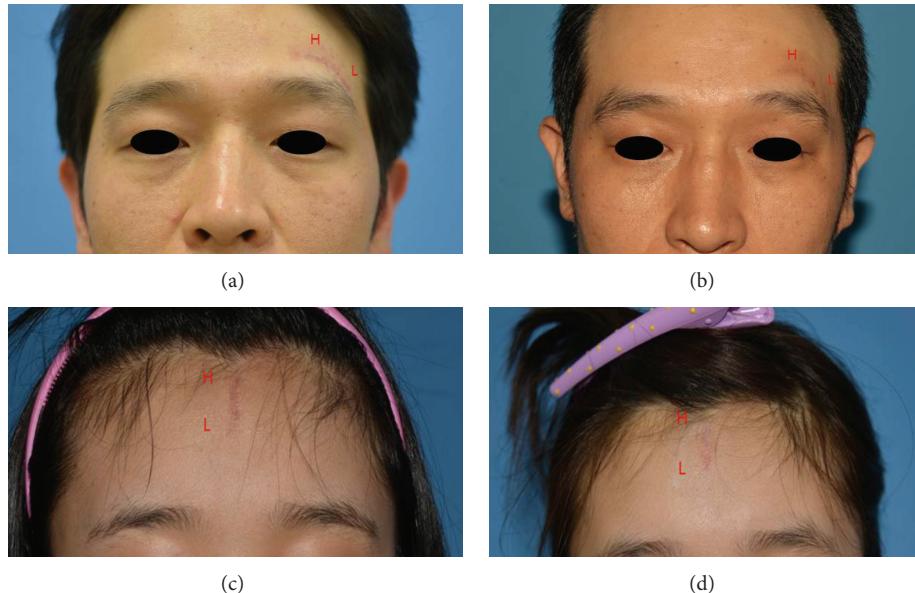


FIGURE 3: Pre- and posttreatment photography of patients 1 and 2. In patient 1, the horizontal scar of left eyebrow region was divided virtually into the left H portion and right L portion (a). Posttreatment photography (b) one month after the final session showed favorable results in the H portion. In patient 2, the vertical scar of forehead was divided virtually into the upper H portion and lower L portion (c). Posttreatment photography (d) one month after the final session showed favorable results in the H portion.

(Figure 3). Complications included erythema, inflammation, discharge, and thermal or radiation burn. However, all complications resolved with conservative treatment.

4. Discussion

In the past decade, many physicians have reported promising results in treatment of scar using carbon dioxide, pulsed dye, and Er:YAG lasers. Although many have reported that lasers are an effective treatment method for scar management, there have been few studies on nonablative fractional lasers. The mechanism for nonablative fractional laser devices involves the creation of microthermal zones of damage in the treated tissue, extrusion of contents, and rapid reepithelialization within 24 hours allowing for rapid epidermal repair via a rapid migratory path for keratinocytes [7, 8].

One of the studies done by Park et al. sought to determine appropriate initial start timing of nonablative fractional laser treatment of scar [9]. They concluded that early treatment (i.e., within 3 weeks) was more effective than delayed treatment (i.e., within 3 months or within 6 months). As studies of other institutions [9–12], we previously performed a study examining the appropriate period for starting laser treatment of facial laceration scar and concluded similarly that early treatment is more effective. Accordingly, we have come to an idea that varying parameters should affect the final cosmetic result of the early treatment. Thus, this paper investigated appropriate energy parameter settings.

The most troublesome aspect of setting up a laser treatment protocol is how to set the case and control criteria to assess the results. Unlike the study led by Park et al.,

where only linear scars of surgical incisions made by the same surgeon were targeted, we investigated random laceration scars. After exclusion of dirty ragged scars from the study, we included relatively clear scars and virtually divided them in half to achieve objective comparison between the two parameter settings. In addition, the length of the scar was included in the exclusion criteria because parts of longer scars might be affected differently by facial muscle function, confounding analysis of scar improvement. Before this study, pilot study comparing nontreated patients and fractional laser-treated patients has been performed in our institute. However, we did not elaborate on the result, since the superiority of final cosmetic result in laser-treated patients over nontreated patients has already been proven in many articles [1, 3–5, 9, 11]. Also, the main purpose of our study is to compare treatment parameters; thus we focused on describing the final outcomes of the different treatment parameters.

Setting up a protocol is another challenge. In the case of a 1,550 nm fractional erbium-glass laser, the total energy is sum of the fluence (pulse energy per effective focal area) multiplied by the total density (treatment spots per square centimeter) in every shot. The purpose of this study was not to evaluate total energy delivered but to compare scar improvement depending on different parameters. Thus, we compared low versus high fluence parameters under constant total energy.

Although narrow-band reflectance spectrophotometry is an alternative manner of assessing scars via the erythema index and melanin index, we excluded this method because it assesses only one aspect of the scar. Moreover, the device is too expensive for widespread use in outpatient departments for clinical follow-up.



FIGURE 4: Depressed scar of nose after four treatment sessions with a fluence of 50 mJ/spot ((a) before laser treatment, (b) one month after the final session).

It is possible that parameters greater than 40 mJ/spot might be more effective for reducing scars. However, in our institution, several patients who were not enrolled in this study received deep second degree burns from parameters greater than 40 mJ/spot (Figure 4). In such cases, conservative dressing will result in healing, but depressed scar formation is possible. Accordingly, further study regarding the effects of parameters greater than 40 mJ/spot on impending hypertrophic scars is needed. In addition, different session numbers might also affect scar treatment. Because our study encompasses only facial lacerations meeting certain criteria, scars in different locations or from different injury mechanisms might require distinct protocols.

5. Conclusion

Laser therapy is a promising method of scar treatment. Our institution compared the effects of high fluence and low fluence 1,550 nm fractional erbium-glass laser treatment for facial scarring in the early postoperative period and revealed that the high fluence parameter was more effective for scar management. Future studies should investigate the optimal number of sessions or protocols for scars in different locations.

Conflict of Interests

None of the authors have any financial interest in this research project or any of the techniques or equipment used.

References

- [1] J. S. Cooper and B. T. Lee, "Treatment of facial scarring: lasers, filler, and nonoperative techniques," *Facial Plastic Surgery*, vol. 25, no. 5, pp. 311–315, 2009.
- [2] C. W. D. Chang and W. R. Ries, "Nonoperative techniques for scar management and revision," *Facial Plastic Surgery*, vol. 17, no. 4, pp. 283–288, 2001.
- [3] J. R. Thomas and M. Somenek, "Scar revision review," *Archives of Facial Plastic Surgery*, vol. 14, no. 3, pp. 162–174, 2012.
- [4] S. Kim, T. H. Choi, W. Liu, R. Ogawa, J. S. Suh, and T. A. Mustoe, "Update on scar management: guidelines for treating Asian patients," *Plastic and Reconstructive Surgery*, vol. 132, no. 6, pp. 1580–1589, 2013.
- [5] M. H. Gold, M. McGuire, T. A. Mustoe et al., "Updated international clinical recommendations on scar management: part 2—algorithms for scar prevention and treatment," *Dermatologic Surgery*, vol. 40, no. 8, pp. 825–831, 2014.
- [6] W. E. Roberts, "Skin type classification systems old and new," *Dermatologic Clinics*, vol. 27, no. 4, pp. 529–533, 2009.
- [7] J.-H. Choe, Y.-L. Park, B. J. Kim et al., "Prevention of thyroidectomy scar using a new 1,550-nm fractional erbium-glass laser," *Dermatologic Surgery*, vol. 35, no. 8, pp. 1199–1205, 2009.
- [8] H. J. Kim, T. G. Kim, Y. S. Kwon, J. M. Park, and J. H. Lee, "Comparison of a 1,550 nm Erbium:Glass fractional laser and a chemical reconstruction of skin scars (CROSS) method in the treatment of acne scars: a simultaneous split-face trial," *Lasers in Surgery and Medicine*, vol. 41, no. 8, pp. 545–549, 2009.
- [9] K. Y. Park, I. Y. Oh, S. J. Seo, K. H. Kang, and S. J. Park, "Appropriate timing for thyroidectomy scar treatment using a 1,550-nm fractional erbium-glass laser," *Dermatologic Surgery*, vol. 39, no. 12, pp. 1827–1834, 2013.
- [10] R. R. Anderson, M. B. Donelan, C. Hivnor et al., "Laser treatment of traumatic scars with an emphasis on ablative fractional laser resurfacing: consensus report," *Journal of the American Medical Association Dermatology*, vol. 150, no. 2, pp. 187–193, 2014.
- [11] S. G. Kim, E. Y. Kim, Y. J. Kim, and S. I. Lee, "The efficacy and safety of ablative fractional resurfacing using a 2,940-Nm Er:YAG laser for traumatic scars in the early posttraumatic period," *Archives of Plastic Surgery*, vol. 39, no. 3, pp. 232–237, 2012.
- [12] T. Ohshiro, T. Ohshiro, and K. Sasaki, "Laser scar management technique," *Laser Therapy*, vol. 22, no. 4, pp. 255–260, 2013.