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Effect of Lyophilized Growth Factors (LGF) Derived from Equine Platelets on Experimentally Induced Skin Wound Healing in Mongrel Dogs

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ABSTRACT

Lyophilized growth factor (LGF) is a novel advanced platelets rich protein growth factor. It has been successfully applied in various fields of regenerative medicine including management of chronic and non-healing wounds and ulcers. Our study aimed to evaluate the effect of subcutaneous infiltration of equine platelets derived lyophilized growth factors on healing of induced full thickness skin wound in dogs. Four healthy mongrel dogs of different ages were used in the current work. Three bilateral critical sized skin wounds were done on the back of each dog. After 24 hours, the right-side wounds were injected in 4 cardinal points around each wound by Saline/lidocaine (control wounds) and simultaneously reconstituted LGF was subcutaneously injected around the left side wounds (LGF treated wounds). Wound contraction was monitored physically and histopathologically. The expression of TGF-β1 and *NF-κB* was evaluated in wound specimens of both groups. A significant reduction in wound size was recorded in LGF treated group compared to saline treated group. The histopathological scoring of the healing progress revealed significant increase in the degree of re-epithelization bridging the wound edges and collagen deposition in LGF-treated wounds compared to control non-treated wounds along the experimental periods. Additionally, the expression of TGF-β1 and *NF-κB* showed significant elevation in LGF-treated wounds compared with their expression in control wounds. In conclusion, LGF therapy could be a superior candidate as a regenerative therapy in skin wounds that can positively impact healing process of the cutaneous wounds.

Key words: Skin wound, Healing, PRP, LGF, Pathology, Dogs

INTRODUCTION

Skin wound healing is a challenging complicated mechanism that occurs during interaction of multiple regulators such as growth factors, integrins, matrix metalloproteinases (MMPs), keratins, cytokines, chemokines and extracellular macromolecules (Lazaro et al. 2016). Platelets play a crucial role in the physiological process of wound healing. There has been a lot of research in the past ten years using platelets-enriched biomaterials in experimental and clinical studies involved in different areas of regenerative medicine as wound healing and tissue engineering. Platelets rich protein (PRP) is widely applied in different clinical approach to accelerate the healing process in wound therapies (Badis and Omar 2018), ophthalmology (Ribeiro et al. 2017), and dentistry (Alishahi et al. 2014). PRP could be prepared by different commercially available systems. Due to the differences in platelet technical details related to the method and time of centrifugation, there are wide variations in the final platelets concentrate obtained. Differences in platelets concentration influence growth factors concentration Moreover, the integrity and quality of the platelets may be damaged (Dhurat and Sukesh, 2014). To overcome the disadvantages of PRP and enhance the wound healing process, several researches have attempted to develop novel devices. Among those devices is the Lyophilized Equine Platelets Growth Factors, a novel, advanced, standardized and refined form of platelets growth factors based on the use of allogenic pathogen free platelets from horses instead of autologous platelets as a source of growth factors. Generally, LGF have a much longer shelf-life of the final lyophilized growth factors when compared to the autologous PRP (12-18 months versus 4-8 hours or even less) (Bausset et al. 2012).

There is a general agreement that, a great deal of homology is present in the structure and functions of growth factors across mammalian species: Fetal bovine

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serum is the most universally applicable cell culture additive for the stimulation of human and other mammalian cell proliferation and biological production, because of its rich content of growth factors (Hemeda et al. 2014). Canine sourced platelet-rich plasma has been successfully used in the management of a feline contaminated cutaneous wound (Gemignani et al. 2017). Human platelet rich plasma had a positive effect on impact-induced chondrocyte apoptosis in rabbits (De Rezende et al. 2011). Accordingly, we decided to test the role of equine platelets derived growth factors in the management of wounds in dogs. Additionally, to the authors knowledge, there is no literature study on the effect of equine platelets derived lyophilized growth factors effect on skin wound healing in dogs, hence, the aim of present study was to evaluate the efficacy of subcutaneous infiltration of equine platelets derived lyophilized growth factors on healing of induced full thickness skin wound in dogs.

MATERIALS AND METHODS

Experimental Animals

Four apparently healthy male mongrel dogs of different ages (1½-2 years), weighing (15-20 kg) were used in the present study. Dogs were housed individually at the kennels of the of the Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Cairo University. Animals were kept under standard environmental conditions of 12 hrs light/dark cycle, 25ºC, 55±5% humidity. The study was approved by the Institutional Animal Care and Use Committee (IACUC), CU/II/108/18.

Preparation of LGF

LGF was produced according to a patented method (Code number: WO2018091713) developed by Dr Hossam M Fahmy, Professor of Laboratory and Transfusion medicine, Ain Shams Medical School, Cairo, Egypt. PRP was collected by pheresis machine. The collected PRP was subjected to UV/Riboflavin treatment by the Mirasol system (Terumo BCT, Lakewood, Colorado, USA), for pathogen inactivation and viral reduction. This was followed by *in vitro* stimulation of platelets by Calcium Chloride and Thrombin for the release of growth factors from the alpha granules. The released growth factors were purified and separated from the cellular debris and fibrin clot, then exposed to a second step of viral inactivation by the Solvent/Detergent method, followed by sterile filtration. Thus, all risks of any potential microbiological contamination have been eliminated. The purified sterile growth factors were aseptically dispensed in sterile vials. Each vial contained a concentration of growth factors equivalent to that obtained from 20 ml of whole blood of a normal donor which ensures the standardization of concentrations of growth factors and clinical effects, within the same batch and between different batches.

Induction of Skin Defect and Experimental Design

After one-week accommodation, general anesthetic regimen was applied on all dogs after overnight fasting; premedication by subcutaneous injection of 0.1mg/kg atropine sulfate (Atropine sulphate ®1%, Adwia Co., Egypt), intramuscular injection of 1mg/kg xylazine HCl (Xyla-Ject ® 2%, Adwia Co., Egypt) followed by intravenous injection of 10mg/kg ketamine (Ketamine ®, Sigmatec, Egypt) (Mckelvey and Hollingshead 2003). Under complete aseptic precautions; three bilateral full-thickness circular critical sized skin wounds (2 cm) were created on the back of each dog using dermal punch (Karayannopoulou et al. 2011). The day 0 was the day of induction of the wound, the right-side wounds were saline/lidocaine treated (Control wound) and the left side wounds were LGF treated (LGF treated wounds).

Treatment with LGF

Twenty-four hours after induction of skin wound, 2 ml reconstituted LGF (using normal saline and lidocaine (lidocaine HCl (Debocaine ®, ADWIC /El-Debeiky, Egypt)) was once injected subcutaneously in 4 cardinal points around the edge of each wound in the LGF treated wounds and simultaneously injected 2ml of normal saline and lidocaine in control wounds. Animals were monitored along the experimental period (14 days) and all care was expressed to decrease animal suffering.

Clinical Evaluation of Wound Healing

Healing of all wounds was monitored daily and digitally photographed for measuring the wound contraction (El-Tookhy et al. 2017). The reduction of wound size was calculated as a percent using the following equation:

$$\text{Percent of the wound size at the day (x)} = \frac{Ai - Af}{Ai} \times 100$$

where Ai is the initial wound area at zero day, and Af is the wound area at day 3 or 7 or 14 post-wound induction (Zhang et al. 2015).

Histological Evaluation of the Wounds

On days 3, 7 and 14, experimental dogs under complete general anesthesia, two tissue samples from the edges of each wound on both sides were harvested using dermal biopsy. Samples were fixed in 10% buffered neutral formalin. Formalin fixed wound specimens were routinely dehydrated in alcohol, cleared in xylene, embedded in paraffin, sectioned into 5µm thickness and finally stained with H&E (Suvarna et al. 2012). The obtained sections were subjected to histopathological examination using the electric light microscope Olympus BH2 (Tokyo, Japan). The presence of polymorphonuclear cells, the degree of re-epithelialization (for formation of new epithelial layer), fibroblasts proliferation and angiogenesis were all scored semi-quantitively between 0 and 4 (where 0 indicates no change and 1, 2, 3 and 4 indicate minimal, mild, moderate and marked changes respectively (Table 1) (Sabol et al. 2012).

Van Gieson's stain was used for evaluation of type 1 collagen fibers deposition. The area percent (expressed as optical density) of the red stained collagen fibers by Van Gieson's was quantified in 5 high power microscopic fields using image analysis software (ImageJ, 1.46a, NIH, USA).

Immunohistochemical Evaluation of TGF-β1and *NF-Kb*

For recognition of TGF-β1 and *NF-Kb* reactive cells, immunohistochemical procedures were carried out on 4

µm paraffin sections of skin wounds specimens of control and LGF-treated groups at 3rd, 7th and 14th days post wounding, using avidin-biotin peroxidase according to the method described by Hsu et al. (1981). Briefly, paraffin sections were deparaffinized in toluene, rehydrated in ethanol, and then incubated with H_2O_2 for blocking the endogenous peroxidase activity. The sections were incubated with a monoclonal antibody for TGF-β1 and *NF-Kb* (Dako Corp, CA, USA) at dilutions recommended by the manufacturer. Hematoxylin (Sigma-Aldrich) was used as a counterstained for visualization of the nuclei. The positive immune reactive cells for each marker were visualized using the chromagen 3, 3-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, MO, USA). Immunohistochemical quantification of TGF-β1 and *NF-Kb* was carried out using image analysis software (ImageJ, 1.46a, NIH, USA) by measuring the optical density of each marker expression in 5 high power microscopic fields, the area for each microscopic field was 18.8913Sqmm.

Statistical Analysis

Statistical analysis was performed by the PASW Statistics, Version 18.0. software (SPSS Inc., Chicago, IL, USA). Statistical analysis of data was carried out by using independent sample T-test. Results was expressed as a mean±SD at $P<0.05$.

RESULTS

Clinical Follow Up

At zero-day post wounding, the size of both control and LGF-treated wounds was the same, while starting from the third day, the LGF-treated wounds showed rapid reduction of wound size compared with the control saline-treated wounds (Fig. 1). The diameter of the induced skin wounds showed significant difference ($P<0.05$) between groups in various evaluation times (Table 2). The percentage of wound size reduction was significantly higher in LGF-treated wounds in comparison with that of the control wounds at days 3, 7 and 14 (Table 2).

Histopathology

Three days post wounding, control wounds revealed complete loss of the epidermal layer, massive necrosis, and heavy polymorphonuclear cells (PMNCs) were seen infiltrating the top of wounds (Fig. 2a) with edema and tiny foci of hemorrhages whereas fibroblasts and macrophages were seen at the base of the wounds (Fig. 2b). While the LGF-treated wounds showed inflammatory reaction and necrosis at the top area and the wound appeared covered with necrotic debris and degenerated PMNCs (Fig. 2c) as well as slightly irregular granulation tissue formation filling the deep wound area with several small vessels formation (angiogenesis) (Figure 2d). At 7 days post wounding, control wounds showed beginning of epithelial proliferation at the wound edges (Fig. 2e) and fibrous connective tissue proliferation that was rich in immature fibroblasts and few angiogenesis (Fig. 2f). However, the LGF -treated wounds 7 days post wounding showed active re-epithelization at which the wound edge was entirely covered by epithelial tissue but sometimes with no connection of the edges of wound (Fig. 3a). Well-

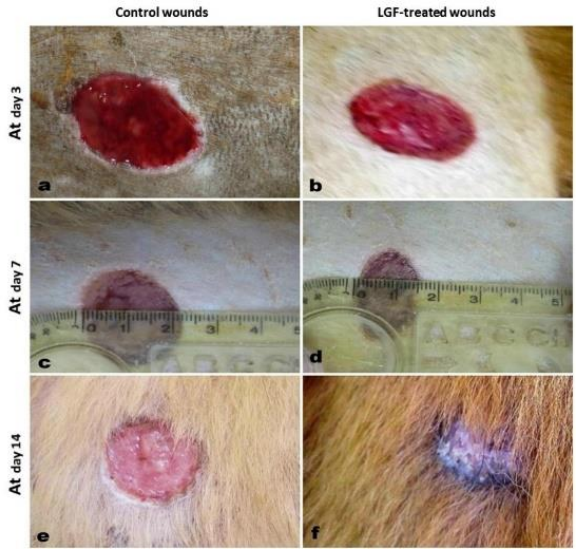

Fig. 1: Clinical evaluation of wound healing in control and LGF-treated wounds at 3, 7- and 14-days post wounding.

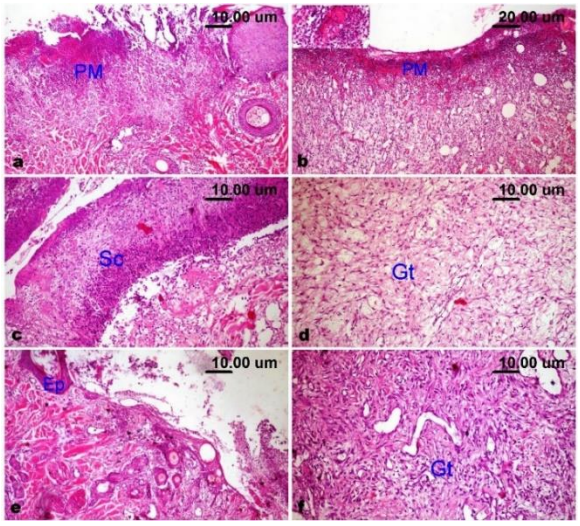

Fig. 2: H&E stained sections of skin of dog, (a and b) Control wound 3 days post wounding showing complete loss of the epidermal layer, massive necrosis, and heavy PMNCs infiltrating the top of wound (PM) (b), whereas fibroblasts and macrophages present at the base of the wounds (b). (c and d) LGF- treated wound 3 days post wounding showing inflammatory reaction and a scab (Sc) of necrotic debris covers the top area of the wound (c) and slightly irregular granulation tissue with several angiogenesis filling the deep wound area (d). (e and f) Control wound 7 days post wounding, showing beginning of epithelial (Ep) proliferation at the wound edges (e) and granulation tissue rich in immature fibroblasts and few angiogenesis (f).

organized granulation tissue was observed beneath the epithelium that was rich in fibroblasts and marked angiogenesis (Fig. 3b). Fourteen days post wounding, control wounds revealed complete covering of the wound gap with irregular re-epithelization (Fig. 3c), subepithelial edema and organized granulation tissue with newly formed blood capillaries (Fig. 3d) and few collagen bundles deposition. Interestingly, the LGF-treated wounds showed significantly advanced re-epithelization with keratinized epithelium covering the wound edges (Fig. 3e) and well-organized collagen bundles having a parallel orientation were densely filled the wound (Fig. 3f).

Table 1: The scoring scale for different histological observations (Sabol et al. 2012)

The score	The indication of the score
Zero	Indicated no epithelization and absence of fibroblasts, PMNL or newly formed blood vessels.
1	Indicated augmentation of the epithelium cut edges with presence of fibroblasts and PMNL in few numbers and diminished number of newly formed blood vessels.
2	Indicated epithelial cells migration, the number of PMNL and fibroblasts is moderate with temperate number of newly formed blood vessels.
3	Indicated epithelial bridging of the wound edges, large number of fibroblasts and PMNL with increased number of newly formed blood vessels.
4	Indicated complete epithelial regeneration, the number of fibroblasts and PMNL is immoderate with extravagant newly formed blood vessels.

PMNL: Polymorphonuclear leukocytes.

Table 2: wound diameter and percentage of wound reduction size compared to initial wound size in both control and LGF-treated groups

		At zero day	At day 3	At day 7	At day 14
Diameter of wound	Control group	2.00±0.00	1.87±0.06^{b}	1.53±0.15^{b}	1.40±0.10^{b}
	LGF-treated group	2.00±0.00	1.47±0.15^{a}	1.27±0.06^{a}	0.87±0.15^{a}
Percentage of wound reduction size	Control group	0	6.5±3^{b}	23.5±8^{b}	30±5 ^{b}
	LGF-treated group	0	26.5±8^{a}	36.5±3^{a}	56.5±8 ^{a}

Data are presented as Mean±SD: $^{a, b}$ Different superscripts indicate significant difference P<0.05 between groups at each evaluation time.

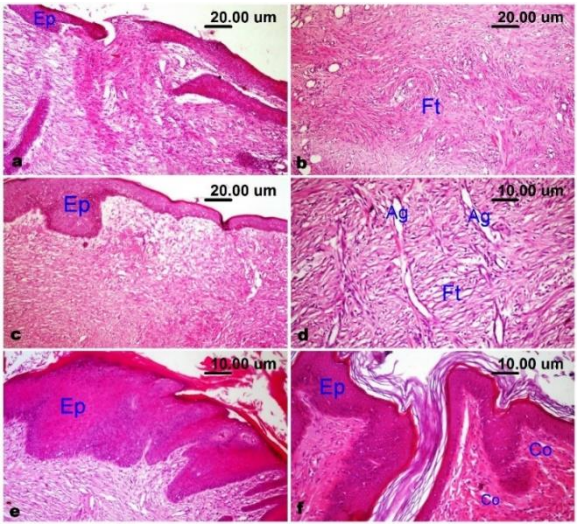

Fig. 3: H&E stained sections of skin of dog, (a and b) LGF-treated wound 7days post wounding showing active re-epithelization (Ep) at the wound edge with no connection (a) and well- organized fibrous tissue (Ft) rich in fibroblasts and marked angiogenesis (b). (c and d) control wound 14-days post wounding showing complete covering of the wound gap with irregular re-epithelization (c), subepithelial edema, organized fibrous tissue (Ft) with newly formed blood capillaries (Ag) and few collagen bundles deposition (d). (e and f) LGF-treated wounds 14-days post wounding revealing advanced re-epithelization (Ep) with keratinized epithelium covering the wound edges (e) with well-organized collagen bundles (Co) having a parallel orientation and densely filled the wound (f).

Generally, the histopathological examination indicated that: there were significant differences in the pattern of wound healing among the two wounds. The scoring of the wound reaction in both wounds (Fig. 4A) at 3days post wounding revealed that PMNCs infiltration was higher in the control wounds, while migrating fibroblasts and angiogenesis were higher in the LGF-treated wounds than in control wounds, the degree of re-epithelization was low in both groups at this period of time. While, 7days post wounding, PMNCs infiltration was lesser in the LGF-treated wounds than the controls, however, the re-epithelization and migrating fibroblasts as well as angiogenesis were significantly higher in the LGF-treated wounds compared with those of controls. 14 days post wounding, the application of LGF enhanced the re-epithelization and angiogenesis as well as increased the number of migrating fibroblasts which all showed significant increase accompanied with significant decrease in the number of PMNCs compared with those of control wounds.

Sections stained with van Gieson's stain revealed that: wounds treated with LGF showed significant deposition of collagen fibers that appeared well organized and of parallel orientation with an obvious no separation between fibers (Fig. 4B). However, control wounds showed inconsistent disorganized collagen with random orientations.

The effect of LGF-Treatment on the Expression of TGF-β1 and NF-kB Activities

Immune-stained skin wounds' sections of both groups at various times intervals revealed increased expression of both TGF-β1 (Fig. 5) and NF-kB (Fig. 6) in LGF-treated wounds compared with control wounds. The intensity of the positive brown color of both markers' expression was significantly higher in LGF-treated wounds compared with control wounds in all the evaluation times (Fig. 7) denotinga significant enhancement of TGF-β1 as well as NF-kB expression was achieved by LGF-treatment starting from the 3rd day of wounding till the 14th day.

DISCUSSION

LGF is a novel advanced growth factor that overcomes the disadvantages of autologous PRP. LGF is a leucocyte free product because of the multiple steps taken in preparation and lyophilization. Recent scientific research has shown the controversial role of white blood cells in a PRP preparation may play. PRP rich in white blood cells is able to promote the healing process, by eliminating the potential microbiological pathogens and stimulating the release of growth factors. Yet, and on the other hand, a big number of leukocytes in a platelet rich suspension may exert an inhibitory effect (Pavlovic et al. 2016). Several research papers have proposed to exclude Leukocytes from PRP preparations used in management of bone defects, chronic tendon injury and osteoarthritis

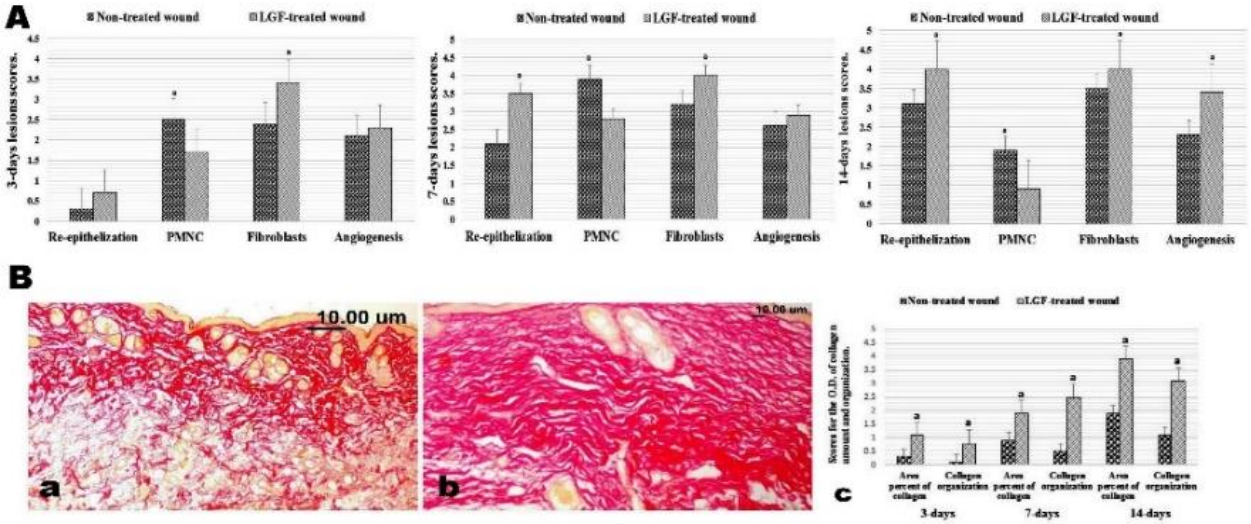

Fig. 4A: The scoring of the wound reaction in both groups at 3days. Fig. 4B: (a) 7days (b) and 14days (c) post wounding showing significant enhancement of re-epithelization, decreased polymorphonuclear cells infiltration as well as significant fibroblasts migration and angiogenesis all in the LGF-treated wounds compared with the control wounds, (image analysis software, ImageJ, 1.46a, NIH, USA). (B) Skin wound sections stained with van Gieson's stain showing inconsistent disorganized collagen fibers in the control wounds (a), deposition of well-organized, of parallel orientation collagen fibers with no separation between fibers in the LGF-treated wound (b) and (c) scores for the area percent of positive red color collagen fibers and its degree of organization at different periods of time in both wounds (image analysis software, ImageJ, 1.46a, NIH, USA). [a] indicates significant difference between groups at P<0.05.

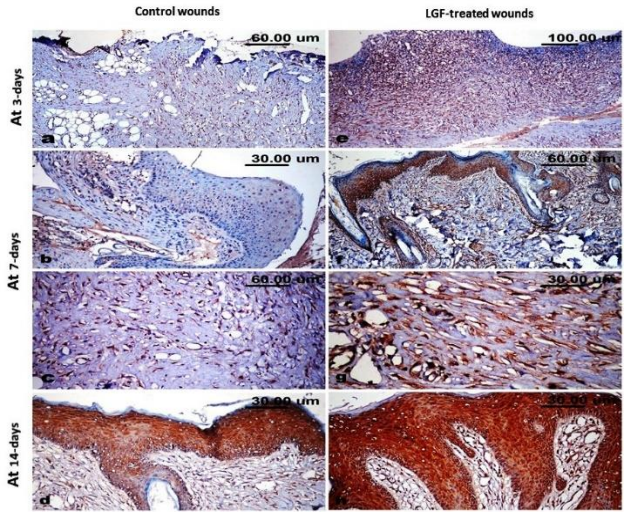

Fig. 5: Immune-stained skin wounds' for TGF-β1 showing increased expression of TGF-β1 (positive brown color) in LGF-treated wounds compared with control wounds at various periods of time.

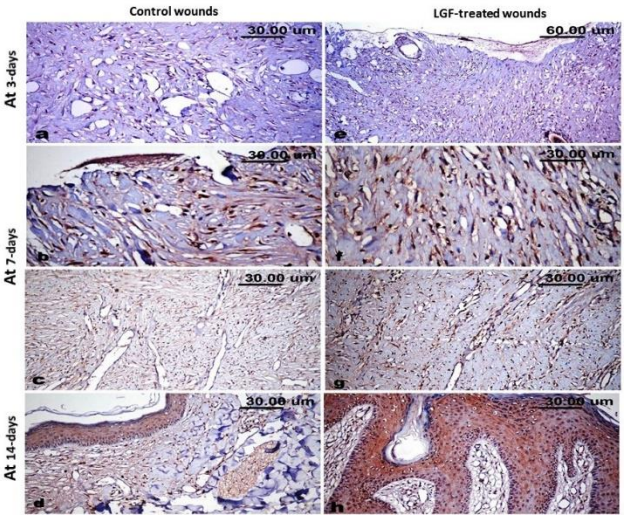

Fig. 6: Immune-stained skin wound for NF-kB showing increased expression of NF-kB (positive brown color) in LGF-treated wounds compared with control wounds at various periods of time.

and osteoarthritis (Wang et al. 2018). However, the ability of the PRP to influence the biological response of different cells as endothelial cells and fibroblasts in vitro, does not rely on the presence of leukocytes (Gusti et al. 2018).

In addition to the absence of white blood cells, LGF preparation is fibrinogen free as well. Although fibrin gel resulting from the interaction between fibrinogen found in the alpha granules and the PRP activator, thrombin and/or calcium chloride, may be useful as a controlled-release carrier of growth factors, the formed fibrin gel reduces the final product volume, hinders the easy injection of PRP, and is not preferable in many clinical situations (Araki et al. 2012). Platelets rich plasma (PRP) for horses are a well-established modality for management of wound healing as well as various degenerative and traumatic injuries of the musculoskeletal system (Carter et al. 2003).

Standardizing the amount of growth factors in each vial to the equivalent to those coming from platelets found in 20ml of whole blood. Having a Fibrinogen depleted, water soluble product for easy administration, with no gel formation was formed. This is particularly significant for intra-articular injections.

In the current study, bilateral full thickness circular wounds were created on the back of six dogs and subcutaneous infiltration of LGF was performed in four cardinal margins of the wound to stimulate the production of excessive inflammatory exudates (Shayesteh 2012). The LGF treatment induced early and better wound contraction and closure compared to control wounds at

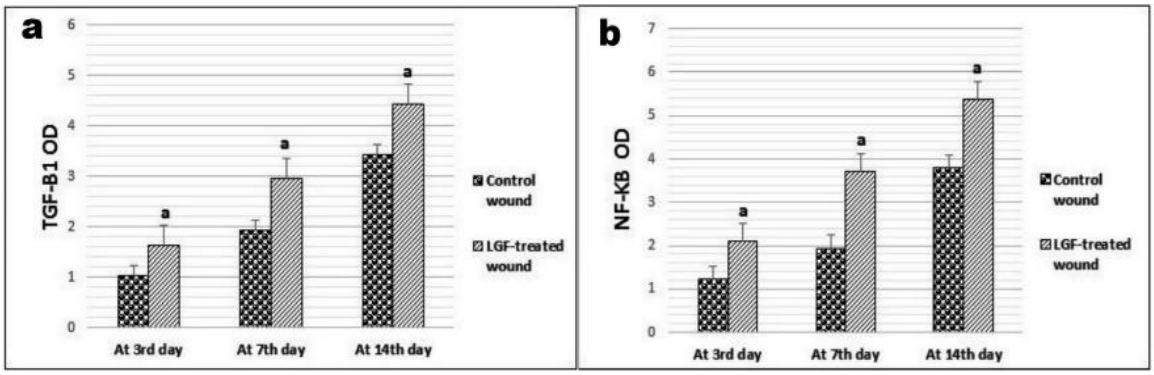

Fig. 7: The area percent of TGF-β1 and NF-kB expression represented by the optical density showing significant enhancement of both markers' expression achieved by LGF-treatment (image analysis software, ImageJ, 1.46a, NIH, USA). [a]indicates significant difference between groups at P<0.05.

days 3, 7, and 14 post-wounding as a result of reduced scar widths and increased collagen maturation. Early deposition of healthy granulation tissue resulted in complete healing and covering the wound with regenerated skin in LGF-treated wounds, while in saline treated wounds there were delayed crustation, deposition of unhealthy granulation tissue and delayed closure of the wound. The narrowest scar widths and largest collagen deposition areas were observed in the LGF group at days 14 post-wounding, compared to the saline treated group, which agreed with the results of Farghali et al. (2017).

The clinical monitoring revealed significant reduction in wound size in the LGF-treated wounds with 26.5, 36.5 and 56.5% at the 3rd, 7th, 14th day, respectively when compared to control wounds 6.5, 23.5 and 30% at the same periods. This could be imputed to the role of LGF in contraction as well as re-epithelization of the wound. Several signaling cytokines and growth factors are supplied to the wound area by the addition of PRP, those factors are important to stimulate angiogenesis and coordinate inflammation and remodeling of the newly formed tissue during wound healing (Andia and Abate 2013). A higher concentration of growth factors can promote the epithelial and endothelial regeneration, stimulates angiogenesis, enhances collagen deposition, and finally accelerates the healing process (Jee et al. 2016).

Histopathologically, 3days post wounding, the deep wound area in the LGF treated wounds showed granulation tissue formation with angiogenesis, while active re-epithelization was noticed at the 7th day post wounding with well- organized granulation tissue and marked angiogenesis. Advanced re-epithelization and keratinization as well as collagen remodelling were all noticed 14 days post wounding with well-organized collagen bundles compared to the control wounds which showed irregular re-epithelization with organized granulation tissue and few collagen bundles deposition.

The scoring of the wound reaction in both groups revealed that the degree of re-epithelization, fibroblasts migration and angiogenesis was significantly higher in LGF-treated wounds than the control ones accompanied with significant decrease in the number of PMNCs in the LGF-treated wounds along the study period, which all indicated a conspicuous LGF-enhancement of wound healing. Platelet rich plasma therapy was considerably preferable for complete wound healing (Marissa et al. 2011).

LGF is a refined form of platelets growth factors that share together in stimulation of wound healing and contraction through several pathways such as promotion of fibroblastic proliferation into myofibroblasts, synthesis of new components of the extracellular matrix and collagen deposition which all pull the skin internally (Golebiewska and Poole 2015). Among those growth factors: the vascular endothelial growth factor (VEGF), TGF-β and platelet-derived growth factor**.** VEGF is well-known to encourage angiogenesis which showed significant increase in our work in wounds treated with LGF which induced earlier neovascularization than in control non-treated wounds (Swaim 2001).

During healing of skin wounds, the neovascularization is essential for the arrival of inflammatory cells with the release of many cytokines that promotes the healing process. While TGF-β was previously reported to promote epidermal regeneration correlated with healing of skin wounds (Jee et al. 2016). In the current work, TGF-β1 showed significant immunopositivity in sections of LGF-treated wounds which showed advanced and accelerated re-epithelization than in the control wounds. That increase in re-epithelization could be mediated by both gelatinase A and gelatinase B expression in wound's area, because both gelatinases play an important role in cell migration and active re-epithelization (Gill and Parks 2008). TGF-β1 and NF-kB were previously reported as chief controllers of inflammatory mediators' release with a subsequent inception of fibroplasia and deposition of collagen**.** LGF treatment in the current study induced significant expression of NF-kB in the treated wounds, which agreed with the findings of El-Hamoly et al. (2014), who pointed out to the relation between the activation of the NF-κB pathway and the excessive deposition of collagen which was confirmed in our work by significant, well-organized collagen deposition on van Gieson's staining in LGF-treated wounds than in control ones. The tensile strength of the healed skin wound is usually evaluated by the amount of collagen deposition and its arrangement which determine the conservation and elasticity of skin (Farghali et al. 2017).Van Gieson's stain in the current study revealed that, in LGF-treated wounds, collagen bundles deposition in the wound area were of parallel

organization, regularly oriented without separation which aids in the increased stretching force of the wound compared with the control wounds which showed randomly organized collagen deposition. Consequently, LGF treatment has a significantly enhanced wound reduction effect compared to that observed in the saline treated group.

Conclusion

On the basis of our results, LGF therapy could be a superior candidate as a regenerative therapy in skin wounds' healing that can positively impact healing process of the cutaneous wounds and the associated signs such as pain, inflammation and infection. Additionally, LGF could be used as such or incorporated in other bioactive formulae as hydrogels and scaffolds for different purposes of regenerative therapies.

Author's Contribution

All authors were involved in the treatment of the experimental cases with LGF. All authors prepared the manuscript and approved the final version of the manuscript.

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