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Evaluation the Effect and Efficacy of Autologous Lyophilized Advanced Platelet- Rich Fibrin on Full Thickness Wound Healing in Dogs

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Abstract

PLATELET RICH FIBRIN (PRF) is a biological and innovative therapeutic which has important role in regenerative injured tissue. The aims of this study were to prepare lyophilized-PRF and determine its applications during wound healing, depending on histopathological and immunohistochemistry parameters. Six healthy, adult local breed dogs were used, the animals were randomly divided into 2 experimental main groups (treated and control) 6 animals for each group, ten ml of whole blood was aspirated from the same animal for platelet rich fibrin preparation and centrifugation of this fresh blood at 3000 rpm for 10 minutes was performed, the resulted PRF layer was removed to prepare lyophilized PRF by freezing and was stored at −80C∘ then the sample was freeze-dried for 12 hours by using Labconco lyophilizer at −51C∘ Surgical full thickness incision wound of 5cm length was performed under general anesthesia on back of dogs under aseptic technique, treated group Lyophilized A-PRF (Right side) was applied subcutaneously while normal saline 0.9% was applied instead of Lyophilized A-PRF in the control group (Left side). The wound was closed using surgical silk suture.

The histological criteria for wound healing, inflammatory cell infiltration, re-epithelialization, and granulation tissue formation were evaluated according to the scoring system (0-4), Furthermore, the results of quantitative analysis of immunohistochemistry of PDGF expression showed that the group treated with Lyph-PRF was moderate, intense and mild cytoplasmic staining reactivity on the first, third and seventh days respectively after PO compared to the control.

Key words: Lyophilization, PRF, Growth factors, immunohistochemistry, surgical wound.

Introduction

Wound healing is a complicated and dynamic process, and a complete understanding of the fundamentals of wound healing physiology is required to adopt chronic wound care concepts [1,2]. Recently, emphasis has been placed on understanding the physiology of wound healing and care, to find innovative and sophisticated approaches for treating acute and chronic wounds to speed up their healing and control the accompanying complications. [3,4]. Wound healing requires the activity of soluble mediators such as growth factors and cytokines, as well as various cell types and the extracellular matrix [5]. Normal wound healing is frequently split into four consecutives but overlapping stages: hemostasis following injury, inflammation, proliferation, and remodeling. On the 3rd day, collagen synthesis starts, and it lasts for three weeks. Increased wound tension strength is caused by the crosslinking of collagens produced from fibroblasts [6,7]. Several growth factors, cytokines, chemokines, and low-molecular-weight substances are released when skin is wounded, starting the repair process right away. The proliferative phase of wound healing begins many hours after damage due to the growth factors, cytokines, and chemokines that are produced by inflammatory cells such neutrophils, monocytes, and lymphocytes [8]. Keratinocyte migration and proliferation begin near the wound edge, followed by dermal fibroblast proliferation in the wound's proximal region. A three to seven days following injury, [9] . Platelet-Rich Fibrin (PRF) is

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composed of a fibrin matrix polymerized in a tetra-molecular arrangement, with the integration of platelets, leucocytes, cytokines, and circulating stem cells. [10]. A novel PRF preparation, known as advanced PRF (A-PRF), was developed in 2014 by lowering the centrifugation force (g) to 100 g for 14 minutes, and A-PRF produced greater growth factors, proliferation, and cell migration of fibroblasts [11]. To retain the biological function of the stored PRF and postpone clinical use. The freeze drying (lyophilization) procedures is a widely used procedure to increase the stability and long-term preservation of proteins used for tissue regeneration. [12]. In addition to having superior stability and storage potential, lyophilized platelet-based products also give developing cells and tissues instant access to growth factors [13]. The aim of the present study was evaluate the effect and efficacy of lyophilized A-PRF in promoting the full- thickness wound healing process based on histopathological and immunohistochemical parameters and this authors of study aimed on developing a procedure for using lyophilized PRF.

Material and Methods

Platelet-rich fibrin preparation

Directly prior to surgical procedure, 10 ml of whole blood was aspirated from the jugular vein of the same experimental animals. A-PRF was prepared by centrifuging the fresh blood without anticoagulants at 3000 rpm for 10 minutes [14, 15]. Following centrifugation, three different layers were obtained. The top layer was Poor platelet plasma (PPP), in the center was platelet-rich fibrin (PRF), while the red blood cells were at the bottom [14, 16]. The PRF layer was removed from the centrifuge tube by surgical scissors from bottom layer and for the preparation of Lyph-PRF, PRF clots were frozen and stored at −80C° and then freeze-dried overnight using a Labconco lyophilizer at −51C° [14, 15] (Fig.1).

Experimental design

The experiment was conducted on 6 healthy dogs of local breeds of both sexes. The experimental animals were housed individually in a place designated for dogs affiliated with the College of Veterinary Medicine, University of Mosul. All animals were treated with ivermectin at dose 0.2 mg /Kg body weight [17] The back of all experimental animals was divided into right and left side, in other words, each animal was considered a control group for itself, In the treated group (right side) Lyophilized A-PRF was applied subcutaneously While normal saline 0.9% was applied instead of Lyophilized A-PRF in the control group (left side).

Wound procedure and experimental design

The dogs were fasted from food for 24 hours prior to surgery. under general anesthesia protocol, the surgical operations were performed by administering atropine sulphate 1% as a premedication at a dose of 0.04 mg /Kg B.W [18] after 10 minutes a mixture of xylazine 2% (VMD-Belgium) and ketamine 10% (Alfasan-Holland) were administered intramuscularly at doses of 10 mg/kg and 2 mg/kg of body weight, respectively [19]. The back of the animal was prepared according to ideal aseptic techniques; A 5 cm longitudinal full thickness incision was made. LA-PRF was applied subcutaneously in the treated group (right side) while 0.9% normal saline was applied instead of lyophilized A-PRF in the control group (left side). The surgical wound is then sutured using (0 USP) silk suture (Figure 2). Animals were examined macroscopically daily for one week.

Histological and immunohistochemistry analysis

Biopsies were taken from the wound site after 1,3 and 7 days postoperative and fixed in buffer formalin 10% for histological technique, H&E staining and detection quantities of platelet derived growth factor (PDGF) antibody in tissue by immunostaining were performed . PDGF antibodies were diluted in PBS at ratio of 1:100, applied to the sections for 12 hours at 4°C, and washed three times with PBS. The negative control section was solely exposed to normal goat serum (Dako) that had been diluted in PBS at ratio of 1:100 for 15 minutes and rinsed three times in PBS. The sections were treated with a secondary antibody solution (biotin-ylated rabbit anti-goat IgG; Vector Laboratories, Inc USA) for 30 minutes before being rinsed three times with PBS. Finally, horseradish peroxidase-conjugated streptavidin-biotin complex (Vector Laboratories, Inc.) was added to the sections for 30 minutes duration, sections were washed three times by PBS and followed by a peroxidase reaction by using 3,3'-diaminobenzidine as a chromogen [20].

Score of immunohistochemistry

By microscopical examination, the immunoreactivity for the PDGF expression in representative tissue sections were evaluated by semi- quantitatively scored for cytoplasmic staining as brown- golden dot, the dominant staining intensity in the cells at the site of wound was scored at the following grade – score design (negative stain = 0), (mild stain = 1), (moderate stain = 2), (intense stain =3) according to the [21], [22].

Results

The wound healing and repair occur in several sequences' events starting by homeostasis and inflammatory phase, tissue re-epithelization, and remodeling, in this study, these phases were variable

according to the groups and day post wounding, however the variable of histopathological features were scored as in Table (1).

The primary differences between the control and Lyph-PRF groups were identified by the histological evaluation of tissue specimens from the wound peripheral areas and beds were began at first day post- wounding. The score and microscopic evaluation of the control group revealed wide wound site with destruction of epithelium layer of mucosa without re-epithelialization (score 0), with the inflammatory crust score (3), that included minor fibrinous exudate and significant inflammatory cells infiltration (score 3), and forming granulation tissue (score 1) (figure 3-A and B) and table (1) in contrast to the histopathological evaluation of the treated group at the same time there was narrowing wound site with precipitated blood clot and sever fibrinous exudate (figure 3-C and D) while the scoring system revealed (3,1, and 2) for inflammatory cells infiltration, slight re-epithelialization and granulation tissue, respectively (Table1).

Skin of control group reveals the mild narrowing site of surgical wound after three days and there was blood clot with moderate fibrinous exudate and inflammatory cells infiltration which occurred as crust (score 3), slight re-epithelialization and granulation tissue scores (1 and 2) respectively, (figure 4A and B), Table (1), while a section of skin from the PRP treatment group revealed complete closure of the wound site with mild infiltration of inflammatory cells as crust (score 1) containing mild fibrinous exudate and re - epithelization as well as mature granulation tissue at the site of the skin wound (score 3) for each these two histopathological features, , (figure 4C and D), Table (1).

The histopathological alterations in the section of control group after seven day post wounding showed incomplete closure of the wound site with presence inflammatory crust containing infiltration of inflammatory cells (score2) , moderate re-epithelization and granulation tissue (score2) for each one (figure 5A and B) Table (1), in the treated group with lyophilized PRF the microscopic examination reveal repair and wound healing characterized by the complete closure of the wound site with mild infiltration of inflammatory cells (score 0) as well as progress and complete re-epithelization (score 4) and well developed mature granulation tissue (score 4) with highly angiogenesis, (Fig. 5 C and D) and Table (1).

Immunohistochemistry:

Immunohistochemistry (IHC) it is an important diagnostic technique used for cell identifying and their distribution in tissue, the results of present study show variable score -grade system of expression PDGF in different groups study with time of postoperative, at 1st post-operative day the microscopic examination revealed that immunohistochemical cytoplasmic staining reactivity for PDGF was mild and intense staining (score 1 and score 3) in control and Lyph-PRF treatment group, respectively as (Fig. 5-A,B) with control negative staining.

Immunohistochemical staining for PDGF revealed moderate and widespread intense immunoreactivity (score 2 and 3) at 1st post-operative day in the cell at the site of wound in both control and Lyph- PRF group respectively, (Fig. 6 C and D).

While intense positive staining for expression PDGF (score 3) was detected in the site of wound after seven-day PO in control group, in comparison to the immunoreactivity staining in the Lyph-PRF group which showed mild positive staining for expression at seven-day PO (Fig. 7 E and F) respectively.

Discussion

Skin wound repairing and healing requires the dynamic processes and many pathological events as hemostasis, coordination of many types of inflammatory cells, re-growth and remolding tissue, thus, methods to modulate the pathological events and cell behaviors via bioactive materials are necessary in the acceleration and improve healing of skin wound such as advanced biological dressing and local Platelet Rich Plasma PRP or PRF implantation [23-25].

Platelet is a vital key role in the coagulation and repair phases of wound healing. Activating factors are stimulate the wounded site, which leads to platelet aggregation, many growth factors are released from platelet play major roles in stopping hemorrhage and improved tissue repair and healing [25 , 27], one source of the cell growth factors is A-PRF. PRF has potential as a new second generation platelet which is developed as biomaterial and source of growth factors as well as cytokines that play a significant part in wound regeneration and healing, bone regenerative and repaired hernia [28-30]. One restriction of the PRF medical application is that it has to be used as soon as possible, in general after ten min of preparation [31], so the lyophilized PRF (L-PRF) a novel approach in regeneration medicine, in our study, the wound healing potential PRF application was inspected in a dog's model of surgical wound defects. After 1 day PO, the wound size reduced; however, gross examination confirmed that wounds treated with PRF was repaired and healed rapidly rather than control group. Microscopic examination showed severe, mild and without infiltration of inflammatory cell during (1, 3 and 7) day PO respectively, newly capillaries and new tissue

formation during early phases of skin wound healing. Complete wound closure at 7day PO referred to well collagen deposition and re-epithelization will occur indicating that PRF is a high biological activity and good source of regenerative medicine [32], in our study the highly angiogenesis has been reported in treated group with PRF in contrast to untreated group at seven days. Therefore, the newly blood capillaries formed could supply nutrients and oxygen to the injured tissue, thereby supporting collagen synthesis. The result of this study come with the result of the study [32-34], according to previous study of [35,36] who reported that freeze dried platelet was accelerating mouse wound healing.

The regenerative activity of L-PRF may be retained to the microstructure features, L-PRF represented the source of fibrin 3- diminution (3D) network which provided blood clot and improve releasing growth factors (GF), as well as prevention of GF from proteolysis [37,38], high density platelet and leukocytes is the one of the main features of L-PRF, [12] reported that L-PRF has large pores size and this will promote cell proliferation and adhesion with enhance tissue engineering.

PDGF is the one of the main two growth factors released from PRF (GF and VEGF), PDGF improves wound healing by accelerating and promoting collagen synthesis and production, as well as, it can stimulate and attracts inflammatory cells, such as macrophages and fibroblast proliferations to the wound site [12] reported that chemical structure of PDGF include presence chain of di- sulphide bond with residue eight -cysteine [39 and 40]. The results of quantitative analysis of immunohistochemistry showed that the group treated with Lyph-PRF was characterized by enhancement and improvement of PDGF expression at the first and third days PO and less intense staining at the seventh day compared with the control, these results shared similar design of PDGF release with 1 hour [11] and one day [12],PDGF evaluation at initial inflammation phase due to activity roles as chemotaxes and even detection at epithelization phase during wound healing due to its ability to coordinate and regulate the growth factors productions.

Conclusion

In conclusion, the result of this study revealed that L-PRF derived from PRF was a novel application in regenerative medicine which play important roles in accelerating and shorting time for wound healing without any complication. Furthermore, it was considered a reservoir for growth factors as PDGF, although Ly-PRF is represented a Potential cells Bank but Future Recommendations and investigations must be conducted to evaluate the benefits and adverse effects of these newer approaches

Highlight

1. Lyph-PRF is the one of the main methods of regenerative medicine
2. Lyph-PRF has activity roles in accelerating wound healing
3. Lyph-PRF is a bank for growth factor
4. PDGF is release immediately in an initial inflammation and epithelization stage of wound healing
5. Immunohistochemistry is diagnostic technique for determined the cells and their spreading

Conflict of interest

The authors state that they have no conflicts of interest.

Ethical approve

The present study has been given approval by the Institutional Animal Care and Use committee of the College of Veterinary Medicine, University of Mosul (UM.VET.2023.059).

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Authors Contribution:

The authors each contributed equally.

Fig. 1. Shows the preparation of A-PRF. A: The centrifuged tubes show three distinct layers. top layer: PPP, center layer: A-PRF, and the bottom: RBC. B: A-PRF was pulled from the centrifuge tube

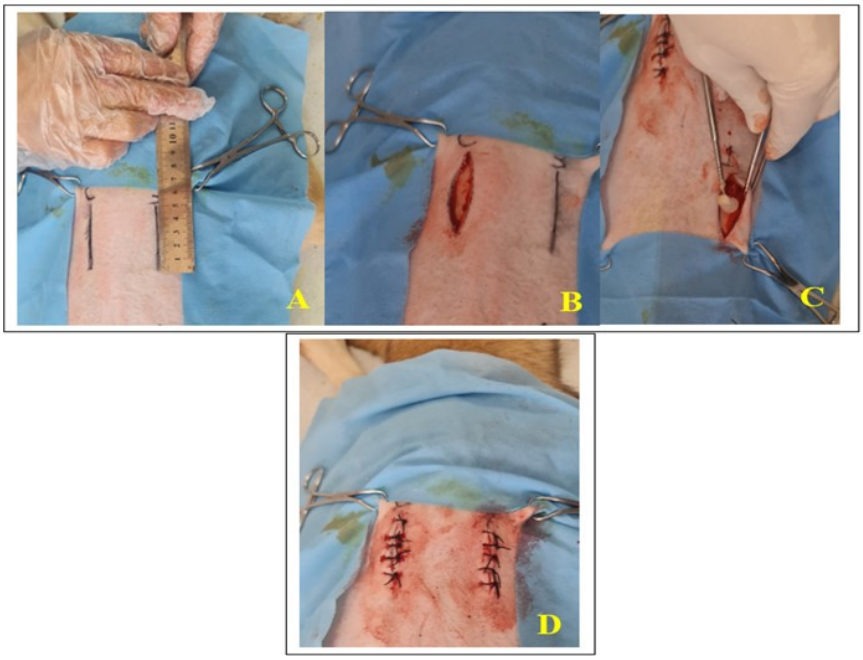

Fig. 2. Shows wound procedure. A: The length of induced wound in skin B: Full thickness incisional wound of 5cm length along on the back of animal. C: LA-PRF application. D: closure of incisional wound.

TABLE 1. The scores of histopathological evaluations of wound healing in the dog skin groups.

Groups	Periods	Inflammation	Re-epithelialization	Granulation tissue
Control group	1 day	3	0	1
	3 days	3	1	2
	7 days	2	2	2
Lyph-PRF group	1 day	2	1	3
	3 days	1	3	3
	7 days	0	4	3

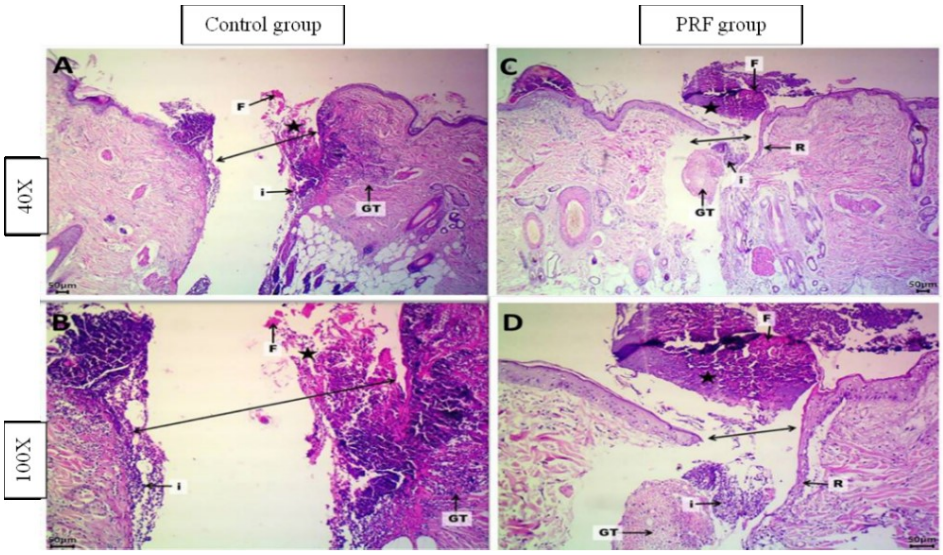

Fig. 3. Histological sections of dog skin of the control group (A&B) and PRF group (C&D)(at first post operative day) Control group (A&B) shows wide wound site (↔) with destruction of epithelium layer of (does skin have mucosa) without re-epithelialization (score 0), with the inflammatory crust (star), containing fibrinous exudate (F), inflammatory cells infiltration (score 3) (i), and granulation tissue (score 1) (GT). PRF group (C&D) shows wound site (↔) with the inflammatory crust (star), containing blood clot (C), fibrinous exudate (F), inflammatory cells infiltration (score 3) (i), slight re-epithelialization (score 1) (R) and granulation tissue (score 2) (GT). H&E staining, (A&C: 40X), (B&D: 100X).

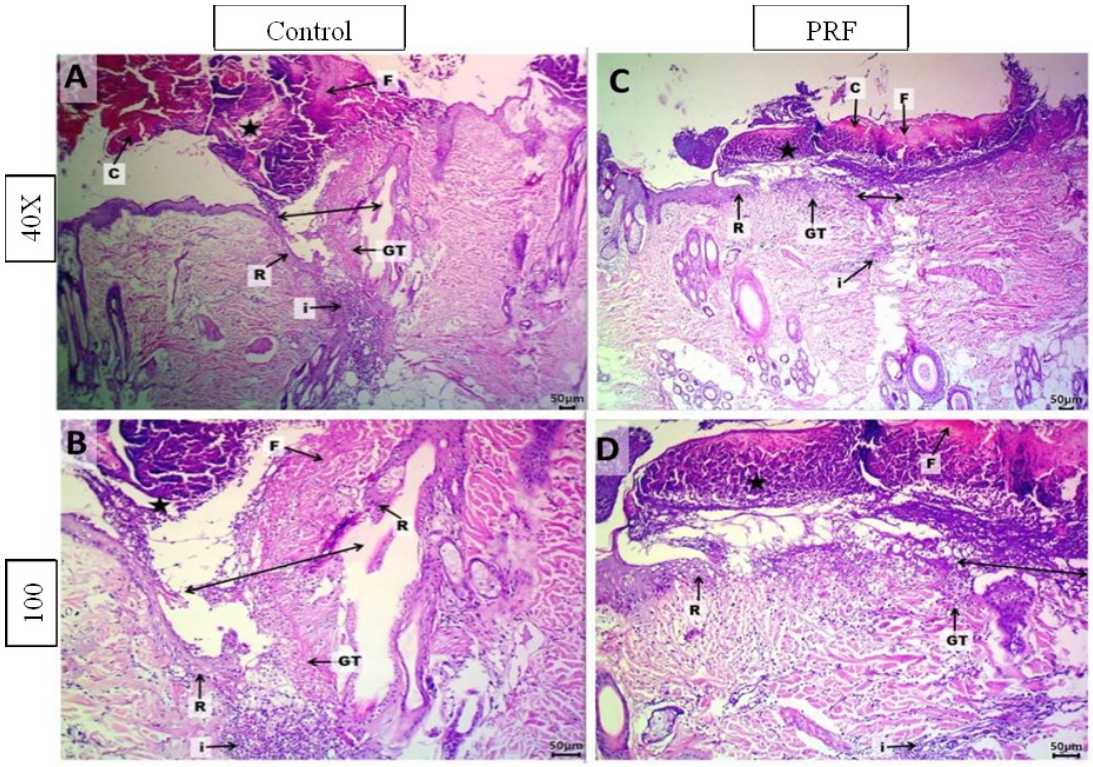

Fig. 4. Histological sections of dog skin of the control group (A&B) and PRF group (C&D) (at 3rd post-operative day). Control group (A&B) shows wound site (↔) with the inflammatory crust (star), containing blood clot (C), fibrinous exudate (F), inflammatory cells infiltration (score 3) (i), slight re-epithelialization (score 1) (R) and granulation tissue (score 2) (GT). PRF group (C&D) shows well closure of the wound site (↔) with the inflammatory crust (star), containing fibrinous exudate (F), mild inflammatory cells infiltration (score 1) (i), well re-epithelialization (score 3) (R) and well granulation tissue (score 3) (GT). H&E staining, (A&C: 40X), (B&D: 100X).

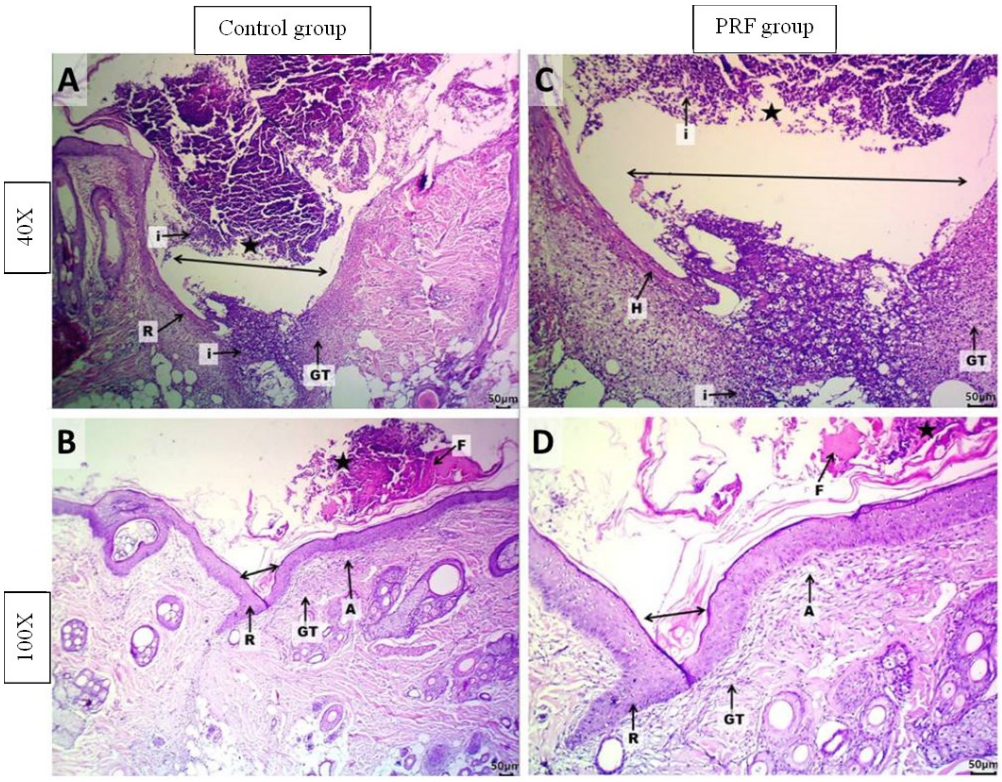

Fig. 5. Histological sections of dog skin of the control group (A&B) and PRF group (C&D) (at 7th post-operative day). Control group (A&B) shows incomplete closure of the wound site (↔) with the inflammatory crust (star), containing inflammatory cells infiltration (score 2) (i), moderate re-epithelialization (score 2) (R) and granulation tissue (score 2) (GT). PRF group (C&D) shows complete closure of the wound site (↔) in very well wound healing with mild inflammatory crust (star), containing mild fibrinous exudate (F), without inflammatory cells infiltration (score 0), complete re-epithelialization (score 4) (R) and well-developed granulation tissue (score 3) (GT) with highly angiogenesis (A). H&E staining, (A&C: 40X), (B&D: 100X).

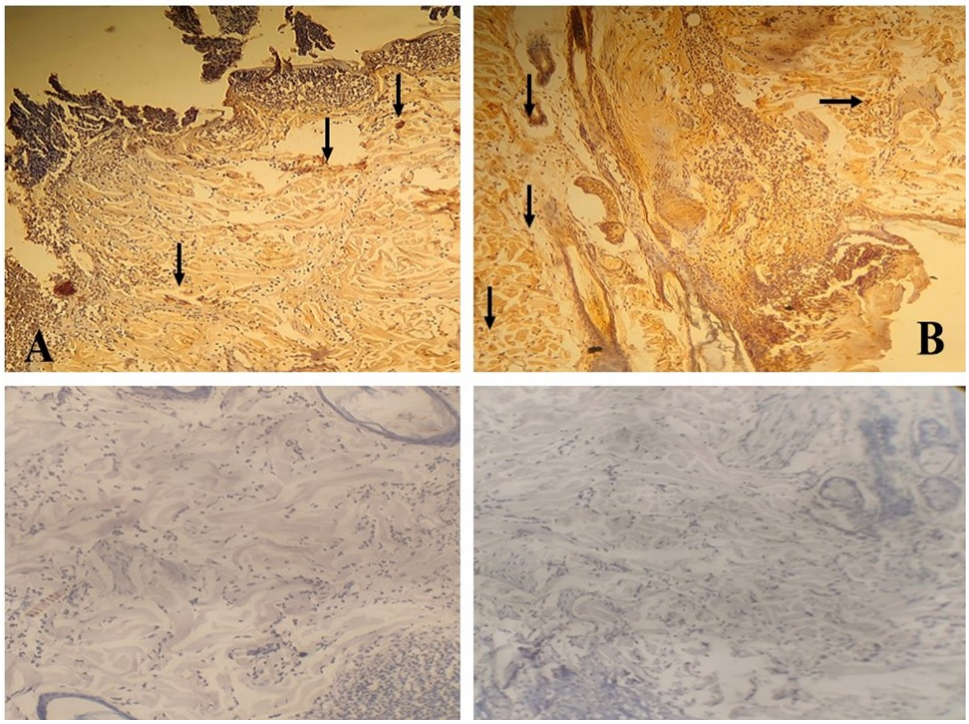

Fig. 6. Immunohistochemical reactivity cytoplasmic staining (brown -gold dot) of PDFG expression at the site of wound at 1st post-operative day , appeared as moderate = score 2 in control group C, while in Lyph- PRF B shows as intensive grade = score 3, 100X with negative control for each group.

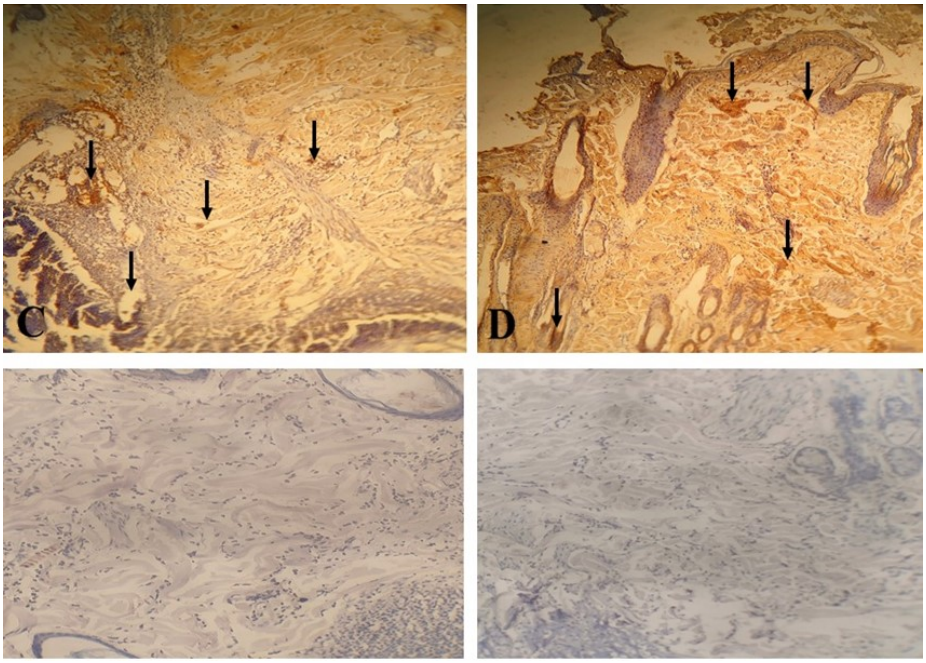

Fig. 7. Immunohistochemical reactivity cytoplasmic staining (brown -gold dot) of PDFG expression at the site of wound at third day PO, appeared as moderate = score 2 in control group C , while in Lyph- PRF B shows intensive grade = score 3, 100X with negative control for each groups

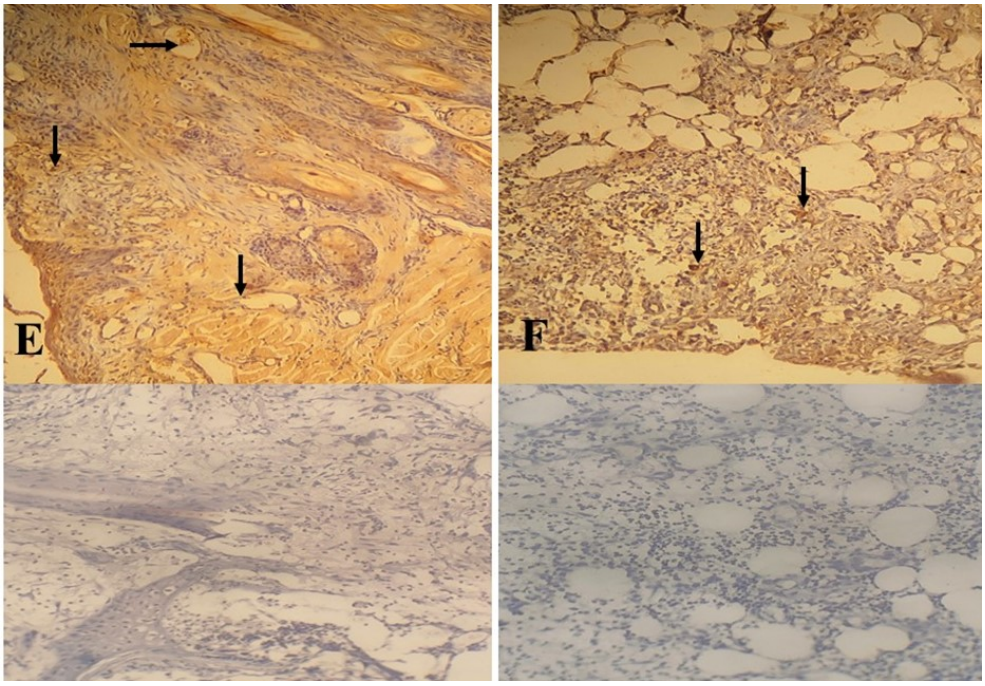

Fig. 8. Immunohistochemical reactivity cytoplasmic staining (brown -gold dot) of PDFG expression at the site of wound at seven-day PO, appeared in control group E intense grade = score 3, while in Lyph- PRF F showed mild grade = score 1, 100X with negative control for each group

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تقييم التأثير والفعالية لليفين المتقدم الغني بالصفائح الدموية الذاتي والمجفف بالتجميد على التئام الجروح الجلدية بسمك تام في الكلاب

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الخلاصة

يعد الليفين الغني بالصفيحات الدموية (A-PRF)علاج بيولوجي مبتكر له دور مهم في تجديد الأنسجة المصابة، وعليه فقد كان الهدف من هذه الدراسة هو تحضير الليفين المتقدم الغني بالصفيحات الدموية والمجفف تحت درجات حرارة منخفضة وتطبيقها على الجروح واعتماد المعايير النسيجية المرضية والمناعية الكيمياء النسيجية لتقييم تأثيرها على التئام الجروح. في هذه الدراسة تم استخدام اثنا عشر كلبا بالغا سليما من السلالات المحلية ومن كلا الجنسين، قسمت حيوانات التجربة بشكل عشوائي إلى مجموعتين رئيسيتين (السيطرة والمعالجة) وبواقع ست حيوانات لكل مجموعة. تم تحضير الجهة الظهرية لحيوانات التجربة تحت ظروف التعقيم القياسية بعدها تم عمل شق جراحي كامل في الجلد وبطول 5 سم، وتم وضع الليفين الغني بالصفائح الدموية والمجفف بالتجميد تحت الجلد في حيوانات مجموعة المعالجة في حين تم وضع المحلول الملحي 0.9٪ في حيوانات مجموعة السيطرة ومن ثم تم إغلاق الجرح باستخدام خيط الحرير الغير ممتص بحجم 0/1

لتقييم التئام الجروح تم أخذ عينات من منطقة الجرح لغرض إجراء الفحص النسجي من جميع حيوانات التجربة في اليوم الاول والثالث والسابع بعد العملية الجراحية. اظهرت نتائج الدراسة لمجموعة السيطرة في اليوم الاول توسع الجرح مع وجود إفرازات ليفية، اما في مجموعة المعالجة كان هناك تضييق في الجرح مع وجود إفرازات ليفية شديدة. ان أفضل نتيجة تم الحصول عليها والمتمثلة بالإصلاح التام والشفاء الكامل للجرح في مجموعة المعالجة كان في اليوم الثالث والسابع مقارنة بنتائج مجموعة السيطرة في اليوم الثالث والسابع بعد العملية الجراحية.

اما بالنسبة لنتائج التحليل الكمي للتغيرات المناعية الكيميائية النسيجية لمجموعة المعالجة بالليفين الغني بالصفيحات الدموية والمجففة بالتجميد فقد ظهر التعبير عن PDGF بشكل تصبغ سيتوبلازمي وبتفاعلات بسيطة ومعتدلة وشديدة في اليوم الأول والثالث والسابع على التوالي بعد العملية الجراحية مقارنة بمجموعة السيطرة. نستنتج مما سبق وبناءً على النتائج النسيجية المرضية والمناعية الكيمياء النسيجية بالإمكان تطبيق الصفائح الدموية الغنية بالليفين المجففة بالتجميد على الجروح كتقنية علاجية جديدة وناجحة من خلال تأثيراتها الحيوية في تسريع التئام الجروح.