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Clinical and histopathological assessment of the combined therapeutic effect of curcumin nanoparticles and PRP on the cutaneous wound repair in rats

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ABSTRACT

Skin wound healing is a complex biological process in which the replacement of dead tissue by a vital one takes place. The aim of this study was to assess the clinical and histopathological modalities of Curcumin nanoparticles and (Platelet-rich plasma) application on excisional skin wound healing activity. Under complete aseptic conditions full-thickness (10 mm) artificial uniform skin wounds were created on the back of twenty anaesthetized male rats (divided into four groups; Control (Group A), Curcumin treatment (Group B), Platelet-rich plasma treatment (Group C), and Curcumin - Platelet-rich plasma treatment (Group D). Tissue sections were stained by hematoxylin and eosin, PAS, and Crossman trichrome for histopathological evaluation of the wound healing properties following the curcumin and PRP topical treatment. Significant skin regeneration including wound closure and histopathological healing was better in Curcumin nanoparticles and PRP treated groups compared to the control untreated one through better reepithelization and coaptation between the epidermis and dermal layers, more vascular angiogenesis, less inflammatory reactions, healthy granulation tissue and better collagen fibers density in the dermal layer. The obtained results proved an effective external therapeutic use of both Curcumin and PRP on cutaneous wound healing progression.

Introduction

Wounds are one of the more perplexing clinical issues since they frequently cause morbidity and mortality and have both early and late consequences (Zarchi *et al*., 2015). Therefore, it is essential to cover the wound site with a suitable dressing to both protect it from external risks and to speed up the healing process by promoting cell proliferation and migration factors (Yang *et al*., 2017; Zhou *et al*., 2018; Song *et al*., 2019). In order to heal a wound, the body goes through four physiological stages in succession, including wound bleeding, an inflammatory response, cell proliferation, and tissue remodeling (Braiman-Wiksman *et al*., 2007; Han and Ceilley, 2017; Akita, 2019). Growth factors, cytokines, plasma-derived proteins, and extracellular matrix (ECM) all work together to start wound healing (Xue and Jackson, 2015). In medical practice, cellulose gauzes, absorbent/surgical cotton, and bandages are typical wound dressings that are economical but have limited benefits because of their dryness and absence of medication. Such dressings are unable to provide the moist, active environment necessary for wound healing, and they cannot keep an infection from spreading to the wound (Karahaliloglu *et al*., 2017). Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptane-3,5-dion), is present in the creeping rootstalk of Curcuma longa and has traditionally been utilized as a herbal medicine due to its antibacterial, anti-inflammatory, and antioxidant properties (Teow *et al*., 2016; Mohanty and Sahoo, 2017; Rezaii *et al*., 2019). The capacity of curcumin to remove Reactive Oxygen Species (ROS) and Lipid Peroxidation (LPx) from the wound region is what gives it its effectiveness as a wound-healing agent (Mohanty *et al*., 2012; Akbik *et al*., 2014). Additionally, curcumin can facilitate the proliferation and migration of fibroblasts, boosts cellular proliferation, collagen synthesis, collagen maturation, collagen cross-linking, and extracellular matrix (ECM) biosynthesis at the wound site (Panchatcharam *et al*., 2006; Novo and Parola, 2012). Although it has powerful pharmacological properties, this yellow hydrophobic polyphenolic compound has drawbacks like hydrophobicity and instability in biological systems. Despite this, it exhibits excellent therapeutic efficacy (Heo *et al*., 2014; Xie *et al*., 2017). Nanotechnology is an exciting, quickly expanding topic that holds a lot of potential for contemporary science and medicine. It deals with extremely small substances called nanoparticles (NPs), which range in size from 1 to 100 nm. This has a large surface area compared to its volume, which enhances the materials' physical, chemical, optical, and electrical capabilities (Hussain *et al*., 2017). Because of their improved solubility, bioavailability, and stability, curcumin nanoforms have been suggested as therapeutic agents against a variety of disorders (Yadav *et al*., 2018). Blending two or more biopolymers may make up for the shortcomings of single-component systems and produce a binary composite system that has excellent desired characteristics. Single-component scaffolds might not be able to impart all the desired properties (Bazli *et al*., 2017; Li *et al*., 2021). PRP is characterized as an autologous biological product made from the patient's blood that, following a centrifugation procedure, yields a plasma fraction with a greater platelet concentration than the blood's circulating level (Ahmad *et al*., 2012). Since platelets have hemostatic properties and contain cytokines and growth factors, they are essential for the healing of wounds (Eppley *et al*., 2004). Numerous growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF1, IGF2), vascular endothelial growth factor (VEGF), transforming growth factor (TGF-), and keratinocyte growth factor (KGF), are known to play a role in the healing of wounds (Grazul-Bilska *et al*., 2003; Falanga, 2005; Arwert *et al*., 2012). Despite various conventional therapeutic strategies aimed at improving

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wound healing, using novel treatments is still a clinical challenge. Clinical and experimental investigations using PRP and Curcumin formulations in cutaneous wound healing have positive in vivo and in vitro findings. Therefore, this trial tries to discuss the area of regenerative skin wound healing with a focus on platelet-rich plasma and nano-Curcumin patch which is novel.

Materials and methods

Experimental Design

Animals

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Veterinary Medicine, South Valley University, Egypt (VM/SVU/23(3)-04). Twenty apparently healthy male Sprague Dawley rats with no previous drug treatment (i.e., 20 wounds) (weighting 250±20 g, 4-5 months old) were housed individually in standard cages at room temperature with normal light/dark cycles, enrolled in an acclimatization period of 7 days in the laboratory environment, and provided with food and water ad libitum. Rats were randomly divided into four equal groups of five animals each: Control (Group A), Curcumin treatment (Group B), Platelet-rich plasma treatment (Group C), and Curcumin - Platelet-rich plasma treatment (Group D). Artificial skin wounds were created at the dorsal thoracic region. The wound was blotted with cotton with gauze, the respective therapeutic treatment was applied to the animals of respective groups starting from the day of the operation (The experiment lasted for 14 days). Wound-healing evaluation and histopathological assessment were made. All rats were closely observed for any gross changes and if they showed signs of infection were separated and excluded from the study.

Excisional Wound Model

Without damaging the skin, the fur from the dorsal surface of rats was removed with electronic hair remover, 24 h prior to the experiment. Before induction of experimental skin wounds, the rats were anaesthetized by intraperitoneal injection of 50 mg/kg ketamine HCl (Troikaa Pharmaceuticals LTD) and 10 mg/kg xylazine HCl (ADWIA Co S.A.E) (Seyhan, 2020), the skin was cleansed with 70% alcohol, touched by povidone iodine 10% solution (Mundipharma AG). Excision wound was created by sterile scalpel and surgical scissors at the dorsal thoracic region. The circular skin from the predetermined area on the depilated back of the animal was excised to its full thickness to obtain a circular wound of 10 mm in diameter.

Curcumin nanoparticles preparation (PVA-CMC patches)

Silver nanoparticles were prepared by the chemical reduction method as reported by Pal *et al.*(2009). Silver nanoparticles were prepared by microwave irradiation of silver nitrate ($AgNO_3$) solution in the ethanolic medium using Polyvinylpyrrolidone (PVP) as a stabilizing agent. Ethanol was observed to act as a reducing agent in the presence of microwave. To get the silver in powder form the solvent was evaporated at a mild temperature then, the suspension was diluted with Carboxymethyl cellulose (CMC) aqueous paste 5% w/v (1 ml silver suspension + 4 ml CMC) to get 100 ppm semi-paste. For Curcumin loaded silver 5 mg of curcumin were dispersed in 1ml silver suspension. Patches were prepared by the esterification crosslinking method as mentioned in a study by Ghorpade *et al.* (2018)with modifications. Polyvinyl alcohol (PVA) and CMC solutions were mixed and stirred for 30 minutes with 10% w/v Curcumin-loaded silver nanoparticles then, a certain concentration of citric acid was added, and then dried in a lab oven for 24 hours at 50 degrees.

Optical Properties: UV-Vis absorption spectra were obtained on an Ocean Optics USB2000+VIS-NIR Fiber optics spectrophotometer. (Figure. 1).

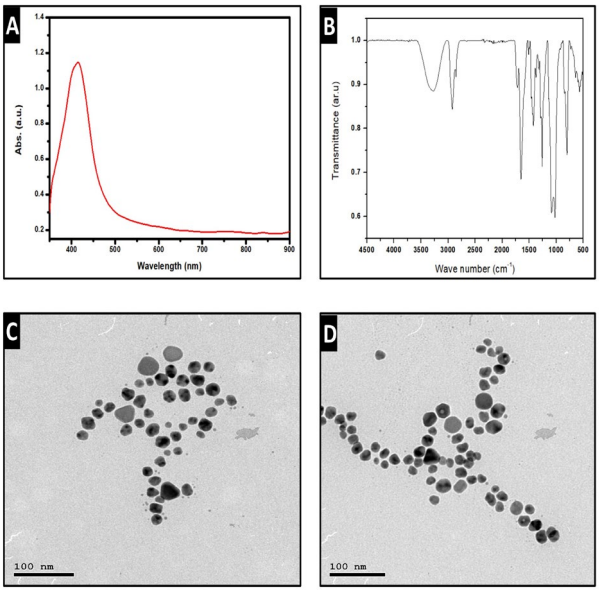

Figure 1. Curcumin nanoparticles preparation, A: the spectrum of AgNPs, B: the FTIR spectrum of final patch, C&D: TEM images of prepared Cur@AgNPs (Size & Shape: TEM were performed on JEOL JEM-2100 high resolution transmission electron microscope at an accelerating voltage of 200 kV, respectively).

Platelet-rich plasma

PRP was prepared by blood collection from another group of rats. Each rat was anaesthetized by diethyl ether, then blood was collected via heart puncture by sterile syringe and rapidly transferred into 9NC coagulation sodium citrate 3.8% tubes (Greiner Bio-One GmbH). Tubes were directly centrifugated using a high speed refrigerated centrifuge (HERMLE LaborTechnik GmbH: Z36HK) at 240 x g for 8 minutes at 16°C for the first spin (Amable *et al.*, 2013). This resulted in the separation of blood constituents into 3 layers; an upper layer (plasma) that contains mostly platelets and leukocytes, an intermediate thin layer (the buffy coat) which is rich in leukocytes, and a lower layer that consists mostly of RBCs. After this, the upper layer and superficial buffy coat were transferred into empty sterile tubes (Eppendorf Tubes: Eppendorf AG). The transferred portion in the new tubes was centrifugated at 700 x g for 17 minutes. The upper two thirds portion of the volume which is known as PPP (platelet-poor plasma) was removed, pellets were homogenized in the lower third by gentle shaking to create the PRP (Platelet-Rich Plasma). PRP tubes were kept without activation until just before use. PRP tubes were stored inactivated at -80°C Ultra-Low Temperature Freezer (Thermo Scientific Revco UxF- Thermo Fisher Scientific Inc.). Activation of PRP was performed by adding calcium gluconate monohydrate 5% / calcium levulinate 3.4% sterile solution (Memphis for pharmaceutical & chemical industries) (Vahabi *et al.*, 2017). The wound was injected by 100 microliter of activated PRP (Xu *et al.*, 2020).

Wound healing evaluation

Wound-healing property was evaluated by wound contraction percentage and wound closure time. All wounds were photographed by a digital camera immediately after wounding (day 0) and on days 4, 7, 10 and 13 post-operation while a ruler was placed near the wounds (Figure 2 A). The wound areas were analyzed by measuring tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc.) and wound contraction percentage was calculated using the following formula:

Percentage of wound contraction = (A0- At) / A0 x 100. Where A0 is the original wound area and At is the wound area at the time of imaging (Yates *et al.*, 2007).

Histopathological Studies

At day 13 post wounding, all animals were euthanized with an overdose of anaesthetic and the whole wound circumference with 1 cm margin from all sides was removed. The harvested tissue samples were fixed in 10% neutral buffer formalin (pH 7.4), passed in alcohols embedded to paraffin wax, cut into 5 µm thickness, histochemical stains as Harries hematoxylin and eosin (Sigma-Aldrich), Crossman trichrome (which can stain collagen fibers bluish green) and Periodic acid–Schiff (McManus, 1948) were conducted for dermatopathological diagnosis and evaluation of connective tissue as epidermal epithelization and cornification, granulation tissue formation especially for angiogenesis in addition to ECM deposition respectively. The slides were examined using a microscope (Olympus BX51) with a camera (Olympus E- 182 330, Olympus Optical Co., Ltd.). Five slides were examined for each block (Bancroft *et al.*, 1996).

The open access software Image J 1.54d (Chen *et al.*, 2021) (Wayne Rasband and contributors, National Institute of Health, http://imagej.org, Java 1.8.0-345 (64-bit) 5280K of 6040MB (1%)) was used for manual computer analysis for the wound closure diagnosis (Figure 2 B).

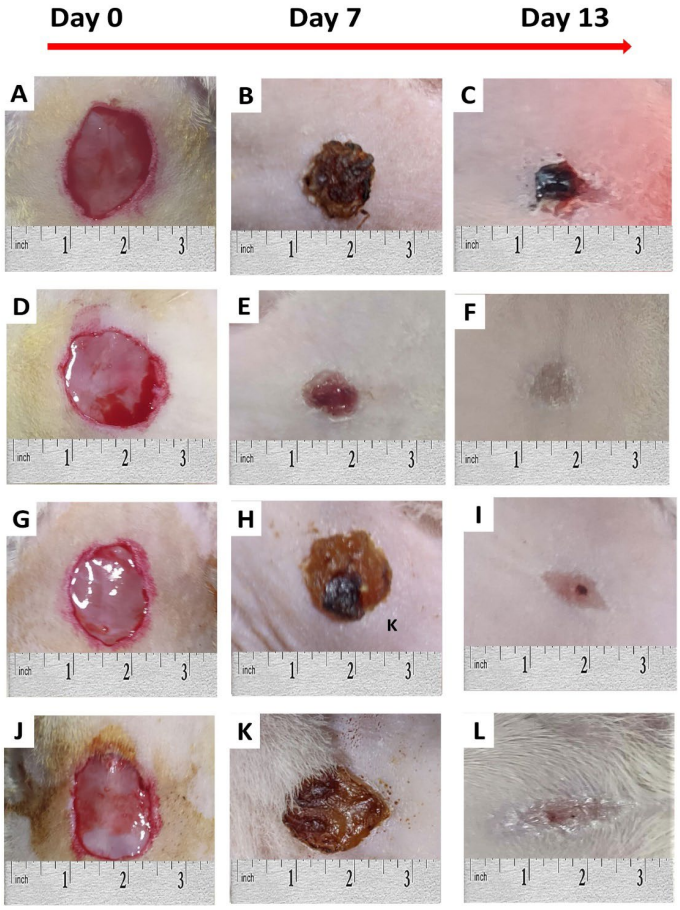

Figure 2 A. Visual inspection by photography of wound closure percentage from day 0 till day 13, skin, Rat. Higher wound contraction percentage was noticed on all treated groups (Group B,C & D) relative to the control one (Group A). While there was no significant difference in wound closure percentage between the treated groups (Group B, C & D) (A&B&C: control group, D&E&F: curcumin nanoparticles treated group, G&H&I: PRP treated group and J&K&L: treated group by both curcumin and PRP).

Many parameters were evaluated, including epidermal epithelization and keratinization, migration of inflammatory cells, and extracellular matrix (ECM) regeneration, especially for collagen fibers and granulation tissue formation (angiogenesis). The histopathological score was evaluated as follows: in epidermal and dermal layers (0- cut edges thickness, 1- epithelization, 2- bridging and collagen migration, 3- complete regeneration) (Figure 3) (Vidinský *et al.*, 2006).

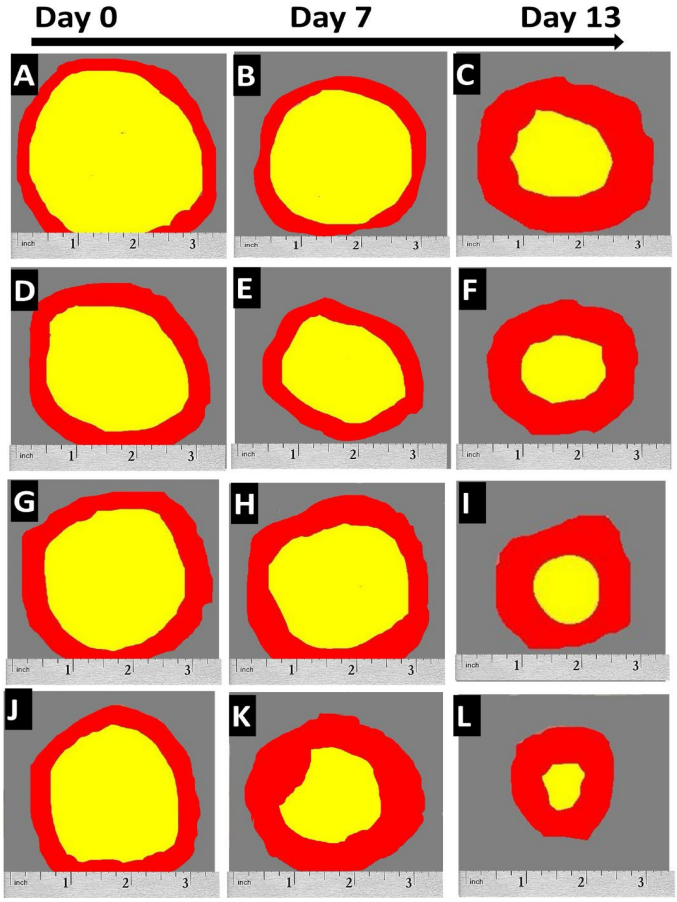

Figure 2 B. Image J manual analysis of wound area, the yellow color revealed the area of the wound healing, it shows better wound contraction in all treated groups compared to control group, (A&B&C: control group, D&E&F: curcumin nanoparticles treated group, G&H&I: PRP treated group and J&K&L: treated group by both curcumin and PRP).

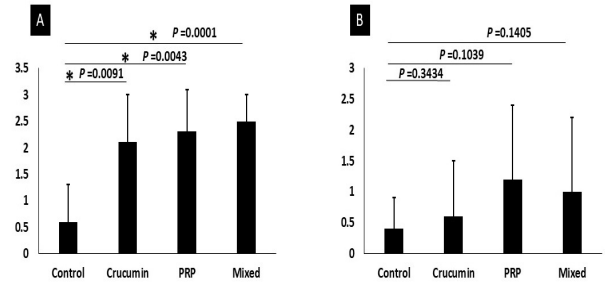

Figure 3. Statistical analysis of histological score of Epidermal (A) and Dermal (B) features of healing. P value <0.05 was considered significantly different.

Statistical analysis

For wound closure %, two-way ANOVA was used (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, www.graphpad.com) and for histological scoring, Student's paired t-test was used and when $P < 0.05$ (*), $P < 0.01$ (**) considered significantly different.

Results

Photographed figures were captured on days 0, 4, 7, 10 and 13 post-operation (at predetermined time) during the experiment (14 days) for all groups for measuring wound contraction percentage. It was noticed that (Figure 4) contraction of wound in Group B, Group C and Group D reached significantly higher percentage of wound closure at day 13 (100%) relative to group A (75%) while there was no significant difference in wound closure percentage between the treated groups at day 13 (groups B, C & D) in which the wound surface area was progressively decreased till reached complete healing by the end of the experiment (Figure 2 A&B). Detailed histological changes on 13th day post healing

were evaluated for regenerative process.

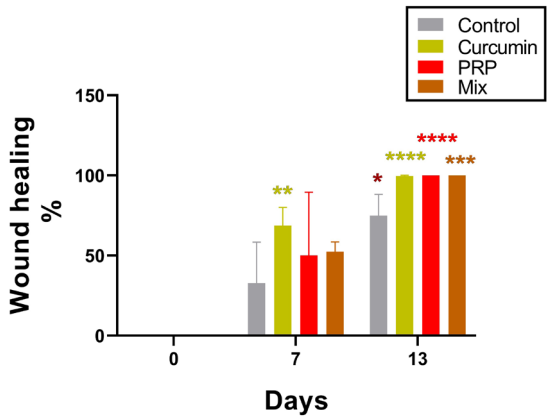

Figure 4. Statistical analysis of wound area reduction. P value <0.05 was considered significantly different.

Applied treatments had great improvement in skin healing process. Histological scoring was done for epidermal and dermal evaluation. There was significant difference between all treated groups and the control one in epidermal healing while there was no significant difference in dermal properties in all groups (Figure 3). Specifically, better epidermal epithelization with complete restoration of the epidermal layer (bridging of the incision was completed by the three layer of newly formed epidermal cells), epidermal thickening and well cornified layer above the superficial epidermal layer as a result of mitotic activity of the basal cell layer (indicating keratinocyte differentiation), some wounds had scab formation under which keratinocyte migration took place, all of the previous changes occurred in nanoparticle Curcumin (Group B), PRP (Group C), and Curcumin - PRP (Group D) than the control one (Group A) in which incomplete epithelization and surface necrosis of skin tissue occurred causing incomplete dermo/epidermal junction and loose dermal matrices. Meanwhile leucocytic infiltration which invade the wound area was scored ((absent /no apparent inflammatory response); mild (< 10% of the area covered by inflammatory cells); moderate (10 to 50% of the area covered by inflammatory cells)) and the control group (Group A) was ranged from moderate to severe while the treated groups (Group B, C & D) had less pronounced inflammatory response ranged from absent to moderate (data not shown) (Figure 5). Regarding histological examination of skin appendage, deep dermal granulation tissue formation and extracellular matrix (ECM) both Crossman trichrome and PAS histochemical stains were used respectively. Results showed significant variation between the treated and untreated groups. In particular, better dermal appendages (include sweat glands, and sebaceous glands), more healthy granulation tissue including abundant regular arranged bluish green collagen bundles deposition giving relative uniform intensity and angiogenesis with considerable number of blood vessels (red blood cells fill the loop of new vessels) indicating the process of healing were observed in the treated received groups (Group B, C & D) compared to the lesions in the control group (Group A) in which lack granulation tissue occurred in some areas, mild appendage restoration occurred and distortion of collagen fibers (without significant quantity) between the cells in the granulation tissue was conducted (Figure 6). Nevertheless, all the treated groups with curcumin nanoparticles, PRP and the mixture of them (Group B, C & D) revealed higher skin recovery and quality (complete epithelization, dermo/epidermal junction, less inflammatory reaction, better skin appendages and good granulation tissue). Cumulatively, the results revealed that using of those topical treatment influence better wound healing process.

The wound area of rats in each group continued to shrink with the extension of the healing time. Complete wound closure was observed in Group B, Group C and Group D in comparison to Group A as we can see from figure 2 A&B. Compared to day 0, the wound contraction was 100% in Group B on day 13 significant **** (P<0.0001), 100% in Group C on day 13 significant **** (P <0.0001), 100% in Group D on day 13 significant ***(P=0.0002) and 75% in Group A on day 13 significant * (P=0.0107) (Figure 4). This indicated that the wound healing rate of all the treated groups was significantly faster than that of control groups.

Discussion

Impairment of wound healing prosperities to wound chronicity comes from the impairment of cell biological and molecular events such as epithelization through cell migration and mitotic activity, deposition of ECM as well as granulation tissue proliferation through angiogenesis and

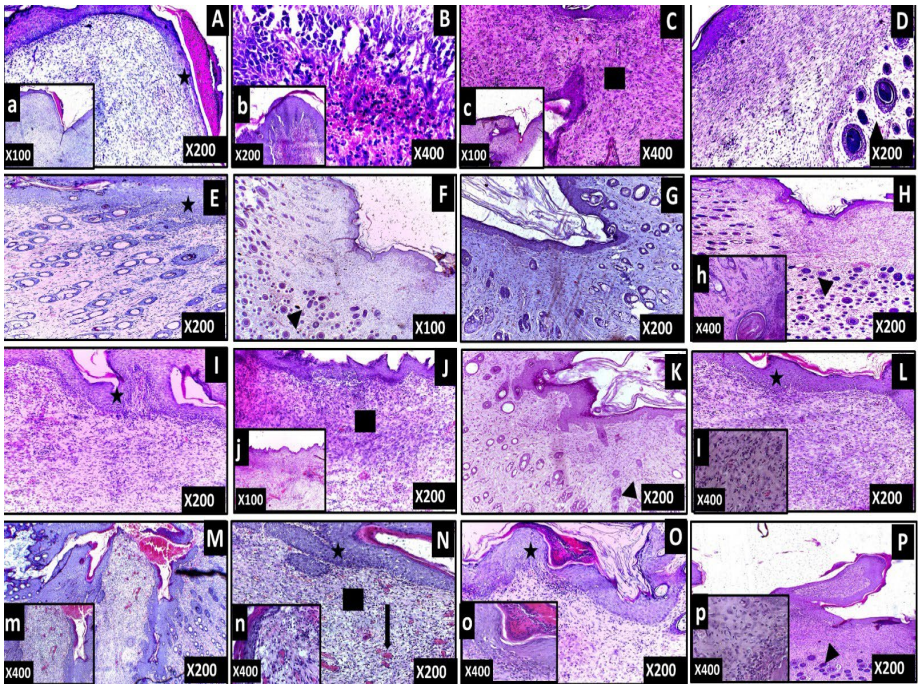

Figure 5. Histopathological examination of wound healing reactivity, epidermal and complete epithelization (Star), mild leucocytic infiltration (Rectangle), skin appendage restoration (Isosceles triangle), well-formed blood vessels (Arrow: down) in all treated groups compared to the control untreated one. (A&B&C&D: Control group, E&F&G&H: Curcumin group, I&J&K&L: PRP group, M&N&O&P: Curcumin - PRP group), (H&E satin).

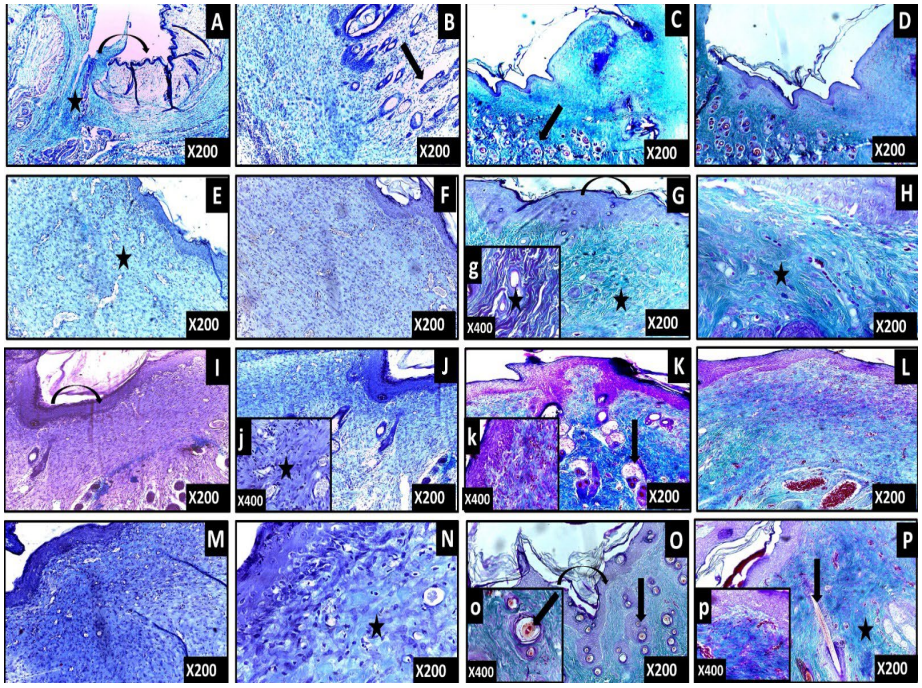

Figure 6. Histopathological examination of dermal granulation tissue formation, complete reepithelization occurred in treated groups while incomplete one noticed in control group (Arrow: curved down), skin appendages (Arrow: down), Collagen bundles (Star) were restored in treated ones and mild restoration occurs in control group (A&B&C&D: Control group, E&F&G&H: Curcumin group, I&J&K&L: PRP group, M&N&O&P: Curcumin - PRP group), (A&B&E&F&I&J&M&N: PAS satin and C&D&G&H&K&L&O&P: Crossman trichrome stain).

collagen deposition in addition to remodeling (Singer and Clark, 1999; Morton and Phillips, 2016), for that, adequate therapeutic interference is much required to overcome the crucial increase of chronic wounds worldwide (Fan *et al*., 2011). Recent studies evaluate the proper usage of some herbal and medical plant wound dressing which have considerable effect on acceleration of the wound healing process (Chua *et al*., 2016). Herein we evaluate the role of Curcumin nanoparticles and PRP on the progression of wound healing. Percentage of wound closure was measured and statistically analyzed after the wounds were photographed on days 0,4,7,10 and 13 post-operation at a predetermined time. We found complete wound contraction with higher wound closure percentage in the treated groups; (Group B) 100%, (Group C) 100% as well as in (Group D) 100% relative to (Group A) in which incomplete wound closure took place (75%). Histological analysis was done for wound repair confirmation. Thickening of epidermal layer revealed complete epithelization and increase mitotic activity and basal cell migration was observed in the treated groups (Group B , C & D) compared with the control untreated one (Group A) in which necrotic surface area with incomplete cornification were diagnosed leading to incomplete dermo epidermal junction. Curcumin has significant antioxidant and anti-inflammatory activity which aids in abundant granulation tissue, regular dermal constructure and noticeable new capillaries helped by recapture active oxygen free radicals (Ahmed *et al*., 2018; Sandri *et al*., 2019). Moreover, curcumin accelerate collagen synthesis and helps in epithelial layer regeneration due to decreased expression of TNFα, IL-1β and MMP-9, which are considered inflammatory cytokines and also increase the expression of anti-inflammatory cytokines as IL-10 (Lim *et al*., 2001; Kloesch *et al*., 2013). MMPs plays important role in cleaning the wound through removing all thickened skin or callus, necrotic and dead tissue, foreign debris through its critical role angiogenesis process, epithelization and remodeling (Kähäri and Saarialho-Kere, 1997; Mun-Bryce and Rosenberg, 1998; Armstrong and Jude, 2002). Curcumin may enhance angiogenesis and new capillary formation as well as higher numbers of myofibroblasts which means improvement of granulation tissue organization (Dai *et al*., 2009). Changing curcumin formulation as a topical dressing enhance its therapeutic effect on skin wounds (Akbik *et al*., 2014). In this study preparation of PRP depends on cold centrifugation at 240 x g for 8 minutes at 16 °C and 700 x g for 17 minutes what makes high concentration of PRP through its activation and act spontaneously (Amable *et al*., 2013). Involvement of PRP in regenerative medicine worldwide spread depending on its source of growth factors and repeat dosage of PRP makes its constituent and the growth factors increase gradually in a constant level instead of sudden elevation of their level (Hammond *et al*., 2009; Pazzini *et al*., 2016). Knox *et al.* (2006) and Choi *et al.* (2012) mentioned that PRP injection resulting in high VEGF expression which is responsible for neo-vascularization and in association of multiple growth factors (five growth factors) thus accelerate in chronic wound healing which associated with some chronic diseases as diabetes. Regular Growth factors secretion such as EGF, IGF-1, FGF-2, PDGF-BB, VEGF, Ang-1, SDF-1, KGF, MMP-9, or cytokines such as TGF-β, IL-1, IL-6, IL-8, and TNF-alpha involve in cellular processes coordination through a complex integration of signals. Insufficient or overproduction of those growth factors may relate to wound chronicity and healing impairment (Badillo *et al*., 2007; Barrientos *et al*., 2008; Kim *et al*., 2012). In particular, PRP affects white blood cells mobilization and release of bactericidal factors from the platelet side helping in infection fighting, as well as many studies suggesting the medical benefits from PRP usage in chronic wound healing on the contrary only few studies described no benefits. To avoid false-negative results of PRP topical application and injection, it must be rich with viable active platelets for healing enhancement (Marx, 2004). Based on microscopical examination, inflammatory response modulation was detected in the treated groups ranging from mild to moderate level on the contrary, in the control group abundant inflammatory cascade (ranged from moderate to severe level) takes place suggesting the effect of curcumin and PRP in regulation of wound inflammatory response. Delayed resolution of the wound inflammatory reaction may be caused by the delay in macrophage infiltration which is responsible for removal of the necrotic tissues and the antibacterial properties at the site of the wounds (Bjarnsholt *et al*., 2008; Khanna *et al*., 2010). The principal component of connective tissue is collagen which plays a pivotal role in wound healing process. PAS and Crossman trichrome stains were applied for the histopathological examination for ECM and granulation tissue formation (Repair hallmark) including collagen fibers which aids in the tensile strength character in the wound area. Likewise, restored skin appendages and good collagen intensity which was arranged in orderly manner associated with neovascularization were noticeable in (Group B, C & D) compared to (Group A) in which loose dermal matrices with distorted collagen bundles in some area and less features of angiogenesis were detected. Collagen stained bluish green and the amount of collagen gives rise to the degree of wound healing, also abundant collagen means promotion of fibroblast proliferation which is responsible for collagen production (Jung *et al*., 2018). This therapeutic function of curcumin and PRP in fastening of the wound healing may come through the early production of granulation tissue followed by angiogenesis. Collagen fibers arrangement and intensity play an aspect in the quality of skin healing and dermal restoration of its mechanical prosperities (Mehanna *et al*., 2015). Survival and keratinocyte migration associated with epithelization and newly formed granulation tissue sustain all depend on the formation of new blood vessels (Fiorina *et al*., 2010; Hong *et al*., 2013). Kant *et al.* (2014) revealed better healing properties in curcumin treated group through collagen synthesis in an arranged manner and tissue remodeling and wound closure. Collagen synthesis is TGF-β1 dependent which is essential for proper healing (Leivonen *et al*., 2005; Werner *et al*., 2007; Brem *et al*., 2009). The results of the present work investigate that the group received a mixture of both curcumin nanoparticles and PRP had complete reepithelization with increased mitotic activity and typical granulation tissue formation compared to the other two treated group and may this

occur under the effect of the growth factors of PRP and the antioxidant and anti-inflammatory effect of curcumin.

Conclusion

It is suggested that the anti-inflammatory and antioxidant potential of curcumin with growth factors effect of PRP cause faster and better wound healing in rats. Curcumin mixed with PRP could be an additional novel therapeutic agent in the management of impaired wound healing.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this publication.

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