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RESEARCH ARTICLE

Beneficial effect of Curcumin Nanoparticles-Hydrogel on excisional skin wound healing in type-I diabetic rat: Histological and immunohistochemical studies

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ABSTRACT

Management of diabetic wounds remains a major challenge in the medical field, mostly due to incompetent outcomes of treatments. Curcumin has been documented as anti-inflammatory, antioxidant, antimicrobial and antineoplastic agent in addition to wound healing activities. However, its poor aqueous solubility and impaired skin permeation handicap its topical pharmaceutical usage. Hydrogel loaded curcumin nanoparticle (Cur-NP/HG) could overcome this pitfall and enable extended topical delivery of curcumin. Rat model of diabetes mellitus (DM) type I was induced using single injection of 70 mg/kg streptozotocin (STZ) followed by full thickness skin wound. Rats were divided into 4 groups. **GpI**: control non-diabetic, **GpII**: diabetic non-treated, **GpIII**: diabetic treated with topical curcumin hydrogel (Cur/HG) and **GpIV**: diabetic treated with topical Cur-NP/HG. Histological assessment of epidermal regeneration, dermo-epidermal junction, leukocyte infiltration and collagen deposition, in addition to immunohistochemical staining for vascular endothelial growth factor (VEGF) and aquaporin-3 (AQP3) were performed. Diabetic rat possessed impaired wound closure, persistence of inflammation and decreased collagen deposition as compared to non-diabetic control. Application of Cur/HG induced partial improvement of the healing process in diabetic rats. Cur-NP/HG treatment provoked obvious improvement of the healing process with complete re-epithelization, intact dermo-epidermal junction, reorganization of the dermis with significantly increased collagen deposition and VEGF and AQP3 expression. These results illustrated that Cur-NP/HG have effectively improved the healing process in diabetic skin wound with substantial differences in the wound healing kinetics compared to wounds that received Cur/HG.

1. Introduction

Typical healing of a skin wound is a dynamic multicellular process that requires incorporation of complex molecular and biological events. It comprises four distinct overlapping phases: hemostasis, inflammation, proliferation and remodelling (Hamed et al., 2010). Corruption of such processes at any phase would lead to impaired healing or even chronicity of the wound. Among various causes, wound chronicity is mainly connected with diabetes, atherosclerosis, vasculitis and trauma (Morton and Phillips, 2016).

Diabetes mellitus is the most prevalent cause of impaired healing of skin wounds. Alarming data showed that 85% of non-healing diabetic foot ulcers eventually require amputation (Lipsky, 1999). There are divers factors that join all diabetic ulcerations and impedes proper wound healing as vascular insufficiency, poor vessel proliferation, impaired immunity (Okonkwo and DiPietro, 2017), and defective growth factors production and collagen accumulation (Xu et al., 2017).

Curcumin (diferuloylmethane), a natural hydrophobic polyphenol, is an orange-yellow crystalline compound and the active ingredient of turmeric. Curcumin has been found to exhibit anti-inflammatory, antioxidant, antimicrobial and antineoplastic properties in addition to wound healing activities (Gupta et al., 2013). However, poor aqueous solubility and impaired skin permeation of curcumin, in addition to its rapid metabolism

Abbreviations: AQPs, aquaporins; Ab, antibody; ANOVA, analysis of variance; AQP3, aquaporin-3; CT, connective tissue; Cur/HG, curcumin hydrogel; Cur-NP/HG, hydrogel loaded curcumin nanoparticle; DM, diabetes mellitus; ECM, extracellular matrix; H&E, hematoxylin and eosin; NPs, nanoparticles; SD, standard deviations; SPSS, Statistical Package of Social Science; STZ, streptozotocin; VEGF, vascular endothelial growth factor.

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profile handicaps its topical pharmaceutical usage (Kumar and Punniamurthy, 2017).

Nanotechnology is a speedily growing interesting field with a great promise in modern science and medicine. It deals with tiny materials known as nanoparticles (NPs) having size ranging from 1 to 100 nm. This possesses high surface area compared to volume which improves the physical, chemical, optical and electronic properties of the materials (Hussain et al., 2017). Recently, NPs have been considered as novel candidates for improving the intracellular drug delivery, subcellular targeting and crossing inaccessible various anatomical and physiological barriers (Dende et al., 2017). Several studies have been developed to provide suitable nanoform delivery system for curcumin as nanosuspension, nanoemulsion, solid-lipid nanoparticles and hydrogel nanoparticles (Dutta and Ikiki, 2013). Curcumin nanoforms have been described for their increased solubility, bioavailability and stability which recommend them as therapeutic agent against a wide spectrum of diseases (Yadav et al., 2018).

Vascular endothelial growth factor (VEGF) is considered as one of the most pivotal growth factor for wound healing. It is produced by keratinocytes, macrophages and fibroblast in response to pro-inflammatory cytokines at early stage of wound healing and acts on angiogenesis and tissue granulation (João De Masi et al., 2016).

Aquaporins (AQPs) are a family of transmembrane proteins that transport water, small solutes such as glycerol and ions across cell membranes. Thirteen AQPs (AQP0-12) have been detected in different organs such as the nervous, renal, cardiovascular, respiratory, reproductive, digestive, musculoskeletal, and integumentary systems (Day et al., 2014).They are categorized into three groups: water channel (AQP0, 1, 2, 4, 5, and 8), aquaglyceroporins (AQP3, 7, 9, and 10), and unorthodox AQPs (AQP6, 11, and 12) (Rojek et al., 2008). AQP3 is a water/glycerol-transporting channel protein expressed in the skin epidermis mainly in the basal layer of normal skin but not the stratum corneum (Hara-Chikuma and Verkman, 2008). Their synthesis occurs early in basal cells with a predominant cytoplasmic accumulation. While keratinocyte differentiation proceeded upwards, AQP3 is trans-located to the plasma membrane. No AQP3 expression is found in skin dermis, where water can diffuse rapidly due to its loose architecture and the highly hydrated glycosaminoglycans contents (Sougrat et al., 2002) and (Sebastian et al., 2015). They have been demonstrated to play a role in cell proliferation, migration, immunity, and wound healing in addition to skin hydration (Ikarashi et al., 2017).

2. Experimental design

2.1. Animals

Forty adult male albino rats weighing (150–200 gm) were included in the study. The animals were bred in the Animal House of Faculty of Medicine, Cairo University. All procedures were held under ethical guidelines of animal care and approved by the Animal Care and Use Committee of Cairo University. The animal were divided into non-diabetic rats (n = 10) and diabetic rats (n = 30).

2.2. Induction of diabetes

Thirty rats were injected with STZ freshly dissolved in 10 mM citrate buffer (pH 4.5) in a dose of 70 mg/kg body weight via the tail vein. During the first 2 days following STZ injection, rats were allowed for free access to glucose 20% orally. After 2 days, the STZ-injected rats were tested for elevated blood glucose levels by blood samples. The animals displaying blood glucose more than 250 mg/dl were considered to be diabetic and included in the present study (Zhang et al., 2016).

2.3. Induction of excisional skin wound

One week after inducing diabetes, full thickness excisional skin wound on the back of all rats were performed. The rats were anesthetized using ketamine (50 mg/kg/rat) and the back hair was shaved. The outline of a standard wound per rat, each 6 mm in diameter, was drawn by a surgical marker pen. Then full thickness excisional wounds were made using scalpel blade reaching down to the muscle layer (João De Masi et al., 2016) and the induced wounds were left open. The rats were administered buprenorphine 0.05 mg/kg intraperitoneal injection, every 8–12 h as post-operative analgesics (Suckow et al., 2017). Continuous observation was done to make sure that the rats did not wipe away the hydrogel. In addition, the wound site was high along their back near the back of the neck, away from their reach.

2.4. Synthesis of Curcumin Nanoparticles-Hydrogel (Cur-NP/HG)

Curcumin (Sigma Aldrish, Egypt) and curcumin nanoparticles (NT-Cur-NP, NanoTech Egypt for Photo-Electronics, City of 6 October, Egypt) were purchased in the form of yellowish powder. The size of Cur-NP is 30 ± 5 nm with spherical like shape and they are freely water soluble. Curcumin and Cur-NP, each was loaded into N,O-carboxymethyl CS/oxidized alginate hydrogel to form a gel-forming composite (Li et al., 2012), with concentration of 7.5 mg/ml (Krausz et al., 2015).

2.5. Groups and treatment

The animals were divided into four groups (n = 10 rats/group) each were kept in separate wire cages at room temperature, fed ad libitum and allowed for free water supply.

- Group I (C): non-diabetic rats with non-treated skin wound.
- Group II (D): diabetic rats with non-treated skin wound.
- Group III (D+ Cur/HG): diabetic rats treated with 1 ml of curcumin hydrogel (Cur/HG) at a dose of 7.5 mg/ml. It was applied topically on the skin wound once daily for two weeks.
- Group IV (D+ Cur-NP/HG): diabetic rats treated with 1 ml of Cur-NP/HG at a dose of 7.5 mg/ml. It was applied topically on the skin wound once daily for two weeks.

3. Evaluation of wound closure

Wound closure size was determined on days 1 and 14. The area of the wound left unhealed was measured using a traditional millimeter graded ruler. All rats were photographed and the percentage (%) of wound closure was assessed using the Wilson's formula stated as % of wound closure = [(Area on 1 day – Area of X days)/Area on 1 day] × 100% (Byrnes et al., 2004). Percentage of wound closure was presented as mean ± standard deviations (SD) and analysed using one-way analysis of variance (ANOVA).

4. Histological studies

The animals were euthanized by an intraperitoneal injection of a pentobarbital overdose (Zhou et al., 2017) after 2 weeks of wound induction. Skin specimens were obtained from the widest area of the wound tissue with surrounding normal skin margin and fixed in 10% formol saline for 1 day. Paraffin blocks were processed and 5 μm thick sections were subjected to the following studies:

1. Hematoxylin and eosin (H&E) staining to determine structural changes.
2. Masson's trichrome stain to determine collagen deposition.

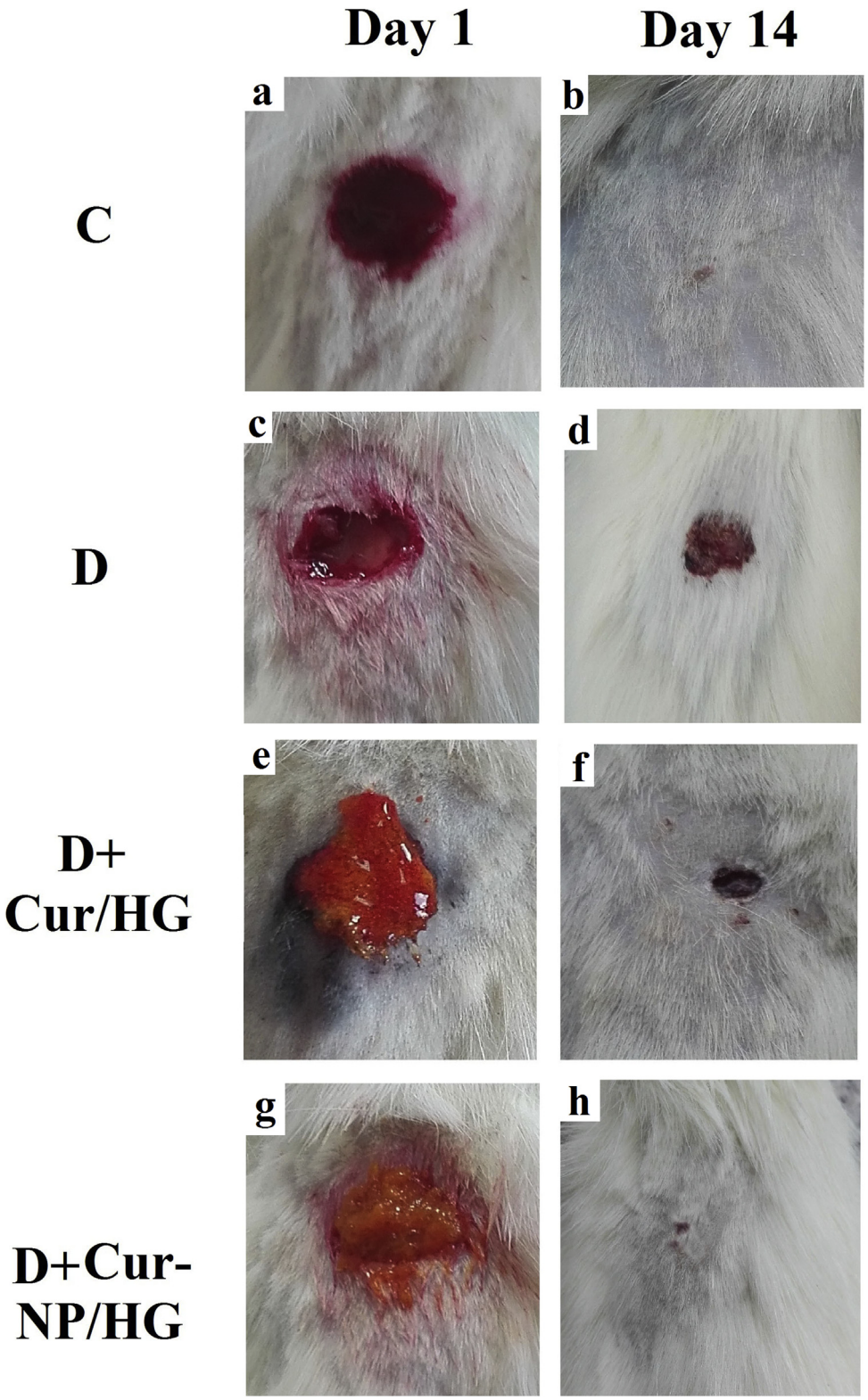

Fig. 1. Gross morphology of diabetic wound size at day 1 and day 14 showing acceleration of the wound healing with Cur/HG (e,f) and Cur-NP/HG (g,h) treatment. (C): control group (a,b), (D): diabetic group (c,d).

3. Immunohistochemical staining for VEGF and AQP3. Polyclonal anti-VEGF antibody (Ab) (Thermo Scientific; Cat. # RB-222-R7), and Polyclonal anti-AQP3 Ab (abcam; Cat. # ab153694) were used. Deparaffinized and rehydrated sections were incubated with 3% H2O2 for 10 min then put into citrate buffer solution (pH 6.0) and heated twice to recover the antigen. Then, washing twice with PBS (pH 7.2–7.6) was done. Application of the primary (1ry) antibodies was followed by incubation in humidity chamber for about an hour at room temperature, and then incubated with biotinylated secondary Ab (Thermo Scientific; Cat. # TP-060-BN). Sections were co-stained with Meyer's hematoxylin to visualize the nucleus.

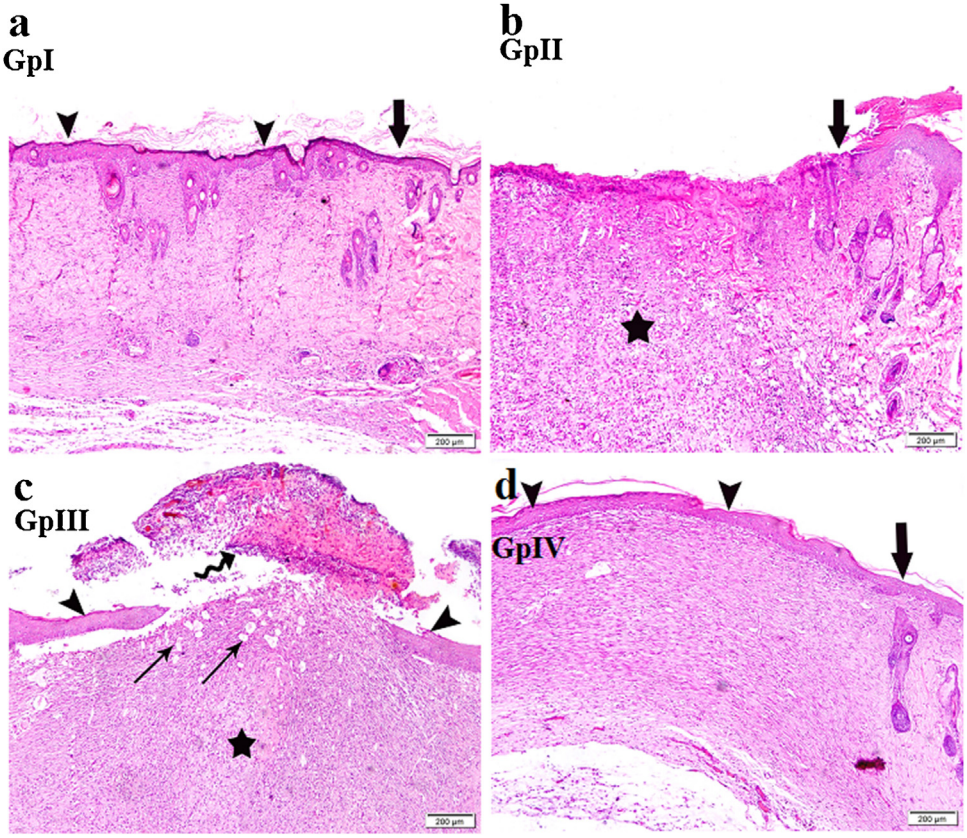

Fig. 2. Photomicrograph of H&E stained sections (×40) showing accelerative effect of Cur-NP/HG on re-epithelization and granulation tissue formation in diabetic skin wound. (a): **GpI** showing complete bridging by newly formed epithelial cells (arrowheads) with superficial horny layer. (b): **GpII** displaying failed re-epithelization of the epidermis and wide disorganized granulation tissue (star) filling the incisional space. (c): **GpIII** demonstrating epithelial cells migration beneath the scab but without bridging the whole incision, inflammatory cells infiltration (wavy arrow) and granulation tissue (star) invaded with newly formed blood vessels (thin arrows). (d): **GpIV** exhibiting complete re-epithelization with complete differentiation of epidermal cells and formation of horny layer over the entire wound. (Thick arrow: the edge of the wound). Scale bar 200 μm.

5. Morphometry and statistical analysis

Using "Leica Qwin 500 C" image analyser (Cambridge, UK), assessment of the followings was performed:

a. The area percent (%) of collagen deposition in 10 non-overlapping low power fields (×10)/rat.
b. The area % of VEGF +ve immunostainig in 10 non-overlapping high power fields (×400)/rat.

Quantitative data were summarized as means ± SD and compared using ANOVA followed by post-Hoc analysis (Tukey test). The probability value < 0.05 was considered statistically significant. Calculations were made on Statistical Package of Social Science software (SPSS), version 19 (Chicago, CA).

6. Results

6.1. Cur-NP/HG accelerated the wound closure

Accelerated wound closure was observed in control and curcumin treated groups as compared to the diabetic non-treated group (33.3%) (Fig. 1). Diabetic rats that received Cur-NP/HG showed a higher rate of wound closure (93.3%) compared to Cur/HG group (58.3%).

6.2. Histological staining

6.2.1. H&E: Cur-NP/HG improved re-epithelization and the reorganization of the dermis in diabetic wound:

The histological analysis of the healing process in control group (**GpI**) demonstrated complete re-epithelization of the wound. The full differentiation of keratinocytes was confirmed by the presence of keratinized layer (cells devoid of nuclei) above the nucleated epithelial cells. In addition, intact dermo-epidermal junction, reorganization of the dermis and formation of appendage-like structures were observed (Figs.2a and 3a). Meanwhile, the skin wound in diabetic non-treated group **(GpII)** showed failed re-epithelization of the epidermis, pronounced leukocytic infiltration and disorganized granulation tissue filling the incisional space (Figs.2b and 3b).

Treatment of the diabetic skin wound with curcumin hydrogel for 2 weeks in **GpIII** accelerated the healing process to some extent provided by partial re-epithelization of the epidermis and detected luminized blood vessels. However, separation at the dermo-epidermal junction and mild inflammatory infiltrate were noted (Figs.2c and 3c).

Application of topical Cur-NP/HG on diabetic skin wound for 2 weeks in **GpIV** exhibited accelerated healing process. This was clarified by complete re-epithelization with complete differentiation of epidermal cells over the entire wound area with intact dermo-epidermal junction and reorganization of the dermis (Figs.2d and 3d).

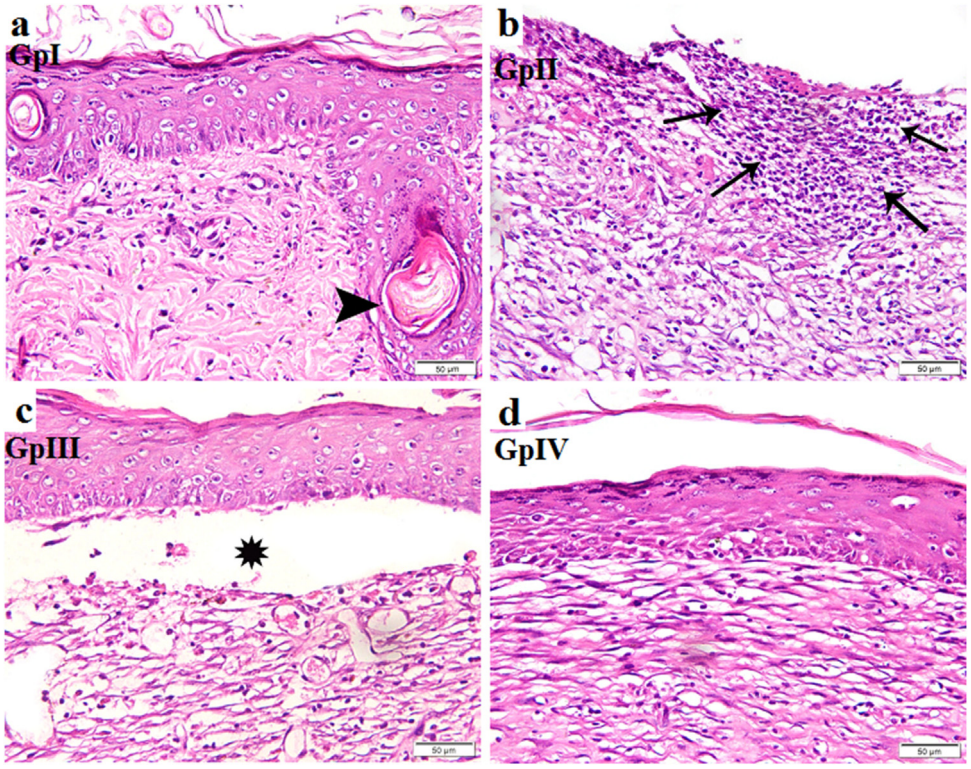

Fig. 3. Higher magnification of H&E stained sections (×200) showing: (a): **GpI** illustrating intact dermo-epidermal junction, reorganization of the dermis and formation of appendage-like structure (arrowhead). (b): **GpII** with pronounced leukocyte infiltration (arrows) on the surface of the wound gap. (c): **GpIII** displaying keratinocytes differentiation with overlaying horny layer, separation at the dermo-epidermal junction (asterix) and arrays of luminized blood vessels. (d): **GpIV** exhibiting nearly complete differentiation of epidermal cells with intact dermo-epidermal junction. Scale bar 50 μm.

6.2.2. Masson's trichrome: Cur-NP/HG enhanced collagen deposition in diabetic wound

Compact collagen bundles formation was found in non-diabetic non treated group **(GpI)**. While diabetic non treated skin wounds demonstrated fine immature collagen deposition exhibiting randomly scattered manner with significant decrease in the mean area % of collagen deposition as compared to other groups. **GpIII** showed fine more organized collagen deposition in the incisional space as compared to **GpII**. While in **GpIV**, more mature properly organized collagen deposition was detected with significant increase in the area % of collagen deposition as compared to **GpII** and **III** (Figs.4 and 6a).

6.3. Immunohistochemistry staining

6.3.1. VEGF: Cur-NP/HG increased VEGF expression

Control and curcumin treated groups revealed significant increase in VEGF +ve immunostaining detected in keratinocytes, endothelium, connective tissue (CT) cells and extracellular matrix (ECM) as compared to diabetic non treated groups. However, there was significant difference between Cur/HG and Cur-NP/HG treated groups (Figs.5 6b).

6.3.2. AQP3: Cur-NP/HG increased AQP3 expression in keratinocytes:

Diabetic skin wound showed negative immunostaining of AQP3. Cur/HG treated group showed +ve AQP3 cells with membranous reaction in startum basale and few intermediate layers. Meanwhile, Cur-NP/HG treated group expressed substantial strong +ve AQP3 membranous reaction in the keratinocytes of the basal and many intermediate layers of the regenerated epidermis that was comparable to the control group. Some intracellular staining was observed in basal cell layer (Fig. 7).

7. Discussion

The present study demonstrates the efficacy of therapeutic topical application of Cur-NP in diabetic skin wound using a gel-based formulation. This work succeeded in modeling Type I diabetes within 2 weeks by using STZ, which was verified by high sugar blood level, before inducing skin wound. Animal models have been documented to assess the healing process of excisional, incisional and burn wounds (Mehrabani et al., 2015). However, the rat model for excisional wound is considered the most common as the wound induction is modest and various stages such as scar formation, epithelialization, and angiogenesis can be distinctly assessed (Zhou et al., 2017).

The diabetic skin wounds untreated after 2 weeks exhibited ulceration, inflammation, defective granulation tissue formation and significant decrease in the collagen deposition with randomly scattered manner. It has been stated that impaired new blood vessels formation in diabetes retards the healing process and induces the ulceration (Bodnar, 2015). Diabetes retards all phases of wound healing; the inflammatory stage through compromising the immunity (Park and Lim, 2011); the proliferative stage via dysfunction of fibroblasts, collagen deposition, and defective neovascularization, and the remodelling stage involving defective reestablishment of tissue structural integrity (Zhou et al., 2017). Diabetic wound is associated with inadequate ECM production with dermal protein profile favouring proteolysis. This was attributed to up-regulation of the activated matrix metalloproteinase. Subsequently, collagen content in diabetic wound decrease and deteriorates the healing process (Xu et al., 2017).

Application of Cur/HG and Cur-NP/HG to diabetic wound for 2 weeks significantly accelerated the healing process, yet in different efficacy rating. Curcumin hydrogel induced partial re-epithelization and luminized blood vessels, the hallmarks of

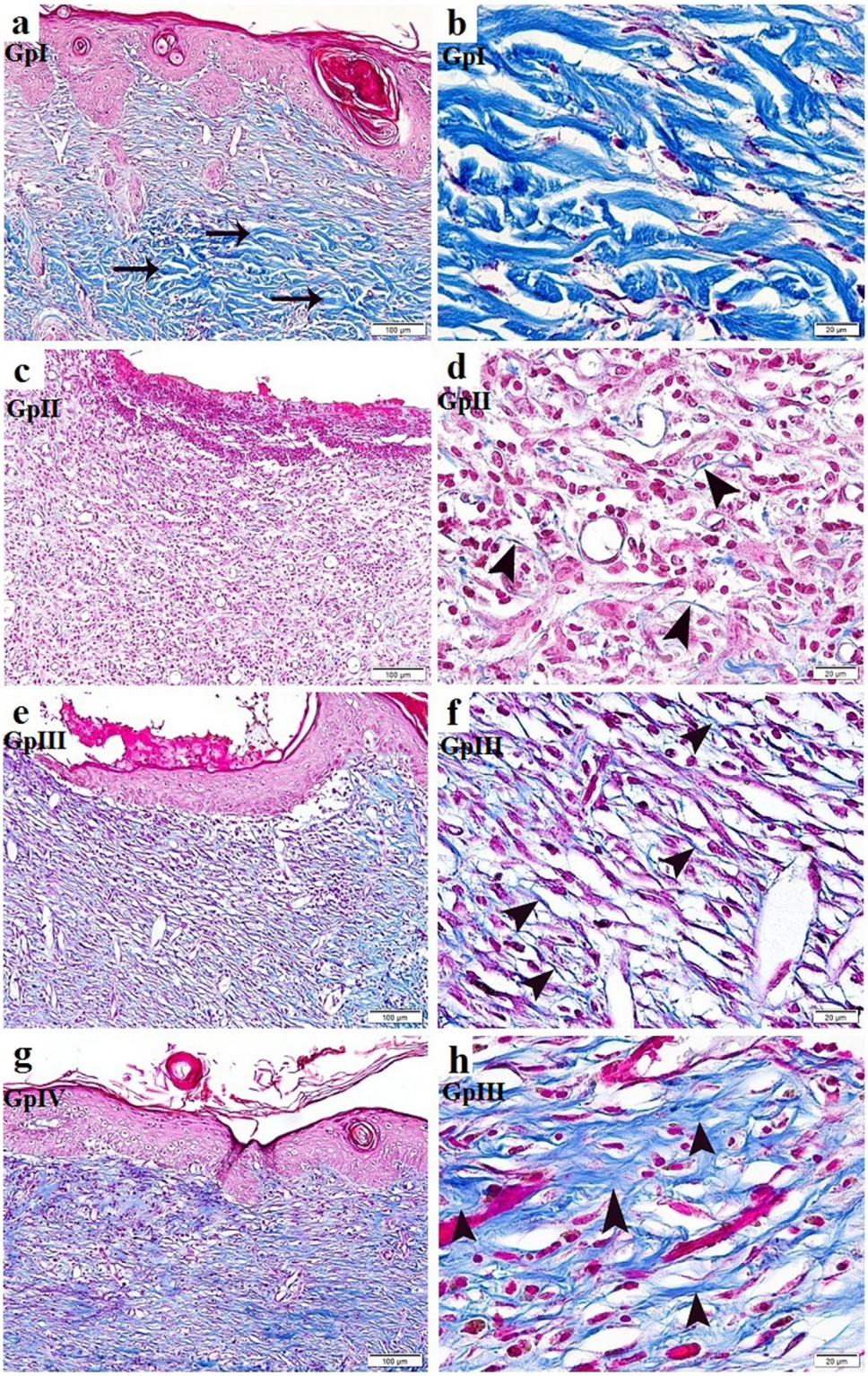

Fig. 4. Photomicrograph of Masson's trichrome stained sections showing improving effect of Cur-NP/HG on collagen deposition in diabetic skin wound. **GpI** (a and b): intense staining of collagen bundle in the reticular layer of the dermis (arrows). **GpII** (c and d): fine sparsely distributed collagen fibrils deposition (arrowheads) in the granulation tissue. **GpIII** (e and f): properly oriented fine collagen deposition (arrowheads) parallel to the epidermis. **GpIV** (g and h): displaying increased density of collagen deposition (arrowheads) intermingling between the cells and the scattered vascular channels. (a, c, e and g ×100, b,d,f and h ×400). Scale bar 100 μm and 20 μm.

proliferative phase. Cur-NP/HG treatment exhibited completely finished re-epithelization and remodelling of the granulation tissue. It was postulated that maturation and remodelling stage of wound healing is recognized by a reduction of the cell population and increasing the collagen organization in granulation tissue to facilitate wound contraction (Kumar et al., 2018).

Curcumin enhance wound healing by alleviating the release of inflammatory cytokines, accelerating formation of granulation tissue and neovascularization, and increasing growth factors

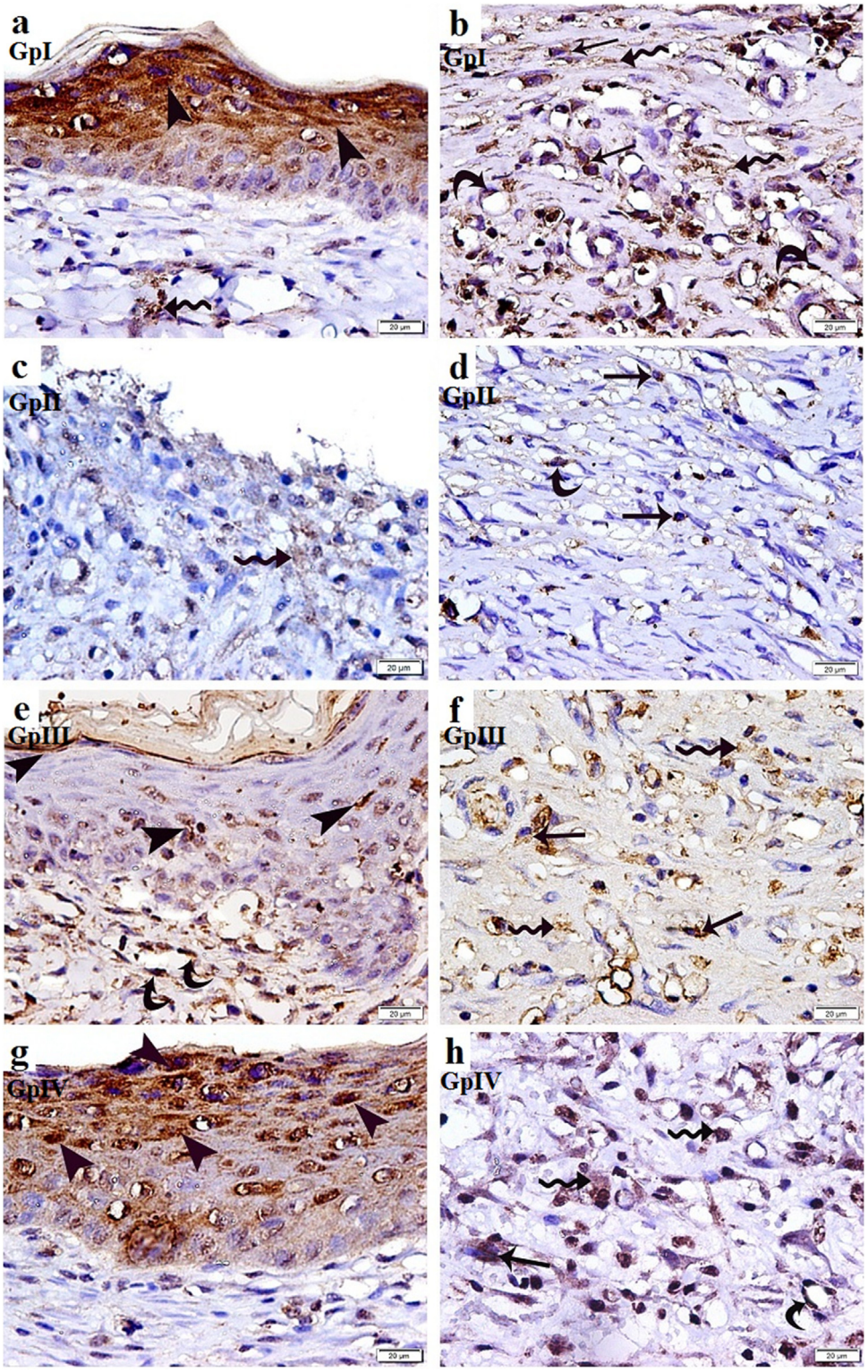

Fig. 5. Photomicrograph of VEGF immunohistochemistry (×400) illustrating showing enhancing effect of Cur-NP/HG on VEGF expression in diabetic skin wound. **GpI** (a and b): extensive VEGF +ve immunostaining in numerous keratinocytes (arrowheads), in addition to endothelium (curved arrows), CT cells (thin arrows) and ECM (wavy arrows). **GpII** (c and d): minimal +ve immunostaining in the endothelial cells (curved arrows), and CT cells (thin arrows) and ECM (wavy arrows). **GpIII** (e and f): +ve immunostainin in many keratinocytes (arrowheads), besides endothelium (curved arrows), CT cells (thin arrows) and ECM (wavy arrows). **GpIV** (g and h): marked VEGF +ve immunostaining in numerous keratinocytes (arrowheads), endothelium (curved arrows), CT cells (thin arrows) and ECM (wavy arrows). Scale bar 20 μm.

biosynthesis and ECM proteins, such as collagen (Kulac et al., 2013). Unfortunately, although its well-known strong anti-oxidant, anti-inflammatory and anti-microbial properties, curcumin dermal delivery is impeded by hydrophobicity, poor skin permeation and rapid degradation profile by hydrolysis (Akbik et al., 2014). This required establishment of suitable vehicle to apply stability and solubility of curcumin with sustained release manner to ensure the

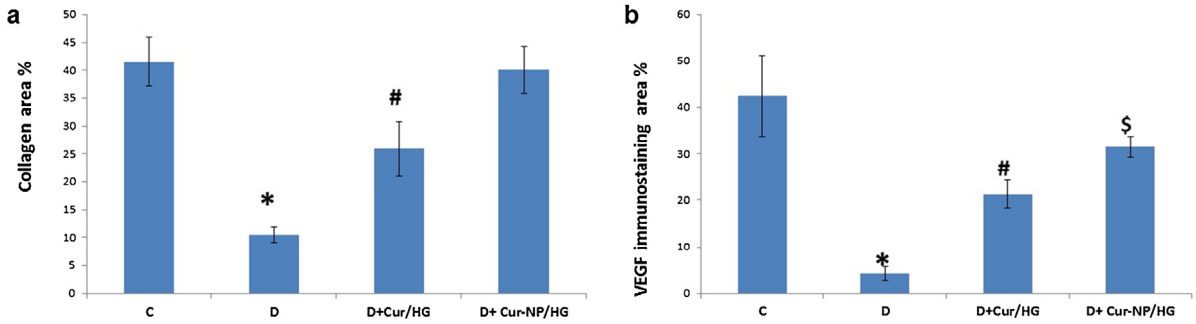

Fig. 6. Quantification of the mean area % of: (a) collagen deposition in Masson's trichrome stained sections, (b) VEGF immunohistochemistry. * Significant decrease as compared to other groups. # Significant decrease as compared to C and D + Cur-NP/HG groups. $ Significant decrease as compared to C.

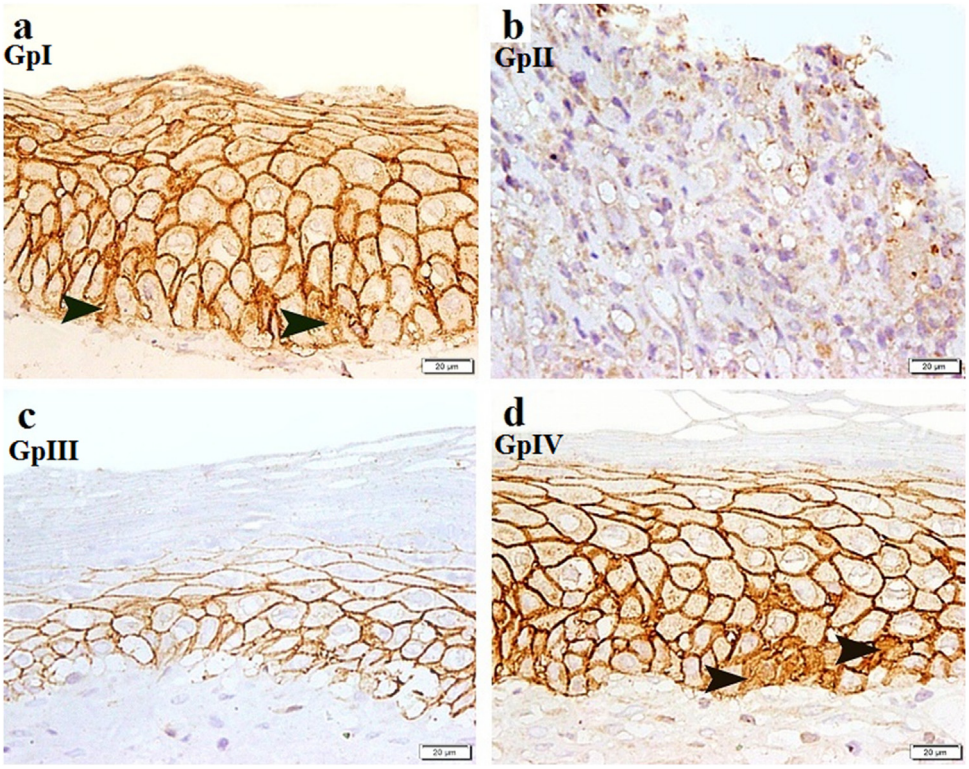

Fig. 7. Photomicrograph of AQP3 immunohistochemistry (×400) showing enhancing effect of Cur-NP/HG on AQP3 expression in diabetic skin wound. GpI (a): strongly stained plasma membranes of keratinocytes in the basal and intermediate layers of the epidermis. Some intracellular staining is observed in stratum basale of the epidermis (arrowheads). GpII (b): negative immunostaining of AQP3 in the wound area. GpIII (c): +ve immunostaining of plasma membranes of keratinocytes in the stratum basale and few intermediate layers. GpIV (d): strong +ve staining of plasma membranes of keratinocytes in the basal and intermediate layers with some intracellular staining (arrowheads). Scale bar 20 μm.

maximum therapeutically effects on skin wounds (El-Refaie et al., 2015).

Hydrogel formulation facilitated topical application of biological molecules, yet rapid release profile of the active ingredients was still observed in this formulation (Demirci et al., 2015). Nanotechnology has provided drug delivery systems with sustained release profile of the molecules. Subsequently, topical application of drug nanoforms alleviated the rapid leakage of the biological molecules from the wound site. Nanogel composite was documented to accelerate skin penetration and dermal localization of curcumin while preventing its degradation (El-Refaie et al., 2015). Moreover, Cur-NP demonstrated antimicrobial activity in in murine burn model (Krausz et al., 2015) and low cytotoxicity in vitro (Li et al. 2016) with enhanced anti-oxidant property indicating its effectiveness and safety administration in wound management (Hussain et al., 2017).

Topical treatment of the diabetic skin wounds with Cur/HG and Cur-NP/HG substantially increased the tissue VEGF compared to non-treated wounds. Diabetes is associated with defective angiogenesis in different organs, with imperfect activity occurring in impaired wound healing. In agreement with the present study, it was reported that delayed diabetic wound closure 12 days post-injury in rat model was associated with reduced VEGF (Hamed et al., 2010). The VEGF stimulates degradation of the ECM of existing blood vessels, by proteases. Thus, providing tube proliferation for endothelial cells and enhance their migration and proliferation for new vessels with subsequent penetration of the wound site (Mohammad et al., 2008). It provides mitogenic and angiogenic effects on the endothelial cells via binding to the membrane (Zhou et al., 2017).

Curcumin was reported to increase micro-vessels density in diabetic wounds associated with up-regulation of VEGF and CD31, the

marker of endothelial progenitors, on day 3, 7 and 14. Thus, indicating curcumin-induced angiogenesis through VEGF pathway (Kant et al., 2015). Recent study postulated that Cur-NP has more powerful effect on neovascularization and endothelial cells migration and proliferation which hasten wound healing (Liu et al., 2018).

In the present work, negative immunostaining of AQP3 was associated with impaired keratinocytes proliferation and migration in diabetic skin wound. Meanwhile, Cur/HG and Cur-NP/HG treatment showed substantial strong +ve AQP3 in the keratinocytes of the regenerated epidermis. AQP3 expression is up-regulated in skin stress indicating enhancement of keratinocyte proliferation (Nakahigashi et al., 2011). Re-epithelialization is a critical step for wound healing that involves migration and proliferation of keratinocytes from the surrounding epidermis and skin appendages like hair follicles and sweat glands. These events are thought to be adjusted mainly by AQP3 (Ishida et al., 2018).

The dermoepidermal junction is a unique anchoring structure which links basal keratinocytes to basement membrane through integrin combinations. The integrin combinations are expressed by mature basal keratinocytes. Collagen VII in basement membrane then is linked to fibrous matrix elements (as: collagens) to complete the anchoring complex to the papillary dermis (Lönnqvist et al., 2015). The separation in this junction could be related to focal incomplete differentiation of keratinocytes. The illustrated difference between GpIII and GpIV in AQP3 expression, which has been demonstrated to play a pivotal role in keratinocytes proliferation, migration and differentiation (Ikarashi et al., 2017), could explain the detected focal separation in GpIII and continuous stability of dermoepidermal junction in GpIV. In addition, the fine collagen deposition detected in GpIII could be another reason for this separation. Meanwhile, GpIV displayed more mature properly organized collagen deposition.

8. Conclusion

Curcumin nanoparticles/hydrogel composite showed much faster recovery of diabetic skin wound via increasing wound closure rate, granulation tissue formation, collagen deposition, VEGF production and AQP3 expression compared with conventional Cur/HG. Cur-NP/HG could be considered as a promising therapeutic candidate for diabetic skin wounds.

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