



Pharmaceutical, clinical, and immunohistochemical studies of metformin hydrochloride topical hydrogel for wound healing application

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Abstract

Wound healing could be categorized as a chronic disorder which needs a prompt and effective medication. Nowadays, vascular problems and wound-healing progression studies rely on the role of metformin; however, the exact mechanisms for its beneficial effects are still not completely understood. The aim of the present work was to study the potential of metformin hydrochloride, MET-HCL hydrogel in wound healing after topical administration. MET-HCL hydrogel was formulated using different hydrogel bases then characterised for its drug content and pH, homogeneity, viscosity, spreadability, and in vitro release. Furthermore, the wound-healing activity was evaluated in rats. Clinical study was also performed in patients showing lower limbs traumatic wounds and cutaneous ulcers. In addition, histopathology and immunohistochemical studies were also investigated. Results showed that MET-HCL hydrogels have a high drug content, and they are homogenous and spreadable with minimum sheer stress and showed null irritation. Furthermore, carbopol showed the optimum gel base for the highest MET-HCL release. Wound healing activity in rats showed a significant ($p < 0.05$) increase in the percentage wound contraction from the day-7 compared with plain hydrogel. Clinical study revealed a complete healing of traumatic wounds and cutaneous ulcers after 21 and 30 days, respectively. Moreover, histopathology investigation showed a complete restoration of connective tissue matrix and re-epithelization of the induced wound. In addition, immunohistochemistry showed a significant expression of transforming growth factor- β 1 (TGF- β 1). In conclusion, MET-HCL hydrogel is a promising formulation for topical treatment of wounds with effective and faster healing activity.

Keywords Metformin hydrochloride · Topical hydrogel · Angiogenesis · Wound healing · Transforming growth factor- β 1

Introduction

Metformin hydrochloride, MET-HCL, is a biguanides derivative and used mainly for the management of diabetes mellitus type 2 long ago with prospective success until this moment [1]. It is a mechanism of action that was already demonstrated via improving glucose tolerance and enhances body sensitivity to insulin [15]. Wounds could be simply defined as a disruption of the cellular anatomic continuity of a tissue. This was followed by the destruction of normal barrier properties of the skin followed by intense invasion of microorganisms [25]. Patients suffer from any form of wounds that need prompt treatment and management in a relatively short time which will protect them from the harmful consequences of these wounds like being chronic as well as increase the risk of infections. In addition, inappropriate healing could also be another important issue which will complicate and delay the

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healing process [43]. The wound-healing action is also a complex cascade process which based on sharing of different processes, elements, and growth factors such as transforming and epidermal growth factors which are responsible for the activation of fibroblast [11]. TGF-beta 1 is a human DNA-derived polypeptide growth factor which helps in normal tissue repair mechanisms, as this growth factor is released by essential cells of the repair process as fibroblasts, monocytes/macrophages, endothelial cells, and platelets [40]. Finally, the different biological responses result in a replacement of normal skin with fibroblast scar tissue.

In general, different approaches were utilized to control different types of wounds such as chronic wounds, deep ulcers, deep burns, and skin loss. These approaches were ranged from traditional therapy, e.g., herbal derived compounds [23], animal-derived compounds [12], and traditional dressing [33], to more advanced methods and techniques such as wound-dressing hydrogel containing sodium salt of fusidic acid [22], stem cells application for healing of chronic wounds [24], cellulose nanomaterials [41], and colloidal metallic nanoparticles such as silver and gold nanoparticles [32].

Nowadays, researchers explored different uses and applications of some FDA-approved medications and drugs. These drugs could be directed to treat another related or non-related disorders. For examples, MET-HCL hydrogel was used successfully for lowering the intraabdominal accumulated fats and help in weight loss when applied into the abdominal area [7]. Sildenafil citrate was formulated into a thermosensitive gel for vaginal administration to treat women having problems in ovulation [39]. Atorvastatin gel has also been used for wound healing applications [9]. Recently, it was proven that MET-HCL has a significant role in wound healing via regulating some signalling pathway which affect macrophages polarization [34]. MET-HCL was formulated into different formulations, e.g., nanoliposomal formulation and nanofibers eluting membranes for wound-healing applications [19, 26]. However, all of these investigated formulations are so what complex, expensive and needs special materials and equipment for their fabrications.

Therefore, the aim of this investigation was to study the potential wound healing activity of locally applied MET-HCL after formulation into a simple hydrogel. The drug was loaded into different hydrogel bases and evaluated for its drug content, pH, homogeneity, spreadability, viscosity, skin irritation performance, and in vitro release. Furthermore, the wound-healing activity was studied in rats. The human clinical study was also performed on patients having lower limbs wounds or ulcers. In addition, histopathological and immunohistochemical changes after gel treatment were also evaluated to demonstrate the effect of the applied formulation on the wound healing.

Materials and methods

Materials

Metformin hydrochloride is purchased from Sigma-Aldrich, Egypt. Carbopol 934, methylparaben were purchased from Merck Company (Darmstadt, Germany). Hydroxypropyl methylcellulose, sodium carboxymethyl cellulose, and sodium alginate were obtained from (Aldrich Chemical Company, USA). The other used chemicals and reagents were of analytical grades and used as received.

Preparation of MET-HCL hydrogels

Different hydrogel bases were utilized for preparation of MET-HCL hydrogel, namely: hydroxypropyl methylcellulose (HPMC) (2.5% w/v), Na-alginate (3.0% w/v), and sodium carboxymethyl cellulose (Na CMC) (2.0% w/v). Hydrogels were prepared by incorporation of gel base to distilled water followed by stirring until a homogenous gel is formed. In case of carbopol gel (2% w/v), the polymer was dispersed in hot-distilled water under stirring until a homogenous gel is obtained. 10 mg of sodium hydroxide pellets were dissolved in distilled water and incorporated into the carbopol hydrogel (0.5% w/v) for neutralization and complete gel formation. Finally, to remove any formed air bubbles, gels were left in the fridge overnight, then MET-HCL (6 mg/g gel) was added to the formed gel with continuous stirring followed by the addition of 0.02% w/v methylparaben as a preservative. The prepared hydrogels were poured and stored into amber-colored containers at ambient room conditions for further characterization.

Characterization of the prepared hydrogels

pH and MET-HCL content

MET-HCL hydrogels were dispersed in distilled water and their pH values were recorded using a digital pH meter (3500 pH meter, Jenway, UK) ($n = 3$). The drug contents of the prepared MET-HCL hydrogels were determined as previously reported [7]. Hydrogel bases did not interfere with the spectrophotometric analysis of MET-HCL.

Viscosity

The viscosity of different MET-HCL hydrogel formulations was performed using a Brookfield digital viscometer (Model

DV2TR Brookfield Engineering Laboratories, USA). Measurements were performed in triplicates at room temperature using spindle No. 94 and at 15.0 rpm.

Spreadability

MET-HCL hydrogels spreadability in (g cm/s) was performed according to the method adapted with Liberman et al. [27]. Briefly, a weight of (20.0 g) was applied to glass slides (6 cm, length) having the formulation; then, the time required to separate the two slides was recorded in seconds using an equation adapted in the previous work [6].

Homogeneity

The homogeneity of MET-HCL hydrogel formulations was tested by inspection of their visual appearance. Furthermore, a small quantity of each gel formulation is pressed between fingers and the consistence of hydrogel was noticed whether homogenous or not [6].

In vitro release study

The in vitro release of MET-HCL from different hydrogel bases was performed at phosphate buffer of pH 7.4 in a thermostatically controlled shaker, 50.0 stroke/min maintained at 37.0 ± 1.0 °C (WiseBath, WSB-45, Korea) as previously published [7]. Briefly, 1-g gel from each formulation was tested and at different time intervals, and samples were taken for the analysis of MET-HCL using UV–Vis spectrophotometry (Jenway-Japan) at λ_{\max} of 233 nm [10]. In vitro release study was performed in triplicate and the average amounts released were calculated.

Wound healing activity in rats

White male Wistar rats (*Rattus norvegicus*), weighing 150 ± 20 g, were housed under the controlled environment of temperature and humidity in individual cages with free access to tap water and diet. The ethical committee approved the wound-healing activity study according to the roles of the Faculty of Medicine, Assiut University, Egypt. Two groups of rats were used in this study, and each group has 15 rats. The first control and received placebo carbopol gel and the second group was received MET-HCL-loaded hydrogel (approximately 50.0 mg hydrogel, containing MET-HCL in a dose of 300 µg). Anesthesia was performed by administration of 4.5 mg of ketamine and 3.0 mg of thiopental, intramuscular, and intraperitoneal, respectively [4]. Rats were kept under stable anesthesia during the 90 min for wound creation. The back of the rat was shaved to remove the hair from the dorsal area between the shoulder blades, and then, the surgical site was wiped three times with fresh sterile cotton tips saturated

with alcohol to sterilize the surgical site. Skin wounds of about 8.0 ± 0.2 mm were performed using 8.0 mm skin biopsy punches on the back of each rat, with two wounds created on each side of the spine of each rat. Hydrogel formulations were applied to the wound injury under non-occlusive conditions. Nevertheless, the wounds were not covered with occlusive dressing.

Clinical study

This prospective open-label study was conducted between October 2017 and December 2018 at the Department of Dermatology, Venereology and Andrology, Assiut University Hospital, Assiut, Egypt. The study design was approved by the Institutional Ethics and Research Committee of the Faculty of Medicine, Assiut University and was carried out in accordance with the guidelines of the Helsinki Declaration. Informed consent was obtained from all patients prior to the study.

Fifty patients with non-healing lower limbs' traumatic wounds or ulcers were screened and 36 patients were enrolled in the study. Six patients skipped treatment, and finally, 30 patients of either gender completed the study. Patients below 12 years of age, those with diabetes mellitus or any active systemic illness, patients on immunosuppressive drugs, immunosuppressed individuals, and those who have received any previous other treatment for the lower limbs wounds or ulcers before enrolment were excluded from the study.

A proper history was taken regarding age, sex, duration, site of the lesions, and previous treatment. A local cutaneous examination was performed. Subsequently, topical MET-HCL hydrogel was applied uncovered on lower limbs' traumatic wounds or ulcers twice daily until clearance of the lesions or for a maximum of 1 month, whichever was nearer. Moreover, clinically pain, edema, granulation tissue formation, and re-epithelialization were recorded. Digital photographs were taken at the beginning and at the end of treatment using digital Canon camera (Model A3100 IS, 12.1 MP, Powershot, Malaysia) to document the improvement in the lesions. The clinical response to treatment was evaluated by the photographic comparison. In addition, side effects from treatment including erythema, edema, and other signs of skin irritation were also recorded. The scores of the erythema and or edema were ranged from 0 for none and 4 for severe erythema and edema.

Histopathological and immunohistochemical studies

Histopathological study

Punch biopsies were obtained before and after treatment using a 3.0 mm punch from the edge of the lesions, and they

were collected from all patients under local anesthesia via administration of 1.0% xylocaine. Then samples were fixed in 10.0% formalin for 24 h at room temperature, embedded in paraffin, and sectioned at 4.0 μm for conventional histopathological examination. Sections were stained with hematoxylin and eosin (H&E), examined and photomicrographs were taken by a digital camera (Nikon DMX1200).

Immunohistochemical study

The paraffin sections are pretreated at 60.0 ± 0.5 °C for 12 min at the microwave then blocked using endogenous peroxidase for 15 min and incubated in the primary antibodies (rabbit polyclonal anti-rat TGF- β 1, 1:100 Abcam) for 60 min. Secondary antibodies were applied for 15 min. Finally, digital images were taken with a digital camera (Nikon DMX1200).

Immunohistochemical evaluations

The cytoplasmic immunostaining of TGF- β was semi-quantitatively evaluated according to the Remmele immunoreactive score (IRS) [35]. The cytoplasmic immunostaining of TGF- β 1 was graded according to its intensity of positivity as negative, weak, moderate, and strong.

Statistical analysis

Student *t* test was applied to investigate the significant difference in the release of MET-HCL from the investigated hydrogel bases and between the tested groups in rats study. Adapting $p < 0.05$ to demonstrate the significant difference. In addition, results are presented as mean \pm standard deviation.

Results

Physical evaluation of MET-HCL hydrogels

The results of pH, drug content, viscosity, and spreadability of MET-HCL hydrogels are presented in Table 1. MET-HCL hydrogels were homogeneous in texture and free from lumps. Moreover, hydrogels retained their physical

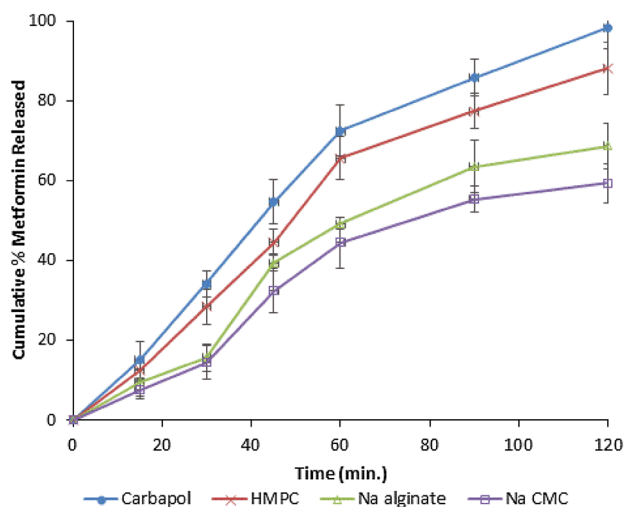


Fig. 1 Cumulative percentage metformin hydrochloride released from the different investigated hydrogel bases at pH 6.8 using modified Franz-diffusion method ($n=5$)

appearance and homogeneity after 1 month of storage at room temperature under ambient conditions. MET-HCL contents were ranged from 98.55 ± 2.2 to $99.6 \pm 1.5\%$, which is considered satisfactory. Moreover, the hydrogels pH values ranged from 6.7 ± 0.08 to 7.0 ± 0.03 , which indicated that they conform with the skin [3, 5, 8]. Viscosity measurements revealed a higher viscosity with Na CMC followed by sodium alginate, HPMC, and lastly carbopol, as depicted in Table 1. In addition, hydrogels showed spreadability values ranged from 4.9 ± 0.5 to 5.9 ± 0.6 g/cm/s.

In vitro release

The performance of MET-HCL released from the investigated hydrogel bases is shown in (Fig. 1). The cumulative percentage of the drug released was in the following order: carbopol hydrogel > HPMC > sodium alginate > Na CMC. It is also worth noting that carbopol showed a significantly ($p < 0.05$) higher amounts of the drug released compared with sodium alginate and Na CMC.

Table 1 Physical evaluation of metformin hydrochloride hydrogels

Hydrogel base	pH	Drug content % \pm SD	Viscosity cPs \pm SD	Spreadability g cm/s \pm SD	Skin irritation
HPMC	6.8 ± 0.02	99.3 ± 2.4	1800 ± 250	5.2 ± 0.3	Null
Carbopol	6.7 ± 0.08	98.5 ± 2.2	1650 ± 130	5.9 ± 0.2	Null
Sodium Alginate	7.0 ± 0.03	99.2 ± 1.8	6000 ± 430	5.2 ± 0.4	Null
Na CMC	6.9 ± 0.01	99.6 ± 1.5	$18,500 \pm 680$	4.9 ± 0.2	Null

The wound healing activity on rats

The wound healing activity was studied on rats to prove the healing activity of MET-HCL hydrogel, a known area of wounds received MET-HCL gel, plain (placebo) carbopol hydrogel. The injured areas were measured after 0, 4, 7, and 14 days. It was found that group who received MET-HCL hydrogel showed a significant decrease ($p < 0.05$) in the wound contraction area compared to the group received placebo hydrogel. MET-HCL gel group showed a whole curative after 7 days as shown the percentage of wound contraction of $99.34 \pm 0.56\%$. Conversely, the wound received a placebo gel showed no improvement on the injured skin even after 14 days, as shown in Fig. 2.

Clinical results

Skin irritation

It was noticed that none of the volunteers shared in the study showed erythema, edema, or any other signs of skin irritation after topical administration of MET-HCL hydrogel during the course of treatment as showed in Table 1.

Demographic data of the patients

Before conducting the clinical studies and evaluations, some demographic data were collected from patients shared in the study. A total of 30 patients (18 patients with lower limbs traumatic wounds and 12 with lower limbs cutaneous ulcers) were treated with topical MET-HCL hydrogel. Male

patients were 19 (63.3%) and females were 11 (36.7%). Age of the patients ranged from 15 to 60 years; mean age was 32.94 years.

Clinical observations

Clinically, it was observed in all patients enrolled in the study, a remarkable decrease of pain and edema within 10–14 days after topical application of MET-HCL hydrogel. Patients with lower limbs traumatic wounds and lower limbs ulcers, the re-epithelization, and formation of new granulation tissue were observed within 7–12 and 13–21 days, and 7–14 and 15–30 days, respectively, as pointed out in Fig. 3a–d.

Histopathological observations

The microscopic observations under H&E staining were done after 1 month of topical MET-HCL hydrogel treatment. Results revealed improvement with the proliferation of both epithelial and dermal elements in the form of re-epithelization of the wound, angiogenesis, fibroplasia, and restoration of the connective tissue matrix (Fig. 4a, b).

Immunohistochemical expression of TGF- β 1

As observed from Fig. 4, a strong positive expression of TGF- β 1 was noticed in fibroblasts in treated group with topical MET-HCL hydrogel (Fig. 4d) in comparison with the pretreated group (Fig. 4c).

Fig. 2 Percentage wound contraction on the induced wound in rats after 14 days treatment using either plain carbopol hydrogel or metformin hydrochloride carbopol hydrogel 0.1% ($n=6$)

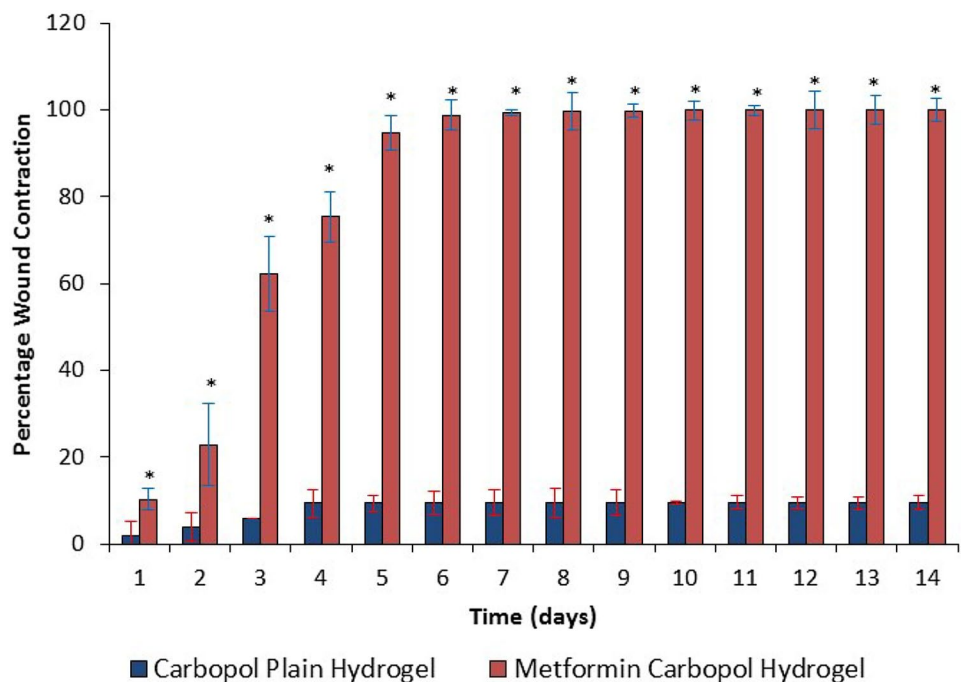




Fig. 3 **a** Traumatic wound on patient's left foot before treatment. **b** Complete healing after 21 days of topical metformin hydrochloride hydrogel treatment. **c** Cutaneous ulcer over the dorsum of patient's left foot before treatment. **d** Complete healing after 30 days of topical metformin hydrochloride hydrogel treatment

Discussion

Wound healing is considered as one of the most important complex processes which need fast and effective treatment [14]; as in different cases, it could be a life-threatening disorder. Cutaneous wound also one amongst soft-tissue injuries which need a relatively long time for complete curing and healing [46]. Different strategies have been investigated to overcome this problem with varying degree of success and time duration such as lyophilized wafers [36], general dressings [45], and topical hydrogels [2]. However, maintaining a moist environment around the wound area is the most preferred strategy [13]. Hydrogels could fulfill this requirement and keep the moist environment around the wound due to their unique three-dimensional network structure as well as they can accelerate the wound-healing process [30]. MET-HCL is widely used all over the world for its anti-diabetic action especially in type II diabetes

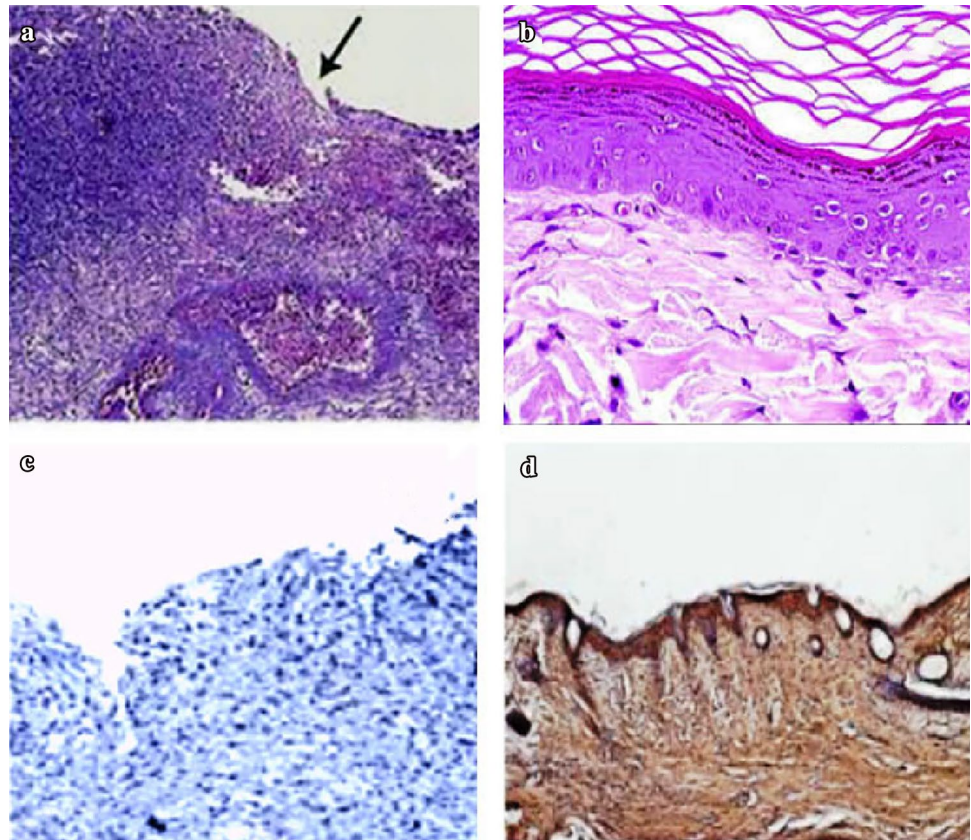
mellitus [20, 38]. In this study, the wound healing activity of topical MET-HCL was explored and evaluated in animals and in humans. Hence, the work was divided into two parts: the first part was the formulation and pharmaceutical characterization of MET-HCL hydrogels and the second part dealing with the clinical, histopathological and immunohistochemical assessment of wound healing after topical treatment with MET-HCL hydrogel.

Hydrogels were prepared using different gelling agents to get the most suitable gelling agent regarding drug release pattern which was observed with carbopol 943. In general, it is known as a higher viscosity of the hydrogel bases the lower percentage of drug released [18]. As shown from Table 1 that carbopol showed a lower viscosity compared with the other investigated bases; hence, a higher percentage of the drug released. Observation of other physical characteristics of the formulated hydrogels revealed their homogeneity and compatibility with skin of the participated volunteers as all of them demonstrated null irritation score.

The easy application of gel into the skin surface is related to a higher extent to its spreadability. The obtained higher values of spreadability revealed the easy and faster application of gel into the skin, which is acceptable for patients. The MET-HCL released from the different hydrogel bases was continued and sustained for about 2 h which is suitable for the wound healing process. The release is slower at the first 60 min, then faster during the second 60 min. A similar release profile was detected by Guerrero et al., where MET-HCL was released from sodium alginate/polyvinyl alcohol hydrogels [29]. This performance could be attributed to the instant dissolution and release of the freely soluble drug during the dissolution of the hydrogel. This fast release of the drug makes the gel could diffuse rapidly when the gel came into contact with the skin or the release medium. The higher water solubility of the drug could also be responsible for the higher release from carbopol base and hence better absorption. The induced wounds in rats showed a time-dependent increase in the percentage of contraction from 22.87 ± 8.46 to 98.80 ± 0.8 from the second day to the sixth-day post-treatment. These results promote the high efficiency of MET-HCL hydrogel for rapid wound-healing activity. It is also worth noting that topical MET-HCL showed more pronounced results in wound closure compared to the previous study performed in rats given oral MET-HCL [44]. MET-HCL could modulate several signaling pathways which are responsible for the vascularization of granulation tissue and promoting angiogenesis [42].

Such finding verified the wound healing action in mice. Similar observations were also recorded with Zhao et al., who studied the effect of local MET in cutaneous wound healing and conclude that MET applied topically strongly promote wound healing compared to resveratrol [46].

Fig. 4 **a** Histopathology. Before treatment: showing ulceration of the epidermis (arrow) (H&E $\times 200$). **b** Histopathology. After treatment: showing re-epithelization of the epidermis, angiogenesis, fibroplasias, and restoration of the connective tissue matrix (H&E $\times 400$). **c** Immunohistochemistry: before treatment: showing negative staining reaction for TGF- $\beta 1$ (DAB $\times 400$). **d** Immunohistochemistry. After treatment: showing strong positive staining reaction for TGF- $\beta 1$ (DAB $\times 200$)



In the clinical study, the reduction of pain and edema within a reasonable time in lower limbs wounds or ulcers after topical application of MET-HCL hydrogel was noted signifies its anti-inflammatory activity. The uncovered topical application of MET-HCL hydrogel was found to have a good therapeutic effect in wound healing in patients with lower limbs traumatic wounds or ulcers. The granulation tissue formation and re-epithelialization were observed in shorter duration of time in patients with traumatic wounds than those with cutaneous ulcers, this may be related to the depth of the lesion, the deeper lesion took a longer duration of healing. Histopathological results showed that topical MET-HCL substantially affected angiogenesis and regulated cellular proliferation. Previously, the effect of MET in tissue regeneration and its pro-angiogenic effects after continuous administration has been reported [16, 28]. TGF- $\beta 1$ plays the main role in wound healing, as it affects the angiogenesis, inflammatory reaction, granulation tissue formation, extracellular matrix deposition, re-epithelization, and remodeling so promoting healing [17]. Immunohistochemical results revealed the significant overexpression of TGF- $\beta 1$ after treatment which accordingly will enhance the tissue vascularity, lowering the inflammation and the granulation tissue. As well as regeneration of epithelium and better wound healing through the promotion of synthesis of some important materials like collagen fibers. Furthermore,

the immunohistochemical findings not only demonstrated the overexpression of TGF- $\beta 1$ in re-epithelialization and connective tissue formation in lower limbs wounds or ulcers after MET-HCL hydrogel treatment but also correlated with the clinical and histopathological findings to interpret the conversion of non-healing wound or ulcer into healing one after such treatment. The exact role and mechanism for MET-HCL in wound healing needs much more investigations as some studies revealed impairment in wound healing process [31], while others accelerate the wound healing both in animal models and in humans [21, 37, 42].

Conclusions

MET-HCL topical hydrogel was efficiently prepared and showed acceptable pharmaceutical behavior, enabling it to be applied easily into the affected wound area. Furthermore, MET-HCL significantly reduced the percentage of wound contraction after 7 days of post-treatment in rats. The clinical study revealed that topical MET-HCL hydrogel was found to be effective and well tolerated in the healing of lower limbs wounds and ulcers in healthy individuals. Moreover, histopathological and immunohistochemical observations showed improvement with the proliferation of both epithelial and dermal elements in form of re-epithelization of the wound

concomitant with a significant overexpression of TGF- β 1 growth factor. These findings promote the MET-HCL topical formulation for further studies in different types of wounds and ulcers and exploration its efficacy in diabetic patients, which will be investigated in future work.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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