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IMPACT OF HYALURONIC ACID ON HEALING OF TONGUE ULCERS IN DIABETIC RATS

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ABSTRACT

Introduction: Wound healing is a highly complicated process achieved through definitely programmed stages. Hyaluronic acid is a natural component present essentially in the skin and other connective tissues, it has been used in many medical fields for acceleration of wound healing.

Aim: This study aimed to investigate the therapeutic effect of hyaluronic acid on healing of tongue ulcer in diabetic rat model.

Material & Methods: Thirty male Albino rats were subjected to diabetes induction and after 1 week the animals were classified into 3 groups, Group I: rats were subjected to tongue ulcer and did not receive any treatment. Group II: rats were subjected to tongue ulcer and the ulcers were treated with 3 times/ day topical applications of commercially used 0.2% high molecular weight hyaluronic acid. Group III: rats were received 1-2 IU/ g/day of insulin NPH injected subcutaneous and then subjected to tongue ulcer after one week from the starting of insulin injection. The animals were sacrificed at 4 and 10 days after ulcer induction, specimens were prepared for histological examination and immunohistochemical staining for α-SMA.

Results: Histological examination revealed better enhancement of ulcer healing and re-epithelization together with reorganization of the epithelium and connective tissue in group II and III samples when compared to group I after 4 and 10 days of ulcer induction.

Conclusion: oral application of hyaluronic acid gel has beneficial effects in accelerating wound healing and improving all phases of wound repair in tongue ulcers in rats.

KEYWORDS: tongue ulcer, hyaluronic acid, insulin, α-SMA.

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INTRODUCTION

Healing of wounds is a physiological, dynamic process in which cellular, molecular and biochemical changes occurs that consequently leads to formation of new connective tissue and fibrous scar with restoration of the anatomical features and functional status of the skin [1]. This process consists of 4 complementary and overlapping stages which are hemostasis, inflammation, proliferation, and tissue remodeling [2]. These stages and their biological functions have specific criteria that it must occur in appropriate order, at a definite time and last for a specific duration at an appropriate environment [3]. There are many factors that can interfere with one or more of stages in this process, thus leads to impaired tissue repair and negatively affect wound healing.

Oral wound healing primarily achieved without scar formation and is faster than that of skin. Oral mesenchyme fibroblasts have a unique phonotypical character as they express high level of α-SMA (alpha smooth muscle actin), increased capacity to contract collagen gel and a more replicative potential than fibroblasts of the skin, finally producing "scar-free" healing process. Growth factors which are present in saliva and crevicular fluid and responsible for wound healing in the oral cavity are epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), and insulin-like growth factor (IL-GF) [4].

Diabetes mellitus is a chronic, lifelong metabolic disorder. It is a worldwide health problem occur due to either inherited or acquired decrease in insulin production from pancreas or the ineffectiveness of the produced insulin, which leads to hyperglycemia. Symptoms of marked hyperglycemia are polydipsia, polyuria, polyphagia, weight loss, blurred vision, increased prone to infections and impaired wound healing [5].

Several studies in both medical and dental literatures reported that the oral cavity is among the affected organ that is extensively affected by diabetes. Significant changes are observed in oral microflora, salivary glands and periodontium. High prevalence of irritation fibromas and persistent oral ulcers were also reported [6].

Impaired wound healing capabilities in both cutaneous and oral ulceration among diabetic patients is the main concern for many researches. They reported that delayed wound healing attributed to various underlying mechanisms includes reduced production of growth factors, little angiogenic response, decreased macrophage function and collagen accumulation, impaired epidermal barrier and keratinocyte function, lastly decrease in fibroblast migration and proliferation [7,8]. Additionally, Diabetic patients are under state of immunosuppression due to accumulation of advanced glycation end products (AGE) causes decrease in number of migrated neutrophils at the site of inflammation [9].

Hyaluronic acid (HA), known as hydrogel; is a natural component present essentially in the skin and other connective tissues, it's glycosaminoglycan in nature and plays an important role in wound healing due to its inherent absorption properties [10]. HA possess an anti-inflammatory, bacteriostatic, antioxidant, anti-edematous, biocompatible and biodegradable properties [11]. It has been used in many fields of medicine [12] and in dentistry [13]. Besides its highly beneficial applications in treating diabetes wound ulcers as reported by many systematic reviews and meta-analyses [14].

Accelerated and safe healing of diabetic wounds with a simple and synergistic strategy was achieved in vitro through using of electro spun thioether grafted (HA)(FHHA-S/Fe) that able to produce a nanofiber fibrous hydrogel, in turns provides intrinsic double modulation of inflammatory mechanisms, with antioxidant properties and transformation of the macrophage phenotype. While in vivo, the average size of diabetic wound area after treatment with FHHA-S/Fe was much reduced in size than that of FHHA/Fe without grafted thioethers, especially during early stage of healing [15].

Tongue ulcer is a common condition, which persists for days or weeks and causes pain and discomfort. In the present study we investigated the effect of hyaluronic acid gel on healing of tongue ulcer in diabetic rat as an experimental animal models by using safety of hyaluronic acid gel as a novel drug and therapeutic approach in the treatment of tongue ulcer.

MATERIALS AND METHODS

The present study used 30 adult male Albino rats (150-200 gm), they were housed in separate cages under appropriate experimental conditions according to the guidelines of the Animal Ethics Committee. The animals were fed standard pellet diet and tap water throughout the study period. Room temperature (22-24°C) and the animals were exposed to 12:12 hours light dark cycles. The present research protocol was approved by the Ethical committee of Faculty of Dentistry, Mansoura University, (A 11070819).

The sample size was estimated with a statistical power of 90% and a significance level of 0.05. The sample size of minimal ten samples per group was calculated based on the primary outcome of a previous study [16].

After one-week acclimatization period, diabetes induction was done, after confirmation of diabetes occurrence through blood samples, table (1) the animals were classified into 3 groups as follow: table (2 and 3):

Group I: 10 rats were subjected to tongue ulcer and did not receive any treatment.

Group II: 10 rats subjected to tongue ulcer and then the ulcers were treated with 3 times/ day topical applications of commercially used 0.2% high molecular weight hyaluronic acid [17].

Group III: 10 rats received 1-2 IU/ g/day of insulin NPH immediately after confirmation of diabetes occurrence, injected subcutaneous [18] and then subjected to tongue ulcer after one week of starting of insulin injection.

TABLE (1) Showing mean ± SD for blood glucose level after induction of diabetes and its statistical analysis results for the different groups:

		Group I	Group II	Group III	ANOVA	
					F	**P value**
One week	Mean	373.34	354.27 ^{B}	189.57 ^{A}	19.07	0.000
	SD	30.46	35.06	72.50		
Two weeks	Mean	441.33 ^{a}	411.67	167.27 ^{A}	15.8	0.000
	SD	44.57	37.24	160.27		
Three weeks	Mean	514.48 $^{a\,b}$	498.82	151.25 $^{A\,a\,b}$	211.6	0.000
	SD	21.31	25.13	64.24		
Four weeks	Mean	518.38 $^{a\,b}$	509.68	139.40 $^{A\,a\,b\,c}$	338.5	0.000
	SD	33.12	18.34	42.93		

All results are expressed as mean ± standard deviation (SD).
Non-significant: at P >0.05. Significant: at P < 0.05.
P value represent significance between Gp. I, Gp. II and Gp. III groups in different weeks
A = significance between group I and group II or group III (in all weeks' groups)
B = significance between group II and group III (in all weeks' groups)
a= significance between week one and other weeks after it (in all groups Gp. I, Gp. II and Gp. III)
b= significance between week two and other weeks after it (in all groups Gp. I, Gp. II and Gp. III)
c= significance between week three and week four (in all groups Gp. I, Gp. II and Gp. III)

TABLE (2) Showing different intervention and treatments applied in all groups

Groups	Intervention	Treatment
Group I	Diabetes induction + tongue ulcer	No treatment
Group II	Diabetes induction + tongue ulcer	Hyaluronic acid gel 3 times/ day
Group III	Diabetes induction + tongue ulcer	Insulin injection

TABLE (3) Showing all time of experiment after confirmation of diabetes occurrence.

Groups	Timing and related intervention
Group I	Tongue ulcer was induced immediately after confirmation of diabetes occurrence.
Group II	hyaluronic acid gel was applied 3 times/ day on the next day of ulcer induction.
Group III	Insulin injection was done immediately after confirmation of diabetes occurrence then ulcer induction was done after one week of starting of insulin injection.

Diabetes induction

Experimental diabetes was induced to all animals after overnight starvation (19) by a single intraperitoneal injection (ip) of streptozotocin (STZ: 45 mg/kg body weight in 0.1 ml citrate buffer, pH 4.5). After 3 days of STZ injection the diagnosis of diabetes was achieved by making blood glucose (non-fasting) analysis, it showed hyperglycemia > 300 mg/dl) [20]. The blood glucose level of the rats was monitored every week and the results were listed in table. Statistical analysis for blood glucose level of all animals was made

HA preparation

A commercially hyaluronic acid gel; Gengigel® was used in this study, (via Egadi,7 -20144 Milano IT). The active ingredient in it is a High Molecular Weight (HMW) hyaluronic acid in a form of sodium hyaluronate. The other ingredients are: Aqua, Xylitol, Celleluse Gum, Alcohol, Peg 40 Hydrogenated Castor Oil, Dichlorobenzyl Alcohol, Carbomer, Aroma, Citric Acid, Sodium Hydroxide and Acidic Blue [17].

Ulcer induction

Rats were generally anesthetized by im. injection of 0.1 ml of ketamine hydrochloride (SIGMATEC Company) in combination with 0.05 ml of xylazine hydrochloride (ADWIA Company), 100/ g body weight of the animal. After anesthesia, the lingual mucosa was antiseptically cleaned with 2% chlorhexidine then a surgical mucosal wound was made by using biopsy punch (Acu-Punch, Acuderm Inc., Ft. Lauderdale, FL, USA) to ensure that all ulcers will have the same size. The ulcer was circular in shape about 4mm in diameter and 2mm in depth [21].

The induced ulcers were left to heal either normally or topically covered with 0.2 % HA gel (Gengigel®) or through subcutaneous injection of insulin, subsequently the rats were kept under observation without any feeding or drinking for 1 hour after topical application of treatments.

Scarification of rats was done by overdose of sodium thiopental at day 4 and 10 respectively [17] after ulcer induction, tongue was excised from each animal and quickly immersed in 10% formalin solution and become ready for histological examination, and immunohistochemically for alpha smooth muscle actin (α-SMA).

Slides were digitized using Olympus® digital camera installed on Olympus® microscope. The resulted images were analyzed on Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for immune-stain quantification.

The software routine of quantification includes:

Step 1: Image acquiring form the camera using a u-tech frame grabber.

Step 2: Target area was manually selected and extracted from the entire field to avoid interference from the un desired area.

Step 3: Target area was converted to grey scale.

Step 4: Grey scale area was digitally scanned to obtain a surface plot which digitally represents the intensity of each pixel in a 3D view. Each pixel represent an intensity ranged from 0 to 255, area with high dye retention represent smaller intensity while areas with faint dye represent larger intensity. The software calculates the mean intensity depending on the intensity distribution within the target area.

RESULTS

Histological analysis

Hematoxylin and Eosin stain

After 4 days of ulcer induction group I showed slight proliferation of the covering epithelium and formation of granulation tissues with heavy inflammatory cell infiltrations, group II showed starting of the healing process via proliferation of the epithelium which shows mitotic figures. Group III showed the migration of the epithelium under the granulation tissue and the granulation tissue appeared the same as group II. (Fig 1)

After 10 days of ulcer induction group I showing the healing process, the covering stratified squamous epithelium proliferate to close the wound margin but the ulcer is still open and the inflammatory cell infiltration still heavy, group II showing complete re-epithelialization of the ulcerative area with the starting of epithelium and connective tissue reorganization, group III showing complete re-epithelialization, covered with the underlying connective tissue stroma which presented increased number of fibroblasts, collagen bundles, and newly formed blood vessels. (Fig 2)

Immunohistochemical analysis, table (4):

After 4 days of ulcer induction group I showing very weak immunohistochemical staining of α-SMA that was presented only in the blood vessels walls, group II the α-SMA was detected in the blood vessel walls and in the granulation tissue fibroblasts, group III was detected in the blood vessels and was begun to be expressed in the fibroblasts. (Fig 3)

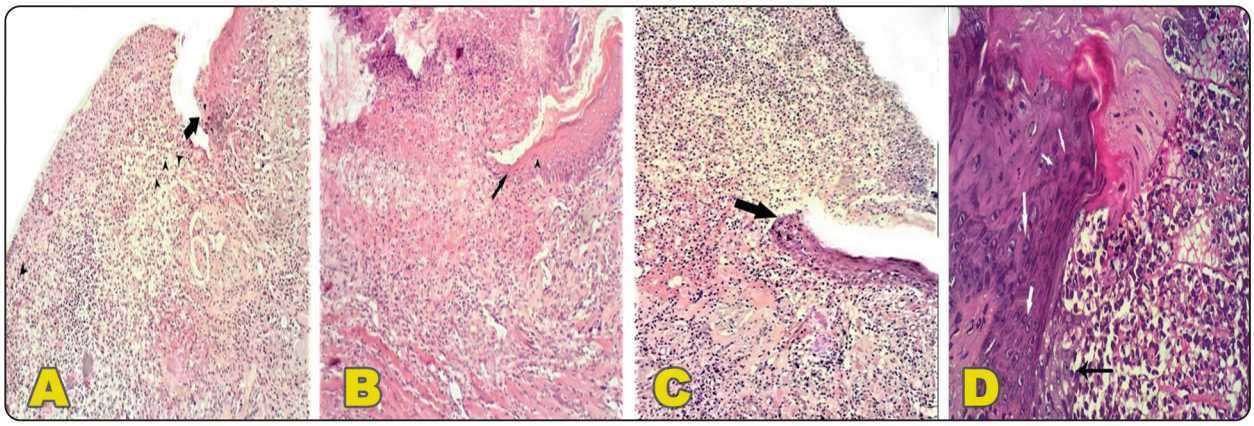

Fig. (1): Photomicrograph showed the ulcer area at 4th day, A showing group I with slight proliferation of the covering epithelium (arrow) and formation of granulation tissues with heavy inflammatory cell infiltrations (arrow heads), B showing group II with proliferation of the epithelium(arrow) which shows mitotic figures (arrow head), C showing group III with migration of the epithelium under the granulation tissue (arrow) and mitotic figures in the epithelium (arrow heads), (H&E x 100). D showing higher magnification of the previous one (C) showing epithelial proliferation (black arrow) and cell mitosis (white arrow). (H&E x 400)

After 10 days of ulcer induction group I showing the α-SMA began to be detected in the blood vessels and fibroblasts in the granulation tissue, group II and III showing increasing in the expression of alpha smooth muscle actin in the fibroblasts and the newly formed blood vessels, group III showing the reaction was strong as in group II. (Fig 4)

TABLE (4) Showing high immunoreactivity in hyaluronic group compared to control and diabetic group

	Group I	Group II	Group III
Area %- 4 days	0.53±0.28	1.32±0.044***	1.54±0.68***
Area %- 10 days	0.84±0.73	1.86±0.056***	1.89±0.74***

Data expressed as mean ± SD, P: Probability: significance <0. 05 Test used: One-way ANOVA followed by post-hoc tukey *P <0.05; **P<0.01; ***P<0.001 vs. Control group.

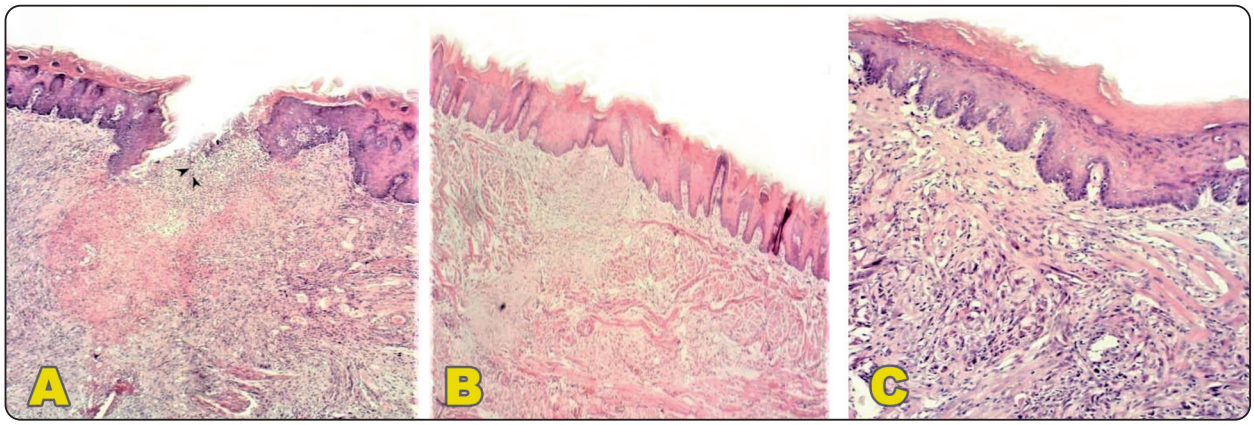

Fig. (2): Photomicrograph showed the ulcer area at 10th day, A showing group I with the covering stratified squamous epithelium proliferate to close the wound margin but the ulcer is still open and the inflammatory cell infiltration still heavy (arrow heads), B showing group II showing complete re-epithelialization of the ulcerative area with the beginning of reorganization of the epithelium and connective tissue, C showing group III complete re-epithelialization and covering of the underlying connective tissue stroma which formed from large number of fibroblasts, collagen bundles, and newly formed blood vessels (H&E x 40).

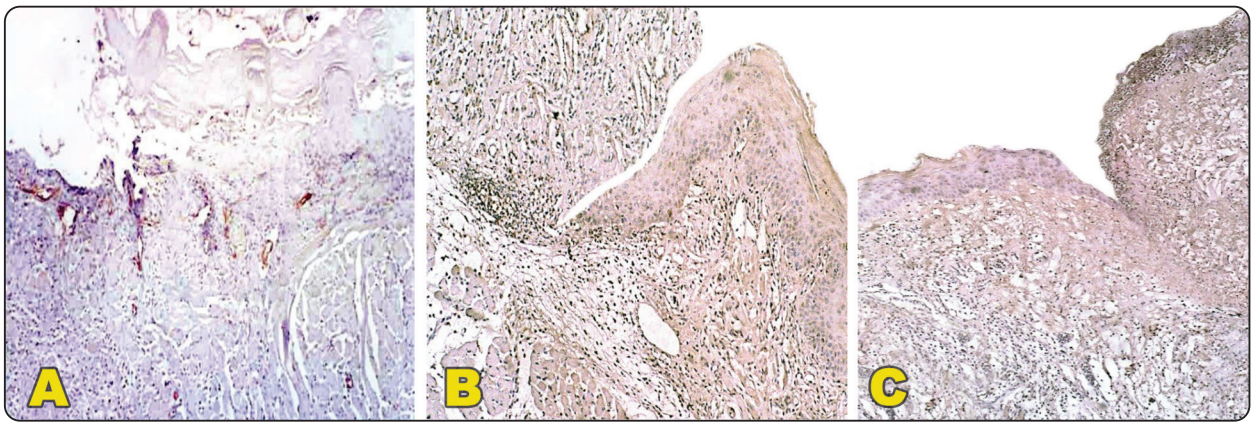

Fig. (3): Photomicrograph showed the ulcer area at 4th day, A showing group I with very weak immunohistochemical staining of α-SMA only in the blood vessels walls, B showing group II with moderate reaction of the α-SMA in the blood vessel walls and in the fibroblasts in the granulation tissue, C showing group III with expression of α-SMA in the blood vessels and was begun to be expressed in the fibroblasts, (IHC staining, alpha SMA, x100).

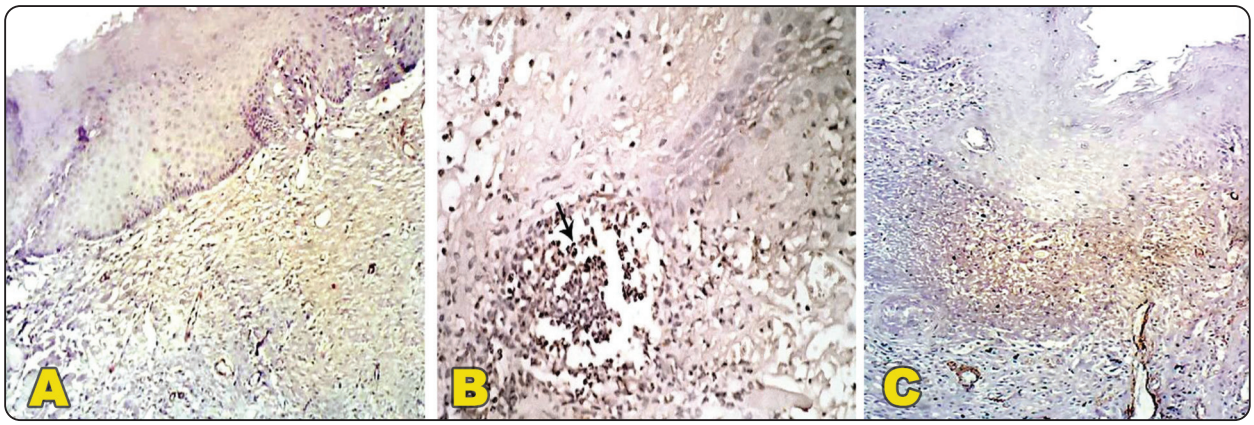

Fig. (4): Photomicrograph showed the ulcer area at 10th day, A showing group I with mild expression of α-SMA in the fibroblasts and the newly formed blood vessels, B showing group II with strong expression of α-SMA that detected in the blood vessels, fibroblasts and inflammatory cells (arrow)in the granulation tissue, C showing group III with moderate expression of α-SMA in the fibroblasts and the newly formed blood vessels, (IHC staining, alpha SMA, x100).

DISCUSSION

Wound healing process is very important to restore the damaged tissue and to inhibit its invasion by pathogens, it involves the following sequences: inflammation, angiogenesis, fibroplasia, wound contraction, and epithelization of the wound defect. These sequences starts immediately after occurrence of injury and continue for variable periods of time depending on the degree of wounding [22].

Hyaluronic acid (HA) is mainly present in the extracellular matrix (ECM) of human tissues, including oral tissues [23]. It has anti-inflammatory effects, moisture preservation and can provide angiogenesis [24]. It was proved clinically that HA enhances oral wound healing e.g. previous study on free gingival grafts stated that HA accelerated re-epithelialization of the donor sites [25]. In other researches, HA was also effective in wound closure after labial frenectomy [26], improving the extraction socket healing in patients with uncontrolled type II diabetes [27].

In the present study the histological finding of group I stained with H&E after 4 days of ulcer induction showed minimal proliferation of the covering epithelium and formation of granulation tissues with heavy inflammatory cell infiltrations, and these findings come in agreements with Rosique RG et al 2015 who stated that oral ulcers in cases of diabetes mellitus prolonged the inflammatory phase period leading to continuous infiltration of neutrophils that released cytotoxic enzymes, free radicals and inflammatory mediators which consequently produces extensive collateral damage to the surrounding tissue [28].

The histological finding of group II and III stained with H&E after 4 days of ulcer induction showed starting of the healing process in the form of proliferation of the epithelium which showed mitotic figures especially in group II, on the other hand after 10 days of ulcer induction group I showed signs of healing process in which the covering stratified squamous epithelium proliferate to close the wound margin but the ulcer is still open and the inflammatory cell infiltration still heavy, group II showed complete re-epithelialization of the ulcerative area with the beginning of reorganization of the epithelium and connective tissue , group III showed complete re-epithelialization, the underlying connective tissue stroma presented large number of fibroblasts, collagen bundles, and newly formed blood vessels, these findings are in agreements with Jeong et al 2022 [29] who investigated the application of Oro-dispersible hyaluronic acid film for oral wound healing in rats and proved that HA groups provides

a clearly better quality of re-epithelialization on day 7. Re-organization of the tissue with new epithelium and resurfacing starts nearly after 1.5 - 2 days from wounding and continue until the third phase of healing [30]. This is mainly occur due to sufficient migration of keratinocytes [31].

These findings assured the obvious histological improvement of HA group in comparison to control group, this mean that the healing is accelerated among HA group than control group, the improvement in hyaluronic acid group may be attributed to many causes, firstly the enhanced water retention, that provides a suitable environment for collagen and elastin fibers formation thus permitting the cells to proliferate and differentiate and finally accelerating the healing process. Secondly, HA can help flow of nutritional materials and aids the wound to get rid of unwanted yields from cell metabolism. Thirdly, HA is truly controlling keratinocyte proliferation and migration to the wounded area [32].

In the current study the obvious histological improvement for group III compared to group I at day 10 after ulcer induction, means that the healing is better among group III than group I, and this finding was in compliance with Soha, 2018 who searched the effect of topical insulin on oral mucosal wound healing and found that hyperplastic Para keratinized epithelium with long rete ridges was present after 7 days in diabetic group while the group treated by placebo showed ulcerative non keratinized epithelium. Moreover, after 14 days the group treated by insulin showed complete healing manifested by hyperplastic orthokeratinized epithelium with prominent granular cell layer and bulbous rete ridges while, the placebo group showed incomplete healing manifested by atrophic non-keratinized epithelium with short rete ridges [33].

Seng Fai, 2017 explained the mechanisms of action of topical insulin in wound healing as insulin is a peptide closely related to insulin-like growth factor (IGF) which is able to stimulate hypotactic migration of human epidermal keratinocytes and is responsible for wound repair of the corneal epithelium [34].

Alpha-smooth muscle actin (alpha-SMA) is the actin isoform that present the main component of vascular smooth-muscle cells, having an important role in fibrogenesis [35]. During healing process, fibroblasts acquire contractile properties i.e. formation of microfilament bundles, and begin to express (α-SMA). These activated cells, called "myofibroblasts" [36] Myofibroblasts secreting large amounts of (ECM) proteins thus sharing in the reparative response [37] and also attributed to wound contraction during healing process [38]. Repair of injured tissues is principally depends on the time of activation and suppression of myofibroblasts i.e. prolonged or excessive myofibroblast activation leads to fibrosis and organ dysfunction [39].

In the current study the reaction of α-SMA in 4 days after ulcer induction in group I was very weak and only in the blood vessels walls while in group II the α-SMA was detected in the blood vessel walls and in the granulation tissue fibroblasts, and in group III the reaction was detected in the blood vessels and was begun to be expressed in the fibroblasts. and these results come in accordance with Ahmed and Salwa, 2018 who investigate the protective effect of Aloe vera and silver nanoparticles on oral ulcer induced by acids in irradiated mice and reported the prominent decrease in the number of activated fibroblasts and mature vascular endothelial cells in the ulcer area of the control group at the 3rd day post ulceration while in the groups of Aloe Vera, silver nanoparticles and Aloe Vera + silver nanoparticles, prominent increase in the number of activated fibroblasts and mature vascular endothelial cells in the ulcer area [40].

After 10 days of ulcer induction α-SMA reaction in group I began to be detected in the blood vessels and in the granulation tissue fibroblasts, group II and III showed increased expression of α-SMA in the fibroblasts and the newly formed blood vessels, and our findings come in compliance with Zaher et

al 2014, who searched for the effect of curcumin on tongue ulcer healing in albino rats and stated that at the 3rd day post ulceration, α-SMA stain presented an improved results due to the presence of myofibroblasts within the granulation tissue of curcumin group sections only and Starting from day 6th post ulceration the myofibroblasts increased in number in curcumin group While sections of control group showed delayed infiltration of myofibroblasts and decreased number of these cells [41].

CONCLUSION

In summary, our study approved that oral application of hyaluronic acid gel has beneficial effects in accelerating wound healing and improving all phases of wound repair in cases of diabetic rats with tongue ulcer, including re-epithelialization, reorganization of the epithelium and connective tissue, collagen and blood vessels synthesis leading to wound closure. Hyaluronic acid gel may therefore be a favorable alternative or an adjuvant approach to standard existing drugs used for treatment of oral ulcers.

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