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Clinical and Histological Efficacy of Nanofat Grafting in the Healing Process of Chronic Ulcers

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Abstract

Background: Chronic ulcers have many bad impacts and are becoming an economic burden. Nanofat is rich origin of fatty-derived stem cells and different precursor cells. This work aimed to assess efficacy of nanofat injection in chronic ulcers and evaluating its effect on the healing process.

Objective: Evaluation of the efficacy of non-fat graft injection in the healing process of chronic ulcers.

Methods: This prospective randomized controlled study was carried out on 20 patients of both sexes aged between 18 - 60 years old with no co-morbidities. We included patients with chronic upper or lower extremity post-traumatic ulcers of 5cm maximum diameter with no healing on conventional dressing for at least 3 months. Patients were randomly divided into two equal groups: Group A (study group) was scheduled for nanofat injection and Group B (control group) with conventional dressing with no plan for surgical intervention.

Results: Vascularity, pigmentation, pliability and height were significantly improved in group A than group B. Time frame for complete healing was significantly delayed in group B than that of group A (median time for complete healing was 16.5 weeks and 3 weeks respectively). Patient satisfaction was significantly increased in group A than in group B. Skin biopsies showed that the epidermal thickness, dermal area % of collagen bundles and elastic fibres and the number of epidermal and dermal proliferating cell nuclei were significantly increased while the inflammatory cell count was markedly decreased in group A compared to ulcers of group B.

Conclusions: Nanofat grafting may be a promising treatment option for patients with traumatic ulcers, as it can improve wound healing outcomes and patient satisfaction.

Key Words: *Chronic ulcers – Nanofat grafting – Fatty-derived stem cells – Healing process.*

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Disclosure: No disclosure.

Introduction

Chronic non healing ulcer are becoming burden on both patients and health care providers. The prevalence of chronic ulcers is increasing dramatically. The appropriate management of chronic wound had become a challenge **[1]**. Nanofat is considered a rich provenance of multiple progenitor cells **[2]**. Also, it induces a reparative effect at injury sites by improving angiogenesis, extracellular matrix remodeling and enhancing immune system modulation. This was assigned to the abundance of adipose-derived precursor cells. According to these facts, cellular therapy in multiple disease was encouraged **[3]**.

The use of nanofat in multiple clinical researches had shown encouraging aesthetic and functional outcomes with marked regenerative capacity. The lesser the manipulation of grafted nanofat and the rapidity of its injection, improving the survival at the targeted site **[4]**.

Minor drawbacks were associated with nanofat harvest techniques like pain at donor site, inflammation, ecchymosis, seroma or hematoma formation and prejudice of the deep framework especially when the aspiration cannula penetrated deep into the peritoneal cavity or underlying muscles **[5]**.

So, in this study we evaluate the efficacy of nanofat injection into the healing process of chronic post-traumatic non healing ulcers.

Patients and Methods

This prospective randomized controlled study was carried out on 20 patients of both sexes aged between 18 years and 60 years old with no co-morbidities. Chronic small sized upper or lower extremity ulcers of a maximum diameter of 5cm were included. Exclusion criteria were suspected or proved malignancy of the ulcer by biopsy, children less than 18 years of age and associated co-morbidities that may affect healing mechanism.

Patients were divided randomly into two equal groups: group A (study group) was scheduled for nanofat injection and group B (control group) for follow up for signs of healing only after conventional management.

All patients were subjected to: History taking, photographic documentation, full labs to exclude other co-morbidities, incisional biopsy to exclude malignancy, follow up patient outcome at day 0 day 3, day 7, day 21.

The study was done in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and after approval from the Ethical Committee Ain Shams University Hospitals, Egypt (approval code: FMASU, MS 858/2022/2023). An informed written consent was obtained from the patients.

Clinical method:

After the infiltration of modified Klein solution in the infra umbilical abdominal region, then aspiration of fatty tissue to prepare nanofat. The aspirated fat was washed with normal saline, followed by instillation out of a sterile nylon cloth (0.5mm pore size) on top of a clean container. Mechanical emulsification of aspirated fat was performed by passing it between 2 syringes linked by a Luer-Lock linkage for a minimum of 30 passages, until the size of the aspirated fat is getting smaller with every passage and finally transformed into an emulsion that appeared whitish. This whitish emulsion was purified once more through the sterile nylon mesh and the effluent was assembled in a sterile reservoir which now is becoming nanofat [2]. The nanofat grafting was injected into the chronic ulcer in a "fanning out" pattern [6]. About 5-10ml were injected into the base of the ulcer and into surrounding margins using 1.2mm injection cannula. Also, we topically apply another 5ml over the ulcer surface. This was performed once for each selected ulcer. Vaseline gauze was applied together with layers of conventional dressing. (Fig. 1) Every other day conventional dressing using antibiotic ointment (fucidic acid) covered by a non-adherent layer (Vaseline gauze) was done for both groups.

Clinical Assessment of wound healing was classified into subjective assessment by following-up signs of healing and vascularity, time frame for complete healing, level of patient satisfaction and objective assessment using Vancouver scar scale.

Two punch biopsies were taken 1st preoperative and the 2nd 1 month later from the ulcer edge using 2mm punch size for histopathological evaluation.

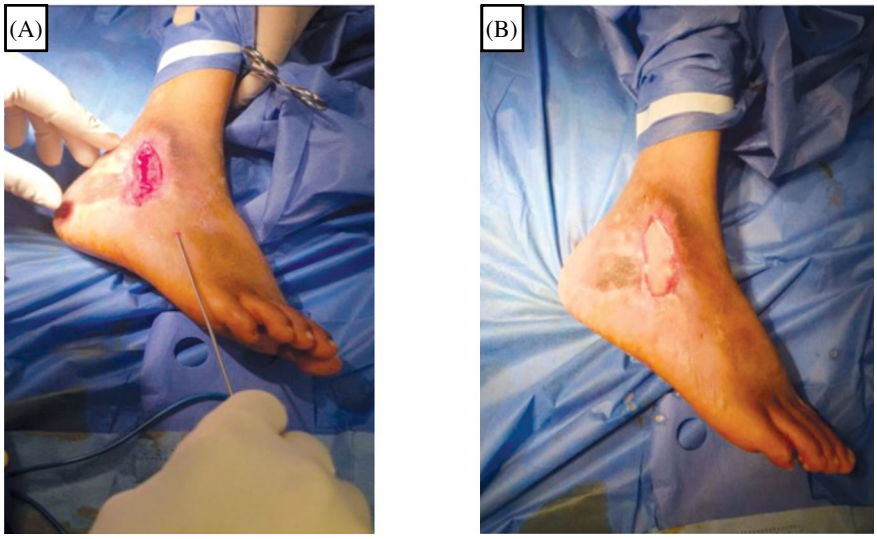

Fig. (1): Fat grafting application to recipient site (A) Injection into ulcer base, (B) Application over ulcer surface.

Histopathological and Immunohistochemical Evaluation:

Punch biopsies of skin specimens were processed for paraffin blocks. Serial skin sections of 6 μm thick were subjected to Hematoxylin and Eosin (H&E) staining to examine the skin integrity, Masson's trichrome staining [7] to assess the collagen bundles deposition and Orcein staining [8] to evaluate the elastic fibers deposition. Anti-proliferating cell nuclear antigen (PCNA) immunohistochem-

ical staining (Sigma-Aldrich Cat# HPA030522, RRID:AB_10602096) was used for the detection of proliferating cells in both the epidermis and dermis. The standard immunohistochemical staining protocol by Sanderson et al. **[9]** was applied to determine the PCNA immunoreactive nuclei. Negative control sections for detection of any unintended background staining were done. Specimens harvested from the ulcer (pre-operative and 1 month post-operative) were examined for wound healing (re-epithelization, neovascularization, collagen, and elastic fibers formation) with statistical comparison between the control group and the study group. Photomicrographs were obtained by Leica DM2500 microscope (Wetzlar, Germany) connected to the photo analyzer Leica Q win V.3 software at the Histology and Cell Biology institute, Faculty of Medicine, Ain Shams University.

Histomorphometric analysis:

Skin layers (epidermis and dermis) calculation were studied from 5 randomly selected areas from parts of each sample. Measurements were assessed using ImageJ 1.53k image analyzer program (https://imagej.nih.gov/ij/, RRID: SCR_003070). Morphometric studies included:

1- Epidermal density was measured at the ulcer margin as the distance between the basement membrane and the keratin layer in sections stained by H&E. The rete ridges thicknesses were not included in the measurement.

2- Quantification of positive PCNA immunostaining in both epidermis and dermis in PCNA immunohistochemically stained sections.

3- Inflammatory cell counts were assessed in the papillary and reticular dermis (stained by H&E stain).

4- Area percentage of collagen bundles deposition in the papillary and reticular dermis (stained by Masson's trichrome stain).

5- Percentage of elastic fibers zone in both dermal layers (stained by Orcein stain).

Results

Age, sex, BMI, WBCs, Hb, HCT, platelets, ALT, AST, urea, and creatinine showed no difference in both groups. All patients had traumatic ulcers. Site, size, and duration of ulcer were almost similar in both groups. Vascularity, pigmentation, and height were significantly improved in group A than group B (Table 1).

Time frame for complete healing was significantly delayed in group B than group A. As regard complications, showed no great variation in both groups. Satisfaction was significantly increased in group A (Table 2).

Table (1): Macroscopic assessment using Vancouver scar scale of the studied groups.

	Group A (n=10)	Group B (n=10)	*p*-value
Vascularity:			
Normal	8 (80%)	2 (20%)	0.030*
Pink	1 (10%)	1 (10%)	
Red	1 (10%)	2 (20%)	
Purple	0 (0%)	5 (50%)	
Pigmentation:			
Normal	8 (80%)	2 (20%)	0.004*
Hypopigmentation	2 (20%)	1 (10%)	
Hyperpigmentation	0 (0%)	7 (70%)	
Pliability:			
Normal	8 (80%)	2 (20%)	0.087
Supple	1 (10%)	1 (10%)	
Yielding	1 (10%)	1 (10%)	
Firm	0 (0%)	3 (30%)	
Ropes	0 (0%)	2 (20%)	
Contracture	0 (0%)	1 (10%)	
Height:			
Flat	8 (80%)	2 (20%)	0.035*
Less than 2 mm	1 (10%)	1 (10%)	
From 2 to 5 mm	1 (10%)	3 (30%)	
More than 5 mm	0 (0%)	4 (40%)	

Table (2): Time frame for complete healing, complications and patient satisfaction of the studied groups.

	Group A (n=10)	Group B (n=10)	*p*-value
Time frame for complete healing (weeks)	3 (2.25-4)	16.5 (11.75-22.25)	<0.001*
Complications:			
Infection	1 (10%)	5 (50%)	0.113
Fat resorption	1 (10%)	0 (0%)	
No	8 (80%)	5 (50%)	
Satisfaction:			
Satisfied	8 (80%)	2 (20%)	0.023*
Unsatisfied	2 (20%)	8 (80%)	

Histopathological Results:

Staining of sections from pre-operative chronic ulcers punch biopsies of group A by H&E stain revealed the ulcer bed occupied by granulation tissue filled with heavy infiltrations of inflammatory cells and several congested blood capillaries without epidermal covering. The margins of the chronic ulcers were seen formed of stratified squamous keratinized epithelium and overlying papillary and reticular dermis with abundant red blood cell and inflammatory cell infiltrations (Fig. 2-A). PCNA immunostained sections showed PCNA immunopositive nuclei appearing as brown nuclear deposits

in the basal layer of the epidermis, scattered in the dermis and in the granulation tissue of the ulcer bed. Almost no PCNA immunoreactivity was detected in endothelial cells nuclei (Fig. 2-D). Compact coarse green collagen bundles were seen at the ulcer bed in sections stained by Masson's Trichrome stain with few thin collagens in the upper dermis (papillary) and some thick collagen in lower dermis (reticular) (Fig. 3-A). Few scattered dermal elastic fibers were detected appearing as very thin red brown threads in Orcein-stained sections (Fig. 3-D).

Post-operative punch biopsies of group A (chronic ulcers treated with nanofat injection) showed complete epidermal covering of the ulcer area in H&E-stained sections with scanty inflammatory cells infiltrations and packed bundles of collagen fibres in the underlying papillary and reticular dermis (Fig. 2-B). Numerous positive PCNA immunoreactive nuclei were spotted in the epidermis, the dermis and in endothelial cells nuclei (Fig. 2-E). Fine interdigitating green bundles of collagen in the upper dermis (papillary) that become coarse dense wavy in the lower dermis (reticular) were seen in specimen stained by Masson's Trichrome dye (Fig. 3-B), while in specimen stained using Orcein dye revealed ample amounts of red-brown thin fibers of elastin in the upper dermis (papillary) that become thick, long and branched in the lower dermis (reticular) (Fig. 3-E).

Group B (control group) H&E-stained sections showed failure of epithelial migration and persistence of inflammatory cell infiltration and capillaries congestion in ulcer bed and in dermis (Fig. 2-C). Scanty immunoreactive PCNA nuclei in both the epidermis and dermis were detected with sparse PCNA positive immunoreactive endothelial cells (Fig. 2-F). Areas devoid of green collagen fibers in the ulcer bed and in the reticular dermis were recognized in Masson's Trichrome-stained sections with thin collagen bundles in the papillary dermis and some greenish wavy bundles of collagen in the deep dermis (reticular) (Fig. 3-C). Only few red brown fibers of elastin in the dermis were seen in specimens stained using Orcein dye but elastic fibers were not identified in the granulation tissue of ulcer bed (Fig. 3-F).

Fig. (2): Histopathological changes in sections from chronic skin ulcers stained by H&E and PCNA immunohistochemical stain:

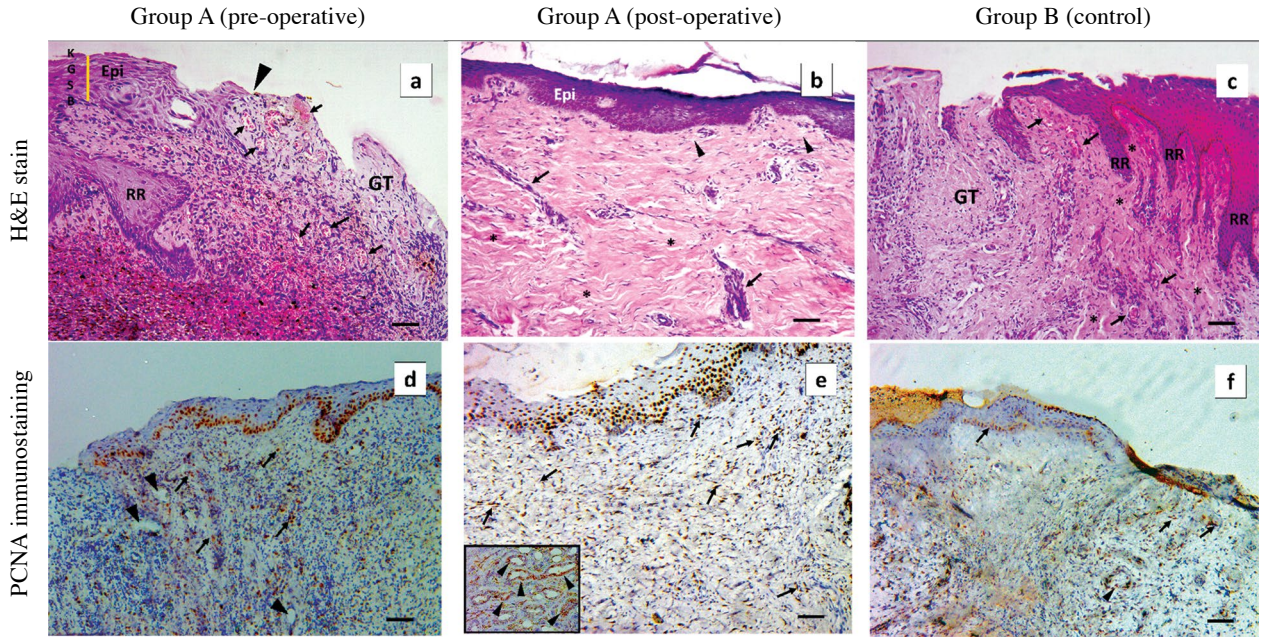

Fig. (2): Representative photographs of chronic ulcers sections from formalin-fixed punch biopsies; (a-c) stained with H&E and (d-f) stained immunohistochemically with PCNA. (a) pre-operative chronic ulcer specimen showing a clear ulcer margin (arrow top), the ulcer bed is seen filled with granulation tissue (GT) with numerous congested blood capillaries (arrows) and multiple inflammatory cell infiltration. The epidermis (Epi) is consisted of stratified squamous keratinized epithelium (yellow line) composed of basal cell layer (B), spinous cell layer (S), granular cell layer (G) and keratin (K). Epidermal rete ridges (RR) are also seen. The underlying dermis is observed full of red blood cell and inflammatory cell, (b) post-operative chronic ulcer specimen showing intact epidermis (Epi) covering the ulcer area. The papillary dermis is packed with thin interdigitating bundles of collagen fibers (arrow tops) and the reticular dermis is rich in densely packed wavy bundles of collagen fibers (*). Scattered aggregations of inflammatory cells (arrows) are also detected, (c) chronic ulcer of control group shows non healed ulcer with failed epithelial migration and persistence of inflammatory cell condensation in granulation tissue (GT) of ulcer bed and in dermis. Collagen bundles appear in the papillary and reticular dermis (*). Congested capillaries are found in the dermis (arrows). The epidermis is seen separated with progressive thinning near the ulcer margin. Many deep epidermal rete ridges (RR) are noted, (d) pre-operative chronic ulcer specimen with +ve PCNA immunoreactive nuclei in the epidermis more at the basal layer and few immunoreactive nuclei in the dermis and in the ulcer bed (arrows). Notice –ve PCNA immunoreaction in endothelial cells nuclei (arrow tops), (e) post-operative chronic ulcer specimen shows many +ve PCNA immunoreactive nuclei in the epidermis and the dermis (arrows). Inset: Nuclei of some endothelial cells show immunopositive PCNA reaction (arrow tops), (f) chronic ulcer of control group showing few immunoreactive PCNA nuclei both the epidermis and dermis (arrows) and little PCNA +ve immunoreactive endothelial cells (arrow top). Magnification: x100. Inset: x400. Scale bar: 50μm.

Fig. (3): Histopathological changes in sections from chronic skin ulcers stained by Masson's Trichrome and Orcein stains:

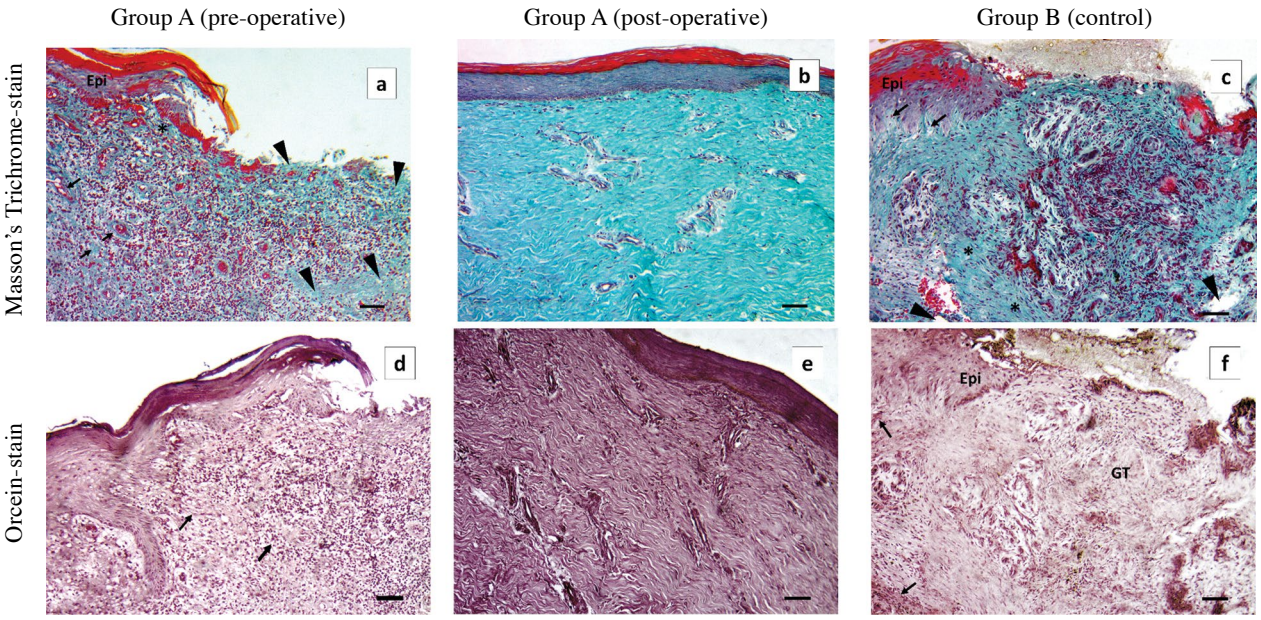

Fig. (3): Representative photographs of chronic ulcers sections from formalin-fixed punch biopsies stained with; (a-c) Masson's Trichrome and (d-f) Orcein stain. (a) pre-operative chronic ulcer section showing dense coarse green collagen bundles (arrow tops) in the ulcer bed without epidermal (Epi) covering. The dermis shows dispersed thin bundles of collagen in the upper dermis (papillary) (*) that become thicker in the deep dermis (reticular) specially around blood vessels (arrows). Notice the heavy cellular infiltration in both the ulcer bed and in the dermis, (b) post-operative chronic ulcer specimen showing loose arrangement of green bundles of collagen in the upper dermis (papillary) that become coarse dense disorganized in the lower dermis (reticular), (c) chronic ulcer of control group shows thin bundles of collagen fibers in the papillary dermis (arrows) underlying the epidermis (Epi) and some coarse wavy loosely arranged bundles of collagenous fibers (*) in the lower dermis (reticular). Empty zones of no collagen (arrow tops) are seen in the reticular dermis and in the ulcer bed. Notice the heavy cellular infiltration in both the ulcer bed and in the dermis, (d) pre-operative chronic ulcer showing few very thin elastic fibers appear red brown scattered in the dermis (arrows), (e) post-operative chronic ulcer specimen showing abundant red brown elastic bundles appearing thin in the upper dermis (papillary) that become thick long branched in the lower dermis(reticular), (f) chronic ulcer of control group showing no apparent elastic fibers in the ulcer bed granulation tissue (GT). few elastic fibers (arrows) are seen in the dermis of the ulcer margin. Magnification: x100. Scale bar: 50μm.

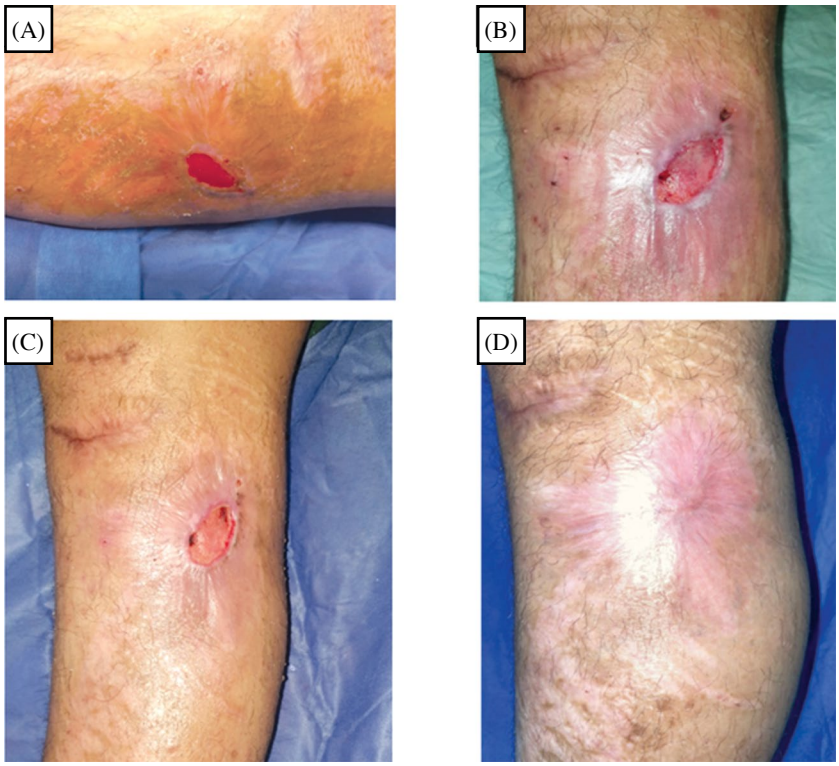

Fig. (4): 19 years old male patient with post-traumatic chronic leg ulcer (A) Pre-operative, (B) After 1st week, C 2nd week, (D) After 3rd post-operative week showing complete reepithelization of the ulcer surface.

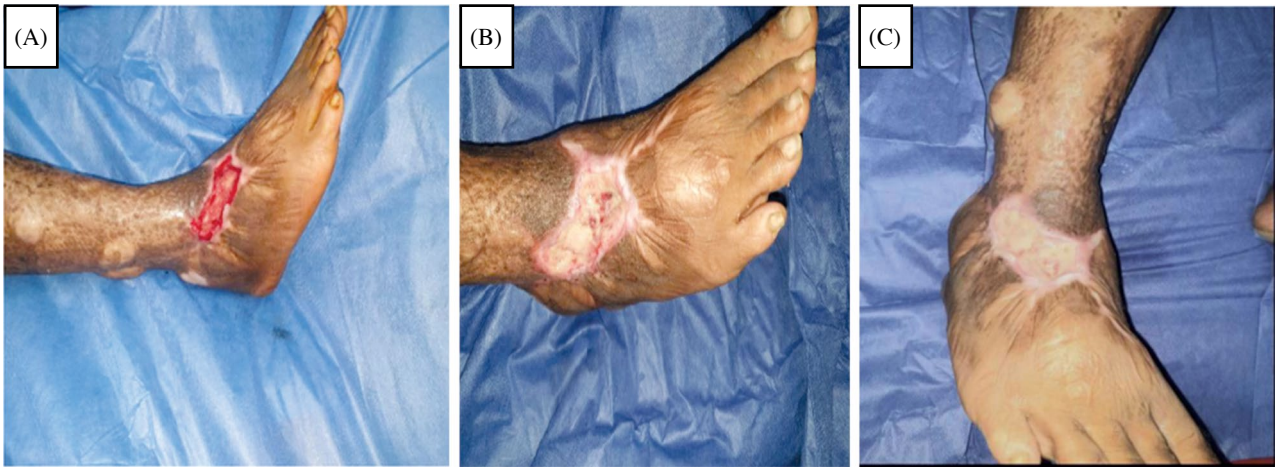

Fig. (5): 23 years old male patient with post-traumatic chronic foot ulcer (A) Pre-operative, (B) After 2nd week, 4th post-operative week showing complete reepithelization of the ulcer surface.

Epidermal thickness of post-operative group A skin biopsies looked of different densities in multiple zones but were collectively significantly more when compared to the epidermal thickness of pre-operative group A ($p<0.05$) and to group B biopsies ($p<0.01$). Quantification of PCNA immunostaining in both epidermis and dermis revealed that the number of proliferating cell nuclei was significantly higher in the post-operative group A sections as compared to that of the group B and to pre-operative group A skin biopsy ($p<0.01$ and $p<0.05$ respectively). Skin biopsies of both group B and the post-operative group A showed a significant decrease in inflammatory cell count ($p<0.01$ and $p<0.001$ respectively) as compared to the pre-operative group A biopsies of chronic skin ulcers. The post-operative group A skin sections exhibited a marked decrease in inflammatory cell count ($p<0.01$) as compared to group B. Skin biopsies of both the group B and post-operative group A revealed a significant increase in the area percent of collagen bundles deposition ($p<0.001$) when compared to pre-operative group A sections of chronic skin ulcers. Area percent of collagen bundles was found to be more significant in the post-operative first group (A) biopsy ($p<0.05$) relative to other group. Skin biopsies of the post-operative group A showed a significant increase in the area percent of elastic fibers ($p<0.05$) when compared to group B. No significant difference was detected in the zone percentage of elastic bundles between group B and the pre-operative group A biopsy (Table 3).

Table (3): Epidermal and dermal histomorphometric measures in all groups.

	Group A (pre-operative) (n=10)	Group A (post-operative) (n=10)	Group B (control) (n=10)	One way ANOVA
Epidermal thickness (μm)	235.81±59.92	389.60±148.99 *##	223.50±82.73	F=7.819
Number of PCNA immunoreactive nuclei	631.7±99.5	954±100.4 *##	464.3±101	F=18.46
Inflammatory cell count	1771±255.7	334.3±127.6 ***##	1062±125.9 **	F=47.65
Area % of collagen bundles	26.38±2.07	49.56±1.17 ***#	43.73±2.68 ***	F=101.6
Area % of elastic fibers	35.56±5.81	42.38±3.27 #	28.54±3.69	F=7.414

Table (3): Shows the differences in the thickness of epidermal layer, total number of immunoreactive nuclei in both epidermis and dermis, dermal inflammatory cell count and area percentage of collagen bundles and elastic fibers in sections from skin punch biopsies of chronic ulcers in all groups. Data is presented as mean ± standard deviation. n= number of observations. Significance was calculated by one-way ANOVA followed by Bonferroni's post hoc test at (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ compared to pre-operative group A skin biopsy, and at (#) $p<0.05$, (##) $p<0.01$ compared to group B (control).

Discussion

In our study, all patients had traumatic ulcers. Site, size, and duration of ulcer were insignificantly different between the studied groups, collagen formation, re-epithelization, and neovascularization were significantly improved in post-operative group A than in group B (control group).

This was corresponded with the results of Elsherbeny et al. (2023) who reported that, the donor site area in the lower limb was used as a test

area where it was subdivided into two equal areas, one area was covered by nanofat graft and Vaseline gauze (group A), the other acting as a control group was covered by Vaseline gauze only (group B) histologically group A showed a statistically significant increased thickness more than group B **[10]**.

In our study, vascularity, pigmentation, and height were significantly improved in group A than group B. While, Elsherbeny et al. (2023) reported that, Group A shows a significant increase in VSS score than group B **[10]**.

In the present study, time frame for complete healing was significantly delayed in group B than group A. The median time for complete healing in Group A was 3 weeks, while the median time for Group B was 16.5 weeks. This agrees with Elsherbeny et al. (2023) who reported that, in group A (study group) the healing time was statistically significant less than group B (control group) **[10]**.

In the current study, patient satisfaction was increased significantly in group A more than group B. Similarly, Tonnard et al. (2013) reported a high level of patient satisfaction, with 75% of patients rating their results as "very good" or "excellent". The authors also reported improvements in skin texture, skin tone, and volume restoration **[2]**.

Regarding the histopathological findings in the current study, punch biopsies from ulcers margin of all groups were examined for skin viability, collagen bundles and orcein fibers content, quantification of proliferating cells in both skin layers (epidermis and dermis) to assess the re-epithelization and neovascularization. Post-operative specimen from group A showed a significant increase in epidermal thickness, a significant decrease in dermal inflammatory cell counts and a significant increase in the zone percent of collagen bundles precipitation when matched to both pre-operative skin sections ($p<0.05$, $p<0.001$ and $p<0.001$ respectively) and group B biopsies ($p<0.01$, $p<0.01$ and $p<0.05$ respectively). Group A (post-operative) specimen also showed a significant increase in the area percent of elastic fibers as compared to group B ($p<0.05$). Also, the number of PCNA immunoreactive nuclei in epidermis and dermis was statistically significant in post-operative first group A in comparison to both group B sections and pre-operative group A skin biopsy ($p<0.01$ and $p<0.05$ respectively). These ameliorative effects of the nanofat injected group are most probably due to the presence of ASCs within the nanofat injections. Adipose tissue-derived stem cells were previously proved to have many beneficial paracrine effects on both epidermis and dermis owing to their regenerative, anti-inflammatory and angiogenic properties **[11]**. A similar outcome was obtained in previous research by Mohammed et al. (2017) as injection of ASCs was found to increase the epidermal thickness and the area percent of both collagen and elastic fibers in animal studies **[12]**. Likewise, Elsherbeny et al. (2023) reported a statistically significant increased epidermal thickness of nanofat treated group as compared to untreated group even in the presence of infection **[10]**. Our results agree with those of Menkes et al. (2020) who extracted ASCs from nanofat procedures and reported an increased dermal vascular density and elastic and collagen bundles compactness **[6]**. This can be explained by the fact that ASCs can differentiate into multiple cell types including keratinocytes and fibroblast that share in healing process greatly, in addition, it promotes revascularization by the paracrine secretions of bioactive molecules **[13]** ASCs are believed to be capable of modifying the dermal pattern owing to their anti-inflammatory effects, which explains the marked reduction in the inflammatory cell count of the study group biopsies in the current study. Similar to our results, Nolan et al. (2022) found that nanofat grafting was significantly more effective in reducing inflammatory cell count and Deng et al. (2019) reported that after 10 days of nanofat injection the lymphocyte infiltration of the deep dermal layers was markedly decreased, neovascualrization was improved and wound healing was accelerated **[14,15]**. As PCNA immunostaining signals cellular proliferation and is found at all stages of cell division **[16]**, the decreased PCNA nuclear immunoexpression in the present study in group B and in pre-operative group A skin specimens indicated poor healing. PCNA expression was previously reported to decrease in ulcers **[17]**. Our findings are consistent with those of Wei et al. (2017) who found that nanofat grafting was significantly more effective in increasing PCNA immunostaining in healing skin ulcers than untreated ulcers. The markedly increased number of PCNA immunopositive nuclei in the post-operative group A specimens of the present work indicate high cellular proliferation that coincides with increased epidermal layer thickness and marked dermal neovascularization **[18]**.

Conclusion:

Nanofat grafting may be a promising treatment option for patients with traumatic chronic ulcers, as it can improve wound healing outcomes and patient satisfaction.

Disclosure: The author confirms that there was no conflict of interest and that no financial support was obtained from anybody.

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