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ORIGINAL ARTICLE

Evaluation of Immediate Nanofat Grafting on VEGF Expression in Acute Wounds in Male Albino Rats

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ABSTRACT

Background: Scarring is one of the most complex outcomes of wound healing which usually replaces normal skin with fibrotic tissue. Scar modulation is a composite biological process involving inflammation, tissue remodeling, and collagen deposition elements. We aimed this study to analyze the expression of Vascular Endothelial Growth Factor (VEGF) in acute wounds and scar modulation following nanofat grafting on an animal model. **Methods:** This experimental study included 24 male Wistar albino rats. The wounds were divided into a group of nano-fat-treated wounds and a control group receiving saline injections (n=12 rats in each group). Nano fat was prepared by emulsifying harvested fat tissue, which was then injected into the incision wounds of the treatment group. Wound assessment and photographic documentation were made on days 15, 21, and 28 post-surgery. Histological and immunohistochemical evaluations were carried out to evaluate fibrosis and VEGF expression. **Results:** Nanofat-treated wounds demonstrated a more rapid and organized healing process compared to the control wounds. On day 21, the scars of nanofat-treated wounds were smoother and had a smaller area. In immunohistochemistry, there was substantially more expression for VEGF in the nano-fat-treated scars, and the capillary density was also higher. The positive area for VEGF and the mean VEGF-positive area in the nanofat-treated group were significantly higher than the control group (118072 µm² ±59937, 4.300416% ± 2.136 vs 10302.3 µm² ±16565, and 0.3644% ± 0.583) (p=0.00044, 0.000025 respectively).**Conclusion:** In conclusion, nanofat grafting improved scar modulation, increased VEGF, and angiogenesis, whereas the quality of scarring increased, and wound-healing time shortened. Detailed mechanisms and potential clinical applications of nanofat grafting in scar treatment require further research.

Keywords: Nanofat grafting; Scar modulation; Wound healing; Angiogenesis.

INTRODUCTION

Scars are the outcome of the intricate process of healing a wound, which usually replaces healthy skin with fibrotic tissue devoid of the original structural integrity [1]. However, scar modulation is the outcome of a balance between inflammatory processes, tissue remodeling, and collagen deposition in the tissue [2].

Nanofat grafting has proven regenerative potential for enhancing skin texture and tissue regeneration ability in regenerative medicine

[3]. This technique leverages adipose-derived stem cells (ADSCs), abundant in fat tissue and known for their regenerative capabilities [4]. In addition to being regenerative, these cells can also differentiate into multiple lineages of cells and secrete various growth factors that contribute to wound healing and remodeling of tissue [5]. The most important of these growth factors is vascular endothelial growth factor (VEGF). Vascular endothelial growth factor is the key factor for angiogenesis, which is the formation of new blood vessels. This is an absolute requirement for delivering nutrients and oxygen to newly healing tissues [6].

The relationship between VEGF and nanofat grafting in scar modulation is particularly interesting. VEGF promotes angiogenesis and increases collagen deposition and other extracellular matrix elements, resulting in improved wound healing and possibly better scar outcomes [7]. Despite the promising potential of nanofat grafting, there are limited reports on its histological effects, particularly concerning VEGF's expression and role in this process. While most studies have focused on clinical outcomes and aesthetic improvements, detailed histological changes and mechanisms involved are still unknown [8].

VEGF expression is oriented on its effects on the fine structure of scars and how this possibly determines the potential of nanofat grafting in improving scar tissue through enhanced vascularization and modulation of tissues [9]. The gap in the literature is the lack of detailed study on how nanofat grafting influences the expression of VEGF and subsequent histological changes in scars. It would offer extremely important information regarding the modulation mechanisms of scars and hence lead to more efficient treatment [1]. In light of these findings, this study aimed to determine the effect of nanofat grafting on scar modulation in acute wounds and its impact on VEGF's expression in an animal model.

METHODS

Study Design and Animal Model

The study included 24 male Wistar albino rats weighing 200–250 gm at Zagazig University Hand and Microsurgery Center (ZUHMC). The animals were kept under controlled environmental conditions with a temperature of 22°C, a light/dark cycle of 12 hours, and free access to standard laboratory food and water. The Institutional Animal Care and Use Committee of Zagazig University approved all experimental procedures (Approval number: ZU-IACUC/3/F/61/2023).

Surgical Procedure and Wound Creation

The rats were anesthetized using intramuscular injections of ketamine hydrochloride at a dose of 60 mg/kg body weight and xylazine hydrochloride at a dose of 5 mg/kg body weight. Part of the abdomen was shaved using a hair clipper, and its skin was scrubbed with iodine solution. Two incisions about 1.5 cm from the midline measuring 2-3 cm long were parallel to the midline on either side of the abdomen. The incision on the right side was treated by autologous nanofat grafting, while the left was used as a control and injected with saline (Figure 1). Only the skin and abdominal muscles were excised, sparing the transversus abdominis muscle so as not to form a hernia in this region (Figure 2). Only the skin layer was closed for maximum tension stress, and the muscle layer was not repaired. The only closed layer was the skin to keep tension stress, while the muscle was not closed with repair. The abdominal area of the wound was dressed. The sutures were removed on the seventh day postoperatively. The rats were distributed in groups, and 10 of them in each

group were euthanized on days 15 and 21 days for clinical evaluation, histological analysis, and immunohistochemistry.

Nanofat Preparation and Grafting

Fat tissue was harvested from the inguinal regions of the rats through 2 cm-long oblique incisions. Approximately 2 grams of fat tissue was collected from each rat. The harvested fat was then processed into nanofat by mechanical emulsification, following a standardized protocol. Initially, the fat tissue was washed with saline solution to remove any blood and debris [10]. It was then mechanically emulsified by repeatedly passing it through progressively smaller gauge needles (starting with an 18-gauge needle and moving to a 27-gauge needle) connected via a Luer lock until a uniform, and injectable consistency was achieved. This process ensures that the resulting nanofat contains a high concentration of stromal vascular fraction (SVF) cells, which is crucial for its regenerative properties. The prepared nanofat was injected subcutaneously into the incision wound on the right side of the abdomen, while the left side received an equivalent volume of saline as a control.

Postoperative Care and Assessment

Postoperative care included wound cleaning with 70% ethanol on days 2 and 4 after surgery. The wounds were examined and photographed at 15-, 21-, and 28-days post-surgery to monitor healing progress and scar formation. The overall healing process was documented through clinical observations and photographs.

Histological and Immunohistochemical Analysis

On the 15th and 21st day post-surgery, rats were sacrificed. The scar tissues were harvested for histological and Immunohistochemical analysis. Additionally, immunohistochemical staining for VEGF was conducted to determine the expression levels and distribution of VEGF in the scar tissues. The specimens were fixed in 10% neutral buffered formalin to observe fatty infiltration of the tissue, and dehydration, clearing, and embedding in paraffin were applied. Sections cut at 4–5 µm were mounted on positively charged slides followed by deparaffinization. Antigen retrieval was carried out using citrate buffer of pH 6.0, preheated in a microwave; endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol, and nonspecific binding was also blocked by incubating sections in 10% normal goat serum. Sections were then incubated overnight at 4°C with the primary antibody against VEGF (1:100 in PBS), followed by incubation with a biotinylated secondary antibody and ABC reagent. Photomicrographs were taken at ×200 and ×400 magnifications, respectively, to evaluate immunoreactivity, and ImageJ analysis software was used to quantify positive signals in photomicrographs [11,12].

STATISTICAL ANALYSIS

The collected data were coded and statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences software version ٢٧.0(IBM Corp., Armok, NY, USA). ImageJ software was used to perform quantitative data analysis of both histological and immunohistochemical data. Parameters measured comprising VEGF expression, VEGF-Positive Area (µm²), and Percentage Positive Area (%) were presented as mean values ± standard deviation; the mean values obtained between wounds treated with nanofat and those treated in the control wounds were compared. Data comparisons were made using suitable statistical tests such as the t-test, Mann-Whitney U Test, or ANOVA as appropriate. The normality of the variables was checked with the Shapiro-Wilk test. The statistical analysis was considered significant at $p < 0.05$.

RESULTS

Clinical Observations and Wound Healing

Clinical assessments showed a significant difference during wound healing in the

nanofat-treated wounds compared to controls. Better outcomes were recorded with the scar aspect, which was narrower with more uniform skin texture and coloration in the nanofat-treated wounds. In contrast, control wounds were wider and less homogeneous in texture, with more manifest scar tissue. From the clinical view, such observations allowed us to suppose that nanofat grafting rejuvenates wound healing by showing better tissue regeneration and reduced scarring (Figure 4). All rats tolerated the surgical procedures and nanofat grafting well during the study without significant postoperative complications.

Histological and Immunohistochemical Analysis

On microscopic examination with H&E staining, there were obvious differences between the nanofat-treated and control scars. Immunohistochemical staining for VEGF showed increased staining in the nanofat-treated scars compared to the control group. The density of VEGF-positive cells was higher in nanofat-treated scars, especially around the newly formed capillaries (Figures 5 & 6).

Quantitative analysis showed the levels of VEGF protein to be significantly higher in nanofat-treated wounds. VEGF immunoreactivity was significantly higher in the nanofat-treated group than in the controls. The positive area for VEGF in the control group had a mean of 10302.3 μm² with a standard deviation of 16565, and the percentage of the positive area in the control group had a mean of 0.3644% with a standard deviation of 0.583. The mean VEGF-positive area in the nanofat-treated group was 118072 μm² with a standard deviation of 59937, whereas the percentage positive area was 4.300416% with a standard deviation of 2.136 (Table 1).

Statistical analysis further confirmed the significant differences between the two groups. The VEGF-positive area showed markedly different results by the Mann-Whitney U test, with a U-statistic of 3.0 and a p-value of 0.00044 for the control and nanofat-treated groups. That is to say, there were significantly different outcomes between the two groups ($p < 0.05$). Similar results were obtained for the percentage positive area in the t-test; the t-statistic was -5.621, and the p-value was equal to $2.471 \times 10{-}5$, also indicating a significant difference between the two groups ($p < 0.05$) (Table 1).

Table 1: Comparing Total Tissue Area, VEGF-Positive Area, and Percentage Positive Area (%) between both groups

Variable	Control				Nanofat			
	Mean	SD	Median	IQR	Mean	SD	Median	IQR
Total Tissue Area (μm²)	2,912,478	182,615	2,895,608	253,576	2,736,784	329,132	2,791,173	377,972
VEGF-Positive Area (μm²)	10,302.30	16,565	2,091.50	7,522.75	118,072	59,937	114,954.50	75,193.70
Percentage Positive Area (%)	0.3644	0.583	0.075	0.2875	4.300416	2.136	4.165	3.15512

Mann-Whitney U Test: Used for non-normally distributed data to compare the ranks of two independent groups (VEGF-Positive Area).

Independent Samples T-Test: Used for normally distributed data to compare the means of two independent groups (Total Tissue Area and Percentage Positive Area).

The p-values in the previous analyses were produced using the following statistical tests:

1. Total Tissue Area (µm²):
   - T-Test: An independent samples t-test was used to compare the means of the total tissue area between the control and nanofat treated groups.
   - P-Value: 0.157
2. VEGF-Positive Area (µm²):
   - Mann-Whitney U Test: This non-parametric test was used because the VEGF-positive area data for the control group was not normally distributed.
   - P-Value: 0.00044
3. Percentage Positive Area (%):
   - T-Test: An independent samples t-test was used to compare the means of the percentage positive area between the control and nanofat treated groups.
   - P-Value: 2.471×10−52.471 \times 10^{-5}2.471×10−5

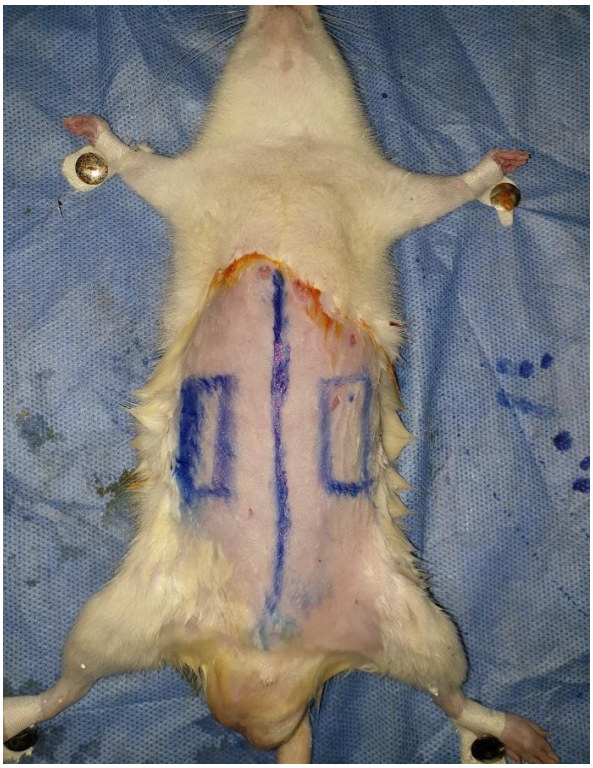

Figure (1): Preoperative Markings of the Incisions

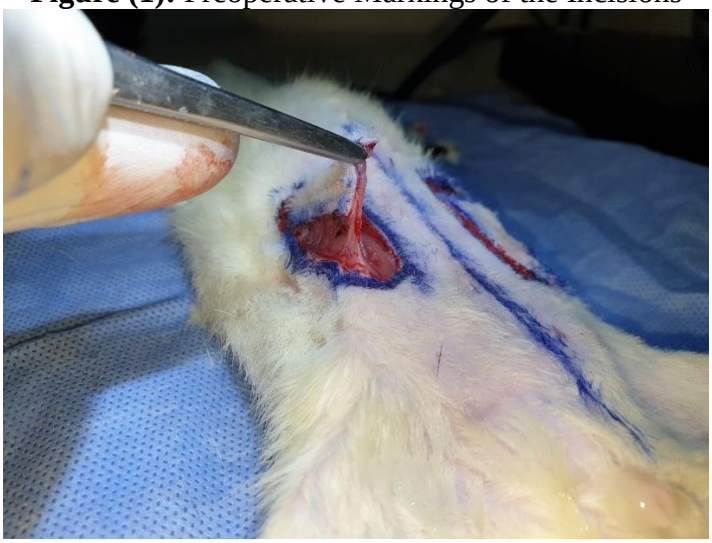

Figure (2): Excision of the skin and abdominal muscles

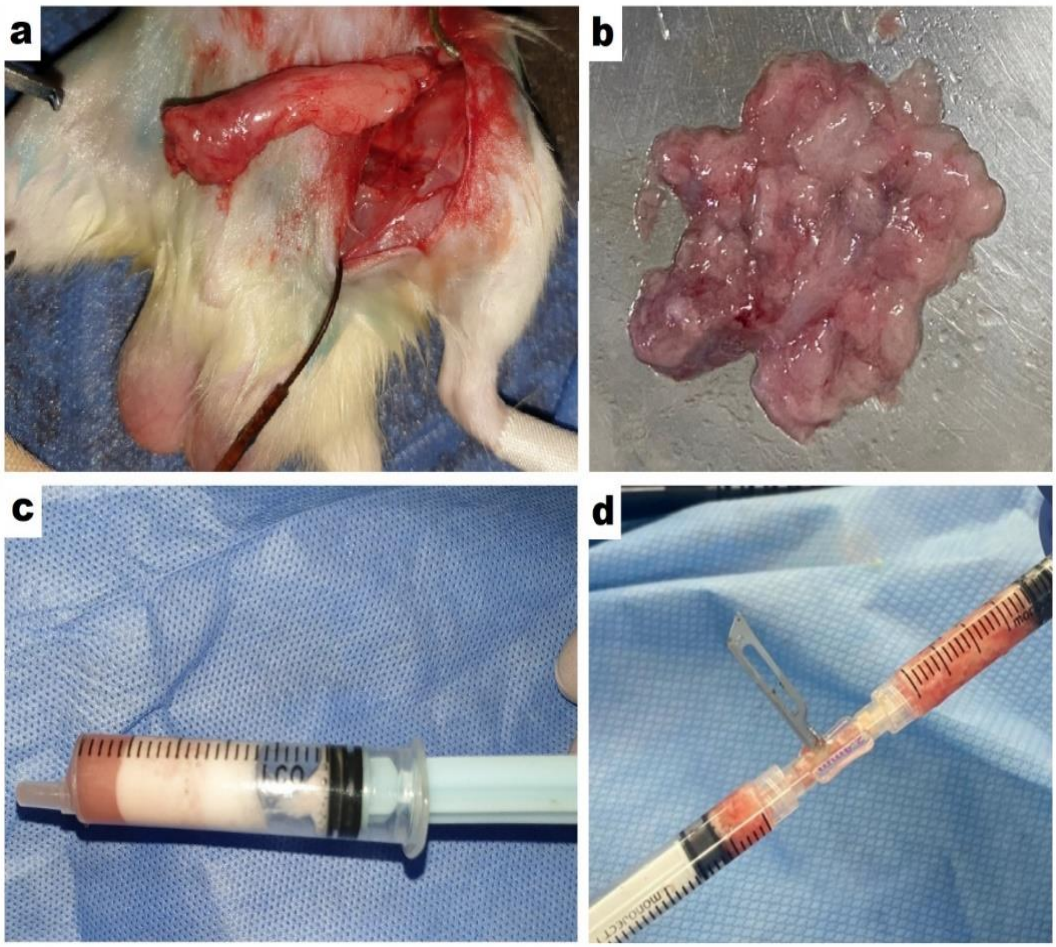

Figure (3): Harvesting and Preparation of Nanofat. **a)** Image showing the surgical procedure for harvesting fat tissue from the inguinal region of a rat. **b)** The tissue is ready to be processed into nanofat. **c)** Image showing a syringe filled with the harvested fat tissue. **d)** The image illustrates the process of transforming the harvested fat into nanofat by mechanical emulsification. The fat tissue is being passed between two syringes connected via a Luer-lock

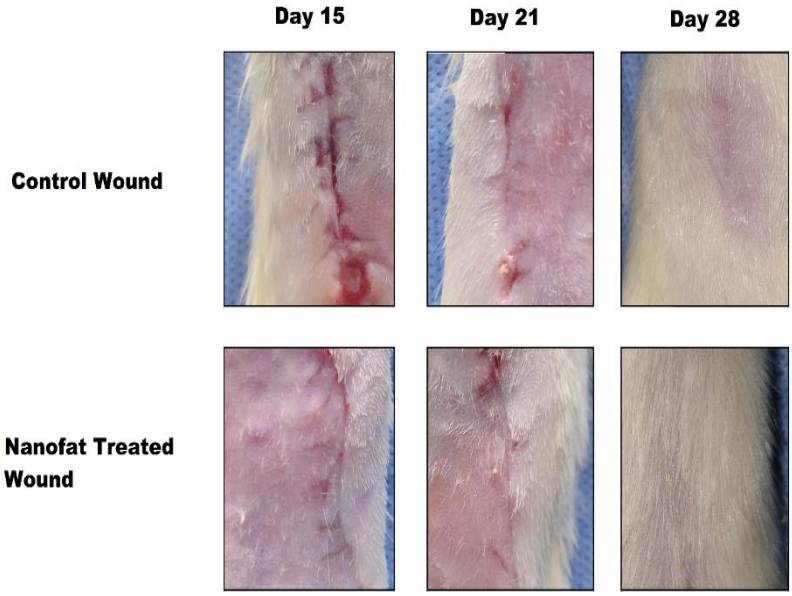

Figure (4): Clinical observation of the scars.

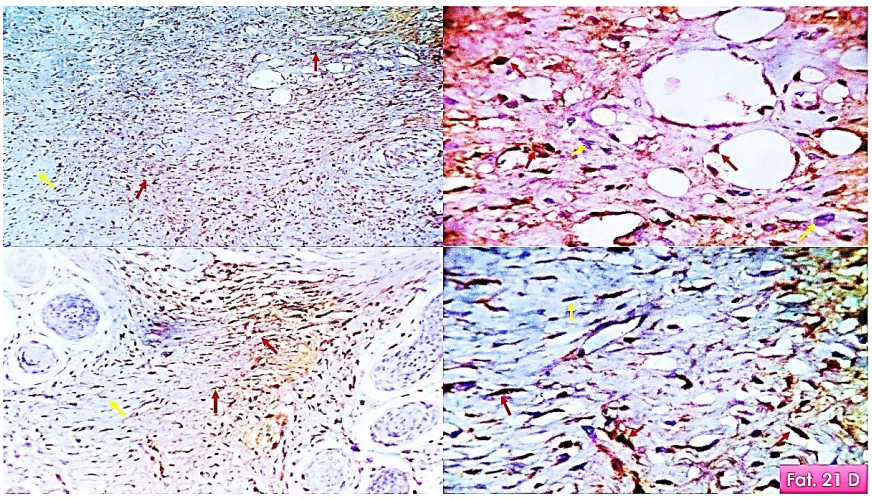

Figure (5): Photomicrograph from fat-treated rats 21 days. Immunostaining by the angiogenetic marker (VEGF) revealed a few to moderate angiogenesis in the well-organized circumscribed scar tissue with a moderate cytoplasmic reaction (red arrows). Negative cells showed blue-stained nuclear and cytoplasm contents (yellow arrows). X200, 400.

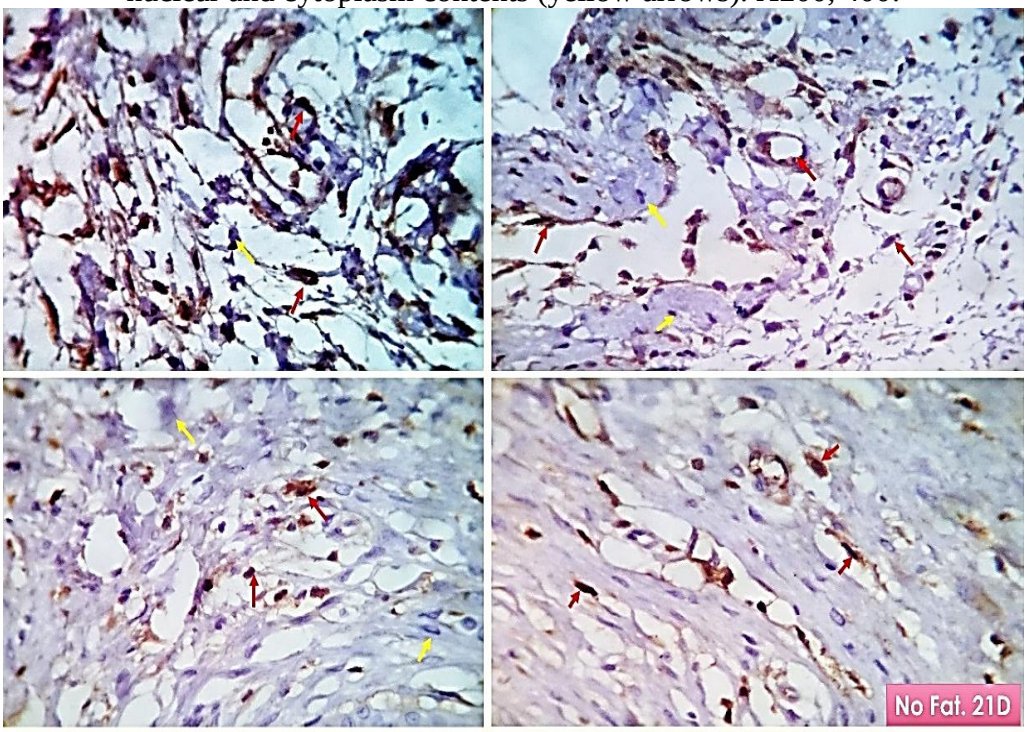

Figure (6). Photomicrograph from the nonfat treated group at 21 days. Immunostaining by the angiogenetic marker (VEGF) shows moderate to marked positive reactivity to the granule-fibroblastic scar tissue's cutaneous activated vascular and nonvascular stromal cells. Expressed cells show moderate to intense brownish cytoplasmic staining reactions (red arrows). Negative cells showed blue-stained nuclear and cytoplasm contents (yellow arrows) X200, 400.

DISCUSSION

The present study aimed to evaluate the immediate effect of nanofat grafting on scar modulation and VEGF expression in acute wounds in male albino rats. This research is meant to establish whether VEGF expression can be significantly improved using nanofat grafting compared to wounds left without any treatment.

The clinical assessment of the wound treated with nanofat grafting demonstrated a remarkably improved wound healing and scar formation outcome. These are further supported by other studies in the literature,

which reported an improved aesthetic appearance of the grafted scars, narrowed due to nanofat grafting, and presenting textural and chromatic homogeneity. Uyulmaz et al. [13] reported marked improvements in the quality of scars with nanofat grafting: the skin appeared softer, with less important discoloration and fewer wrinkles; all the patients were highly satisfied with aesthetic results. Similarly, Tran et al. [14] reported that the final appearance of scars and wrinkles from the result of nanofat grafting improved significantly as proven by the increase in skin thickness, collagen, and elastic fibers in histology.

The effect of nanofat grafting on the improvement of scar appearance has been compared to other studies. For example, the use of condensed nanofat, along with fat grafting for treatment of atrophic facial scars, was examined by Gu et al. [15]. Scars' color, thickness, and pliability were all significantly improved, hinting at the effectiveness of combined therapy for both aesthetic and functional improvement of scar treatment. Moreover, Gentile et al. [16] conducted a systematic review of fat tissue engineering in scar treatment. The study concluded that autologous fat grafting, combined with adipose-derived mesenchymal stem cells, aids in enhancing wound healing and decreasing scar formation, in comparison to classical methods.

In the present study, quantitative analysis clearly revealed significant differences in VEGF expression between groups treated with nanofat and the respective controls. The increased VEGF expression in the nanofat treatment group correlates with findings from other studies that showed fat grafting could improve skin quality and reduce fibrosis by increasing VEGF levels and vascularization. Since VEGF levels in nanofat-treated wounds are elevated, they cause angiogenesis, the main process in tissue repair and regeneration. Our findings agree with those of Garza et al. [17] and Sultan et al. [18] as they showed similar increases in both VEGF expression and vascularization following fat grafting to irradiated tissue.

This marked upregulation of VEGF expression in the nanofat-treated wounds is substantiated by several studies within the literature. For instance, a study by Liang et al. [4] indicated that stromal cells isolated from the nanofat significantly improved VEGF levels, which was associated with superior vascularization and better skin quality in treated regions. This is consistent with our finding that the nanofat-treated scars were higher in VEGF-positive cell density.

The quantitative analyses from other studies have validated the current study. For example, the significant elevation in positivity in VEGF areas of nanofat-treated wounds when compared to controls. Other studies showed the same trend, like that of Bahammam and Attia [19] with significantly higher bone densities and vascularization of VEGF-rich platelet-rich fibrin in periodontal defects. Indeed, their study showed increased VEGF expression, which had a positive effect on capillary density and the healing of the wound. Their results further support this critical role of VEGF in increasing vascular structures and tissue regeneration, similar to what we found with nanofat-treated wounds.

Moreover, a study by Morelli et al. [20] aimed at identifying angiogenic biomarkers related to the VEGF during the wound repair of soft tissue reconstructive procedures. The authors concluded that living cellular constructs showed significantly higher VEGF expression than autografts in all comparisons.

This supports that similar increased VEGF expressions would also occur in our nanofat-treated wounds.

These results of the current study showed that nanofat treatment greatly accelerated the process of wound healing by elevating the VEGF-positive expression in treated wounds. The current results, therefore, suggest that nanofat treatment led to a better quality of the wound-healing process through better tissue regeneration and an increased level of VEGF expression, generating more effective and aesthetically pleasing results.

CONCLUSION

In conclusion, in light of the findings of this study, nanofat grafting increases VEGF levels during the wound healing process, leading to better angiogenesis and overall better wound healing. This study established that nanofat treatment led to more VEGF expression, a higher capillary density, and good tissue remodeling compared to those of untreated scars. These are consistent with the literature postulations of the potential role of nanofat grafting in the modulation and regeneration of scars. The detailed mechanisms of the modulation of VEGF by nanofat and other possible clinical usages require further investigations. Future studies must be conducted concerning the long-term effects of nanofat grafting in the clinic and its safety, as well as applications in different types of wounds and surgeries.

CONFLICT OF INTEREST

No potential conflict of interest is to be reported by the authors.

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