Original Article

Histological Analysis of the Effect of Nanofat Grafting in Scar Rejuvenation

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Abstract

Introduction: The morphology and tissue response to macro- and micro-fat grafting have been widely studied in both clinical and experimental settings; the histological effects of the nanofat graft, however, remain largely unexplored. **Aims:** This study was carried out to evaluate the histological changes leading to scar rejuvenation in a fine scar following nanofat grafting. **Materials and Methods:** This was an experimental study carried out on guinea-pig fine-line scar models. Nanofat prepared from abdominal fat of the animal was injected into scar on right legs (NFG) at 1 month whereas left acted as controls (CG). Punch biopsies from all scars were analyzed at 2, 4, and 6 months by Hematoxylin&Eosin, Masson's trichrome, and Picrosirius red stains to evaluate dermal/epidermal regeneration, collagen fiber orientation, pattern of distribution, and amount of mature and immature collagen. **Results:** Nine animals were included in the final analysis of the study. On histological analysis, the amount of inflammatory infiltrate, collagen fiber orientation, pattern and total histological score at 2, 4, and 6 months were similar between the groups. There was an increased trend for earlier appearance of organized and mature forms of collagen in the NFG group. The distribution of collagen was similar at 2 months; however, there was a significant increase in collagen distribution in NFG at 4 months (NFG: 46.11±11.6, CG: 31.16±9.9; $P = 0.010$) and at 6 months (NFG: 63.48± 6.6, CG: 49.9 ±8.8; $P = 0.002$). **Conclusion:** Nanofat grafting is associated with an accelerated and increased production of mature collagen with proper alignment in fine-line scars.

Keywords: Collagen, fat grafting, Picrosirius red, scar remodeling, Tonnard

Introduction

Fat grafting is one of the useful adjuvant modalities for management of scar in the armamentarium of plastic surgeons.[1,2] Fat being autologous, widely abundant, easily accessible, and low immunogenicity is an ideal volumizing agent.[3,4] Wide research has demonstrated its immunomodulatory and regenerative potential as well. There has been an increasing interest in utilizing lipofilling for delicate areas using fine cannulas.[5-8] Tonnard described "nanofat" for use in further superficial planes and hence the role in rejuvenation of finer scars and rhytids.[9]

Nanofat does not contribute to volume as classical fat grafting and hence is relatively lacking in viable adipocytes. Its role as a filler is limited and acts by enhancing the skin quality.[10,11] There is considerable evidence to support the role of fat-grafting in neo-collagen synthesis, potentially via adipocyte-derived stem cells.[9,12-24] Although the adipocytes are eliminated during processing in nanofat, adequate amounts of mesenchymal stromal cells are present which are believed to produce the rejuvenating effects.[9] The morphology and tissue response to macro- and micro-fat grafting have been widely studied both in clinical and experimental settings; the histological effects of the nanofat graft, however, remain largely unexplored.[15-19] Deficiency of a matching experimental model with similar scar-forming properties remains a challenge. Nanofat grafting is clinically used for very fine scars and rhytids.[20] Procuring tissue biopsy from such scars in humans is practically not feasible owing to ethical issues and hence is a potential reason for the dearth of histological evidence. To the best

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Access this article online

Quick Response Code:

Website: www.jcasonline.com

DOI: 10.4103/JCAS.JCAS_106_21

How to cite this article: VinayKumar DM, Mohsina S, Tripathy S, Sharma RK, Bhatia A. Histological analysis of the effect of nanofat grafting in scar rejuvenation. J Cutan Aesthet Surg 2022;15:147-53.

of our knowledge, there are no reports on direct histological effects of nanofat grafting. Hence, this study was carried out to evaluate the histological changes in a fine scar, leading to scar rejuvenation following nanofat grafting in an animal model.

Materials and Methods

This was a prospective experimental study carried out from March 2018 to October 2018 on scar models created in guinea pigs at a tertiary care hospital. The study was approved by the Institute Research Committee and Institute Animal Ethics committee (89/88/IAEC/599R).

Five-month-old Dunkin-Hartley guinea pigs weighing around 700–800 g, kept at a constant temperature (25°C) under a 12-h light/dark cycle with free access to food and water in Animal Research Facility of the Institute, were selected for the assessment of eligibility. A fine-line surgical wound was created on both the legs of the animal. Guinea pigs with a fine-line scar at 1 month were included in the study.[21,22] Animals were excluded if any abnormal scar sequelae such as hypertrophic scarring were noted.

Study procedure

Creation of scar models

All animals were nursed as per standard protocols and were handled with utmost care. Intraperitoneal ketamine hydrochloride and xylene were administrated in a dose of 90 and 10 mg/kg, respectively, to induce anesthesia. The success of anesthesia was determined by absence of the wink reflex and by lack of reaction to pinching of the foot. Under strict aseptic precautions, a straight full thickness skin incision 1 cm long was made using a15G surgical blade on both limbs. The incisions on both legs were ensured to be at similar location and of same length. These were then sutured with three to four interrupted simple sutures with 5-0 nylon and allowed to heal [Figure 1]. The sutures were removed on day 7.

At 1 month, the presence of fine-line scar was assessed as per inclusion criteria. Under similar anesthesia techniques, a small chunk of lower abdominal fat (approximately 6–8 cc) was harvested [Figure 1]. This was mechanically emulsified by passing between two 10 cc syringes connected to each other by a female-to-female Luer-Lok connector of 2.4 mm diameter. The emulsified fat solution after 30 passes was strained on a nylon cloth and the effluent nanofat solution was utilized.[9] Nanofat (approximately 0.5–1 mL) was injected into intradermal and subdermal levels underneath the scar on the right leg using a 100 U insulin syringe fitted with a 1 in, 27-gage needle as shown in Figure 1.[9,23,24]. The end-point of injection was visible blanching of the scar.

Histological assessment

After 2, 4, and 6 months of nanofat grafting, 4 mm punch biopsies were taken from the scar site of both the legs. The

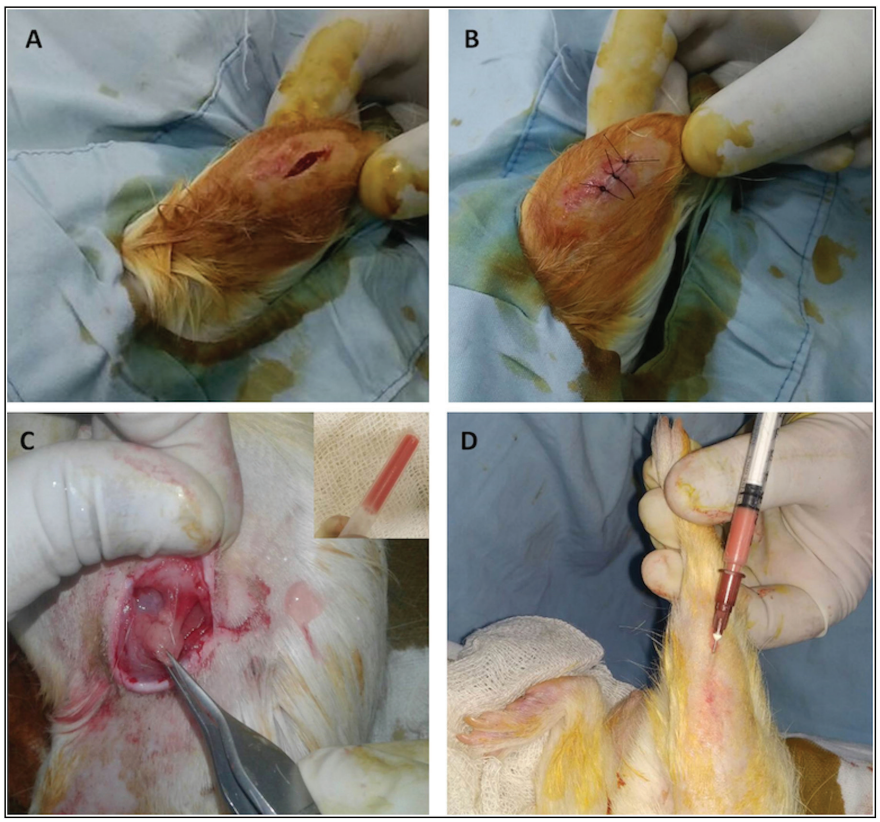

Figure 1: Study procedure. (A) Full thickness surgical incision; (B) sutured surgical wound; (C) harvest of abdominal fat. Inset: Prepared nanofat solution after emulsification of fat harvested. (D) Nanofat injection in the fine-line scar at intradermal and subdermal levels

punch biopsy tissues were fixed immediately in formalin, paraffin embedded, and cut in to 4-µm thick sections. Parts of the sections were stained with Hematoxylin & Eosin (H&E) and Masson's trichrome stain (MTC). The sections were examined initially by a senior registrar of the Department of Pathology and reviewed by a senior consultant from the same department. Both evaluators were blinded to the nature of intervention in the scar specimens. Part of the section was stained with Picrosirius red stain to evaluate the amount of collagen fibers.[25]

To determine the dermal and epidermal regeneration and collagen deposition, a histological scoring system on maxillofacial wounds was utilized.[26] Parameters evaluated for scoring comprised amount of granulation tissue (profound—1, moderate—2, scanty—3, absent—4), inflammatory infiltrate (plenty—1, moderate—2, a few—3), collagen fiber orientation (vertical—1, mixed—2, horizontal—3), pattern of collagen (reticular—1, mixed—2, fascicle—3), amount of early collagen (profound—1, moderate—2, minimal—3, absent—4), and amount of mature collagen (profound—1, moderate—2, minimal—3). The total score was obtained by adding the individual score and was used to grade the healing status as good (16–19), fair (12–15), and poor (8–11).[26]

Picrosirius red-stained biopsy sections were evaluated for the presence of collagen under a polarized light microscope.[25] The collagen fibers exhibit green/greenish yellow to yellowish-orange through orange to red polarizing colors with Picrosirius red stain, depending on the thickness of the fibers.[27,28] The quantitative analysis was carried out by analyzing the photographs of polarized light microscopy sections using IMAGE J software to calculate the percentage of area of the total field comprised of collagen.[29]

Statistical analysis

Statistical analysis was carried out using SPSS version 22. The quantitative variables were expressed as mean and categorical variables were expressed as proportions. The categorical variables were analyzed using χ^2 or Fisher's exact test, and quantitative variables were analyzed using independent *t*-test or Mann–Whitney *U*-test. A *P*-value <0.05 was considered as significant.

Results

A total of 10 guinea pigs were included in the study; however, one animal died during the study period and hence was excluded. Both the study and control groups were comparable as two different limbs of the same animal were utilized.

Histological assessment of scar by a blinded investigator

The amount of granulation tissue was scored as "absent" in all the sections from both the legs of all the nine animals. Inflammatory infiltrate was found to be "moderate" in both the legs of one animal and "few" in all the other animals at 2 months. The inflammatory infiltrate was "few" and comparable between the legs in all the animals by 4 months. The collagen fiber orientation, pattern and amount of early and mature collagen, and total histological score at 2, 4, and 6 months are shown in Table 1. Although there was a relatively increased trend for earlier appearance of organized and mature forms of collagen, this was not statistically significant. The light microscopy changes of collagen at 2, 4, and 6 months are shown in Figure 2. The mean total histological scores in the fat-grafted group at 2, 4, and 6 months were 15.33± 0.71, 15.89±0.78, and 16± 0.86, respectively, thus indicating fair-to-good healing. The mean total scores between the two groups were also similar [Table 1].

Table 1: Comparison of histological parameters between the nanofat-grafted and control groups

	2 months			4 months			6 months		
	NFG (*N*=9)	Control (*N*=9)	*P*-value	NFG (*N*=9)	Control (*N*=9)	*P*-value	NFG (*N*=9)	Control (*N*=9)	*P*-value
Collagen fiber orientation									
Vertical	0	0	0.410*	1	1	0.412*	0	0	0.234*
Mixed	9	9		7	6		4	3	
Horizontal	0	0		1	2		5	6	
Amount of early collagen									
Profound	1	2	0.788*	0	0	0.277*	0	0	0.222*
Moderate	4	3		3	2		4	5	
Minimal	4	4		6	7		5	4	
Amount of mature collagen									
Profound	2	1	0.614*	5	6	0.234*	5	4	0.222*
Moderate	4	3		4	3		4	5	
Minimal	3	5		0	0		0	0	
Pattern of collagen									
Reticular	0	1	1.059*	0	1	2.485*			0.222*
Mixed	9	8		2	4		5	4	
Fascicle	0	0		7	4		4	5	
Mean total score	15.33±0.71	15.44 ±0.72	0.747**	15.89±0.78	15.56±0.72	0.363**	16± 0.86	16±0.83	0.582**

NFG = nanofat group. *Fisher's exact test; **Mann–Whitney *U*-test

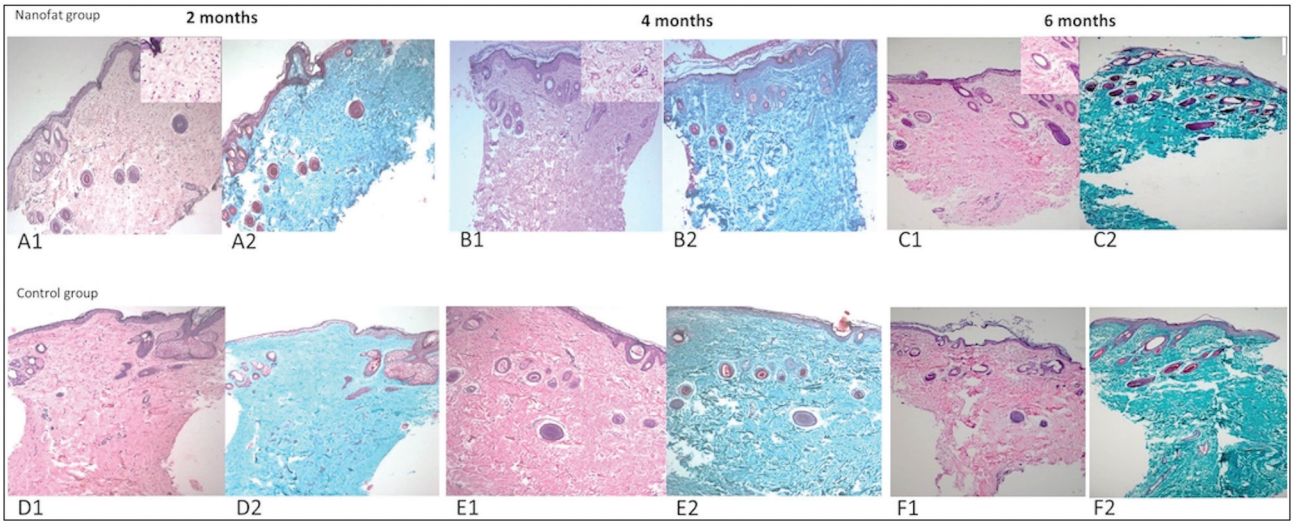

Figure 2: Microphotographs of the punch biopsy specimens (10×, H&E) and MTC staining. Bright field microscopy showing collagen changes at 2 (A, D), 4 (B, E), and 6 (C, F) months. *Upper row*: Nanofat-grafted scar demonstrating increase in amount and maturation of collagen with time. (A) At 2 months; (A1): immature collagen with mixed arrangement of fibers with no fascicles. Few inflammatory cells noted in the upper dermis. A2: same is evident in MTC stain. (B) At 4 months; (B1): increase in mature collagen with appearance of fascicles in the lower dermis, (B2): same is evident in MTC stain. (C) At 6 months; (C1): the collagen is more dense with more number of fascicles, (C2): MTC stain depicting the same. Inset in (A1), (B1), and (C1) shows zoomed images to better depict the collagen arrangement. *Lower row*: Collagen changes in the control group (D) at 2 months; (E) at 4 months; (F) at 6 months. No significant differences noted in the two groups except for more immature collagen in the latter group at 2 months

Assessment of amount of collagen

The amount of collagen identified by Picrosirus red staining was found to be similar at 2 months between the two groups. The mean percentages of collagen in the nanofat-grafted and control groups at 2 months were 46.11±11.6% and 31.16±9.9%, respectively. At 4 months, the mean amount of collagen was 63.48± 6.6% in the nanofat group and 49.9 ±8.8% in the control group. There was a significant increase in the amount of collagen between the two groups at 4 and 6 months, as shown in Figure 3. Comparison of the serial biopsies from the scar which was evaluated using Picrosirius stain is shown in Figure 4. The Picrosirius staining shows a significant increase in the amount of collagen in the nanofat-grafted group.

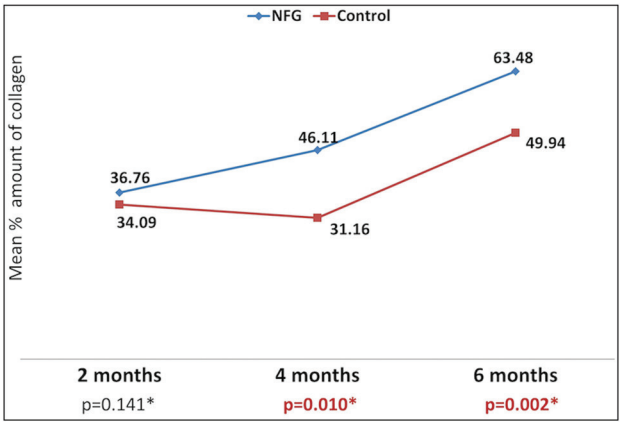

Figure 3: Comparison of trend of amount of collagen showing significant increase at 4 ($P = 0.010$) and 6 months ($P = 0.002$); *Mann–Whitney *U*-test

Discussion

Adequate and satisfactory management of scars is a never-ending challenge facing the plastic surgeons. A variety of treatment modalities have been widely studied; however, there is no gold standard method. Nanofat grafting is a novel modality of rejuvenating fine scars which has gained interest over the past few years.[9] Although there are few reports demonstrating its clinical efficiency, there is a dearth of histological evidence of scar remodeling.[9,10,20] In the present study, we have prospectively demonstrated the accelerated and increased production of mature collagen following nanofat grafting, which can potentially explain the scar rejuvenation properties of nanofat.

There are few reports on effect of lipofilling on scar histology carried out on radiation-damaged rodent models.[23,24] In another recent report, better scar appearance after fat grafting on full thickness burn wounds in mice was demonstrated.[30] Although these studies demonstrated the volumizing and rejuvenating role of fat cells on deep and extensive scars, the effect of fat grafting on finer scars has not been widely studied. The effect of nanofat on scar rejuvenation is believed to be mediated by adipocyte stem cells.[9] There has been a recent shift from the regular animal models such as mice and rat to newer models on guinea pigs, hamsters, pigs, etc. in the literature owing to the increased similarity in the skin properties to humans.[21] A recent study demonstrated that adipose tissue in guinea pigs contained mesenchymal stem cells which share similar properties to human bone marrow-derived mesenchymal stem cells and suggested the role of guinea pigs as a valuable source of multipotent stem cells

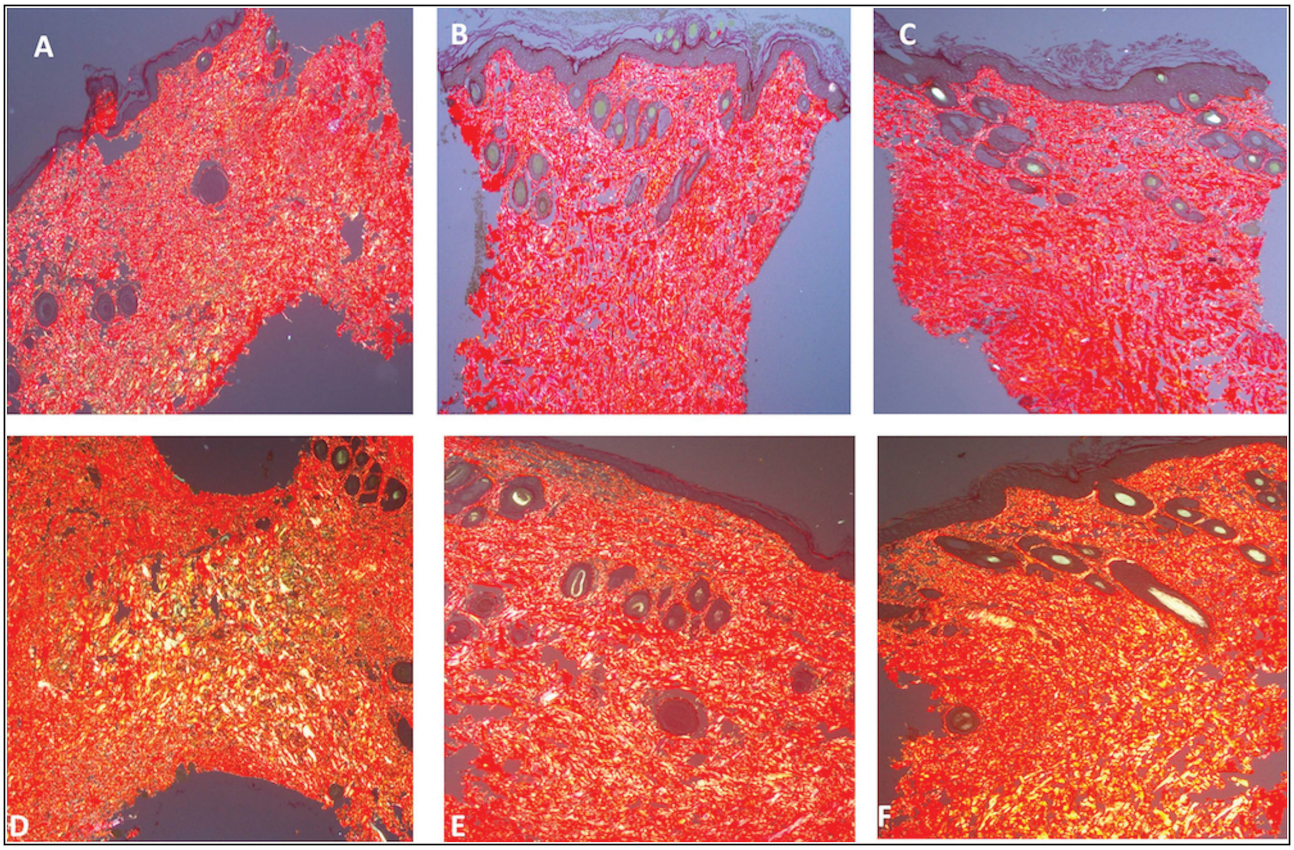

Figure 4: Picrosirus red staining (under polarized light microscope) of the punch biopsy specimens showing red collagen fibers with yellow green immature fibers. *Upper row*: Nanofat-grafted scar demonstrating significantly increased amount of collagen (mainly red fibers depicting mature collagen). (A) At 2 months; (B) at 4 months; (C) at 6 months. *Lower row*: The control group demonstrating lesser amount of collagen when compared with the nanofat group with time. (D) At 2 months; (E) at 4 months; (F) at 6 months. Note that the majority of the collagen noted are yellow green immature fibers.

for use in experimental and preclinical studies in animal models.[31] Similarly, in the present study, the authors have utilized a guinea pig model to create a fine-line scar which was treated with and without nanofat grafting. Aksoy *et al.*,[22] in a recent report, concluded guinea pigs to be new, practical, and economical experimental models with scarring properties similar to those of humans. The use of two limbs of the same animal helped in avoiding the confounding factors that can interfere in wound healing.

Mojallal *et al.*[32] grafted human fat tissue into mice scar model and assessed the histological changes. They demonstrated that fat tissue grafting stimulated a neosynthesis of collagen fibers at the recipient site and makes the dermis thicker. In another recent report on irradiated scar model, an increase in vascular density and a decrease in collagen levels to normal were noted 8 weeks following fat-grafting.[24] Similar findings were noted in our study as well with a significant increase in the amount of collagen in the nanofat-grafted scars by 4 months. On light microscopy histological scoring, the majority of the scars demonstrated "profound" amounts of mature and "minimal" amounts of immature collagen by 4 months. Although there was a relatively favorable trend noted in the nanofat-grafted group when compared with controls, this difference in type of collagen between the two groups was not statistically significant. This may probably be attributed to the limited sample size. Similar reports of neo-collagen deposition were demonstrated in clinical studies on patients with post-burn hypertrophic scars following classical fat grafting.[33]

The scoring system chosen for histological analysis was previously used in the histological study of scars in the maxillofacial region, which have a good healing potential which is similar to that of the fine-line scars in the animal models under study.[26] The evaluation of tissue biopsies by two blinded pathologists and use of software for quantification of Picrosirius red-stained images helped to reduce the subjectivity in the scoring. Recent experimental studies have demonstrated that Picrosirius stain cannot distinguish between the type of collagen as the amount of light absorbed depends on the thickness and orientation of collagen bundles.[27,28] The role of Picrosirius stain in identifying type of collagen is controversial and hence an attempt for quantifying type of collagen with Picrosirius stain was not made in this study. In the present study, there was an increase in the fascicular pattern and a

better orientation of collagen fibers by 4 and 6 months in both the groups by light microscopic evaluation. Also, on Picrosirius staining, the amount of collagen was significantly higher in the fat-grafted group at 4 and 6 months. Although quantification of the types was not carried out using Picrosirius stains, there was relatively larger amounts of red fibers suggestive of mature collagen at 4 and 6 months when compared with the control group. Similar results of better collagen alignment with fat grafting were reported in full thickness burn wound models by 8 weeks.[24,30] The effect of nanofat grafting as seen in the present study is delayed and becomes prominent by 4 months as opposed to macro- and micro-fat grafting. Similar effect was described by Tonnard *et al.*[9] with a delay of up to 3 months for the clinical effects to appear. This delay is probably attributed to the fact that the effect of nanofat is by soft-tissue rearrangement due to effect of stem cells rather than acting only as volumizing agent.

Previous studies have demonstrated increased blood flow in fat-grafted models at 4 weeks but not at 8 weeks.[23,24] However, these were on scar models with excessive fibrosis. In the present study, there was minimal or no granulation tissue and inflammation noted as early as 2 months. Also, the total score in both the groups was >15 at 2 months, indicating fair healing of the wound. This probably may be due to the inclusion of only fine scar following a surgically created wound. The histological score between the two groups was similar at 2, 4, and 6 months; however, a slightly increased trend was noted in the nanofat group when compared with controls. The limited sample size or use of a scar model with good healing properties may have contributed to the non-significant difference in this healing process.

The improvement in collagen distribution and pattern of alignment and type in the nanofat-grafted group when compared with the controls suggests the role of nanofat in stimulating these histological changes, thus leading to scar remodeling. However, the study is limited by a small sample size and short follow-up period. A longer follow-up period might be required to demonstrate significant changes in the histological scores as it may take up to 12 months for the changes brought about by fat grafting to stabilize. Further studies are needed to determine the long-term effects.

Conclusion

The collagen fiber orientation, pattern and amount of early and mature collagen, and total histological score at 2, 4, and 6 months by light microscopy were similar between the two groups. Nanofat grafting was found to be associated with an accelerated and increased production of collagen in fine-line scars when compared with the control group which did not undergo any form of lipofilling. This scar remodeling can potentially explain the histological mechanism of nanofat in scar rejuvenation.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Financial support and sponsorship

Nil.

Conflicts of interests

There are no conflicts of interest.

Author contributions

S. T. and R. K. S. supervised and designed the study. V. K.-D. M., S. M., and A. B. carried out the research. S. M.,V. K.-D. M., S. T., and A. B. analyzed and interpreted the data. V. K.-D. M., S. M., S. T., R. K. S. and A. B. wrote and edited the manuscript. V. K.-D. M., S. M., and A. B. arranged the clinical and histological photographs. All authors have seen and approved the final version of the manuscript.

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