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RESEARCH ARTICLE

Can Viscoelastic Materials Prevent Fibrosis in Incisional Skin Wounds? An Experimental Study in a Mouse Model

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A B S T R A C T

This study was designed based on the hypothesis that synovial fluid (SF) can reduce scar formation in incisional skin wounds, because of rich hyaluronic acid (HA) contents and viscoelastic properties. For this purpose, the efficacy of intralesional injection of SF in a primary closure model of incisional skin wound was investigated. A total of 36 male mice, 8–12 weeks old and weighing 39–44g, were randomly divided into two equal groups, i.e., an untreated control group (Group-C) and an SF group (Group-S). A 3-cm long skin incision involving all the layers was made at the back of each mouse. The skin wound was closed with a simple interrupted suturing technique. In Group-S only, SF was injected into the wound area before its closure. Six animals in each group were sacrificed at 7, 14, and 21 days of incision and underwent macroscopic and histopathological examination. For the histological examination, tissue sections were stained with Crossmann's modified triple staining technique. Results showed that wound healing was faster in the SF group than in the control group. This difference was more evident at 14 days after the incision. In addition, the significant decrease in both the number of inflammatory cells and granulation tissue at 14 days after the incision in the SF group than the control indicated that SF accelerated the healing phase. In conclusion, the present study reveals that the use of SF as a source of HA accelerates wound healing and is effective in preventing fibrosis in incisional skin wounds.

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INTRODUCTION

A wound is defined as the disruption of the cellular, anatomical, and functional integrity of a living tissue caused by physical, chemical, thermal, microbial, or immunological factors (Masson-Meyers *et al.,* 2020). Wound healing involves inflammation, proliferation, and epithelialization processes that are modulated by various types of cells and mediators that interact with each other in a considerably complex temporal order (Özaydın *et al.,* 2018; Kurt *et al.,* 2018).

Healing of all skin wounds, except fetal wounds, results in fibrous scar formation, which can cause permanent cellular, morphological, and functional disorders of the skin. Therefore, preventing or minimizing scar formation in surgical wound management is of utmost importance, and research in this field is in progress (Aya and Robert, 2014; Sorg *et al.,* 2017; Gündüz *et al.,* 2019). A meticulous surgical procedure, use of proper suture material and suturing technique, and regular wound management can reduce chances of scar formation (Sorg *et al.,* 2017). In addition, other methods such as silicone gel application, use of various wound care products, and intralesional corticosteroid injections can also minimize chances of scar formation (Çolak *et al.,* 1995; Sorg *et al.,* 2017).

Hyaluronic acid (HA) is a polymer naturally found in the body, and its concentration in the wounded area increases following wound formation. It is involved in all stages of wound healing and tissue repair and has a positive effect on events such as inflammation, cell proliferation (mitosis), vascularization and

epithelialization. Due to these properties, HA has found widespread use in preventing scar formation in surgery (Aya and Robert, 2014; Özaydın *et al.*, 2014).

Being rich in HA, synovial fluid (SF) is used in wound studies as a viscous material (Neuman *et al.*, 2015). The present study was designed to investigate the efficacy of intralesional SF injection in preventing fibrotic scar formation, by studying all stages of wound healing (inflammation, proliferation, and re-epithelialization), in a primary closure model of incisional skin wound in mice.

MATERIALS AND METHODS

Ethical approval: This study was conducted by obtaining approval from the Animal Experiments Local Ethics Committee of Kafkas University (Kars, Turkey).

Animals: A total of 36 male *M. musculus* mice, 8–12 weeks old and weighing 39–44 g, were included in the study. These mice were housed in individual cages under standard laboratory conditions (12 hours darkness/12 hours daylight, 45–55% humidity, and room temperature of 20–22°C). Animals were fed standard diet and water *ad libitum*. These mice were randomly divided into two main groups, i.e., a control group (Group-C) and an SF group (Group-S), with 18 mice in each group. The mice of Group-S were injected synovial fluid intradermally and subcutaneously. The synovial fluid was collected from the tarsal joint of healthy cattle by arthrocentesis and centrifuged at 3,500 rpm for 10 min before administration.

Surgical procedure: The surgery was conducted under general anesthesia induced by intraperitoneal injection of a mixture of 10 mg/kg xylazine HCl (Rompun, 2%, Bayer®) and 100 mg/kg ketamine HCl (Ketalar, 50 mg/mL, Pfizer®). After shaving the animals on the back and skin preparation (povidone iodine + 70% ethanol), a 3-cm long skin incision involving all layers was made.

In Group-C, the incisional skin wound was closed with a simple interrupted suturing technique. In Group-S, SF (1 mL) was injected into the wound area intralesionally so that it could spread into the skin surface, skin, and associated subcutaneous structures. This was followed by closure of the incisional wound with a simple interrupted suturing technique. In all groups, an absorbable suture material (4/0 polyglactin 910, Vicryl®) was used for wound closure.

Postoperative care: All mice were individually housed in standard cages under standard laboratory conditions, and they were fed an appropriate diet and provided water regularly for 21 days.

Macroscopic examination : Six animals from each main group were sacrificed at 7, 14, and 21 days of treatment, following high-dose pentobarbital sodium intraperitoneal euthanasia. Then the wound area was macroscopically examined, skin and subcutaneous tissues were excised and preserved in 10% formaldehyde solution for histopathological examination.

Histopathological examination: For histological examination, the tissue samples taken from both the control group and the SF group were subjected to the routine histological tissue processing procedures, using the Crossmann's modified triple staining technique (triple staining) (Luna, 1968). The tissues were evaluated histologically, using a semi-quantitative scoring method (Gupta and Kumar, 2015). For this purpose, tissue samples were scored on a scale of 0–3 (0: absent, 1: minimal, 2: moderate, and 3: intense) based on the epithelialization, number of inflammatory cells, granulation tissue, fibroblast proliferation, neovascularization, and amount of collagen tissue.

Statistical analysis: For statistical analysis, the data were subjected to Independent-Samples T Test, using the SPSS 16.0 software package. A p-value of <0.05 was considered statistically significant.

RESULTS

Clinical observations: During the experiment, no adverse events associated with the animals or the wound area were encountered. The wound healing process was clinically uneventful in all groups at postoperative days 7, 14, and 21.

Macroscopic findings: There were no macroscopic signs of infection or necrosis in mice of any group. Adhesion and tissue thickening were observed in the subcutaneous connective tissues and muscles at days 7 and 14 in the control group (Group-C), whereas no such changes could be seen at days 7 and 14 in the SF group (Group-S).

Histopathological findings: In the control group, re-epithelialization in the epidermis and granulation tissue formation in the dermis was observed at day 7 after the incision (Fig. 1). The cellular components of the granulation tissue included mononuclear cells, fewer fibroblasts and neutrophils. Neovascularization was noted to be intense, whereas collagen synthesis was low. In 7th day it was observed that there was no difference between the groups in all areas. In the SF group, re-epithelialization was noted in the epidermis at day 7 after the incision. Granulation tissue formation was observed in the dermis. The cellular components of the granulation tissue mostly included mononuclear cells, and neutrophils and fibroblasts to a lesser extent, as was the case in the control group. There was a small amount of collagen synthesis in the granulation tissue. Neovascularization was intense during this period (Fig. 4). Thickening of the epidermal layer due to epithelial proliferation was observed at day 14 after the incision in the control group (Fig. 2). There was a decrease in the number of inflammatory cells in the granulation tissue at day 14, whereas there was an increased fibroblast migration and proliferation. Collagen synthesis and accumulation had increased significantly at day 14 compared to day 7 (Fig. 1,2). Neovascularization was observed to a lesser extent at day 14 in the control group (Fig. 2). There was remarkable healing at day 14 after the incision in the SF group than in the control group. During this period, re-epithelialization was deemed as completed in the epidermis; furthermore, there was a significant decrease in the number of inflammatory cells in the granulation tissue

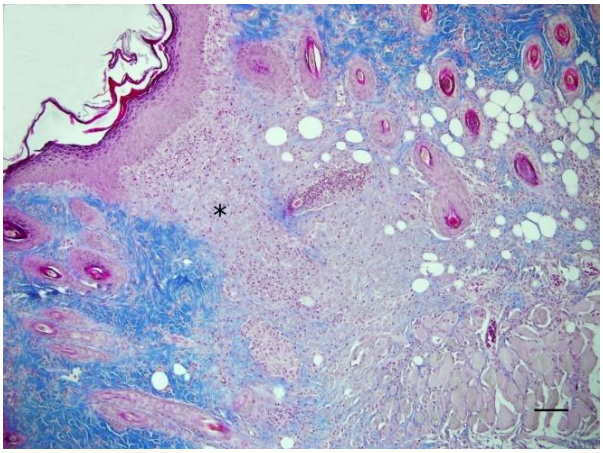

Fig. 1: Histological View of Skin in Control Group at 7th Day. *:wound line, bar: 200 µm. Triple staining.

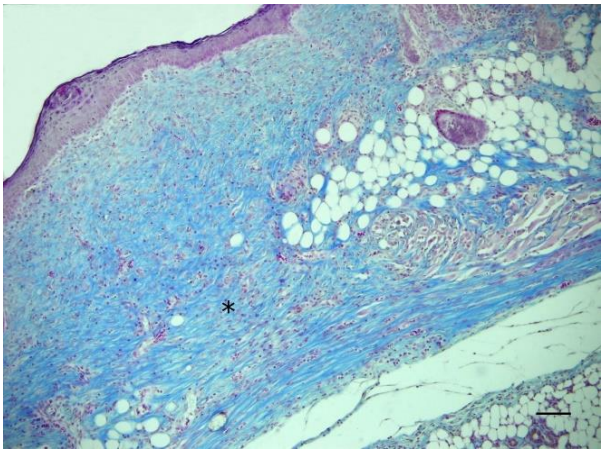

Fig. 2: Histological View of Skin in Control Group at 14th Day. *:wound line, bar: 200 µm. Triple staining.

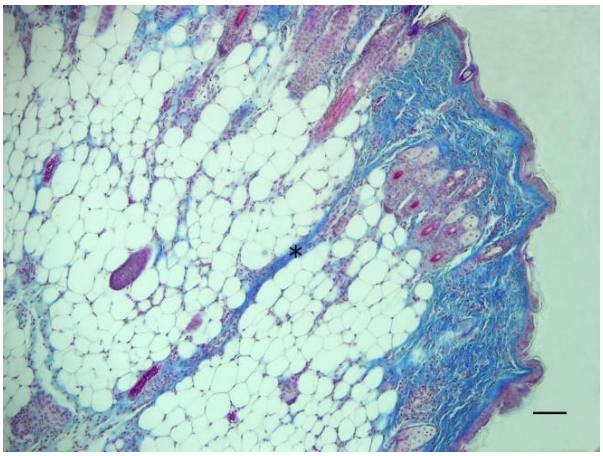

Fig. 3: Histological View of Skin in Control Group at 21st Day. *:wound line, bar: 200 µm. Triple staining.

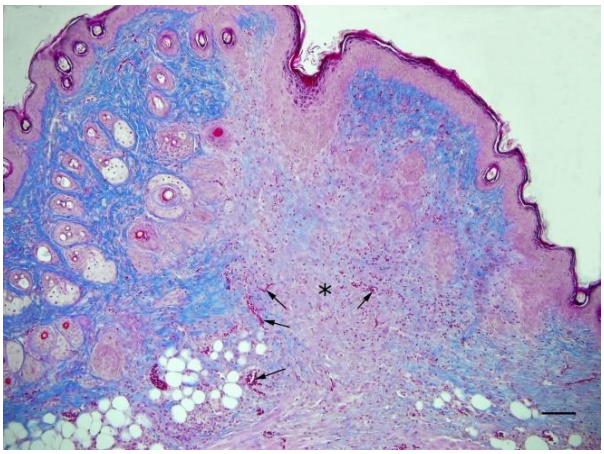

Fig. 4: Histological View of Skin in Synovial Fluid Group at 7th Day. *:wound line, arrow: neovascularization. Bar: 200 µm. Triple staining.

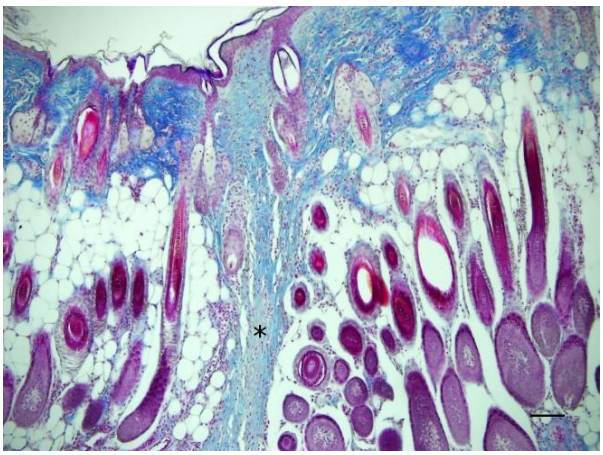

Fig. 5: Histological View of Skin in Synovial Fluid Group at 14th Day. *:wound line, Bar: 200 µm. Triple staining.

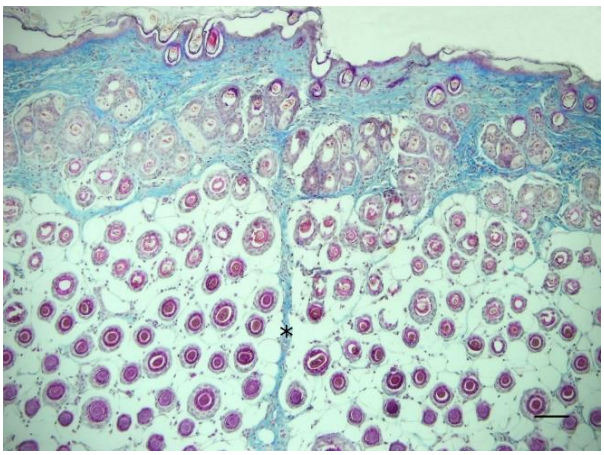

Fig. 6: Histological View of Skin in Synovial Fluid Group at 21st Day. *:wound line. Bar: 200 µm. Triple staining.

Table 1: Histopathologic evaluations of control and synovial fluid groups

Proses	Control Group			Synovial Fluid Group		
	7th Day	14th Day	21th Day	7th Day	14th Day	21th Day
Epithelialization	2.66±0.51^{a}	2.83±0.40^{a}	3.0±0.0^{a}	3 .0±0.0^{a}	3.0±0.0^{a}	3.0±0.0^{a}
Inflammatory cell amount	2.50±0.54^{a}	1.83±0.63^{bA}	0.5±0.54^{c}	2.33±0.51^{a}	1.16±0.40^{bB}	0.16±0.40^{c}
Granulation tissue	2.50±0.54^{a}	2.33±0.51^{aA}	0.83±0.75^{b}	2.66±0.51^{a}	1.50±0.54^{bB}	0.5±0.54^{c}
Fibroblast proliferation	1.33±0.51^{b}	2.66±0.51^{a}	1.5±0.54^{b}	1.50±0.54^{b}	2.66±0.51^{a}	1.0±0.63^{b}
Neovascularization	2.66±0.51^{a}	1.33±0.51^{bA}	0.66±0.81^{b}	2.66±0.51^{a}	0.66±0.5^{bB}	0.16±0.40^{b}
Amount of collagen	0.66±0.51^{b}	2.33±0.81^{a}	2.33±0.5^{aA}	1.0±0.63^{a}	1.83±0.75^{a}	1.50±0.5^{aB}

$^{a, b}$:Different letters on the same row between days are significant within the groups for different days. $^{A, B}$:The letters on the same row are significant between the two groups for the same day.

in the dermis, increase in fibroblast proliferation, decrease in neovascularization, and increase in collagen synthesis (Fig. 5). In the control group, the thickness of the epidermal cell layer decreased and returned to normal and epithelial maturation occurred, inflammatory cells in the dermis disappeared, and there was a decrease in the number of fibroblasts at day 21 after the incision. The granulation tissue was replaced by scar tissue and the healing process was largely completed at day 21 (Fig. 3). The maturation of epithelial layer occurred at day 21 in the SF group. The inflammatory cells completely disappeared in this period in the dermis. There was a decrease in the number of fibroblasts and collagen deposition. It was noted that the granulation tissue was replaced by the scar tissue, but the amount of scar tissue was less compared with that in the control group (Fig. 6). The healing process was noted to be faster and better in the SF group.

In the statistical analysis, it was observed that there was no significantly difference between the groups in terms of epithelialization in all days. It was determined that the amount of inflammatory cells and granulation tissue gradually decreased in both groups. However, on the 14th day, it was noted that there was a significantly decrease in the amount of granulation tissue and inflammatory cells in the synovial fluid group compared to the control group. It was determined that neovascularization reached the highest level on the 7th day and fibroblast proliferation reached the highest level on the 14th day in both groups. On the 14th day, it was determined that neovascularization was a significantly decreased in the synovial fluid group compared to the control group. On the 21st day, it was observed that there was no difference between the groups in terms of epithelialization, inflammatory cell, granulation tissue, fibroblast proliferation and neovascularization, however, it was significantly determined that the amount of collagen in the wound area was less in the synovial fluid group compared to the control group. The results of the histological examinations in the groups across the assessment points are presented in Table 1.

DISCUSSION

The results show that SF accelerates wound healing and is effective in minimizing the scar formation.

HA is a glycosaminoglycan that has a regulatory role in mitosis, cell migration, inflammation, and immune system, and HA is known to be derived from the joint fluid, cockscomb, umbilical cord, spinal cord, corpus vitreum, bacteria, and sea creatures (Tan *et al.,* 2001; Tırnaksız and Kaymak, 2008; Tuygun and Atasoy, 2016). In our study, SF collected from the tarsal joint of cattle was used as a source of HA to prevent adhesion and fibrosis in incisional skin wounds created in mice fed under appropriate conditions.

It is known that HA acts as an antioxidant by capturing free oxygen radicals and has a protective effect by removing enzymes that destroy tissues in inflammation. Owing to its viscous structure, it helps delaying viral and bacterial passage in the pericellular region that is rich in HA, and it is used in bandages as a potential material to prevent bacterial contamination and promote wound healing (Chen ve Abatangelo, 1999 ; Lequeux *et al.,* 2014 ; Çantay *et al.,* 2021). In macroscopic examination in our study, no signs of necrosis or abscess were found and wound healing was uneventful in both groups. There were adhesions and tissue thickening in the subcutaneous connective tissue and muscles at days 7 and 14 in Group-C, and the healing of the skin, subcutaneous connective tissue, and muscles was uneventful at day 21 in both groups. There was no evidence of adhesion or tissue thickening at days 7 and 14 in Group-S. The absence of abscess and necrosis in both groups indicates that the viscous structure of the SF acts as a barrier in the wound area, preventing infection and therefore the development of necrosis and abscess. The uneventful tissue healing that occurred at days 7 and 14 in Group-S is considered to be because of the fact that HA makes the wound matrix more permeable for fibroblast migration and promotes faster repair of the wound.

Histologically, wound healing involves hemostasis, inflammation, proliferation, and maturation phases. Although the healing process basically involves the same stages, it can vary depending on many extrinsic and intrinsic factors (Gantwerker and Hom, 2011; Gupta and Kumar, 2015; Tepebaşı and Calapoğlu, 2016). The hemostasis stage is the first stage after injury. At this stage, vasoconstriction, platelet aggregation, and angiogenesis occur. In the second phase, which is called the inflammatory phase, many cell types such as neutrophils, macrophages, and fibroblasts migrate to the wound area (Diegelmann and Evans, 2004; Broughton *et al.,* 2006; Gupta and Kumar, 2015; Tepebaşı and Calapoğlu, 2016). Consistent with the literature, neovascularization and granulation tissue formation were observed at day 7 after the incision. At this stage, mononuclear cells were predominant in the wound site, and neutrophils and fibroblasts were also present to a lesser extent. In the proliferation phase, fibroblast migration and matrix synthesis occur while angiogenesis continues (Diegelmann and Evans, 2004; Broughton *et al.,* 2006; Gupta and Kumar, 2015; Tepebaşı and Calapoğlu, 2016). In addition, consistent with the literature, the present study found a decline in the number of inflammatory cells in the wound site and an increase in the number of fibroblasts and collagen synthesis at day 14 after the incision. Collagen is the most abundant protein in mammals and is the most important factor that promotes wound healing (Gantwerker and Hom, 2011). The collagen synthesized by fibroblasts plays an important role in wound healing and promoting tissue integrity and strength (Tepebaşı and Calapoğlu, 2016). The tensile strength in wound healing is initially provided by collagen synthesis, and in the later stages, the wound strength is provided by the maturation and organization of the collagen as well as the bonds between the fibers (Gantwerker and Hom, 2011; Gupta and Kumar, 2015; Gündüz *et al.,* 2019). Although the maturation phase, which is the last phase in wound healing, takes a longer time, the granulation tissue is replaced by the scar tissue in this phase. The initial scar tissue consists of irregular collagen bundles. Over time, this is replaced by a more regular collagen organization, while the number of fibroblasts and macrophages gradually decreases. The durability of the newly formed tissue also increases

(Gupta and Kumar, 2015; Tepebaşı and Calapoğlu, 2016). In our study, the reconstruction and maturation phase began at day 21 after the incision. In histological examination, the wound healing process is evaluated using criteria such as epithelialization, number of inflammatory cells, granulation tissue, fibroblast proliferation, neovascularization, and the amount of collagen (Gupta and Kumar, 2015). The same criteria were used in histological examination in the present study.

It has been reported that HA has positive effects on wound healing (Tırnaksız and Kaymak, 2008; Neuman *et al.,* 2015). Consistent with the literature, the administration of SF that is rich in HA content positively affected wound healing. In our study, wound healing was faster and better in the SF group than in the control group. Furthermore, the decrease in the amount of collagen in the wound site on histological examination performed at day 21 after the incision supports the idea that SF application would contribute to less scar tissue formation during the wound healing process (Manuskiatti and Maibach, 1996). Every new study in this field will contribute to the determination of new treatment methods associated with wound healing.

Conclusions: The present study concludes that the use of SF as a source of HA is effective in preventing the formation of fibrosis and adhesions, which can contribute positively to the wound healing process, and will have important implications in clinical practice in the management of incisional skin wounds.

Authors contribution: UA, İÖ, TA, CŞE and MC designed the study and conducted the experiment. SKT, DGE and SİA analyzed tissue samples. All authors interpreted the data, critically reviewed the manuscript for important intellectual contents and approved the final version.

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