

Received: 2016.02.09
Accepted: 2016.03.15
Published: 2016.04.01

Effects of Silk Sericin on Incision Wound Healing in a Dorsal Skin Flap Wound Healing Rat Model

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Data Interpretation D
Manuscript Preparation E
Literature Search F
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Source of support: Departmental sources

Background: The wound healing process is complex and still poorly understood. Sericin is a silk protein synthesized by silk worms (*Bombyx mori*). The objective of this study was to evaluate *in vivo* wound healing effects of a sericin-containing gel formulation in an incision wound model in rats.


Material/Methods: Twenty-eight Wistar-Albino rats were divided into 4 groups (n=7). No intervention or treatment was applied to the Intact control group. For other groups, a dorsal skin flap (9×3 cm) was drawn and pulled up with sharp dissection. The Sham operated group received no treatment. The Placebo group received placebo gel without sericin applied to the incision area once a day from day 0 to day 9. The Sericin Group 3 received 1% sericin gel applied to the incision area once a day from day 0 to day 9. Hematoxylin and eosin stain was applied for histological analysis and Mallory-Azan staining was applied for histoimmunochemical analysis of antibodies and iNOS (inducible nitric oxide synthase), and desmin was applied to paraffin sections of skin wound specimens. Parameters of oxidative stress were measured in the wound area.

Results: Epidermal thickness and vascularization were increased, and hair root degeneration, edema, cellular infiltration, collagen discoloration, and necrosis were decreased in Sericin group in comparison to the Placebo group and the Sham operated group. Malonyldialdehyde (MDA) levels were decreased, but superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were increased in the sericin group.

Conclusions: We found that sericin had significant positive effects on wound healing and antioxidant activity. Sericin-based formulations can improve healing of incision wounds.

MeSH Keywords: **Antioxidants • Sericins • Wound Healing**

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/897981>

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Background

A wound is a result of a physical injury that causes damage and penetrates into the layers of the skin [1]. Wound healing is important in terms of ensuring anatomic integrity of the skin and protecting its functional structure, and it occurs with the repair of the skin and other soft tissues [2]. Wounds start as a response to an injury and end with the re-establishment of the integrity and functioning of the injured area [2].

Wound healing is a preprogrammed process consisting of 4 continuous phases [3]: bleeding controlled by hemostasis, inflammatory response (0–3 days), cellular proliferation (3–12 days), and remodeling (3–6 months) [1,4,5]. The interruptions, aberrancies, and prolongations that may occur during these phases can interrupt the wound healing process and lead to non-healing chronic wounds [3].

Hemostasis starts immediately after the injury and fibrin clots are formed together with vasoconstriction. Proinflammatory cytokines and growth factors – fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF- β) – are expressed from the clot and the surrounding tissue after hemostasis. Inflammatory cells migrate to the inner side of the wound after hemostasis and start the inflammatory phase. Neutrophils, macrophages, and lymphocytes migrate to the tissue in this phase [3].

The proliferative phase follows and overlaps with the inflammatory phase. The proliferative phase is characterized by epithelium proliferation and the migration of this epithelium to the temporary matrix in the wound (re-epithelization) [3]. The main components of the proliferative phase are angiogenesis, deposition of collagen, granulation tissue formation, epithelization, and wound contraction. During angiogenesis, new blood vessels are formed from endothelium cells [6]. Fibroblasts and endothelium cells are the main cells that have a role in processes of capillary growth, collagen formation, and granulation tissue formation in the wound area in reparative dermis. Fibroblasts synthesize glycosamine and proteoglycan, as well as collagen, which is the main component of the newly developing extracellular matrix [3]. After this process, epithelial cells migrate to the other side of the wound bed and cover the wound, and the wound is covered completely by myofibroblasts, contracting the edges of the wound [6,7]. After the quick proliferation and extracellular matrix synthesis, the last phase, remodelling, will begin, which may last for years [1].

In these processes, there is a coordinated influx of many neutrophils to the wound area, especially in the inflammation phase. The characteristic respiratory burst activity, which occurs due to the properties of these cells, triggers free radical

formation [8]. The non-phagocytic cells localized in the wound area produce the free radicals, which are in the non-phagocytic NAD(P)H oxidation mechanism. This situation gives rise to the increase in oxygen and nitrogen in the wound area. The appearance of these radicals results in oxidative stress that leads to lipid peroxidation and DNA breakage, as well as to the inactivation of some enzymes, including free radical scavenger enzymes [1]. Topical application of antioxidants in wound healing has become common practice, and it has been demonstrated that components with free radical scavenging characteristics clearly improve wound healing and protect the wound from oxidative stress [9].

Wounds caused by mechanical trauma, acute and chronic burn injuries, and pressure and foot ulcers may pose important difficulties for surgeons and emergency physicians [10,11]. Many studies have been conducted all over the world for the purpose of accelerating wound healing and preventing amputation or serious complications [12]. Many chemicals and herbal substances have been tried for wound healing and ensuring maximum recovery [2,4,6,7,13,14]. Sericin, which is a protein synthesized by the domestic silkworm, *Bombyx mori*, is one of these substances. The silk protein, which is a natural polymer, is bio-synthesized from the epithelium cells in *Bombyx mori*. The silk proteins that are expressed to the lumens of these glands are converted into silk protein fibers [16]. Silk is well characterized and easy to obtain.

Sericin becomes gel by itself, and forms a film when combined with some polymers by cross-linking and therefore can be used in manufacturing cosmetics and pharmaceuticals. It has been proven that sericin does not cause immunological reaction with materials that have more widespread acceptance in biological applications [16]. In addition, it has some unique characteristics, such as UV resistance, antioxidation, moisture absorbance, and biocompatibility [17–22]. It has been determined in previous empirical studies that sericin decreases the peroxyl radical activity and tyrosinase [21], and suppresses lipid peroxidation [22,23]. Sericin contributes to these antioxidant effects with the chelation to the trace elements copper and iron, and hydroxyl groups [23]. Sericin's chemical structure is shown in Figure 1.

We conducted a literature review using the keywords “sericin”, “wound healing”, and “antioxidants”, and found that there have been very few clinical and empirical studies conducted on the effects of silk protein sericin in wound healing models [16,24].

The present study was conducted using histochemical, immunohistochemical, and biochemical methods to determine whether sericin affects wound healing.

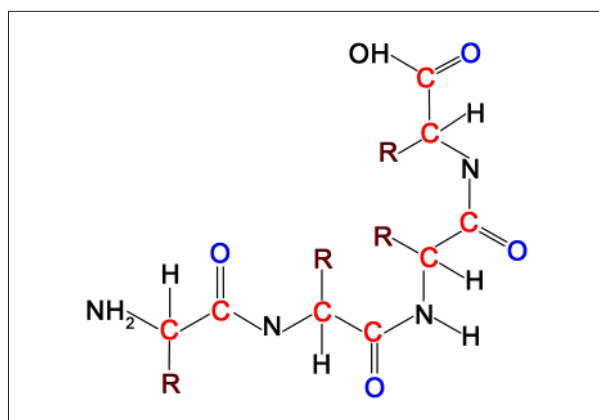


Figure 1. Chemical structure of sericin.

Material and Methods

Extraction of sericin from cocoon

Sericin was extracted from silkworm cocoons and used as a calibration standard. To do this, the cocoons were first cut into small pieces. Then, water was added and they were autoclaved at 120°C for 1 h. The sericin solution was filtrated through a 1.6- μ m filter (Whatman GF/A), then cold ethanol was added. The final ethanol concentration was 75% (v/v). The supernatant of ethanol was discarded and the settled sericin was frozen at -80°C. Then, it was dried in a lyophilizer to obtain sericin powder [25–27]. Sericin's chemical structure is shown in Figure 1.

Analytical methods

We used the Shimadzu Prominence Model High-Performance Liquid Chromatography (HPLC) system to perform quantitative analysis of sericin and to determine its molecular weight distribution. The HPLC system contains a Nucleogel aqua OH-40-8 gel permeation chromatography (GPC) column, and a buffer solution containing 0.05 M phosphate and 0.3 M NaCl. The analyses were all done at 30°C and their ultraviolet absorbances (UVA) were read at 230 nm [27]. In sericin analysis, the flow rate of mobile phase was adjusted to 1 mL/min and it was decreased to 0.3 mL/min for the determination of molecular weight distribution. For filtration of all samples, a 0.45- μ m filter (Millipore Millex-HV) was used, then the solution was injected into the system using a 20- μ L syringe. For standardization of the calibration, we used sericin extracted from native silk cocoons [29].

Preparation of formulations

Preparation of placebo gel

Placebo gel formulation was prepared by dispersing 7.5% w/w Na-CMC in distilled water to form a homogeneous dispersion under continuous stirring until a homogeneous gel was formed.

Preparation of gel containing sericin

Gel formulation was prepared by dispersing 7.5% w/w Na-CMC in distilled water to form a homogeneous dispersion. We dissolved 1% sericin in distilled water, then the solution was added to the Na-CMC dispersion while stirring until the solution became a homogeneous gel [30].

Animal studies

Animals

Twenty-eight male Wistar-Albino rats weighing 200–220 g were used in this study. All experiments were performed after obtaining permission from the Ege University Animal Ethics Committee and according to its regulations (Permission Number: 2014-062). National guidelines on the care and use of laboratory animals were strictly followed. The animals were kept under standard conditions, with 25 \pm 1°C room temperature, 12-h light/dark cycles, in individual cages, and fed pellet diet and water ad libitum. The rats were randomly divided into 4 groups with 7 rats in each group. No intervention or treatment was applied to the Intact control group. For the Sham operated group, the Placebo group, and the Sericin group, a dorsal skin flap (9 \times 3 cm) was drawn and pulled up with sharp dissection. The Sham Control group received no treatment. The Placebo group received a placebo gel applied to the incision area. The Sericin group received a gel containing 1% sericin applied to the incision area. The rats were treated immediately in the Placebo group with placebo gel and the Sericin group was treated with sericin gel. All formulations were applied to animals once a day from day 0 until day 9.

Incision wound model

Anesthesia were used for all the animals prior to and during the experimental interventions. Dorsal fur of the rats was shaved before infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using ketamine hydrochloride (75 mg/kg) and xylazine (8 mg/kg) anesthesia intraperitoneally. On the dorsal side of rats, a caudally-based 9 \times 3 cm (27 cm²) dorsal skin flap was drawn and pulled up by sharp dissection as described by Khouri et al. [31]. The entire flap was undermined below the level of the *panniculus carnosus*. Incisions were closed with 4/0 silk surgical sutures placed 1 cm apart to the parted skin of the animals. The wounds were left undressed. Total operation time was 15 min for every rat [32].

Wound healing

Under ether anesthesia, gel formulations were applied over the flaps once a day and repeated every day for 9 days. The gel

formulations were evenly applied in sufficient amounts covering all the surface of the wound area.

On the 9th day after the initial operation, flap viability was evaluated. Rats were reanesthetized for evaluation of the viability of the flap tissues on the 10th postoperative day. Six slices were randomly selected from each individual animal in a group and 5 separate measurements were made in each slice to determine epidermal thickness and necrotic area. The dorsal skin flaps were photographed with a digital camera (Canon A 610, Japan). A mechanism using a tripod was used to standardize all images, and images were taken from equal distances. By calculation of the necrotic tissue percentage, necrotic skin surface was defined by the necrotic skin borders, and total flap areas were delineated by surgical borders of the flaps.

The animals were sacrificed by high-dose ketamine hydrochloride administered intramuscularly. When the animals were sacrificed, a skin biopsy was taken from the beginning of the 3 cm distal of the flap base and 1 cm width. For the histopathological analysis, we investigated tissue degeneration of skin biopsy samples fixed in 10% buffered formalin under a light microscope.

Histopathological analysis

After fixation of the skin biopsy samples in 10% buffered formalin for 24 h, routine paraffin wax embedding procedures were used and samples were blocked. About 5- μ m sections were cut using a Leica RM 2145 model microtome (Germany) then stained with both hematoxylin and eosin and Mallory azan staining. Sections were investigated at a magnification of 40 \times and examined the thickness of their epithelium. After taking digital photos (Olympus BX51 Light Microscope, Olympus C5050 Digital Camera, Japan) at a magnification of 20 \times , necrotic skin and total flap area border were determined with the aid of a software program (Image Pro Express Version 4.5.1.3., Media Cybernetics Inc., 2002, USA).

Immunohistochemical analysis

To analyze the immunohistochemical expressions, anti-iNOS and anti-desmin antibodies were used. Paraffin sections were immersed in xylene overnight and incubated in methanol containing 3% H₂O₂ to reduce endogenous peroxidase activity. Sections were heated in sodium citrate solution in a microwave oven at 90 W for 5 min and at 360 W for 15 min. Subsequently, sections were incubated in primary antibodies (anti-desmin, Bioss, bs-1026R, USA; 1/100 and anti-iNOS, Santa Cruz, Sc-651, USA; 1/100) for 24 h at 4°C. Antibody detection was performed with the Histostain-Plus Bulk kit (Bioss, Inc) against rabbit IgG, and 3,3' diaminobenzidine (DAB) was used to visualize the final product. Immunoreaction was assessed by

light microscopy (Olympus BX-51 light microscope, Olympus C-5050 digital camera) at 40 \times magnification.

Histological scoring

The tissue edema and collagen discoloration grading were scored as: normal=0; mild increase=1; mild to moderate increase=2; moderate increase=3; moderate to marked increase=4; and marked increase=5. For the purpose of expressing the cellular infiltration grading, the scoring obtained by counting the total inflammatory cells (lymphocytes, neutrophils, macrophages, eosinophils, plasma cells, and mast cells) for each section per high-power field (HPF) (400 \times). According to this scoring, a score of 0 = <3 inflammatory cells, 1 = 3–10 inflammatory cells, 2 = 11–20 inflammatory cells, 3 = 21–30 inflammatory cells, 4 = 31–40 inflammatory cells, and 5 = >41 inflammatory cells [33].

Biochemical analysis

After the routine skin tissue homogenization and centrifuge processes, the BioVision brand Glutathione Peroxidase Activity Colorimetric Assay Kit was used to determine the GSH. The reading was performed using a BMG LABTECH 96 Microplate Reader at 340 nm.

To determine the MDA in the tissue, the BioVision brand Lipid Peroxidation (MDA) Colorimetric/Fluorometric Assay Kit was used, and the measurements were used with the Colorimetric method. The reading was performed using a BMG LABTECH 96 Microplate Reader at 532 nm. A BioVision brand Catalase Activity Colorimetric/Fluorometric Assay Kit was used with the Colorimetric method to determine the catalase in the tissue. The reading was performed with a BMG LABTECH 96 Microplate Reader at 570 nm. We used a Beckman Coulter AU640 brand auto-analyzer applique and a Randox Ransod brand commercial kit to determine the SOD activity in the tissue.

Statistical analysis

Data are expressed as percentages, numbers, median, and mean \pm SD. Statistical comparison of the groups was performed using SPSS Version 22.0 (Chicago, Illinois, USA). The Kruskal-Wallis test was used to analyze the significant difference between groups in number of total hair roots, number of degenerated hair roots, number of vessels, epidermal thickness and percentage of the necrotic area around the flap, biochemical analysis of GPx, SOD, catalase, and MDA levels in the flap biopsy. When significance was determined, the Mann-Whitney U test was used for post hoc analysis. A *P* value below 0.05 was considered to be statistically significant.

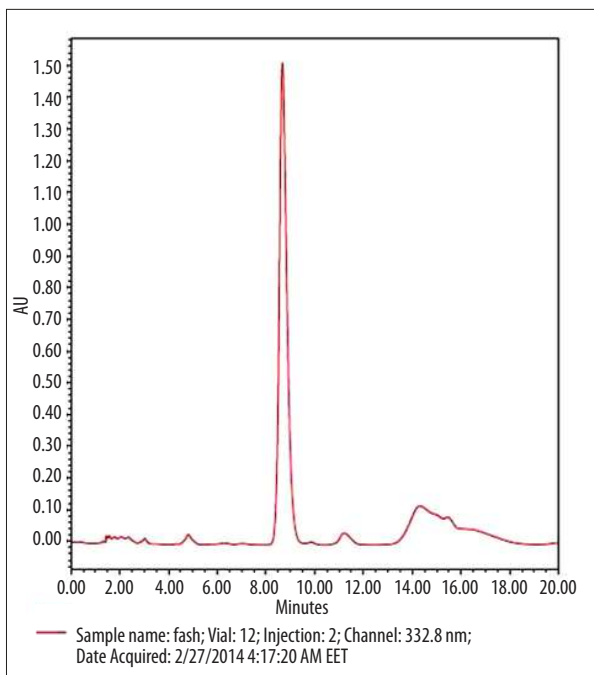


Figure 2. HPLC spectrum of sericin (concentration=2.5 mg/mL).

Results

Analytical methods

HPLC was used for quantitative analysis of sericin and determining its molecular weight distribution. The HPLC chromatogram is shown in Figure 2. The sericin was eluted as a broad peak between 6.5 and 11.0 min.

Histopathological results

Intact Control Group: Normal skin histological structure was observed. Five different layer structures were determined, stretching from the basal to the stratum corneum in epidermis. Normal histologic structure was observed in the glandular structures, which are called *glandula sebacea*, expressing their holocrine degenerative secretions to hair follicles. The hair follicles started from the dermis and extended through the epidermis and opened to the surface. The glandular structures were located in the bottom area of the hair follicles. The irregular tight collagen tissue, which hosted collagen bundles stretching every direction in the dermis, was determined to be normal. The structure, which is rich in fat tissue and connects the skin defined as hypodermis to the tissues in deeper areas, was normal (Figures 3A, 4A, 5A, 6A).

Sham Operated Group: Histologic structure of the wound lips was determined. There was decreased epidermis thickness. Degeneration was determined in the hair roots. The degenerative findings in the sebaceous glands in the hair roots and

the areas hosting the hair roots were observed to be smaller than those in the Intact control group. We also observed that the amount and distribution of collagen in collagen bundles stretching in every direction in the dermis were significantly greater than the intact control group, and that this was in the direction of scar formation (Figures 3B, 4B, 5B, 6B).

Placebo Group: We determined that the Placebo Group was similar to the Intact control group in terms of histopathology, and the epidermis was thinner than in the Intact control group. The scar formation, collagen discoloration, decrease in the hair roots, and the histopathological image in the *glandula sebacea* structures were similar to those in the Sham operated group (Figures 3C, 4C, 5C, 6C).

Sericin Group: We determined that the epidermis thickness was closer to that of the Intact control group, and was thicker than in the Sham operated group and the Placebo group. The degenerative findings in the hair roots were less than in the Sham operated group and the Placebo group, and was closer to that of the Intact control group, as was the case with epidermis thickness. There were similarities in collagen distribution, scar formation, and collagen discoloration (Figures 3D, 4D, 5D, 6D).

After the histopathological evaluation of the samples, the parameters on wound healing were evaluated. The total number of the hair follicles, the number of the degenerated hair follicles, and the number of the unaffected vessels are given in Table 1; the epidermis thickness and necrotic tissue percentage are given in Table 2; and the edema, cellular infiltration, and collagen discoloration are given in Tables 3–5, respectively.

The number of the total hair roots

When the samples that were obtained after the tenth day were compared in terms of the number of the total hair roots, a significant difference was found among the 4 groups ($KW\chi^2$, $p < 0.0001$) (Table 1). The groups were compared as pairs using the Mann-Whitney U test as post hoc analysis. After these comparisons, we determined that there was a statistically significant difference between the Intact control group and the Sericin group ($p = 0.002$) and between the Sericin group and the Sham operated group ($p = 0.003$) and the Placebo group ($p = 0.008$). At the end of the tenth day, the Sericin group had more hair roots than the Sham operated group and the Placebo group.

Number of degenerated hair roots

When the samples taken were examined in terms of the number of degenerative hair roots, we observed that there was significant difference among the 4 groups ($KW\chi^2$, $p = 0.001$) (Table 1). There was no hair root degeneration in the Intact control group, but there was a statistically significant difference between the

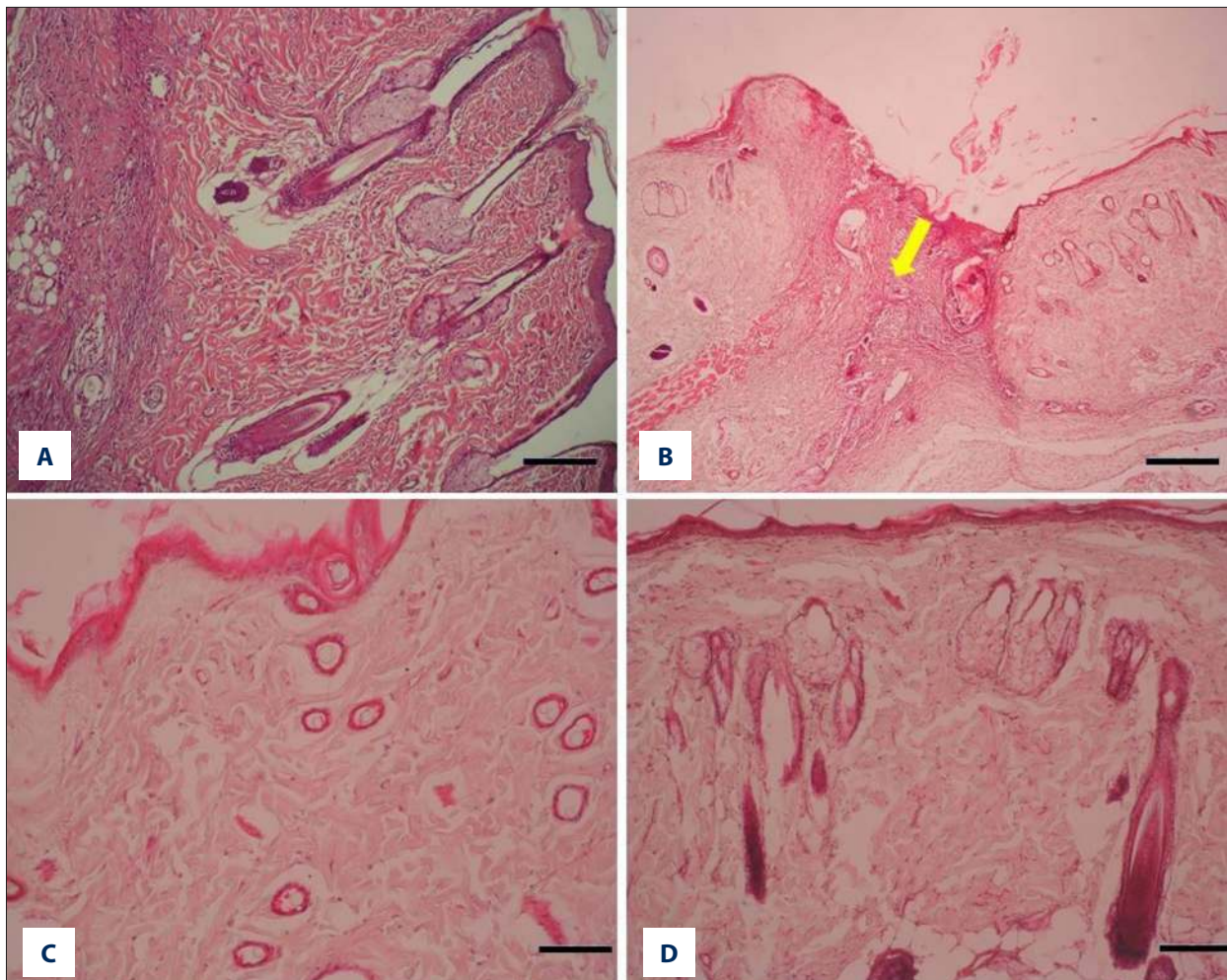


Figure 3. Light microscopic images of dorsal skin flap: (A) Intact control group, (B) Sham operated group, (C) Placebo group, (D) Sericin group. H&E staining, original magnification $\times 20$, scale bar=250 μm . Yellow arrow shows location of incision line.

Sericin group and the Sham operated group ($p=0.002$) and the Placebo group ($p=0.002$) after post hoc analyses (Mann-Whitney U test). On the tenth day, there were less degenerated hair roots in the Sericin group than in the Sham operated group and the Placebo group (Table 1).

Number of vessels

When the sections were examined according to the number of the vessels, we found a significant difference among all the groups in terms of the total number of the vessels ($KW\chi^2$, $p<0.0001$) (Table 1). The groups were compared as pairs by using the Mann-Whitney U test as post hoc analysis, showing a statistically significant difference between the Intact control group and the Sericin group ($p=0.002$), and between the Sericin group and the Sham operated group ($p=0.002$) and the Placebo group ($p=0.002$). There were more intact vessels in the Sericin group than the Sham operated group and the Placebo group (Table 1).

Epidermal thickness

When the samples taken from the study groups were compared in terms of the epidermal thickness values, there was a statistically significant difference among all the groups ($KW\chi^2$, $p<0.0001$) (Table 2). The groups were compared in pairs by using the Mann-Whitney U test as post hoc analysis. It was determined after the comparisons that there was a statistically significant difference between the Intact control group and the Sericin group ($p=0.004$), and the between the Sericin group and the Sham operated group ($p=0.002$) and the Placebo group ($p=0.002$). At the end of the tenth day, the Sericin group had more epidermal thickness than the Sham operated group and the Placebo group.

Percentage of the necrotic tissue

Statistically significant differences were determined between the Sham operated group, the Placebo group, and the Sericin group in terms of necrotic tissue percentage in the section

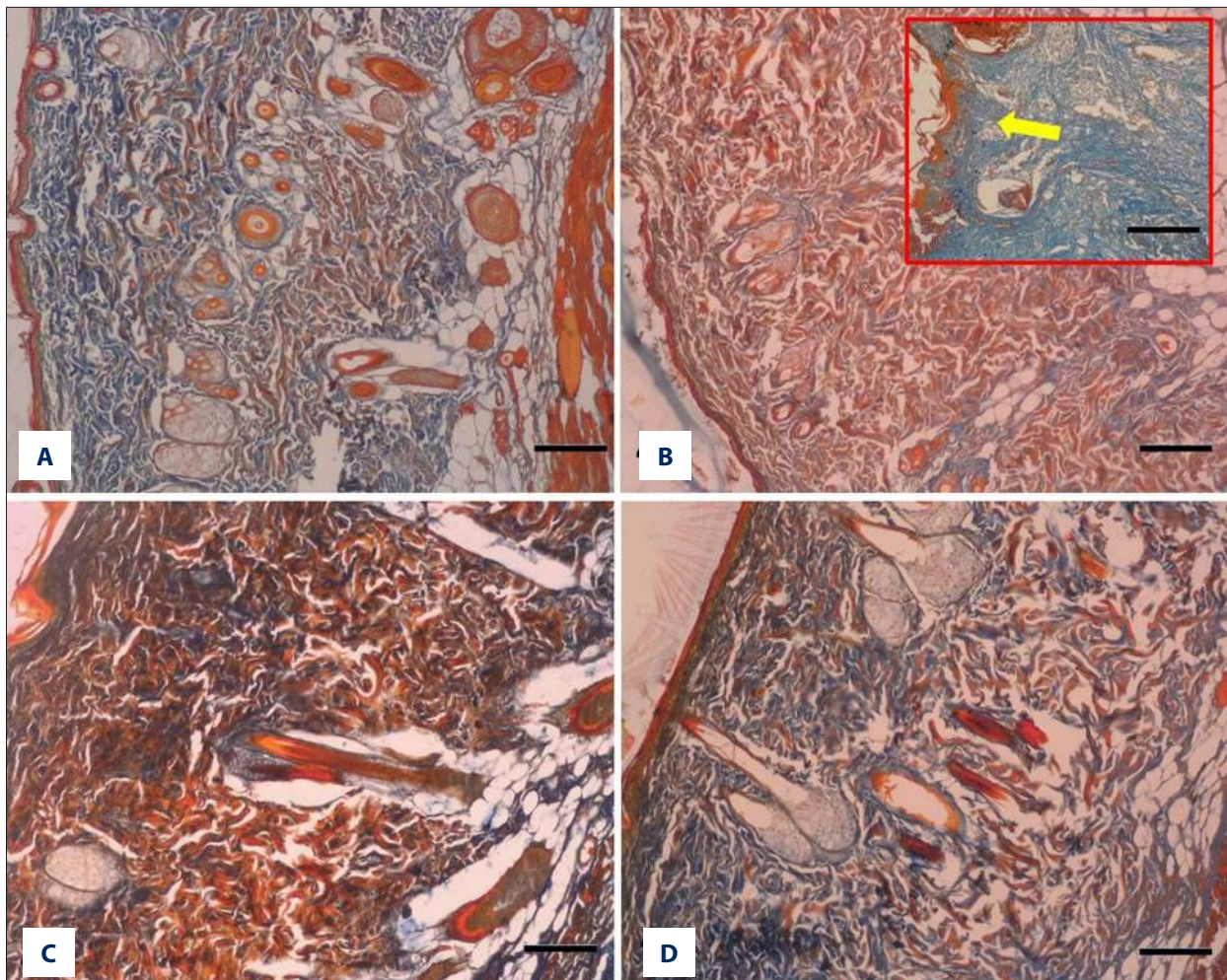


Figure 4. Mallory Azan staining of the sections images of dorsal skin flap: (A) Intact control group, (B) Sham operated group, (C) Placebo group, (D) Sericin group. Original magnification $\times 20$, scale bar=250 μm . Small picture magnification $\times 10$, scale bar=500 μm . Yellow arrow shows location of incision line.

areas ($KW\chi^2$, $p < 0.0001$) (Table 2). No necrosis was observed in the Intact control group because there were no incisions. The groups were compared as pairs by using the Mann-Whitney U test as post hoc analysis, showing that the necrotic tissue percentage in the Sericin group was lower than in the Placebo group ($p = 0.002$) and the Sham operated group ($p = 0.002$). At the end of the tenth day, the sericin application limited the formation of necrotic tissue in the sections.

Cellular infiltration

There were significant differences among the study groups in terms of cellular infiltration scores. The infiltration scores in the Intact control group were 0–1, the infiltration scores in the Sericin group were 1–2, the scores in the Sham operated group were 3–5, and the scores in the Placebo group were 3–4. We found less cellular infiltration in the Sericin group than in the Placebo group and the Sham operated group (Table 3).

Degree of edema

The edema was graded in all groups. The edema scores according to group are given in Table 4. When the study groups were compared in terms of edema grades, the scores were 0–1 in the Intact control group; 0–3 in the Sericin group (but mostly 1–2), and the edema scores in the Sham operated group and the Placebo group were distributed in a balanced manner at higher scores (4–5). The edema scores were distributed at lower levels in the Sericin group than in the Sham operated group and the Placebo group (Table 4).

Collagen discoloration

Collagen discoloration observed in the tissues in all study groups was scored and the results are given in Table 5. Scores were 0–1 in the Intact control group and 1–2 in the Sericin group, but in the Sham operated group and the Placebo group

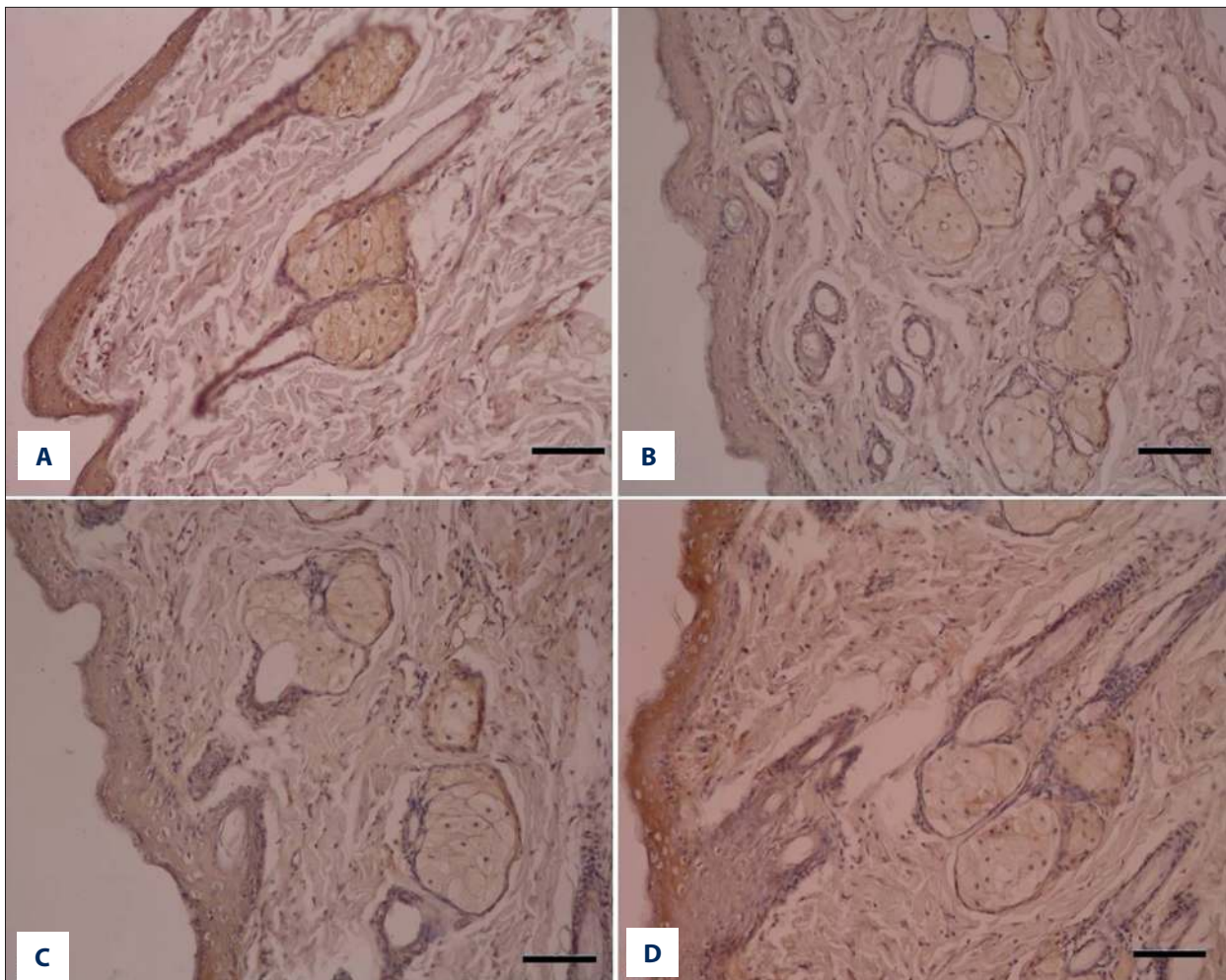


Figure 5. Anti-iNOS staining of the sections images of dorsal skin flap: (A) Intact control group, (B) Sham operated group, (C) Placebo group, (D) Sericin group. Original magnification $\times 20$, scale bar=250 μm

the scores were mostly 3–4. The collagen discoloration scores in the Sericin group were lower than in the Sham operated group and the Placebo group.

Immunohistochemical results

Anti-iNOS staining

Anti-iNOS staining in the Control group showed immunoreaction in sebaceous glands, hair follicles, and epidermis. While there was intense immune-positivity in the Intact control group after the incision wound, there was less immune-positivity in the Sham operated group and the Placebo group. The Sericin group showed an immunohistochemical reaction similar to that in the Control group (Figure 5).

Anti-desmin staining

It was observed in the control group that the muscle that is immune-positive in anti-Desmin staining *musculus erector pili* was located in the hair roots in longitudinal form. It was determined that these muscles were placed in a short and flat manner in the Placebo group, and had degenerative findings in parts. In the Sericin group, on the other hand, it was observed that this structure was similar to that of the Control group, and that it was long and the muscle structure was protected (Figure 6).

Biochemical results

At the end of the biochemical analyses, the oxidative stress markers and the antioxidant enzyme levels were determined. According to these analyses, the MDA (malonyldialdehyde), which appears after lipid peroxidation and which is an oxidative stress marker, was determined as being significantly lower in the Sericin group than in the Placebo and Sham operated group (Table 6; $p < 0,001$).

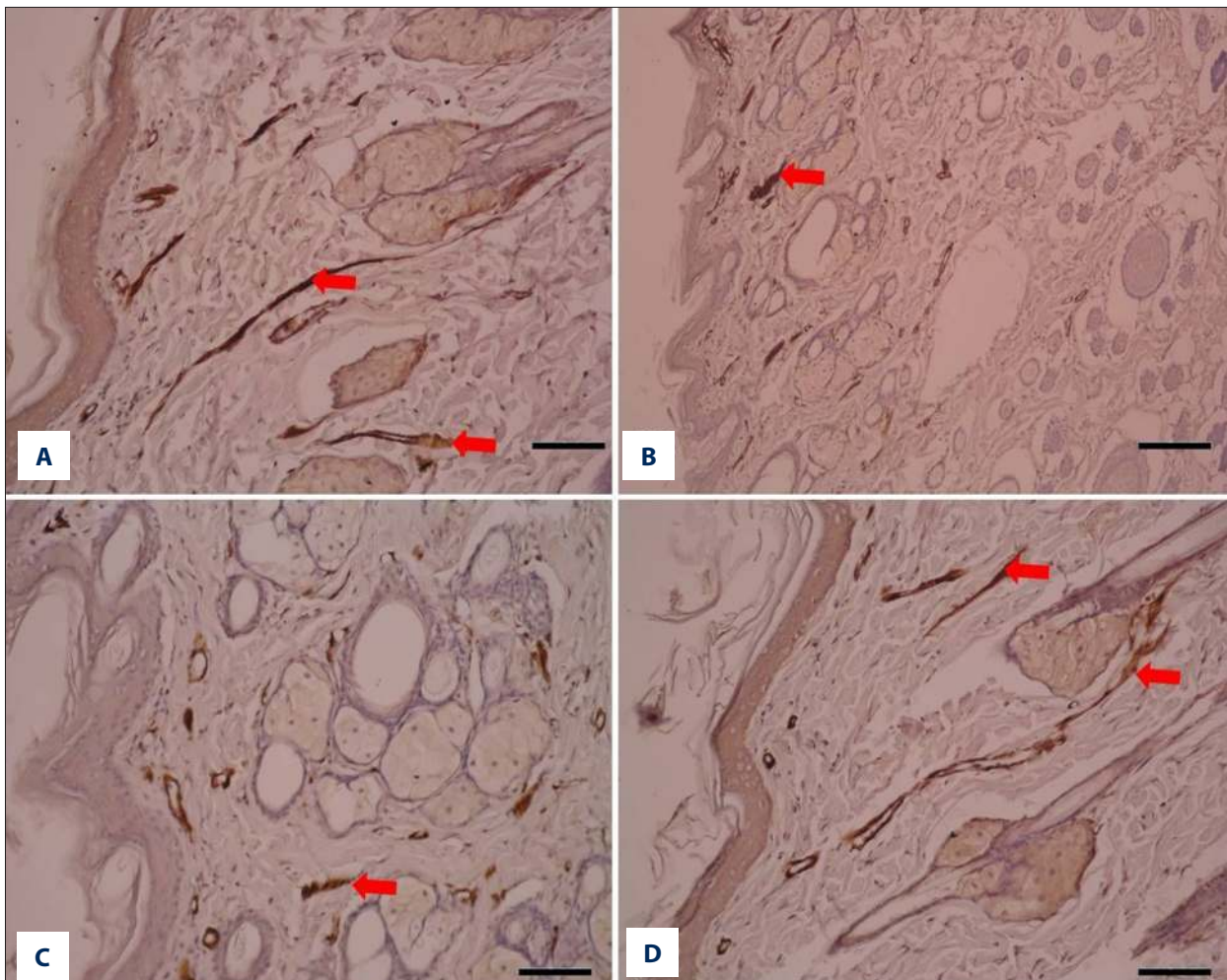


Figure 6. Anti-desmin staining of the sections of dorsal skin flap: (A) Intact control group, (B) Sham operated group, (C) Placebo group, (D) Sericin group. Original magnification $\times 20$, scale bar=250 μm . Red arrows show *M. arrector pillia*.

GPx (Glutathione Peroxidase), SOD (Superoxide Dismutase), and catalase, which are important antioxidant defense enzymes, were significantly higher in the Sericin group than in the Sham operated group and the Placebo group ($p < 0.001$).

Discussion

Wound healing is a natural response to tissue damage. The healing process includes cellular activity that forms a complex cascade. After this activity, the resurfacing, reconstruction, and the restoration of the wound tensile power is ensured [34]. Limitation of keloid formation is important as well as the development of wound tensile strength [35]. Healing of incision wounds without leaving scars is the goal of medical and surgical practices. The basic aim in wound healing is the restoration of the connective tissue and fast wound closure. The desired outcomes in this process are minimal pain, minimal discomfort, and minimal scar formation [7]. Various

medications and medical dressing materials have been developed and used to accelerate wound healing and optimize the healing process [2,4,6,7,10,13,36].

Bombyx mori synthesizes silk that consists of 2 types of proteins: fibroin and sericin. Fibroin has a fibril structure and accounts for 70% of the structure of pure silk. Sericin, on the other hand, is a sticky water-soluble protein and surrounds and connects the fibroin fibers [37]. Fibroin is used in production of raw textiles and in some bio-material applications. On the other hand, sericin is regarded as waste material in the textile industry [29]. It has been demonstrated in recent years that sericin has proliferative effects on some cell membranes, and also has various other biological activities. In previous *in vivo* and *in vitro* studies, sericin has been used in gauze bandages or medical dressings and was applied as ointment [19–22,38–40].

Sericin increases the attachment and proliferation of skin fibroblasts, osteoblasts, and keratinocytes [19,41–43], and clearly

Table 1. Intergroup comparison of biopsy samples for total and degenerated hair roots and number of vessels.

	Group	n	Median	Min	Max	KW χ^2 *	p
Number of total hair roots	Intact control**	7	43	38	46	21.993	<0.0001
	Sham**	7	24	19	29		
	Placebo**	7	28	20	30		
	Sericin**	7	33	29	36		
Number of degenerated hair roots	Intact control	7	–	–	–	13.775	0.001
	Sham**	7	24	19	19		
	Placebo**	7	24	19	31		
	Sericin**	7	9	8	12		
Number of vessels	Intact control**	7	40	37	45	23.227	<0.0001
	Sham**	7	9	6	10		
	Placebo**	7	9	7	12		
	Sericin**	7	23	19	30		

* Kruskal Wallis Chi Square; ** Post-Hoc p<0.05.

Table 2. Intergroup comparison of biopsy samples for epidermal thickness and necrotic tissue percentage.

	Group	n	Mean	Standart deviation	Min	Max	KW χ^2 *	p
Epidermal thickness (μ m)	Control**	7	39.97	1.18	37.94	41.68	22.440	<0.0001
	Sham**	7	27.52	1.44	25.79	29.64		
	Placebo**	7	27.82	1.95	24.64	29.88		
	Sericin**	7	36.11	1.96	33.65	38.99		
Necrotic tissue percentage (%)	Control	7	–	–	–	–	13.471	<0.0001
	Sham**	7	31.77	1.95	31.77	29.45		
	Placebo**	7	31.47	1.55	31.77	29.45		
	Sericin**	7	7.90	1.11	8.34	6.57		

* Kruskal Wallis Chi Square; **Post-Hoc p <0.05.

increases collagen production and epithelization [16,44,45]. In addition, sericin can be used an antioxidant, antibacterial, coagulant, and moisturizer [18,44,46–48].

A recent study determined that silver sulfadiazine preparations containing sericin increased the collagen production in burn patients and decreased the pain [38]. Aramwith et al. investigated the use of 8% sericin formulation in empirical incisional wound models with a different empirical method and reported decreased inflammation in the wound area and reduced wound

size [16]. There were no ulcerations, and, as the amount of collagen was increased, wound healing was accelerated [16].

The present study, which used a wound healing model formed with a dorsal graft, showed that sericin was superior to the placebo in all criteria. Hair root degeneration was prevented in rats treated with sericin, and epidermal thickness and number of vessels, which is an important finding of wound healing, was significant higher than in the Placebo group and Sham operated group. Epidermal thickness provides clues about scar formation. If epidermal thickness is less, the chance of scar formation

Table 3. The scores for the degree of cellular infiltration.

Groups	Cellular infiltration score						Total
	0	1	2	3	4	5	
Intact control	3	4	0	0	0	0	7
Sham operated	0	0	0	2	3	2	7
Placebo	0	0	0	2	5	0	7
Sericin	0	4	3	0	0	0	7
Total	3	8	3	4	8	2	28

A score of 0 = ≤ 3 inflammatory cells, 1 = 3–10 inflammatory cells, 2 = 11–20 inflammatory cells, 3 = 21–30 inflammatory cells, 4 = 31–40 inflammatory cells, and 5 = ≥ 41 inflammatory cells.

Table 4. The scores for the degree of edema.

Groups	Score for degree of edema						Total
	0	1	2	3	4	5	
Intact control	3	4	0	0	0	0	7
Sham operated	0	0	0	1	3	3	7
Placebo	0	0	0	1	3	3	7
Sericin	1	3	2	1	0	0	7
Total	4	7	2	3	6	6	28

A score of 0 = normal; 1 = mild increase; 2 = mild to moderate increase; 3 = moderate increase; 4 = moderate to marked increase; 5 = marked increase.

Table 5. The scores for the degree of collagen discoloration.

Groups	Scores for collagen discoloration						Total
	0	1	2	3	4	5	
Intact control	3	4	0	0	0	0	7
Sham operated	0	0	0	2	4	1	7
Placebo	0	0	0	3	4	0	7
Sericin	1	3	3	0	0	0	7
Total	4	7	3	5	8	1	28

A score of 0 = normal; 1 = mild increase; 2 = mild to moderate increase; 3 = moderate increase; 4 = moderate to marked increase; 5 = marked increase.

is greater [2]. We found that epidermal thickness was clearly greater in rats treated with sericin. The optimization of collagen synthesis as a result of fibroblast activity [40] increased by sericin might have accelerated the epidermal healing process.

The necrotic tissue area percentage around the incision areas in Sericin group rats was significantly lower compared with the Placebo group and the Sham operated group, suggesting that

sericin limits necrosis, perhaps by limiting lipid peroxidation in the tissues. MDA, a marker of oxidative stress, was significantly lower in the Sericin group. The enzymes GPx, SOD, and catalase help the body deal with oxidative stress and were detected at much higher levels in the Sericin group. Depending on the decrease in oxidative stress in wound healing studies, there is a direct relationship between wound healing and prevention of scar tissue formation [2,13,16,19–22,49,50].

Table 6. Malonyldialdehyde (MDA), glutathion peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) activities in the wound area of the experimental groups.

	Sham operated group	Placebo group	Sericin group
MDA (nmol/mg)	0.255±9.3	0.248±8.9	0.177±10.2*.#
GPx (mU/ml)	7.6436±12.3	7.772±11.5	11.9708±8.3*.#
SOD (U/ml)	8.029±11.2	8.105±10.4	9.715±8.9*.#
Catalase (U/ml)	0.3346±10.7	0.3567±12.7	0.7832±10.6*.#

* p<0.001, when compared to sham-operated group; # p<0.001, when compared to placebo group. GPx – glutathion peroxidase; MDA – malonyldialdehyde; SOD – superoxid dismutase.

After incision injuries, additional tissue damage and edema are caused by disruption of vascular integrity, initial neutrophil infiltration to tissue in the inflammation phase, and cells in this area producing protease and reactive oxygen species (ROS) to eliminate infection and cellular debris in the wound area [3]. When the 4 study groups were evaluated in terms of edema and cellular infiltration, the lowest edema and cellular infiltration scores were found in the Sericin group. Another secondary activity of macrophages is the clearing of apoptotic cells (mainly neutrophils) and ensuring the resolution of inflammation [3]. Previous studies reported that sericin increases the activity and number of macrophages [40]. The observed decrease in edema might have been contributed to by the limitation of cellular infiltration by sericin due to its antioxidant effects and ROS scavenging activity [19,22]; the limitation of effects of mediators like plasma-activated C3a and C5a; transforming growth factors β and α , and PDGF [51], which cause infiltration; and the sequestration by the increased number of macrophages.

One of the important conditions for wound healing is sufficient circulation to correct tissue ischemia [2]. Angio-neogenesis increases oxygen levels and promotes collagen synthesis and cell proliferation [52]. When we consider the results of the study, the positive effects of sericin are clearly observed in terms of vascularization. Vascularization was at a much higher level in sections obtained from rats treated with sericin compared with the other groups. The histopathological examination in this study explored the effects of sericin on wound healing by applying a dorsal skin flap model directly to the incision area, showing that the sections of the Sericin group were similar to the Intact control group in terms of collagen distribution, scar formation, and collagen discoloration. These results reveal the positive effects of sericin on wound healing.

The collagen discoloration, which is an important parameter of wound healing, was limited in the Sericin group, and did not reach the deeper areas of the dermis from the epidermal layer. Collagen discoloration was clearly increased in the Sham operated group and the Placebo groups, which did not receive

sericin. In addition, Mallory-Azan staining (Figure 4) showed that the collagen was in a more effective form and in a structure that supported connective tissue formation. Motta et al. revealed that collagen led to properly modulated collagen lysis and prepared the way for healthier fibroblast proliferation [53]. The optimization of collagen use in the Sericin group might be explained by increased collagen synthesis [40]. As a last item, the decrease observed in the Sericin group in terms of collagen discoloration might be caused by increased scavenger activity [22]. It was used more effectively in connective tissue formation (Figure 4) because of the collagen-optimized fibroblast activity [53], and an image supporting the connective tissue formation emerged.

Nitric oxide (NO) has a role in wound healing similar to that of the conductor of an orchestra. It has been shown in *in vitro* [54] and *in vivo* studies that it influences macrophages, fibroblasts, and keratinocytes, and ensures coordination among cells during wound healing [55]. NO is an important cellular signaling protein which has a role in many vital processes [56]. NO is synthesized in 3 different forms by nitric oxide synthetase from the amino acid L-arginine [57]. Many cells can synthesize NO with cellular endothelial or inducible nitric oxide synthase (iNOS) enzymes in wound healing [58,59]. Zhao et al. showed that iNOS may play crucial roles in all wound healing processes [60].

It has been revealed in many studies that the decrease in NO synthesis decreases collagen deposition and increases wound fragility [61–63]. The effects of NO on keratinocytes were tested in photo-damaged skin, in different models formed in skin incisions, and in burn injuries, showing that decreased NOS activity led to decrease in epithelium proliferation in the wound types in all models [55].

Sericin might have directed the function of collagen in wound healing with its antioxidant activity and with the increase in NO levels [57,60]. Using anti-iNOS staining, we found that the i-NOS activity in the tissue occurs in epidermis and *glandula sebaceas*.

While the anti-iNOS expression in the Intact control group and the Placebo groups was limited, in the Sericin group we found that the i-NOS activity in tissues was increased. The high NO levels detected in tissues show the positive effect of decreasing oxidative stress in wound healing [60].

Desmin, vimentin, and vimentin were expressed in *arrector pili* and smooth muscles. Desmin is a key sub-unit in the intermediate filaments of the cardiac, skeleton, and smooth muscles, and is a muscle-specific protein [64]. The *arrector pili* muscles are small muscles that are connected to hair follicles in mammals and are responsible for piloerection [65]. These muscles and sebaceous glands (follicular unit structures) are important markers in healthy skin structure [66]. For this reason, evaluation of these muscles in skin biopsies is a strong information source. Desmin *arrector pili* is a marker used in showing the muscle damage [67]. Clarkson and Tremblay used immune expressions of desmin for measurement of muscle damage [68].

We found more intact hair roots in and around the incision area in the Sericin group together with anti-desmin staining. The Sericin group had more hair roots than the other incision groups. In addition, we observed in the histological examination that there were more hair roots in the Sericin group. We found that the condition of the *m. erector pili* located near the hair roots was due to the protection against oxidative stress after sericin application. Therefore, the condition of *m. erector pili* in the Sericin group was closer to that of the Control group than the other incision groups.

Free radicals and antioxidants negatively influence wound healing by triggering oxidative stress. This ROS elimination may be a critical point in wound healing [69]. There are some studies in the literature showing the antioxidant effects of sericin [21,22,49]. Sericin exhibits high ROS-scavenging activity [20,22].

MDA, which appears due to lipid peroxidation, is a very harmful substance that influences the ion change in the cell membranes and disrupts the permeability, gains a mutagenic character by having a reaction with DNA bases, and blocks cellular metabolism, leading to cytotoxicity [50,67]. In the present study, the MDA levels were found to be significantly lower higher in the Placebo group and the Sham operated group. Our results agree with the results reported by Dash et al. [19].

Different defense mechanisms act in preventing lipid peroxidation. These mechanisms are the structures that decrease the activity of free oxygen radicals or limit the reactions of the structures like hydrogen peroxide or hydro-peroxides, and are called antioxidants [70]. An important part of the antioxidant defense consists of special enzymes clearing O_2^- and H_2O_2 . These are the superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate

dehydrogenase enzymes [71]. The enzymes whose levels were determined in the present study – glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) – are the antioxidant enzymes. While glutathione shows its antioxidant activity by joining to the free radicals in the medium and by preventing the oxidative damage of the cell, it also shows its activity by holding the sulfhydryl groups in the proteins at a reduced status, and by preventing inactivation that may appear in the proteins and enzymes [72]. In our study, the Sericin group had higher GPx levels. SOD is the enzyme that provides the first defense in oxygen toxicity against the production of free oxygen radicals and catalyzes the conversion of O_2 to H_2O_2 [73]. This enzyme level was significantly higher in the Sericin group. As the last item, the catalase levels were also clearly higher in the Sericin group.

All of these biochemical tests show that sericin limits the effects of oxidative stress at the cellular level. Although epidermal thickness, controlled collagen use, and vascularization are important factors in wound healing, sericin can prevent oxidative stress and affect wound healing in a positive way [19,22,40]. However, the main positive effect of sericin in wound healing may come from its potent anti-oxidative characteristics [40].

Limitations

Our study focused on short-term wound healing effects of sericin, which overlaps with the proliferation phase. However, to evaluate the long-term effects of sericin on wound healing, other parameters need to be investigated, such as scar formation, decrease in infection rates, keloid formation ratio, skin integrity, and tensile strength of the wound. Further studies are needed to investigate parameters involved in the remodeling phase of wound healing.

Conclusions

In the Sericin group we found increased epidermal thickness and vascularization and decreased necrosis, hair root degeneration, cellular infiltration, and collagen discoloration. Histopathological analysis showed that the Sericin group had values similar to those obtained in the Control group in terms of collagen distribution, discoloration, and scar formation in wound healing.

In addition to these results, the clearly lower levels of oxidative stress markers in the Sericin group emphasize the potent antioxidant characteristics of sericin and provide strong proof that sericin may be useful as an agent to optimize wound healing via its anti-oxidative effect. Our study of the effects of sericin on wound healing used histopathological and biochemical methods, and showed that sericin has positive effects in

the wound healing process. These positive effects of sericin in wound healing must be evaluated in human clinical studies.

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Conflicting interests

The authors declare no conflicting interests.

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