ORIGINAL ARTICLE

The effects of topical collagen treatment on wound breaking strength and scar cosmesis in rats

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H Sinno, M Malhotra, J Lutfy, et al. The effects of topical collagen treatment on wound breaking strength and scar cosmesis in rats. Can J Plast Surg 2012;20(3):181-185.

BACKGROUND: Topical application of collagen has been suggested to enhance wound healing; however, its long-term effect on wounds has not been studied in a rat model.
HYPOTHESIS: Topical application of collagen type I will not facilitate incision healing or cosmesis in rats up to 28 days postwounding.
METHODS: The effects of bovine collagen type I (6 mg/mL) on the rat surgical paired skin incision model were examined. Each rat served as its own control in which topical collagen was applied to one incision while normal saline (0.9%) was applied to the other incision. Rats were euthanized three (n=6), seven (n=6) and 28 (n=5) days after wounding. Tissue harvested from each time point was examined for maximal breaking strength, and for biochemical and histological analysis.
RESULTS: There were no statistically significant differences (ie, P<0.05) in maximum wound breaking strength between the collagen- and saline-treated wounds at all time points. Histological analysis revealed a similar infiltration of inflammatory cells and fibroblasts in the wound edges of all incisions when matched with time of wounding. Western blot analysis revealed no differences in fibronectin or collagen I content in all wounds in each rat.
CONCLUSIONS: The topical application of collagen did not facilitate wound healing from three to 28 days in the rat wound model.

Key Words: *Collagen; Scar formation; Topical application; Wound healing*

Les effets d'un traitement au collagène topique sur la résistance des plaies à la rupture et sur l'esthétique des cicatrices chez des rats

HISTORIQUE : L'application topique de collagène améliorerait la guérison des plaies. Cependant, on n'a pas étudié son effet à long terme sur les plaies d'un modèle de rat.
HYPOTHÈSE : L'application topique de collagène de type I ne facilitera pas la cicatrisation ou l'esthétique d'une incision chez les rats jusqu'à 28 jours après la formation de plaies.
MÉTHODOLOGIE : Les auteurs ont examiné les effets du collagène bovin de type I (6 mg/mL) sur un modèle de double incision cutanée chez des rats. Chaque rat était son propre sujet témoin, car du collagène topique était appliqué sur une incision, et une solution physiologique normale (0,9 %), sur l'autre. Les rats étaient euthanasiés trois (n=6), sept (n=6) et 28 (n=5) jours après la création des plaies. Les auteurs ont prélevé des tissus à chacun de ces moments et les ont examinés pour établir leur résistance maximale à la rupture et pour procéder à une analyse biochimique et histologique.
RÉSULTATS : Il n'y a pas de différence statistiquement significative (c'est-à-dire P<0,05) quant à la résistance maximale à la rupture des plaies traitées au collagène et de celles traitées au moyen d'une solution physiologique à l'un de ces trois moments. L'analyse histologique a révélé une infiltration similaire des cellules inflammatoires et des fibroblastes dans les lèvres de la plaie de chacune des incisions par rapport au moment de création de la plaie. Le transfert de Western n'a révélé aucune différence dans le contenu en fibronectine ou en collagène I des plaies de chaque rat.
CONCLUSIONS : L'application topique de collagène ne facilite pas la cicatrisation des plaies au bout de trois à 28 jours dans un modèle de plaies chez les rats.

Collagen is the most abundant protein in the body, and is responsible for skin strength and is a key player in tissue healing. Various applications of collagen, such as a filler for cosmetic enhancement of skin wrinkles and regeneration of youthful appearance, have been proposed. When used appropriately, collagen has been shown to be safe and reliable.

There is suggestive evidence that the topical application of collagen enhances wound healing in human and animal models (1). Glasgold et al (1) reported subjective enhancement of wound closure and cosmesis in an uncontrolled clinical experience involving 12 patients who underwent Mohs micrographic surgery. They correlated their finding with studies performed in guinea pigs showing rapid infiltration of fibrous tissue and endothelial cells. In contrast, Becker et al (2) prospectively examined 39 patients and found no evidence of enhanced wound closure or final cosmetic results with the topical use of bovine collagen in facial wounds. Importantly, the results of these studies were based on subjective outcomes and were not blinded or randomized.

In addition to its cosmetic uses, collagen has also been shown to be useful as a vehicle for the administration of growth factors to wounds in animal models. A purified and hypoallergenic viscous suspension of collagen has been shown to facilitate a slow release mechanism of dissolved growth factors without itself interfering with early wound healing in rats (3). Bovine collagen type I was further studied in an oral surgery application in dogs. Al-Kateeb et al (4) found that collagen-treated wounds demonstrated an improved rate of early wound healing and had a topical hemostatic effect at the time of application, with no adverse reactions to the collagen treatment, consistent with the literature. Pharmacological effects of collagen on wound healing, including a hemostatic effect, interaction with platelets and fibronectin, and effects on fluid exudates, have been suggested (5-8).

The hypoallergenic and safe properties of collagen, and its potential use as a vehicle for topical application to wounds, are very attractive for experimental and future clinical applications. There is, however, controversy regarding the long-term effects of collagen on wound healing and cosmesis. The present study aimed to determine the effects of collagen on early and late wound healing in the rat incisional skin model.

METHODS

Materials

Type I collagen (PureCol, Inamed BioMaterials, USA) was purchased at concentrations of 6 mg/mL. Normal saline solution (0.9% NaCl, 300 mOsm/L) was prepared in the laboratory using double-distilled water and other laboratory chemicals and surgical equipment (Fisher Scientific, Canada).

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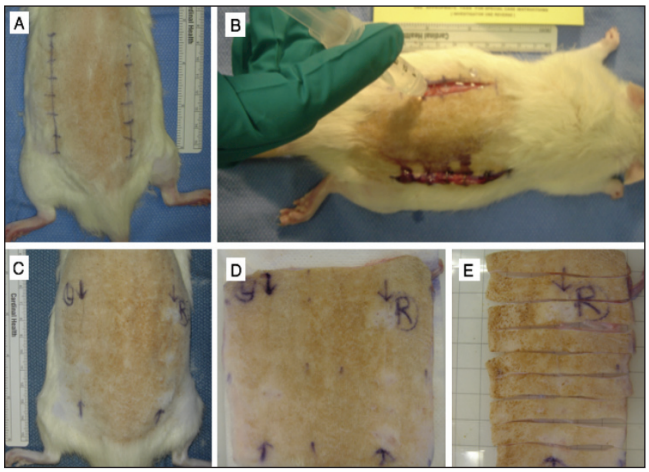

Figure 1) A *Operative image of the paired dorsal incisional rat model. This model has been shown to be a reliable and reproducible method for studying wound healing in rats.* **B** *Intraoperative image of topical application of the saline or collagen solution on the open incisional wound. The contralateral wound was closed with five equidistant cutaneous staples.* **C** *Image of the healed dorsal wounds after 28 days. There are no obvious subjective aesthetic differences between the morphology and characteristics of the two scars (scars can be seen as thin white lines on the dorsum of the rat between the blue arrows drawn on the skin).* **D** *The skin has been removed from the rat body and laid flat in preparation for biochemical, histological and mechanical analysis.* **E** *The skin was cut precisely into six 10 mm strips, as shown by the 10 mm grid on which the skin is placed*

Animal study

All procedures were performed in accordance with guidelines approved by the McGill University Animal Ethics Committee and Veterinary Care Services (Montreal, Quebec). Before surgery, adult male Sprague-Dawley rats (Charles River, Canada) weighing 300 g to 350 g were housed for one week for acclimatization in separate clean cages and fed water and standard rat chow ad libitum. Before performing the surgical procedure, the rats were anesthetized with isoflurane gas (4% to 5% for induction, 1% to 2% for maintenance) and subcutaneous injection of carbofen (5 mg/kg). The surgical protocol was adapted and performed as described by Mustoe et al (3). Briefly, the dorsum was shaved with an electric hair clipper and disinfected with 70% alcohol. Two full-thickness, 6 cm linear skin incisions were made in the median plane (2 cm on either side of the midline) beginning 1 cm below the inferior edge of the scapula using sterile No. 10 surgical scalpel blades. One wound received a single topical application of control solution (0.9% normal saline) and the other wound received the same volume of the experimental solution (ie, bovine type I collagen). This enabled each rat to serve as its own control. The incisions were reapproximated with five equidistant surgical clips and the rats were monitored under heat lamp for 1 h postoperatively. Groups of animals were euthanized at three, seven and 28 days. The central 3 cm of the wounds were harvested for tensometry measurements. The remaining wounds were prepared for histological analysis and western blotting. Representative in vivo images of surgical markings, drug application, final scar cosmesis and skin preparation for tensometry, western blotting and histological analysis are presented in Figures 1A to 1E.

Cosmetic analysis

All wounds and scars were photographed preoperatively, intraoperatively, during recovery and at days 3, 7 and 28. The photographs were acquired using a digital camera from a height of 10 cm from the skin surface. A blinded assessment of the cosmetic appearance of the scars (raised borders, colour, width and general appearance) was tabulated for all scars at all time points using a grading scheme from 1 to 3.

Blood analysis

Blood samples were drawn from the right saphenous vein during the recovery period, on the day of wounding and just before euthanization. A complete blood count (CBC) and differential of the cell count was performed at different time points. Serum inflammatory markers (C-reative protein [CRP], complement [C] 3 and C4) were analyzed at all time points.

Tensometry

The maximum wound breaking strength (MWBS) was calculated from tensometer (Tensometer 10, Monsanto Co, USA) measurements of three 10 mm strips excised from each wound. The 10 mm strips were precisely cut using a preformed instrument consisting of two microtome blades separated by a steel beam 10 mm wide. The skin strips were placed vertically between the clamps of the tensometer with the wound at the centre (2 cm from each jaw). A force was applied with a constant speed of 10 mm/s until rupture. The forces were plotted by computer software and the MWBS was measured as the greatest force attained before rupture of the wounds. Tensile strength is the force per unit of cross-sectional area. Because the cross-sectional area was made constant in all skin strips (10 mm wide, 40 mm jaw space and similar adjacent skin width), the MWBS was in direct proportion to tensile strength; these terms are used interchangeably in the present article.

Histological examination

The skin specimens excised from incisional wounds of the animals were fixed in 10% phosphate-buffered formalin, processed and embedded in paraffin. The specimens were cut at 5 µm intervals perpendicular to the long axis of the wound surface using a microtome (Olympus, USA). Hematoxylin and eosin staining was used to visualize the gross microscopic cellular architecture. A pathologist blinded to the treatments examined the slides for assessment of wound extent, inflammation and cellular infiltration. A grading scheme was used to quantify differences between the specimens: grade I – few fibroblast infiltrates; grade II – moderate fibroblast infiltration; and grade III – maximal fibroblast infiltration. The same grading scheme was used for extent of inflammation and fibrosis.

Western blotting

Rat skin harvested from incisional wounds was analyzed for protein expression by western blot. On harvesting the skin, the tissue was immediately flash frozen in liquid nitrogen and subsequently stored at –80°C. Tissue was homogenized in a RIPA buffer (20 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulphate, 10.0% glycerol, 10 mM $Na_2HPO_4 \cdot 7H_20$, 1% sodium deoxycholate), supplemented with protease inhibitor, vanadate and phenylmethylsulfonyl fluoride (Roche Diagnostics, Canada). Following centrifugation at 10,000 rpm for 10 min at 4°C, the supernatant was collected and protein concentration was determined using the Lowry protein assay (BioRad, USA). Cell lysates were subsequently diluted in 5× electrophoresis sample buffer and run on a sodium dodecyl sulphate polyacrylamide gel (7.5%) at 100 V. Proteins were then transferred to a nitrocellulose membrane (Whatman, USA) and the membrane was stained with Ponceau red (Sigma-Aldrich, Canada) to verify effective transfer of the protein. Nonspecific sites were blocked in 5% nonfat milk in TBST (Tris buffer saline, 1% Tween) for 1 h at room temperature and the membranes were incubated overnight at 4°C with anti-collagen I (1:2000) (Abcam, USA), antifibronectin (1:2000) and β-actin (1:1000) antibodies diluted in 5% milk. The membranes were then washed three times in TBST for 10 min per wash, incubated with an appropriate horseradish peroxidase-linked secondary antibody for 1 h at room temperature and washed three times in TBST. The membranes were subjected to chemiluminescence analysis using an ECL protein detection system (Amersham, USA) and developed on photographic film. Membranes were stripped using 0.2 M glycine (pH 2.8) for 30 min and reprobed with anti-β-actin (Santa Cruz, USA) antibody to demonstrate equal protein loading. The western blotting was performed in

TABLE 1
Effect of topical collagen on wound breaking strength

	Breaking strength, g		
Treatment	**3 days**	**7 days**	**28 days**
Collagen	490±57	673±53	3326±169
Saline	478±86	713±58	3440±230

Data presented as mean ± SD. There were no statistical differences between the collagen-treated wounds and the saline-treated wounds measured at days 3, 7 and 28. P<0.05 calculated using the paired t test comparing the mean maximal wound breaking strength in the same rat (ie, control side versus the experimental side)

triplicate using samples from different rats to confirm the consistency of the results and band density.

Bias elimination
Computer software available online was used to randomly assign the rats into groups on the day of surgery. Two symmetrical wounds were made on either side of the rat dorsum to enable each rat to serve as its own control. The sidedness of solution application was established randomly using online computer software. A laboratory technician prepared the solutions to apply on the wounds and labelled them with alphabetical designation corresponding to the randomized wound. The identity of the solutions (ie, control versus experimental) was kept blinded from the investigators until the end of the study.

Statistical analysis
A paired Student's *t* test was used to compare the means of experimental data between the same rats. P<0.05 was considered to be statistically significant. The data are expressed as the mean ± SE.

RESULTS

Cosmetic and tensometry analysis
The long-term effects of collagen on the rat skin incisional model were analyzed (Figure 1A and 1B). The blinded subjective assessment of the cosmetic appearance of the scars (raised borders, colour, width and general appearance) at all time points revealed no differences between the collagen-treated wounds compared with the saline-treated wounds (Figure 1C). Table 1 presents the values for MWBS. Values are presented in g and are proportionally representative of wound tensile strength. The topical application of collagen had no effect on MWBS compared with control saline-treated wounds at all time points (collagen side: day 3, 490±57 g; day 7, 673±53 g; and day 28, 3326±169 g versus saline side: day 3, 478±86 g; day 7, 713±58 g; and day 28, 3440±230 g) (Figure 2). There was a statistically significant increase (up to 49%) in MWBS from day 3 to day 7 (P<0.0001). The MWBS increased 395% to 482% from day 7 to day 28 (P<0.0001) for both saline-and collagen-treated groups. The largest increase in MWBS was seen between days 3 and 28 (578% to 619% [P<0.0001]).

Blood analysis
To assess the effect of topical application of collagen on systemic hematological and inflammatory markers, a CBC, and measurement of CRP, C3 and C4 levels was performed after the surgery and on the day of euthanization. Figure 3 illustrates the differences observed in the blood tests at different time points in all rats. Results indicated a general increase in CRP, C3 and C4 levels from day 0 to day 3 (day 0: CRP, 6.31±0.77 mg/L; C3 0.51±0.08 g/L, C4 0.122±0.01 g/L versus day 3: CRP, 8.28±0.28 mg/L; C3 0.62±0.03 g/L, C4 0.17±0.01 g/L; P<0.05, P=0.08 and P<0.01, respectively) to day 7 (CRP, 7.95±0.32 mg/L; C3 0.57±0.02 g/L, C4 0.18±0.02 g/L; P<0.05, P=0.22 and P=0.62, respectively). The inflammatory values appeared to revert back to preoperative values at day 28 (CRP, 1.49±0.88 mg/L; C3 0.49±0.03 g/L, C4 0.118±0.002 g/L; P<0.05, P<0.05 and P=0.71, respectively).

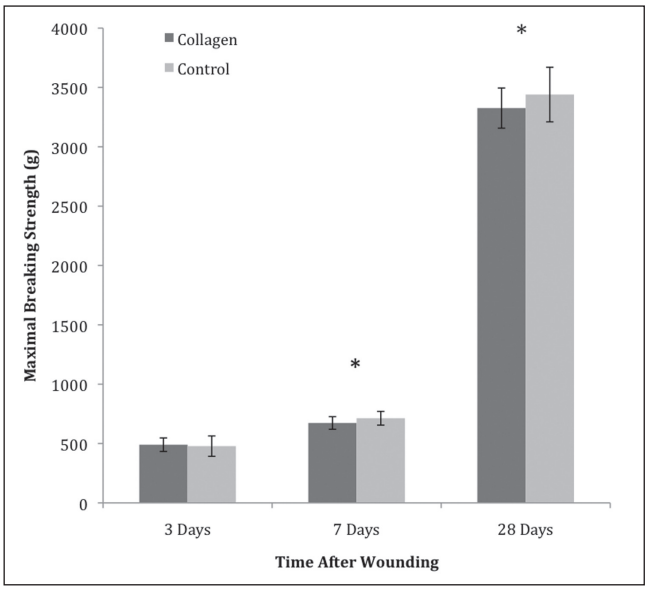

Figure 2) *Effects of topical collagen on wound strength. Dark grey bars represent the collagen-treated wounds and the light grey bars represent the saline-treated side (control) in the same rat. The maximum wound breaking strength was not statistically different between collagen-treated wounds and saline-treated wounds measured at days 3, 7 and 28. However, a significant increase was observed from day 3 to day 7, and from day 3 to day 28. *P<0.0001*

Values of the changes in the CBC and differential for all time points are presented in Table 2. There appeared to be significant increases in platelet count and monocyte count between day 0 and day 3. The hemoglobin level decreased over this time interval. When comparing day 0 with day 7, the hemoglobin level and platelet count appeared to increase, in contrast to the white blood cell count. At day 28, only the monocyte count was elevated. Neutrophil, lymphocyte and eosinophil counts were not different when compared between day 0 and days 3, 7 and 28.

Histological analysis
On histological analysis, there were no significant differences according to visual assessment of the extent of cellular infiltration, fibroblast deposition, collagen content and wound architecture in the collagen- and saline-treated wounds (Figure 4).

Western blot analysis
The fibroblast wound healing-associated proteins, collagen I and fibronectin, were chosen to biochemically identify differences in treatments. Western blotting of cell lysates with an antifibronectin antibody (1:2000) demonstrated that fibronectin levels were not different in wounds treated with collagen compared with saline (Figure 5 day 3 [left panel] and day 7 [right panel]). Using anticollagen I (Abcam, 1:2000), the middle bands showed no differences in collagen I content in wounds treated with collagen compared with saline. The lower panel shows Ponceau staining of the actin bands demonstrating that equal amounts of protein were loaded into each lane.

DISCUSSION

Normal wound healing is characterized by fibroblast deposition of collagen type I and III. However, in many patients, this process can be deleteriously altered, causing wound dehiscence, increased morbidity and death. An understanding of these steps and the ability to increase initial hemostasis, antibacterial properties and tensile strength of wounds can revolutionize the approach to chronic wounds, surgical incisions and traumatic lacerations.

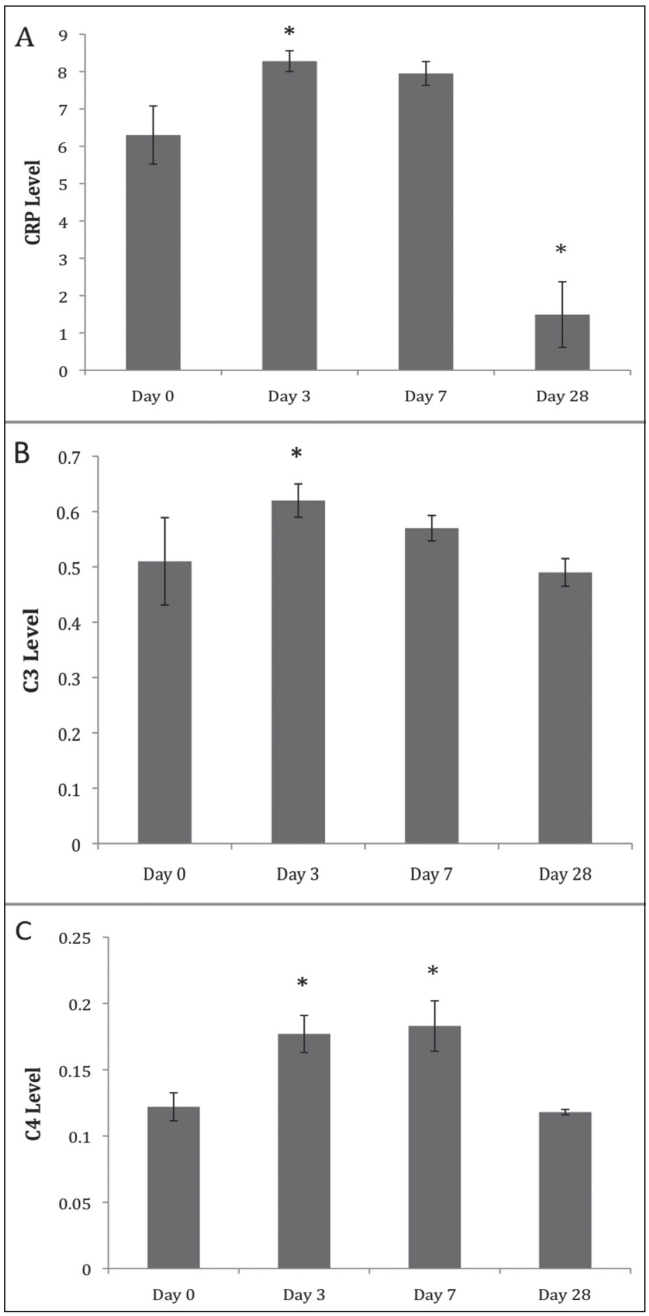

Figure 3) *Levels of C-reactive protein (CRP), complement (C) 3 and C4 after topical administration of collagen in rats. Serum inflammatory markers (CRP, C3, and C4) were measured on days 0, 3, 7 and 28 after the topical application of collagen to the wounds.* **A** *CRP levels comparing day 0 with days 3, 7 and 28.* **B** *C3 levels comparing day 0 with days 3, 7 and 28.* **C** *C4 levels comparing day 0 with days 3, 7 and 28. *P<0.05*

Some reports have suggested that the topical application of collagen to wounds may improve wound healing and, ultimately, cosmetic results. Furthermore, other experimental studies have used the viscous properties of collagen solution to be used as a vehicle for the topical application of growth factors to wounds. The long-term effect of collagen on wounds, however, has not been studied in the rat incisional model and, therefore, cannot be used for experimentation in long-term wound healing.

In our study, we showed that there are no significant increases in MWBS or cosmetic appearance in the collagen-treated wounds compared with the control saline-treated wounds. To minimize potential

TABLE 2
Complete blood count in the different groups

A	Day 0	Day 3	P
Hemoglobin, g/L	166±3.2	146±1.7	<0.01
Platelets, $\times 10^9$/L	610±117	1401±116	<0.01
Monocytes, %	0.076±0.04	0.488±0.14	<0.05
B	**Day 0**	**Day 7**	**P**
Hemoglobin, g/L	152±21	156±3.8	<0.05
WBC, $\times 10^9$/L	11.8±2	9.4±0.7	<0.05
Platelets, $\times 10^9$/L	530±125	1380±93	<0.01
C	**Day 0**	**Day 28**	**P**
Monocytes, %	0.074±0.32	0.718±0.14	<0.01

Data presented as mean ± SD unless otherwise indicated. **A** *White blood cell (WBC), neutrophil, eosinophil and lymphocyte counts did not change between day 0 and day 3. Hemoglobin level, and platelet and monocyte counts between day 0 and day 3, however, decreased.* **B** *Neutrophil, WBC, monocyte, eosinophil and lymphocyte counts did not change between day 0 and day 7. Hemoglobin level and platelet counts, however, increased.* **C** *Hemoglobin content, WBC, platelet, neutrophil, eosinophil and lymphocyte counts were not different when compared between day 0 and day 28; only monocyte count was significantly increased*

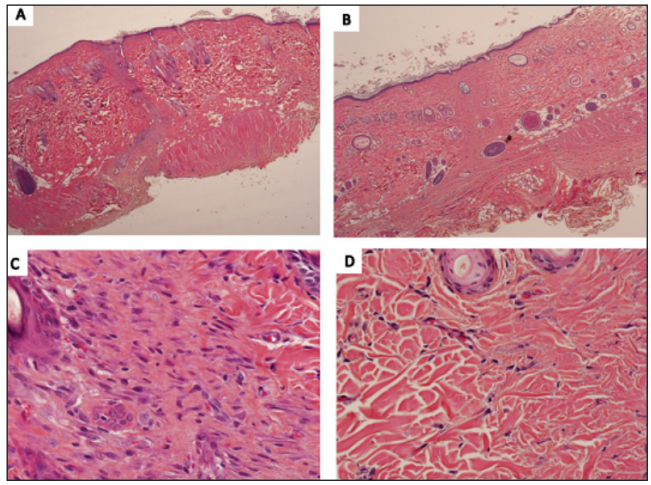

Figure 4) *Histological evaluation of wound healing and the effect of collagen (hematoxylin and eosin stain, blue colour indicates nuclear staining). Representative wound at day 3* **(A)** *and at day 28* **(B)** *(original magnification ×4). Representative wound at day 3* **(C)** *and at day 28* **(D)** *(original magnification ×40). No differences between collagen- (experimental) and saline- (control) treated wounds were seen at any time point.* **A** *and* **C** *are from the control-treated incisions, and* **B** *and* **D** *are from the experimental group of incisions*

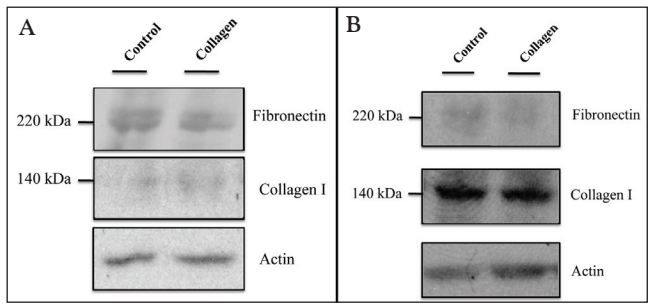

Figure 5) *Western blot demonstrating the effects of collagen on fibronectin and collagen type I postwounding.* **A** *Day 3.* **B** *Day 7. Biochemical analysis revealed no differences in fibronectin (upper bands at 220 kDa) and collagen I (middle bands at 140 kDa) in collagen-treated wounds compared with normal saline- (control) treated wounds at postwounding for both days 3 and 7. Actin was used as a loading control (lower bands)*

bias, we used a blinded experimental methodology that compared wounds in the same rats. The significant increase in MWBS with time was expected because the normal wound healing process did not appear to be disturbed by the collagen treatment. We have shown that collagen does not change wound healing strength up to 28 days in the rat incisional model. Furthermore, we demonstrated that there were no significant cosmetic differences between collagen-treated wounds and control saline-treated wounds at all time points. Our histological and western blot analysis confirmed our macroscopic findings, and revealed no significant changes in cellular infiltration and collagen deposition in all wounds in the same time interval.

The inflammatory markers and the results of the CBC in our model have been generally consistent with the trends seen in patients. There appeared to be increases in the levels of inflammatory blood markers CRP, C3 and C4 at days 3 and 7 after wounding. Predictably, at the maturation phase of wound healing at 28 days, levels of the inflammatory markers trended downward and levelled off. The CBC results also showed predictable trends as the hemoglobin levels decreased early postoperatively at day 3 but then increased again at day 7, and were unchanged at day 28. The platelet counts were representative of the inflammatory reaction after wounding and increased early after wounding up to day 7 and then normalized at day 28.

CONCLUSION

We have shown that the topical application of collagen to wounds does not affect wound strength or cosmetic appearance of scars up from early to the late phases of wound healing. Furthermore, we have confirmed the safety and predictability of the use of collagen in wounds.

ACKNOWLEDGEMENTS: The authors acknowledge financial support from Dr Bruce Williams Division of Plastic Surgery Research Funds and the Canadian Institute of Health Research (CIHR) to Dr S Prakash. They also acknowledge the National Sciences and Engineering Research Council of Canada (NSERC) and the McGill University Surgeon Scientist Fast Family Research Fund, and the Division of Plastic Surgery Research Fund to Dr Hani Sinno. The authors also thank Pina Sorrini for her excellent secretarial help, and Genevieve Berube, Karen Zwiker and the McGill University Animal Research Center Staff for their professional help with animal handling. They also acknowledge the technical support of the McGill University Orthopedic Research Laboratory and the Small Animal Imaging Laboratory staff for their excellent technical support.

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