Nitric Oxide Synthase Activity and Angiogenesis Measured by Expression of CD34 in Burns Treated With Chitosan Films

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Abstract: Nitric oxide (NO) signaling appears to play a vital role in wound healing associated to improve collagen and angiogenesis. A burn wound model was used to evaluate the effects of a chitosan films on histopathological features, nitric oxide synthase (NOS) activity, and quantification of neofomed capillaries assessed with CD34. Methods. Bilateral burns (n = 16) were made on adult Sprague-Dawley rats. The lesions on the right side of the rats were treated with chitosan films, and the lesions on the left side of the same rats were treated with gauze with NaCl 0.9% as a control. Results. Histological analysis revealed accelerated burn wound healing supported by significant differences in acute inflammation, collagen, and granulation tissue formation in chitosan-treated burns. Additionally, chitosan-treated burns were associated with higher CD34 immunoreactivity antibody supported by significant differences. This analysis of NOS activity was statistically significant on treated burns in the second treatment week. NOS results are associated with the highest collagen deposition, granulation tissue formation, and new capillary formation. Conclusion. The use of chitosan on burns promoted re-epithelialization by means of angiogenic and NO release associated with higher cell infiltration into the wound bed during the proliferative phase.
able way with minimal foreign-body reactions. Chitosan’s effectiveness on animal and human tissue healing is outstanding. Chitosan promotes greater inflammatory cell infiltration that encourages wound healing with controlled granulation tissue production and minimal scarring. Its versatility when used with other elements such as growth factors, allows its association with angiogenic processes contributing to rapid wound healing and the control of fibrotic processes and adherences. The key process for wound healing is the formation of blood vessels. Among the many factors stimulating the formation of blood vessels, nitric oxide (NO) reduces the interaction between the extracellular matrix and endothelial cells. This favors fluctuations in the resistance of cell movement and inhibits endothelial cell adhesion. Individual cells are transformed in presence of vascular endothelial growth factor (VEGF) and a vectorial movement is added. In endothelial nitric oxide synthase (eNOS) knockout mice, a decrease in wound healing directly related to the absence of angiogenesis was observed, suggesting that NO plays an important role in the formation of granulated tissue and the healing of burns through regulating cell processes of the skin. This study evaluated the effect of chitosan films on burn wound models in rats. The histological changes, angiogenesis and NOS activity were examined following the application of chitosan on the wounds. Measurements were carried out at different stages post-burning by using enzymatic and immunohistochemical techniques.

**KEYPOINTS**

- When chitosan and its derivatives are applied to live tissue, they act in a non-toxic and biodegradable way with minimal foreign-body reaction.
- The burns on the right side of the rats were treated with chitosan films (chitosan treated group), and the lesions along the left side of the same rats were treated with gauze with NaCl 0.9% (control group). The burns were covered bilaterally with Coban.

**Methods**

**Rat burn wound model.** Adult Sprague-Dawley rats weighing 250 g–350 g were individually housed and maintained on a 12-hour light/dark cycle at constant room temperature (22°C) with food and water *ad libitum*. All experiments were performed in accordance with the institutional guidelines and National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Rats were anaesthetized by intraperitoneal injection (40mg/kg ketamine, 5mg/kg xylazine) for induction of the burns. The dorsal supra-abdominal region of 16 rats was shaved and disinfected with chlorhexidine. Afterward, the rats were scalced for 10 seconds with water at 100°C to lead to 30% total body surface area with third-degree burns (confirmed by pathologic examination). The burns on the right side of the rats were treated with chitosan films (chitosan treated group), and the lesions along the left side of the same rats were treated with gauze with NaCl 0.9% (control group). The burns were covered bilaterally with Coban. The burns on the right side of the rats were treated with gauze with NaCl 0.9% (control group). The burns were covered bilaterally with Coban. An Elizabethan collar was added to prevent biting and scratching at the wounds. Additionally, each rat had an Elizabethan collar to prevent biting and scratching at the wounds. The samples from burned areas were collected on days 3, 7, 14, and 21 post burning. Both skin collected groups included the normal skin and burns to histological examination. All animals were euthanized after sampling with an overdose of chloral hydrate (0.1 mg/kg) administered intraperitoneally.

**Chitosan film preparation.** Chitosan (95% deacetylation degree) solutions at 1% (w/v) in 1% lactic acid were prepared and then mixed with sorbitol and Tween 20 under magnetic stirring for 1 hour. The films were obtained by casting on previously washed glass plates with relative humidity of 55% at room temperature and pH adjusted to 5.5. The films were then rinsed with high performance liquid chromatography (HPLC) grade water and dried, then irradiated on both sides with 20 W of ultraviolet light for 3 minutes in a clean area in order to sterilize the surface. They were then immediately stored in sterilized sealed aluminum bags.

**Histopathologic analysis.** The samples were fixed in 10% formalin at pH 7.4 and processed in an automatic tissue processor Leica TP1020 machine (Leica Microsystems, Wetzlar, Germany). Two-millimeter thick sections were obtained and stained with hematoxylin-eosin (H&E) using a Micron HM325 microtome (Microm International GmbH, Walldorf, Germany). Microscopic analysis was performed using an Olympus CX 31 microscope (Olympus Co., Tokyo, Japan), which was operated by a senior pathologist who had no knowledge of the animals’ serial numbers.

The histopathological description included acute inflammation (polymorphonucleic cells [PMN]), chronic inflammation (macrophages, lymphocytes, plasma cells), collagen deposition, and maturation of granulation tis-
The Abramov's histologic scoring system (modified Greenhalgh's scoring system) was used. The Greenhalgh's scoring system compiled several histological parameters simultaneously to create a single score, Abramov's system assessed each parameter independently and gave a score of 0–3. Acute and chronic inflammatory infiltrates, the amount of granulation tissue, and collagen deposition were graded as: 0 (none), 1 (scant), 2 (moderate), and 3 (abundant). The maturation of granulation tissue was graded as either: 0 (immature), 1 (mild maturation), 2 (moderate maturation), and 3 (fully matured). Capillary density was calculated by CD34 on the basis of five high magnification fields per sample and averaged among five fields per sample using a 400x light microscope.

Immunohistochemical protocol for the detection of CD34. Samples were kept in paraffin blocks, were cut into 5-µm thick pieces, and then mounted in xylanized slides. Sections were then treated using SC-2053 Goat ImmunoCruz Staining System (Santa Cruz, CA). The anti-CD34 goat polyclonal antigen (SC-7045) was used as the primary antibody. Each section underwent a treatment with H2O2 and phosphate buffered saline (PBS) for five to block the activity of endogenous peroxidase. The recovery or antigenic exhibition was carried out using sodium buffered citrate 0.01M, pH 6.0 for 30 minutes at 96°C, then left to cool for 20 minutes. They were then blocked with 5% serum block (normal goat serum) for 20 minutes. The primary antibody CD34 (1:200) was then applied for 60 minutes and the sections were then washed with PBS and incubated with the biotinyl secondary antibody for 30 minutes at room temperature. Following incubation, HRP-Streptavidin for 30 minutes. Antibody binding was visualized with 3,3 diaminobenzidine in the absence of light. Hematoxylin was used to counter stain. Finally, they were mounted with Flo-texx (Thermo Fisher Scientific, Lerner Laboratories, Waltham, MA) for microscopic observation.

Microvessel counting. Capillary counting was calculated on the basis of the four most highly vascularized
areas detected by CD34 using a 40x field and 400x field light microscope. Highest magnification was used to count microvessels in each of these areas. Single ECs or clusters of ECs, with or without lumen, were considered to be individual vessels. The mean value of 4 field counts was recorded as Microvessel density (MVD).

Measuring of NOS activity using L-[3H]-arginine and L-[3H]-citrulline conversion assay. Protein extraction was based on a method developed by Bredt et al. Several skin pieces were taken from the obtained biopsy and the tissue was immediately homogenized in lysis buffer (Tris-HCl 50 mM, pH 7.4, sucrose 0.32 M, EDTA 1 mM) with protease inhibitors (PMSF, 100 mM). The homogenate underwent ultrasound for 30 seconds and was then centrifuged at 11,000 revolutions per minute for 20 minutes at 4 °C. Afterwards, the concentration of proteins from the obtained supernatant was calculated using the Bradford method. Cofactors (2 mM CaCl2, 1 μM calmodulin, 15 μM FAD, 10 μM THB, 1 mM NADPH, 1 mM DTT) and substrate (3H-arginine-1 μCi/mL) required for the reaction were added to a 200 μg aliquot of protein and then incubated in reaction buffer (Tris-HCl 50 mM, pH 7.4, EDTA 1 mM) for 30 minutes. The reaction was arrested by adding 1 mL of stop buffer (Tris-HCl 100 mM, pH 5.5, 10 mM EDTA). Following incubation, an ion exchange resin column (Dowex 50WX8-400), previously activated with NaOH 1N was used to remove arginine from samples. The L-[3H]-citrulline samples were eluted using 2 mL of deionized water. Aliquots of eluate were taken and mixed with scintillation liquid and then quantified with a scintillation counter. Data are presented as disintegrations per minute (DPM) per mg tissue protein.
Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). Differences between groups were tested by one-way ANOVA test with a Newman-Keuls multiple comparison test when comparing the group means at the same point in time. $P < 0.05$ were considered to be statistically significant.

Results

Burn wound healing. During the first few days of the experiment, the burns showed a large amount of necrotic tissue with acute cell infiltrates localized in the periphery in treated and control (Figure 1).

Acute inflammation (Figure 2) peaked on post burn day 14 in both groups; however, there was a significant difference between the two groups in terms of acute inflammation on post burn days 3 and 7 ($P = 0.003$). While chronic inflammation peaked on post burn day 7 in both groups, a significant difference between the two groups was not observed (Figure 3).

During the first 7 days, the control burns showed fissures from epidermis to dermis, this was also observed in some chitosan-treated burns; however, these fissures were observed only in the epidermis (Figure 1). The amount of granulation tissue peaked on post burn days 21, and there were significant difference between the two groups on post burn days 14 and 21 (Figure 4). Collagen deposition (Figure 5) gradually increased beginning on post burn day 3 and peaked on post burn day 14 in chitosan groups. Collagen deposition exhibited variable progress in control groups by increasing post burn day 3, however, after that the collagen deposition downing on post burn day 7, and then continuing to increase until peaking on post burn day 21 in control groups. A significant difference between the groups was observed on post burn day 21.

![Image](image.png)

Figure 6. Reactivity of endothelial cells with CD34 antibodies in skin post burn. CD34 shows positive staining in burns (A, B, C, D). Few endothelial cells showed CD34 expression associated with severe cellular infiltration in control burns on day 14 (A). CD34 is expressed on several endothelial cells on day 14 in chitosan-treated burns (B). On day 21, wide distribution of CD34 in the dermis was observed in control burns (C). Staining was positive for CD34, but was absent from capillaries because the healing process was ending (D). Bar = 50 μm

KEYPOINTS

- Accelerated burn wound healing was observed and supported by significant differences in acute inflammation, collagen, and granulation tissue formation in chitosan-treated burns.

Capillary density. During the first 7 days, isolated capillaries were observed however, the staining with CD34 showed a higher amount of buds in the chitosan-treated burns (Figure 6). In the observations carried out at day 14, a higher number of dermal capillaries were observed in the chitosan-treated burns with a clear tendency to decrease toward day 21 (Figure 7). In control burns, a lower number of capillaries were counted in all
control compared to chitosan-treated burns (Figure 7). In chitosan-treated burns, the CD34 stain showed a higher presence of angiogenic processes on post burn day 14 and capillaries of a lower caliber on post burn day 21. A significant difference between the groups was observed on days 7 and 14 (Figure 6).

**Measuring NOS activity in burns.** The results obtained through the measurement of NOS activity on post burn day 3 revealed a higher activity in chitosan-treated burns compared to their controls; these significant differences remained until day 14, and on day 21 they decreased below the activity level showed by controls. This is linked to the end of the healing process and decrease of inflammation infiltrate into the wound zone (Figure 8).

**Discussion**

Many studies have demonstrated the therapeutic properties of chitosan (eg, dressings or scaffolds for tissue engineering). However, the effects of chitosan on angiogenesis remain unclear. The present study found that chitosan enhances angiogenesis and NO release. In addition, the chitosan treated burns yielded positive effects on the overall healing response. The use of chitosan to cover burn wounds may accelerate healing by inhibiting water loss, since it creates a layer of high adherence capacity on the wound, and stimulates a higher infiltration of PMNs. The infiltration and migration of PMNs during the first days of healing is an important event in the complex process of tissue repair. This event is triggered at the beginning of the healing process by the release of several growth factors contained in the platelets. Chitosan plays an important role in adhesion, aggregation, and platelet-leukocyte aggregation agent. *In-vitro* studies revealed that and large amounts of leukotriens and prostaglandins in chitosan-treated wound exudates due to increase the PMN’s infiltration and macrophages containing inflammation mediators and stimulates phagocytosis. Histopathological samples taken from burn area revealed that acute inflammation peaked at post-burn day 14 in both groups. However, the chitosan group’s score was higher. Chronic inflammation peaked at post-burn day 7 in both groups. The control group had the highest score for chronic inflammation. Ueno et al suggest that chitosan enhances the functions of inflammatory cells, such as PMNs stimulating phagocytosis and production of osteopontin and leukotriene 4; macrophages increased the production of interleukin-1, TGF-β1, and platelet-derived growth factor (PDGF); and fibroblasts increased the production of IL-8. With the results shown for acute inflammation and chronic inflammation, it is possible to associate these with the highest score in collagen deposit and granulation tissue in the chitosan group.

Increased inflammatory reaction causes more collagen production and scar formation due to the increased number of fibroblasts. It has been reported that chitin- and chitosan-treated wounds have a higher collagen...
With the present results that demonstrate an association with higher NO release and collagen deposit with a higher cell infiltration, especially in chitosan-treated burns. The activity of the NOS enzyme detected in the authors’ experiments was highest in the treated burns. NOS activity was concentrated after day 7 and extended to day 14. On these days significant differences were observed and such findings can be linked to the type of infiltration described in the histopathological analysis, where a notable number of PMNs and macrophages were found during the previously mentioned period of time. Consequently, the increase in the NOS enzyme activity would not be caused by a direct effect of chitosan, but an indirect effect promoted by the higher cell infiltration in wounds, which could lead to a higher release of NO. The present results demonstrated that the highest NOS activity was during the inflammatory and proliferative stages. The lowest activity of NOS was found on day 21. This presumably associated to iNOS activity can be downregulated by the resolution of the inflammatory response or by cytokine signaling. In the present study NOS activity was not differentiated between eNOS or iNOS, but it can be assumed that most or nearly the entire released NO was caused by the iNOS activity associated to cellular infiltration. The previous data are supported by experiments carried out by Carter et al10 who found a significant iNOS increase on day 21 in rat burns. The beneficial effects of NO on wound repair may be attributed to its functional influences on angiogenesis and inflammation.

A higher number of capillaries per field were observed in treated burns compared to controls. According to statistical analysis, there were significant differences by direct chitosan treatment influence. This agrees with the results obtained by Fukumura et al11 who associated neovascularization with proportional presence of NO. Also, the present study confirmed their results that VEGF increased NO production at the gene expression level and that the angiogenic effects of VEGF appear to be dependent on NO.

**Conclusion**

NO plays a meaningful and important role in angiogenesis, suggesting a mechanism that causes upregulation of v3 integrins, which facilitate cell adhesion to the base of the matrix and stimulate cell migration, which would explain the relationship between the increase of NOS activity in chitosan-treated lesions and the increased number of counted capillaries. In vivo studies...
using CD31 demonstrated that the treatment of burns using NO increases angiogenesis significantly. In the present study, the use of CD34 was preferred to determine the number of capillaries, because this marker had shown more specific results on the capillary endothelium compared with other markers such as CD31 and vWF (Factor von Willenbrand).

At day 21 the control stills displayed a persistently slow angiogenic response and dissociated extracellular matrix. Meanwhile, chitosan-treated burns achieved the restoration of skin architecture. The presented findings show that chitosan displayed a significant increase of capillary density and extracellular matrix. These results could explain the effects of chitosan on burns to reduce the time to regenerate the skin damaged mainly because it stimulates cell infiltration, allowing a higher NO release and angiogenesis.

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References


