Topical and Systemic Effects of N-acetyl Cysteine on Wound Healing in a Diabetic Rat Model

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ABSTRACT

Objective. This study evaluates the effects of topical and systemic N-acetyl cysteine (NAC) treatment on wound healing in a diabetic rat model. **Materials and Methods.** A total of 48 male Wistar Albino rats were randomly divided into 4 groups of 12. Diabetes was induced with an intraperitoneal injection of 60 mg/kg streptozotocin. A 2-cm x 1-cm full-thickness wound was created on the back of each animal. In group 1 (control) and group 3 (systemic NAC), the wounds were closed with 0.9% sodium chloride-treated sterile gauze. In group 2 (topical NAC) and group 4 (topical + systemic NAC), the wounds were closed with sterile gauze treated with 3 mL (300 mg) of NAC. The animals in groups 3 and 4 were administered 200 mg/kg of NAC once daily through an orogastric tube. On days 1 and 14, the wounded areas were measured. Tissue and blood samples were taken on day 14 for histopathological and biochemical examination. **Results.** On day 14, the wounded area in groups 2, 3, and 4 was found to be smaller than in group 1 (control). Histopathologically, epithelialization and fibrosis scores were significantly lower, whereas the inflammation score was higher in group 1 than in the other groups. Tissue oxidative stress parameters (malondialdehyde, fluorescent oxidation products, total oxidative stress) were higher in the control group than in the other groups. In groups 3 and 4 (which received systemic NAC), the oxidative stress parameters in serum samples were lower than those of the control group and group 2. Serum sulphydryl levels were the lowest in group 1. **Conclusions.** The results of this study show that both topical and systemic administration of NAC improved wound healing in a diabetic rat model. This effect of NAC may be related to its antioxidant properties since a reduction in oxidative stress parameters in both tissue and serum were shown in the present study.

KEY WORDS

N-acetyl cysteine, wound healing, diabetes mellitus, rat, antioxidant, animal model

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Wound healing is a complex process involving hemostasis, inflammation, proliferation, and remodeling. Diabetes is well known to be associated with poor wound healing, causing an accumulation of advanced glycation end products, a decreased vascular supply to the wounded area, and a predilection for infections. Patients with diabetes often exhibit poor wound healing and thus chronic wounds; chronic inflammation, oxidative stress, impaired keratinocyte proliferation and migration, reduced angiogenesis, chronic infections, and abnormal expression of matrix metalloproteinases are the main factors that can contribute to poor wound healing in these patients. In addition, hyperglycemia induces increased oxidative stress, and the high levels of reactive oxygen species (ROS) result in poor wound healing.[1-4]

N-acetyl cysteine (NAC) is a derivative of the amino acid L-cysteine that is characterized by antioxidant properties.[5] Although NAC was first used for its mucolytic activity in the treatment of respiratory diseases, detoxifying, antioxidant, and anti-inflammatory properties have been discovered in recent years.[6-9]

Its antioxidant property can be attributed to its specific structure that contains L-cysteine and an acetyl group attached to the amino group. Amino acids, including sulphur group with L-cysteine, are characterized by antioxidant properties. The anti-inflammatory action of NAC inhibits the activity of various proinflammatory cytokines. Due to its antioxidant and anti-inflammatory properties, NAC has been investigated in the treatment of many diseases, including cancer, arthritis, cardiovascular diseases, diabetes, respiratory distress syndrome, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease, experimentally and clinically.[5] The positive effects of NAC on wound healing also have been shown in several studies,[10-13] however, the wound healing

Table 1. The epithelialization scoring system

SCORE	EPITHELIALIZATION DEGREE
0	No epithelialization, superficial ulcers
1	Incomplete reepithelialization, focal epidermal hyperplasia
2	Complete reepithelialization

Table 2. The inflammation scoring system

SCORE	INFLAMMATION DEGREE
0	No inflammation
1	Few lymphocytes, plasma cells, and giant cells in dermis
2	Vascular proliferation, eosinophils, plasma cells, neutrophils, and giant cells in dermis
3	Many inflammatory cells, vascular proliferation, microabscess formation

Table 3. Fibrosis scoring system

SCORE	FIBROSIS DEGREE
0	No fibrosis
1	Mild and weak
2	Moderate intensity
3	Severe intensity

activity of NAC has not yet been evaluated in diabetic rats.

In light of its anti-inflammatory and especially antioxidant activities, the current study aims to evaluate the possible effects of topical and systemic NAC on wound healing in a diabetic rat model. To the best of the authors' knowledge, this is the first study in the literature evaluating wound healing properties of NAC in diabetic rats.

MATERIALS AND METHODS

Animals and experimental protocol

The study used 48 male Wistar Albino rats (Kobay Deney Hayvanları Laboratuarı San. Tic. A.S., Ankara, Turkey), each weighing 250 g to 300 g (age, 5–6 months) and raised under the same environmental conditions. Rats were housed at 20°C to 21°C with a 12-hour light/dark cycle and free access to water until 2 hours before the anesthesia procedure. Animals were divided randomly into 4 groups, each containing 12 rats.

After an overnight fast, rats were given an intraperitoneal injection of streptozotocin (Sigma-Aldrich, St Louis, MO) at a dose of 60 mg/kg body weight in 0.05 mol/L sodium citrate buffer (pH 4.5). Streptozotocin possesses a diabetogenic property characterized by selective destruction of pancreatic islet β-cells. It causes hyperglycemia, insulin deficiency, polyuria, and polydipsia, all of which mimic human type 1 diabetes mellitus.[14] Blood glucose concentration was measured with a OneTouch glucometer (LifeScan, Milpitas, CA) on samples taken from the tail vein. The development of diabetes was confirmed by measurement of blood glucose > 200 mg/dL at 3 days and 1 week post streptozotocin injection.

A full-thickness wound measuring 2 cm x 1 cm, involving the panniculus carnosus, was created on the back of each animal by surgical excision. In all groups, the wounds were cleaned daily with 0.9% sodium chloride (NaCl). In group 1 (control) and group 3 (systemic NAC), the wounds were covered with 0.9% NaCl-treated sterile gauze. In group 2 (topical NAC) and group 4 (topical + systemic NAC), the wounds were covered with sterile gauze treated with 3 mL (300 mg) of NAC (Asist 10%, 3 mL, 300 mg, intravenous form, Husnu Arsan, Turkey). The animals in groups 3 and 4 received an additional NAC through an orogastric tube at a dose of 200 mg/kg daily for 14 days. All animals were euthanized on day 14 by using high-dose anesthetic.

Evaluation of wound contraction rates

The wounded areas were observed for 14 days. On days 1 (1 day post wound creation) and 14, the wounds were drawn onto acetate paper. After scanning the drawings, the surface area was calculated using a scientific image processing program (ImageJ, Version 1.45; National Institutes of Health, Bethesda, MD).

Histopathological evaluation

The wounded areas, excised together with the surrounding scar tissue just until healthy tissue outside scarring on day 14, were fixed in 10% phosphate-buffered formaldehyde solution and kept at room temperature for 24 hours. Histopathological examinations were performed by a pathologist blinded to the groups. The prepared specimens were washed under running tap water and dehydrated through a series of graded concentrations of alcohol (70%-40 min, 75%-40 min, 80%-45 min, 85%-45 min, 90%-50 min, 96%-50 min). After dehydration, specimens were placed into xylene to obtain transparency and embedded in paraffin. Then, 5-µm thick tissue sections were cut with a microtome (Leica RM 2125 RT; Leica Biosystems, Buffalo Grove, IL) from the paraffin blocks. These preparations were stained with hematoxylin and eosin and Masson's trichrome. The degrees of epithelialization, inflammation, and fibrosis were evaluated using semiquantitative scoring systems as shown in **Tables 1–3**.

Evaluation of oxidative stress parameters

The oxidative stress parameters of malondialdehyde (MDA), fluorescent oxidation products (FOP), and total oxidative stress (TOS) were measured in the tissue samples. Levels of MDA were measured using the spectrofluorometric method defined

by Wasowicz et al.[15] Total sulfhydryl (SH) groups were measured spectrophotometrically using the Sedlak and Lindsay method.[16] Tissue homogenates extracted using ethanol-ether for FOP measurements were calculated with a spectrofluorometer at wavelengths of 360/430 nm.[17] The TOS measurement was performed using the calorimetric method based on the cumulative oxidation of the molecules from ferrous ion to ferric ion. Data were stated as μmol H_2O_2 equivalent per L.[18]

Statistical analysis

All data analyses were applied using SPSS for Windows version 15.0 software (SPSS Inc, Chicago, IL). All variables were found to be distributed normally around the mean, and the data were given as mean ± standard deviation. In the present study, nonparametric tests were used because the number of rats in the groups was < 30. Kruskal-Wallis variance analysis was used for evaluation of the differences between the groups. If the *P* values obtained from the variance analysis were statistically significant, the Mann-Whitney *U* multiple comparison test was applied to determine from which group the difference originated. A value of $P < .05$ was considered statistically significant.

RESULTS

Wound contraction rates

There was no difference between the groups in respect to the wounded area at day 1. On day 14, the wounded areas in groups 2, 3, and 4 were found to be smaller than those in group 1 ($P < .05$), with no significant difference determined between groups 2, 3, and 4 ($P > .05$). The mean unhealed wounded area was smallest in group 4 (topical + systemic NAC) (**Table 4**).

Histopathological results

The epithelialization, inflammation, and fibrosis scores are shown in **Table 5** and also in **Figures 1** and **2**. The epithelialization and fibrosis scores were significantly lower, and the inflammation score was higher in group 1 compared with the other groups. Inflammation scores were lowest and epithelialization and fibrosis scores were highest in group 4, but the difference was not statistically significant when compared with groups 2 and 3 ($P > .05$).

Oxidative stress parameters

The oxidative stress parameters (MDA, FOP, and TOS) measured in the tissue samples were higher in the control compared with the other groups ($P < .05$). In groups 3 and 4 (NAC administered systemically), the oxidative stress parameters in the serum samples were lower than those of the control and group 2 ($P < .05$). Serum sulfhydryl levels were lower in group 1 than in the other groups ($P < .05$) (**Tables 6, 7**).

Table 4. Group wound areas (mm^2) on days 1 and 14

GROUP	DAY 1	DAY 14
1 (control)	179.88±39.50	129.82±19.99
2 (topical NAC)	190.77±18.01	84.45±35.32[a]
3 (systemic NAC)	185.01±37.21	75.98±25.95[a]
4 (systemic + topical NAC)	171.39±25.63	59.44±22.98[a]

[a] Significantly different, control vs. other groups, *P*<.05.
NAC: N-acetyl cysteine

Table 5. The mean histopathological scores of the groups

GROUP	EPITHELIALIZATION	INFLAMMATION	FIBROSIS
1 (control)	0.40±0.18	2.60±1.21	1.40±0.68
2 (topical NAC)	1.28±0.54[a]	1.85±0.99[a]	2.14±0.77[a]
3 (systemic NAC)	1.42±0.57[a]	1.71±0.83[a]	2.28±0.86[a]
4 (systemic + topical NAC)	1.50±0.72[a]	1.50±0.72[a]	2.38±0.94[a]

[a] Significantly different, control vs. other groups, *P*<.05.
NAC: N-acetyl cysteine

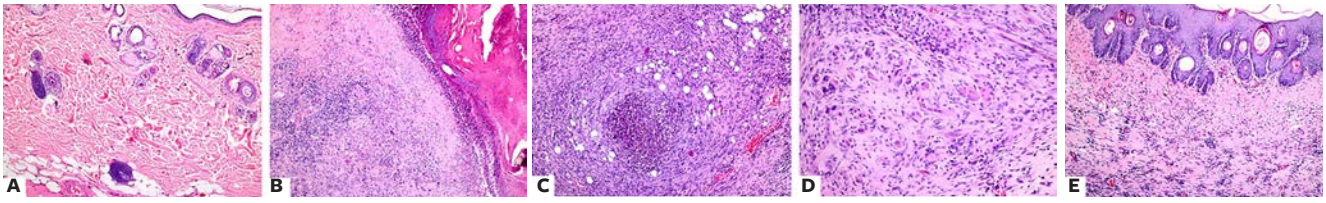

Figure 1. (A) Normal skin-pilosebaceous units in epidermis and dermis (x100, hematoxylin and eosin stain [H&E]); (B) epidermis is ulcerated with no epithelialization (epithelialization score = 0) (x100, H&E); (C) numerous inflammatory cells and microabscess formation are observed (inflammation score = 3) (x100, H&E); (D) foreign body type multinuclear giant cells accompanied by fibrosis as well as inflammatory granulation tissue composed of a small number of lymphocytes and plasma cells (inflammation score = 2) (x200, H&E); and (E) complete reepithelialization (epithelialization score = 2); epidermal hyperplasia, a small number of inflammatory cells, and signs of intense fibrosis/healing (inflammation score = 1) (x100, H&E).

DISCUSSION

Delayed wound healing is well established in the presence of diabetes[19] and entails a significant health burden, including diabetic foot ulcers and postoperative complications in patients with diabetes.[20] One of the molecular mechanisms underlying poor wound healing is increased oxidative stress, which is associated with the accumulation of advanced glycation end products.[21]

As a strong antioxidant, NAC may play a potential role in the improvement of states characterized by the generation of free oxygen radicals.[22] It has been shown to modulate oxidative stress and inflammation through different pathways. L-cysteine is a precursor of reduced glutathione (GSH), one of the major intracellular antioxidants; NAC is thought to contribute to the augmentation of the GSH pool.[23] Therefore, NAC can normalize disturbed redox status of cells and influence redox-sensitive cell signaling and transcription pathways. The SH group in NAC directly scavenges ROS, which may have toxic properties.[24] In addition, NAC has anti-inflammatory properties manifested by the inhibition of proinflammatory cytokines such as interleukin 8 (IL-8), IL-6, and tumor necrosis factor α.[25]

In addition to the effects on oxidative stress parameters, several studies[10,26,27] have investigated the role of NAC in wound healing. Aktunc et al[26] showed that NAC promoted wound healing in a diabetic mouse model, probably by reducing oxidative stress parameters and upregulating vascular endothelial growth factor expression (an angiogenic growth factor). In another experimental animal study by Thaakur et al,[27] NAC was reported to accelerate wound healing by increasing both enzymatic superoxide dismutase and catalase and nonenzymatic GSH antioxidants. N-acetyl cysteine also has been shown to improve anastomotic wound healing after radiotherapy in a rat model.[10]

Improved healing of amputation stumps with NAC has been demonstrated in a diabetic murine model with hind-limb ischemia amputation.[28] Dhall et al[29] investigated the effect of NAC and α-tocopherol in reversing the chronicity of wounds in a db/db mouse model in which antioxidant enzymes catalase and glutathione peroxidase were inhibited at the time of wound generation. The authors observed the development of biofilm-producing microbiota in chronic wounds that remained open for months. The application of NAC and α-tocopherol was seen to reduce oxidative stress, increase sensitivity to antibiotics, and improve tissue remodeling.[29] Similarly, in the current study, a reduction in oxidative stress parameters was observed along

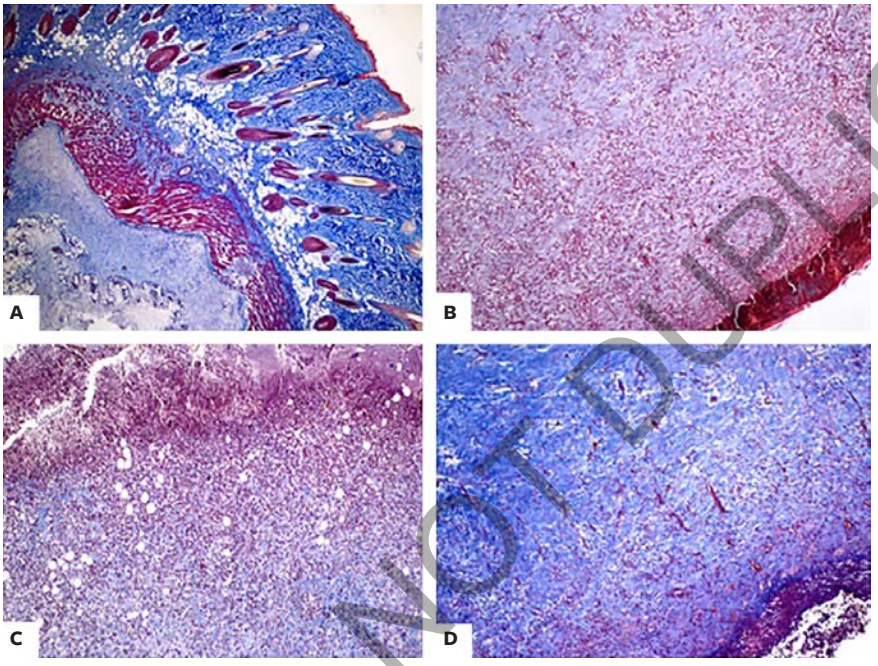

Figure 2. Development of collagen-type fibrosis is seen as blue-colored fibrils (histochemical trichrome staining). (A) Normal skin (collagen is blue and muscle is red); (B) mild fibrosis development (fibrosis score = 1); (C) moderate fibrosis development (fibrosis score = 2); and (D) severe fibrosis development (fibrosis score = 3). Magnification x100, Masson's trichrome stain.

Table 6. The mean plasma oxidative stress parameters

GROUP	PLASMA MDA	PLASMA FOP	PLASMA TOS	PLASMA T-SH
1 (control)	337.00±34.87	578.00±64.18	58.05±6.04	86.80±20.33
2 (topical NAC)	281.00±67.06	512.71±37.07	51.82±5.10	101.85±39.04[a]
3 (systemic NAC)	95.85±27.19[a,b]	428.14±121.53[a,b]	31.23±7.38[a,b]	144.57±53.39[a,b]
4 (systemic + topical NAC)	72.62±40.53[a,b]	423.00±52.72[a,b]	30.85±8.23[a,b]	154.50±55.46[a,b]

[a] Significantly different, control vs. other groups, $P<.05$.

[b] Significantly different, topical NAC vs. other groups, $P<.05$.

MDA: malondialdehyde; FOP: fluorescent oxidation product; TOS: total oxidative stress; T-SH: total sulphydryl; NAC: N-acetyl cysteine

with an improvement in epithelialization in diabetic rats with systemic or topical application of NAC.

Several clinical and experimental studies have investigated the effects of NAC as a therapeutic agent against insulin resistance, type 2 diabetes, and the associated complications.[30-33] Kaneto et al[30] demonstrated the protective effects of NAC on pancreatic β cells in diabetic db/db mice. It also has been shown to have a modulatory action on oxidative stress biomarkers in alloxan-induced diabetic rats.[31] In addition, NAC treatment in insulin-resistant rats fed a high carbohydrate diet has been reported to decrease insulin resistance as assessed by the Homeostasis Model Assessment of Insulin Resistance index.[32] Xia et al[33] demonstrated a significant decrease in blood glucose in streptozotocin-induced diabetic rats supplemented with NAC for a period of 8 weeks. The beneficial effect of NAC may be related to its effects on glycemic control in a diabetic rat model. However, this effect could not be shown in the current study as the design was not to compare the groups in respect to the degree of glycemic control or insulin resistance. In the current study, it was decided that the positive effects of NAC on wound healing in diabetic rats were mainly due to its antioxidant activity.

LIMITATIONS

The limitation of this study is the fact that this is an animal model and is not assured to be reproducible in humans in the current situation. There needs to be further human studies before NAC can be used clinically for improving wound healing. The role of NAC in angiogenesis and on inflammatory mediators, as well as the contribution to glycemic control, also should be evaluated in order to strengthen the data about the mechanism of action of NAC.

CONCLUSIONS

The results of this study showed that both topical and systemic administration of NAC improved wound healing in the diabetic rat model. This effect of NAC may be related to its antioxidant properties, as a reduction in oxidative stress parameters was observed in both tissue and serum. Further studies are needed to understand other mechanisms of NAC in diabetic wound healing, with possible areas of investigation being the role of NAC in angiogenesis and its effect on inflammatory mediators in addition to the contribution to glycemic control.

Table 7. The mean tissue oxidative stress parameters of the groups

GROUP	TISSUE MDA	TISSUE FOP	TISSUE
1 (control)	199.66±43.52	132.12±26.66	2919.60±466.26
2 (topical NAC)	51.84±10.89[a]	91.07±23.21[a]	2197.42±403.12[a]
3 (systemic NAC)	42.24±17.64[a]	78.92±32.06[a]	2189.14±452.37[a]
4 (systemic + topical NAC)	33.43±10.63[a,b]	72.92±11.61[a]	2168.12±313.28[a]

[a] Significantly different, control vs. other groups, $P<.05$.
[b] Significantly different, topical NAC vs. other groups, $P<.05$.

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