Original Article

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N-acetylcysteine Treatment Protects Intestinal Mitochondria in a Surgical Stress Model

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Abstract

Surgery-associated small intestine damage is a clinical complication. It has been found that opening the abdominal cavity during surgery and manipulation of organs, including the intestine, could lead to intestinal barrier disintegrity and the entrance of pathogens to the systemic circulation. Hence, finding agents to protect the intestine during surgical manipulation could have clinical value. Oxidative stress and enterocytes mitochondrial dysfunction and energy (ATP) crisis are the proposed mechanisms for surgery-induced intestinal damage. N-acetylcysteine (NAC) is a thiol reducing agent and radical scavenging molecule which is widely investigated for its pharmacological properties. The current study was designed to evaluate the effects of NAC treatment on the surgery-induced mitochondrial dysfunction in an animal model. Rats were treated with NAC (500 and 1000 mg/kg, oral) and underwent surgical stress. Afterward, the small intestine mitochondria were isolated and assessed. The effects of surgical stress on small intestine mitochondrial was revealed as a significant decrease in mitochondrial dehydrogenase activity, mitochondrial depolarization, decreased mitochondrial ATP levels, and mitochondrial permeabilization. Moreover, the level of alkaline phosphatase secretion from the intestinal brush border was increased. It was found that NAC treatment significantly alleviated ALP levels, and improved mitochondrial indices when this drug was pre-treated (1 week) to rats. Collectively, it could be concluded that NAC treatment might be a therapeutic approach against surgery-induced intestinal damage. The effects of NAC on mitochondrial function seem to has a pivotal role in its protective mechanism of action.

Keywords: Antioxidant, Energy crisis, Mitochondrion, Stress, Surgery

1. Introduction

The small intestine is a susceptible tissue in which a large number of different compounds such as drugs and nutrients are absorbed through its effective circulatory system and transporters. Intestine also acts as an effective barrier against the translocation of pathogens to the systemic circulation. Any stress and damage to the intestine could lead to various clinical complications. Therefore, preserving the integrity and function of the intestine is critical.

Previous studies found that surgical operations in which the abdominal cavity is opened and the manipulation of different organs, including the intestine, occurs could lead to the damage of intestinal tissue, decreasing enterocytes population, and disruption of intestine effective physiological function (1-4).

Various mechanisms have been proposed

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for surgical manipulation-induced intestinal injury (3, 5). The production of reactive species and induction of oxidative stress is the acritical mechanism for surgery-induced intestinal damage (3, 5). Mitochondrial impairment is another new mechanism for surgical operations-associated intestinal injury (3, 5). Hence, the administration of antioxidant agents might have a protective role. Mitochondrial function and energy (ATP) metabolism is a vital mechanism for adequate absorption of nutrients to the systemic circulation, which is mostly an active energy-dependent mechanism. The impaired mitochondrial function also could affect intestinal barrier integrity (3, 5). Based on these data, different compounds that positively affect mitochondrial function could prevent surgery-associated intestinal damage.

N-acetylcysteine (NAC) is widely investigated for the pharmacological properties of NAC (6). Although NAC is clinically used as the antidote of acetaminophen, several other therapeutic effects such as its neuroprotective, effects on the cardiovascular system, protective effects against liver disease, and its effects on the toxicity of other xenobiotics in biological systems have been proved (7-13). The effects of NAC on cellular antioxidant systems, as well as its radical scavenging activity, are the main mechanisms of its cytoprotection (14, 15). On the other hand, several investigations mentioned the positive effects of NAC on cellular mitochondria (10, 16-18).

The current study was designed to evaluate the effects of NAC treatment on mitochondrial impairment as one of the pivotal mechanisms involved in the pathogenesis of surgery-induced small intestine damage. Rats underwent surgical stress, and different mitochondrial indices were evaluated.

2. Material and Methods

2.1. Reagents

Rhodamine123 (Rh 123), 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 3 (N-morpholino) propane sulfonic acid (MOPS), N-acetyl cysteine, coomassie brilliant blue, bovine serum albumin (BSA), D-mannitol, sodium succinate, sucrose, and trichloroacetic acid (TCA) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). The kit for evaluating alkaline phosphatase (ALP) was obtained from Pars Azmun® (Tehran, Iran). All salts used for making buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Animals

Male Sprague-Dawley rats (n = 40; 200-250 g weight) were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Animals were housed in plastic cages over hardwood bedding. There was an environmental temperature of 23±1ºC and a 12L: 12D photoschedule along with a 40% of relative humidity. The rats were allowed free access to a regular chow diet and tap water. All the experiments were performed in conformity with the guidelines for care and use of experimental animals approved by an ethics committee at Shiraz University of Medical Sciences, Shiraz, Iran (# 98-01-36-21408).

2.3. Surgery stress model

Animals were anesthetized (10 mg/kg of xylazine and 70 mg/kg of ketamine, i.p). A midline incision was made, and the small intestine from the duodenum to ileum was mildly taken out from the abdominal cavity, then returned to its place again (1, 19). The sham operation (control group) consisted of laparotomy without manipulation of the small intestine.

2.4. Experimental setup

Animals were equally allotted into six groups (8 rats/group). The treatments were as follows:

1) Sham-operated (Vehicle-treated);
2) Surgical stress;
3) Surgical stress + NAC pre-treatment (500 mg/kg, oral, 24 hours before stress);
4) Surgical stress + NAC pre-treatment (1 g/kg, oral, 24 hours before stress);
5) Surgical stress + NAC pre-treatment (500 mg/kg, oral, 7 days before stress); and
6) Surgical stress + NAC pre-treatment (1 g/kg, oral, 7 days before stress).

2.5. Intestinal lavage fluid

After isolation of the small intestine, 5

mL of ice-cooled normal saline was used to wash the intestine. Afterward, the intestinal lavage fluid (ITF) was centrifuged (12000 g, 10 min, 4 ºC), and the supernatant was collected. The activity of alkaline phosphatase was measured in ILF using commercial kits and an autoanalyzer (20).

2.6. Mitochondria isolation from the rat small intestine

Rat small intestine was washed with ice-cooled normal saline and minced in the ice-cold isolation buffer containing 70 mM mannitol, 2 mM HEPES, 220 mM sucrose0.5 mM EGTA and 0.1 % BSA (pH=7.4) (21, 22). Minced tissue was transported into fresh isolation buffer (5 mL buffer: 1 g tissue) and homogenized. Mitochondria were isolated by the differential centrifugation method (23-25). First, unbroken cells and nuclei were pelleted at 1000 g for 10 min at 4 ºC; second, the supernatant was centrifuged at 10,000 g for 10 min at 4 ºC to pellet the mitochondria fraction. This step was repeated (3 times) using a fresh buffer medium. Finally, mitochondrial pellets were resuspended in a buffer (5 mL buffer/g tissue) containing 70 mM mannitol, 220 mM sucrose, and 2 mM HEPES (pH=7.4) (26, 27). The mitochondria fractions used to assess mitochondrial permeabilization and mitochondrial depolarization were re-suspended in mitochondria permeabilization buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH=7.2), and depolarization assay buffer (220 mM sucrose, 10 mM KCl, 68 mM Mannitol, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 µM EGTA, and 10 mM HEPES, pH=7.2) (23, 26, 28). The Bradford method was used to assess samples of proteins for the standardization of data.

2.7. Mitochondrial dehydrogenases activity

Based on a previously described method, the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases was determined (29-33). Briefly, samples of mitochondrial suspension (1 mg protein/mL) were incubated with 40 µL of MTT (0.4% w: v, 37 °C, 30 min, in the dark) (34, 35). Then, samples were centrifuged (16,000 g, 10 min) and the pellet (product of purple formazan crystals) was dissolved in 1 mL of dimethyl sulfoxide. Finally, the optical density (OD) of samples was measured (EPOCH plate reader, λ=570 nm, Bio-Tek® Instruments, Highland Park, USA) (26, 30, 34, 36).

2.8. Mitochondrial ATP levels

Based on a previously reported protocol, mitochondrial ATP level was assessed by HPLC (37, 38). Briefly, isolated mitochondria (1 mg protein/mL) were treated with 100 µL ice-cooled phosphoric acid (50 % w: v, 4 ºC) and centrifuged (10 min, 15,000 g, 4 ºC). Afterward, the supernatant (100 µL) was treated with its equivalent volume of ice-cooled 1 M KOH solution (22). Samples (25 µL) were injected into an HPLC system consisted of an LC-18 column (µ-Bondapak, 25 cm). The mobile phase was composed of potassium phosphate buffer (100 mM KH_2PO_4, pH=7 adjusted with KOH), tetrabutylammonium hydroxide (1 mM), and acetonitrile (2.5 % v: v). The flow rate was 1 mL/min, and the UV detector was set at λ=254 nm (21, 37).

2.9. Mitochondrial depolarization

Mitochondrial uptake of the cationic dye rhodamine 123 was applied for the evaluation of mitochondrial depolarization (39-44). Rhodamine 123 accumulates in the mitochondrial matrix by facilitated diffusion. In the current investigation, the mitochondrial fractions (0.5 mg protein/mL; in the depolarization assay buffer) were incubated with 10 µL of rhodamine 123 (Final concentration of 10 µM; 15 min, 37 ºC, in the dark) (22, 28, 33, 44, 45). Afterward, samples were centrifuged (15,000 g, 10 min, 4 ºC) and the fluorescence intensity of the supernatant was monitored with a multifunctional microplate reader (FLUOstar Omega®; BMG Labtech, Germany; $\lambda_{excitation}$=485 nm and $\lambda_{emission}$=525 nm) (39, 40, 42, 46).

2.10. Mitochondrial permeabilization and swelling

Mitochondrial swelling was estimated by analyzing the changes in optical density at λ=540 nm (46-48). Briefly, isolated mitochondria (0.5 mg protein/ml) were suspended in swelling buffer (125 mM Sucrose, 65 mM KCl, 10 mM HEPES, pH=7.2). The absorbance was monitored (25 °C,

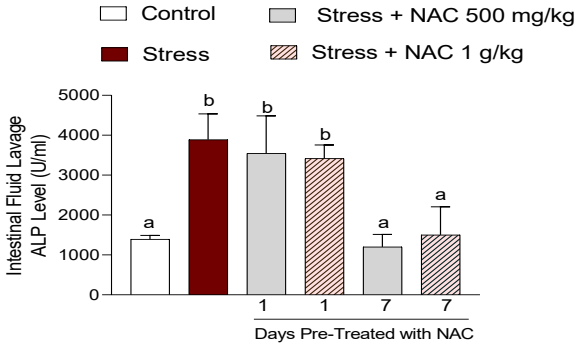

Figure 1. The alkaline phosphatase (ALP) level in the intestinal fluid lavage. Data are represented as mean±SD (n=8). Columns with different superscripts are significantly different (P<0.01).

during 30 min of incubation), using an EPOCH plate reader (Bio-Tek® Instruments, Highland Park, USA) (49). A decrease in absorbance is associated with an increase in mitochondrial swelling. The results are reported as maximal mitochondrial swelling amplitude (ΔOD 540 nm) (47-49).

2.11. Statistical analysis

Data are represented as mean±SD (n=8). The comparison of data sets was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as the post hoc test. Values of P<0.05 were considered statistically significant.

3. Results

The induction of surgical stress by opening the abdominal cavity and the manipulation of the small intestine significantly increased the secretion of ALP from the brush border of the intestinal tissue (Figure 1). Pre-treatment of animals with NAC (500 and 1000 mg/kg, oral), 24 hours before surgical stress showed no significant effect on this parameter (Figure 1). On the other hand, the administration of NAC (500 and 1000 mg/kg/day) for one week before surgical stress significantly decreased the ILF level of ALP (Figure 1).

Mitochondrial indices were significantly changed in the rats with surgical manipulation (Figure 2). It was found that mitochondrial dehydrogenases activity, ATP content, and membrane potential were significantly decreased in mitochondria isolated from the intestine of the surgically-stressed group (Figure 2). Moreover, Ca^{2+}-induced mitochondrial permeabilization was significantly decreased in the surgical stress group (Figure 2). Although the effects of NAC administration 24 hours before surgery was not significant

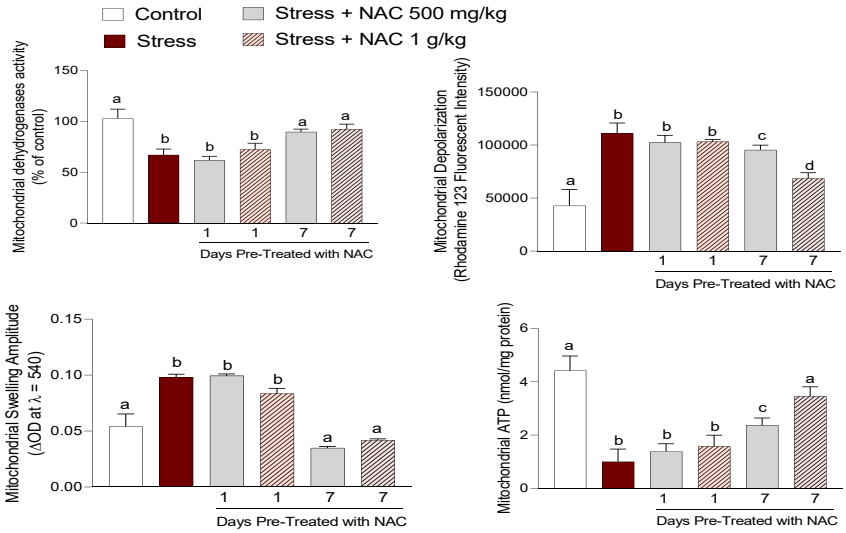

Figure 2. Mitochondrial indices assessed in the intestinal of rats underwent surgical stress. Data are represented as mean±SD (n=8). Columns with different superscripts are significantly different (P<0.01).

on mitochondrial indices (Figure 2), the pre-treatment of animals with NAC (500 and 1000 mg/kg, oral) for one week before surgical manipulation, significantly improved intestinal mitochondrial function (Figure 2).

4. Discussion

The intestinal epithelium is an essential barrier for decreasing the entrance of pathogens to the systemic circulation. On the other hand, a large number of different chemicals and nutrients are absorbed through the intestine, mostly by an energy-dependent and transporters-mediated manner. It has been found that surgical manipulation, including the opening of the abdominal cavity, could affect intestinal tissue and affect its physiological function (5, 50-52). In the current study, we found that pre-treating rats before the surgical operation could effectively improve mitochondrial indices of functionality. Based on these data, NAC might be a candidate for decreasing surgical operation complications, including intestinal damage.

Previous studies mentioned the occurrence of mitochondrial impairment as a pivotal mechanism in the pathogenesis of enterocytes injury and death in laparotomy (52, 53). It has been reported that surgical manipulation influences cell numbers (53). Although the damage to the intestinal epithelium might be reversible in most cases, there is always a risk for the translocation of pathogens to the systemic circulation (51, 54). Surgery-induced intestinal barrier impairment might lead to serious complications such as sepsis, systemic inflammatory response, or even multi-organ failure (19).

Previous studies mentioned the potential therapeutic effects of agents such as glutamine on surgery-induced gut damage (1). Zhou et al. also revealed that the administration of antioxidant polyphenol molecules such as chlorogenic acid could significantly ameliorate intestinal injury in an animal model (55). Chlorogenic acid is an antioxidant which its positive effects on mitochondrial function also has been mentioned in various experimental models (24, 56-58). Our data on the effects of NAC on mitochondrial function in the intestinal tissue of surgically-stressed rats is in line with these investigations. The effects of NAC on mitochondrial function have been repeatedly mentioned in previous studies (10, 59-62). NAC could effectively preserve mitochondrial matrix redox balance (63-65). Mitochondrial matrix redox status is an essential factor for the inhibition of mitochondrial permeabilization (66, 67). It has been reported that an oxidized mitochondrial matrix leads to the opening of mitochondrial membrane permeabilization pore, and finally, the release of cell death mediators from this organelle (66, 67). Hence, NAC might protect enterocytes by inhibiting their mitochondrial permeabilization (mitochondrial swelling; Figure 2).

Impaired function of cellular mitochondria also has been reported in other intestinal diseases such as ulcerative colitis and irritable bowel (IBD) syndrome and Crohn's disease (68-70). Based on our data in the current investigation, NAC might have a therapeutic effect in these situations. Surprisingly, Amrouche-Mekkioui et al. revealed that NAC (150 mg/kg) given in drinking water for 45 days) significantly improved dextran sulfate-induced ulcerative colitis in an animal model (71). It seems that the protective effects of NAC in ulcerative colitis are associated with the positive effects of this thiol reducing and antioxidant molecule on mitochondrial function (71).

Collectively, our data indicate that NAC supplementation (Pre-treatment) could significantly ameliorate surgical manipulation-associated small intestine injury. Protecting enterocytes' mitochondria seems to have a role in its mechanism of action. Indeed, future studies could reveal the precise mechanism of action of this drug in gastrointestinal disorders as well as using NAC in clinical settings.

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Conflict of Interest

None declared.

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