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Intestinal ischemia–reperfusion induced diaphragm contractility dysfunction: Electrophysiological and ultrastructural study in a neonatal rat model

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ABSTRACT

Aim: To evaluate the remote effect of intestinal ischemia reperfusion (IR) injury mediated by tumor necrosis factor alpha (TNF-α) on diaphragm contractility functions and whether administration of NAC may counteract the possible detrimental effects in an experimental neonatal rat model.

Methods: 40 Wistar rat pups were randomized into four groups; ten animals in each. Intestinal ischemia was conducted by obstructing mesentery of intestines by a silk loop. In the control group; only laparotomy was performed. After 1 h ischemia, reperfusion was conducted for 1 h in 1 h group, 24 h for 24 h group and 24 h for 24 h + NAC group but administration of NAC (150 mg/kg/day) intraperitoneally twice a day was performed. Inflammatory response was evaluated by tissue TNF-α level and contractility functions by mechanic activity studies of the diaphragm. Electrophysiology of the diaphragm and the phrenic nerve was conducted to determine neuropathy or myopathy and transmission electron microscopy was performed to evaluate ultrastructural changes in the phrenic nerve.

Results: Diaphragm tissue TNF-α level significantly increased in 1 h and 24 h groups ($P = 0.004$, $P = 0.0001$; respectively). Diaphragm mechanic activation force and duration significantly decreased at 1 h and 24 h ($P = 0.004$, $P = 0.02$ and $P = 0.0001$, $P = 0.0001$; respectively). NAC administration significantly prevented decrease in the maximal contraction and the duration ($P < 0.001$). Phrenic nerve compound action potential (CMAP) amplitude significantly decreased in 1 h group ($P < 0.0001$) and NAC administration significantly prevented this decrease when compared with 24 h group ($P < 0.001$). In diaphragmatic needle electromyography, the duration of motor unit potentials (MUP) was prolonged significantly when compared with control group. Contractility and electrophysiological studies were indicating primarily neuropathy in diaphragm dysfunction. Histopathology revealed axonal and myelin degeneration in the 1 h and 24 h group, but less injury in the NAC administered group.

Conclusions: Intestinal IR induced elevation of TNF-α level in the diaphragm. Impairment in the diaphragm contractility and neuropathic changes in the phrenic nerve occurred even in the first hour of reperfusion. NAC administration prevented these detrimental effects.

Intestinal ischemia–reperfusion injury (IR) is a complex pathology of surgical practice characterized by reduction or cessation of the mesenteric blood flow. Pediatric surgeons dealing with the disorders of neonatal intestinal surgery are prone to having their patients have post-surgical mechanical ventilation support. Disorders such as malrotation, congenital bands, midgut volvulus, internal herniation, acute mesenteric ischemia, necrotizing enterocolitis, small bowel transplantations, or collapse of the systemic circulation as in hypovolemic or septic shock causing mesenteric IR are associated with respiratory insufficiency in intensive care units [1]. Activated neutrophils, oxygen derived free radicals and proinflammatory cytokines play an important role not only in the local effects but also in the remote effects of IR injury such as impairment in the gas exchange system and the development of diaphragm dysfunction [2–5]. Among these, TNF-α has emerged as a potential mediator of contractile dysfunction of diaphragm [6]. The diaphragm contracts rhythmically throughout life with the help of intact neuromuscular activity. Although neuromuscular abnormalities including neuromuscular transmission defects, myopathies, neuropathies and any combination of these pathologies have been shown in sepsis or hypoxic models in the literature, to the best of our knowledge no study has been performed to identify the intestinal IR induced diaphragmatic injury by means of myopathy or neuropathy [7–10].

N-Acetylcysteine (NAC) serves as a glutathione precursor; stimulates vasodilatation, and suppresses the proinflammatory cytokines.

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NAC has been shown to reduce inflammatory response by decreasing TNF-α level in an experimental lung IR model [11]. NAC also has been shown to reduce the diaphragmatic dysfunction in hypoxic, septic and IR animal models, inhibit muscle fatigue of diaphragm in in vitro studies and improve human diaphragmatic strength and fatigability [8,9,12,13]. In order to understand whether cessation and restoration of blood flow in the intestinal system have an effect on diaphragmatic functions, we hypothesized that intestinal IR may affect the diaphragm contractility functions either myopathy or neuropathy mediated by TNF-α. The second goal of this study was to determine if administration of NAC can change these alterations. Therefore, biochemical, histopathological and electrophysiological examinations of diaphragm and phrenic nerve were conducted in an experimental intestinal IR newborn rat model.

1. Materials and methods

The present experimental protocol was approved by the Animal Care and Use Committee. The rat pups were maintained according to the recommendations of the National Institutes of Health's guidelines for the care and use of laboratory animals [14]. Seven-day-old spontaneously delivered Wistar male rat pups weighing between 15 and 20 g were used in this experimental study. 40 rats were randomized into four groups of ten animals each and subjected to one hour mesenteric ischemia and subsequently reperfusion. control group: rats underwent a sham surgical procedure with laparotomy only; 1 h group: ischemia and reperfusion for 1 h, 24 h group: ischemia for one hour and reperfusion for 24 h, 24 h + NAC group: same procedure with group 24 h but with 150 mg/kg/day NAC intraperitoneally injected.

1.1. Drug preparation and administration

N-Acetyl-L-cysteine (Asist, Bilim, Istanbul, Turkey) was dissolved in saline at a concentration of 3 mg/mL and neutralized at pH 7.0. The NAC solution was given intraperitoneally with insulin injector between 37 and 50 U (0.37–0.5 mL) twice a day according to the body weight of rat pups in group 4. The initial injection of NAC is performed after the ischemic period and the second dose is injected 12 h after the first injection. Animals received a total dose of 150 mg/kg NAC treatment per day.

1.2. Protocol for the intestinal ischemia reperfusion injury

Intestinal IR was produced by silk loop technique. Briefly; rats were placed over a heating pad to protect the body temperature and anesthetized with sevoflurane inhalation. Animals were not ventilated and were allowed to breathe spontaneously throughout the experiment. A mid-line abdominal incision was performed, intestines were exteriorized and the root of the mesentery was identified. A 2/0 silk loop was passed through 4 cm of 8 F feeding tube to create the loop. The loop was encircled over the mesenteric root of bowels and constricted with the help of hemostatic clamp for one hour and then reperfused. Ischemia was recognized by the existence of pulseless and pale color of bowel. The intestine was replaced into the abdomen and incision was closed with 5/0 running silk suture. Rat pups were placed in cages. After the reperfusion period relaparotomy was performed, bowels were exteriorized and electrophysiological and mechanical studies of diaphragm and phrenic nerve were conducted initially and then specimens were taken for the histopathological analyses.

1.3. Electrophysiological procedures

1.3.1. Phrenic nerve conduction study

All electrophysiological studies were recorded with Biopac-MP 100 data collection system (Biopac-MP 100; Biopac, Santa Barbara, USA) as described by Atıs et al. [7]. Modified MacLean and Mattioni technique was performed for recording the phrenic nerve compound action potential (CMAP) with the help of active and reference needle Ag/AgCl electrodes and a disc ground electrode [15]. Active electrode was introduced into the diaphragm muscle, the reference surface electrode was placed closed to the intercostal space and the ground electrode was placed on the sternum (Medelec, Oxford, UK). The phrenic nerve was stimulated (intensity 1 V, duration 0.5 ms) at a supramaximal voltage by a standard bipolar surface stimulator (Medelec small bipolar nerve stimulator, Reference no. 6894T, Oxford, England). The amplitude from the baseline to the negative peak, the duration and the latency of CMAP were measured.

1.3.2. Diaphragmatic needle electromyography

To record motor unit action potential (MUP) of diaphragm modified Koepke technique was performed [16]. The monopolar needle electrode (Biopac-EL 400; Santa Barbara, USA) was inserted into the diaphragm muscle, the reference electrode was inserted adjacent to costal margin, and the ground electrode (Medelec disk electrodes, Reference no. 017K006, Oxford, England) was placed over the sternum. Motor unit recruitment pattern and duration, frequency, amplitude of MUPs and the number of MUPs in 10 s were recorded by BIOPAC Acknowledge Analysis Software (ACK 100 W).

1.3.3. Measurement of mechanical activation of diaphragm

Approximately 5 mm of diaphragm muscle strip prepared from the left hemidiaphragm and intact fibers inserted at the ribs and central tendon was used for measurements of isometric contractile measurements described as previously [17]. Free end of diaphragm strip was attached to the isometric force transducer (MAY, FDT-10, TR) and stimulated with needle electrode. The duration, maximal force and the curve of contractions were recorded (Biopac-MP 100; Biopac, Santa Barbara, USA). After all electrophysiological and mechanical studies, all rats were euthanized by decapitation. Diaphragm muscle and phrenic nerve were removed for histopathological studies.

1.4. Measurement of tissue TNF-α level

The samples were stored at −20°C until the enzyme-linked immunosorbent assay was performed. Tissue was homogenized with 0.5 ml, 10 mM PBS (pH 7) for 60 s and then centrifuged at 20,000 rpm for 15 min. Specific TNF-α enzyme-linked immunosorbent assay kit (Biosource Rat TNF-α kit; lot. no. KRC3011) was then used for the tissue TNF-α assays according to the manufacturer's instructions and guidelines. The results were calculated as pg/g per tissue after the production of concentration/absorbance curve from the standards as pg/ml was used to determine the TNF levels. Samples were analyzed according to the manufacturer's instructions, and all samples were run in duplicate.

1.5. Phrenic nerve histology

A midline incision was made through the sternum to the abdomen. After removing the skin over the chest and the abdomen, the thorax was opened along the left side of the sternum and the abdominal surface of the left hemidiaphragm was exposed. After completing the mechanical activation studies of the diaphragm, the right hemidiaphragm was excised close to the sternum and the rib cage taking care not to damage the phrenic nerve. Diaphragmatic tissue specimens including the phrenic nerve were prefixed in 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide. Graded ethanol series were used for dehydration. Fixed tissues underwent propylene oxide/resin combination for transparency and embedded in epoxy resin (Araldite, EMS). The samples were cut into semi thin (1 μm) and ultrathin sections (50–70 nm) by a microtome (Leica Ultra cut UCT–125, Leica Microsystems GmbH, Vienna, Austria). After polymerization with resin for one day, the toluidine blue stained semi thin sections were examined in light microscope to detect the region for phrenic nerve ultrathin sections. Ultrathin sections were stained with uranyl acetate/lead

Table 1
TNF-α level of diaphragm.

Variable	Control	1 h	24 h	24 h + NAC	P
TNF-α (pg/g)	108.6 ± 78.3	712.9 ± 314.1[a]	626.1 ± 114.5[b]	573.9 ± 548.3	0.05

[a] $P = 0.004$, compared with control group.
[b] $P = 0.0001$,compared with control group.

citrate and examined and digital images were captured by using a transmission electron microscope (JEOL JEM1011, Tokyo, Japan).

1.6. Statistical analysis

Data were analyzed after confirmation of normal distribution by Kolmogorov–Smirnov test and expressed as mean ± S.D. The comparisons of the electrophysiological data were made by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The SPSS 11.5 statistical packet program was used for analyzing the data and the Statistica 6.0 for the figures of the differences. The statistical significance of differences was set at $P < 0.05$.

2. Results

All rats in the ischemia reperfusion group manifested tachypnea, respiratory distress and became progressively more lethargic. One rat from the group 1 h, two rats from the group 24 h and three from group 24 h + NAC died secondary to respiratory arrest during the experiment before the studies and measurements were conducted. These animals were excluded from the study.

2.1. Tissue TNF-α level

In 1 h and 24 h groups, the TNF-α levels of the diaphragm significantly increased from 108.6 ± 78.3 to712.9 ± 314.1 and 626.1 ± 114.5, respectively ($P = 0.0001$ and $P = 0.004$) pg/g tissue, compared with the control group ($P = 0.01$ and $P = 0.004$). The NAC treatment prevented this increase, but no statistical difference was found, compared with the 24 h group (Table 1).

2.2. Mechanical activation of diaphragm

The maximal contraction force and the duration of contractions are shown in Table 2. In 1 h and 24 h groups maximal contraction force and the duration of contractions significantly decreased when compared with control group ($P < 0.001$). The NAC treatment significantly prevented this decrease in the maximal contraction and the duration ($P < 0.001$).

2.3. Electrophysiology

2.3.1. Phrenic nerve conduction study

The amplitudes and the distal latencies of CMAPs are shown in Table 3. In 1 h group, the amplitudes of CMAPs significantly decreased compared with sham group ($P < 0.001$). The NAC treatment significantly prevented this decrease when compared with 24 h group ($P < 0.001$) (Fig. 4). The distal latency of CMAPs was steadily prolonged compared with control group and became significant in the 24 h group ($P < 0.001$).

2.3.2. Diaphragmatic needle electromyography

The amplitude, the duration and frequency of MUPs are shown in Table 4. In all groups, the duration of MUPs was prolonged significantly when compared with sham group. The NAC treatment reduced this duration but no statistical difference was found. In 1 h and 24 h groups the amplitude of MUPs increased when compared with sham group but no statistical difference was found. After NAC treatment it decreased but there was no statistical difference found when compared with 24 h. The frequency of MUPs was reduced but no statistical difference was found when compared with sham. NAC treatment significantly increased the reduction of frequency of MUPs ($P = 0.028$).

All electrophysiological and mechanical activation studies are summarized in Fig. 1.

2.4. Ultrastructure findings of phrenic nerve

In the control group the normal architecture of phrenic nerve was preserved. Electron lucent axons were surrounded by electron dense regular concentric lamellar structures of myelin sheath. Nucleus and cytoplasmic processes of Schwann cells and free ribosomes were adjacent nerve fibers. Capillaries, endothelial cells and basal lamina were normal (Fig. 2a).

In the 1 h group, degradation of lamellar myelin sheath and invagination to axons were seen. Axons were disrupted, degenerated, swollen and exhibited the disruption of organelle structures. There were vacuole like spaces between axon and myelin sheath due to axonal shrinkage. Schwannian cells were composed of clumped chromatin, electron dense appearance and organelle disruption (Fig. 2b).

In the 24 h group, although myelin sheath, axon and Schwannian cells displayed structural deficits similar to 1 h group, some fields exhibited preserved axons (Fig. 2c).

In the 24 h + NAC group, although there were slight differences in the morphological details, the overall architecture of of myelin and axonal structures were preserved (Fig. 2d).

3. Discussion

This present study demonstrated a) elevated TNF-α level in diaphragm tissue, b) mechanical contractility dysfunction of the diaphragm, c) electrophysiological and histopathological abnormalities of phrenic nerve and d) attenuation of these detrimental effects by administration of NAC in an experimental intestinal IR newborn rat model.

Intestinal IR has systemic effects and has been shown to increase level of TNF-α in serum and in remote organ tissues. TNF-α stimulates muscle wasting and induces contractile dysfunction without muscle wasting [18]. TNF-α is an important predictor of acute respiratory distress syndrome and has been shown to impair diaphragm contractility resulting in difficulty in weaning from mechanical ventilation and even high mortality in the intensive care units [19,20].

In the present study, to mimic the pathophysiological changes seen in acute mesenteric ischemia, internal herniation and obstruction, congenital band anomalies or necrotizing enterocolitis we used a silk loop technique and constricted the mesenteric root of intestines for one hour. The excessive productions of cytokines initiate secondary responses in remote organs, increase tissue damage and are accepted as the principal reason for diaphragmatic contractility dysfunction [21–23].

Neuromuscular abnormalities including neuromuscular transmission defects, myopathies, acute neuropathies, or any combination of

Table 2
Mechanical activation of diaphragm.

Variable	Control	1 h	24 h	24 h + NAC	P
Force (g/cm^2)	36.89 ± 1.23	11.42 ± 1.82[a]	16.37 ± 2.79[a]	41.31 ± 6.8[b]	<0.001
Duration (ms)	335.61 ± 5.19	177.45 ± 18.29[a]	181.87 ± 19.11[a]	335.93 ± 6.51[b]	<0.001

[a] $P < 0.001$, compared with control group.
[b] $P < 0.001$, 24 h + NAC group compared with 24 h group.

Table 3
Phrenic nerve conduction study findings.

Variable	Control	1 h	24 h	24 h + NAC	P
CMAP Amplitude (mV)	5.94 ± 2.50	2.43 ± 1.80[a]	5.07 ± 0.93	7.73 ± 1,41[b]	<0.001
Distal latency (ms)	2.45 ± 0.41	3.01 ± 0.49	3.64 ± 0.53[a]	2.87 ± 0.75	<0.001

[a] $P < 0.0001$ compared with control group.
[b] $P < 0.001$ 24 h + NAC compared 24 h group.

these pathologies have been described in systemic inflammatory response system (SIRS) or sepsis by the help of endotoxins, free oxygen radicals or increased inflammatory cytokine levels [23,24].

In the present study, there was a significant increase in the diaphragm TNF-α level in the first hour and 24 h after intestinal IR demonstrating an inflammatory activity in a time course. TNF-α levels increased in 1 h and 24 h. Systemic administration of exogenous TNF-α to anesthetized dogs has been shown to impair diaphragm force generation beginning within 3 h [6]. In the present study mechanical activation study of the diaphragm showed a significant decrease not only in the maximal contraction force but also in the contraction duration in the first and 24th hours of reperfusion in accordance to the elevated tissue TNF-α level. Additionally, administration of NAC significantly increased force generation and contraction duration in 24 h group. Diminishing diaphragm contractions and the elevation of TNF-α level in the diaphragm tissue indicated that elevated TNF-α levels induced diaphragm contractility dysfunction as in previous studies [18,25]. TNF-α directly effects muscle fibers and contractile functions by the chemical and electrical signals with the changes in intracellular calcium release and also induce muscle wasting [26]. However, studies on transgenic mice with the overexpression of TNF-α levels and rise in circulating TNF-α levels decreased diaphragm force to half-normal despite normal muscle mass and ultrastructure [27]. Atıs et al. showed phrenic nerve lipid peroxidation and Naycı et al. showed axonal and demyelinating phrenic nerve neuropathy without a clear myopathy in an intra-abdominal sepsis model. Electrophysiological investigations including needle electromyography of the diaphragm and phrenic nerve conduction studies are the gold standard to determine neuropathy and myopathy [28]. To differentiate the cause of alterations in the contractile functions of the diaphragm, phrenic nerve conduction studies and needle electromyography of the diaphragm were also performed. In the present study all animals were anesthetized with inhalation anesthetics without mechanical ventilation to standardize contractile functions. Phrenic nerve conduction study findings revealed a significant reduction in the compound muscle action potential amplitudes (CMAP) in the IR 1 h group. CMAP decreased in 1 h group, compared with sham group ($P < 0.001$) and NAC treatment significantly increased the amplitude reduction when compared with IR 24 h group ($P < 0.001$). The distal latency steadily prolonged and became significant in the 24 h group compared with control group ($P < 0.001$) indicating electrophysiological abnormalities in the phrenic nerve. Moneley et al. showed that lower torso ischemia reperfusion induces diaphragmatic dysfunction and also IR injury results in a significant impairment in diaphragmatic twitch and tetanic functions as early as 2 h following the initial injury [29]. On the nerve conduction studies abnormal spontaneous muscle activity, reduced action potentials and relatively normal conduction velocities indicate axonal neuropathies. In demyelinating neuropathies nerve myelin sheath is affected. Slowing in the nerve conduction velocity, elongation in the duration and no spontaneous muscle activity can be seen in the nerve conduction studies [30,31].

Diaphragm needle electromyography study revealed decreased motor unit potential (MUP) in 1 h and 24 h group respectively compared with control group ($P < 0.001$). NAC treatment decreased duration and increased MUP in 24 h group significantly. Increase in the durations indicated primarily demyelinating and axonal neuropathy without a diaphragmatic muscle involvement. In the present study, phrenic nerve conduction and diaphragm needle electromyography findings all indicated an axonal injury and demyelinating neuropathy more than a myopathy within the first hour and slight recovery in a time course and NAC administration significantly increased the recovery.

In addition to axonal injury and demyelinating neuropathy in electrophysiological and nerve conduction studies, electron microscopic evaluation of phrenic nerve was also carried out. In 1 h group, lamellar myelin sheath degradation and invaginations to axons were seen. Additionally, axons were swollen, degenerated and exhibited disruptions of organelle structures which also indicated an axonal and demyelinating neuropathy of the phrenic nerve. In 24 h group, although myelin sheath, axon and Schwannian cells displayed structural deficits similar to 1 h group, some fields exhibited preserved axons. It has been reported that unlike central nervous system peripheral nervous system is able to regenerate and this regeneration starts immediately after injury [32]. Systematic or local administration of TNF-α antagonists has been shown to enhance axonal regeneration in animal models [33,34]. In the present study the group intraperitoneally administered with NAC showed slight difference in morphological details of myelin and axonal structures but preserved overall architecture. These ultrastructural changes were all relevant with the findings of phrenic nerve conduction studies and diaphragmatic needle electromyography. In clinical practice, surgical disorders causing intestinal ischemia–reperfusion may result in respiratory insufficiency and necessitates ventilatory support due to elevated cytokine levels. Reducing cytokine levels particularly TNF-α levels by TNF-α antagonists or neuro-protective agents to prevent destructions in the phrenic nerve may help to achieve better respiratory pump action which can result in better oxygenation in tissues and reduce mechanical ventilation support in intensive care units.

The present study has some potential limitations that should be considered. First of all NAC treatment timing, dose and the route are important to achieve an effective antioxidant treatment. Pharmacologic NAC concentrations in plasma affect anti-inflammatory response and a high dose of NAC increases mortality [35]. NAC also has been shown to have direct temperature dependent effect on diaphragm function [36]. In the current study, all experiments were conducted at room temperature and a heating pad was used for the rat pups to maintain body temperature at 37 °C and further investigations to investigate optimum dose, route and

Table 4
Diaphragmatic needle electromyography findings.

Variable	Control	1 h	24 h	24 h + NAC	P
Amplitude (mV)	3.43 ± 0.89	3.83 ± 0.95	3.83 ± 1.51	3.04 ± 0.96	0.459
Duration (ms)	3.12 ± 1.21	22.74 ± 4.40[a]	26.09 ± 5.61[a]	21.27 ± 6.85[a]	<0.001
MUPs	39.60 ± 11.37	36.34 ± 5.77	32.14 ± 8.33	43.58 ± 10.45[b]	0.110

[a] $P < 0.001$ compared with control group.
[b] $P = 0.0028$, 24 h + NAC group compared with 24 h group.

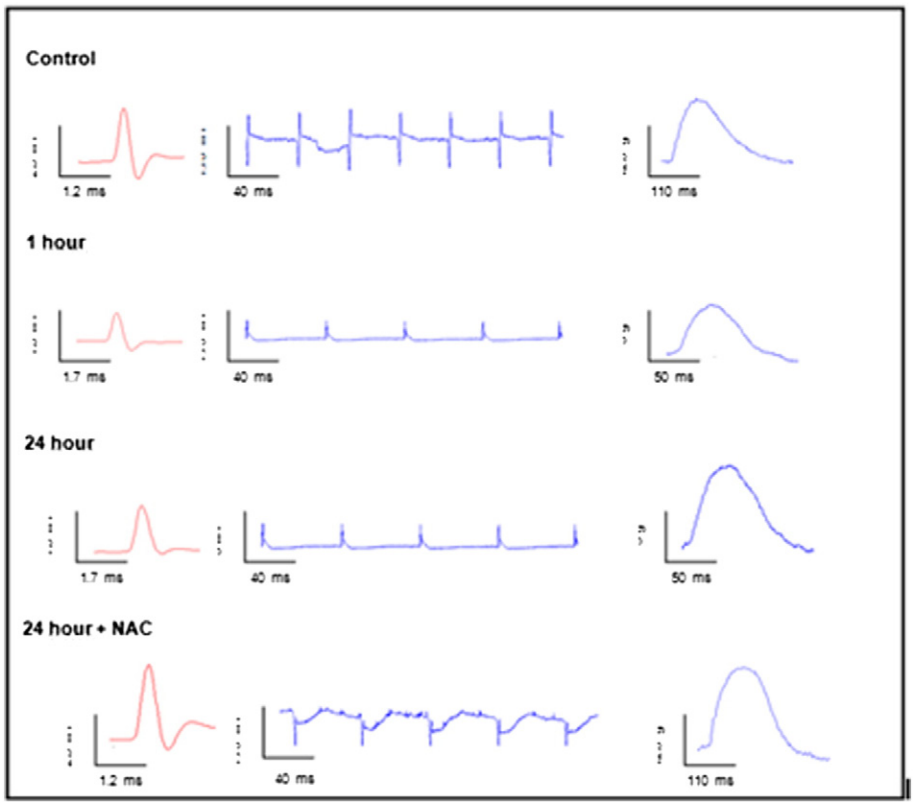

Fig. 1. Diaphragm muscle electromyography and phrenic nerve conduction study data. a) Phrenic nerve compound muscle action potentials. b) Diaphragm needle electromyography findings. c) Mechanical activation of diaphragm muscle.

temperature are needed. Small size of the groups is the second limitation of the present study. 6 rat pups were excluded during study due to sensitive, fragile body of newborn rats. Additionally, translating data from in vitro rat experiments to clinical application to human is difficult.

In conclusion, this study demonstrated that intestinal ischemia–reperfusion induced a diaphragmatic contractility dysfunction which is primarily caused by phrenic nerve neuropathy. NAC treatment ameliorated neuropathy and also diaphragmatic contractility dysfunction by inhibiting TNF-α.

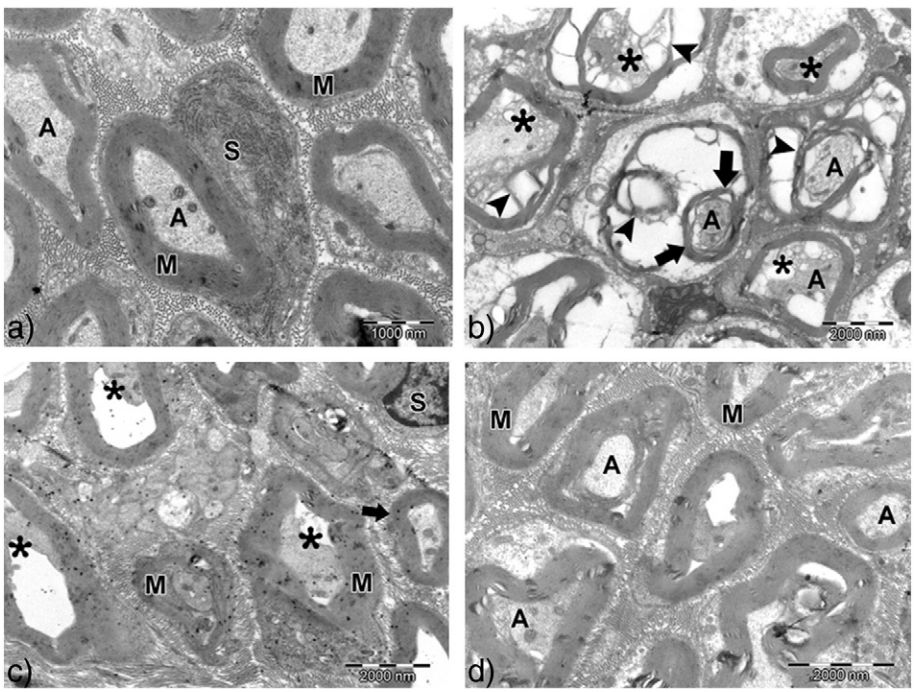

Fig. 2. Phrenic nerve electron microscopy. a) (control group) A: axon, M: myelin sheath, S: schwann cell. b) (1 h group) A: axon, *: axonal degeneration, arrow: degradation of lamellar myelin sheath and invagination to axons arrow head: myelin sheath degeneration. c) (24 h group) M: myelin sheath, S: schwann cell, *: axonal degeneration, arrow: protected areas of axonal and myelin structure. d) (24 h + NAC group) A: axon, M:myelin sheath.

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