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ORIGINAL ARTICLE

Is there a role for antioxidants in prevention of pulmonary hypoplasia in nitrofen-induced rat model of congenital diaphragmatic hernia?

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Abstract
Background/purpose Many studies suggest a role for antioxidants in the prevention of lung hypoplasia in nitrofen-induced rat models with congenital diaphragmatic hernia (CDH). This study investigates the oxidative status and the histological outcome of prenatal administration of vitamins E and C with synergistic effect, and effect of *N*-acetylcysteine (NAC) to improve lung maturation of nitrofen-induced rats.
Methods CDH was induced by maternal administration of a single oral dose of nitrofen on day 9.5 of gestation, and the Sprague–Dawley rats were randomly divided into five groups: nitrofen (N), nitrofen + vitamin C (NC), nitrofen + vitamin E (NE), nitrofen + vitamin C + vitamin E (NCE) and nitrofen + NAC (NNAC). A control group in which only vehicle was administered was included. Cesarean section was performed on day 21. Body weight (BW) and total lung weight (LW) of all fetuses with CDH were recorded; lung histological evaluation was performed, and protein content of lungs, determination of thiobarbituric acid reactive substances, and the protein carbonyls in tissue samples were determined.
Results A total of 133 rat fetuses with CDH were investigated. The body weight and the lung weight of fetuses of all groups that were exposed to nitrofen were significantly decreased than of the control group ($P < 0.05$). The animals exposed to nitrofen with different antioxidants showed increased protein levels in lung tissue. However, in the NCE and the NNAC groups, protein levels were significantly increased than in the others. Malondialdehyde levels significantly decreased in the NCE and the NNAC groups when compared with the NC and the NE groups. In addition, the NCE and NNAC groups decreased protein oxidation to control levels, and no significant difference was observed between control and these two antioxidants groups. The N, NC, NE and NNAC groups showed minimal improvement in lung histology; the NCE groups showed the most improvement in lung histology when compared with the other nitrofen plus antioxidant groups.
Conclusion Prenatal administration of NAC and vitamin E in combination with vitamin C represented the best effects to avoid oxidative damage and protein content of the lungs in rat pups with CDH at birth.

Keywords Nitrofen · CDH · Pulmonary hypoplasia · Antioxidant vitamins · *N*-acetylcysteine · Rat · Protein oxidation · Lipid peroxidation

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Introduction

Despite recent advances in neonatal care, congenital diaphragmatic hernia (CDH) remains a major clinical problem involving severe respiratory failure in newborns, and the mortality rate for CDH is still high [1]. The main cause of

this high mortality is pulmonary hypoplasia and pulmonary hypertension. Both lungs are small in size, and are associated with decreased alveolar branching, thickened arterioles and biochemical immaturity [2]. Fetal surgery, postnatal early surgical repair or other interventions such as high-frequency ventilation and extracorporeal membrane oxygenation have been used for improvement in prognosis of CDH recently, but still treatment of CDH remains difficult and challenging as no single treatment has yet proved to be very effective [3–6].

Previously, there was a well-established animal model of CDH and pulmonary hypoplasia involves exposing the pregnant rat to oxidant herbicide 2, 4-dichlorophenyl-*p*-nitrophenylether (nitrofen) induces in rat embryos [7, 8]. The mechanism of the action of nitrofen is not known completely. It was shown that early and late gestational lung underdevelopment is caused by nonmechanical and mechanical factors [7, 9]. A study indicates that the mechanism of the action of nitrofen could be intracellular oxidative stress and subsequent changes in the molecular signaling pathways [10]. In a recently described rat model of CDH, prenatal administration of antioxidant vitamins improved lung maturation [2, 11–13]. Moreover, we hypothesized that prenatal administration of antioxidant vitamins E and C would be more effective with synergistic effects to prevent lung hypoplasia. The aim of the present study was to investigate the preventive effect of antioxidant vitamins E and C alone, combination of these vitamins, and *N*-acetylcysteine (NAC) using morphometric and biochemical evaluation, various oxidative status parameters, and histological evaluation in lung hypoplasia induced by nitrofen.

Materials and methods

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Dicle University (approval no. 2005-45/2006).

Female 200–250 g Sprague–Dawley rats were mated with fertile male rats overnight, and vaginal smears were examined under light microscope. Observation of positive smears was considered day 0 of gestation. At 9.5 days of gestation (term, 22 days), 100 mg nitrofen (Riedel-de Haën, Germany) dissolved in 1 mL of olive oil was given intragastrically, and the animals were randomly divided into five groups: nitrofen (N), nitrofen + vitamin C (NC), nitrofen + vitamin E (NE), nitrofen + vitamin C + vitamin E (NCE) and nitrofen + NAC (NNAC). A control group (C) in which only vehicle was administered was included. A total of 150 IU Vitamin E (α-tocopherol, Sigma–Aldrich, Switzerland) was administered; each dose was dissolved in 1 mL of olive oil and given intragastrically in four doses on days 16–20 to the NE groups. A total of 150 IU Vitamin C (Ascorbic Acid, Merck, Germany) was administered; each dose was dissolved in 1 mL of distilled water and given intragastrically on the same days to the NC groups. To the NCE groups, the same doses of both vitamins were given by the methods explained above. To the NNAC groups, 800 mg/kg/day NAC (Asist, Adeka, Turkey) was given intraperitoneally in four doses on days 16–20. Cesarean section was performed on day 21, and the fetuses were weighed and examined internally for the presence of CDH. The lungs were dissected. The body weight (BW) and the total lung weight (LW) of all fetuses with CDH were recorded. The lungs of ten fetuses from each group were frozen at −80°C for further studies. Only the lungs of fetuses with CDH from the experimental groups were investigated.

Biochemical analysis

The lungs were removed immediately and stored at −80°C until further analysis. The lung tissue samples were homogenized with 120 mM KCl, 50 mM phosphate buffer, pH 7.4 (1:10 w/v). The homogenates were centrifuged at 700 *g* at 4°C for 10 min and the supernatant was kept at −20°C until use.

Determination of thiobarbituric acid reactive substances in the tissue samples

TBARS were determined calorimetrically [14]. Briefly, 1 mL of each sample was mixed with 1 mL of trichloroacetic acid (TCA) 10% and 1 mL of thiobarbituric acid (TBA) 0.67% and then heated in a boiling water bath for 15 min. Tubes were chilled on ice and the rose-colored trimethin-complex was extracted into 3 mL of *n*-butanol. The organic phase was separated by centrifugation for 10 min at 3,000 RPM; malondialdehyde (MDA) an intermediate product of lipoperoxidation was determined by absorbance at 535 nm. A standard curve for TBARS was prepared with 1,1,3,3-tetramethoxypropanol in a concentration range of 0.1–10 nmol.

Determination of protein carbonyls in tissue samples

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH); 0.5 mL of tissue homogenates was mixed with 2 mL dinitrophenylhydrazine (0.2% in 2.5 mol/L HCL) and incubated for 1 h at room temperature. Then 5 mL TCA solution (20%) was added to the samples for protein precipitation. The tubes were cooled in an ice bath for 10 min and subsequently centrifuged at 10,000×*g* for 5 min to collect the protein fraction. The protein pellets were washed once with 4 mL

TCA (10 g/L) and three times with 4 mL of a mixture of ethanol/ethyl acetate (1:1, v/v). The resulting protein precipitates were dissolved in guanidium HCl (6 mol/L in 20 mmol/L potassium phosphate, pH 2.3) and incubated for 10 min at 37°C. The carbonyl content was calculated from the peak absorbance 370 nm using an absorption coefficient (ε) of 22,000 [$(mol/L)^{-1}$ cm^{-1}] [15].

Protein assay

Total protein concentration was determined according to the method described by Lowry et al. [16] with bovine serum albumin as the standard.

Histological evaluation

The lungs were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin–eosin (H–E) and trichrom masson (T–M).

Statistical analysis

Data were expressed as the mean ± SD. Significant differences among the groups were analyzed by one-way ANOVA, and Duncan post hoc test was used if the results of ANOVA were significant. Statistical evaluation was carried out by using SPSS 15.0 (SPSS Inc., IL, USA) software package program. The results were considered significant, if the value of P was <0.05.

Results

Lung morphometry

A total of 133 rat fetuses with CDH were investigated. The body weight and the lung weight of fetuses of all groups that were exposed to nitrofen decreased significantly when compared with the control group ($P < 0.05$). In addition, there was no statistical significance among the nitrofen plus antioxidant groups (NC, NE, NCE and NNAC) and the nitrofen group (Fig. 1).

Protein content

An amount of 1 mg of protein/g of lung tissues in different groups is shown in Fig. 2. The nitrofen-exposed lungs alone had less protein than the control and the four antioxidant groups. The animals exposed to nitrofen with different antioxidants showed increased protein levels in lung tissue. However, in the NCE and the NNAC groups, protein levels were significantly increased than in the others ($P < 0.05$).

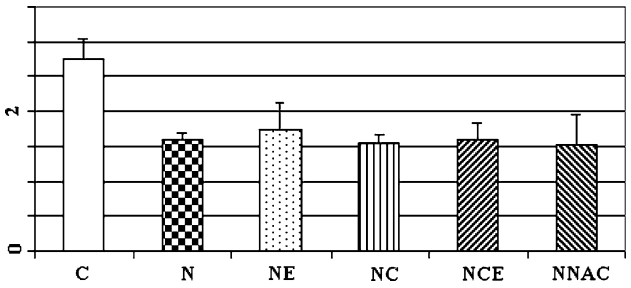

Fig. 1 Lw/Bw % of all groups (mean ± SD). There is no significance among nitrofen treated groups ($P > 0.05$). *C* contol *N* nitrofen, *NE* nitrofen + vitamin E, *NC* nitrofen + vitamin C, *NCE* nitrofen + vitamin C + vitamin E, *NNAC* nitrofen + *N*-acetylcysteine

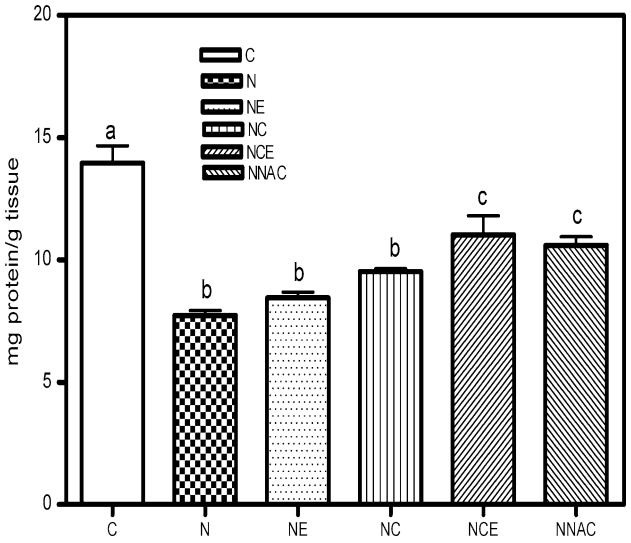

Fig. 2 Comparison of lung protein levels among groups. Data are expressed as mean ± SD of the mean of ten animals. Means with different letters at a time differ significantly, $P < 0.05$, while the values sharing common letters are not significantly different, at $P > 0.05$. *C* contol ($n = 10$), *N* nitrofen ($n = 10$), *NE* nitrofen + vitamin E ($n = 10$), *NC* nitrofen + vitamin C ($n = 10$), *NCE* nitrofen + vitamin C + vitamin E (n=10), *NNAC* nitrofen + *N*-acetylcysteine ($n = 10$)

Lipid peroxidation levels

The levels of TBARS as an indicator of lipid peroxidation are shown in Fig. 3. The MDA levels in nitrofen groups significantly increased when compared with the control and the nitrofen plus groups. However, MDA levels in the NCE and the NNAC groups significantly decreased than in the NC and the NE groups.

Protein oxidative damage

Figure 4 shows protein oxidative damage between the groups. The treatment with antioxidants acted positively in a significant manner. However, the NCE and the NNAC

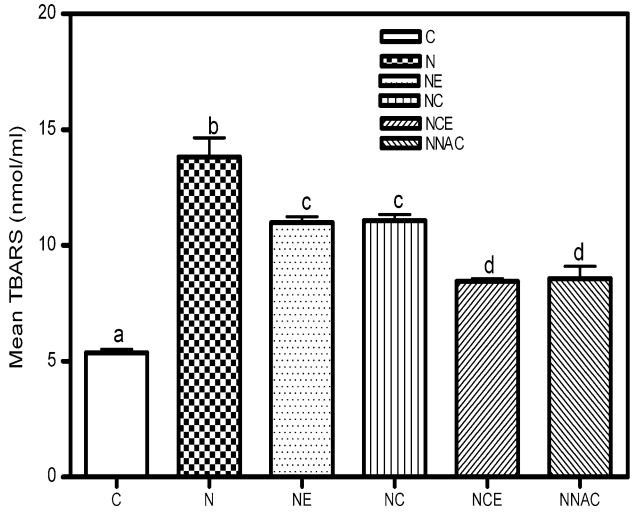

Fig. 3 Comparison of lung thiobarbituric acid reactive substances (TBARS) levels among groups. Data are expressed as mean ± SD of the mean of ten animals. Means with *different letters* at a time differ significantly, $P < 0.05$, while the values sharing common letters are not significantly different, at $P > 0.05$. *C* contol ($n = 10$), *N* nitrofen ($n = 10$), *NE* nitrofen + vitamin E ($n = 10$), *NC* nitrofen + vitamin C ($n = 10$), *NCE* nitrofen + vitamin C + vitamin E (n=10), *NNAC* nitrofen + *N*-acetylcysteine ($n = 10$)

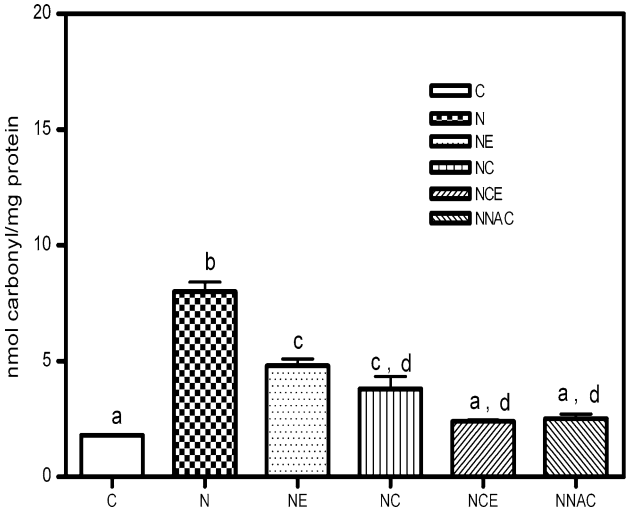

Fig. 4 Oxidative protein damage among the groups. Data are expressed as mean ± SD of the mean of ten animals. Means with *different letters* at a time differ significantly, $P < 0.05$, while the values sharing *common letters* are not significantly different, at $P > 0.05$. *C* contol ($n = 10$), *N* nitrofen ($n = 10$), *NE* nitrofen + vitamin E ($n = 10$), *NC* nitrofen + vitamin C ($n = 10$), *NCE* nitrofen + vitamin C + vitamin E (n=10), *NNAC* nitrofen + *N*-acetylcysteine ($n = 10$)

groups decreased protein oxidation to control levels. No significant difference was observed between control and these two antioxidant groups.

Histology

Histological findings of pulmonary hypoplasia as poorly formed saccules and thickened septal walls were seen in the nitrofen groups. Although the N, NC, NE and NNAC groups showed minimal improvement in lung histology, the NCE groups showed the most improvement in lung histology when compared with the other nitrofen plus antioxidant groups (Fig. 5).

Discussion

In an effort to reduce mortality of newborns with CDH, in utero surgical interventions such as open fetal surgery and tracheal occlusion have been proposed in fetal lambs and human conditions [6, 17]. However, many investigators proposed that prenatal pharmacological therapy, which is less invasive than in utero surgical intervention, has also improved pulmonary maturity in experimental studies [2, 11, 12, 18, 19]. The rat model of nitrofen-induced CDH is very useful for examining the effect of prenatal agents on hypoplastic fetal lungs. There are several agents used for testing the probability of favorable action of some antioxidant substances on lung hypoplasia associated with CDH. Glucocorticoid, dexamethasone, thyrotropin-releasing hormone (TRH), epidermal growth factor, and finally antioxidant vitamins (A, E and C) have been used prenatally to improve pulmonary maturation of fetal lungs in the nitrofen-induced CDH rat model [2, 11–13, 18–20]. All of the earlier studies reported that the antioxidant vitamins had beneficial effects on pulmonary hypoplasia [2, 11–13]. However, to our knowledge, none of the earlier studies reported the effect of the antioxidant vitamins in combination. Therefore, the current study is the first in vivo study to evaluate the effects of NAC and combination of vitamins C and E on nitrofen-induced CDH rat lungs.

It is known that reactive oxygen species (ROS) damage proteins, but due to rapid turnover of proteins they are considered to contribute less prominently to total cellular damage. It has been shown that oxidatively modified proteins accumulate during aging and in some pathological conditions [21]. The exposure of proteins to $^{\cdot}OH$, $O_2^{\cdot-}$ or both leads to gross structural modifications. These oxidatively modified proteins may undergo spontaneous protein fragmentation and cross-linking or exhibit a substantial increase in proteolysis. The principles of protein modification by ROS and the characterized reaction products of protein interactions with $^{\cdot}OH$ and $O_2^{\cdot-}$ are well established. In addition to fragmentation, the oxidation of lysine, arginine, proline and threonine residues may yield carbonyl derivatives. The presence of carbonyl groups has therefore been used as a marker for ROS-mediated protein oxidation [22]. The end products

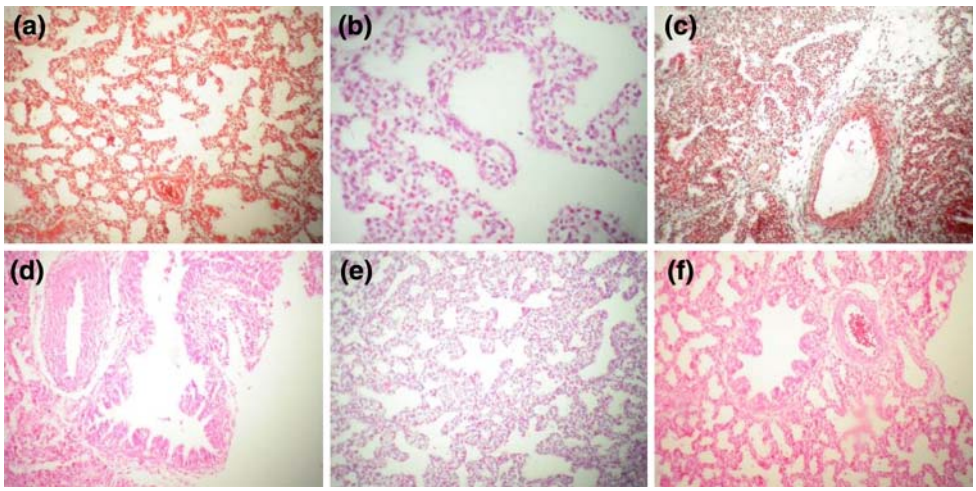

Fig. 5 Histological findings of lungs: **a** control animals (T–M, ×40). **b** Typical findings of pulmonary hypoplasia as poorly formed saccules and thickened septal walls were seen in nitrofen groups (H–E, ×40). **c–e** Findings of pulmonary hypoplasia were continued in nitrofen plus vitamin C, vitamin E, and NAC groups (H–E, ×40, T–M, ×80, T–M, ×80, respectively). **f** In nitrofen plus combined vitamin C and E groups showed well-differentiated saccules and thin septal walls like control group (H–E, ×40)

of lipid peroxidation such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) as well as products from polyunsaturated fatty acids cause protein damage [23]. TBARS as an indicator of lipid peroxidation, and protein carbonyl as an indicator of protein oxidation were evaluated in this study.

Antioxidants are a chemically diverse group of compounds sharing the property of preventing or reducing injury induced by singlet oxygen [24]. Multiple free radicals are generated ordinarily as products of oxygen metabolism. The most widely studied dietary antioxidants are vitamin C, vitamin E and beta-carotene. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids, as it is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E is a major lipid-soluble antioxidant, and is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation [25]. Gonzales-Reyes et al. [2] reported that administration of antioxidant vitamins in the last 5 days of gestation to mothers' rats of fetuses with CDH was accelerated lung development and maturation. In their study, all vitamins were administered to mother rat alone. One useful concept related to antioxidants is synergism. Synergism is, in general, the phenomenon in which a number of compounds, when present together in the same system, have a more pronounced effect than that which would be derived from a simple additive concept [25]. Moreover, we hypothesized that the combination of vitamin C and vitamin E might be more effective to accelerate pulmonary maturity. Therefore, vitamin C and vitamin E were used both alone and combined in the current study. Our study showed that vitamin C or E supplementation alone is less effective than combined vitamin C and E. Although in the NCE groups LW/BW% did not significantly increase than in the nitrofen groups, other values (protein content and TBARS) and histological findings showed that the combination use of vitamins was more effective than vitamin alone groups. In addition, protein oxidative damage in the NCE groups was also lesser than vitamin alone groups. In many studies, another widely used antioxidant is NAC [10, 26, 27]. NAC is a glutathione precursor and direct antioxidant. NAC is easily deacetylated to form cysteine, which efficiently supports glutathione biosynthesis [28]. As a potent antioxidant, NAC directly scavenges hydrogen peroxide, hydroxyl free radicals, and hypochloric acid in vitro [29]. In this study, NAC was chosen as an antioxidant for prenatal treatment. Fisher et al. [10] reported that antioxidant NAC administration has accelerated fetal pulmonary growth in vitro. In their study, after nitrofen-induced CDH model fetal lungs were harvested on day 13.5 and placed in organ culture and the experimental group received NAC in vitro. They concluded that NAC stimulates nitrofen-induced hypoplastic fetal lung growth. The result of the current study has shown that NAC decreased oxidative damage, and increased protein content on nitrofen-induced CDH lungs in vivo.

In conclusion, serious oxidative damage was done in fetus' lungs of nitrofen-induced CDH rats. All antioxidants which were used for prenatal treatment in this study had beneficial effect of this condition. However, prenatal administration of NAC and vitamin E in combination with vitamin C showed the best effects to avoid oxidative damage and protein content of the lungs in rat pups with CDH at birth. We speculate that our findings of prenatal antioxidant therapy in rat fetuses extend to CDH patients as well. If that hypothesis is correct, prenatal administration of the combination of vitamin E and vitamin C will offer better prospects for randomized trials in prenatally diagnosed children with CDH than the administration of vitamin alone.

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