3-*n*-Butylphthalide reduces the oxidative damage of muscles in an experimental autoimmune myositis animal model

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Abstract. 3-*n*-Butylphthalide (NBP) protects the mitochondria and reduces apoptosis in multiple disease models. However, it remains to be determined whether NBP can protect muscle cells from oxidative stress, lipid peroxidation and apoptosis in myositis. In the present study, a myosin immunization protocol was applied to induce experimental autoimmune myositis (EAM) in guinea pigs. After 4 weeks, a low- or high-dose NBP solution was injected intraperitoneally into the guinea pigs, with saline solution serving as the negative control. After 10 days, the guinea pigs were sacrificed and muscle cells were isolated for analysis. The results revealed that NBP increased the superoxide dismutase and catalase activity, and reduced malondialdehyde activity in the EAM model. Furthermore, NBP enhanced ATPase activity in muscle mitochondrial membranes and muscle fiber membranes, reduced the number of apoptotic cells, and differentially regulated the Bcl-2, Bax and BAD mRNA and protein expression levels in muscle tissues and sera. NBP directly protects muscle mitochondria and muscle cells from oxidative damage. Notably, NBP reduced muscle cell apoptosis. Thus, it is speculated that, as an antioxidant treatment, NBP may benefit individuals with myopathy.

Introduction

Inflammatory muscle diseases or idiopathic inflammatory myopathies (IIMs) are systemic connective diseases that consist of three major heterogeneous categories: Polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM). IIMs are characterized by proximal and symmetric muscle weakness, and chronic muscle inflammation (1). The etiology of IIMs remains unclear, however, it has been hypothesized that IIMs result from immune-mediated damage to muscles in genetically susceptible individuals and are triggered by environmental factors or infectious agents (2). The immune involvement in IIMs is supported by the presence of multiple immune cells in muscle tissues, including T cells, B cells, macrophages and dendritic cells, and of associated myositis-specific autoantibodies in IIM patients (3). Currently, immunotherapy for IIMs primarily involves corticosteroids with frequent addition of immunosuppressive drugs; however, the effectiveness of these therapies is limited by side effects (4,5).

Skeletal muscle is an organ with high mitochondrial content. Mitochondrial disorder manifests as a pathology of IIMs and may provide evidence for the underlying pathogenic mechanisms (6). The mitochondria are essential organelles that are indispensable for normal cell processes, such as cell proliferation and programmed cell death (7). Mitochondrial respiration not only generates cellular energy, but is also the main source of reactive oxygen species (ROS) in most tissues (8). In normal cells, a low amount of ROS and other free radicals, including superoxide anions, nitric oxide (NO) and hydrogen peroxide (H_2O_2), are beneficial to cells and are involved in physiological redox signaling and cell function (9). However, with mitochondrial dysfunction, high concentrations of ROS results in oxidative stress, which damages biological macromolecules and eventually causes cell death (7). For instance, oxidative stress-induced lipid peroxidation can cause DNA damage and directly inhibit various proteins, such as Na^+/K^+ ATPase and glutamate transporters (10). Malondialdehyde (MDA), the final product of lipid breakdown caused by oxidative stress, is a useful biomarker for oxidative damage, as well as a prognostic maker for septic patients (11,12). Nevertheless, enzymatic and non-enzymatic antioxidant defenses exist to remove free radicals in cells. Superoxide dismutase (SOD) is the only eukaryotic antioxidant enzyme that catalyzes the dismutation of highly reactive superoxide anions to O_2 (13). Catalase (CAT) is another common antioxidant enzyme that protects cells from H_2O_2 and reduces the oxidative stress in mitochondria (14). ROS and antioxidant ratios can be used to evaluate muscle damage.

3-*n*-Butylphthalide (NBP) is a synthetic botanical medicine that is commonly used in China for the treatment of ischemic stroke (15). Previous studies have suggested multiple neuroprotective mechanisms of NBP in brain damage, including

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reducing oxidative stress and lowering lipid peroxidation (16), preventing neuronal apoptosis (17,18), and inhibiting neutrophil infiltration (19). In a cellular model of Parkinson's disease, NBP ameliorated ROS production and attenuated mitochondrial transmembrane potential loss (20). However, whether or not NBP can protect muscle cells remains unclear. Therefore, in the present study, guinea pigs were immunized with rabbit myosin to induce experimental autoimmune myositis (EAM) (21). The present study results revealed that NBP treatment significantly enhanced the mitochondrial SOD and CAT activity levels in injured muscle cells and reduced muscle cell apoptosis. Thus, NBP may be useful for in the treatment of IIM.

Materials and methods

Animals. Healthy albino Dunkin-Hartley English short-haired female guinea pigs (n=40; age, 6-8 weeks; weight, 250-300 g) were purchased from the Laboratory Animal Center, Academy of Military Medical Sciences (Beijing, China). The animals were bred at the Fengtai Facility of the Laboratory Animal Center, Academy of Military Medical Science. The indoor temperature was 18-22˚C and humidity was 45-60%. The animals were kept in a 12-h light/dark cycle. Noise, cleanliness, and other breeding environments followed the national standards. The guinea pigs were fed a special lab diet, and were intermittently supplied with fresh cabbage for vitamin C. They were anesthetized with 1% pentobarbital (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany, 0.5 ml/100 g) for experiments. The study was approved by the Animal Ethics Committee of the People's Liberation Army General Hospital and Academy of Military Medical Sciences. All the procedures involving laboratory animals were performed according to the National Institutes of Health (NIH) standards for the care and use of animals.

Reagents. Purified rabbit myosin (M1636), complete Freund's adjuvant (CFA; F5881; containing 1 mg/ml *Mycobacterium tuberculosis* powder) and pertussis toxin (P7208) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Mycobacterium tuberculosis* (Mtb) toxin was obtained from the Tuberculosis Research Center of the People's Liberation Army 309 Hospital (Beijing, China), and 600 mg inactivated bacteria were diluted in 5 ml normal saline with repeated freezing and thawing. NBP (1 g/ml) was provided by CSPC Pharmaceutical Co., Ltd. (Shijiazhuang, China). A Bradford protein assay kit (PA102) was purchased from Tiangen Biotech Co., Ltd. (Beijing, China), and 50% acetic acid was obtained from the Material Supply Center at the Academy of Military Medical Sciences. TRIzol reagent, RevertAid First-Strand cDNA Synthesis kit (cat. nos. K1621 and K1622) and Maxima SYBR Green/ROX qPCR master mix (2X, cat. no. K0222) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Tween-80 and ELISA kits for B-cell lymphoma-2 (Bcl-2, cat. no. KN-E92878), Bcl-2-associated X protein (Bax, cat. no. KN-E92877) and Bcl-2-associated death promoter (BAD, cat. no. KN-E92879) were obtained from Kainuo Bio, Ltd. (Beijing, China). A DeadEnd™ Colorimetric TUNEL system was purchased from Promega Corp. (Madison, WI, USA).

Myosin immunization and NBP treatment. The myosin immunization protocol used in the present study was performed as described previously (22,23). Briefly, 0.3 μl purified rabbit myosin (10 mg/ml), 0.1 ml CFA and 10 μl Mtb toxin (120 mg/ml) were added to 0.2 ml of saline and vortexed into a white emulsion. The emulsion (0.3 ml) was then injected subcutaneously into the animal backs at multiple sites, once a week for a total of four immunizations. During the first two immunizations, each guinea pig was also injected intraperitoneally with 200 μl of 500 ng pertussis toxin (50 μg/500 μl stock solution diluted 40-fold with normal saline to obtain a final concentration of 0.0025 μg/μl).

NBP solution was prepared as described previously (24-26). Briefly, Tween-80 was dissolved in normal saline to obtain a final concentration of 0.5%. Next, 0 μl, 40 μl (40 mg) and 80 μl (80 mg) NBP were added to 10 ml Tween-80 to prepare the NBP solutions for the saline, low-dose and high-dose groups, respectively. After 4 weeks of EAM induction, NBP solution was injected intraperitoneally into the guinea pigs twice a week for 2 weeks. At 10 days after the last NBP or saline injection, the animals were sacrificed for analysis.

Preparation of muscle tissue homogenates. To prepare muscle samples for analysis, 0.2-1 g muscle tissues were washed in cold normal saline to remove the blood, dried on filter paper, weighed and placed in 10-ml Eppendorf tubes. The tissues were minced with ophthalmic scissors and quickly transferred to a small mortar on ice. The volume of 0.9% sodium chloride injection was then calculated, which was nine times the weight of the tissue blocks. Two-thirds of the injection solution was directly added to the mortar and the remaining one-third was added after flushing the residual tissues in the Eppendorf tube. Tissues were ground repeatedly with a pestle for 10-15 min until they were fully homogenized. The homogenized tissues were then transferred to a new Eppendorf tube with a pipette, and centrifuged at 700 x g at 4˚C for 10-15 min. Following centrifugation, the collected supernatant was diluted with 0.9% sodium chloride to obtain a suspension with 10% muscle tissue homogenate.

Preparation of skeletal muscle cell membrane (27). The 10% muscle tissue homogenate was centrifuged at 1,500 x g at 4˚C for 10 min, then the supernatant was centrifuged at 8,000 x g at 4˚C for 60 min. The collected precipitate was centrifuged at 10,000 x g at 4˚C for 60 min after being suspended with 0.05 mol/l Tris-HCL (pH 7.4). The collected precipitate was suspended in 1.2 ml of 0.05 mol/l Tris-HCL (pH 7.4) and preserved at -20˚C for ATPase detection, the supernatant was collected following centrifugation at 350 x g at 4˚C for 5 min.

Mitochondrial isolation. To isolate mitochondria, the 10% muscle homogenate was centrifuged at 700 x g at 4˚C for 10 min, and then the supernatant was centrifuged at 8,000 x g at 4˚C for 15 min to precipitate the mitochondria. The isolated mitochondria were suspended in ice-cold homogenization medium (0.01 M Tris-HCl, 0.0001 M EDTA-2Na, 0.01 M sucrose and 0.8% NaCl), homogenized by hand or repeatedly frozen and thawed (to lose partial enzyme activity). The fragmented mitochondrial suspension was then used for further assays.

Bradford protein assay. The protein was quantified in muscle tissue homogenates or mitochondrial suspensions using the Bradford assay. First, Coomassie blue solution was equilibrated to room temperature and 1 mg/ml of bovine serum albumin (BSA) was sequentially diluted and used as a standard solution. Each sample was placed in a 96-well plate at 10 μl/well, and each sample was assayed in duplicate. Then, 200 μl of Coomassie blue was added to each well, mixed, and left at room temperature for 5-10 min. The optical density (OD) at 595 nm was measured with a spectrophotometer (T6 new century; Beijing Purkinje General Instrument Co., Ltd.) and quantified using a standard curve; a sample without BSA was used as the blank.

ELISA assay for determination of Bcl-2, Bax and BAD proteins. According to the ELISA kit instructions, 50 μl standard protein or 10 μl of each sample with 40 μl dilution buffer were added to each well of an antibody-pre-coated plate. The plates were then incubated for 1 h at room temperature, washed three times with buffer, followed by addition of 100 μl horseradish peroxidase (HRP)-conjugated antibodies to the wells. The plate was then sealed with a closure plate membrane and incubated at 37˚C for 60 min. Blank wells were incubated without protein and antibodies. The plate was washed five times, patted dry with absorbent papers and allowed to sit for 1 min with washing buffer. Next, 50 μl substrate A and 50 μl substrate B were added to each well, and the plate was incubated in the dark at 37˚C for 15 min, followed by addition of 50 μl stop solution to each well. After 15 min, the OD values at 450 nm were the measured and quantified.

RNA purification and cDNA synthesis. Fresh muscle tissues were weighed, immersed in liquid nitrogen and powdered in a mortar. Next, 50-100 mg tissues were added to 1 ml TRIzol reagent in a 1.5-ml Eppendorf tube, and the tubes were incubated at room temperature for 5 min. Subsequently, 0.2 ml chloroform was added to the lysed tissues, and the tube was shaken for 15 sec and incubated for 2-3 min at room temperature. Following centrifugation at 12,000 x g at 4˚C for 15 min, small quantities of the upper aqueous phase were extracted several times. Isopropyl alcohol (100%; 4˚C) was added to the aqueous solution in a new Eppendorf tube, mixed and incubated at room temperature for 10 min. The sample was then centrifuged at 12,000 x g for 10 min at 4˚C, and the supernatant was discarded. Next, the sample was suspended in 1 ml of 75% ethanol, and the RNA was precipitated by centrifugation at 7,500 x g for 5 min at 4˚C and air-dried in a sterile hood. The RNA was dissolved in RNase-free water, divided into aliquots and stored at -80˚C. The absorbance of the obtained RNA samples was measured at 260 and 280 nm via ultraviolet spectroscopy to determine the concentration of RNA, as follows: RNA concentration (μg/ml)=OD_{260} x fold dilution x 40 μg/ml. Samples with >1 μg/μl RNA and OD_{260}/OD_{280} ratios ranging from 1.9-2.0 were used in subsequent experiments.

For cDNA synthesis, 0.1-5 μg total RNA was mixed with 1 μl random primers and added to nuclease-free water (final volume, 12 μl). Next, 4 μl of 5X reaction buffer, 1 μl RiboLock RNase inhibitor (20 U/μl), 2 μl of 10 mcM dNTP mix and 1 μl RevertAid M-MuLV reverse transcriptase (200 U/μl) were added to obtain a reverse transcription reaction volume of 20 μl. This was mixed gently and centrifuged at 200 x g at room temperature for 1 min. The mixture was incubated for 5 min at 95˚C, followed by 60 min at 42˚C. The reaction was terminated by heating at 70˚C for 5 min. The transcripts were directly used in polymerase chain reaction (PCR) analysis, stored at -20˚C for 1 week or stored long-term at -70˚C.

Detection of Bax, Bcl-2 and BAD by PCR. Primers for Bax, Bcl-2 and BAD were designed after searching the Pubmed gene coding regions, and they were confirmed by BLAST (http://blast.ncbi.nlm.nih.gov/). The primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). Fragment lengths of the PCR products of Bax, Bcl-2 and BAD were 182, 208 and 179 bp, respectively. The primer sequences were as follows: Bax (GenBank no. NT-176349) forward, 5'-CCTTTTGCTTCAGGGGATGA-3', and reverse, 5'-TGATCAGCTCGGGTACCTTG-3'; Bcl-2 (GenBank no. NT-176405) forward, 5'-GGTGAACTGGGGGAGGATTG-3', and reverse, 5'-AAACAGAGGCCGTACACTGG-3'; BAD (GenBank no. NT-176377) forward, 5'-TGAAAGACCCCGATTCCAGC-3', and reverse, 5'-AGCGTCACCTTAGAAAGGGC-3'. GAPDH was used as an internal control: Forward, 5'-GCCGCATCGGTATTCCTTCT-3', and reverse, 5'-GCGTCCAATACGGCCAAATC-3'. The PCR conditions were as follows: 95˚C for 10 min; 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec; final extension at 72˚C for 30 sec; and a melt curve between 55 and 95˚C with 0.5˚C increments for 30 min. A quantitative PCR instrument was used and Bio-Rad IQ5 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was employed for quantification analysis.

Serum preparation. Blood was collected from the heart via an injection needle, and 5-10 ml of blood was transferred to a separation gel pro-coagulant vacuum blood collection tube, and the tube was immediately inverted gently 3-5 times to mix the blood thoroughly with the coagulant. After the blood solidified (~18 min), the separation gel tube was centrifuged at 1,000-1,200 x g for 5-10 min to obtain the serum. Isolated serum was placed in 1.5-ml Eppendorf tubes and stored at -80˚C.

SOD in muscle mitochondria and serum. The procedure followed in the present study to determine the SOD content in the mitochondria and serum was according to instructions of the SOD kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In this reaction, the amount of enzyme that inhibited 50% SOD corresponded to one SOD activity unit (U). The formula used to determine the SOD inhibition was as follows: SOD inhibition rate=[(control A-control A_{blank})-(sample A-sample A_{blank})]/(control A-control A_{blank}) x100%. Subsequently, the SOD activity in the mitochondria samples was calculated as follows: Tissue SOD activity (U/mg)=SOD inhibition rate ÷ 50% x (reaction volume 0.24 ml/fold dilution 0.02 ml)/sample protein concentration (mg/ml). Similarly, SOD activity in the serum was calculated using the following equation: Serum SOD activity (U/ml serum)=SOD inhibition rate ÷ 50% x (reaction volume 0.24 ml/fold dilution 0.02 ml) x serum dilution ratio.

MDA measurement in muscle mitochondria and sera. MDA is a degradation product of lipid peroxidation that integrates with

thiobarbituric acid to form a red product (maximum absorption, 532 nm) (28). The detailed MDA assay in the tissue and serum samples was conducted as per the instructions of the MDA kit (Nanjing Jiancheng Bioengineering Institute). The MDA content in muscle mitochondria was calculated as follows: Tissue MDA content (nmol/mg)=[(OD_{sample}-$OD_{control}$)/($OD_{standard}$-OD_{blank})]x[standard concentration/sample protein concentration (nmol/mg)]. Similarly, the MDA content in the serum was determined as follows: Serum MDA content (nmol/ml)=[(OD_{sample}-$OD_{control}$)/($OD_{standard}$-OD_{blank})]x [standard concentration x sample fold dilution]. A standard concentration of 10 nmol/ml was used in the calculations.

CAT activity in muscle mitochondria and serum. Procedures were performed according to the instructions of the CAT kit (Nanjing Jiancheng Bioengineering Institute) in order to determine the CAT content in the mitochondria and serum of the guinea pigs. Addition of molybdate (Nanjing Jiancheng Bioengineering Institute) rapidly terminated the decomposition of H_2O_2 by catalase (CAT). The remaining H_2O_2 interacted with ammonium molybdate to produce a pale yellow complex, which was measured at 405 nm to calculate the CAT activity. One unit of CAT activity was defined by the decomposition of 1 μmol/l H_2O_2 per second per ml of protein. CAT content in the mitochondria was calculated as follows: Tissue CAT content (U/mg)=($OD_{control}$-OD_{sample}) x 271/[60 x sample volume x protein concentration (mg/ml)]. Similarly, the serum CAT content (nmol/ml) was measured as follows: ($OD_{control}$-OD_{sample}) x sample fold dilution x 271/(60 x sample volume).

Ca^{2+}-Mg^{2+} ATPase and Na^{+}-K^{+} ATPase assays. According to the instructions of the ATPase kit (Nanjing Jiancheng Bioengineering Institute), 1 unit of ATPase activity was defined as the decomposition of ATP producing 1 μmol inorganic phosphorus per hour per mg protein. The ATPase activity in skeletal muscle cell membrane and mitochondria was calculated as follows: [OD_{sample}-$OD_{control}$)/$OD_{standard}$ x standard concentration (1 μmol/ml) x sample fold dilution x 6/protein concentration (mg/ml).

TUNEL assay. A TUNEL assay was conducted according to the kit instructions (cat no. G7130, Promega Corp.). Briefly, slides with sliced muscle tissues were fixed in 4% paraformaldehyde for 15 min at room temperature. After washing twice with phosphate-buffered saline (PBS), the slides were incubated with 100 μl of 20 μg/ml proteinase K for 10-30 min at room temperature, followed by incubation with PBS for 5 min and then 4% paraformaldehyde for 5 min at room temperature. Next, slides were washed with PBS and placed in 100 μl equilibration buffer for 5-10 min at room temperature. Subsequently, 100 μl biotinylated nucleotide mixed with rTdT enzymes was added to the slides, and incubated at 37˚C for 60 min. The slides were washed twice with saline sodium citrate and PBS, followed by incubation with 0.3% H_2O_2 for 3-5 min at room temperature. Finally, HRP and DAB were added to stain the apoptotic cells. The slides were mounted with a coverslip for microscopic observation, and TUNEL-positive cells were defined as cells presenting brown or sepia nuclei. The apoptosis index was calculated as follows: Positive cell number/total cell number x 100%.

Statistical analysis. Data are represented as the means ± standard deviation. SPSS version 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for one-way analysis of variance, least significant difference test and Dunnett's t-test.

Results

NBP enhances SOD and CAT activities in muscle mitochondria and serum. The antioxidant function of NBP in injured muscle cells in EAM was initially measured. The group that received high-dose NBP treatment had significantly higher SOD and CAT activities in their muscle mitochondria compared with the saline-treated group (P<0.05; Fig. 1A). In addition, the saline and low-dose NBP groups had significantly lower SOD and CAT activities compared with the control group (P<0.01 and P<0.05, respectively; Fig. 1A). There were no significant differences in the SOD and CAT activities between the low- and high-dose NBP groups, between the high-dose NBP and the normal control groups, and between the saline and low-dose NBP groups (Fig. 1A). These results indicated that high-dose NBP may enhance or normalize the antioxidant activity in injured muscle cells.

Similarly, the serum SOD and CAT activities were significantly higher in the high-dose NBP group as compared with the saline group (P<0.05 and P<0.01, respectively; Fig. 1B). Furthermore, the serum SOD and CAT activities were significantly lower in the saline and low-dose NBP group in comparison with the control group (P<0.01 and P<0.05, respectively; Fig. 1B). No significant differences were observed in the serum SOD and CAT activities between the low- and high-dose, high-dose NBP and normal controls, and the saline and low-dose NBP groups (Fig. 1B). Thus, NBP had a dose-dependent effect on the SOD and CAT activities in EAM animals.

NBP reduces MDA in muscle mitochondria and serum. Next, the MDA levels were measured in order to study the role of NBP in lipid peroxidation. As shown in Fig. 2A, in muscle mitochondria, the MDA level in the low- and high-dose NBP groups was significantly lower compared with that in the saline group (P<0.05 and P<0.01, respectively). However, the MDA level was significantly higher in the low-dose NBP and saline groups than in the controls (P<0.05 and P<0.01, respectively). There was no significant difference in the MDA level between the low- and high-dose NBP groups or between the high-dose NBP and the normal control group.

Similarly, the serum MDA levels (Fig. 2B) were lower in the low- and high-dose NBP groups compared with the saline group (P=0.056 and P<0.05, respectively), but did not differ between the two NBP groups. When compared with the control, the MDA level was evidently higher in the saline group (P<0.01), but did not differ significantly in the low-dose and high-dose NBP groups. Thus, NBP can protect against lipid peroxidation and reduce the MDA levels in injured muscles.

ATPase activity in muscle mitochondrial membranes and muscle fiber membranes. Na^{+}-K^{+} ATPase and Ca^{2+}-Mg^{2+} ATPase maintain the plasma membrane potential and calcium sequestration. ATPase is sensitive to peroxidative reactions due

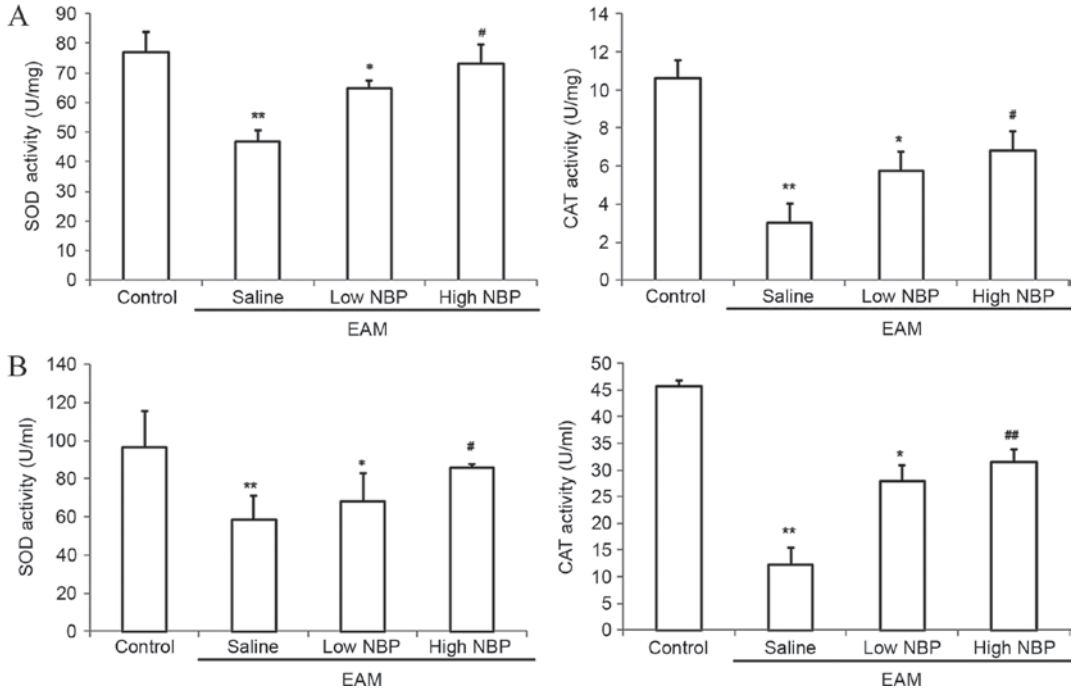

Figure 1. Effects of NBP on SOD and CAT activities in the (A) muscle mitochondria and (B) serum of guinea pigs in the control and three EAM groups, treated with saline, low-dose NBP (40 mg/kg body weight) and high-dose NBP (80 mg/kg body weight). Samples were obtained on day 10 after treatment and measured by ELISA. Values are presented as the mean ± standard deviation (10 animals per group; analyzed in duplicate). *P<0.05 and **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. saline group (Dunnett's method). NBP, 3-*n*-butylphthalide; EAM, experimental autoimmune myositis; SOD, superoxide dismutase; CAT, catalase.

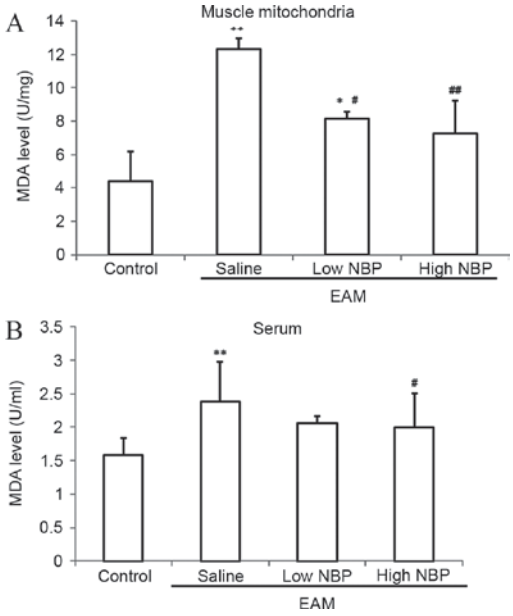

Figure 2. Effects of NBP on MDA levels in the (A) muscle mitochondria and (B) serum. Samples were obtained on day 10 after treatment from guinea pigs in the control and three EAM groups, treated with saline, low-dose NBP (40 mg/kg body weight) and high-dose NBP (80 mg/kg body weight), and were measured by ELISA. Values are represented as the means ± standard deviation (10 animals per group; analyzed in duplicate). *P<0.05 and **P<0.01 vs. the control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the saline group (Dunnett's method). NBP, 3-*n*-butylphthalide; EAM, experimental autoimmune myositis; MDA, malondialdehyde.

to its association with the plasma membrane (29). NBP treatment increased the ATPase activity in muscle mitochondrial membranes (Fig. 3A), as well as in muscle fiber membranes (Fig. 3B) compared with the saline group. Fig. 3 shows higher Na^{+}-K^{+} and Ca^{2+}-Mg^{2+} ATPase activities in the high-dose NBP group compared with the saline group in muscle mitochondrial membranes and muscle fiber membranes (P<0.05 for both). The Na^{+}-K^{+} and Ca^{2+}-Mg^{2+} ATPase activities were significantly lower in the saline (P<0.01 for both), low-dose NBP (P<0.05 and P<0.01, respectively) and high-dose NBP groups (P<0.05 for both) in comparison with the control group in the mitochondrial and fiber membranes.

Apoptosis of muscle cells. In apoptotic cells, DNA endonucleases are activated to break down genomic DNAs. The 3'-OH end of broken DNAs can be detected using TUNEL assay. Among atrophic muscle cells, the number of TUNEL-positive apoptotic cells increased in the saline group compared with the control group (Fig. 4A and B). NBP treatment evidently reduced apoptosis-positive cells compared with the saline group; however, the number of apoptosis-positive cells in the NBP group remained higher than that in the control group. There was no evident difference between the low-dose (Fig. 4C) and high-dose (Fig. 4D) NBP groups. Thus, the present results revealed that NBP evidently reduced apoptotic cells in the EAM model.

BAD, Bax and Bcl-2 mRNA expression in muscle tissue. Apoptosis is strictly regulated by a group of proteins. Pro-survival proteins, such as Bcl-2 and Bcl-xL, inhibit apoptosis, whereas pro-apoptotic proteins, including BAD and Bax, initiate the apoptotic process (30). NBP may affect the expression of these apoptosis-associated proteins to regulate muscle cell apoptosis. In the present study, it was observed that the BAD, Bax and Bcl-2 mRNA expression levels in muscle

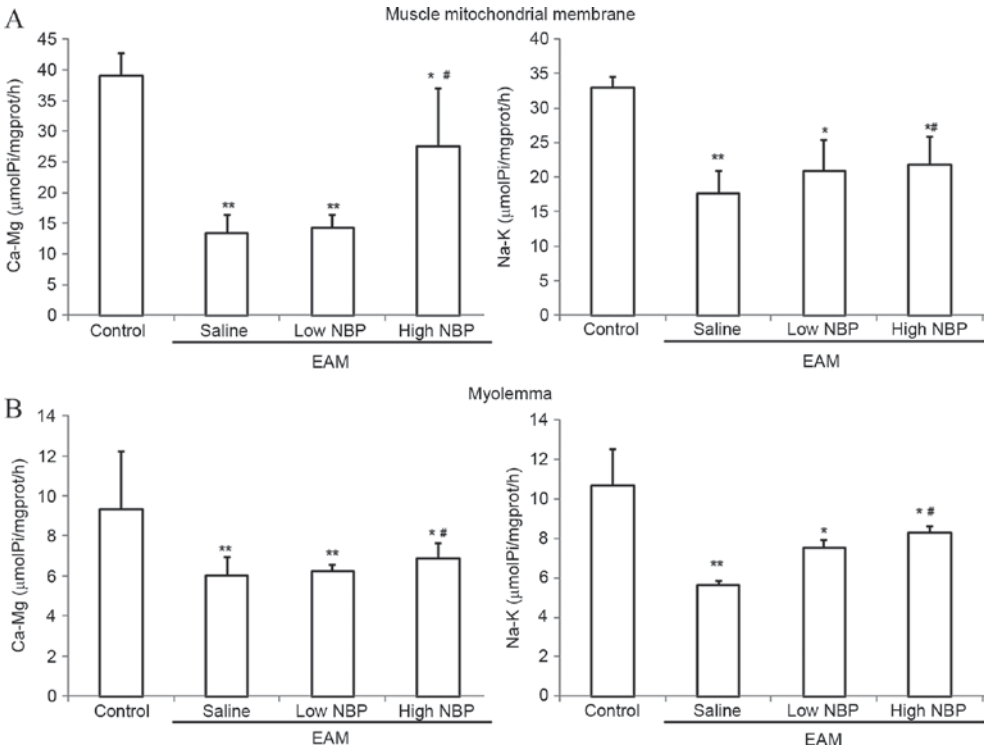

Figure 3. Effects of NBP on Ca^{2+}-Mg^{2+} and Na^{+}-K^{+} ATPase activities in the (A) muscle mitochondrial membrane and (B) myolemma. Samples were obtained on day 10 after treatment from guinea pigs in the control and three EAM groups, treated with saline, low-dose NBP (40 mg/kg body weight) and high-dose NBP (80 mg/kg body weight), and were measured by ELISA. Values are represented as the means ± standard deviation (10 animals per group; analyzed in duplicate). *P<0.05 and **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the saline group (Dunnett's method). NBP, 3-*n*-butylphthalide; EAM, experimental autoimmune myositis.

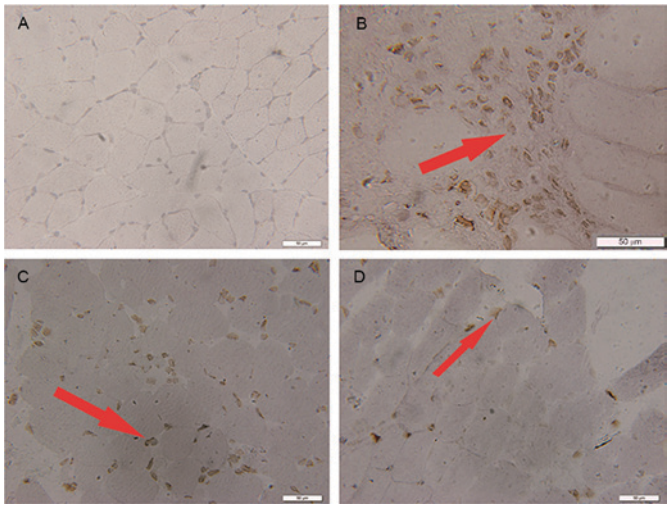

Figure 4. Effects of NBP on TUNEL staining of apoptotic cells. Samples were obtained on day 10 after treatment and frozen muscle sections were stained with TUNEL. Levels of apoptotic cells are shown for the (A) control, (B) EAM + saline. (C) EAM + low-dose NBP (40 mg/kg body weight) and (D) high-dose NBP (80 mg/kg body weight) groups. Arrows indicate brown circular nuclei (TUNEL positive cells). Magnification, x200. NBP, 3-*n*-butylphthalide; EAM, experimental autoimmune myositis.

tissue were significantly higher in the saline group than in the control group (P<0.01; Fig. 5A-C). However, their expression in the muscle tissue was significantly lower in the NBP-treated groups compared with in the saline group (P<0.01; Fig. 5A-C). Furthermore, the BAD, Bax and Bcl-2 mRNA expression levels in the low-dose NBP group were significantly lower compared with the saline group (P<0.01; Fig. 5A-C). Compared with the control, mRNA expression in the low-dose NBP group was significantly higher for Bax (P<0.05), but no significant difference was observed for BAD (P=0.065) and Bcl-2 (P=0.087). In the high-dose NBP group, the BAD, Bax and Bcl-2 mRNA expression levels were significantly lower compared with the saline group (P<0.01), but did not differ when compared with the low-dose NBP or control groups (Fig. 5A-C).

The Bax/Bcl-2, BAD/Bcl-2 and BAD/Bax mRNA ratios in muscle tissue were lower in the NBP group compared with the saline group (P<0.05; Fig. 5D-F), suggesting that BAD and Bax declined to a greater extent than Bcl-2. In addition, Bax declined to a lower extent than BAD. Thus, the protein decline was in the order Bcl-2 <Bax< BAD. Furthermore, the Bax/Bcl-2, BAD/Bcl-2 and BAD/Bax mRNA ratios in muscle tissue were higher in the saline group compared with the control group (P<0.05), indicating that the BAD and Bax levels increased to a greater extent than Bcl-2, and that BAD mRNA increased to a greater extent than Bax. The order of these protein modifications in the saline group were as follows: BAD >Bax> Bcl-2 (Fig. 5D-F). Thus, NBP may differentially regulate the Bcl-2, Bax and BAD mRNA expression levels to modulate cell apoptosis.

BAD, Bax and Bcl-2 protein expression in muscle tissue and sera. To understand the effect of NBP on apoptotic protein expression, the BAD, Bax and Bcl-2 protein levels in the muscle cells of EAM animals were measured. Compared with the saline group, the NBP-treated groups presented higher BAD, Bax and Bcl-2 protein expression levels in the muscle tissue

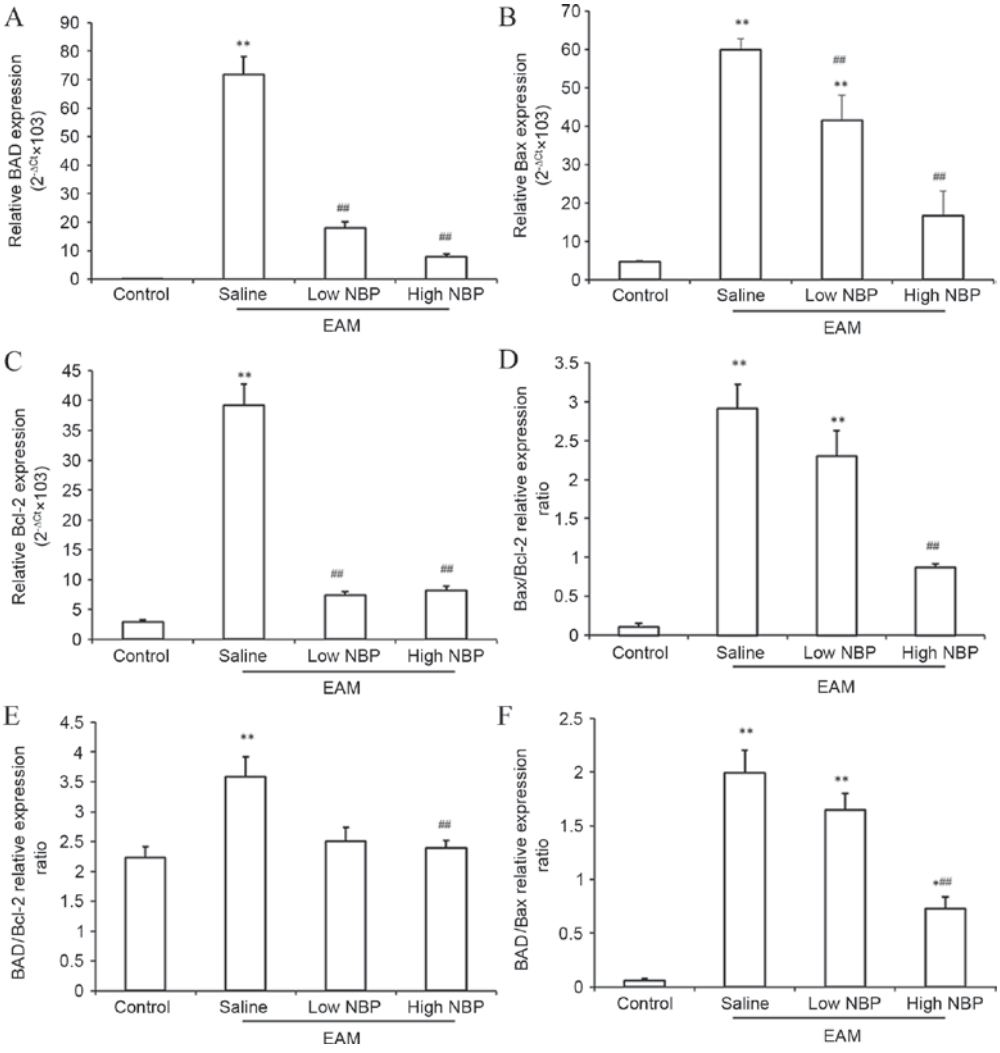

Figure 5. Effects of NBP on the muscle mRNA expression levels (relative value, $2^{-\Delta\Delta Cq}$ x10^3) of (A) BAD, (B) Bax and (C) Bcl-2. Relative mRNA expression ratios of (D) Bax/Bcl-2, (E) BAD/Bcl-2 and (F) BAD/Bax are also shown. mRNA levels in the control and three EAM groups, treated with saline, low-dose NBP (40 mg/kg body weight) and high-dose NBP (80 mg/kg body weight), were measured by quantitative polymerase chain reaction. Values are represented as the means ± standard deviation (10 animals per group; analyzed in duplicate). *P<0.05 and **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the saline group (Dunnett's method). NBP, 3-*n*-butylphthalide; EAM, experimental autoimmune myositis; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; BAD, Bcl-2-associated death promoter.

(Fig. 6A-C) and serum (Fig. 6D-F); however, the differences were not statistically significant. In addition, BAD, Bax and Bcl-2 protein expression levels in the serum and muscle tissue were lower in the saline group compared with the control group, but the differences were not statistically significant (Fig. 6). Thus, it is suggested that NBP did not regulate the BAD, Bax and Bcl-2 proteins in the muscle cells of EAM animals.

Discussion

The therapeutic effects of NBP have been described in multiple models of human diseases, including stroke, Alzheimer's disease and Parkinson's disease (16,19,20,31,32). NBP has been demonstrated to decrease oxidative stress and mitochondrial damage by increasing the activity of mitochondrial Na^+-K^+ and Ca^{2+}-Mg^{2+} ATPases and mitochondrial SOD, as well as by reducing the level of MDA (16,33). In IIMs, muscle tissues are locally inflamed and this occurs simultaneously with cell degeneration and necrosis, dysregulated oxygen metabolism, production of free radicals and oxidative stress. In the present study, the protective role of NBP in inflamed muscle cells in an EAM animal model was investigated. NBP significantly enhanced the antioxidant enzyme and ATPase activities in muscle cells. Overall, NBP-treated EAM animals presented reduced muscle cell apoptosis, indicating that NBP may be a potential therapy for IIMs.

SOD is an important antioxidant that protects aerobic cells from free radical species (13). CAT also contributes to reducing mitochondrial oxidative stress (14). The current study observed that NBP treatment significantly enhanced SOD and CAT activity, but reduced MDA in muscle mitochondria and serum, and these findings are consistent with previous findings in neuronal cells (16). Furthermore, the results of the present study suggested that NBP may directly or indirectly regulate antioxidant enzyme expression in order to reduce oxidative stress. However, the involvement of SOD in myopathy may be complex. There are two main SOD isoforms in human tissues: Copper-zinc SOD (Cu/Zn SOD) and manganese SOD (Mn SOD) (34). High serum levels of Mn SOD have been reported in patients with PM/DM, and thus this has been

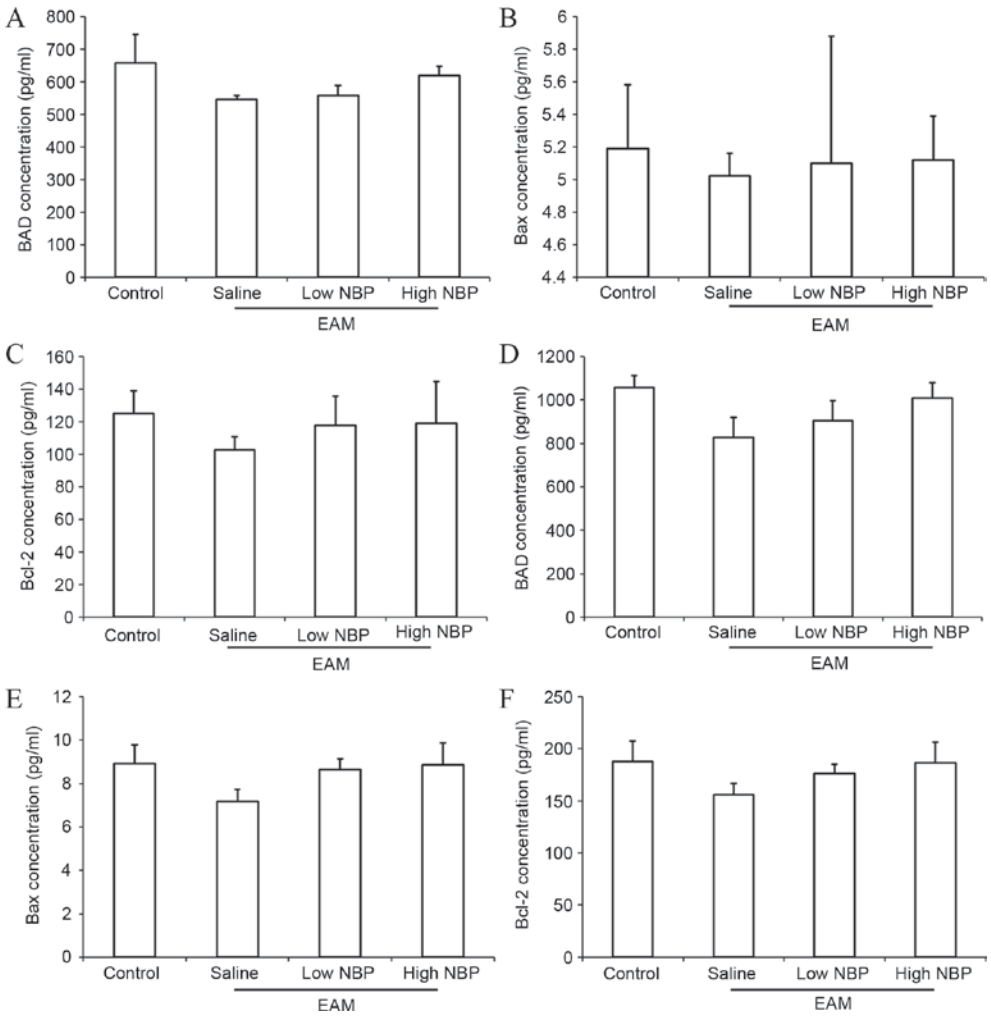

Figure 6. Effects of NBP on (A) BAD, (B) Bax and (C) Bcl-2 protein levels in muscle homogenates, as well as the serum protein levels of (D) BAD, (E) Bax and (F) Bcl-2. Samples were obtained on day 10 after treatment. Protein concentrations in guinea pigs in the control and three EAM groups, treated with saline, low-dose NBP (40 mg/kg body weight) and high-dose NBP (80 mg/kg body weight), were measured by ELISA. Values are presented as the mean ± standard deviation (10 animals per group; analyzed in duplicate). NBP, 3-*n*-butylphthalide; EAM, experimental autoimmune myositis; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; BAD, Bcl-2-associated death promoter.

considered to be a useful clinical marker for PM/DM (35). In IBM, the expression levels of the two SOD isoforms may have different roles for cell protection in myopathies (36). Further studies are required to confirm whether NBP differentially regulates the two SOD isoforms to prevent oxidative stress.

Mitochondria are critical for cell proliferation and metabolism control, providing energy by ATP synthesis, as well as regulating the membrane potential and calcium storage, and controling cell apoptosis (7,37,38). The Na^+K^+ and $Ca^{2+}Mg^{2+}$ ATPases consume ATP to maintain the membrane potential and balance intracellular calcium (39,40). Contractility and excitability of a working skeletal muscle requires abundant functional Na^+K^+-ATPase pumps (41), while Ca^{2+} ATPase sequesters calcium from the cytosol to maintain muscle contractile functions (42). The current study reported that intraperitoneal injection of 40 or 80 mg NBP/kg body weight significantly increased the activity of Na^+-K^+ and Ca^{2+}-Mg^{2+} ATPase in a guinea pig model of EAM, suggesting that NBP protects mitochondria and plasma membrane damage in EAM.

In patients with PM and DM, Fas was detected in muscle fibers and infiltrating mononuclear cells, and its expression was associated with muscle cell apoptosis (43). Bcl-2, Bax and BAD are proteins involved in cell apoptosis and pro-survival. Bcl-2 is mainly expressed in regenerating fibers, whereas Fas is expressed by non-regenerating muscle fibers (44,45). However, there was no correlation between Bcl-2 expression and muscle cell apoptosis (45,46). In the present study, NBP significantly decreased apoptotic cells in EAM. NBP treatment also reduced the Bax, BAD and Bcl-2 mRNA levels in muscle cells; however, the pro-apoptotic proteins Bax and BAD were found at higher levels in comparison with Bcl-2. In addition, NBP did not affect the levels of soluble Bcl-2, Bax and BAD proteins. Thus, NBP may act on other apoptotic proteins to promote muscle cell survival, while Bcl-2, Bax and BAD may change their cellular distribution to be similar in abundance after apoptotic processes.

In conclusion, NBP was demonstrated to improve the antioxidant enzyme activity and reduce oxidative damage in an EAM model. Furthermore, NBP mediated this protective effect by enhancing the Na^+-K^+ ATPase and Ca^{2+}-Mg^{2+} ATPase activities in muscle mitochondria and muscle fiber membranes. Additionally, NBP was found to reduce the apoptotic muscle

cells in EAM. Therefore, NBP may be a potential novel therapeutic agent for treating patients with IIM.

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