

DL-3-n-Butylphthalide Improves Physical and Cognitive Performance of Animals with Acute and Chronic Hypobaric Hypoxia

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Research

Keywords: DL-3-n-butylphthalide, hypobaric hypoxia, physical function, cognitive function, oxidative stress, energy metabolism

Posted Date: July 6th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-39309/v1>

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Version of Record: A version of this preprint was published on March 25th, 2021. See the published version at <https://doi.org/10.1186/s40779-021-00314-7>.

Abstract

Background: Studies revealed the protective effect of DL-3-n-butylphthalide (NBP) against ischemic hypoxia diseases. However, the role of NBP in animals with hypobaric hypoxia is elusive. This study investigated the effect of NBP on animals with acute and chronic hypobaric hypoxia.

Methods: SD rats and Kunming mice administrated with NBP (90, 180 and 360 mg/kg for mice, and 60, 120, and 240 mg/kg for rats) were located in 10,000 m hypobaric hypoxia chamber. And survival analysis of animals implied that NBP could significantly improve survival percent at 30 min. Then, treated animals were evaluated for exhaustive time and exhaustive distance in forced exercise wheel-track treadmill and treadmill running experiments at 5,800 m for 3 or 21 days, to evaluate physical functions. Rats were also evaluated for times of active escape, average time of active escape, time of passive escape, and average time of passive escape in a shuttle-box experiment at 5,800 m for 7 or 28 days, to evaluate cognitive functions. ATP level was evaluated in the gastrocnemius muscle and malonaldehyde (MDA), superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), lactate, and glutathione peroxidase (GSH-Px) measurements and routine blood tests were detected.

Results: Exhaustive time for rats (NBP, 120 and 240 mg/kg) and exhaustive time and distance for mice (NBP, 90 mg/kg) significantly increased under acute hypoxia. And NBP treatment significantly increased the exhaustive time for rats under chronic hypoxia. Moreover, NBP of 120 and 240 mg/kg significantly increased the average time of passive escape under acute and chronic hypoxia. These results suggested that NBP could improve physical and cognitive functions under acute and chronic hypobaric hypoxia. Furthermore, the levels of MDA and H_2O_2 decreased but those of SOD and GSH-Px increased under acute and chronic hypoxia. Furthermore, the content of ATP significantly increased, while lactate level significantly decreased. The results presented that NBP could regulate redox homeostasis and improve energy metabolism.

Conclusion: NBP could improve physical and cognitive functions under acute and chronic hypobaric hypoxia by increasing anti-oxidative capacity and energy supply.

1. Background

Exposure to high altitude environment may lead to an obvious decrease in work and cognitive performances [1–2]. At an altitude of 4,500 m, the maximum working capacity was found to reduce to 50% of that observed at low altitude [2]. People exposed to hypobaric hypoxia conditions showed significant alterations in cognitive processes, including attention, short-term memory, decision-making, simple and complex reaction time, and mood [1]. The changes in physical and cognition functions in response to hypobaric hypoxia may greatly affect work and normal life. Therefore, development of drugs and methods to alleviate physical and cognition impairment is imperative for migrators in high altitude.

DL-3-n-butylphthalide (NBP), a racemic mixture of an optical isomer extracted from the seeds of *Apium graveolens* Linn (Fig. 1), is widely used to treat patients with ischemic stroke [3]. NBP is thought to inhibit

inflammation and oxidative as well as endoplasmic reticulum stress and promote angiogenesis in cerebral ischemic animals and patients [4]. NBP was recently shown to exert neuroprotective effects by alleviating vascular cognitive impairment [5] and promoting neuroplasticity and motor recovery after cerebral ischemia [6] and chronic intermittent hypoxia-hypercapnia [7]. Whether NBP exerts beneficial effects in other hypoxic conditions, such as hypobaric hypoxia is, however, unclear.

In this work, we investigated the effects of NBP on the physical and cognitive abilities of animals in hypobaric hypoxia conditions at 5,800 m altitude. The role of NBP on animal behavior was evaluated through exhaustive exercise and shuttle-box experiments. We evaluated the potential mechanism of NBP by collecting muscle and blood samples from treated animals and analyzing the levels of ATP, malonaldehyde (MDA), superoxide dismutase (SOD), hydrogen peroxide (H₂O₂), lactate, and glutathione peroxidase (GSH-Px) as well as by performing routine blood tests.

2. Materials And Methods

2.1. Experimental animals and NBP administration

Male pathogen-free Sprague-Dawley (SD) rats (6 to 8 weeks old, weighing 180–220 g) and male pathogen-free Kunming (KM) mice (6 weeks old, weighting 18–20 g) were used in this study. Rats and mice were obtained from the Laboratory Animal Center of Army Medical University, and the animal study protocol was approved by the Animal Care and Use Committee Guidelines of the Army Medical University. NBP (purity, 99.6%) was obtained from Shijiazhuang Pharma Group NBP Pharmaceutical Co., Ltd (Shijiazhuang, Hebei, China). Animals were randomly assigned to four experimental groups as follows: control group (n = 10), NBP low dose-treated group (60 mg/kg for rats and 90 mg/kg for mice, n = 10), NBP intermediate dose-treated group (120 mg/kg for rats and 180 mg/kg for mice, n = 10), and NBP high dose-treated group (240 mg/kg for rats and 360 mg/kg for mice, n = 10). NBP was intragastrically administered once every day. Animals had free access to food and water.

2.2. Survival analysis in 10,000 m hypobaric hypoxia chamber

NBP were given to SD rats and KM mice by intragastric administration at 1 ml/100 g body weight for 7 days. After 1.14 hours from last administration, animals were placed in hypobaric hypoxia chamber. The high altitude of chamber ascended to 10,000 m by around 1,000 m/min. The survival of animals were observed and recorded and the experiment stopped at 30 min in 10,000 m.

2.3. Standard hypoxia tolerance time of mice

NBP were given to KM mice by intragastric administration for 3, 5, or 7 days. After 1.14 hours from last administration, each mouse was put into a bottle with a nominal volume size of 125 ml, and 5 g of soda lime, respectively, was added to absorb the carbon dioxide and water vapor produced by breathing. Then, the lid was tightly sealed until the mouse's breathing movement stopped. The ST, i.e., the time from when

the mouse was sealed in the bottle to its death, was recorded. STT was calculated according to formulas [8].

$$STT = ST/(V-BW/0.94)$$

STT: standard hypoxia tolerance time (min/ml), ST: survival time (min), V: bottle volume (ml), BW: body weight of mice (g).

2.4. Gasping duration time of mice after decapitation

NBP were given to KM mice by intragastric administration for 7 days, twice per day. Mice were made to fast with free access to drinking water 12 h before the experiment. After 1.14 hours from last administration, the mice were decapitated, and the gasping duration of the isolated head was determined.

2.5. Treadmill running experiment for acute hypoxic rats

NBP was intragastrically administered to SD rats for 3 days at low altitude. Rats were then subjected to treadmill exercise everyday as follows: 5 min for adaptation, followed by 15 m/min for 10 min and 20 m/min for 20 min. On day 4, rats were placed in a 5,800 m hypobaric hypoxia chamber, administered NBP and subjected to the above exercise regimen for 3 days. On day 7, exhaustive exercise was conducted by a treadmill running experiment. The experimental plan included 5 min for adaptation, followed by 15 m/min for 3 min, 20 m/min for 3 min, 25 m/min for 30 min, and then 30 m/min up to exhaustive status. Rats were anaesthetized and their arterial blood was collected for the analysis of MDA, SOD, H₂O₂, lactate, and GSH-Px levels as well as to perform routine blood tests. In addition, the gastrocnemius muscle tissues was excised and used for ATP detection. MDA, SOD, H₂O₂, lactate, and GSH-Px levels were detected by Nanjing Jiancheng assay agents (A003-1, A001-3, A064-1-1, A020-1-2, and A005, respectively). ATP level was analyzed by a Beyotime ATP assay kit (S0026). Blood routine tests were performed at the Xinqiao Hospital, Chongqing, China.

2.6. Forced exercise wheel-track treadmill experiments for mice

KM mice were intragastrically treated with NBP twice per day for 7 days at low altitude. On day 4, mice were exercised on a wheel-track treadmill (YLS-10B, Shandong Academy of Medical Sciences, Jinan, China) for 3 days under following conditions: 10 min/day, 0.8 mA for motor current limitation, 3 s for maximum electronic shock time, 30 s rest after electronic shock, and five times rest in 10 min as exhaustive standard. On the day 7, mice were placed in 5,000 m hypobaric hypoxia chamber and treated with NBP after 30 min. Following 1.14 h, exhaustive exercise was conducted through wheel-track treadmill running experiment and exhaustive time was recorded.

2.7. Treadmill running experiment for chronic hypoxic rats

Rats were placed in 5,800 m hypobaric hypoxia chamber and intragastrically administered with NBP for 14 days from day 8. Rats were exercised on treadmill as above for 3 days from day 18. On day 21,

exhaustive exercise was conducted in treadmill running experiment. At exhaustive status, rats were anaesthetized and their arterial blood samples were obtained to analyze MDA, SOD, H₂O₂, lactate, and GSH-Px levels and perform routine blood test. Further, gastrocnemius muscle was excised for ATP detection.

2.8. Shuttle-box experiment for acute hypoxic rats

NBP was intragastrically administered to SD rats for 7 days in 5,800 m hypobaric hypoxia chamber. On day 4, rats were subjected to an exercise regimen in a shutter box for 3 days under following conditions: 2.2 mA of current, 10 s interval, 10 s rest for sound and light stimuli, and 10 s for electronic stimulus (50 times every day). On day 7, the cognition ability of rats was investigated through the shuttle-box experiment in 5,800 m hypobaric hypoxia chamber. Rats were then anaesthetized and their arterial blood was collected to measure MDA, SOD, H₂O₂, and GSH-Px levels.

2.9. Shuttle-box experiment for chronic hypoxic rats

Rats were placed in 5,800 m hypobaric hypoxia chamber and administered with NBP via intragastric route for 21 days from day 8. From day 25, rats were subjected to the exercise regimen mentioned above for 3 days. On day 28, cognition ability was studied through the shuttle-box experiment. At the end of the experiment, rats were anaesthetized and arterial blood was collected for the analysis of MDA, SOD, H₂O₂, and GSH-Px levels.

2.10. Statistical analysis

Statistical analysis was carried out using the SPSS 19.0 software. For survival analysis, a Kaplan-Meier curve was generated using the log-rank test. Other experiments were analyzed using an independent-samples *t* test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. NBP could improve hypoxia tolerance ability of rats and mice

To investigate whether NBP could benefit animals in hypobaric hypoxia, we firstly studied the effect of NBP on the survival time at 10,000 m exposure with 30 min as stopped time. The survival curve of experiments showed that 120 and 240 mg/kg NBP administration could significantly improve the survival time of rats instead of mouse (Fig. 2A, B). Compared to control group, the death percent at 30 min in 120 and 240 mg/kg group for rats and 180 mg/kg group for mice was declined (Fig. 2C, D). Moreover, the standard tolerance time with closed hypoxia (Figure S1) and gasping duration time after decapitation (Figure S2) of mice also markedly extended by 360 mg/kg NBP administration for day 5 and day 7 separately. The results indicated that NBP could improve the ability of hypoxia tolerance of rats and mice.

3.2. NBP improved physical ability under acute and chronic hypoxia

As hypobaric hypoxia is known to impair physical and cognitive functions in humans, we studied the role of NBP on the physical and cognitive behaviors of animals. The physical abilities of rats and mice were evaluated through treadmill running and forced exercise wheel-track treadmill experiments.

Under acute hypoxic conditions, NBP treatment at 120 and 240 mg/kg concentrations significantly increased the exhaustive time of rats, and 90 mg/kg NBP dose significantly improved the exhaustive time and distance for mice (Fig. 3). Moreover, NBP at 60, 120, and 240 mg/kg concentrations could significantly increase the exhaustive time for rats under chronic hypoxia (Fig. 4). These results suggest that NBP may improve the physical ability of animals under acute and chronic hypoxia.

To clarify the mechanism underlying the NBP-mediated acceleration in physical activity under acute and chronic hypoxic conditions, we evaluated the levels of MDA, SOD, H₂O₂, lactate, and GSH-Px in the blood and ATP in the gastrocnemius muscle. The levels of MDA and H₂O₂ decreased but that of SOD increased under acute hypoxia following treatment with various doses of NBP (Fig. 5B–D). Under chronic hypoxia, levels of MDA and H₂O₂ decreased, but GSH-Px expression was upregulated (Fig. 6B, D, F). Thus, NBP may increase the antioxidant capacity and exert opposite effects on oxidant capacity. The content of ATP significantly increased under chronic hypoxia (Fig. 6A) and lactate level significantly decreased under acute and chronic hypoxia (Fig. 5E and 6E). These observations suggest NBP promoted ATP production via oxidative phosphorylation instead of glycolysis. NBP at 240 mg/kg dose significantly decreased red blood cell, hemoglobin, and platelet counts as well as hematocrit level under acute hypoxia (Table S1). NBP significantly increase white blood cell count at 60 mg/kg concentration and decreased platelet count at 120 mg/kg concentration under chronic hypoxia (Table S2).

3.2. NBP improved cognition ability under acute and chronic hypoxia

The cognition functions of rats were evaluated through the shuttle-box experiment. Under acute and chronic hypoxia, NBP at 120 and 240 mg/kg doses could significantly increase the average time of passive escape (Figs. 7 and 8). However, NBP had no effect on time of active escape, average time of active escape, and time of passive escape. The beneficial effect of NBP on cognitive function was not as evident as that on physical activity. Furthermore, MDA, SOD, H₂O₂, and GSH-Px levels were analyzed in animal blood samples. The level of H₂O₂ decreased and the expression of GSH-Px was upregulated under acute hypoxia (Fig. 9C–D). Under chronic hypoxia, the levels of MDA and H₂O₂ decreased and the expression of GSH-Px increased (Fig. 10A, C). Thus, NBP could also increase the antioxidant capacity and decrease the oxidant capacity in animals.

4. Discussion

High altitude is characterized with decline in air pressure and partial oxygen pressure. Physical and cognitive functions may be severely affected following exposure to high altitude. However, the number of people staying at high altitude is increasing and their work performance and daily life are greatly affected by high altitude. Therefore, there is an urgent need to develop methods that alleviate physical and cognitive impairments at high altitude under hypobaric hypoxia environment.

Several studies have demonstrated that NBP ameliorates ischemic injury and promotes neuroplasticity and recovery from injury. The therapeutic mechanisms underlying these effects were closely associated with various processes, including anti-oxidant, anti-inflammation, angiogenesis, anti-thrombosis, neurogenesis and metabolic reprogramming. Our previous results have shown that the effects of NBP on hypobaric hypoxia were mainly related to anti-oxidant properties and metabolic reprogramming. The failure of human physiological responses to hypobaric hypoxia may result in increased inflammation, oxidative stress, and metabolic adjustment. Exposure to high altitude results in upregulation in the expression of inflammatory cytokines and downregulation anti-inflammatory cytokines expression [9]. Moreover, hypobaric hypoxia induces oxidative damage and decreases antioxidative functions [10]. Considering metabolic modulation, glycolytic capacity is promoted and oxidative metabolism is suppressed in response to hypoxia [11–12]. A recent study showed the relationship between oxidative stress and accelerated cognitive decline in chronic mountain sickness [13], suggesting that these molecular changes induced by hypobaric hypoxia may lead to behavioral abnormality. Given the effect of NBP on hypobaric hypoxia, we conclude that NBP reverses the alteration in hypoxia-induced oxidative stress and metabolism, thereby possibly reversing physical and cognitive changes. Whether NBP could improve the effect of hypoxia on inflammation warrants further studies.

While NBP played an important role in improving physical and cognitive functions of animals under hypobaric hypoxia, it could not ameliorate all the abnormal changes. Further, the administrative dosage was relatively higher than that employed in other studies. Therefore, it is imperative to improve the role of NBP and develop new therapeutics for reducing hypobaric hypoxia-induced physical and cognitive decline.

5. Conclusion

In summary, we demonstrate the NBP-mediated improvement in physical and cognitive functions of animals under acute and chronic hypobaric hypoxia, as evident from the elevation in the anti-oxidative functions and promotion of oxidative phosphorylation instead of glycolysis.

Abbreviations

BW

body weight

GSH-Px

glutathione peroxidase

H₂O₂
hydrogen peroxide
KM
Kunming
MDA
malonaldehyde
NBP
DL-3-n-butylphthalide
SD
Sprague-Dawley
SOD
superoxide dismutase
ST
survival time
STT
standard hypoxia tolerance time
V
volume

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All co-authors have read and approved of its submission to this journal.

Availability of data and materials

Not applicable.

Conflict of interest statement

The authors have no conflict of interest.

Funding

This research was supported by grants from National Science and Technology Major Project (2014ZX09J14102-05B and 2018ZX09J18109).

Author contributions

GX performed experiments, interpreted the data, and wrote the manuscript. YS, BS, GE, and QH raised animals and performed animal experiments. LL, SH, and JZ participated in experiments of blood samples. BL and JC participated in data analysis and manuscript writing. YG and EZ conceived the study design, experimental plan, and manuscript writing. All authors discussed the results and critically reviewed the manuscript.

Acknowledgements

Not applicable.

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Figures

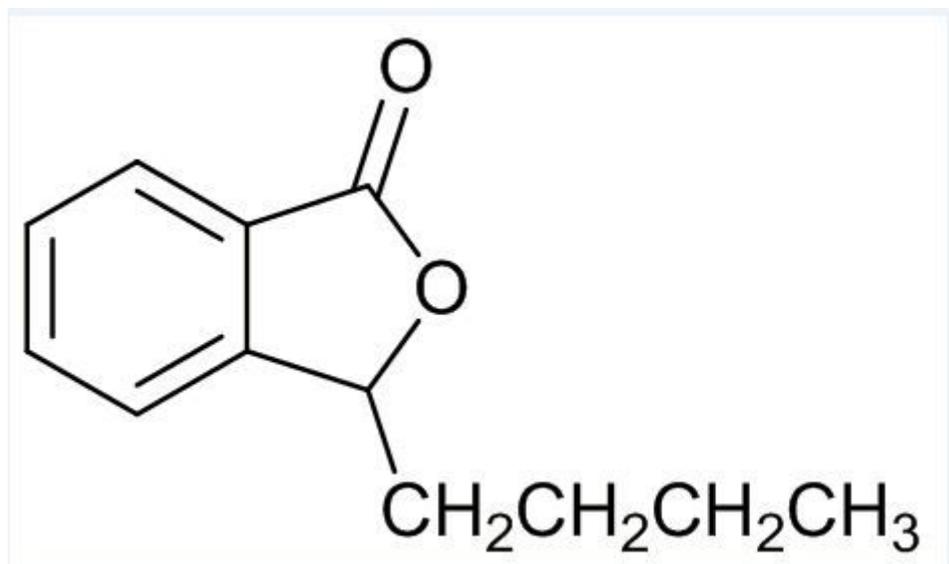


Figure 1

Structure of DL-3-n-butylphthalide.

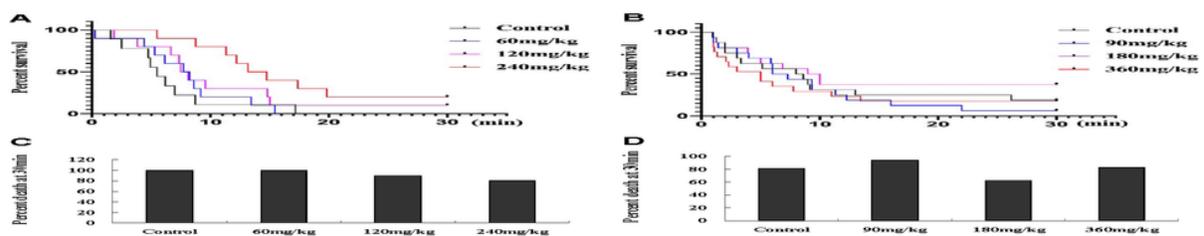


Figure 2

Survival analysis of rats and mice with administration of NBP at 10,000 m exposure. The survival curve of rats (A) and mice (B) at 10,000 m exposure after NBP administration for 7 days with 30 min as stopped time. The death percentage of rats (C) and mice (D) after 30 min exposure at 10,000 m.

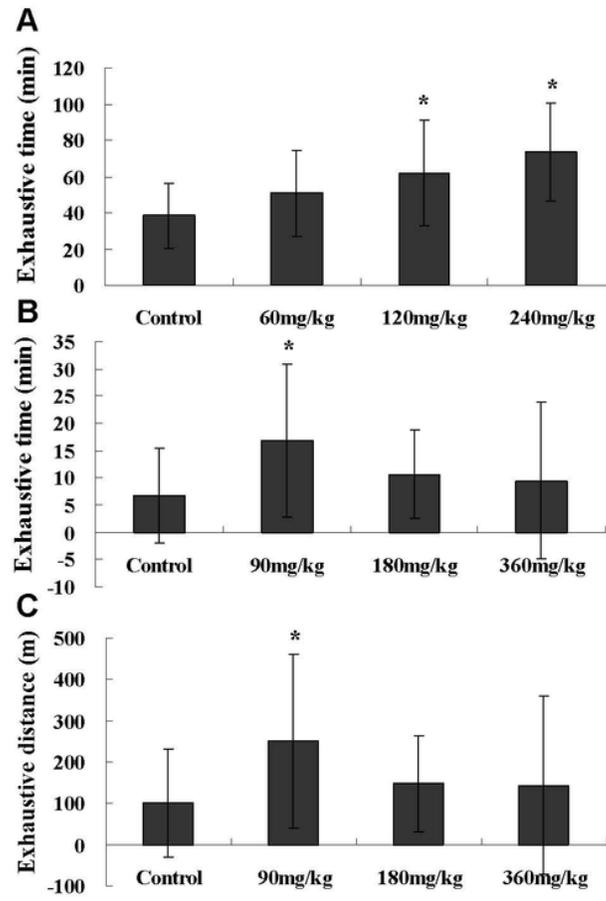


Figure 3

Effects of NBP on the exhaustive motor of rats and mice under acute hypoxia. (A) Exhaustive time of rats in treadmill running experiment at 5,800 m for 3 days. Exhaustive time (B) and exhaustive distance (C) of mice in forced exercise wheel-track treadmill experiment at 5,000 m for 1 day. *, $p < 0.05$ vs. control group.

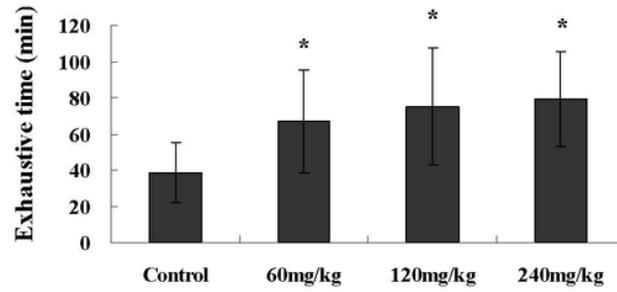


Figure 4

Effects of NBP on the exhaustive motor of rats under chronic hypoxia. Exhaustive time of rats in treadmill running experiment at 5,800 m for 21 days. *, $p < 0.05$ vs. control group.

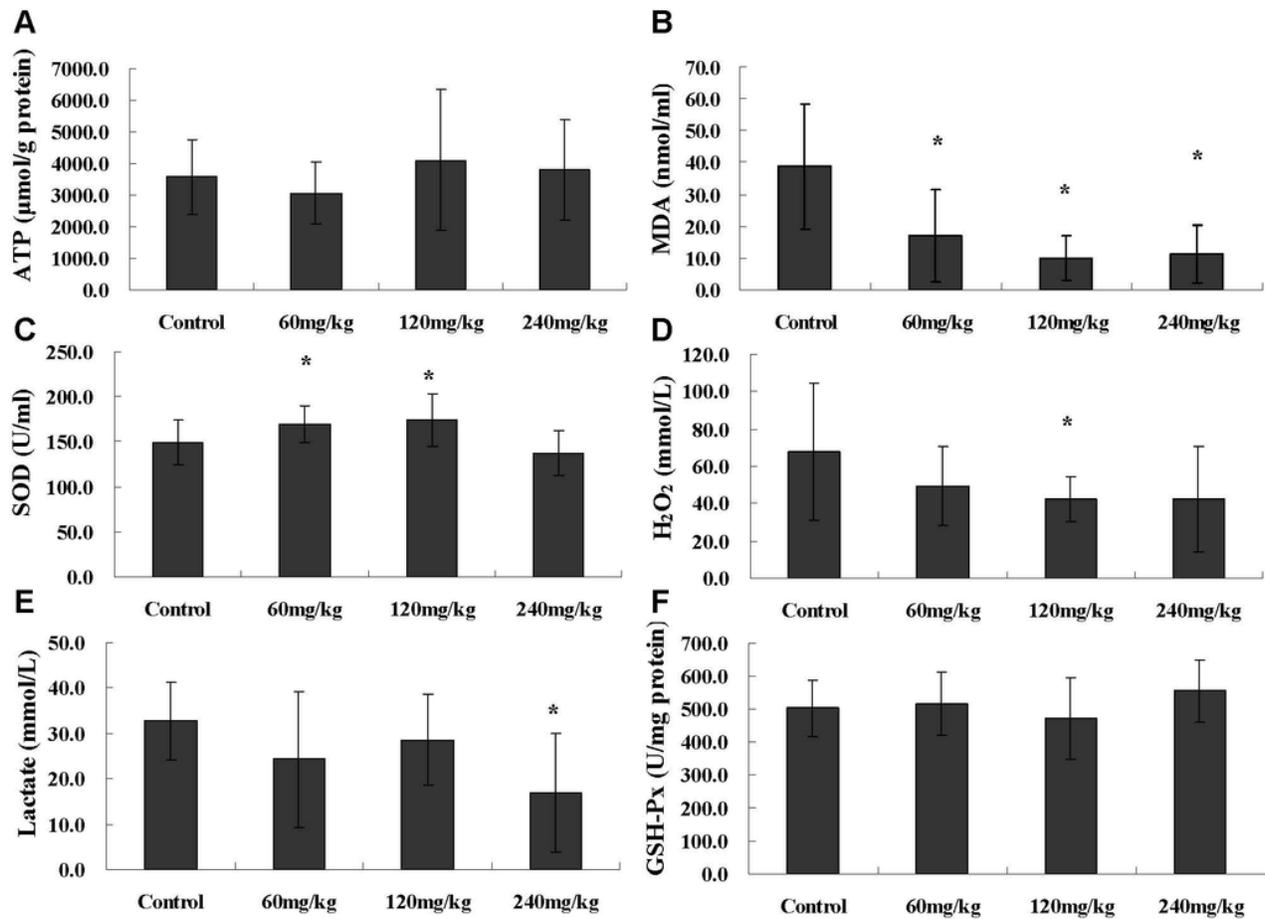


Figure 5

Effects of NBP on the energy metabolism and oxidative stress of exhaustive rats under acute hypoxia. ATP level in the gastrocnemius muscle (A) and MDA (B), SOD (C), H₂O₂ (D), lactate (E), and GSH-Px (F) levels in the serum were detected. *, $p < 0.05$ vs. control group.

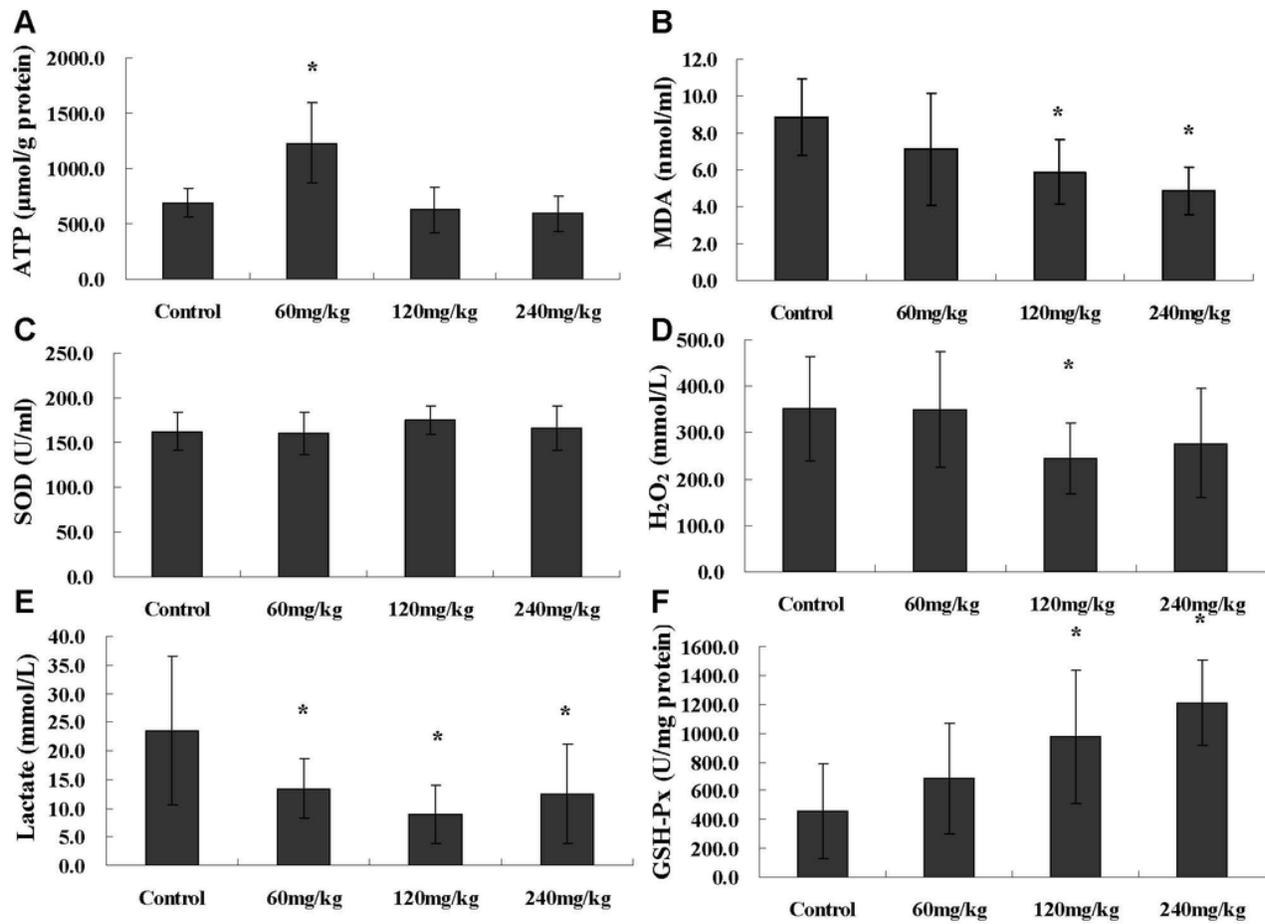


Figure 6

Effects of NBP on the energy metabolism and oxidative stress of exhaustive rats under chronic hypoxia. ATP level in the gastrocnemius muscle (A) and MDA (B), SOD (C), H₂O₂ (D), lactate (E), and GSH-Px (F) levels in the serum were detected. *, $p < 0.05$ vs. control group.

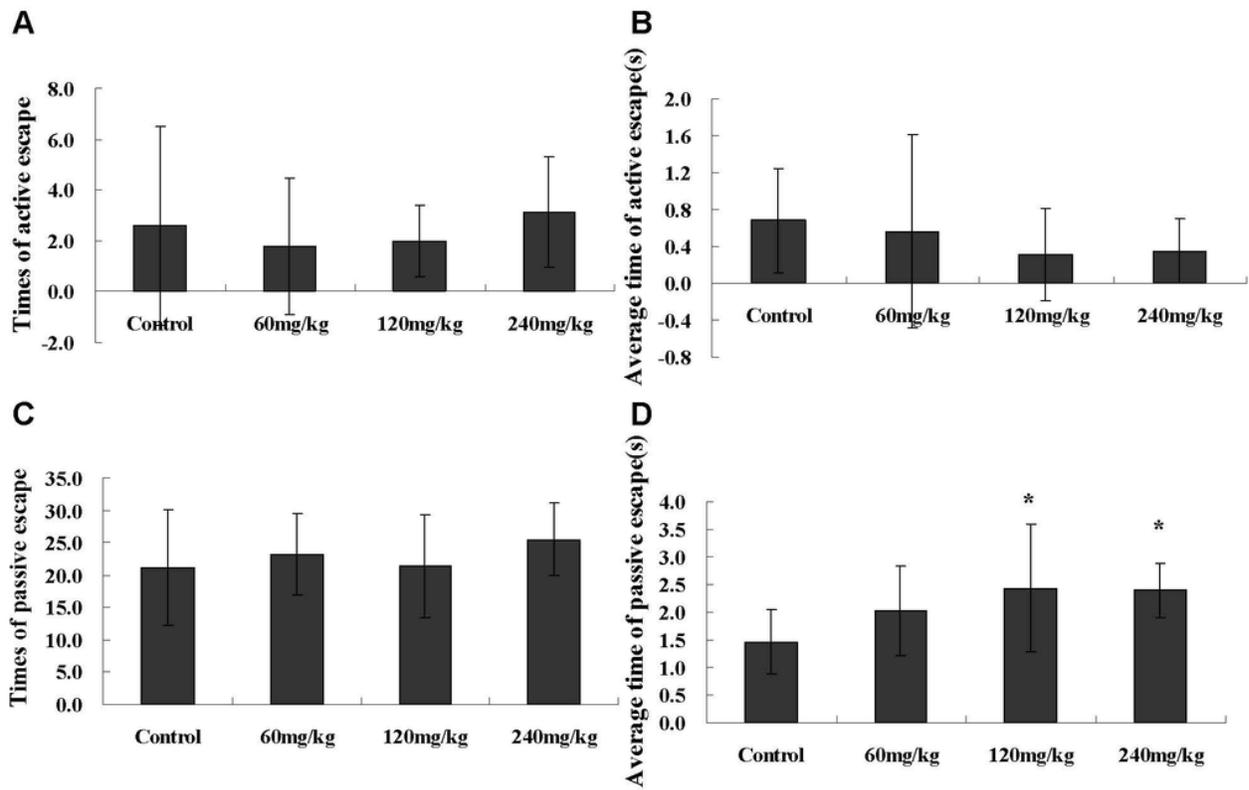


Figure 7

Effects of NBP on rats under acute hypoxia in shuttle-box experiment. Time of active escape (A), average time of active escape (B), time of passive escape (C) and average time of passive escape (D) for rats at 5,800 m for 7 days in the shuttle box experiments. *, $p < 0.05$ vs. control group.

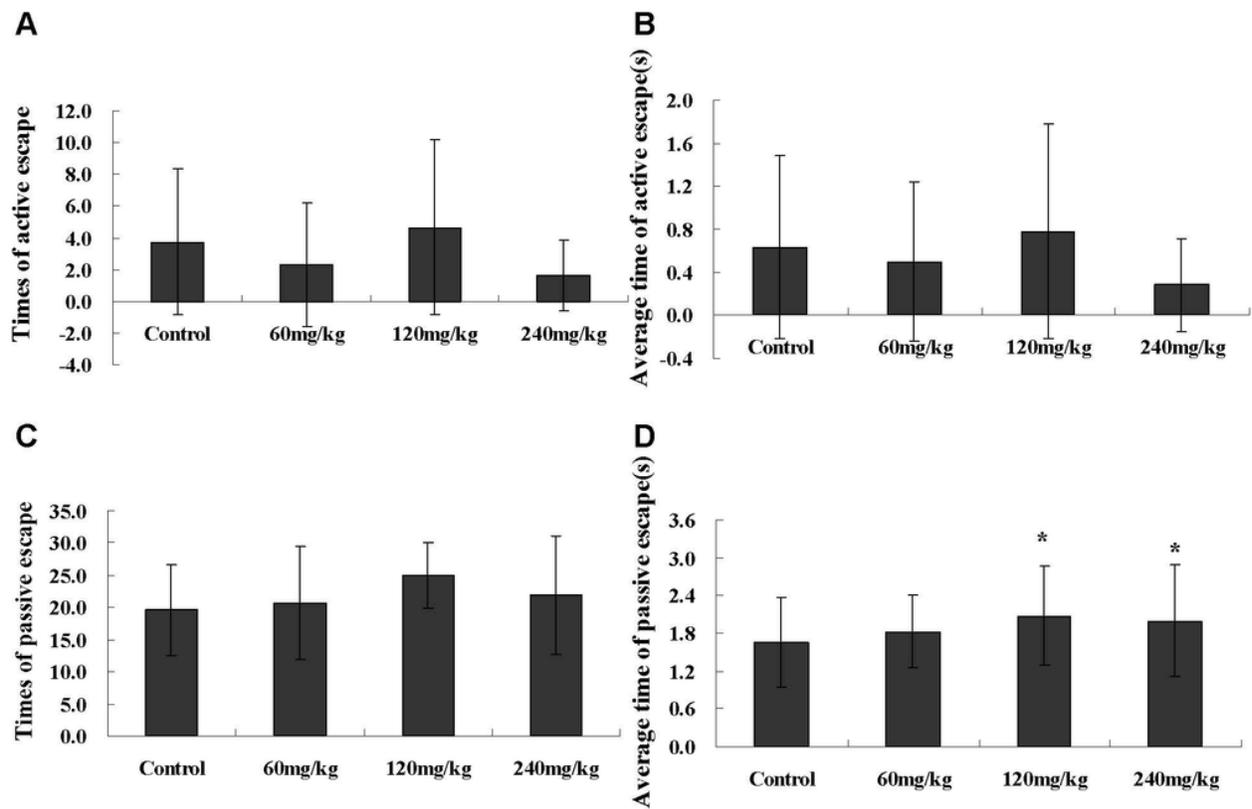


Figure 8

Effects of NBP on rats under chronic hypoxia in shuttle-box experiment. Time of active escape (A), average time of active escape (B), time of passive escape (C) and average time of passive escape (D) for rats at 5,800 m for 28 days in shuttle-box experiments. *, $p < 0.05$ vs. control group.

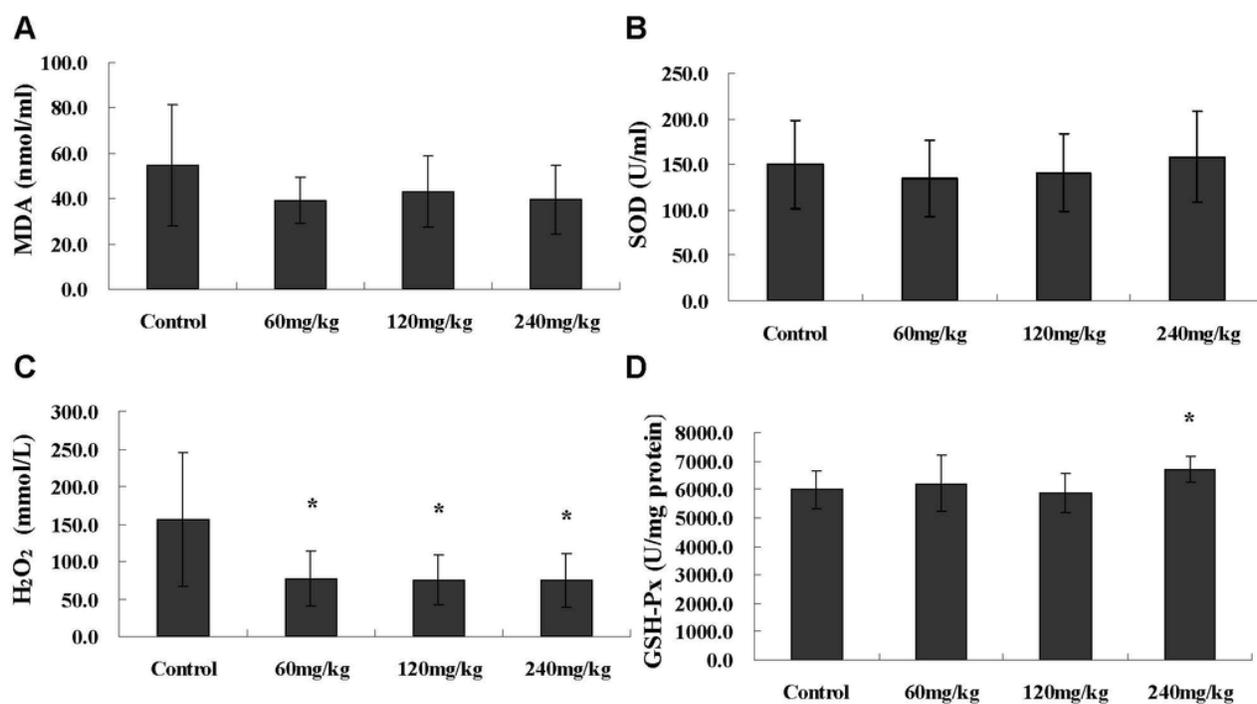


Figure 9

Effects of NBP on the oxidative stress of rats under acute hypoxia after shuttle-box experiment. Levels of MDA (A), SOD (B), H₂O₂ (C), and GSH-Px (D) in the serum were detected. *, p<0.05 vs. control group.

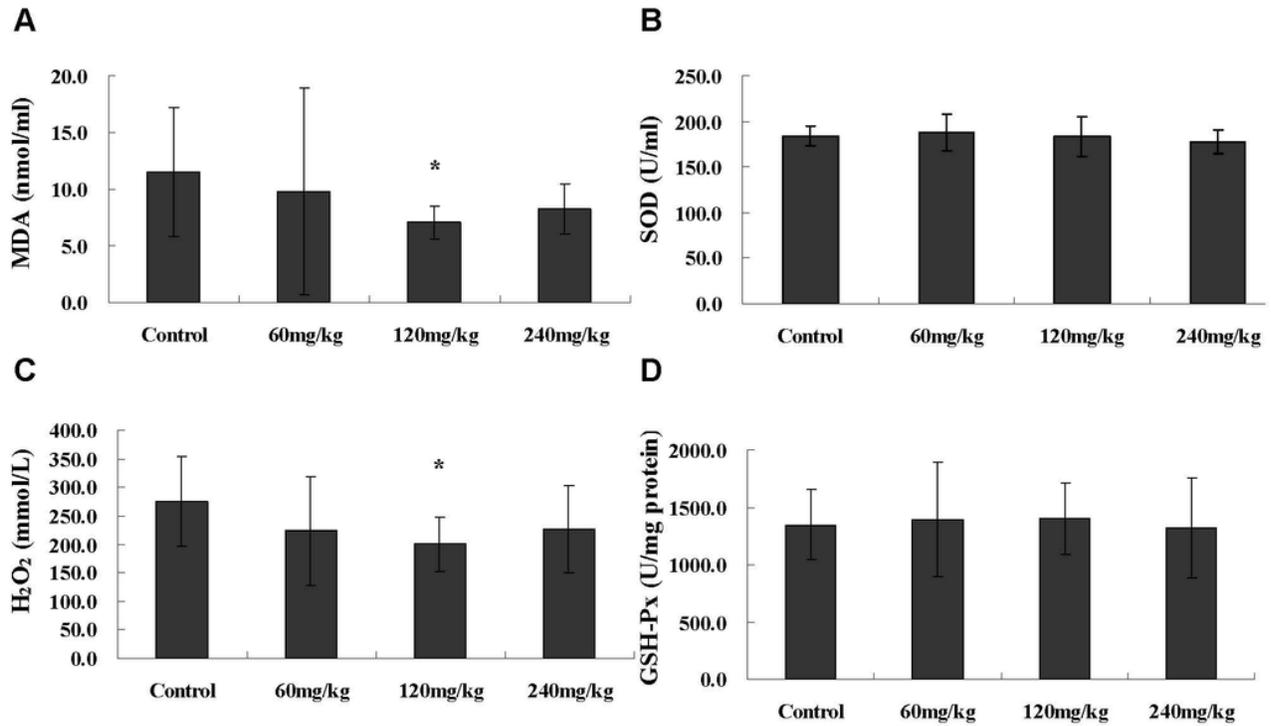


Figure 10

Effects of NBP on the oxidative stress of rats under chronic hypoxia after shuttle-box experiment. Levels of MDA (A), SOD (B), H₂O₂ (C), and GSH-Px (D) in the serum were detected. *, $p < 0.05$ vs. control group.

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