

The neuroprotective effects of grape seed proanthocyanidin on rat brain injury caused by chronic intermittent hypoxia

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Abstract

Chronic intermitted hypoxia including sleep breathing disorder leads to brain injury. This study explores the potential therapeutic effects of grape seed proanthocyanidin as a neuroprotective agent. A rat model of chronic intermittent hypoxia was employed, and the animals were given low or high doses of grape seed proanthocyanidin. The ultrastructure changes in the brain, the biochemical components, and the animal behavior were examined. The results showed that with hypoxia exposure, neuronal mitochondria exhibited injuries at ultrastructural level, with increased malondialdehyde (MDA) content and reduced superoxide dismutase (SOD) activity. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining revealed increased cell apoptosis in hippocampus. In Morris water maze the animals showed decreased learning abilities, when compared to normal control. The administration of grape seed proanthocyanidin treatment reversed all these observed changes, and improved the learning behavior. We concluded that grape seed proanthocyanidin could alleviate the brain injury caused by hypoxia from sleep breathing disorder.

INTRODUCTION

Sleep breathing disorder, especially obstructive sleep apnea syndrome (OSA), leads to hypoxia in the circulation, with possible brain injury, grey matter loss, hippocampus shrinkage, and cognition impairment.^{1,2} It is believed that the brain injury is caused by oxidative stress and activation of a series of signaling pathways such as c-Jun N-terminal kinase (JNK).³⁻⁵ Therefore targeting the oxidative stress may be helpful in treatment against the associated brain injuries caused by chronic hypoxia.

Grape seed proanthocyanidin is a bioactive compound extracted from grape seed. It has been previously found to have strong anti-oxidation effect in treatments of different diseases, especially the cardiovascular diseases.⁶⁻⁹ Whether grape seed proanthocyanidin is also effective in protecting brain injury caused by hypoxia, is however not been previously investigated. Here we test the hypothesis in a rat model of chronic intermitted hypoxia, to determine whether the grape seed proanthocyanidin is able to protect against such brain disorders, and possibly other brain degenerative diseases.

METHODS

Animals and the hypoxia exposure

Eighty male Sprague Dawley (SD) rats (Beijing Weitong Lihua Co.) (Animal license: SCXK(Jing)2002-003) (Weighted 310-350 g) were used in this study. The animals were randomly assigned to four groups with 20 animals each: control, hypoxia (50 ml/L oxygen), hypoxia with low dose proanthocyanidin (orally fed 100mg/kg) and hypoxia with high dose proanthocyanidin (orally fed 200mg/kg).

For intermitted hypoxia, the animals were kept in intermittent hypoxia room from 8:00 am to 4:00 pm (8 hours) every day for 2 or 6 weeks (10 animals for each duration). N₂ and O₂ air were delivered to the intermittent hypoxia room in controlled manner. In the hypoxia group, the N₂ was given for 30 seconds, with lowest O₂ concentration at 5% (50 ml/L). Then the O₂ concentration was allowed to recover to 21% (210 ml/L). The cycle was repeated every 2 minutes. In the control group, compressed air was given.

Morris water maze

The Morris water maze was performed as previously described. The animal was trained five times in the morning, tested six times at noon and six times in the afternoon. The time the animal took to find the hidden platform, and the time the animal spent in the area of hidden platform after the platform was removed were recorded.

Histology

After the experiments, 2 random animals from each group were sacrificed and perfused with 2% paraformaldehyde (PFA) / 2.5% glutaraldehyde following washout of blood with saline. The brain was then removed for preparation of electron microscopy (EM) sections. The sections were examined under transmitted EM (TEM) (H-7650, Rili) at 80 kV.

In order to compare and quantify the changes in the EM pictures, the shape and size of mitochondria were observed; the ribosome and nuclear membrane were examined, and the cytoplasm examined for potential particle formation.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Four random animals from each group were sacrificed and perfused with 4% Paraformaldehyde (PFA) following washout of blood with saline. Then the brain was removed for preparation of paraffin sections. For TUNEL staining, TUNEL kit from Zhongshen Biotechnol Co. was used. Four sections from each animal were used, with 4 images taken randomly from each slice at 200 X. The positive cells were counted double-blindly and averaged.

Mitochondria function measurement

For Mitochondria function measurement, 4 random animals from each group were sacrificed and cortex/hippocampus regions from both sides were removed. Mitochondria was extracted from the supernatant of the homogenized brain tissues, and then tested for malondialdehyde (MDA) content as well as superoxide dismutase (SOD) activity. The measurement was repeated two times each (2 samples from each animal).

Statistics

The data were analyzed with SPSS 13.0 software (Chicago, US) and examined with repeated standard variation analysis. The data were represented with mean \pm SD, and $P < 0.05$ was considered as statistically significant.

RESULTS

Morris water maze

We first examined the learning ability of animals in each group with Morris water maze. The time taken to find the hidden platform reflected the learning ability and the short term memory; while the time spent/number of crosses in the original region of the removed platform reflected the stability of the memory. In compared to the control group, the hypoxia group exhibited longer time to find the hidden platform, and reduced number of crosses after the platform was removed. With proanthocyanidin treatment, the time to find the hidden platform was shortened, and the number of times the animals swam cross the original position of the removed platform increased ($P < 0.05$) (Table 1).

Table 1: The Morris water maze results ($\bar{x} \pm s$)

Group	Time to find the hidden platform		Number of crosses	
	2w	6w	2w	6w
Control	25.66 \pm 2.97	25.40 \pm 3.00	11.57 \pm 2.18	10.94 \pm 2.40
Hypoxia	62.15 \pm 7.44*	68.42 \pm 7.91*	4.42 \pm 1.25*	2.87 \pm 0.92*
Low dose treated	49.17 \pm 8.87* Δ	56.47 \pm 6.98* Δ	5.81 \pm 1.60* Δ	6.32 \pm 1.12* Δ
High dose treated	32.76 \pm 2.02* $\Delta\Delta$	38.86 \pm 3.18* $\Delta\Delta$	7.68 \pm 1.61* $\Delta\Delta$	9.12 \pm 1.18* $\Delta\Delta$

*in compared to control group, $p < 0.05$;

Δ in compared to hypoxia group, $p < 0.05$;

$\Delta\Delta$ in compared to low dose treated group, $p < 0.05$

Ultrastructural changes following hypoxia were reversed by proanthocyanidin treatment.

In hippocampal neurons from control group, the mitochondria were enriched in round/eclipse shape, with clear and patterned internal membrane, showing attachment of enriched ribosomes. The nuclear membrane was intact with chromatin evenly distributed. In 2 weeks or 6 weeks hypoxia treated group, there were plaques formed in the cytoplasm, with mitochondria structure appearing blurred. However with proanthocyanidin treatment, especially the high dose group, the changes were significantly less, with identifiable mitochondria structure and less number of dense particles (Figure 1).

The MDA content and SOD activity

We investigated whether there was any biochemical changes at ultrastructure level with the therapeutic intervention. In compared to the control group, the hypoxia group showed increased MDA content and decreased SOD activity, the severity of which depended on the hypoxia time. This was also reversed with the proanthocyanidin treatment, especially in the high dose group (Table 2).

The neuronal apoptosis in hippocampus

We then examined whether the proanthocyanidin treatment prevented neuronal apoptosis induced by chronic intermittent hypoxia. When compared to control, the hypoxia group showed increased neuronal apoptosis in hippocampus, which also varied according to the duration of hypoxia. This was reduced by proanthocyanidin treatment, especially at high dose (Table 2).

DISCUSSION

Neuronal mitochondria are extremely sensitive to hypoxia and ischemia. The chronic hypoxia leads to increased levels of reactive oxygen species and therefore overloads the endogenous clearing system, for example by SOD.¹⁰⁻¹² The antagonism of oxidative stress (such as with proanthocyanidin from the grape seeds) could protect the neurons from the hypoxia-caused injury, as evidenced by previous reports.¹³ The present study demonstrated the usefulness of this product in neuroprotection at ultrastructural and molecular levels, and helped to preserve the cognitive function.

Previous studies showed that proanthocyanidin could improve the learning and memory abilities

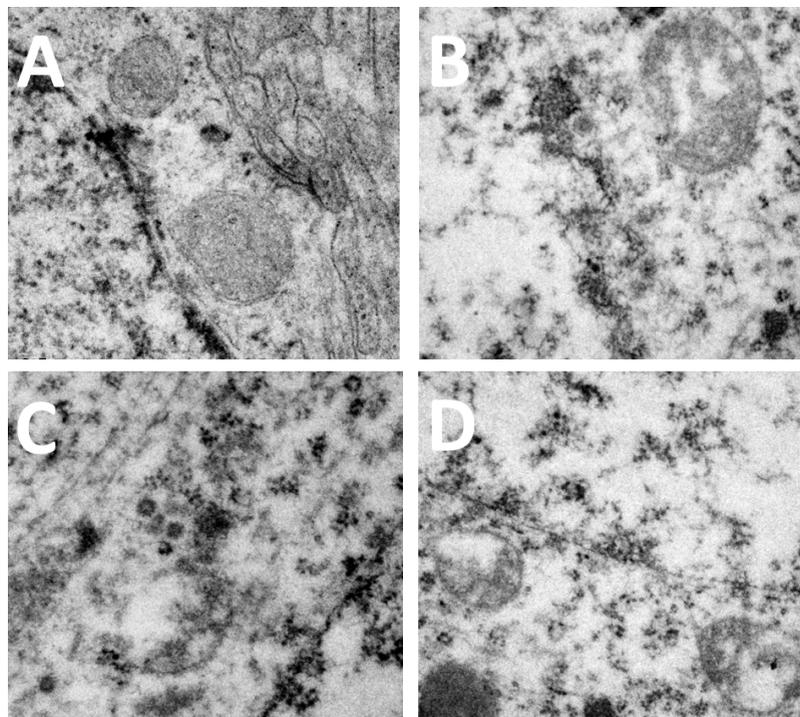


Figure 1. The mitochondrial changes in each group (X 20,000). A: Control group; B: 2 weeks hypoxia; C: 6 weeks hypoxia; D: high dose treated

Table 2: TUNEL positive cells, MDA content and SOD activity in hippocampus (x±s)

Group	TUNEL positive cells		MDA content		SOD activity	
	2w	6w	2w	6w	2w	6w
Control	2.40±0.42	2.42±0.38	71.51±2.08	71.56±2.10	84.56±5.42	86.48±5.30
Hypoxia	9.68±0.79*	15.9±2.92*	79.86 ±2.52*	88.26±2.86*	70.67±6.70*	64.26±7.86*
Low dose treated	6.60±0.69* [△]	9.54±1.36* [△]	76.38 ±1.96* [△]	82.16±2.02* [△]	76.20±6.86* [△]	70.58±6.86* [△]
High dose treated	4.39±0.73* ^{△△}	6.10±0.72* ^{△△}	73.10 ±1.94* ^{△△}	76.94±1.98* ^{△△}	80.62±5.86* ^{△△}	76.56±5.48* ^{△△}

*in compared to control group, $p < 0.05$;

[△]in compared to hypoxia group, $p < 0.05$;

^{△△}in compared to low dose treated group, $p < 0.05$

of animals suffering from ageing and other types of diseases¹⁴⁻¹⁸, partially mediated by its anti-oxidative functions. These results are consistent with our data, and we believe that proanthocyanidin may also be useful in other oxidative stress triggered brain diseases, such as neurodegeneration diseases. Proanthocyanidin exhibited strong anti-oxidative function (similar to SOD), which is 50 times of Vitamin E and 20 times of Vitamin C.^{6,7} As a natural product, it has good safety profile. It will be important to investigate the use of this product to treat other brain diseases, and to compare the neuroprotective efficiency of proanthocyanidin with other natural products. It is also important to understand if proanthocyanidin could antagonize the molecular signaling pathway of oxidative stress, such as JNK signaling. In addition, whether proanthocyanidin is able to restore the systematic dysfunction at advanced stage of disease should also be explored.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the support of their departments in this project.

DISCLOSURE

Conflicts of interest: None

REFERENCES

1. Tsai JC. Neurological and neurobehavioral sequelae of obstructive sleep apnea. *NeuroRehabilitation* 2010; 26:85-94.
2. Lal C, Strange C, Bachman D. Neurocognitive impairment in obstructive sleep apnea. *Chest* 2012; 141:1601-10.
3. Row BW. Intermittent hypoxia and cognitive function: implications from chronic animal models. In: Roach RC, et al, eds: Hypoxia and the circulation. New York: Springer, 2007:51-67.

4. Jun J, Savransky V, Nanayakkara A, et al. Intermittent hypoxia has organ-specific effects on oxidative stress. *Am J Physiol Regul Integr Comp Physiol* 2008; 295:R1274-81.
5. Xie H, Yung WH. Chronic intermittent hypoxia-induced deficits in synaptic plasticity and neurocognitive functions: a role for brain-derived neurotrophic factor. *Acta Pharmacologica Sinica* 2012; 33:5-10.
6. Bagchi D, Bagchi M, Stohs S, et al. Cellular protection with proanthocyanidins derived from grape seeds. *Ann NY Acad Sci* 2002; 957:260-70.
7. Preuss HG, Bagchi D, Bagchi M. Protective effects of a novel niacin-bound chromium complex and a grape seed proanthocyanidin extract on advancing age and various aspects of syndrome X. *Ann NY Acad Sci* 2002; 957:250-9.
8. Bertelli AA, Das DK. Grapes, wines, resveratrol, and heart health. *Journal of Cardiovascular Pharmacology* 2009; 54:468-76.
9. Wang CZ, Mehendale SR, Calway T, et al. Botanical flavonoids on coronary heart disease. *The American Journal of Chinese Medicine* 2011; 39: 661-71.
10. Wang Y, Zhang SX, Gozal D. Reactive oxygen species and the brain in sleep apnea. *Respiratory Physiology & Neurobiology* 2010; 174: 307-16.
11. Raghurir R, Nakka VP and Mehta SL. Endoplasmic reticulum stress in brain damage. *Methods Enzymol* 2011; 489:259-75.
12. Nijboer CH, Bonestroo HJ, Zijlstra J, et al. Mitochondrial JNK phosphorylation as a novel therapeutic target to inhibit neuroinflammation and apoptosis after neonatal ischemic brain damage. *Neurobiol Dis* 2013; 54: 432-44.
13. Kim JY, Lee HK, Hwang BY, et al. Neuroprotection of Ilex latifolia and caffeoylquinic acid derivatives against excitotoxic and hypoxic damage of cultured rat cortical neurons. *Archives of Pharmacal Research* 2012; 35: 1115-22.
14. Bagchi D, Sen CK, Ray SD, et al. Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutation Research* 2003; 523-524:87-97.
15. Ariga T. The antioxidative function, preventive action on disease and utilization of proanthocyanidins. *BioFactors* 2004; 21:197-201.

16. Sano T, Oda E, Yamashita T, *et al.* Anti-thrombotic effect of proanthocyanidin, a purified ingredient of grape seed. *Thrombosis Research* 2005; 115:115-21.
17. Cho ML, Heo YJ, Park MK, *et al.* Grape seed proanthocyanidin extract (GSPE) attenuates collagen-induced arthritis. *Immunology Letters* 2009; 124:102-10.
18. Karaaslan O, Ulusoy MG, Kankaya Y, *et al.* Protective effect of grape seed extract against ischaemia/reperfusion injury in a rat epigastricflap model. *Journal of Plastic,Reconstructive & Aesthetic Surgery JPRAS* 2010; 63: 705-10.