Pakistan J. Zool., vol. 55(2), pp 849-854, 2023 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20201111181147

Effect of Caffeine Citrate with Aminophyline on Apoptosis and Respiratory Endothelial Cell Dysfunction in Premature Infants

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ABSTRACT

This study was aimed to investigate the mechanism of continuous positive airway pressure (CPAP) combined with caffeine citrate and aminophylline in the treatment of premature infants with apnea. in present study, sixty premature SD mice were randomly divided into blank control group (n = 20), intermittent hypoxia group (n = 20) and drug administration group (n = 20). Establishment of intermittent hypoxia model: the oxygen concentration in the cabin of mice in intermittent hypoxia group was maintained at 40%, then decreased to 10% and maintained for 3 min. After that, it was continuously increased to 40%, and was decreased to hypoxia after 8 min. The operation was repeated 3 times every 3h. The drug administration group was treated with continuous CPAP during modeling. The expressions of malondialdehyde (MDA), superoxide dismutase (SOD) and prostaglandin (PGE) in lower respiratory tract were detected by the kit; the expressions ofprotein kinase B (Akt), Bax and Bcl-2 were detected by Western blot. According to our findings after drug treatment, the body weight and respiratory ventilation of mice returned to normal level compared with the intermittent hypoxia group. Compared with the blank control group, after intermittent hypoxia, the contents of MDA, SOD and PGE in the lower respiratory tract of SD mice were significantly lower than those in the normal air group (P<0.05); after the premature mice were treated with drugs, the contents of MDA, SOD and PGE in the lower respiratory tract of SD mice were almost normal compared with those in the intermittent hypoxia group. RT-PCR results showed that compared with the blank control group, the expression of VEGF gene in the lower respiratory tract of SD mice increased after intermittent hypoxia (P<0.05). WB results showed that compared with the blank control group, the expressions of Akt and Bax protein in the lower respiratory tract of SD mice in intermittent hypoxia group were increased. The expression of Bcl-2 was decreased, and the difference was statistically significant (P<0.05). for conclusion, CPAP combined with caffeine citrate and aminophylline could improve the expressions of Akt, Bax and bcl-2 protein to treat premature infants with apnea.

Article Information
Received 11 November 2020
Revised 26 December 2020
Accepted 22 January 2021
Available online 06 May 2022
(early access)
Published 02 January 2023

Authors' Contribution
HG collected the samples, analysed the data, conducted the experiments and analysed the results. All authors discussed the results and wrote the manuscript.

Key words
Continuous CPAP, Caffeine citrate, Aminophylline, Premature infants with apnea, Apoptosis, Endothelial dysfunction

INTRODUCTION

In recent years, there are more and more additives in the diet, and the living environment is getting worse. The incidence of premature babies in China is increasing gradually. Premature babies refer to newborns with gestational age below 37 weeks (Bairam *et al*., 2019; Ye C *et al*., 2019), and their respiratory central system is obviously immature after delivery (Fenouillet *et al*., 2019; Sofien *et al*., 2019). Compared with full-term infants, premature infants have immature body function, which leads to their low resistance (Armanian *et al*., 2016). As a result, they are more likely to suffer from tissue and organ infection and pathological changes. Apnea is a very dangerous disease caused by tissue and organ damage. It has become a typical factor affecting the early survival rate of premature infants (Atik *et al*., 2017; Peter *et al*., 2010). The treatment and disposal of premature infants with apnea involves many aspects in clinic. Caffeine and aminophylline are the most commonly used prescription drugs (Lee *et al*., 2011) for premature infants, which can reduce the incidence of lung by improving the early neural development of premature infants and exerting beneficial effects on respiratory mechanics. In recent years, caffeine citrate combined with continuous CPAP has gained more and more clinical recognition (Butler *et al*., 2014). However, the mechanism of its effect on premature infants with apnea is still unclear. Therefore, in view of the urgent problems to be solved in the medical field, it is extremely necessary to study the treatment mechanism of caffeine citrate combined with continuous CPAP for premature infants with apnea, reduce the lung dysplasia of newborn bronchus, improve the survival rate and living quality of premature infants,

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0030-9923/2023/0002-849 $ 9.00/0

create social benefits and reduce the burden on families and society.

MATERIALS AND METHODS

Experimental animals

Sixty healthy SD mice pregnant for 15 days were selected. After weighing, they were anesthetized with chloral hydrate. The anesthetized pregnant mice were fixed on the special fixing plate for surgery. Their abdominal skin and muscle tissue were exposed with surgical instruments, and their abdominal cavity was exposed. Fetal rats were taken one by one after cesarean section, and were quickly dried. Soft hose was used to stimulate their breathing. The oxygen concentration in the cabin of mice in intermittent hypoxia group was maintained at 40%, then decreased to 10% and maintained for 3 min. After that, it was continuously increased to 40%, and was decreased to hypoxia after 8 min. The operation was repeated 3 times every 3h. The drug administration group was treated with continuous CPAP during modeling. The mouse anti-human AKT, Bax and Bcl-2 monoclonal primary antibodies were from CST Company, an American company. And the corresponding secondary antibodies are from Biyuntian Company. MDA, SOD, PGE kits, PBS and other reagents were all from Tianjin Standard Technology Co., Ltd., and the related primers of *VEGF* gene were designed by Tianjin Thermo Fisher Scientific Company.

Detection of biochemical indicators

The expression of VEGF gene in respiratory tract tissue was detected by q-PCR, and the changes of important protein content were detected by Western blot. MDA, SOD and PGE test kits were used and operated according to the instructions.

Design of Gene primers for mice in each group

After DNA removal, the extracted RNA was reverse transcribed at 60 °C for 40 min, at 70 °C for 5 min and at 4°C for 30 min. VEGF and β-actin primers (Table I) were provided by Tianjin Thermo Fisher Scientific Company.

Determination of protein content by western blot (WB)

The cells in each group were washed with PBS at 4°C for 2-3 times, collected and centrifuged at 10000 rpm. After centrifugation, the supernatant was removed and the mixture was added with 10 mL of RIPA lysate and PMSF for ice lysis for 15 min. After centrifugation for 15 min, the supernatant was taken. The Coomassie method was used to detect absorption value of each tube. The protein concentration of each group was adjusted to the same level, and then the protein was loaded. For tissue samples, after cutting them into piece, lysate was added. The mixture was centrifuged to collect supernatant, namely tissue protein. The loading conditions were: 80 v, 30 min; 120 v, 30 min. After membrane transfer, NC membranes with different molecular weights were collected corresponding to marker, and the NC membranes were put into a pre-marked plate. After that, skimmed milk powder was added, and the mixture was sealed in the dark on a shaker for 1h. After the milk powder was discarded, the NC membrane was taken out and washed in PBST for 3 times. Then, 1 mL of mouse anti-human AKT, Bax and Bcl-2 primary antibodies diluted by 1:1000 times PBS was added from left to right and the mixture was incubated overnight at 4°C. The NC membrane was taken out the next day, washed in TBST solution for 4 times, and then incubated with 1: 1000 times diluted mouse anti-rabbit 2 antibody solution for 1 hour. Then, it was washed in TBST solution for 4 times. 1.5 mL of luminescent liquid was prepared. Liquid a and liquid b were prepared in a ratio of 1:1 when it was in need, and were slowly dripped onto the membrane from left to right. 150 μL of luminescent liquid was dripped onto each membrane. Then, the membrane was transferred to a darkroom for film processing.

Statistical analysis

SPSS 20.0 software was used for statistical analysis, and all the data were expressed by "mean±standard deviation" ($\bar{x}$±s). After variance homogeneity test, LSD test was used for comparison between groups. Pearson Correlation test was used to test the standards. α=0.05; $\chi 2$ analysis was used for comparison between groups; $P<0.05$indicated statistically significant difference.

RESULTS

Body weight and ventilation of mice

Table II shows the weighed and ventilation capacity of different experimental groups. After intermittent hypoxia, the development of SD mice was lower than that of the blank control group, and their body weight was significantly different from that of the normal air group (P<0.05); when premature mice were treated with CPAP combined with citric acid and caffeine, the body weight of mice recovered significantly compared with that of intermittent hypoxia group, and basically returned to normal level. The results of respiratory ventilation showed that after intermittent hypoxia, the respiratory ventilation of SD mice was lower than that of normal control group (35.74 ±2.75, P<0.05). However, after therapeutic intervention, the respiratory capacity recovered obviously, close to that of the blank control group. The results indicated that CPAP combined with citric acid and caffeine could improve the apnea symptoms of premature mice and strengthen the protection of respiratory tract tissue.

Table I. Primer sequence during RT-PCR (n=10).

Gene name	Anterior primer	Rear primer	Length (bp)
VEGF	ATGCTAGCTAGCTAGCAAG	CTAGCTAGTCAGAATCGATC	113
β-actin	TCGATACGCTAGTTCTACTA	CTAGTTGCTAGATGCATAGT	109

Table II. Effect of caffeine citrate and aminophyline on body weight, ventilation, MDA, SOD, PGE, VEGF gene and Bax and Bcl-2 protein expression and AKT protein expression of SD mice (n=10).

	Blank control group	Intermittent hypoxia group	Drug administration group	F value	P value
Weight (g)	5.31±0.43	3.60±0.24**	5.01±0.22	20.763	0.007
Ventilation capacity (mL)	35.74±2.75	21.36±3.57	29.58±3.26	27.352	0.009
MDA (nmol/mL)	6.27±0.52	9.46±1.32	7.52±1.11	39.483	0.001
SOD (U/mg)	79.38±2.47	47.74±5.27	62.31±4.62	38.646	0.001
PGE (pg/mL)	83.56±3.99	52.27±6.86	73.75±5.64	42.145	0.001
VEGF(gray value)	1.00±0.03	0.42±0.09	0.74±0.07	47.352	0.001
AKT (gray value)	1.00±0.03	3.12±0.17	1.64±0.23	49.258	0.001
Bax (gray value)	1.00±0.04	6.36±0.32	3.84±0.45	66.978	0.001
Bcl-2 (gray value)	1.00±0.07	0.32±0.02	0.51±0.06	50.817	0.001

MDA, SOD and PGE expression

Table II also shows that compared with the blank control group, the contents of MDA, SOD and PGE in the lower respiratory tract of SD mice decreased after intermittent hypoxia compared with those in the normal air group, with statistical significance (*P<0.05*). When premature mice were treated with CPAP combined with citric acid and caffeine, the contents of MDA, SOD and PGE in lower respiratory tract of mice were basically restored to normal levels compared with those in intermittent hypoxia group. This showed that CPAP combined with citric acid and caffeine could improve respiratory tract injury and restore respiratory tract barrier in premature mice Table II.

VEGF *gene expression*

The results of RT-PCR showed that compared with the blank control group, the expression of VEGF gene in the lower respiratory tract of SD mice increased after intermittent hypoxia, and the difference was statistically significant (P<0.05). Compared with the intermittent hypoxia group, the expression of VEGF gene in the treatment group recovered significantly. The results further indicated that CPAP combined with citric acid and caffeine could reduce the apoptosis of respiratory skin cells and achieve the purpose of treating apnea (Table II).

AKT protein expression

The WB results showed that compared with the blank control group, the expression of AKT protein in the lower respiratory tract of SD mice increased after intermittent hypoxia, and the difference was statistically significant (P<0.05). Compared with the normal control group, there was no significant difference in AKT protein expression in the administration group, but it recovered obviously compared with the intermittent hypoxia group. This indicated that premature apnea was related to endothelial cell apoptosis, and Akt pathway was involved in the process of CPAP combined with citric acid and caffeine to inhibit endothelial cell apoptosis Table II and Figure 1.

Expressions of Bax and Bcl-2 protein

The WB results showed that compared with the blank control group, the expressions of Bax and Bcl-2 protein in lower respiratory tract of SD mice increased and decreased respectively after intermittent hypoxia, and the difference was statistically significant (P<0.05). The expressions of Bax and Bcl-2 protein in the tissues of the administration group recovered significantly compared with those of the intermittent hypoxia group. This indicated that premature apnea was related to endothelial cell apoptosis. CPAP combined with citric acid and caffeine inhibited endothelial cell apoptosis by inhibiting Bax overexpression and up-regulating Bcl-2 protein expression, thus finally achieving the purpose of treating premature apnea (Table II, Fig. 2).

Fig. 1. AKT protein expression detected by western blot.

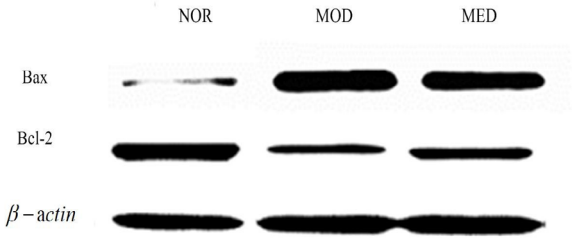

Fig. 2. Bax and Bcl-2 protein expression detected by western blot.

DISCUSSION

Premature infants with apnea is the main clinical problem in neonatal intensive care (Amin and Wang, 2015). Once the birth weight of a newborn is less than 1kg, the risk of apnea will be as high as 80%. Repeated apnea attacks of premature infants can lead to respiratory failure, intracranial hemorrhage, pulmonary hemorrhage and other serious complications (Fiore *et al*., 2013), thus it is vitally important to find the key treatment. It has been proved that antioxidants play a major role in eliminating the formation of free radicals (Wu *et al*., 2019; Yin *et al*., 2019; Zhang *et al*., 2020) and offsetting the excessive free radicals produced in the process of disease formation in many respiratory diseases. In the experiment, we found that the contents of MDA and SOD in lower respiratory tract of premature apnea mice were lower than those in normal air group. After drug treatment, the contents of MDA, SOD SOD in the lower respiratory tract of mice recovered to normal levels compared with the intermittent hypoxia group. The results indicated that CPAP combined with citric acid and caffeine could improve the respiratory tract injury of premature mice and protect the integrity of respiratory tract cells.

Prostaglandin E(PGE) is involved in improving the synthesis of gastric mucin, promoting angiogenesis and triggering the proliferation of mucosal cells (Shukla *et al*., 2016). Therefore, PGE plays an important role in regulating the process of apoptosis and regeneration. It was found that after intermittent hypoxia, the content of PGE in the lower respiratory tract of SD mice decreased compared with that in normal air group. Compared with the intermittent hypoxia group, the content of PGE in tissues of premature mice recovered to normal level after drug treatment. This may be related to the restoration of respiratory barrier. Epidermal growth factor (VEGF), not only an epithelial growth factor, but also used in endothelial cells, may play a role in cell protection, which is beneficial to mucosal and submucosal angiogenesis (Mole *et al*., 2014). The results of RT-PCR showed that compared with the blank control group, the expression of VEGF gene in the lower respiratory tract of SD mice decreased after intermittent hypoxia. Compared with the intermittent hypoxia group, VEGF in the drug administration group recovered significantly. This result fully confirmed that CPAP combined with citric acid and caffeine could reduce the apoptosis of respiratory epithelial cells. In addition, this study also detected the expression level of MAPKs to explore its working mechanism. All MAPKs members are activated by oxidative stress, and MAPK signal cascade is the main stress signal pathway of oxidative stress. AKT is an important protein of MAPK family, playing an important role in cell differentiation, proliferation and apoptosis. In addition, it has been reported that AKT activation induced by oxidative stress plays an anti-apoptosis role in maintaining steady state in vivo and regulating cell differentiation and growth pathway (Zhang *et al*., 2019). This study proves for the first time that apnea can participate in signal cascade reaction, and is especially related to AKT pathway. We found that AKT protein increased and Bcl-2 protein expression decreased in the lower respiratory tract of premature mice with apnea. However, the expressions of Bax and Bcl-2 protein in the administration group recovered significantly compared with the intermittent hypoxia group. Mitochondria participated in regulation of apoptosis pathway and integrated and circulated apoptosis signals during the regulation. Apoptosis pathway mediated by mitochondria is called intrinsic pathway. This apoptosis is controlled by Bcl-2 family proteins, including two types: pro-apoptosis and anti-apoptosis (Anselmo *et al*., 2020). The pro-apoptotic proteins (Bax, Bak, Bok, Bid) and anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-w, etc.) of Bcl-2 family proteins keep balance in the body (Zhang *et al*., 2019), and determine the fate of cells by up-regulating and down-regulating apoptosis or survival in pathophysiology (Joseph *et al*., 2019).

The WB results showed that compared with the blank control group, Bax protein increased and Bcl-2 protein expression decreased in the lower respiratory tract of SD mice after intermittent hypoxia. The expressions of Bax and Bcl-2 protein in the tissues of the drug administration group recovered obviously compared with those of the

intermittent hypoxia group, showing that the drug inhibited apoptosis.

To sum up, premature infants with apnea would be accompanied by mucosal injury and apoptosis. CPAP combined with citric acid and caffeine could obviously alleviate the oxidative damage caused by apnea. By inhibiting the expression of apoptosis pathway protein AKT and Bax, up-regulating the expression of anti-apoptosis protein Bcl-2, rebuilding the cell barrier, and inhibiting respiratory tract injury, it could achieve the purpose of treating premature infants with apnea.

CONCLUSION

The findings of our study showed that for the treatment of premature infants with apnea, CPAP combined with caffeine citrate and aminophylline could enhance the expressions of Akt, Bax and bcl-2 proteins.

Statement of conflict of interests

The authors have declared no conflict of interest.

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