Neuroprotective effect of 1,25-dihydroxyvitamin D₃ against hyperoxia-induced brain injury in premature rats

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Studies have shown that vitamin D plays a crucial role in brain development, brain metabolism and neuroprotection. There is little evidence for the neuroprotective effect of 1, 25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) on various brain injury models. The aim of this study was to investigate the neuroprotection effect of 1,25-(OH)₂D₃ against hyperoxia-induced brain injury in premature rats. Sprague-Dawley rats were exposed to 95% oxygen or room air for 24 h and treated with 1,25-(OH)₂D₃ or normal saline for 14 consecutive days. The histopathological changes of optic chiasma tissue were observed by hematoxylin-eosin staining. Immunohistochemistry, qRT-PCR, and western blot were performed to detect the expression of integrin-β1 and yes-associated protein (YAP) in the organization of the optic chiasm. Histopathological sections of optic chiasma showed visible optic nerve swelling, expanded nerve fiber space, uneven staining, obvious oligodendrocyte proliferation and disordered cell arrangement accompanied by inflammatory cell infiltration and exudation after 7 days and 14 days of hyperoxia exposure. The hyperoxia group treated with 1,25-(OH)₂D₃ showed improvement of brain injury with reduced inflammatory exudation, uniform nerve fiber staining and less obvious oligodendrocyte proliferation. Immunohistochemical staining, qRT-PCR and western blot indicated that 1,25-(OH)₂D₃ treatment upregulated the expression of integrin-β1 and YAP in the hyperoxia group on day 7. However, the expression of YAP was significantly increased compared with control group and treatment with 1,25-(OH)₂D₃ reduced the expression of YAP in the hyperoxic group on day 14. 1,25-(OH)₂D₃ may regulate the expression of integrin-β1 and YAP to alleviate hyperoxia-induced brain injury in premature rats.

Key words: 1,25-(OH)₂D₃, hyperoxia, preterm brain injury, integrin-β1, yes-associated protein

INTRODUCTION

Neonatal intensive care has significantly improved survival rates, but in extremely premature infants have immature lungs at birth, making them vulnerable to respiratory failure, therefore oxygen therapy is usually needed (Chawanpaiboon et al., 2019). However, continuous oxygen therapy can cause prematurity-related diseases such as retinopathy of prematurity and bronchopulmonary dysplasia (Shah et al., 2016; Day and Ryan, 2017). Furthermore, the brains of these infants are immature and vulnerable to harmful stimuli such as oxygen poisoning and inflammation. As a result, these infants remain at high risk of short-term and long-term neurological complications (Volpe, 2009). Hyperoxia is extremely damaging to the immature retina, and hyperoxia-induced brain injury can lead to neurodevelopmental abnormalities, including defective myelin formation (Obst et al., 2022). At present, the mechanism of neonatal brain injury induced by hyperoxia remains to be further elucidated.

Integrin-β1, as a regulator, is involved in the regulation of multiple mechanisms of brain injury, such as inner retinal injury, blood-brain barrier (Tang et
al., 2020; Abbasi et al., 2021). Expression changes of integrin-β1 will affect the proliferation and differentiation of glial cells (Pan et al., 2014). In the nucleus, yes-associated protein (YAP) transcription regulates cell proliferation and apoptosis (Szymaniak et al., 2015). After tissue injury, the location of YAP was significantly changed, and the expression of YAP was correlated with the increase of regeneration (Choi and Kwon, 2015; Gong et al., 2021). YAP has been demonstrated to be involved in a variety of brain injury mechanisms, although the role of YAP in hyperoxia-induced brain injury in premature rats remains unclear.

1α, 25-dihydroxyvitamin D3 (1,25-(OH)₂D₃) is widely found in human organs and tissues and exerts the steroid effect in the whole body. Its neuroprotective effect has been paid more and more attention by scholars (Zhang et al., 2022). In this study, a brain injury model of preterm mice was constructed through hyperoxia induction to further elucidate the neuroprotective effect of 1,25-(OH)₂D₃ on brain injury, and further elucidate the protective effect of 1,25-(OH)₂D₃ on optic chiasm under hyperoxia conditions.

METHODS

Animal model and study design

All procedures and protocols were approved by the Ethics Committee of Guizhou Provincial People’s Hospital on 13th August 2019 [2019Y08M13D; EC review (Animal) 2019-009]. Sprague-Dawley rats (20 weeks old, 350-450g, male: female=1:1 mating) were provided by Shanghai Sipur-Bikai Experimental Animal Co., Ltd and Hunan Slacker Jingda Experimental Animal Co., Ltd. Rats born naturally without external factors interfering with gestation of less than 19 days were premature rats and randomly selected, and the experiment was started within 12 h after birth. The premature rats were divided into four groups and treated as follows: control group (n=6) (Premature rats); control plus 1,25-(OH)₂D₃ treatment group (n=6); hyperoxia group (n=6) (Preterm rats placed in a hyperxia environment); and hyperoxia plus 1,25-(OH)₂D₃ treatment group (n=6).

The premature rats in control and control plus 1,25-(OH)₂D₃ treatment group were kept in room air, while hyperoxia and hyperoxia plus 1,25-(OH)₂D₃ treatment group were fed hyperoxia (O₂≥95%) as described previously (Ladha et al., 2005). The premature rats in two 1,25-(OH)₂D₃ treatment groups were intraperitoneally injected with 1,25-(OH)₂D₃ (0.3 μg/ml per 0.1 ml/10 g, Sigma, St. Louis, MO, USA), while control group and hyperoxia group were intraperitoneally injected with 0.9% normal saline (0.1 ml/10 g), respectively. Three rats from each group were injected continuously for 7 days and 14 days for follow-up experiment.

Tissue preparation

The rats were killed by intraperitoneally injecting with 5% chloral hydrate (0.16 ml/20 g) on postnatal day 7 and day 14, the tissue of 5 mm³ before and after optic chiasma was taken for subsequent experiments. After cardiac perfusion of normal saline, the tissue was fixed with 4% polymethyl for immunohistochemistry experiments.

Histology

The optic chiasma tissue of rats was rinsed with water for several hours and stained by standard HE method for histological and morphometrical examinations. Images were taken with CKX41 microscope (Olympus Corporation, Tokyo, Japan). Histopathological analysis was performed on 400 x magnification images using Image Pro software.

Immunohistochemistry

The prepared slices were dewaxed and hydrated with gradient ethanol, and then citric acid buffer was added for antigen repair. The slices were added with 3% hydrogen peroxide to remove the endogenous peroxidase blocking solution, and were incubated for 10 min, and fully rinsed with PBS. The slices were added with 5% BSA and sealed at 37°C for 30 min after immersing with PBS for three times.

Diluted primary antibody CD31 (Abcam, USA, 1:200) was added and incubated at 37°C for 30 min after immersing with PBS for three times. The slides were differentiated into blue using acid alcohol after being redyed with hematoxylin for 3 min. After differentiation, the tissue samples were rinsed under running water for 1 min. Dehydration, clearing, cover-slipping were carried out and the slides were finally examined under microscope. Expressions of integrin-β1 and YAP in optic chiasma tissues from the four groups of were observed under a microscope.
Western Blot

According to the manufacturer’s protocol (Beyotime, Shanghai, China), total proteins were extracted from optic chiasma tissue. The protein concentration was determined according to BCA kit (Beyotime, Shanghai, China). The protein was denatured and electrophoresed in 10% SDS-PAGE (Beyotime, Shanghai, China), then transferred to PVDF membrane (IPVH00010, Millipore). Afterwards, the membrane was incubated with primary antibodies overnight at 4°C and incubated with secondary antibodies lasting 2h. The antibodies information was shown as follows: β-actin (Abcam, USA, 1:100), integrin-β1 (Abcam, USA, 1:100), YAP (Abcam, USA, 1:100). Then, the ECL substrate (Thermo Scientific, USA) was dropped on the membrane and exposed in the chemiluminescence imaging system. The integrated optical density of the protein band was measured using ImageJ software (version 1.8.0).

Statistical analyses

All analyses were performed using Graphpad Prism software (version 7.0). One-way analysis of variance (ANOVA) was used to compare the statistical differences between multiple groups. The results were showed as mean ± standard deviation. Each experiment was conducted in triplicate. P<0.05 was considered statistically significant. The specific research process is shown in Fig. 1.

RESULTS

1,25-(OH)2D3 improve optic chiasmatic tissue morphology

As shown in Fig. 2, the rats killed in the control group and treated with 1,25-(OH)2D3 group at 7 days and 14 days showed uniform staining of nerve fibers, regular arrangement of oligodendrocytes, and no inflammatory cells infiltration. After 7 days and 14 days of hyperoxic exposure, swelling of the optic nerve in rats in the hyperoxic group, expanded nerve fiber space, uneven staining, obvious oligodendrocyte proliferation and disordered cell arrangement accompanied by inflammatory cell infiltration and exudation. The histomorphological changes of optic chiasma tissue in the hyperoxia plus 1,25-(OH)2D3 treatment group were improved, with reduced inflammatory exudation. Nerve fiber staining was uniform, and oligodendrocyte proliferation was not obvious.

1,25-(OH)2D3 reduces the expressions of integrin-β1 and YAP in the optic chiasm of rats with hyperoxic brain injury

To investigate the neuroprotection effect of 1,25-(OH)2D3 on hyperoxia-induced brain injury, the expression of integrin-β1 and YAP in optic chiasma tissue was detected by immunohistochemistry (Fig. 3). As shown in Fig. 4, western blot results showed that on day 7, the expression of integrin-β1 in the hyperoxic

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![Fig. 1. Research flowchart.](image-url)
group and the hyperoxic+1,25-(OH)_2D_3 group was significantly increased compared to the control group (t_{4, hyperoxic} = 32.39, P < 0.0001) (t_{4, hyperoxic+1,25-(OH)_2D_3} = 17.40, P < 0.0001) (F_{3,8} = 772.0, P = 0.0001). YAP expression in the hyperoxic group and the hyperoxic+1,25-(OH)_2D_3 group was significantly increased on day 7 compared to the control group (t_{4, hyperoxic} = 52.73, P < 0.0001) (t_{4, hyperoxic+1,25-(OH)_2D_3} = 25.30, P < 0.0001) (F_{3,8} = 1143, P = 0.0001). On day 7, the expression of integrin-β1 (t_{4, hyperoxic+1,25-(OH)_2D_3} = 22.32, P < 0.0001) and YAP (t_{4, hyperoxic+1,25-(OH)_2D_3} = 26.56, P < 0.0001) in the hyperoxic+1,25-(OH)_2D_3 group was significantly reduced compared to the hyperoxic group, which was consistent with immunohistochemistry. On day 14, the expression of integrin-β1 in the control+1,25-(OH)_2D_3
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The expression trend of integrin-β1 and YAP in the hyperoxic group and the hyperoxic+1,25-(OH)2D3 group was consistent with that in the control group compared with that on day 7 (t4control+1,25-(OH)2D3 =-22.46, P=0.0001) (t4hyperoxic =85.14, P=0.0001) (F3,8 =6.161, P=0.0035) (F3,8 =5992, P=0.0001). On day 14, the expression trend of YAP in the hyperoxic group and the hyperoxic+1,25-(OH)2D3 group was consistent with that in the control group compared with that on day 7 (t4hyperoxic =49.44, P=0.0001) (t4hyperoxic+1,25-(OH)2D3 =32.94, P=0.0001) (F3,8 =1829, P=0.0001). On day 14, integrin-β1 expression was significantly reduced in the hyperoxic+1,25-(OH)2D3 group than that in the hyperoxic group (t4hyperoxic+1,25-(OH)2D3 =127.8, P=0.0001). The expression trend of YAP in the hyperoxic+1,25-(OH)2D3 group compared with the hyperoxic group was also the same as on day 7 (t4hyperoxic+1,25-(OH)2D3 =35.22, P=0.0001).

**DISCUSSION**

Extremely premature infants with gestational age of less than 28 weeks are highly likely to have multiple organ injuries and developmental abnormalities, mainly involving lung and brain (Jobe and Bancalari, 2001; Volpe, 2009). To explore the role of 1,25-(OH)2D3 in hyperoxia-induced brain injury, we established this animal model which showed that treatment with 1,25-(OH)2D3 in premature rat pups exposed to hyperoxia attenuates brain injury. Premature rat pups within 24 h after birth were exposed to hyperoxia for 7 days and 14 days continuously, resulting in brain injury, visible swelling of optic nerve, expansion of nerve fiber space, uneven staining, obvious oligodendrocyte proliferation, disorder of cell arrangement accompanied by inflammatory cell infiltration and exudation.
Vitamin D is mainly synthesized from 7-dehydrocholesterol in response to skin exposure to ultraviolet light, but can also be obtained through dietary supplements (Holick, 2007). To be biologically active, vitamin D must first be converted to 1,25-(OH)$_2$D$_3$. Once in this active form, vitamin D can bind to vitamin D receptors located on a variety of cell types, including white blood cells, endothelial cells, astrocytes, and neurons (Provvedini et al., 1983; Merke et al., 1989; Langub et al., 2001; Lee et al., 2008). Vitamin D is best characterized by promoting calcium absorption in the small intestine, but recent findings have suggested that it may also control the expression of a large number of genes, particularly those involved in inflammatory processes (Lugg et al., 2015). The active form of vitamin D, 1,25-(OH)$_2$D$_3$, has attracted more and more attention in recent years. In the present study, we demonstrated that 1,25-(OH)$_2$D$_3$ reduced hyperoxia-induced brain injury, according to histopathological examination of optic chiasma and related protein expression.

Presence of integrins in the axon correlates with the regenerative capacity of neuronal pathways (Nieuwenhuis et al., 2018). Integrin-β1 plays a role in cell differentiation in nerve cells and the developing nervous system (Brooker et al., 2016). Yes-associated protein, a major downstream effector of the Hippo signaling pathway, plays a critical role in inflammation (Tian et al., 2020). The results showed that hyperoxia exposure increased the expression of integrin-β1 and YAP, whereas 1,25-(OH)$_2$D$_3$ treatment significant-
ly reduced the expression of integrin-β1 and YAP on day 7 and day 14. The results suggested that neonatal brain injury may affect the optic chiasma nerve through integrin-β1 and YAP. However, the underlying mechanisms need further elucidation.

Studies have found that 1,25-(OH)_{2}D_{3} reduced subsequent brain injury and inflammation associated with ischemic stroke and may play an immunomodulatory role through a variety of cellular and molecular mechanisms (Zeitelhofer et al., 2017; Anrather and Iadecola, 2016; Evans et al., 2018). This is consistent with the results of this study that YAP can participate in the regulation of inflammation related pathways (Choi and Kwon, 2015). In addition, 1,25-(OH)_{2}D_{3} has a protective effect on the injury of human umbilical vein endothelial cells induced by high glucose (Wu et al., 2021). 1,25-(OH)_{2}D_{3} is considered to be a neuroprotective agent by participating in the regulation of signal pathways, promoting blood vessel formation and playing a neuroprotective role (Zhang et al., 2022). Vitamin D has shown some advantages in eye diseases (Marampon et al., 2016; Hernandez et al., 2021). Besides, 1,25-(OH)_{2}D_{3} has been shown to protect retinal pigment epithelial cells and promote angiogenesis under hyperoxia exposure (Murugeswari et al., 2020). This is consistent with the results of this study, that 1,25-(OH)_{2}D_{3} treatment can reduce inflammatory damage (Fig. 2).

This study has some limitations and can only preliminarily reveal the protective effect of 1,25-(OH)_{2}D_{3} on the optic chiasma tissue in hyperoxia-induced brain injury, and its related potential mechanism remains to be further clarified.

CONCLUSION

This study demonstrated that 1,25-(OH)_{2}D_{3} may protect against hyperoxia-induced brain injury. 1,25-(OH)_{2}D_{3} may ameliorate hyperoxia-induced brain injury by decreasing integrin-β1 and YAP expression in optic chiasma tissues and reducing inflammatory exudation. However, the specific mechanism of the protective effect of 1,25-(OH)_{2}D_{3} on optic chiasma tissue needs further studies.

ACKNOWLEDGEMENTS

This study was supported by Guizhou Science and Technology Planning Project, Guizhou Science and Technology Integration Foundation (ZK [2021] General No. 370) and Doctor Foundation of Guizhou Provincial People’s Hospital (GZSYBS [2018] No. 04).

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