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Vitamin D Supplementation Reduces Functional Impairment of Hippocampus Caused by Dexamethasone

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The use of dexamethasone in premature infants has adverse effects on neurological function. Vitamin D is considered to be vital nutrient for neurological diseases. Pups were randomly divided into the control, model and treated groups. Treated group received vitamin D on postnatal day1 (60,000 IU/kg) . Following, model and treated group received dexamethasone from postnatal day 2 to postnatal day 4 following tapering doses (0.5, 0.3, and 0.1 mg/kg.d, respectively). Pups' neurological function was assessed by wire-hanging test and Morris water maze task. And apoptotic cells in hippocampus were counted. Vitamin D effectively improved spatial learning and memory impairment induced by dexamethasone. The protective effects of vitamin D may be related to the modulation of apoptosis. Vitamin D may therefore have a role in bronchopulmonary dysplasia treatment process.

Keywords: bronchopulmonary dysplasia; caspase-3; dexamethasone; hippocampus function; vitamin D
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Introduction

Bronchopulmonary dysplasia is the most common chronic lung disease in preterm infants. Immature newborn depends on oxygen concentration of more than 21% for 28 days or longs were diagnosed as bronchopulmonary dysplasia (Jobe and Bancalari 2001). Any factors which can enhance lung injury lead to the happen of bronchopulmonary dysplasia (Jobe and Bancalari 2001). Nowadays, contributed to higher survival rate of preterm newborns, the incidence of bronchopulmonary dysplasia is sustained growth. As a consequence, bronchopulmonary dysplasia becomes an important public health concern. There are many adverse health outcomes for bronchopulmonary dysplasia. The respiratory system, nervous system and growing development show poor performances in bronchopulmonary dysplasia patients. The hospital readmission rate of very-low-birthweight infants with bronchopulmonary dysplasia was twenty times higher than full-term infants (Monte et al. 2005). Additional, neurodevelopmental delay especially in hearing and vision deficits were obviously shown in most preterm infants with bronchopulmonary dysplasia in 24 months of life (Barrington and Finer 1998). Growth failure was also found by short-term follow-up studies, there were weight and height deficiency in bronchopulmonary dysplasia patients in comparison to normal infants (Barrington and Finer 1998). Therefore, effective and safe treatment methods for bronchopulmonary dysplasia are explored by neonatologists.

Dexamethasone therapy has been known as an effective treatment method for bronchopulmonary dysplasia since 1972. Dexamethasone can play an effective anti-inflammatory effect. Meanwhile, edema and fibrosis of lung can be prevented by dexamethasone. These consequential pathological changes lead to the improvement of lung function. As a result, dexamethasone therapy is one effective to reduce the incidence and severity of bronchopulmonary dysplasia. However, the neurologic impairment causing by dexamethasone has been recognized (Yeh et al. 2004). Newborn who was treated by dexamethasone would show learning and memory capability deficit at the juvenile age (Feng et al. 2015). Many studies showed that exposure to dexamethasone lead to the reduction in cerebral tissue volume in premature infants (Murphy et al. 2001).

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Dexamethasone increases cell death in brain (Yu et al. 2008). And dexamethasone induces impairs neurogenesis and apoptosis (Kanagawa et al. 2006). There were much more prominent apoptotic changes in the subependymal ventricular zone and dentate gyrus in hippocampus (Bhatt et al. 2013). Dexamethasone can induce caspase-3 activation (Feng et al. 2009). More cleaved caspase-3 positive cells have been found in the dexamethasone treated developing brain (Feng et al. 2015). And oxidative stress-induced cell death in cerebellar granular cells and hippocampal neurons would increase when exposure to dexamethasone *in vitro* (Ahlbom et al. 2000). It resulted in the neurodegenerative effects in newborn rats. Given this, dexamethasone has an adverse effect on long-term neurological function. In conclusion, dexamethasone therapy is an effective treatment on bronchopulmonary dysplasia along with many long-term adverse outcomes. So now, it is still a controversy on the use of dexamethasone in preterm infants.

Vitamin D is an important steroid which affects many aspects of human physiology. Researchers found that calcitriol has an essential role in cell life in different tissue. It regulates various physiological pathways. So, vitamin D is considered as potential therapeutic strategy for various diseases. Recently, vitamin D has been demonstrated to be a potent neurohormone in neuronal development and dysfunction. And the wide distribution of vitamin D receptors in brain suggests that vitamin D may play important role in brain functions (Mokhtari-Zaer et al. 2020). Vitamin D-deficient mothers' infants are at a high risk of abnormal brain development (Eyles et al. 2013). And vitamin D-deficient neonates are vulnerable to brain injury. This is also shown in animal studies (Eyles et al. 2003). Vitamin D is an important role in inflammatory processes. It takes part in immunomodulatory actions through cellular and molecular mechanisms (Zeitelhofer et al. 2017). Vitamin D could reduce infarct volume in ischemic stroke patients by reducing expression of inflammatory factors (Evans et al. 2018). Further, vitamin D can enhance antioxidant ability by decreasing expression of nicotinamide adenine dinucleotide phosphate oxidase enzymes and increasing expression of superoxide dismutase and glutathione (Dong et al. 2012). Vitamin D3 might improve hippocampus function by antioxidant and anti-inflammatory pathway (Mokhtari-Zaer et al. 2020). At the same time, vitamin D inhibited expression of cleaved caspase-3 significantly (Evans et al. 2018). So vitamin D exerted neuroprotective effects in many studies. Therefore, we reasoned vitamin D can be one important therapeutic strategy to improve neurological diseases in connection with oxidative stress and/or inflammation.

Vitamin D receptor and glucocorticoid receptor are distributed in human body. Both of vitamin D and glucocorticoid are important steroids for human. There is interaction effect between vitamin D and glucocorticoids. Vitamin D can suppress glucocorticoid-induced osteoporosis and attenuate excessive glucocorticoid receptor signaling (Compston 2010). Meanwhile, glucocorticoids play an influence on intracrine of vitamin D. And dexamethasone can increase vitamin D and vitamin D receptor in a glucocorticoid receptor-dependent manner (Hidalgo et al. 2011). The interaction between these two in many aspects of human physiology is pervasive. Dexamethasone-induced side-effects are attenuated with vitamin D (Mehta et al. 2015). Given this, vitamin D may be an effective to inhibit dexamethasone side effects on nervous system. In this study, we test the hypothesis that bronchopulmonary dysplasia infants treatment with dexamethasone along with vitamin D could be reduce dexamethasone -associated side-effects on brain development.

Methods

Animals

Ten pregnant Sprague-Dawley rats (Wenzhou medical University, Wenzhou, China) with timed gestations were individually housed under standard conditions: a 12:12-h light-dark cycle, with lights on at 7:00 a.m.; temperature maintained at 21 ± 2˚C; and the provision of food and water. All animal experiments were approved by the Ethics Committee for Animal Experimentation of The First Affiliated Hospital of Wenzhou Medical University. And all animal procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of The First Affiliated Hospital of Wenzhou Medical University. All pregnant rats delivered on the third gestational weeks. The birthday was assigned postnatal day 0. Healthy male rat pups, weighing 5-8 g, were divided into three groups: dexamethasone group (model group, n = 24), dexamethasone with vitamin D group (treated group, n = 24), control group (control group, n = 12).

Drug administration

Model group received a three-day tapering doses dexamethasone on postnatal day 2 through postnatal day 4 (0.5, 0.3, and 0.1 mg/kg.d, respectively, by intraperitoneal injection). Treated group received a combination of vitamin D on postnatal day 1 (60,000 IU/kg, by intraperitoneal injection) and dexamethasone on postnatal day 2 through postnatal day 4 (0.5, 0.3, and 0.1 mg/kg.d, by intraperitoneal injection). Control group received equivalent volumes of saline in the same manner from postnatal day 1 to postnatal day 4.

Tissue collection

During the treatment period, rats were euthanized on postnatal day 28. Rats were weighed on birthday and date of death. Their brain tissue was weighed and dissected on date of death. Brain was further studied by immunohistochemistry.

Behavioral tests

Wire-hanging test: The wire-hanging test was carried out to assess motor tone on postnatal day 15. A glass rod,

0.2 cm in diameter and 25 cm long, was placed 25 cm above table on this test. Animals were required to hang on the rod by their forelimbs. The time that animals were hanging on the rod was recorded. A scale of 1 to 5 based on duration was used to assess motor tone: 1 (less than 10 seconds), 2 (10-30 seconds), 3 (30-120 seconds), 4 (120-300 seconds) and 5 (more than 300 seconds).

The morris water maze task: The spatial learning ability of rats was assessed by the Morris water maze task on postnatal day 21. A circular pool filled with water on this test. It is a black pool, 120 cm in diameter and 50 cm in height, was filled with water to a depth of 47 cm. The temperature of water maintained at 24°C-26°C for a comfortable temperature. And the water was made opaque by ink. The pool was divided into four quadrants. And a circular platform which was 10 cm diameter and 45 cm height was placed on the third quadrant. The activity of rats was recorded by video and analyzed by DigBehv animal behavior analysis system (Shanghai Jiliang Software Technology limited company). The experiment lasted six days. The first step of procedure was pretraining for the first five days. The rats were gently placed into pool in one of the four quadrants randomly in the pretraining step. They swam in pool freely to reach the platform and climb up out of the water in 60 seconds. The rats could have a rest on platform for 10 seconds. On the other hand, the rats were introduced to platform if they could not find platform in 60 seconds by themselves. At the sixth day, the platform was removed from the pool. Each rat was released into pool for the exploring test separately. They were allowed to explore the pool for 60 seconds. The route of rats was record by video. The times of rats across the target site, the percentage of time spent in swimming and total distance traveled were analyzed.

Immunohistochemistry

All rats were deeply anesthetized and perfused through left ventricle with normal saline followed by 4% phosphate-buffered paraformaldehyde. Then the brain was removed carefully and stored in 4% paraformaldehyde in 4°C refrigerator for 24 hours. And brain samples were processing by histochemical procedures. The paraffin sections contain hippocampus were collected. Primary antibody was caspase-3 antibody (1:200, Cell Signaling, 9662). Then rabbit reinforced polymer detection system kit (ZSGB-BIO, PV-9001) were used to detect primary antibody according to the protocol. All images were record by camera and analyzed by imagepro plus. Caspase-3 positive cells in the subgranular zone (SGZ) and the granule cell layer (GCL) of the hippocampus were counted in the three groups. The sections in the same animal which were selected in the analysis were spaced five sections apart. Results were expressed as the average number of caspase-3 positive cells in SGZ and GCL of the dentate gyrus of the hippocampus.

Statistical analysis

All data are reported as mean ± standard error (SEM) of the mean. A repeated measures analysis of variance (ANOVA) was used to analyze the result of Morris water maze task. The other data was analyzed by one way ANOVA followed by LSD post hoc test. P values < 0.05 were considered to be statistically significant.

Results

Vitamin D and dexamethasone make negative impact on weights

The body weights of animals were recorded at their birthday. There was no significant difference among three groups ($F_{(2,57)} = 0.349$, $p = 0.640$) (Fig. 1a). And after treatment, the growth of body weights in three groups was different. The body weight in control group was greater than model group ($p = 0.026$) and treated group ($p = 0.026$) (Fig. 1b). But there was no significant difference between treated group and model group in body weight ($p = 0.997$) (Fig. 1b). In addition, brain weight in control group also was bigger than the other groups (treated group, $p = 0.033$; model group, $p = 0.033$) (Fig. 2). However, there was also no significant difference between treated group and model group in brain weight ($p = 1.0$) (Fig. 2).

Vitamin D and dexamethasone exert little influence on neuromotor function

To determine whether dexamethasone could induce disorders of neuromotor function, wire hanging test were performed to assess motor tone. However, there was no significant difference among three groups ($F_{(2,57)} = 0.803$, $p = 0.453$). Compared with the control group, the model group did not show significant motor tone defects ($p = 0.568$). And we observed that the model group got a lower score compared with the treated group, nevertheless, there also was no statistical significance between model group and treated group ($p = 0.211$) (Fig. 3).

Vitamin D acts against dexamethasone-induced hippocampal function impairment

To evaluate the effects of dexamethasone and vitamin D on hippocampal function, we detailed recorded and analyze motion trail in Morris water maze task. The escape latency of rats in model group was significantly longer than control group ($p = 0.000$). But following the treatment of vitamin D, the treated group showed shorter escape latency than model group ($p = 0.010$). And after therapy, escape latency of treated group was close to control group ($p = 0.125$) (Fig. 4). In addition, the time spent in target quadrant and the number of times they crossed the site of platform on probe trial were significantly difference among groups. The model group showed less time in target quadrant and frequency crossing the target location. There were statistic difference between model group and control group in time in target ($p = 0.028$) (Fig. 4) and the number of times crossing the site of platform ($p = 0.001$) (Fig. 4).

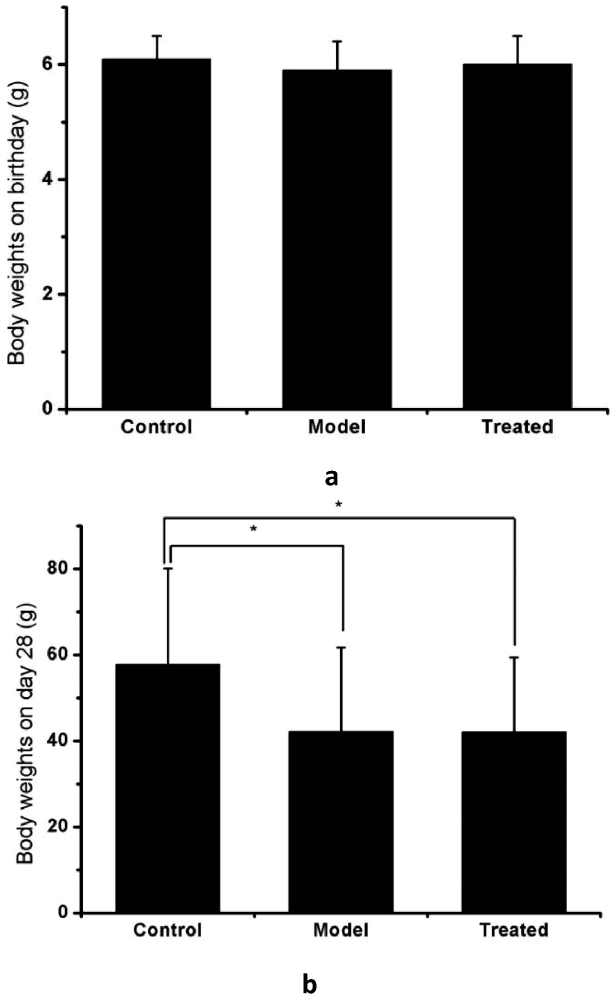

Fig. 1. Effects of vehicle, dexamethasone and/or vitamin D on body weight.
(a) The birth weights of three groups. Bar graphs represent mean ± SEM. There were no significant differences among groups. (b) Effects of vehicle, dexamethasone and/or vitamin D on body weight at postnatal day 28. Data are represented as mean ± SEM. Significance differences was found at *p < 0.05.

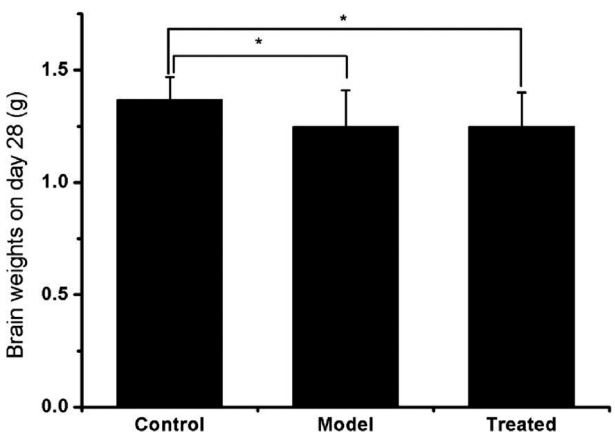

Fig. 2. Effects of vehicle, dexamethasone and/or vitamin D on brain weight.
Data are represented as mean ± SEM. Significance differences was found at *p < 0.05.

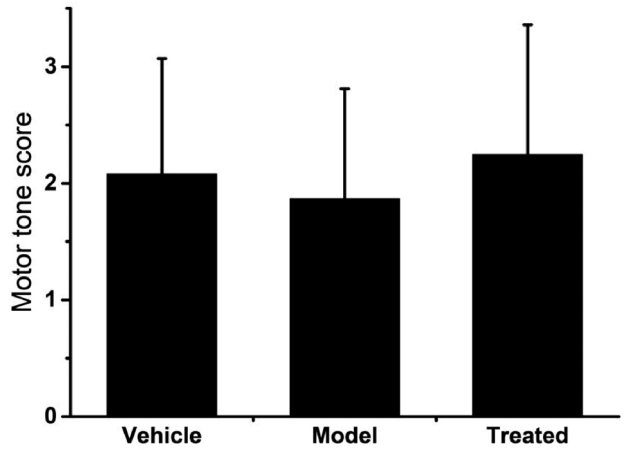

Fig. 3. Effects of vehicle, dexamethasone and/or vitamin D on wire-hanging test.
There is no significant difference among groups.

Meanwhile, Vitamin D improved the hippocampal function which was hurt by dexamethasone. The treated group spent more time in target quadrant than model group ($p = 0.039$) (Fig. 4). Similarly, the number of times crossing the site of platform in the treated group was more than the model group ($p = 0.007$) (Fig. 4). These results indicated that vitamin D effectively improved spatial learning and memory impairment induced by dexamethasone.

Vitamin D suppress dexamethasone-induced hippocampal cell apoptosis

In the control group, model group, and treatment group, the number of apoptotic cells in the GCL (granule cell layer) was higher than that in the SGZ (subgranular zone) area. However, the increase in apoptotic cells in the GCL was more pronounced in the model group compared to the SGZ area ($p = 0.000$) (Fig. 5). There were statistically significant differences in the number of apoptotic cells in the SGZ and GCL areas of the hippocampus between the model group and the control group ($p = 0.000$) (Fig. 5). However, treatment with vitamin D significantly reduced the number of apoptotic cells in both the SGZ and GCL areas of the hippocampus. There was a statistical difference between the model group and the treatment group in the SGZ area ($p = 0.000$), as well as in the GCL area ($p = 0.000$) (Fig. 5). Furthermore, there was no significant difference in the number of apoptotic cells in the SGZ area between the control group and the treatment group ($p = 0.068$). Similarly, there was no significant difference in the number of apoptotic cells in the GCL area between the control group and the treatment group ($p = 0.690$) (Fig. 5). These results suggest that dexamethasone can induce apoptosis in hippocampal cells in baby rats, and vitamin D can effectively inhibit cell apoptosis induced by dexamethasone.

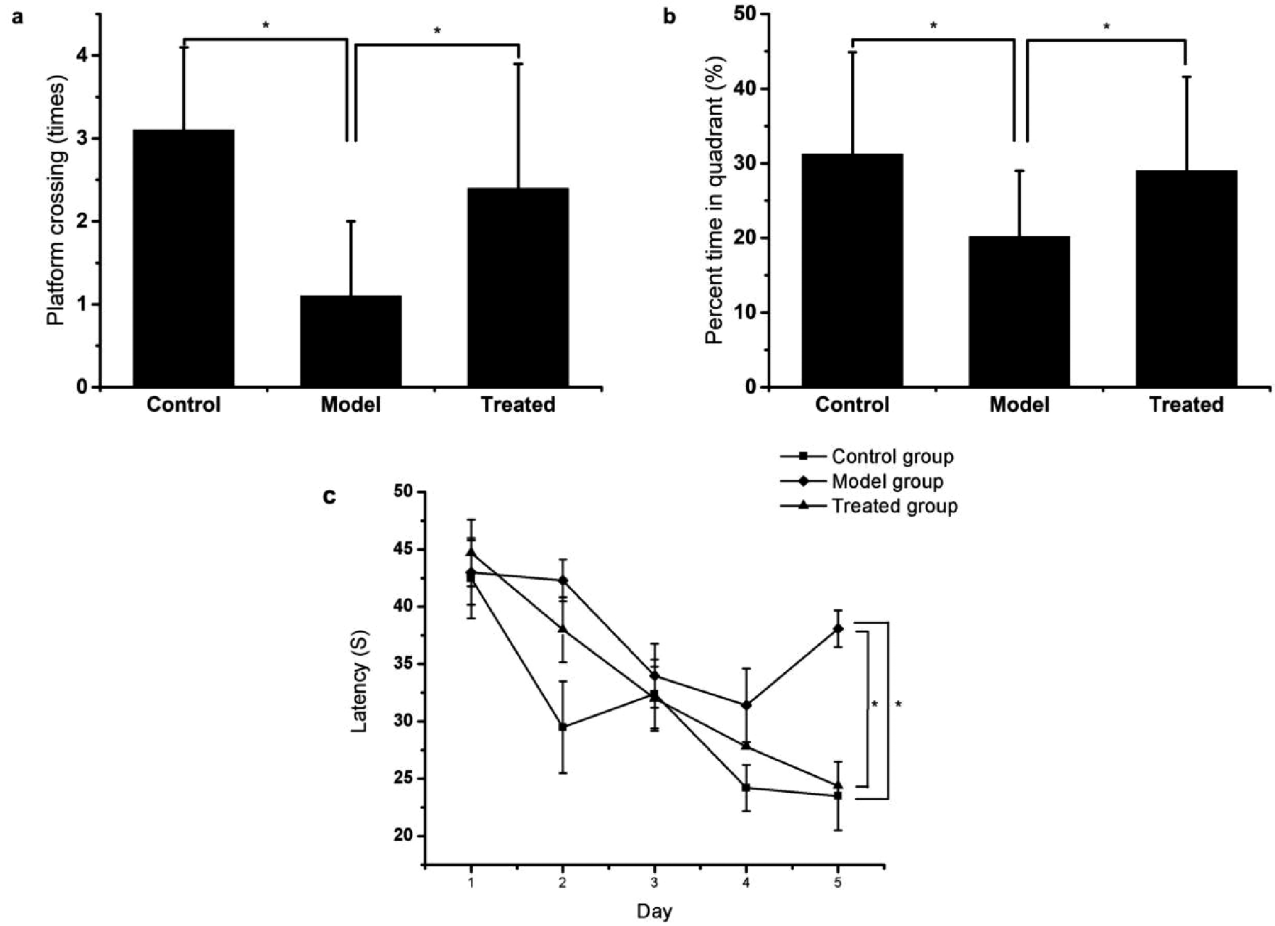

Fig. 4. Vitamin D attenuated dexamethasone-induced cognitive impairment in pups.
(a) Platform crossing, (b) Time spent in the target quadrant, (c) Escape latency was measured in each group. Data are represented as mean ± SEM. Significance differences was found at *p < 0.05.

Discussion

Dexamethasone so far is one important therapy for bronchopulmonary dysplasia. However, dexamethasone is associated with high incidence of cerebral palsy in premature infant (Yeh et al. 2004). It still lacks a potent treatment to overcome dexamethasone side effect on nerve system. Our data presented here showed that dexamethasone has a significant negative effect on hippocampus function for premature infants. The model group in Morris water maze task need more time to find the platform and showed longer escape latency in the task. Besides, the time spent in target quadrant and the frequency they crossed the target site were less than control group. The pups had worse learning and memory ability after dexamethasone injection. But, we found vitamin D effectively prevent side effect of dexamethasone on nervous system. All observing index in Morris water maze task got better after vitamin D treatment and were closed to control group. Vitamin D had a positive effect on improving hippocampus impaired function. Since vitamin D was previously demonstrated to be an important neuroactive steroid, we followed its role on developing brain and discovered vitamin D maybe overcome dexamethasone side effect.

To our knowledge, dexamethasone can significantly increase cell apoptosis in brain. This is obviously observed in developing brain (Bhatt et al. 2013). The level of caspase-3 expression in different brain zone was high after dexamethasone strategy. This was also found in our result. Caspase-3 positive cells in hippocampus increased after exposing to dexamethasone. However, the intervention of vitamin D suppressed the number of caspase-3 positive cells. The vitamin D treated group in our study showed small quantity of caspase-3 positive cells in hippocampus. The level of caspase-3 positive cells was similarly between treated group and control group. And this was accord with result in animal behavioral experiment. We reasoned that vitamin D may prevent dexamethasone adversely affects brain development by reducing cell apoptosis. In the past years, numerous studies proved that dexamethasone enhance oxidative stress induced cell death by increasing free radical damage (Ahlbom et al. 2000). Also, it can intervene and restrain cell cycle to decrease cell proliferation (Crochemore et al. 2002). Dexamethasone induce

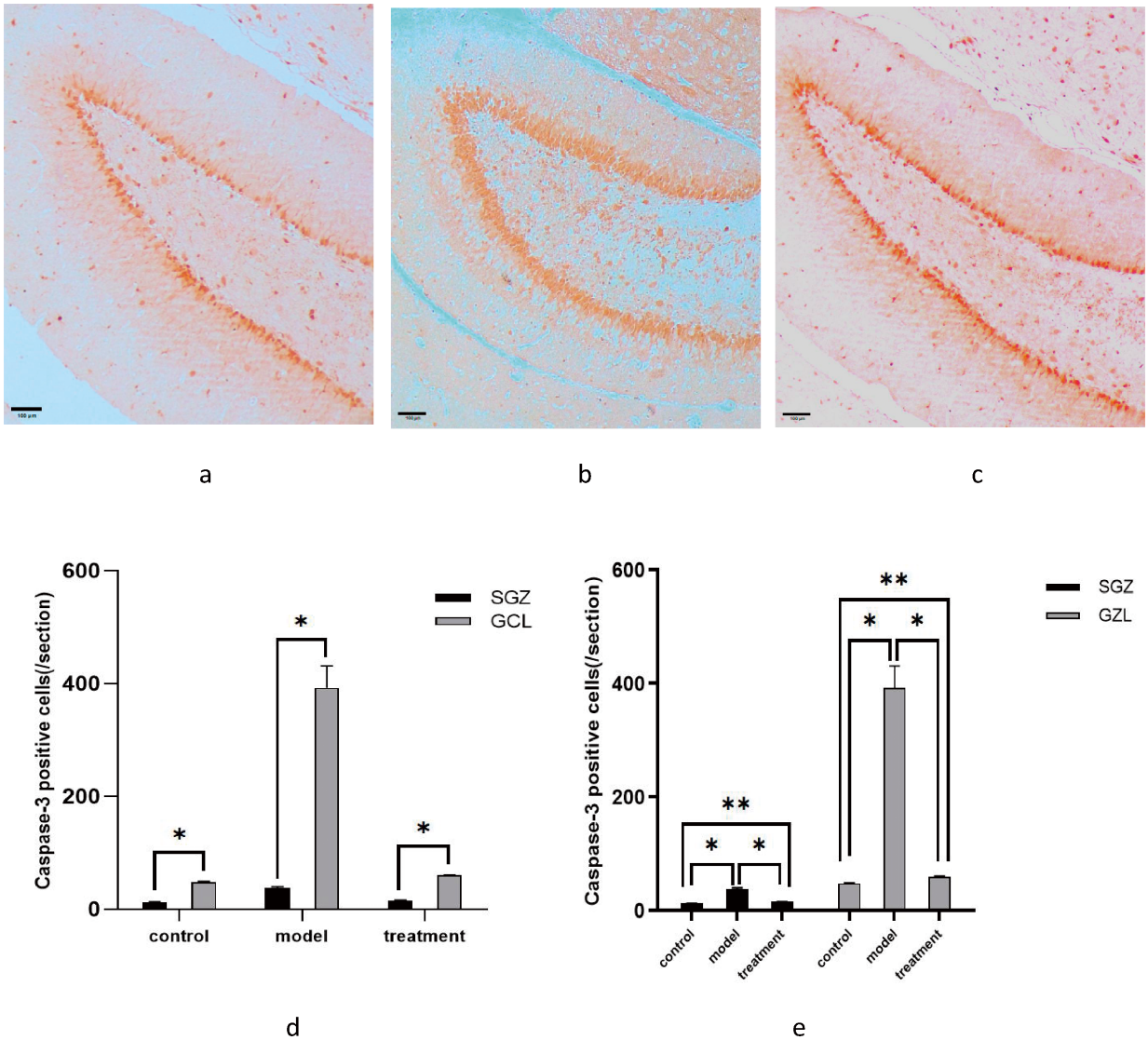

Fig. 5. Vitamin D protects against methadone-induced cell death in hippocampus.
Caspase-3 positive cells in SGZ and GCL of hippocampus were observed under light microscope. (a) Control group, (b) Model group, (c) Treated group. (*) indicates a significant difference ($p < 0.05$); (**) indicate a lack of significant difference ($p > 0.05$).

apoptosis cascade by many pathways. As we known, vitamin D may regulate various physiological pathways to protect nerve cell, such as immune modulation, oxidation resistance and cell cycle. So, there should be cross-talk in different pathways between vitamin D and dexamethasone. In our study, caspase-3 was one site where they interaction. According to other studies, vitamin D can increase phosphorylated CREB to protect hippocampus function (Nadimi et al. 2020). Detoxification pathways and neurotrophin synthesis are where vitamin D plays a role to protect neurons (Nadimi et al. 2020). In addition to these, to inhibit inflammation and oxidative stress are effective approaches for vitamin D to protect neurological function (Mokhtari-Zaer et al. 2020). Those also are the action sites of dexamethasone. So we hypothesis there still are many interaction between vitamin D and dexamethasone in nervous system need to explore in further study.

Unexpectedly, dexamethasone-treated animals showed normal motor tone compared with controls in our study. In wire-hanging test, the time hanging on the rod was similar among three treatment different groups. Neuromotor function was no affected by dexamethasone and vitamin D. It is different with other scholars' results which revealed dexamethasone has adverse effects on neuromotor function in clinical research (Yeh et al. 1998).

Dexamethasone therapy with or without vitamin D in stage of brain development exerts negative influence on body and brain development trend. It was also conformed to some current opinions. In present study, neonatal dexamethasone treatment would reduce pups' body and brain weight (Camm et al. 2011). However, vitamin D did not change the effect caused by dexamethasone in growth and

development. We think more time may be needed to observe.

Beyond that, we found extra vitamin D probably not have an obvious impact on normal growth and development. As just described, pups went through dexamethasone treatment showed normal neuromotor function. Further, vitamin D did not change this situation even lead to worse neurological function. In spite of evidence from the literature on vitamin D, there is to our knowledge few studies focus on relationship between vitamin D overdose and nervous system. Nowadays, there has been increasing attention to vitamin D treatment in different diseases. However, so far, the risks of vitamin D long term therapy even vitamin D overdose were rarely mentioned. There were only scattered reports described vitamin D overdose resulted in hypercalcemia and hypertension (Narsaria et al. 2016). It is controversial whether multiple organs would be further impaired. Our results did not found vitamin D played significant side effects on growth and development. Of course, vitamin D treatment duration and dose in this study were not enough to be statistically meaningful. A longer follow-up is needed. Safe and effective treatment plan for vitamin D can be gradually perfect.

Conclusion

Dexamethasone still is one useful drug for bronchopulmonary dysplasia. But the proper therapeutic strategy remains to be defined to avoid side effect. Vitamin D can effectively improve hippocampus function deficit causing by dexamethasone. Vitamin D can reduce the level of caspase-3 in hippocampus with dexamethasone. Our work highlights the important role of vitamin D on dexamethasone for bronchopulmonary dysplasia. The data provide the proof of concept that vitamin D is useful and safe to join in bronchopulmonary dysplasia treatment. But the study of mechanism research in our work is not enough. It is now important to investigate the crosstalk between vitamin D and dexamethasone in therapy and find the best therapeutic strategy.

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Conflict of Interest

The authors declare no conflict of interest.

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