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Original Article

Effect of glucocorticoids on SCN injury and the PI3K/AKT/mTOR signaling pathway

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Abstract: Objective: This study aimed to investigate the effect of glucocorticoids on spinal cord neuron (SCN) injury and the PI3K/AKT/mTOR signaling pathway. Methods: In total, 50 Sprague Dawley rats were purchased, of which 10 were randomized into the blank control group (BCG), and the other 40 were modeled for spinal cord injury by rubrospinal tract (RST) cross section method. After which, 10 of these were included into the model control group (MCG) on a random basis, and the other 30 were given dexamethasone, and equally divided into the low dose group (LDG) (n=10) (0.3 mg/kg), medium dose group (MDG) (n=10) (0.5 mg/kg) and high dose group (HDG) (n=10) (0.8 mg/kg) according to the dose of dexamethasone. The MTT method was used to observe the SCN activity in each group, western blot was used to detect the expression level of PI3K/AKT/mTOR signal pathway, qPCR was used to detect the level of autophagy-related (ATG) genes' mRNA (LC3II and Beclin-1) and apoptosis genes' mRNA (Caspase-3), AO fluorescent staining was used to detect the autophagy expression of SCN, and transmission electron microscope (TEM) was used to detect the autophagy and apoptosis in each group. Results: SCN in the MDG demonstrated higher activity than that in the HDG and LDG ($P<0.05$); the expression levels of proteins PI3K, AKT and mTOR, LC3II, Beclin-1 and Caspase-3 mRNA in the MDG were lower than those in the HDG and LDG ($P<0.05$); the expression of proteins PI3K, AKT and mTOR had no statistical difference in the MDG and BCG ($P>0.05$). According to the results of fluorescent staining, the HDG and LDG had no clear difference in fluorescence intensity (FI), while the FI of the LDG was slightly lower than that of the HDG and the MDG; according to the results of TEM, with a few number of cells experiencing autophagy and apoptosis, the HDG and the LDG had no clear difference, but both exceeded the MDG in terms of cell autophagy ($P<0.05$). SPSS Pearson correlation analysis results showed that glucocorticoid use in patients with spinal cord injury was negatively correlated with PI3K protein, AKT protein, mTOR protein and PI3K/AKT/mTOR signal pathway ($P<0.05$). Conclusion: Glucocorticoid, at a medium dose, is advantageous to SCN injury by reducing the expression level of ATG genes according to a mechanism related to PI3K/AKT/mTOR signaling pathway.

Keywords: Glucocorticoid, SCN injury, ATG genes, PI3K/AKT/mTOR signal pathway

Introduction

As the most serious complication of spinal injury, spinal cord injury (SCI) refers to severe dysfunction of the extremities under the injured segment, which not only causes damages to the patients physically and psychologically, but also imposes a heavy financial burden on them [1]. The peripheral nerve consists of spinal nerves, the nerve cord and innervation of the peripheral target organs. Central neurons are responsible for maintaining the shape of peripheral target muscles as modulators of functions of the motor end-plate [2]. Some studies by foreign scholars [3] have demonstrated that calcitonin gene related peptides (CGRP) played a vital role in the regenerative process after SCI. Dexamethasone, a member of the glucocorticoids, is extensively applied in the treatment of injuries to the central and peripheral nerves. It can alleviate the inflammation of the injured site, prevent and slake edema. However, clinically there is no unified criterion for the dosage of dexamethasone. As the mechanism of high dose glucocorticoid on acute SCI is complicated, the role of hormones in SCI is unknown [4].

Autophagocytosis, or autophagy, is a process of decomposing and digesting the components of excessive or injured cells by lysosomes [5]. It is an important part in normal cell regulation, and a response to internal and external pressure stimulation [6]. Clinical studies [7] have revealed the important roles of autophagocytosis in cell development, tissue remodeling,

cellular immunity, and adaption to the environment. Mammalian target of rapamycin (mTOR) is a non-typical serine/threonine protein kinase with great importance in the regulation of cell growth; while TOR, a negative regulator, functions as a gate in autophagocytosis by leveraging the two regulation mechanisms to inhibit this process [8, 9]. Foreign scholars [10] have found in their studies that apoptosis and autophagy play an important role in SCN injury; but considering the complicated autophagy process and the direct participation of PI3K/AKT/mTOR signal pathway in regulating autophagy level, their contribution has not been established yet.

Therefore, with Sprague Dawley (SD) rats as the subjects, this study explored the effect of a glucocorticoid on SCN injury and the PI3K/AKT/mTOR signaling pathway.

Materials and methods

Animals

Fifty SD rats were purchased, of which 10 were randomized into the blank control group (BCG), and the other 40 were modeled for SCI by rubrospinal tract (RST) cross section method. After which, 10 of them were included into the model control group (MCG) on a random basis, and the other 30 were given dexamethasone, and equally divided into the low dose group (LDG) (n=10), medium dose group (MDG) (n=10) and high dose group (HDG) (n=10) according to the dose of dexamethasone. All of them were purchased with the qualification certificate number of SCXX-2005-0001 and kept in the experimental animal room of a medical university with routine housing and exposure to light. This study was approved by Fujian Medical University Union Hospital.

Reagents and instruments

Breathing apparatus for small animals (Medical Instrument Factory of Zhejiang University School of Medicine), multi-channel electrophysiologic signal data acquisition system (MESDAS) (Chengdu Instrument Factory), pentobarbital sodium (Hubei Hongyunlong Biotechnology Co., Ltd.), Olympus bx-50 inverted phase contrast microscope (Olympus, Japan), biological image processing system (Nikon, Japan), BCA protein assay kit (Beijing Biyuntian Biotechnology Co., Ltd.), horse radish peroxidase (HRP)-labeled sheep anti-mouse secondary antibody (Wuhan Boster Bioengineering Co., Ltd.) and rabbit anti-rat PI3K, Akt, mTOR and p-mTOR antibodies (CST, the United States) were used in this study.

Methods

Grouping and modeling. Fifty SD rats were purchased, of which 10 were randomized into the BCG, and the other 40 were modeled for SCI by rubrospinal tract (RST) cross section method. The specific operations were as follows [11, 12]: all rats were accurately weighed and anaesthetized by intraperitoneal injection of 10.0% chloral hydrate at the dose of 4 mL/kg, after which, they were fixed at the supine position with the cervical flexure projecting upward. In the process of the surgery, the soft tissues and superficial muscles were sufficiently separated under the microscope to expose the large amount of muscles attached to the C_2 spinous process. By removing part of the erector muscle of spine attached to the right side of C_2 spinous process, C_3 and C_4 vertebral pedicles, and ligamentum flavum were sufficiently exposed. The ligamentum flavum was removed to fully expose the spinal cord. After identifying the dorsal root of spinal nerve, a No. 12 surgical knife was used to cut open the area from the junction of dorsal root outward until the posterolateral cord of the right half spinal cord so as to complete the SCI modeling. The wound was sutured layer by layer, and the surgery was then ended. As the rats woke up, they were found with the right forelimb flexed and close to the body, with uncoordinated movement, while the right paws were difficult to unfold, indicating a successful result of modeling. All rats were housed in cages separately, and the wound was cleaned and dressed every day. The cages were maintained and kept dry and clean.

Intervention. Rats in the MCG and BCG were injected with normal saline of equal amount (5 uL), and the other 30 animals were divided into the LGD, MDG and HDG according to the doses of dexamethasone, which were 0.3 mg/kg, 0.5 mg/kg and 0.8 mg/kg respectively, by way of intragastric injection administration, and 5 uL (10 ug) once a day. During intragastric administration, the needled was retained for 5 min, and then those rats were administered with distilled water by the same method continuously for 8 d. Both groups were intramuscularly injected with 100,000 U of penicillin for continuously for 3 d.

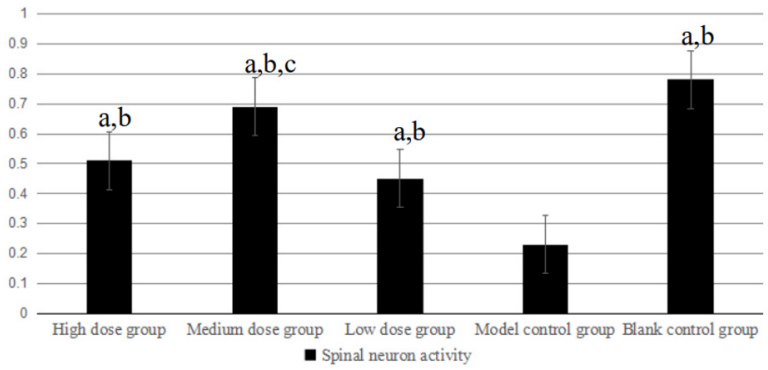

Figure 1. Comparison of SCN activities among the 5 groups. Compared with the blank control group, [a]P<0.05; compared with the model control group, [b]P<0.05; compared with the high-dose group and low-dose group, [c]P<0.05.

Observation indexes

SCN activity. The SCN activity of each group was observed by MTT method. Eight days after intervention, 2 rats were selected from the five groups and sacrificed by cervical dislocation. Tissues were taken from the spinal cord and centrifuged to obtain the cell suspensions which were routinely cultivated and mixed with 10 uL of 10 mg/L MTT solution for 4 h of cultivation. After the cultivation, the culture supernatants were removed from the wells, and then each well was mixed with 100 uL of DMSO for color development on a table. The absorbency was determined at the wavelength of 595 nm [13].

Expression of PI3K/AKT/mTOR signal pathway. Western blot was adopted to detect the expression level of PI3K/AKT/mTOR signal pathway by the following methods. Spinal cord tissues from the five groups were accurately weighed and added with 1 mL of lysate. Then, the mixture was placed at room temperature for 30 min and centrifuged for 15 min at the speed of 15000 rpm. The supernatant was reserved and detected for protein content of the tissues by BCA protein assay kit. As the protein was quantified, 60 ug of protein sample was added into each well for electrophoresis and trarsmembrane. After that, the primary antibodies Anti-PI3K, Akt, mTOR and β-actin were added and stayed overnight at 4°C. One h after culturing with second antibody, the mixture was exposed in ECL chemiluminescence reagent, and image analysis was done [14].

Expression levels of LC3II, Beclin-1 and Caspase-3 mRNA. qPCR was used to detect the level of autophagy-related (ATG) genes' mRNA (LC3II and Beclin-1), apoptosis genes' mRNA (Caspase-3).

SCN autophagy expression. The method AO fluorescent staining was applied to detect the SCN autophagy expression of each group. From the spinal cord tissue obtained, neurons were separated, and those that have grown robustly were selected to inoculate the 24-well plate at 5×10^4. As cells covered the whole well plate and after mechanical injury processing, AO with final concentration of 10 umol/L was added into the wells for staining, and SCN was observed under the fluorescence microscope.

SCN autophagy and apoptosis. The separated samples of spinal cord were taken and placed on the slide for detection of SCN autophagy and apoptosis of each group by TEM.

Correlation analysis. SPSS Pearson correlation analysis software was adopted for correlation analysis between glucocorticoid and PI3K/AKT/mTOR signal pathway.

Statistical analysis

Statistical analysis was performed with SPSS 18.0. In case of enumeration data expressed as [n (%)], comparison studies were carried out through X^2 test. In case of measurement data, t test was adopted. All the data conformed normal distribution. One-way ANOVA was used for the comparison of multiple groups of data, which were expressed as $\bar{x} \pm s$. SPSS Pearson correlation analysis software was adopted for correlation analysis between glucocorticoid and PI3K/AKT/mTOR signal pathway. For all statistical comparisons, significance was defined as $P<0.05$.

Results

Comparison of SCN activities among the five groups

No statistical difference was found between the HDG and the LDG, and the MDG and the BCG in terms of SCN activity ($P>0.05$); the MDG demonstrated higher SCN activity that the HDG and the LDG ($P<0.05$, **Figure 1**).

Table 1. Comparison of expressions of the PI3K/AKT/mTOR signal pathway in the 5 groups

Group	n	PI3K	AKT	mTOR
HDG	10	0.73±0.12[a,b]	0.90±0.17[a,b]	0.81±0.16[a,b]
MDG	10	0.45±0.11[b,c]	0.51±0.13[b,c]	0.44±0.10[b,c]
LDG	10	0.74±0.13[a,b]	0.89±0.16[a,b]	0.80±0.14[a,b]
MCG	10	0.86±0.16	0.84±0.17	0.89±0.17
BCG	10	0.44±0.10	0.50±0.12	0.43±0.09
P		0.013	0.019	0.033

[a]*P*<0.05 as compared with the BCG, [b]*P*<0.05 as compared with the MCG, and [c]*P*<0.05 as compared with the HDG and the LDG.

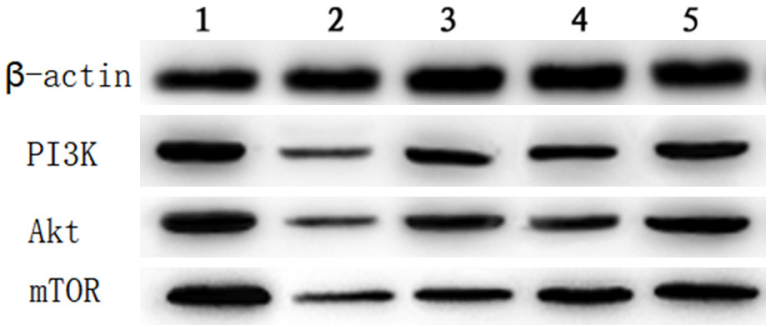

Figure 2. Expressions of PI3K/AKT/mTOR signaling pathway in the 5 groups. Note: in the figure, MCG is expressed as 1, BCG as 2, HDG as 3, MDG as 4 and LDG as 5.

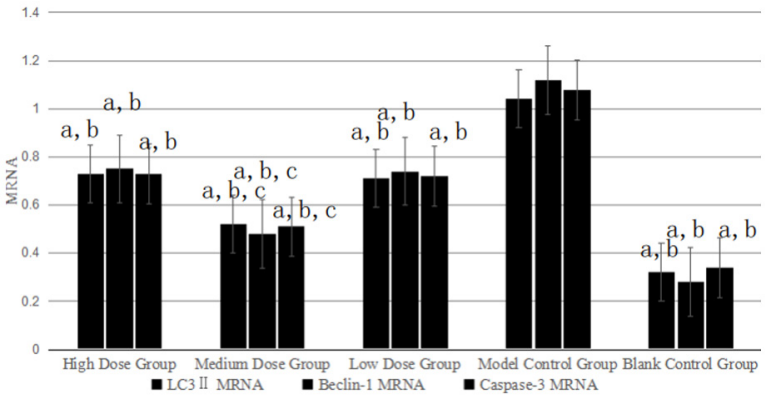

Figure 3. Comparison of the expression levels of LC3II, beclin-1 and caspase-3 mRNA amongst the 5 groups. Compared with the blank control group, [a]P<0.05; compared with the model control group, [b]P<0.05; compared with the high-dose group and low-dose group, [c]P<0.05.

Comparison of expression of PI3K/AKT/mTOR signal pathway among the five groups

In terms of the expressions of PI3K, AKT and mTOR, no statistical difference was observed between the HDG and the LDG (*P*>0.05), and between MDG and BCG (*P*>0.05), while the MDG demonstrated lower levels as compared with the HDG and the LDG (*P*<0.05, **Table 1** and **Figure 2**).

Comparison of expressions of LC3II, Beclin-1 and Caspase-3 mRNA

For the expressions of LC-3II, Beclin-1 and Caspase-3 mRNA, there was no statistical difference between the HDG and the LDG, and between the MDG and the BCG (*P*>0.05), while the MDG yielded a lower expression level as compared with the HDG and the LDG (*P*<0.05, **Figure 3**).

Comparison of SCN autophagy expression among the five groups

According to the results of fluorescent staining, MCG achieved a significance reinforcement in the red and green FI as compared with BCG, while the FI in HDG was not significantly different from that in the LDG (*P*>0.05), which, in turn, was slightly lower than that of the HDG and MDG (*P*<0.05, **Figure 4**).

SCN autophagy and apoptosis in the five groups

TEM results have revealed that in the BCG, the cell structure was integrated with little autophagy and apoptosis, while in the MCG, autophagy dominated and apoptosis was comparatively severe. Between the HDG and the LDG, no significant difference was observed in cell autophagy, but slight cell autophagy and apoptosis showed a higher level than that of the MDG (*P*<0.05, **Figure 5**).

Correlation analysis

SPSS Pearson correlation analysis results showed that glucocorticoid use in patients with

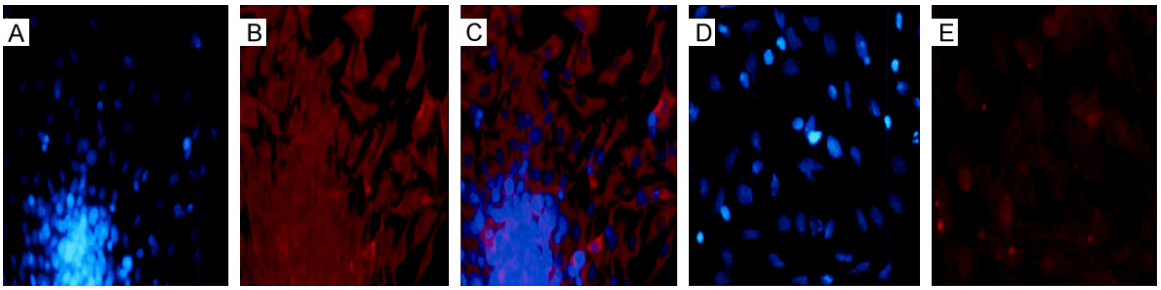

Figure 4. SCN Autophagy expression in the 5 groups. A: Fluorescent staining of BCG; B: MDG; C: MCG; D: LDG; E: HDG.

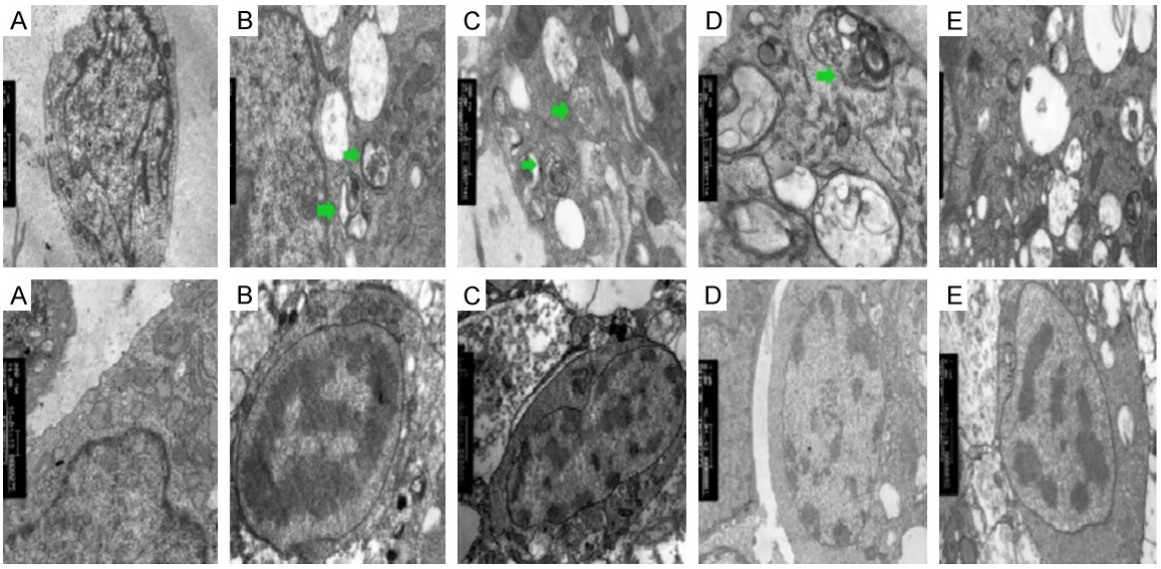

Figure 5. SCN autophagy and apoptosis in the 5 groups. A: BCG; B: MCG; C: HDG; D: LDG; E: MDG.

Table 2. Correlation analysis (r, P)

Correlation	PI3K	AKT	mTOR	PI3K/AKT/mTOR
r	0.734	0.669	0.711	0.793
P	0.000	0.000	0.000	0.000

spinal cord injury was negatively correlated with PI3K protein, AKT protein, mTOR protein and PI3K/AKT/mTOR signal pathway ($P<0.05$, **Table 2**).

Discussion

SCI is a severe complication accompanied by sequelae to various degrees. Clinically, SCI is divided into primary injury and secondary injury. As the primary SCI is unpreventable and irreversible, taking effective measures to reduce the secondary injury and protect the residual neurons becomes a hot point of study. So far, medication is the main clinical treatment for SCI. Though high-dose methylprednisolone pulse therapy can achieve a good efficacy, the long-term prognosis is not so satisfactory. Some foreign scholars have performed an experiment, and results indicated that the high dose hormone can inhibit the autophagy of injured SCNs. Based on these findings, they concluded that high dose hormone can reduce SCI and inhibit the processes of lipid peroxidation and formation of oxygen free radicals so as to control inflammatory reactions [15]. In this study, the MDG demonstrated higher SCN activity and lower expression of LC3II, Beclin-1 and Caspase-3 mRNA as compared with the HDG and the LDG ($P<0.05$), indicating that the medium dose dexamethasone can achieve a better efficacy in rats with SCI by alleviating injuries and promoting recovery. Dexamethasone is a synthetic steroid hormone with strong anti-inflammation effects. It is extensively applied in the treatment of nerve system injury and inflammation, including the early treatment of brain injury and SCI.

However, there are disputes over its dose. In this study, the results of fluorescent staining indicated that between the HDG and the LDG, there was no difference in FI ($P>0.05$), but FI of the LDG was slightly lower than that of the HDG and the MDG; TEM results demonstrated that between the HDG and the LDG, no significant difference was observed in cell autophagy, but cell autophagy and apoptosis showed a higher level than that of the MDG ($P<0.05$), indicating that medium dose dexamethasone can reduce cell autophagy and apoptosis.

The studies of foreign scholars have demonstrated that [16] the PI3K/AKT signaling pathway played a key regulatory role in autophagy, and satisfactory achievements have been made in antitumor activities with selective inhibitory drugs targeting this channel. Clinical studies have shown that the PI3K/AKT signaling pathway can regulate the synthesis of aliphatic acid, while the transcription factor FOXo1 works on lipid metabolism. According to this study, the expressions of proteins PI3K, AKT and mTOR in the MDG were at a lower level as compared with the HDG and the LDG ($P<0.05$), but had no statistical significance ($P>0.05$), indicating that dexamethasone can improve the SCN injury and protect rats. As the PI3K/AKT signaling pathway was blocked, the body's protective mechanism gave rise to the effect of autophagy and was inhibited, indicating that the PI3K/AKT signaling pathway may be a protective channel by dexamethasone to reduce SCN injury [17-19]. The PI3K/AKT signaling pathway is a classical pathway to inhibit apoptosis and promote survival signal transduction. When the signal is activated, effective inhibition is exerted on inflammation and oxidative stress to cause apoptosis of nerve cells, so as to improve the pathological state and neurological symptoms of rats with SCN injury [20-22]. Studies of domestic and foreign scholars have revealed that [23-25] apoptosis and autophagy were both the major pathological foundations of SCN injury, and the intervention based on dexamethasone can improve autophagy, which provides a new thought for the treatment of SCN injury.

In conclusion, medium dose of glucocorticoid can achieve a good effect on SCN injury, which can reduce the expression level of ATG genes, and its mechanism is related to the PI3K/AKT/mTOR signaling pathway.

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Disclosure of conflict of interest

None.

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