Tropical Journal of Pharmaceutical Research January 2021; 20 (1): 47-51
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i1.8

Original Research Article

Expression levels of apoptotic factors in a rat model of corticosteroid-induced femoral head necrosis

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Sent for review: 16 October 2020 *Revised accepted: 29 December 2020*

Abstract

Purpose: *To study the expression levels of apoptotic factors in corticosteroid-mediated femoral head necrosis (FHN) in rats.*
Methods: *Sprague-Dawley (SD) rats (n = 60) bred adaptively for one week were randomly assigned to control and model groups (30 rats/group). A rat model of corticosteroid-induced femoral head necrosis was established. Then, 3 mL of blood drawn from the inferior vena cava of each rat was used for the assay of the expression levels of osteoprotegerin (OPG) and osteoclast differentiation factor (RANKL) in each group using enzyme-linked immunosorbent assay (ELISA). The caspase-3- and Bcl-2-+ve cells in each group were determined with immunohistochemical method.*
Results: *Relative to control, serum OPG level of model group was significantly decreased, while the RANKL level was markedly raised ($p < 0.05$). The degree of empty lacunae in the model rats was markedly increased, relative to control. Caspase-3-+ve cells were more numerous in the model group than in control, while Bcl-2-positive cells were markedly decreased compared to control ($p < 0.05$).*
Conclusion: *Apoptosis occurs in the rat model of femoral head necrosis. Glucocorticoids may regulate the apoptotic process by upregulating caspase-3 and inhibiting Bcl-2. This provides a novel lead for FHN therapy.*

Keywords: *Femoral head necrosis, Corticosteroid, Glucocorticoid, Apoptosis*

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INTRODUCTION

Femoral head necrosis (FHN), a frequently-encountered lesion, is a disease that causes collapse and dysfunction of femur head as a result of death of cells and changes in its structure. Once FHN occurs, it causes buckling of femur head. This increases the biomechanical instability of hip joint, impairs repair of femoral head necrosis, and causes hip subluxation and destruction of the associated cartilage, resulting in osteoarthritis [1]. Femoral head necrosis caused by corticosteroids is a pathological process in which the active components of the femur head such as bone cells, bone marrow hematopoietic cells and fat cells die due to excess hormones [2].

Glucocorticoids have been in use in clinical practice as early as the 1940s. The first case of femoral head necrosis caused by glucocorticoids was reported in the 1950s [3]. With extensive clinical application of glucocorticoids, the incidence of femoral head necrosis is on the rise. Presently, there are many clinical treatment methods for femoral head necrosis. However, these treatments are not very effective against femoral head collapse or femoral head regeneration. In the absence of any recognized ideal treatment strategies, most patients resort to artificial joint replacement surgery. However, post-surgery complications are frequent in corticosteroid-induced femoral head necrosis patients who are mostly young and middle-aged persons, with the attendant serious impact on them and their families [4]. Therefore, it is important to identify effective treatment methods at the early stage of the disease so as to prevent its occurrence.

Some scholars have discovered that there are a large number of apoptotic cells in the bone tissue of patients with steroid-induced femoral head necrosis, implying that apoptosis may be crucial in pathogenesis of the disease [5]. The aim of this research was to study the expression levels of apoptosis-related factors in glucocorticoid-induced femoral head necrosis in rats.

EXPERIMENTAL

Materials

Healthy Sprague-Dawley (SD) rats (n = 60) of mean weight 208 ± 18 g were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co. Ltd (SCXK [Beijing] 2016-0011). The rats were raised in the laboratory at a mean temperature of 22 ± 2 °C and mean humidity of 52 ± 12 % in an environment with equal hours of day and night, and were allowed *ad libitum* access to feed and water.

This research was approved by the Animal Ethical Committee of The First Hospital of Shanxi Medical University (approval no. 20193621572) and was conducted according to the guidelines of Principles of Laboratory Animal Care [6].

Equipment and reagents

The equipment and reagents employed, and their suppliers (in parenthesis) were: biological microscope (Suzhou Jingtong Instrument Co. Ltd, model: XSP-136SM); paraffin microtome (Shanghai Fuze Trading Co. Ltd, Model: ASONE); electronic balance (Dongguan Pubiao Experimental Equipment Technology Co. Ltd, model: YP6001); low-temperature, high-speed centrifuge (Beijing Qianming Gene Technology Co. Ltd, model: MC-15); -80 ℃ ultra-low temperature refrigerator (Guangzhou Hangxin Scientific Instrument Co. Ltd, model: DW-86L828W); methylprednisolone sodium succinate injection (Chongqing Huabang Pharmaceutical Co. Ltd., production batch number: 20183136, specification: 40mg); real-time fluorescent quantitative PCR detection kit (Shanghai Yumeibo Biotechnology Co. Ltd), and immunohistochemistry kit (Zhoubeiyuanxin Biological Technology Co. Ltd).

Animal grouping

All rats were bred adaptively for one week, after which they were randomly divided into the control group and model group, with 15 rats in each group. Corticosterone-induced femoral head necrosis was set up in rats which were injected with endotoxin at a dose of 20 μg/kg twice at an interval of 24 h. Then, prednisolone sodium succinate was injected 3 consecutive times at a dose of 40 mg/kg at 1-week intervals. Control rats received equivalent volume of saline in place of drug.

Histological studies

Rat inferior vena cava blood (3 mL) was taken and centrifuged at 3000 rpm. The supernatant was used for the assay of osteoprotegerin (OPG) and RANKL in each group, with ELISA. At 3, 6, and 9 weeks post-drug administration, 5 rats were taken from each group. Under anesthesia, the abdomen of each rat was cut, and the knee joint plane was excised to completely separate the femoral head. The surrounding soft tissue and the bilateral femoral heads were removed. The tissues were processed for histological examination via preparation of paraffin sections, dewaxing in xylene, dehydration in alcohol and staining with hematoxylin and eosin (H & E). Pathological changes in the femoral heads of rats in each group were examined under a light microscope, and the percentage of empty lacunae was calculated.

Biochemical assays

Levels of Bcl-2 and caspase-3 were estimated immunohistochemically. Four (4) slices were randomly selected in each slide for counting of 100 cells, and percentage of positive cells was determined. After the experiment, the rats were sacrificed.

Statistical analysis

Measured data are presented as mean ± SD, while enumeration data are presented as numbers/percentages [n (%)]. Two-group comparison of enumeration data was performed with χ^2 test, while measured data were compared with independent sample *t*-test. All statistical analyses were done with SPSS 21.0 software. Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Changes in serum expression levels of OPG and RANKL in rats

Compared with the control group, the serum OPG level in the model group was significantly reduced, while the RANKL level was significantly increased ($p < 0.05$). These results are shown in Table 1.

Table 1: Serum OPG and RANKL expression levels of rats

Group	OPG (pg/mL)	RANKL (pg/mL)
Control	85.82 ± 30.31	8.95 ± 4.38
Model	55.17 ± 22.44	25.75 ± 4.28
t	3.148	10.625
P-value	0.004	< 0.001

Data are shown as mean ± SD

Pathological changes in femoral head of rats

The trabecular bone structure of rats in the control group was clear and neatly arranged. Clear bone cells were seen in the trabecular lacuna. The cartilage surface was smooth, and the thickness was normal. In contrast, the thickness of trabecular bone structure in the model group was decreased, and there was evidence of osteocyte necrosis. Moreover, the number of empty bone lacunae was increased significantly, the cartilage thickness was decreased, and some cartilage surfaces were peeled off. These results are shown in Figure 1.

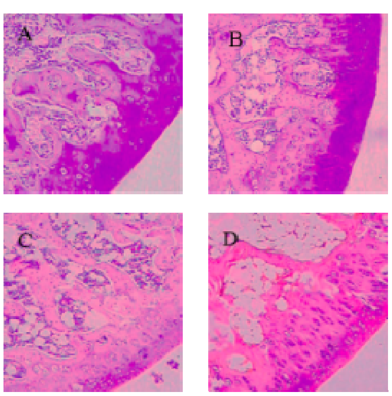

Figure 1: Pathological changes in the femoral head of rats in each group. A: Control group; B: model group at 3 weeks; C: model group at 6 weeks; D: model group at 9 weeks

Empty lacunae in rats

As shown in Table 2, no marked time-dependent variation in % empty lacunae was observed in control group. In contrast, there was marked increase in percentage of empty lacuna in the model group. The percentage of empty lacunae in model rats markedly exceeded that in control rats.

Table 2: Empty lacunae in rats

Group	Empty lacuna (%)		
	3 weeks	6 weeks	9 weeks
Control	1.95 ± 0.92	1.81 ± 0.97	1.63 ± 0.92
Model	3.27 ± 0.84	7.33 ± 1.84	22.18 ± 2.50
T	4.104	10.278	25.512
P-value	< 0.001	< 0.001	< 0.001

Data are shown as mean ± SD (n = 10)

Serum levels of Bcl-2 and caspase-3

Figure 2 and Table 3 show that, compared with the control group, the caspase-3-positive cells in the model group were markedly increased, while the Bcl-2-positive cells were appreciably reduced ($p < 0.05$).

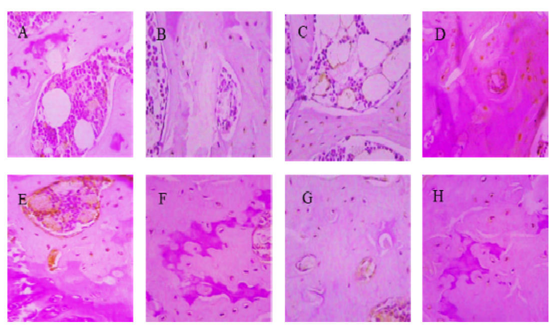

Figure 2: Cellular expression levels of caspase-3 and Bcl-2 in rats in each group A: caspase-3-positive cells in control group; B: caspase-3-+ve cells in model at 3 weeks; C: caspase-3-+ve cells in model at 6 weeks; D: caspase-3-+ve cells in model at 9 weeks; E: Bcl-2-+ve cells in control; F: Bcl-2-+ve cells in model at 3 weeks; G: Bcl-2-+ve cells in model at 6 weeks; H: Bcl-2-+ve cells in model at 9 weeks.

DISCUSSION

Femoral head necrosis caused by cortical steroid refers to the pathological process arising from death of the bioactive components of the femur head such as bone tissue, bone marrow hematopoietic cells and fat cells due to the use of high-dose hormones.

Table 3: Expression levels of caspase-3 and Bcl-2 of rats

Group	Caspase-3			Bcl-2		
	3 weeks	*6 weeks*	*9 weeks*	*3 weeks*	*6 weeks*	*9 weeks*
Control	38.33 ± 2.17	39.80 ± 2.76	38.47 ± 2.03	12.75 ± 2.45	12.29 ± 1.89	12.06 ± 2.76
Model	45.56 ± 3.83	55.74 ± 3.13	65.13 ± 5.69	10.37 ± 1.54	9.94 ± 1.63	10.02 ± 1.68
t	6.361	14.794	17.091	3.185	3.647	2.445
P-value	< 0.001	< 0.001	< 0.001	0.004	0.001	0.021

Data are shown as mean ± SD (n = 10)

The large-scale use of clinical hormones has led to increases in the incidence of corticosteroid-induced femoral head necrosis which currently ranks first among non-traumatic necrotic changes in the femoral head [7]. Studies have revealed that hormones induce the differentiation of bone marrow stromal cells into adipocytes, and reduce their differentiation into osteoblasts. This leads to vascular intimal inflammation, increased vascular permeability, disorders in intraosseous fat metabolism, local inflammation and edema, and increased intraosseous pressure, all of which result in femoral head necrosis [8,9]. In the present study, combined injection of endotoxin and methylprednisolone sodium succinate was used to induce femoral head necrosis in rats, and the expression levels of apoptosis factors were determined.

It is known that OPG and RANKL are factors closely associated with the formation of osteoclasts. The ratio of OPG to RANKL in the bone marrow microenvironment determines the degree of osteoclast development. Osteoprotegerin (OPG), a negative regulator of osteoclast production synthesized by B cells, competitively blocks the combination of RANKL and RANK, thereby inhibiting the differentiation and maturation of osteoclasts, and blocking bone resorption [10]. Receptor activator nuclear factor kappa B ligand (RANKL) is currently the only factor that induces osteoclast differentiation and bioactivity, while inhibiting its apoptosis. It belongs to TNF group synthesized in bone marrow mesenchymal cells and osteoblasts for the enhancement of differentiation and maturation of osteoclasts [11]. Studies have revealed that glucocorticoids have strong inhibitory and stimulating effects on OPG and RANKL, respectively [12].

It has been shown that apoptosis is involved in the etiology of steroid-induced femoral head necrosis [13]. Apoptosis refers to the processes involved in programmed cell death under certain physiological or pathological conditions. Mitochondria are important in the cell death pathway in that they contain apoptosis-inducing and caspase-activating factors. The pathway mediated by mitochondria is the mitochondrial excitation pathway or the caspase-9 pathway. Caspase-3, a member of the interleukin-1 (IL-1) family, is also known as death protease, and is one of the downstream factors of caspase-9. The activation of caspase-3 by caspase-9 triggers the caspase cascade reaction which results in apoptosis [14]. This pathway is also used by Bcl-2 protein family to regulate apoptosis. In the outer mitochondrial membrane, Bcl-2 forms ion channels, improves membrane stability and regulates the release of apoptotic proteins, thereby playing an important role in protecting cellular functions. Reports have shown that the expression level of Bcl-2 determines the sensitivity of cells to glucocorticoids [15]. The results of the present study show that glucocorticoids inhibited bone cell apoptosis. This effect may be due to glucocorticoid-induced disorder in fat metabolism and impairment of differentiation of bone stromal cells into adipocytes bone stromal cells, leading to intramedullary fat accumulation, increased intraosseous pressure, and apoptosis of osteoblasts and bone cells [16].

CONCLUSION

Apoptosis occurs in the rat model of corticosteroid-induced femoral head necrosis. Glucocorticoids inhibit the apoptotic process by enhancing the expression of caspase-3 and inhibiting the expression of Bcl-2. This provides a novel lead for FHN therapy.

DECLARATIONS

Acknowledgement

This study was supported by study on the correlation between different viscera Qideficiency symptom sand intestinal flora (no. 188805).

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This study was done by the authors named in this article, and the authors accept all liabilities

resulting from claims which relate to this article and its contents. The study was conceived and designed by Haibiao Sun; Yongliang Fu, Xiaoqiang Han, Jiangang Xue, Hao Bai, Haibiao Sun collected and analyzed the data; Yongliang Fu and Xiaoqiang Han wrote the manuscript. All authors read and approved the manuscript for publication. Yongliang Fu and Xiaoqiang Han contributed equally to this work and should be considered as co-first authors.

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