



# Roles of oxidative DNA damage of bone marrow hematopoietic cells in steroid-induced avascular necrosis of femoral head

Z.Q. Zhao<sup>1\*</sup>, R. Bai<sup>1\*</sup>, W.L. Liu<sup>1</sup>, W. Feng<sup>2</sup>, A.Q. Zhao<sup>1</sup>, Y. Wang<sup>1</sup>, W.X. Wang<sup>1</sup>, L. Sun<sup>1</sup>, L.S. Wu<sup>1</sup> and S.X. Cui<sup>1</sup>

<sup>1</sup>Department of Pediatric Orthopedics,  
The Second Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China

<sup>2</sup>Department of Orthopedics,  
The Second Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China

\*These authors contributed equally to this study.

Corresponding authors: W.L. Liu / W. Feng

E-mail: liuwanlinmg@sohu.com / 49753192@qq.com

Genet. Mol. Res. 15 (1): gmr.15017706

Received September 23, 2015

Accepted December 3, 2015

Published March 24, 2016

DOI <http://dx.doi.org/10.4238/gmr.15017706>

**ABSTRACT.** An animal model of steroid-induced avascular necrosis of femoral head (SANFH) was established to investigate the role of oxidative DNA damage of bone marrow hematopoietic cells in SANFH. Forty-five-month-old Japanese white rabbits (male or female,  $2.5 \pm 0.5$  kg) were randomly divided into groups A (methylprednisolone + *Escherichia coli* endotoxin), B (methylprednisolone alone), C (*E. coli* endotoxin alone), and D (blank control). The animals were sacrificed two and four weeks after administration of the last dose (N = 5 each group and each time). Left and right femoral heads were fixed and decalcified. Empty lacunae were counted by hematoxylin and eosin staining and oxidative DNA damage of bone marrow hematopoietic cells was detected by immunohistochemistry. At week 2, the rate of oxidative DNA damage in bone marrow hematopoietic cells was significantly higher in group A than in groups B, C, and D ( $P < 0.01$ ), while there was no significant difference between groups B, C,

and D. At week 4, the rate of oxidative DNA damage in bone marrow hematopoietic cells was significantly higher in group A than in groups B, C, and D ( $P < 0.01$ ), while there was no significant difference among groups B, C, and D. Thus, oxidative DNA damage of bone marrow hematopoietic cells appears to play an important role in SANFH.

**Key words:** Hormone; Femoral head; Necrosis; Oxidative DNA damage

## INTRODUCTION

Steroid-induced avascular necrosis of femoral head (SANFH) mostly affects young and middle-aged people. In most patients, the disease is bilateral and affects a large area. It is much more serious than idiopathic avascular necrosis and results in high disability rates. SANFH is a complex biological process and its pathogenesis remains unclear. Therefore, the etiology of SANFH remains the focus of research in this field. Potential vasculitis, vascular injury, and pro-coagulation can occur in SANFH (Yin et al., 2006; Takano-Murakami et al., 2009; Niizuma et al., 2010). Several studies have shed some light on the pathogenesis SANFH and laid a foundation for probing into the etiology of SANFH. However, many questions about pathological changes in osteocytes and osteoblasts in early SANFH and the potential causes of these changes, particularly the manner of death of the two cell types and factors that may reverse this process, remain unanswered. Studies have indicated that oxidative DNA damage plays an important role in cell apoptosis (Nishida et al., 2008; Kalyuzhny, 2011). However, the pathological changes that occur in bone marrow hematopoietic cells, an important component of the femoral head, during early SANFH are not known. Furthermore, it is unclear whether oxidative DNA damage occurs during SANFH. In order to address these questions, we examined SANFH in a rabbit model. The relationship between SANFH and oxidative DNA damage was examined in order to investigate its role in the pathogenesis of SANFH.

## MATERIAL AND METHODS

### Experimental materials

#### *Experimental animals*

Forty healthy adult Japanese white rabbits (male or female,  $2.5 \pm 0.5$  kg) were fed a standard diet, provided by Beijing Fuhao Animal Breeding Center, and were raised alone in one cage. The animals were certified by the National Institute of Medical Experiments of China (Certificate No. 20090137).

#### *Experimental instruments*

Hitachi H-700H electron microscope (Hitachi, Japan), LKB2800 ultramicrotome (Meyer Burger, Sweden), JD801 image analysis system (Xircom, Jiangsu, China), Millipore ultrapure water purifier, and Olympus microscope (IX83, Olympus, Japan) were provided by the Molecular Biology Research Center and Electron Microscopy Center of Inner Mongolia Medical College.

## **Reagents**

*Escherichia coli* endotoxin (provided by China National Institutes for Food and Drug Control), methylprednisolone (Pharmacia & Upjohn, Belgium), N45.1 monoclonal antibody oxidative DNA damage detection kit (Sigma-Aldrich, Japan), and TUNEL apoptosis detection kit (Roche, Germany) were used. Hematoxylin and eosin (H&E) staining, ethylenediamine tetraacetic acid decalcifying solution, and other conventional reagents were provided by the Molecular Biology Research Center of Inner Mongolia Medical College.

## **Experimental methods**

### ***Animal grouping and drug administration***

Experimental animals were randomly divided into four groups (N = 10 each) using a random number table. Group A received two intravenous injections of *E. coli* endotoxin (100 µg/kg) 24 h apart. The second injection of *E. coli* endotoxin was followed by three intramuscular injections of methylprednisolone (20 mg/kg) 24 h apart. Group B received two intravenous injections of *E. coli* endotoxin (100 µg/kg) 24 h apart. The second injection of *E. coli* endotoxin was followed by three intramuscular injections of saline (20 mg/kg) 24 h apart. Group C received two intravenous injections of saline (100 µg/kg) 24 h apart. The second injection of saline was followed by three intramuscular injections of methylprednisolone (20 mg/kg) 24 h apart. Group D received two intravenous injections of saline (100 µg/kg) 24 h apart. The second injection of saline was followed by three intramuscular injections of saline (20 mg/kg) 24 h apart. Group A is the SANFH model group and groups B, C and D are controls. The animals were sacrificed by air embolism two and four weeks after the last injection (N = 5 from each group at each time). Left and right femoral heads were obtained from animals under relatively sterile conditions.

### ***Preparation of specimens***

One side of the removed femoral heads was fixed with 5% gluteraldehyde for one week, and the other side was treated with 10% formaldehyde solution. All femoral heads were then decalcified with 15% EDTA for 45 days and embedded in paraffin.

### ***Measurement of oxidative DNA damage of bone marrow hematopoietic cells by immunohistochemical assay using a monoclonal antibody N45.1***

Sections were incubated with 0.3% H<sub>2</sub>O<sub>2</sub> at 37°C for 30 min then digested with 0.1% trypsin for 15 min. The digested sections were then incubated with one drop of N45.1 monoclonal antibody at 37°C for 2 h. The sections were developed with DAB for 5 min and counterstained with H&E.

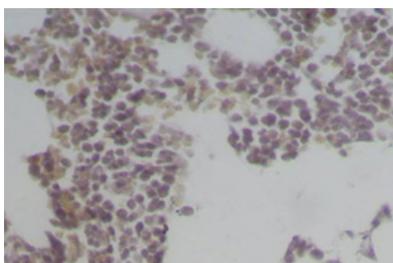
## **Statistical analysis**

All data are reposted as means ± standard deviation and analyzed using the SPSS 13.0 software (Chicago, IL, USA). Statistical tests of oxidative DNA damage rate of bone marrow hematopoietic cells were performed using analysis of variance (ANOVA). Pairwise comparisons between sample means were performed using the least significant difference (LSD) test. A linear

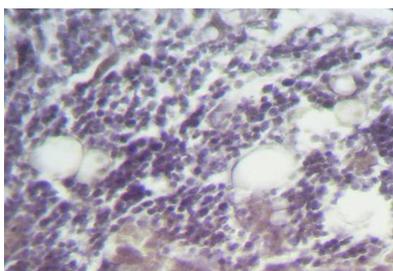
relationship between the two variables is indicated by  $-1 \leq r \leq +1$ .  $P < 0.05$  was considered statistically significant.

## RESULTS

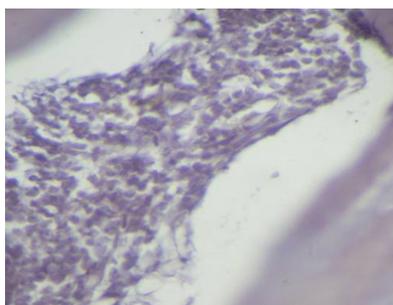
At week 2, the rate of oxidative DNA damage in bone marrow hematopoietic cells was significantly higher in group A than in groups B, C, and D ( $F = 38.18$ ,  $P = 0.00$ ), while there was no significant difference between groups B, C, and D. The rate of oxidative DNA damage in bone marrow hematopoietic cells was  $(29.80 \pm 1.26)\%$ ,  $(8.05 \pm 1.31)\%$ ,  $(8.24 \pm 1.44)\%$ , and  $(8.75 \pm 1.47)\%$  in groups A, B, C, and D, respectively (Figures 1-4). At week 4, the rate of oxidative DNA damage in bone marrow hematopoietic cells was significantly higher in group A than in groups B, C, and D ( $F = 189.56$ ,  $P = 0.00$ ), while there was no significant difference between groups B, C, and D. The rate of oxidative DNA damage in bone marrow hematopoietic cells was  $(41.60 \pm 1.67)\%$ ,  $(7.80 \pm 1.78)\%$ ,  $(8.20 \pm 1.48)\%$ , and  $(7.80 \pm 1.64)\%$  in groups A, B, C, and D, respectively (Figures 5-8).



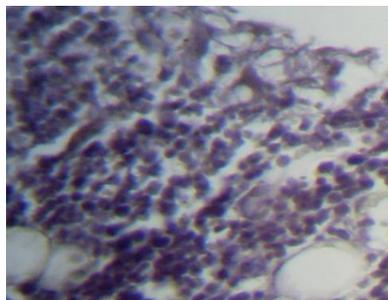
**Figure 1.** High rate of oxidative DNA damage to bone marrow hematopoietic cells at week 2 in group A (400X).



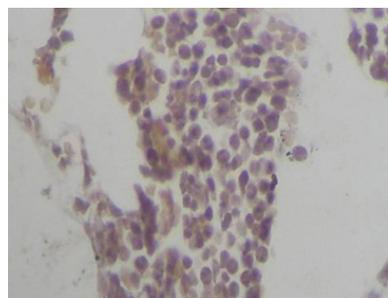
**Figure 2.** High rate of oxidative DNA damage of bone marrow hematopoietic cells at week 2 in group B (400X).



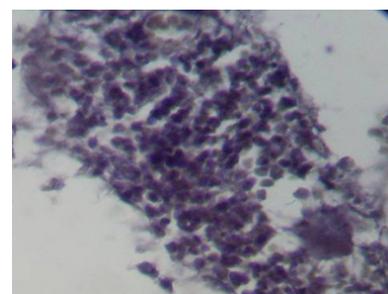
**Figure 3.** High rate of oxidative DNA damage of bone marrow hematopoietic cells at week 2 in group C (400X).



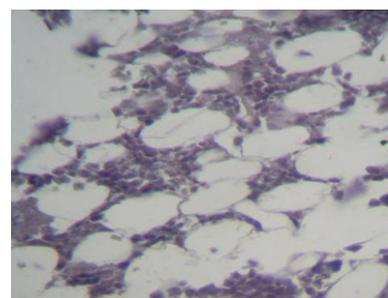
**Figure 4.** High rate of oxidative DNA damage of bone marrow hematopoietic cells at week 2 in group D (400X).



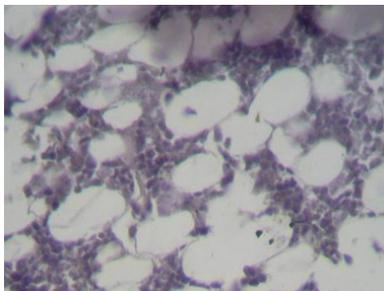
**Figure 5.** Oxidative DNA damage of bone marrow hematopoietic cells at week 4 in group A showing a high rate of oxidative DNA damage 400X.



**Figure 6.** Oxidative DNA damage of bone marrow hematopoietic cells at week 4 in group B showing some evidence of oxidative DNA damage (400X).



**Figure 7.** Oxidative DNA damage of bone marrow hematopoietic cells at week 4 in group C showing no oxidative DNA damage (400X).



**Figure 8.** Oxidative DNA damage of bone marrow hematopoietic cells at week 4 in group D showing some oxidative DNA damage (400X).

## DISCUSSION

Avascular necrosis of femoral head induced by long- or short-term intensive use of adrenocortical hormone has attracted wide attention in the medical profession. SANFH mostly affects young and middle-aged people, is mostly bilateral, and involves a large area. It is much more serious than idiopathic avascular necrosis, resulting in high disability rates. In particular, a large number of SANFH cases are diagnosed after atypical pneumonia (Srilaxmi et al., 2010) and the etiology of SANFH is currently a focus of research in this field. Therefore, many researchers have attempted to develop animal models of human SANFH. Among these efforts is the methylprednisolone + *E. coli* endotoxin-induced osteonecrosis rabbit model by Yamamoto et al. (2007). The rabbit model of SANFH exhibits similar histological changes to humans, can be established in a short period (as short as four weeks), and shows good reproducibility.

Oxidative DNA damage plays an important role in aging and death of somatic cells. Free radicals are considered one of the main factors that cause DNA damage. Hydroxyl radicals and superoxide anions can directly react with DNA molecules, leading to oxidative DNA damage such as DNA strand breaks, base modifications, and DNA-protein cross-linking. The most critical mutagenic damage is transversion mutation of guanine to thymine at C-8, producing 8-hydroxy-deoxyguanosine. The pathological changes of bone marrow hematopoietic cells, an important hematopoietic cell in the femoral head, will inevitably affect the development of avascular necrosis. The present study demonstrated that oxidative DNA damage of bone marrow hematopoietic cells was an early molecular event in SANFH, occurring even before osteocyte apoptosis.

In the present study, it was concluded that the rate of oxidative DNA damage in bone marrow hematopoietic cells increased during SANFH and that this phenomenon is involved in the pathology of SANFH. In short, SANFH appears to have more than one cause of pathogenesis, one of which may be oxidative DNA damage of bone marrow hematopoietic cells.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

Research supported by the National Science Foundation of China.

**REFERENCES**

- Kalyuzhny AE (2011). Combination of TUNEL assay with immunohistochemistry for simultaneous detection of DNA fragmentation and oxidative cell damage. *Methods Mol. Biol.* 682: 15-27. [http://dx.doi.org/10.1007/978-1-60327-409-8\\_2](http://dx.doi.org/10.1007/978-1-60327-409-8_2)
- Niizuma K, Yoshioka H, Chen H, Kim GS, et al. (2010). Mitochondrial and apoptotic neuronal death signaling pathways in cerebral ischemia. *Biochim. Biophys. Acta* 1802: 92-99. <http://dx.doi.org/10.1016/j.bbadis.2009.09.002>
- Nishida K, Yamamoto T, Motomura G, Jingushi S, et al. (2008). Pitavastatin may reduce risk of steroid-induced osteonecrosis in rabbits: a preliminary histological study. *Clin. Orthop. Relat. Res.* 466: 1054-1058. <http://dx.doi.org/10.1007/s11999-008-0189-4>
- Srilaxmi P, Sareddy GR, Kavi Kishor PB, Setty OH, et al. (2010). Protective efficacy of natansnin, a dibenzoyl glycoside from *Salvinia natans* against CCl<sub>4</sub> induced oxidative stress and cellular degeneration in rat liver. *BMC Pharmacol.* 10: 13. <http://dx.doi.org/10.1186/1471-2210-10-13>
- Takano-Murakami R, Tokunaga K, Kondo N, Ito T, et al. (2009). Glucocorticoid inhibits bone regeneration after osteonecrosis of the femoral head in aged female rats. *Tohoku J. Exp. Med.* 217: 51-58. <http://dx.doi.org/10.1620/tjem.217.51>
- Yamamoto T, Miyanishi K, Motomura G, Nishida K, et al. (2007). [Animal models for steroid-induced osteonecrosis]. *Clin. Calcium* 17: 879-886.
- Yin L, Li YB and Wang YS (2006). Dexamethasone-induced adipogenesis in primary marrow stromal cell cultures: mechanism of steroid-induced osteonecrosis. *Chin. Med. J. (Engl.)* 119: 581-588.