RESEARCH ARTICLE

# Oxidative Stress Induced Osteocyte Apoptosis in Steroid-Induced Femoral Head Necrosis

Zhen-Qi Fan, MM[1†], Shu-Cai Bai, MM[2†], Qian Xu, MM[3], Zhi-Jun Li, PhD, MD[1], Wen-Hao Cui, PhD[4,5], Hui Li, MD[1], Xiao-Hui Li, MD[2], Hua-Feng Zhang, MD[1]

*[1]The Department of Orthopedics, Tianjin Medical University General Hospital, [2]Department of Orthopedics, Tianjin Hospital and [3]Tianjin University of Traditional Chinese Medicine, Tianjin and [4]Department of Endocrinology, The Shenzhen Second People's Hospital, Health Science Center, The First Affiliated Hospital of Shenzhen University, Shenzhen, China and [5]Department of pharmacology, Kyoto Prefectural University of Medicine, Kyoto, Japan*

**Objective:** To investigate the effect and mechanism of Glucocorticoids (GCs) induced oxidative stress and apoptosis on necrosis of the femoral head in patients and rats.

**Methods:** Eight patients with steroid-induced avascular necrosis of the femoral head (SINFH) and eight patients with developmental dysplasia of the hips (DDH) were enrolled in our study. In animal model, twenty male Sprague-Dawley rats were randomly divided into two groups (SINFH group and NS group). The SINFH model group received the methylprednisolone (MPS) injection, while control group was injected with normal saline (NS). MRI was used to confirm SINFH rat model was established successfully. Then, the rats were sacrificed 4 weeks later and femoral head samples were harvested. Histopathological staining was preformed to evaluate osteonecrosis. TUNEL staining was performed with 8-OHdG and DAPI immunofluorescence staining to evaluate oxidative injury and osteocyte apoptosis. Immunohistochemistry staining was used to detect Nox1, Nox2, and Nox4 protein expression.

**Results:** MRI showed signs of typical osteonecrosis of femoral head in SIHFH patients. Histopathological staining showed that the rate of empty lacunae in SINFH patients was significantly higher (56.88% ± 9.72% *vs* 19.92% ± 4.18%, $T = -11.04$, $P < 0.001$) than that in DDH patients. The immunofluorescence staining indicated that the TUNEL-positive cell and 8-OHdG-positve cell in SINFH patients were significantly higher (49.32% ± 12.95% *vs* 8.00% ± 2.11%, $T = -7.04$, $P = 0.002$, 54.6% ± 23.8% *vs* 9.75% ± 3.31%, $T = -4.17$, $P = 0.003$) compared to the DDH patients. The immunohistochemistry staining showed that the protein expression of NOX1, NOX2 and NOX4 in SINFH patients were significantly increased (64.50% ± 7.57% *vs* 37.58% ± 9.23%, $T = -3.88$, $P = 0.018$, 90.84% ± 2.93% *vs* 49.56% ± 16.47%, $T = -5.46$, $P = 0.001$, 85.46% ± 9.3% *vs* 40.69% ± 6.77%, $T = -8.03$, $P = 0.001$) compared to the DDH patients. In animal model, MRI showed signs of edema of femoral head in MPS group, which represents SINFH rat model was established successfully. Histological evaluation showed the rate of empty lacunae in MPS group was significantly higher (25.85% ± 4.68% *vs* 9.35% ± 1.99%, $T = -7.96$, $P < 0.001$) than that in NS group. The immunofluorescence staining indicated that the TUNEL-positive cell and 8-OHdG-positve cell (in MPS group were significantly increased (31.93% ± 1.01% *vs* 11.73% ± 1.16%, $T = -32.26$, $P < 0.001$, 47.59% ± 1.39% *vs* 22.07% ± 2.45%, $T = -22.18$, $P < 0.001$) compared to the NS group. The immunohistochemistry staining showed that the expression of NOX2 in MPS group was significantly increased (76.77% ± 8.34% *vs* 50.32% ± 10.84%, $T = -4.74$, $P = 0.001$) compare with NS group.

**Conclusion:** Our findings indicated that GC-induced NOXs expression may be an important source of oxidative stress, which could lead to osteocyte apoptosis in the process of SINFH

**Address for correspondence** Hua-Feng Zhang, MD, The Department of Orthopedics, Tianjin Medical University General Hospital, Anshan Road No. 154, Heping District, Tianjin, China 300052 Tel: +86 18622496267; Fax: 00862260362255; Email: tijmuhua516@163.com
[†]These two authors contributed equally to this work.
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## Introduction

Glucocorticoid (GC) usage is the most common cause of non-traumatic femoral head osteonecrosis[1,2]. The theory of vascular endothelial injury, oxidative stress, fat metabolism disorder, apoptosis and osteoporosis has been proposed[1,3]. However, the exact mechanism of steroid-induced avascular necrosis of the femoral head (SINFH) is still unknown. Since Weinstein and his colleagues reported osteocyte apoptosis in necrosis of human femoral head[4], the theory of apoptosis has been widely studied[5]. Zalavras *et al.* suggested that glucocorticoid can induce osteoblast and osteocyte apoptosis, which is an important pathogenic factor, could lead to the occurrence of SINFH[6]. In addition, by reducing the pro-apoptotic actions of GC on osteoblasts and osteocytes, the deleterious effects of GC on the skeleton will be decreased and bone mass can be increased[7]. Our previous study has been confirmed that reactive oxygen species (ROS) mediated oxidative injury plays a pivotal role in high-dose dexamethasone-induced osteoblast and osteocyte apoptosis in vitro[8].

Bone is a powerful self-repairing tissue that maintains remodeling *via* the process of osteoclastic resorption and osteoblastic formation[9]. However, after long-term usage of high-dose GCs, the pathological processes included of adiposis of mesenchymal stem cells, weakening of osteogenesis, and destructive microstructure of osteocyte and perilacunar/canalicular (PLR) will emerge[10,11]. Previous reports have reported that GC-induced bone loss due to excessive bone resorption of osteoclasts in early stage, and in later phase in which bone is lost due to inadequate bone formation[12,13]. In addition, GCs directly inhibit cellular proliferation and differentiation of osteoblast lineage cells, reduce osteoblast maturation and activity and induce osteoblast and osteocyte apoptosis, which could affect the balance of bone metabolism[14]. Wang *et al.*[7,15] has reported that preventing the pro-apoptotic effect of GC in osteoblasts and osteocytes may serve as a novel strategy for the prevention of GC-induced osteoporosis and osteonecrosis. Recent studies have shown that the imbalance of bone metabolism in the process of glucocorticoid induced femoral head necrosis is closely related to oxidative stress[16]. It can effectively decrease osteoblast and osteocyte apoptosis and protect bone tissue by reducing glucocorticoid induced oxidative injury. However, the mechanism is still unclear, and more experimental studies are still needed.

*In vitro*, it has been reported the administration of GC increased oxidative injury and influence metabolism microenvironment in a dose-dependent manner[17]. Intracellular reactive oxygen species (ROS) as an important molecular has been widely studied in the process of oxidative stress. Previous study has been confirmed that glucocorticoids can increase the production of ROS in osteoblasts and osteoclasts.

Under physiological conditions, ROS, as an important second messenger, play an important role in intercellular signal transduction, cell proliferation and differentiation[18,19]. However, pathological excessive generation of ROS can result in oxidative damage, which is related to rheumatoid arthritis, osteoarthritis, osteoporosis and steroid induced necrosis of the femoral head[20,21]. Tao *et al.*[5] has reported that exosomes derived from human platelet-rich plasma prevent apoptosis induced by glucocorticoid-associated oxidative stress in rat osteonecrosis of the femoral head *via* the Akt/Bad/Bcl-2 signal pathway. Sato *et al.*[22] has been confirmed that prevention of glucocorticoid induced-apoptosis of osteoblasts and osteocytes by protecting against oxidative stress *in vitro* and *in vivo* in female mice. In addition, ROS, as an important biomolecule mediating oxidative stress, can be an important target for the therapy of steroid-induced femoral necrosis[23]. However, the source of ROS is still unclear.

It has been confirmed that Nicotinamide adenine dinucleotide phosphate, reduced form NADPH oxidase(NOX), mitochondrion and endoplasmic reticulum stress are related to the generation of ROS[5,8,24]. Recently, NOX, as an important source of ROS generation, has been widely studied[25,26]. Several isoforms including NOX1–5 and dual oxidase (DUOX)1–2 have been identified, which are closely associated with regulating vascular remodeling, genetic immune disorders and endothelial cell function[26,27]. NADPH oxidase (NOX) plays an important role in the process of regulation of vascular endothelial cell function, cytoskeleton remodeling, cell growth and differentiation[25,26]. In addition, accumulating evidence has indicated that ROS derived from NOX1, NOX2, and NOX4 is closely related to skeletal metabolism[25,28]. Previous studies has been reported that reactive oxygen species derived from NOX4 mediate BMP2 gene transcription and osteoblast differentiation[28]. In addition, it suggested that NOX1 and NOX4, as downstream targets of CSF-1 and NOX1 as an important mediator that contributes to RANKL expression in osteoblasts. NADPH oxidase may represent targets for treatment of bone disease[29]. Our previous study has also confirmed that NOX1- and NOX4-derived ROS plays a pivotal role in high-dose dexamethasone-induced pre-osteoblast apoptosis by increasing phosphorylated ASK1 and p38 *in vitro*. However, the NOX-induced oxidative stress and apoptosis of osteocyte in the process of SINFH *in vivo* is still needed further study.

Therefore, the mechanism related oxidative stress and apoptosis of osteocyte in steroid-induced avascular necrosis of the femoral head prompted us investigate the study of the femoral head of patients and animal model. We hypothesized that: (i) GCs-induced oxidative stress is involved in the process of SINFN; (ii) oxidative stress is an important factor of osteocyte apoptosis in SINFH; and (iii) GCs can increase the level of oxidative stress in femoral head by inducing the expression of NOXs.

## Materials and Methods

### *Ethics Statement*

This study was approved by the ethics committee of the Tianjin Medical University General Hospital Institutional Review Board and complied with the World Medical Association Declaration of Helsinki. All patients signed informed consent forms. The animal experiment was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals, and the Institutional Animal Care and Use Committee of Tianjin Medical University and Tianjin hospital approved all animal protocols.

### *The Patients*

The inclusion criteria were: (i) adult SINFH patients with ARCO IV with extreme impairment of daily life. Adult DDH patients with Crowe type IV DDH with extreme impairment of daily life; (ii) patients who underwent THA by a single surgeon in the department of orthopedic of Tianjin Medical University General Hospital; (iii) patients were divided into two groups (SINFH group and DDH group); (iv) outcome measures are based on MRI, pathological changes, oxidative stress and apoptosis of osteocyte; and (v) prospective study. The exclusion criteria included: (i) patients with femoral head fracture; and (ii) patients with a history of drinking.

Eight SINFH and eight DDH patients were recruited in our study. All patients received total hip replacement (THA). Human specimens of femoral head were obtained from total hip arthroplasty. The severity and volume of necrosis of the femoral head were evaluated using ARCO system. The femoral head of SINFH and DDH groups were extracted during the operation. All bone specimens were harvested around the center of femoral head.

### *The SINFH Rat Model*

All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Twenty male adult Sprague–Dawley rats (12 weeks old; body weight, 400–450 g) were obtained from the Tianjin Medical University experimental animal center.

The rats were fed for 1 week to adapt to the environment. The SINFH model was induced using methylprednisolone sodium (MPS) as a protocol reported previously[16]. The rats were randomized into two groups ($n = 10$ per group): NS (normal saline) group and MPS (methylprednisolone) group. The methylprednisolone sodium (Pharmacia & Upjohn, Peapack, NJ, USA) was subcutaneously injected at a dose of 21 mg/kg per day for 4 weeks. The rats in the NS group only received normal saline. Dynamic MRI was performed for bilateral proximal and distal femora at 4 weeks after the last injection of MPS, using a 1.5 T superconducting system (MRI, GE Medical System, Fairfield, CT, USA). The rats were placed in supine with the lower limb flexed and fixed by adhesive tape. a human knee joint coil was used on the target site. Preliminary sagittal and oblique axial images were obtained to define the femoral longitudinal axis, then coronal and axial MRI images (T2W, FOV (14 cm), repetition time [TR]·/ echo time [TE] = 3495/65.4 ms) was used for analysis of the target side.

For sample harvest and tissue process, the femoral head samples were harvested and fixed by 4% polyformaldehyde after euthanasia. EDTA decalcifying solution (PH 7.2) were used to decalcify. Then the femoral head samples were dehydrated by a graded ethanol. After dehydration, the tissue was transparentized by Xylene. Finally, the femoral head samples were embedded in paraffin and cut into 4 μm-thick sections along the coronal plane.

### *Histological Examination*

Hematoxylin and eosin (H&E) staining was performed for histological observations. Characteristic histopathological features of SINFH were defined as a diffuse presence of empty lacunae or pyknotic nuclei of osteocytes in the trabeculae accompanied by surrounding increasing and hypertrophic adipocytes in bone marrow. The number of positive cells per field was evaluated in five fields per section and five sections per femoral head.

### *Immunofluorescent Staining*

The procedure of triple fluorescence staining is as follows. The 4 μm sections were deparaffinized in xylene, and rehydrated through a graded ethanol series. A TUNEL staining kit (Roche *In Situ* Cell Death Detection Kit, POD) was used to detect DNA strand breaks according to the manufacturer's instructions. For immunofluorescent staining, sections were incubated at 37°C in the dark for 1 h with 8-OHdG (ab183393, Abcam). Then, sections were incubated with DAPI (ab104139, Abcam) staining in the dark for 15 min at room temperature. Immunofluorescence was detected using a confocal microscope (FV1000, Olympus, Tokyo, Japan). The number of positive cells per field was evaluated in five fields per section and five sections per femoral head.

### *Immunohistochemical Staining*

For immunohistochemical analyses, the 4 μm sections were incubated with Anti-NOX1 (Thermo Fisher Scientific, PA5-79752), Anti-NOX2 (Thermo Fisher Scientific, PA5-72435) and Anti-NOX4 (Thermo Fisher Scientific, MA5-32090) overnight at 4°C. After washing with PBS, the sections were further incubated with a horseradish peroxidase-conjugated Rabbit anti-goat (ZSGB-BIO, PV-9003). At last, generally DAB stain, hematoxylin slightly stain, neutral balata fixation. The number of positive cells per field was evaluated in five fields per section and five sections per femoral head.

### *Main Outcome Measures*

*The Empty Lacunae Percentage.* The pathological change was showed by HE staining. The empty lacunae percentage is defined as the ratio of the number of empty lacunae to the total number of osteocytes. The empty lacunae percentage with broken trabeculae was used to evaluate the occurrence

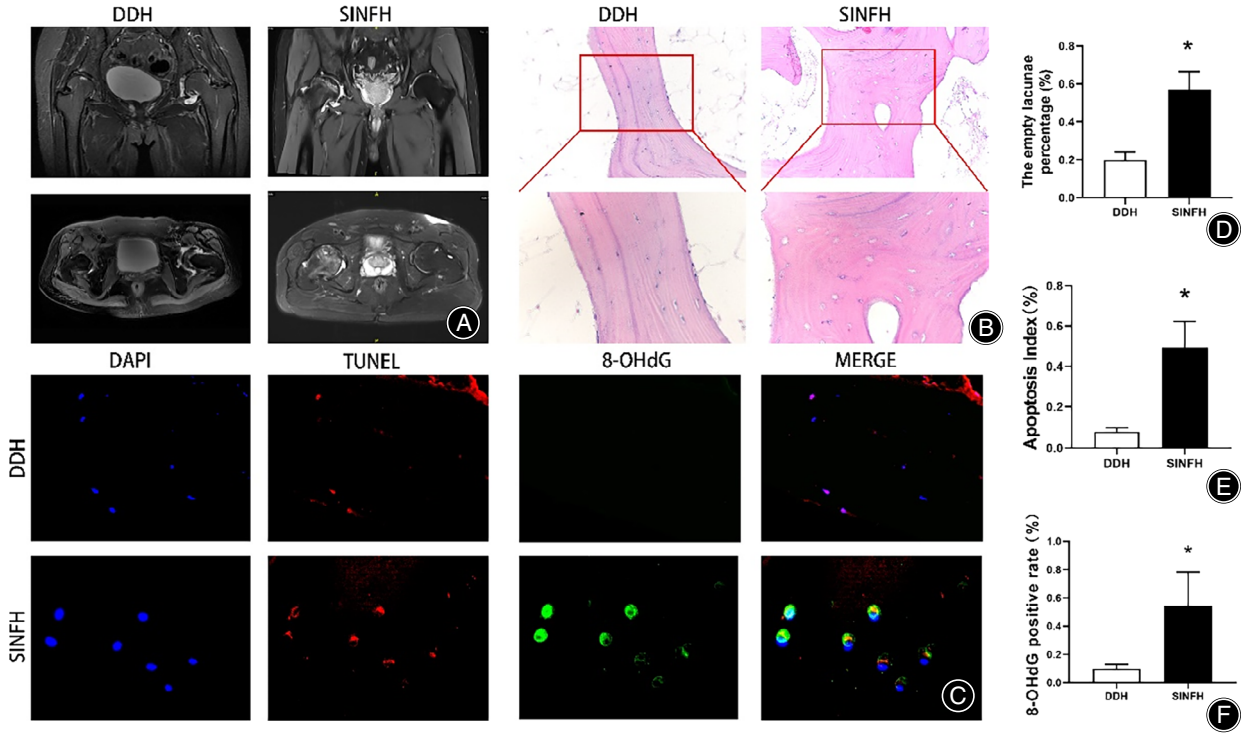


**Fig. 1** Dynamic MRI, histological examination and immunofluorescent staining in the femoral head tissues of SINFH and DDH patients. (A) Representative MRI of hip joint of patients in the DDH and SINFH groups. (B) Representative HE staining images in the DDH and SINFH groups. (C) Representative immunofluorescent staining images of osteocyte apoptosis (red) and 8-OHdG positive cells (green) in the DDH and SINFH groups. (D) The empty lacunae percentage in the DDH and SINFH groups. The Apoptosis index of osteocyte (E) and 8-OHdG positive rate (F) in the DDH and SINFH groups. (*: $P < 0.05$ *vs* DDH group).

of osteonecrosis of femoral head and confirm SINFH rat model was established successfully.

*TUNEL Stain and Apoptosis Index.* As the occurrence of apoptosis, The DNA fragment breaks and 3′-OH terminal will be exposed, which can bind to fluorescein-dUTP under the catalysis of terminal deoxynucleotidyl transferase (TdT). Fragmented DNA in the nucleus, which also represents apoptotic activity, can be observed by fluorescence staining. Apoptosis index, which refers to the percentage of positive staining cells in the total number of cells, can represent the intensity of apoptotic activity. The increased Apoptosis Index indicates the increase of osteocytes apoptosis.

*8-OHdG Positive Rate.* Oxidative stress could result in the oxidative injury of DNA. Hydroxylation of deoxyguanosine by the addition of an -OH group at the C-8 position leads to 8-hydroxy-2'deoxyguanosine formation(8-OHdG). 8-OHdG has extensively been used to reflect the degree of oxidative damage to DNA. Anti-DNA Damage antibody binds with high specificity and affinity to 8-OHdG, which can be observed by fluorescence staining. The 8-OHdG positive rate, which refers to the percentage of positive staining cells in the total number of cells, represents the degree of oxidative stress. The increased 8-OHdG positive rate indicates a high level of oxidative stress.

*Immunohistochemical and NOXs Positive Rate.* Immunohistochemical staining is used to determine antigen qualitatively, locatively and quantitatively through the chromogenic reaction of the complex formed by antigen–antibody reaction. NOXs are related to the generation of ROS. NOXs Positive Rate refers to the percentage of positive staining cells in the total number of cells. The increased NOXs Positive Rate, which represents a increased expression of NOXs, can illustrate the source of ROS in SINFH.

### *Statistical Analysis*

Each experiment was conducted at least three times. Data are expressed as the mean ± SD from *n* independent experiments. Student's *t*-test was used to compare differences between two groups (SINFH *vs* DDH, MPS *vs* NS). SPSS software (version 26.0, IBM, Armonk, NY, USA) was performed for statistical analysis. $P < 0.05$ was considered statistically significant.

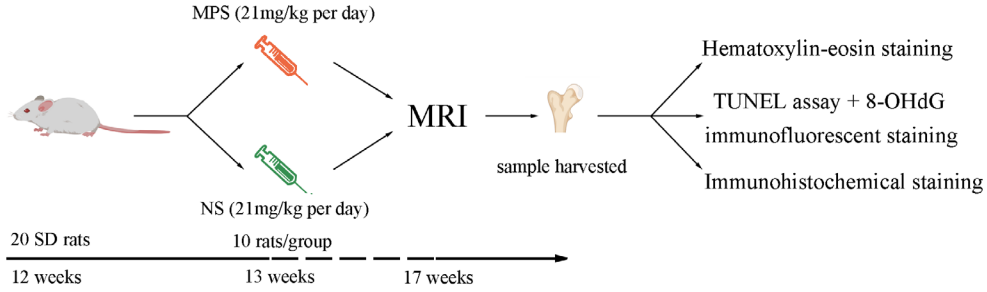


**Fig. 2** Steroid-induced avascular necrosis of the femoral head (SINFH) induction protocol. MPS, methylprednisolone; NS, normal saline.

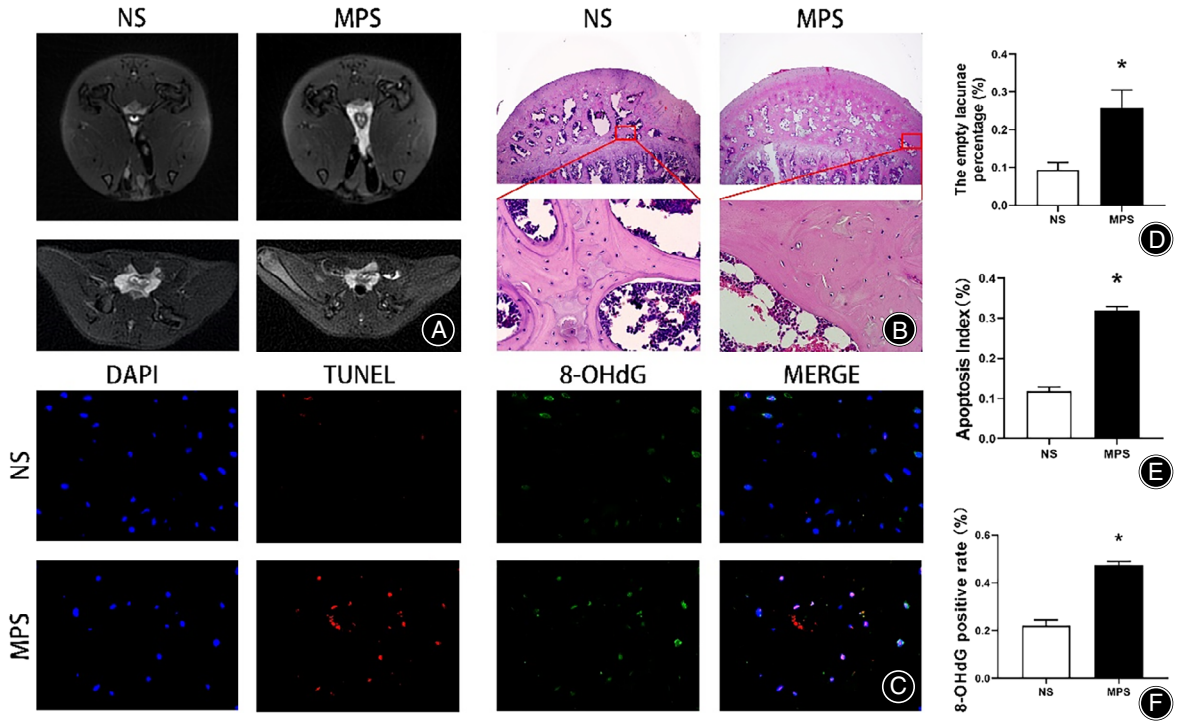


**Fig. 3** The MRI, HE staining and immunofluorescent staining in the femoral head tissues of rats with SINFH. (A) Representative MRI of hip joint of rats in the NS and MPS groups. (B) Representative HE staining images in the NS and MPS groups. (C) Representative immunofluorescent staining images of osteocyte apoptosis (red) and 8-OHdG positive cells (green) in the NS and MPS groups. (D) The empty lacunae percentage in the NS and MPS groups. The Apoptosis index of osteocyte (E) and 8-OHdG positive rate (F) in the NS and MPS groups. (*$P < 0.05$ *vs* NS group).

## Results

### *The Presentment of MRI and HE Staining of Femoral Head in SINHF and DDH Patients*

Dynamic MRI was performed on bilateral femoral to assess femoral head necrosis. As shown in Fig. 1A, the MRI analysis result showed that bone marrow edema, collapse of the femoral head, and narrowing of the hip joint. HE staining was performed to observe histological changes and confirmed osteogenesis. The histopathological appearance of femoral head of SINFH patients is depicted in Fig. 1B. Compared with DDH group, the results of HE staining in SINFH patients showed increased empty lacunae and broken trabecular accompanied by surrounding myelofibrosis. The rate of empty lacunae in SINFH patients was 56.88% ± 9.72%, which was significantly higher than that in DDH patients (56.88% ± 9.72% *vs* 19.92% ± 4.18%, $T = -11.04$, $P < 0.001$) (Fig. 1D).

### *Oxidative Stress Induced Osteocyte Apoptosis of Femoral Head in SINHF and DDH Patients*

In order to further study the effect of glucocorticoid on osteocytes of femoral head of SINFH patients. TUNEL and 8-OHdG staining were used to detect oxidative stress and apoptosis of

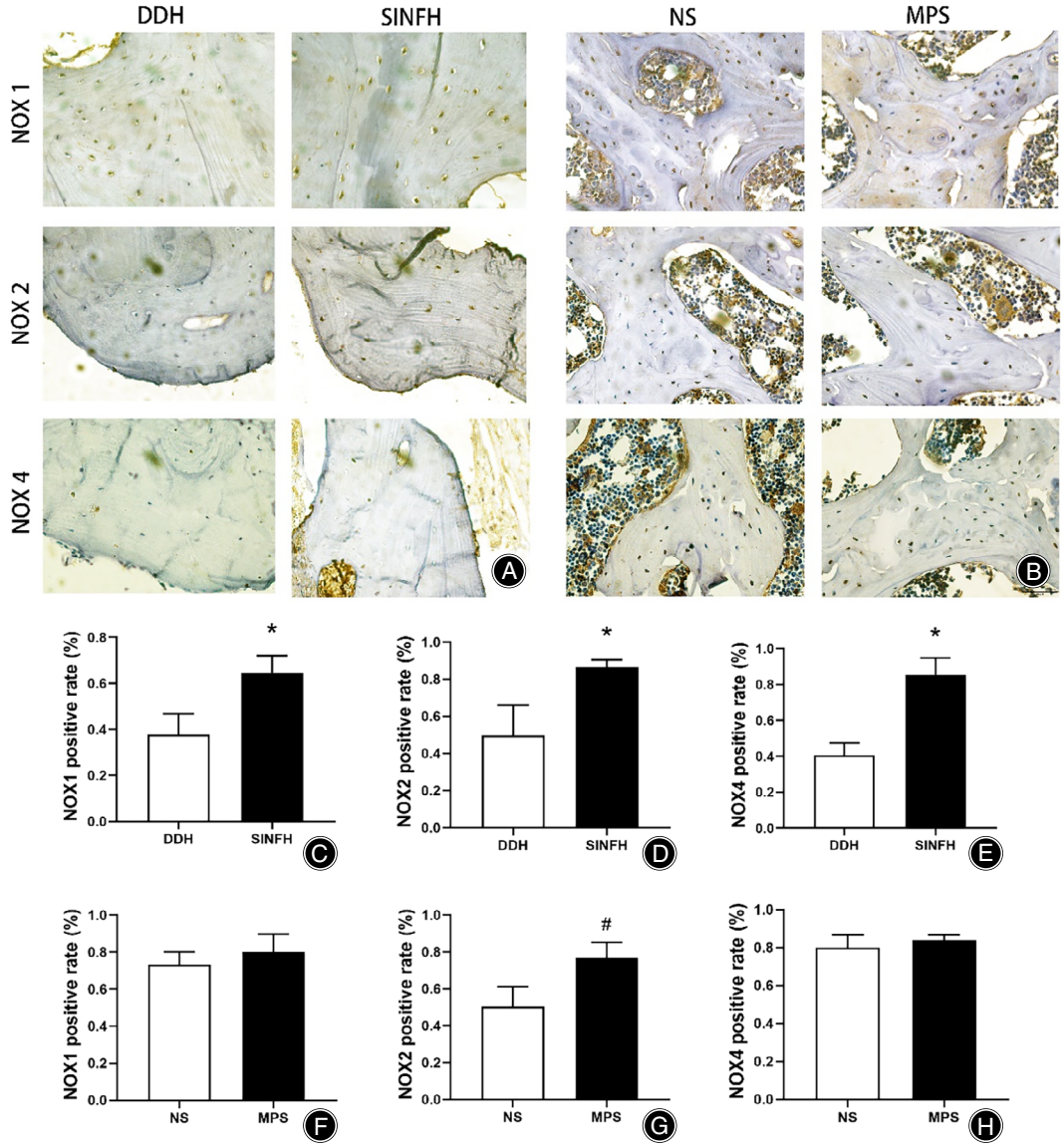


**Fig. 4** NOXs are involved in the process of GCs-induced oxidative injury. (A) Representative immunohistochemistry staining images of NOX1, NOX2 or NOX4 in the DDH and SINFH groups. (B) Representative immunohistochemistry staining images of NOX1, NOX2 or NOX4 in the NS and MPS groups. The NOX1 positive rate(C), NOX2 positive rate(D) and NOX4 positive rate(E) in the DDH and SINFH groups. The NOX1 positive rate(F), NOX 2 positive rate (G) and *NOX4* positive rate (H) in the NS and MPS groups. (*$P < 0.05$ *vs* DDH group, #$P < 0.05$ *vs* NS group).

osteocytes. Compared to the results of DDH group, long-term usage of GCs will increase the oxidative injury of osteocytes, and lead to cell apoptosis (Fig. 1C,E,F). It showed that the TUNEL-positive cell (6.2-fold) and 8-OHdG-positve cell (5.6-fold) in SINFH group were significantly higher compared to the DDH group ($49.32\% \pm 12.95\%$ *vs* $8.00\% \pm 2.11\%$, $T = -7.04$, $P = 0.002$, $54.6\% \pm 23.8\%$ *vs* $9.75\% \pm 3.31\%$, $T = -4.17$, $P = 0.003$). These findings indicated that the administration of GCs will induce oxidative injury of osteocyte, and lead to cell apoptosis.

### *The Presentment of MRI and HE Staining of Femoral Head in SINFH Rats*

To confirm GCs-associated oxidative injury and apoptosis of osteocytes *in vivo*, we established the SINFH rat model (Fig. 2). The MRI results of rats are shown in Fig. 3A. At 4 weeks after MPS injection, it showed high-intensity T2W1 signals, which means the signs of edema in femoral head. In addition, the femoral heads of NS group had no changes. The incidence of osteonecrosis at 4 weeks was 70.0% (7/10) in the MPS group and 0% (0/10) in the NS group based on MRI results ($P < 0.05$).

Pathological change was evaluated by hematoxylin–eosin (HE) staining (Fig. 3B). Fat cells were obviously showed in MPS group compared with NS group. The rate of empty lacunae in MPS group was 25.85% ± 4.68%, which was significantly higher than that in NS group (25.85% ± 4.68% *vs* 9.35% ± 1.99%, T = −7.96, $P < 0.001$) (Fig. 3D). The incidence of osteonecrosis at 4 weeks was 90.0% (9/10) in the MPS group and 0% (0/10) in the NS group based on histological examination ($P < 0.05$).

### *Oxidative Stress Induced Osteocyte Apoptosis of Femoral Head in SINHF Rats*

To confirm the role of glucocorticoid in osteocyte oxidative injury and apoptosis, we performed TUNEL and 8-OHdG staining. It showed that the TUNEL-positive cell (2.7-fold) and 8-OHdG-positve cell (2.6-fold) in MPS group were significantly increased compared to the NS group (31.93% ± 1.01% *vs* 11.73% ± 1.16%, T = −32.69, $P < 0.001$, 47.59% ± 1.39% *vs* 22.07% ± 2.45%, T = −22.18, $P < 0.001$) (Fig. 3C,E,F). These findings indicated that GC-induced oxidative injury and apoptosis of osteocyte, which confirmed our findings in SINFH patients. Our study provides important information on oxidative injury and apoptosis in the pathogenesis of SINFH.

### *NOXs are Involved in the Process of GC-Induced Oxidative Stress*

*In vitro* experiments, we have confirmed that NOXs are involved in the process of oxidative injury of osteoblast. Therefore, we further evaluated the effect of GCs on the expression of NOXs in SINFH patients and *in vivo* experiment. The protein expression levels of *NOX1, NOX2* and *NOX4* were detected by immunohistochemistry (IHC). The results showed that the protein expression of NOX1 (1.7-fold), NOX2 (1.8-fold) and NOX4 (1.4-fold) were significantly increased compared to the DDH group (64.50% ± 7.57% *vs* 37.58% ± 9.23%, T = −3.88, $P = 0.018$, 90.84% ± 2.93% *vs* 49.56% ± 16.47%, T = −5.46, $P = 0.001$, 85.46% ± 9.3% *vs* 40.69 ± 6.77%, T = −8.03, $P = 0.001$) (Fig. 4A,C–E). In addition, In the specimens from rat model of SINFH, it showed that the expression of NOX2 (1.5-fold) was significantly increased compare with NS group (76.77% ± 8.34% *vs* 50.32% ± 10.84%, T = −4.74, $P = 0.001$) (Fig. 4B,F–H). The protein level of NOX1 and NOX4 has no difference between MPS group and NS group. This is probably because species difference between human and rat. Taken together, the results confirm that *NOX1, NOX2* or *NOX4* is closed associated with GC-induced oxidative injury of osteocytes.

## Discussion

Oxidative stress was closely related to the process of steroid-induced necrosis of the femoral head (SINFH). Pathological examination of SINFH patients showed long-term usage of glucocorticoids (GCs) could lead to oxidative injury and cell apoptosis of osteocyte. To investigate GC-induced pathological changes *in vivo*, we established the rat model with SINFH. Further investigation confirmed oxidative injury and apoptosis of osteocyte in specimens with femoral head necrosis *in vivo*. In addition, we further evaluated the effect of GCs on the expression of NOXs and confirmed NOX1, NOX2 or NOX4 is closed associated with GC-induced oxidative injury of osteocytes.

### *GCs-Induced Oxidative Injury Lead To Osteocyte Apoptosis*

Since Weinstein and colleagues demonstrated osteocyte apoptosis in GC-induced osteonecrosis of the hip[4], accumulating evidence has performed for further research into the role of apoptosis in the development of osteonecrosis of the femoral head[30]. *In vitro* experiment, it has been reported that excessive GCs induce reactive oxygen species (ROS) generation and lead to cell apoptosis of osteocyte and osteoblast[17]. In the present study, the comprehensive effect of GCs on bone metabolism microenvironment and trabecular changes will result in osteoporosis of the femoral head. We revealed nucleic acid damage and cell apoptosis of osteocyte in specimens with femoral head necrosis by 8-OHdG staining and TUNEL assay. Because of the ischemic changes of femoral head in patients with femoral neck fracture, we prefer patients with developmental dysplasia of the hips (DDH) as the control group. The results of immunofluorescent staining indicate that oxidative injury could lead to osteocyte apoptosis, which involved in the process of GC-induced osteonecrosis of femoral head. In addition, it has reported that the special mechanical environment in the weight-bearing area of the femoral head aggravates osteonecrosis and eventually leads to the occurrence of steroid necrosis and collapse of the femoral head[31].

### *Oxidative Injury and Apoptosis in SINFH Rat Model*

To confirm the role of oxidative stress and apoptosis in the process of SINFH *in vivo*. We established steroid-induced osteonecrosis model by subcutaneously injected with high-dose MPS for 4 weeks as previous studies[32]. MRI scanning showed signs of edema in femoral head in MPS group and the ON incidence was 70.0%, suggesting that this method successfully induced SINFH in rat. In addition, GC-induced histopathological changes were further confirmed by sections of HE staining. In contrast to control group, the broken trabecular, increasing and hypertrophic adipocytes, rate of empty lacunae of osteocyte in MPS group were significantly higher, which confirmed the comprehensive effect of GCs on necrosis of femoral head. In the pathogenesis of the femoral head necrosis, the activity of MSCs as well as the osteogenic differentiation potential were attenuated by GCs[33]. Our results of HE staining confirmed the significant process of bone marrow fat in MPS group. The increased marrow fat suggests that GCs possibly shifts the differentiation of bone marrow stromal cells to favor adipocyte over osteoblast[34], which will lead to thrombus formation in the process of SINFH[34]. In addition, osteocyte apoptosis, induced by oxidative injury was also observed in femoral heads of SINHF rats, which confirmed

our previous founding in SINFH patients. These findings indicate an important oxidative stress–induced mechanism involved in osteocyte apoptosis that could lead to SINFH.

### *NOX Isoforms Participate in GCs-Induced Oxidative Injury*

NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) oxidase (NOX), including NOX1–5 and dual oxidase (DUOX)1–2, which exerts a predominant role in ROS generation. Previous studies have been reported that ROS derived from NOX1, NOX2, and NOX4 is closely related to oxidative stress in our skeletal metabolism[25]. We investigated NOX1, NOX2, and NOX4 protein expression to clarify the NOX isoforms in the process of GC-induced oxidative injury. The results suggested that NOX1, NOX2 and NOX4 played a significant role GC-induced oxidative injury of osteocyte in SINFH patients. *In vivo*, the results showed the expression of *NOX2* was significantly increased. The different results between patient and rat with SINFH may be due to the differences between species.

In summary, our study indicates that oxidative injury and apoptosis of osteocytes are involved in the process of SINFH, which may provide viable means of preventing GC-induce oxidative stress to prevent SINFH. In addition, the underlying mechanism of this condition may be related to GC-induced NOX expression. Further experimental studies are still needed.

### *Informed Consent*

The study was approved by the Institutional Review Boards of the Tianjin Medical University General Hospital, Tianjin, China.

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