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Full Length Research Paper

Effects of an herbal formula on the prevention of steroid-induced osteonecrosis in rabbits

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The objective of this study is to investigate the alterations of inflammation in the steroid-induced avascular osteonecrosis of femoral head with/without an intervention agent. Twenty-seven healthy New Zealand rabbits were included and randomized into 3 groups. Group A (the control group, n=9) received a single gluteal injection of physiologic saline at a dosage of 20 mg/kg. Group B (the steroid group, n=10) received a single gluteal injection of methylprednisolone at a dosage of 20 mg/kg. Group C (the intervention group, n=8) received intervention agent continuously at a dosage of 2.13 g/kg/day for 12 days, and were given a single gluteal injection of methylprednisolone at a dosage of 20 mg/kg on the seventh day. Tumor necrosis factor alpfa (TNF-α) and interleukin 1 beta (IL-1β) were measured before and after treatment. Tissues from the steroid group were stained with haematoxylin and eosion to undergo histopathologic examination on the 4th weeks after the injection. Both TNF-α and IL-1β levels increased relative to the baseline, and were greater than the control group at each time point. No positive finding was observed in the histopathologic examination. High-dose glucocorticosteroid increases the levels of TNF-α and IL-1β, which are related to intravascular coagulation and thus play an important role in steroid-induced osteonecrosis. The intervention agent appeared to have the efficacy to decrease the levels of inflammation.

Key words: Osteonecrosis, glucocorticosteroid, inflammation, intervention.

INTRODUCTION

Osteonecrosis has frequently been reported to occur in patients who have received steroids for spinal cord, collagen diseases or immunosuppression after organ transplantation (Peng et al., 2008; Asada et al., 2008; Kabata et al., 2005; Yamaguchi et al., 2011). Abnormal metabolism of lipids, and hypertrophy and proliferation of the bone marrow fat cells which are simulated by glucocorticoids are related to steroid-induced osteonecrosis of the femoral head (ONFH) (Peng et al., 2008). Besides that, the interruption of the bone vascular supply and bone ischaemia are also responsible for ONFH (Yamaguchi et al., 2011). Untreated ONFH is a debilitating and progressive collapse disease which often requires surgical treatments. The non-surgical method has been shown to improve symptoms and function, delay the progression of disease in patients, thus it is a better chance for patient with early-stage osteonecrosis (Yamaguchi et al., 2011; Garino and Steinberg., 1997; Kang et al., 2010; Liu et al., 2005). Non-surgical approaches for ONFH include protected weight-bearing, physical therapy, and pharmacological agents (Agarwala et al., 2005). Weight bearing with protection is recommended to reduce the pain for patients with ONFH.

A case was reported with all clinical features of early ONFH resolved (Rooney et al., 2009). However, whether weight bearing with protection can prevent collapse of the femoral head is controversial (Sun et al., 2011). Physical therapy includes electrical stimulation (Malizos et al., 2007), extracorporeal shock wave and high frequency

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magnetic field therapy. They are commonly useful for alleviating pain and promoting recovery of necrotic bone (Sun et al., 2011). Drug treatment is suitable for the pre-collapse stage of ONFH (Yoon and Kim, 2009). A combination treatment with an anticoagulant (warfarin) plus a lipid-lowering agent (probucol) was proved to have effects on the prevention of ONFH in rabbits (Motomura et al., 2004). Chinese medicine for treating thrombophilia or hypofibrinolysis, alendronate for preventing collapse of the femoral head, vasodilator drugs and drugs for elimination of bone marrow edema are all useful for ONFH. Several Chinese herbal formula exhibit effects of tonifying kidney, bone-invigorating, activating blood and dissolving stasis, thus they are usually used for ONFH in China (Liu et al., 2005; Li et al., 2002).

Therefore, the prevention intervention drug for osteonecrosis is an optional strategy for patients. Recent studies usually introduced rabbit models of methylprednisolone-induced osteonecrosis with high reproducibility of the necrosis by using methylprednisolone (Yamaguchi et al., 2011; Mikami et al., 2010; Ikemura et al., 2010). In this study we used this kind of models to investigate whether the lipid-lowering and anti-platelet formula (China's Kangguzhengsheng Capsules) can prevent or decrease the incidence of steroid-induced osteonecrosis.

MATERIAL AND METHOD

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Peking Union Medical College, China.

Animals and experimental groups

Thirty healthy adult New Zealand rabbits of either sex weighting about 2.78±0.26 kg were maintained on adaptive feeding for 1 week. 3 were dead; the remaining 27 rabbits were randomly divided into three groups with no group differences in body weight.

Group A had 9 rabbits weighting 2.8±0.2 kg which received gluteal injections of physiologic saline (PS, Kelun Pharmaceutical, Sichuan, China) at a dosage of 20 mg/kg for 1 time. Group B had 10 rabbits weighting 2.7±0.2 kg which received gluteal injections of methylprednisolone (MPSL, Pfizer Inc, China) at a dosage of 20mg/kg for 1 time. Group C had 8 rabbits weighting 2.9±0.2 kg which received gavage of China's Kangguzhengsheng Capsules (KGZS, Jiangsu Kangyuan Pharmaceutical Co., Ltd., China) at a dosage of 2.13 g/kg/day for 12 days, and were given gluteal injections of methylprednisolone at a dosage of 20 mg/kg for 1 time on the seventh day. KGZS used for intervention was an herbal formula. Every 350 g of KGZS was extracted from 1005g of 9 herbs which were Rehmanniae Radix Praeparata (175 g), Cistanches Herba (117 g), Cibotii Rhizome (117 g), Ligustri Lucidi Fructus (70 g), Epimedii Folium (117 g), Spatholobi Caulis (117 g), Raphani Semen (58 g), Drynariae Rhizome (117 g), and Achyranthis Bidentatae Radix (117 g). Thus 2.87 g of 9 raw herbs was equivalent to 1 g of KGZS granule. The rabbits were fed with a standard diet and allowed to do free exercises.

Hematological examination

For group A and C, blood samples were collected immediately prior to drug administration (0 day) and on days 1, 3, 7, 14, and 28 after injection. For group B, blood samples were collected before intervention gavage (-7 day), before drug administration (0 day) and on days 1, 3, 7, 14, and 28 after injection. Blood samples were collected from the marginal vein of rabbit ear using vacuum blood collection set (vacuum blood collection needles and tubes; Vacutainers, BD, Meylan, France). Blood were collected at the same time for each time point of blood sampling (generally at 3:00 pm). Blood samples were shaken up and reacquired if there was blood clot.

Detection of inflammatory proteins by Array-ELISA

The serum collected was standing for 1 h, then centrifuged at 2,500 rpm for 15 min at 24°C to remove red blood cells. The supernatant was transferred to the 1.5 ml EP tube and stored at -80°C until it was evaluated.

ELISA kits were used for the quantitative determination of TNF-α and IL-1 β in supernate according to manufacturer's instructions. Human TNF-α ELISA Test Kit (DTA00C, R and D Systems, MN) was used for TNF-α, and Human IL-1β ELISA Test Kit (DLB50, R and D Systems, MN) was used for IL-1β. In briefly, TNF-α/IL-1 β in the samples was bound to monoclonal human antibodies which were immobilized on the surface of the microtiter plate. After a washing step, to remove all interfering substances, the quantification of the bound TNF-α/IL-1 β was carried out by adding a second monoclonal antibody and a horseradish peroxidase labeled conjugate. The amount of the converted substrate by the peroxidase was directly proportional to the amount of bound TNF-α/IL-1 β and could be determined hotometrically at 450 nm. The reference wavelength in this study was 620 nm. The dilutions of serum were RD6-35 and RD6C, respectively.

Tissue sample preparation

Four weeks after methylprednisolone injection (Group B), three animals were painless sacrificed. For light microscopic examinations, bilateral femur head and peripheral joint capsule were obtained at the time of death and fixed for 24 h with a 10% formalin solution. Bone samples were decalcified with 10% EDTA for 4 weeks. Subsequently specimens were embedded in paraffin, cut along the coronal plane into 5 μm sections, and stained with hematoxylin and eosin.

Statistical analyses

PSAW v 17.0 (SPSS Inc., Chicago, IL, USA) was used to plot figures and for data analysis. Results are expressed as means ± SE; a P value <0.05 was considered significantly different.

RESULTS AND DISCUSSION

Macroscopic and histologic examination

Usually in rabbit models, 20mg/kg of MPSL as a high concentration was used to cause corticosteroid-induced osteonecrosis (Asada et al., 2008; Lu and Li., 2011; Miyanishi et al., 2008). The frequency for osteonecrosis began to increase after injection and reached a plateau at the second week (Kabata et al., 2005). Results of histologic examination usually showed no significant difference among different time points after two weeks. In this study,

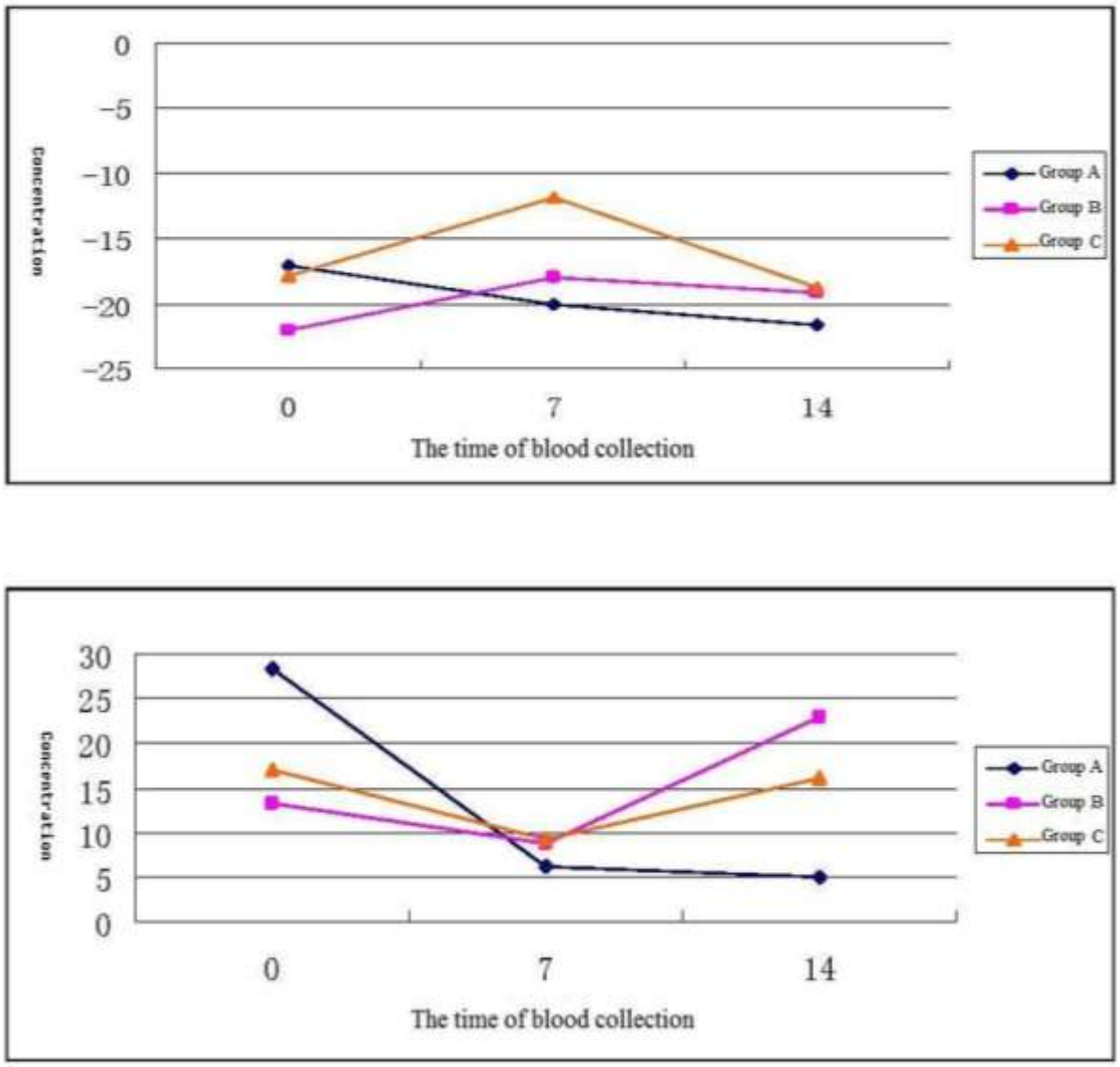

Figure 1. The line charts representing changing trend of TNF-α and IL-1β.

histologic examination of three rabbits in group B was observed four weeks after injection. Femoral heads from three rabbits in group B exhibited normal bone morphology, no bones collapse, no proliferative synovial membrane of joint capsule. With microscopic examinations, the bone trabeculae had no empty lacunae, bone fatty degeneration did not exist, and the bone marrow was not filled with intramedullary fat cells and fat tissue. This was mainly because the frequency of necrosis of the femoral head was 43~95.2% in MPSL group (Yamamoto et al., 1997; Kuribayashi et al., 2010; Nishida et al., 2008; Murata et al., 2007). Thus it was normal that there were no obvious histologic results of three rabbits. However, MPSL did have some effects on proinflammatory cytokines which was related to steroid-induced osteonecrosis according to the following results.

Effect of KGZS on the expression of proinflammatory TNF-α and IL-1β

The line charts representing changing trend of TNF-α reached the peak at the 7th day and declined slightly, there were no significant differences at baseline between time points for group B and C. The TNF-α production of group B and C were both higher than the control group A at 7 and 14 day after administration (Figure 1a). A substantial increase in IL-1β production was found and peaked at 14 day and there were no significant differences at baseline between time points for group B and C. The IL-1β production of group B and C were both higher than the control group A at 7 and 14 day after administration (Figure 1b).

The r^2 values curves of TNF-α and IL-1β were more

Table 1. Results of regression analysis on calibration curves and detection limits.

Compounds	Regression equation	r^2
TNF-α	$Y = 0.0026\ X + 0.1085$	0.9986
IL-1β	$Y = 0.008\ X + 0.0172$	0.9946

Y- Value of ultraviolet absorption, *X*- *C*oncentration of TNF-α and IL-1β, r^2 - Correlation coefficient of the equation.

Table 2 Serum levels of TNF-α and IL-1β in rabbits before and after administration.

	0d		7d		14d	
	TNF-α	**IL-1β**	**TNF-α**	**IL-1β**	**TNF-α**	**IL-1β**
group A	-17.00	12.10	-20.05	6.25	-21.60	4.90
group B	-21.95	13.15	-18.00	8.70	-19.10	22.95
group C	-19.48	17.05	-19.03	9.10	-20.35	15.95

*Linear curves were fitting according to concentration gradients and ultraviolet absorption, thus there were sample concentration with negative values.

than 0.9900 with *r* values more than 0.9950. This demonstrated that the linear was good and met the requirement of experiment (Table 1). Serum levels of TNF-α and IL-1β in rabbits before and after administration were shown in Table 2.

TNF-α is the key pro-inflammatory cytokine to activate an inflammatory response and the main factor inducing endothelial cell injury (Lam et al., 2000; Louis et al.,1998). Besides that, IL-1β is also an important inflammatory cytokine, which leads to endothelial cell injury, participates in immune regulation and regulates inflammatory response (Jung et al., 2003). IL-1 can stimulate synthesis and secretion of TNF- α and platelet-derived growth factor to collaboratively produce biological effects, which leads to tissue damage (Esmon, 1999; Hata et al., 2004). IL-1 and TNF- α also could induce mRNA expression of tissue factor which further increased the risk of thrombosis and ONFH (Brown et al., 2009; Tesfamariam and DeFelice et al., 2007; Kaur et al., 2007). In this study, inflammation in steroid-induced osteonecrosis is found in group B, and the inflammatory response has been reduced to some extent due to drug intervention in group C.

Although there are no significant differences for expression of TNF-α and IL-1β between group C and B because of sample size limitations and hemolysis, there are obvious better trend towards remission in group C than B. It is the characteristics of Chinese medicine which action is warm and slow. The advance of using this kind of Chinese Medicine is little by little. If there is enough experiment time the results may be different.

China's Kangguzengsheng Capsule has been recorded in Pharmacopoeia of the People's Republic of China; its main bioactive component is icariin. It usually used for osteoarthritis (Ren et al., 2006), and calcaneal bone hyperplasia (Liu et al., 2009) in Clinical Practice. This formula can regulate matrix metalloproteinase-1 and its inhibitor TMP (Yin et al., 2011), block IL-1β -mediated chondrocyte apoptosis (Song et al., 2008), and inhibit the contents of TNF-α and IL-1β in degenerated cervical inter vertebral disc (Liu et al., 2005). Thus, KGZS can alleviate endothelial damage, improve damaged endothelial function, inhibit inflammatory response and protect endothelial function. In accordance with previous study, our study found that KGZS improved the inhibition of inflammatory factors in rabbits with osteonecrosis to play a therapeutic effect.

However, the protective effect of KGZS on steroid-induced osteonecrosis of the femoral head in vivo remains to be further observed. In conclusion, this finding supports the beneficial effect of KGZS on steroid-induced osteonecrosis, and provides a theoretical basis for the clinical application of KGZS.

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