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Original article

Role of hypercoagulability in steroid-induced femoral head necrosis in rabbits

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Abstract

Background. The pathogenesis of femoral head necrosis is still uncertain. Both steroid treatment and hypercoagulopathy are considered risk factors. To investigate possible changes in coagulability and histology during steroid-induced osteonecrosis, we used a combination of the Shwartzman reaction and corticoid injections in rabbits to develop an animal model of femoral head necrosis. We studied blood coagulability and histopathological characteristics of the femoral head and liver.

Methods. A total of 30 rabbits were divided into three groups. In group A, rabbits were given two injections of lipopolysaccharide (40 μg/kg) at an interval of 24 h and were then immediately given one injection of prednisolone acetate (20 mg/kg). In group B, 10 rabbits were given one injection of prednisolone acetate (20 mg/kg). In group C, 10 rabbits were given no treatment and served as controls. At 1, 3, 7, 14, and 21 days after prednisolone injection, coagulability, blood lipid levels, and blood platelet levels were measured; and the femoral head and liver were removed for histopathological examination.

Results. At 24 h after the prednisolone injection in group A, coagulability and blood lipid levels were increased ($P < 0.01$), and blood platelet levels were decreased ($P < 0.01$). These abnormal levels were maintained throughout the entire observation period. Histologically, degeneration and necrosis of hepatocytes and osteocytes were found at day 21 after prednisolone injection in group A. In group B, coagulability and blood lipid levels were significantly increased by day 3 after treatment.

Conclusions. Abnormal hypercoagulability might potentiate the thrombotic status and induce thrombus formation in the presence of steroid-induced necrosis of the femoral head.

Introduction

Osteonecrosis (ON) of the femoral head is a potentially debilitating disease that frequently affects young patients. The exact pathogenesis of ON remains uncertain. Corticosteroid therapy — as given, for instance, to treat uveitis — is the most widely recognized risk factor for nontraumatic ON.[1] Various pathophysiological mechanisms have been proposed for this disease, including fat emboli, microfracture, microvascular tamponade, and retrograde embolization of the marrow fat.[2–4] Data from one epidemiological survey in humans indicated that thrombophilia was a common feature in patients experiencing steroid-induced femoral head ON.[5] Jones[6] suggested that corticosteroid treatment enhanced hypercoagulability, induced thrombus formation, damaged blood circulation, and led to ON of the femoral head. In agreement with this idea, reduction of blood flow to the femoral head and hypercoagulability of plasma were observed in pigs subjected to 24 h of treatment with high-dose methylprednisolone.[7]

Abnormalities in hemorheology usually play an important role during thrombus formation and disease development, and the extent of the abnormality is positively correlated with thrombotic disease severity and prognosis.[8–11] The high level of plasmin–α_2-plasmin inhibitor complex in the coagulation–fibrinolysis system seen in patients after high-dose corticosteroid therapy suggests that a sustained hemostatic abnormality participates in the development of osteonecrosis in this context.[12] To clarify the relation among hypercoagulability, blood platelets, and blood lipids, it is necessary to elucidate the mechanisms underlying steroid-induced ON.

The development of a suitable animal model is important for clarifying the pathogenesis of steroid-induced ON. A classic inductive protocol that is relevant to corticosteroid-induced ON is commonly used for establishing ON in rabbits. This method uses two injections of high-dose lipopolysaccharide (LPS) combined with a subsequent injection of corticosteroid, which induces a greater incidence of ON in experimental animals.[12–14] However, as yet, there are few reports that elucidate the

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relation among hemorheology, pathology, and pathophysiology in this model.

Based on existing data, this study was designed to combine the Shwartzman reaction with a single corticosteroid treatment to investigate changes in hemorheology as well as the pathology and pathophysiology of ON in a rabbit model.

Materials and methods

In this model, LPS was injected to provoke a Shwartzman reaction. In the context of the resulting hypercoagulable state, steroids were used to induce osteonecrosis of the femoral head.

Animals, grouping, and treatment

Adult Japanese white rabbits, weighing 3.0 ± 0.5 kg, were maintained on a standard laboratory diet and water. This experiment was approved by the institutional review board for Animal Research at Shanghai Ninth People's Hospital, affiliated with Shanghai Jiao Tong University. The national rules for laboratory animals were strictly observed. All experiments on animals were conducted in accordance with the guidelines of the Animal Center of the authors' institution.

The animals were divided into three groups. In group A, 10 rabbits were given two intravenous injections of LPS (40 μg/kg each) (Bangdingtaike Biotechnology, Beijing, China) at an interval of 24 h and were then immediately given one intravenous injection of prednisolone acetate (20 mg/kg) (Hubei Pharmaceutical, Guangshui, China). In group B, 10 rabbits were given one intravenous injection of prednisolone acetate (20 mg/kg). In group C, 10 rabbits given no treatments were used as controls. At 1, 3, 7, 14, and 21 days after prednisolone injection, hemorheological characteristics, blood lipid levels, and blood platelet levels were measured. After the last measurement, the rabbits were sacrificed, and the femoral head and liver were removed for histological examination.

Hemorheological examination

After an overnight fast, 8-ml blood samples were collected into EDTA tubes (2.0 mg K_3 EDTA/ml blood) from each rabbit, through the vein in the ear, immediately before injection of LPS and at 1, 3, 7, 14, and 21 days after the injection of prednisolone acetate. Plasma was obtained by centrifuging the EDTA blood (1750*g*/3000 rpm at 4°C for 20 min). Blood viscosity (BV), plasma viscosity (PV), and red blood cell aggregation (RCA) were determined immediately after blood sampling. PV and BV were measured at 37°C using a rotation viscosimeter (LBY-N6' Precil, Tianjin, China) at continuously increasing shear rates, from 1/s to 200/s. The temperature was kept at a constant 37°C by means of an automatic heating control unit. The shear rates were recorded by a scanner integrated into the viscosimeter. PV and BV results are given at two representative shear rates (low shear 40/s and high shear 200/s).

Blood platelet, triglyceride, and total cholesterol examination

The timing and collection method for blood samples were identical to those used for hemorheological analyses. All blood samples were collected in 4.5-ml vacuum tubes containing 0.5 ml of 3.8% sodium citrate anticoagulant. Blood platelet levels were measured with a blood analyzer (1400; Abbott Japan, Tokyo, Japan). Triglycerides and total plasma cholesterol were determined enzymatically using an autoanalyzer (7170A; Hitachi, Tokyo, Japan).

Histological examination

All of the rabbits were sacrificed by an overdose of anesthetic 21 days after prednisolone acetate injection. For light microscopy (Olympus, Tokyo, Japan), tissue samples were obtained from the femoral head and liver and were fixed with 10% formalin/0.1 M phosphate buffer, pH 7.4. The bone samples were decalcified with 25% formic acid and neutralized with sodium sulfate buffer. The specimens were embedded in paraffin, cut into 6-μm sections and stained with hematoxylin and eosin following routine protocols.

Measurement of necrotic rate

Empty lacunae in bone trabeculae and pyknotic or fragmented osteocyte nuclei were defined as histological osteonecrosis. The necrotic rate (dead cells and empty lacunae/the total number of osteocytes, dead cells, and empty lacunae) was calculated for each femoral head using a coronal section taken at the maximal femoral width. A computerized image analysis program (VNT Quant Lab-ST Visual New Technology Developing, Beijing, China) was used for this calculation. The system functioned as follows: An image of the sections was picked up by the microscope and directed to the image processor, digitalized into numerical values, and interpreted by a digital computer.

Statistical analysis

Data were reported as means with standard deviations, and one-way analysis of variance (ANOVA) (LSD)

tests with 95% confidence intervals (CI) ($P < 0.05$) were done.

Results

Hemorheological examination

In group A, the BV, PV, and RCA were markedly increased at 24 h after treatment ($P < 0.01$), and these high levels were maintained during the observation period. In group B, the BV, PV, and RCA were significantly increased compared with those values for group C ($P < 0.01$) by day 3 and continued to increase throughout the experiment (Table 1).

Blood platelet, triglyceride, and total cholesterol examination

In group A, blood platelet levels were significantly decreased at 24 h after treatment ($P < 0.01$) and then gradually recovered by day 21. In group B, blood platelet levels were significantly decreased at day 3 and recovered from days 7 to 21 ($P < 0.01$) (Table 2). In group A, triglyceride and total cholesterol concentrations were sharply increased at 24 h after treatment ($P < 0.01$) and remained elevated at days 3, 7, 14, and 21 ($P < 0.01$). In group B, triglycerides and total cholesterol were significantly increased at day 3 and continued to increase until the observation period ended at day 21 ($P < 0.01$) (Table 3).

Table 1. Hematoplasma viscosity, whole blood viscosity, and erythrocyte aggregation index at various time points for the three groups

Parameter, by group	Time				
	24 h	3 d	7 d	14 d	21 d
PV (mPa.s)					
A	2.15 ± 0.16	2.55 ± 0.17	2.60 ± 0.14	2.37 ± 0.14	2.15 ± 0.13
B	1.11 ± 0.11**	1.51 ± 0.20**	1.67 ± 0.14**	1.91 ± 0.15**	2.05 ± 0.13
C	1.13 ± 0.08$^{\blacktriangle\blacktriangle}$	1.06 ± 0.13$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.01 ± 0.08$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.08 ± 0.09$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.06 ± 0.11$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$
BV (mPa.s, 40s^{-1})					
A	5.88 ± 0.21	5.82 ± 0.14	5.26 ± 0.13	5.18 ± 0.13	5.11 ± 0.13
B	4.51 ± 0.12**	4.85 ± 0.21**	5.21 ± 0.18*	5.54 ± 0.19**	5.16 ± 0.19
C	4.14 ± 0.17$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	4.13 ± 0.12$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	4.09 ± 0.08$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	4.11 ± 0.10$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	4.19 ± 0.16$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$
BV (mPa.s, 200s^{-1})					
A	2.65 ± 0.09	3.01 ± 0.13	2.56 ± 0.06	2.60 ± 0.07	2.78 ± 0.06
B	2.39 ± 0.17**	2.45 ± 0.14**	2.40 ± 016**	2.68 ± 0.11	2.63 ± 0.08**
C	2.29 ± 0.10$^{\blacktriangle\blacktriangle}$	2.26 ± 0.06$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	2.24 ± 0.06$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	2.28 ± 0.08$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	2.23 ± 0.09$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$
RCV					
A	2.22 ± 0.06	1.93 ± 0.07	2.05 ± 0.08	1.99 ± 0.06	1.84 ± 0.10
B	1.89 ± 0.14**	1.99 ± 0.19	2.18 ± 0.18*	2.07 ± 0.07*	1.93 ± 0.10*
C	1.81 ± 0.04$^{\blacktriangle\blacktriangle}$	1.83 ± 0.05$^{\blacksquare\blacksquare}$	1.83 ± 0.05$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.80 ± 0.04$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.88 ± 0.08

Data values are the means ± SD
PV, plasma viscosity; BV (mPa.s, 40s^{-1}), blood viscosity at lower shear rates; BV (mPa.s, 200s^{-1}), blood viscosity at high shear rates; RCV, red cell aggregation
Based on one-way analysis of variance (ANOVA) (LSD) tests. A to B: $^{\star}P < 0.05$, $^{\star\star}P < 0.01$; A to C: $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$; B to C: $^{\blacksquare}P < 0.05$, $^{\blacksquare\blacksquare}P < 0.01$.

Table 2. Blood platelet counts at various time points for the three groups

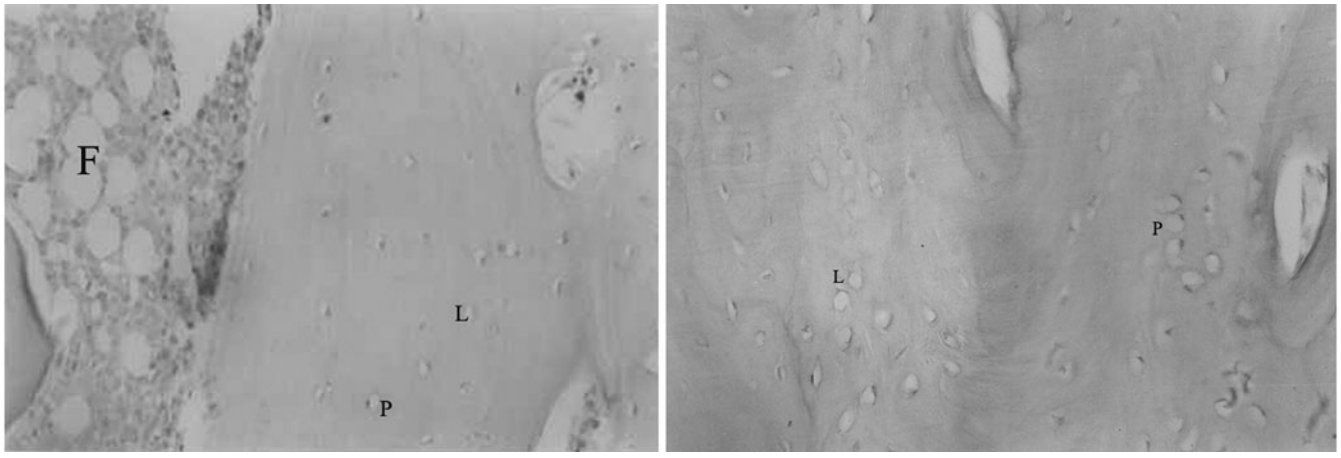
Group	Platelet count (10^9/l)				
	24 h	3 d	7 d	14 d	21 d
A	100 ± 13	140 ± 11	210 ± 20	238 ± 12	274 ± 11
B	312 ± 17**	290 ± 17**	220 ± 18	260 ± 13**	281 ± 9
C	314 ± 13$^{\blacktriangle\blacktriangle}$	311 ± 13$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	306 ± 13$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	306 ± 12$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	310 ± 8$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$

Data values are the means ± SD
Based on one-way ANOVA (LSD) tests. A to B: $^{\star}P < 0.05$, $^{\star\star}P < 0.01$; A to C: $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$; B to C: $^{\blacksquare}P < 0.05$, $^{\blacksquare\blacksquare}P < 0.01$

Table 3. Serum triglycerides and total cholesterol at various time points for the three groups

Parameter	24 h	3 d	7 d	14 d	21 d
Serum TGs (mmol/l)					
A	1.45 ± 0.10	1.38 ± 0.14	1.49 ± 0.08	1.68 ± 0.08	1.88 ± 0.09
B	1.07 ± 0.08**	1.23 ± 0.10**	1.69 ± 0.07**	2.15 ± 0.14**	1.93 ± 0.13
C	1.03 ± 0.11$^{\blacktriangle\blacktriangle}$	1.06 ± 0.09$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.09 ± 0.08$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.01 ± 0.10$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.00 ± 0.08$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$
Total Chol (mmol/l)					
A	2.49 ± 0.12	2.54 ± 0.12	2.19 ± 0.10	2.24 ± 0.10	2.43 ± 0.08
B	1.59 ± 0.11**	1.77 ± 0.14**	2.00 ± 0.12**	2.35 ± 0.12	2.56 ± 0.15*
C	1.56 ± 0.09$^{\blacktriangle\blacktriangle}$	1.57 ± 0.06$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.54 ± 0.13$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.54 ± 0.15$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$	1.53 ± 0.08$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$

Data values are the means ± SD
TG, triglycerides; Chol, cholesterol
Based on one-way ANOVA (LSD) tests. A to B: $^{\star}P < 0.05$, $^{\star\star}P < 0.01$; A to C: $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$; B to C: $^{\blacksquare}P < 0.05$, $^{\blacksquare\blacksquare}P < 0.01$



a b

Fig. 1. a, **b** Histological sections of the femoral head from an animal in group A at 3 weeks. Hypertrophic fat cells (*F*), empty lacunae in bone trabeculae (*L*), and osteocytes with pyknotic nuclei (*P*) were observed. Living osteoblasts around the dead trabeculae were rare. H&E, ×200

Histological examination

Macroscopically, neither collapse nor deformity of the femoral head was seen in any animals in the three groups. In group A, histological examination of the necrotic area demonstrated hypertrophic fat cells, bone trabeculae with empty lacunae, and osteocytes with pyknotic nuclei and nuclear fragmentation. Living osteoblasts with empty lacunae around the dead trabeculae were rarely seen (Fig. 1). In group A, thrombi were noted in some intraosseous blood vessels of the femoral head (Fig. 2). Focal hepatocyte necrosis with fatty metamorphosis was noted in group A (Fig. 3). In group B, the rabbits demonstrated fatty metamorphosis of the hepatocytes (Fig. 4).There were no abnormalities seen in the liver or bone of rabbits in group C.

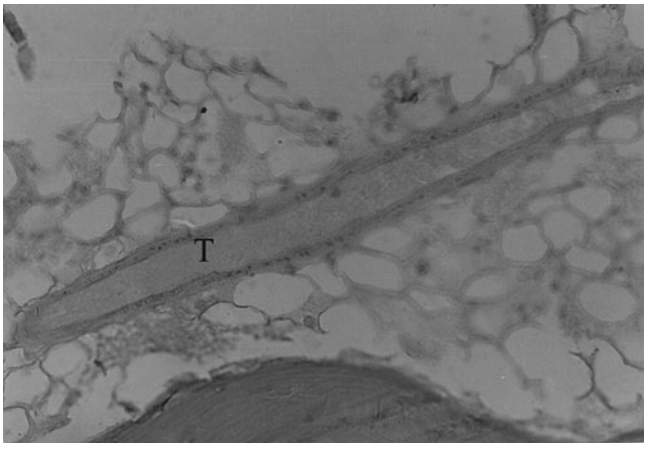


Fig. 2. Histological section of the femoral head from an animal in group A at 3 weeks. Thrombi (*T*) were noted in the intraosseous blood vessels of the femoral head. H&E, ×200

Measurement of necrotic rate

The necrotic rates at 21 days for groups A and B were significantly higher than those for group C ($P < 0.01$) (Table 4).

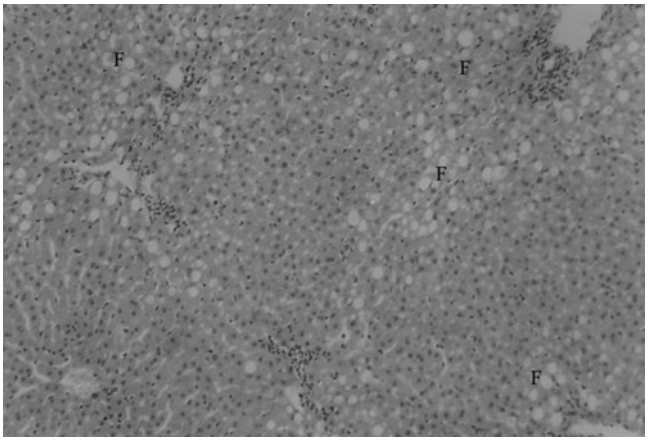


Fig. 3. Histological section of the liver from an animal in group A at 3 weeks. Focal hepatocyte necrosis and fatty metamorphosis (*F*) were seen. H&E, ×200

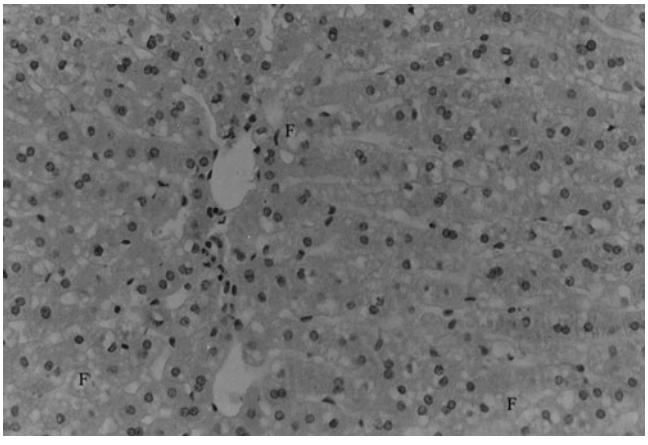


Fig. 4. Histological section of the liver from an animal in group B at 3 weeks. Focal hepatocyte necrosis and fatty metamorphosis (*F*) were seen. H&E, ×200

Table 4. Femoral head necrosis rates at day 21 for the three groups

Group	No. examined	Necrosis rate (%)
A	20	36.2 ± 1.78
B	20	30.0 ± 1.35**
C	20	16.8 ± 0.81$^{\blacktriangle\blacktriangle\blacksquare\blacksquare}$

Data values are the means ± SD
Based on one-way ANOVA (LSD) tests. A to B: $^{\star}P < 0.05$, $^{\star\star}P < 0.01$; A to C: $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$; B to C: $^{\blacksquare}P < 0.05$, $^{\blacksquare\blacksquare}P < 0.01$

Discussion

Current reports suggest that intravascular coagulation (IC), as an intermediary mechanism, is the most common pathway by which intraosseous embolism causes nontraumatic ON.[1,6] Some studies have found direct histological evidence of intraosseous thromboses in animal models of ON and in patients with this condition.[1,5,6,12,14,15] Hypercoagulability plays an important role in the process of thrombosis formation, so the study of coagulability is necessary to elucidate the pathogenesis of steroid-induced ON.

In the present study, based on the animal model established by Yamamoto et al.,[16] we successfully created a steroid-induced osteonecrosis model in the rabbit, with histological features clearly representative of osteonecrosis. The pathological changes were similar to those seen in patients in the clinic.[17–19] Short-course therapy with high-dose corticosteroids has been assumed to cause decreased blood flow and a hypercoagulable state by blocking the reticuloendothelial system and reducing the fibrinolytic activity of endothelial cells.[4,7] In group A, the BV, PV, and RCA were markedly increased 24 h after treatment compared with the values in group B ($P < 0.01$), and this difference was maintained throughout most of the observation period, indicating that LPS treatment induced abnormal hypercoagulability and that corticosteroid treatment exacerbated thrombotic status. At the same time, in group A, blood platelet levels were significantly decreased 24 h after corticosteroid injection ($P < 0.01$), compared with the levels in group B and recovered very slowly during the observation period, indicating the occurrence of a consumption coagulopathy. Thrombus formation in capillaries confirmed this finding. Therefore, it appears that LPS causes a number of pathological changes (e.g., local or systemic inflammation, a hypercoagulable state, and a hypofibrinolytic state) by activating not only endothelial cells but also macrophages and platelets.

High-dose corticosteroids appear to potentiate the Shwartzman reaction and the magnitude of osteonecrosis, perhaps by increasing endothelial damage and hypercoagulability of intraosseous and extraosseous vessels that subsequently become thrombosed.[16] These findings were consistent with those from previous studies.[5,6,13,16] However, Séguin et al. suggested that nontraumatic ON is not associated with a specific thrombophilic abnormality and that there is a potential association between regional endothelial dysfunction and ON.[20]

Blood viscosity is a basic hemorheologic parameter, and BV at low shear is considered the best marker of blood coagulability. Because blood is a non-Newtonian fluid, coagulability is affected by many factors, such as RCA and PV. As a main determinant of blood viscosity, increased RCA could directly lead to abnormal hemodynamics and disturb blood flow. PV also significantly influences BV. Large protein molecules (size and shape) and high protein content in blood plasma are the most important determinants of PV. At the same time, we found a defined hypertriglyceridemia and hypercholesterolemia, which were closely associated with increased

PV. Several reports showed positive associations between PV and triglycerides, as well as total cholesterol.[21,22] Short-course, high-dose corticosteroids can produce a hyperlipidemic state, and our experiments confirm this. Hyperlipidemia can produce high PV, degrade erythrocyte deformability, enhance platelet aggregation, and damage blood vessel endothelium. All of these impairments could directly damage osteoblasts and induce thrombogenesis in many ways.[13,19]

In the microcirculation, hemokinesis follows Poiseuille's law ($Q = \Delta R\pi r^4/8\,\eta l$), which illustrates an inverse correlation between the volume of blood flow and the viscosity, such that when viscosity is increased, circumfusion is correspondingly reduced. In bone tissue, the bone blood flow is obviously affected by viscosity.[23] The microcirculation of the femoral head represents a terminal branch of the circulation system. Capillaries leave the parent stem at an angle of approximately 90° and follow a course directed perpendicular to the joint surface. They subsequently bend back at an angle of approximately 180°. As the slender afferent vessels debouch into the capacious sinusoids, the rate of blood flow therein is severely decreased.[24] This sluggish circulation could easily contribute to the formation of microthrombi and the induction of avascular necrosis in the femoral head. Thus, abnormal hypercoagulability could potentiate thrombotic status and induce thrombus formation in steroid-induced necrosis of the femoral head.

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