# Cholesterol- and lanolin-rich diets may protect against steroid-induced osteonecrosis in rabbits

Garida Zhao, Takuaki Yamamoto, Goro Motomura, Ryosuke Yamaguchi, Satoshi Ikemura, Kenyu Iwasaki, and Yukihide Iwamoto

Department of Orthopaedic Surgery, Kyushu University, Fukuoka, Japan.
Correspondence: yamataku@ortho.med.kyushu-u.ac.jp
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**Background and purpose** **It remains controversial how hypercholesterolemia influences the development of steroid-induced osteonecrosis (ON). We investigated the role of hypercholesterolemia induced by a cholesterol-rich diet on the development of ON in rabbits.**

**Methods** **40 adult male Japanese white rabbits were randomly divided into 2 groups. 20 rabbits were maintained on a cholesterol-rich diet for 2 weeks before receiving steroid treatment (the CHOL group). The other 20 rabbits were maintained on a standard diet (the control (CTR) group). 2 weeks after the start of the study, all 40 rabbits were injected with methylprednisolone acetate (MPSL) into the right gluteus medius muscle (20 mg/kg body weight). 2 weeks after the steroid injection, both the femora and humeri were examined histopathologically for the presence of ON. Hematological analysis of the serum lipid levels was performed every week. Based on the same protocol, we also investigated the effects of lanolin, a primary component of a cholesterol-rich diet, in another group (the LA group).**

**Results** **The incidence of ON in the CHOL group (3/20) was lower than that observed in the CTR group (15/20) ($p < 0.001$). During the whole experiment, the levels of total cholesterol and the ratio of low-density lipoprotein to high-density lipoprotein in the CHOL group were higher than those observed in the CTR group ($p < 0.001$). The LA group also had a lower incidence of ON (2/20), and the lipid levels in the LA group showed similar changes to those observed in the CHOL group.**

**Interpretation** **Our findings suggest that preexisting hypercholesterolemia itself induced by a cholesterol-rich diet does not increase the risk of developing steroid-induced ON, but rather seems to diminish it. Lanolin may be the active anti-ON component of the cholesterol diet.**

Osteonecrosis (ON) of the femoral head is often caused by administration of corticosteroids for underlying diseases, such as systemic lupus erthematosus (SLE) and nephrosis syndrome (Mont and Hungerford 1995). Although several possible factors have been suggested to be involved in the pathogenesis of ON, based on human and animal studies (Yamamoto et al. 1997, Pritchett 2001, Motomura et al. 2004, Yamaguchi et al. 2011), the precise pathological mechanisms of steroid-induced ON remain unclear.

Lipid metabolism abnormalities have been suggested to be a possible mechanism of steroid-induced ON in both human and animal studies (Wang et al. 1978, Yamamoto et al. 1997, Pritchett 2001, Motomura et al. 2004, 2008, Kang et al. 2008). Wang et al. (1978) reported that hypercholesterolemia occurs in steroid-treated rabbits, and that adipocytes in the femoral head of such rabbits show a 25% greater increase in fat content. Pritchett (2001) reported that statin therapy reduces the risk of ON in patients receiving steroid treatment for underlying diseases, by improving hyperlipidemia. On the other hand, neither familial hypercholesterolemia nor hypertriglyceridemia has been reported to be associated with ON (Goldberg et al. 2011, Klop et al. 2012). Thus, the exact effects of hypercholesterolemia on the development of ON have not yet been clarified. In addition, to our knowledge there have been no reports on the effects of preexisting hypercholesterolemia on the development of steroid-induced ON.

In this study, we examined the role of hypercholesterolemia induced by a cholesterol-rich diet on the development of steroid-induced ON in rabbits.

## Material and methods

### *Experiment 1*

All experiments were conducted in accordance with the Guidelines for Animal Experiments at our institution (date of


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issue: January 29, 2010; registration number: A22-104-0), in accordance with Japanese law (no. 105), and in accordance with notification no. 6 of the Japanese government.

### Animals

We first studied 40 adult male Japanese white rabbits (Kyudo, Saga, Japan) with ages ranging from 28 to 32 weeks. The mean body weight of the rabbits was 3,549 (SD 203) g. The animals were kept at the animal center of our institution.

### Treatment

40 rabbits were randomly divided into 2 groups as follows. The hypercholesterolemia (CHOL) group (n = 20) was maintained on a standard diet containing 1% cholesterol (Riken Vitamin, Chiba, Japan) based an established protocol to induce hypercholesterolemia via diet (Ohara et al. 1993). The control (CTR) group (n = 20) was maintained on a standard diet. 2 weeks after the start of the study, all 40 rabbits had methylprednisolone acetate (MPSL; Pfizer, New York, NY) injected into the right gluteus medius muscle (20 mg/kg body weight). 2 weeks after injection, the rabbits were anesthetized with an intravenous injection of pentobarbital sodium (25 mg/kg body weight). They were then killed by exsanguination via an aortectomy, and tissue specimens were prepared. The steroid-induced ON rabbit model has been reported to be precisely reproduced (Yamamoto et al. 1997).

### Tissue preparation

For light microscopy, tissue samples were obtained from the femur and humerus at the time of death and fixed for 1 week with 10% formalin in 0.1 M phosphate buffer, pH 7.4. The bone samples were decalcified with 22.5% formic acid for 3 days, and were then neutralized with 0.35 M sodium sulfate for 3 days. The specimens were embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin.

### Evaluation of ON

The diagnosis of ON was based on previously reported histopathological criteria (Yamamoto et al. 1997, Motomura et al 2004). The stained specimens were obtained from the proximal one-third and distal condyles of both femora and humeri (8 regions). The presence of ON was assessed blindly by 3 of the authors (GZ, TY, and GM), based on the presence of diffuse empty lacunae or pyknotic nuclei of osteocytes within the bone trabeculae accompanied by surrounding bone marrow cell necrosis. If the diagnoses differed between the 3 investigators, discussions were held regarding the histological findings without knowing which group the specimen was obtained from until consensus was reached. Rabbits with at least 1 ON lesion over the 8 areas were considered to have ON.

### Examination of the laboratory data

We collected 5-mL blood samples from the auricular arteries in the early morning while the animals were in a fasting state. The samples were obtained at week 0 and weeks 1, 2, 3, and 4 after the start of feeding. We examined the following serum lipid levels: total cholesterol (TC) and the ratio of low-density lipoprotein to high-density lipoprotein (LDL/HDL ratio).

### Experiment 2

To analyze the results of experiment 1 in greater depth, an additional experiment was conducted in which we investigated the effects of lanolin—a major component of cholesterol-rich diets—on the development of steroid-induced ON.

20 adult male Japanese white rabbits, the lanolin (LA) group, were maintained on a standard diet containing 3% lanolin (Riken Vitamin, Chiba, Japan). The mean body weight of the rabbits was 3,707 (SD 104) g. 2 weeks after the start of the study, all 20 rabbits had methylprednisolone acetate (MPSL; Pfizer, New York, NY) injected into the right gluteus medius muscle (20 mg/kg body weight) 2 weeks after injection, the rabbits were sacrificed and tissue specimens were prepared.

Tissue preparation, evaluation of ON, and examination of the laboratory data were performed according to the methods used in Experiment 1.

### Statistics

The numbers of ON-positive rabbits in the CHOL and LA groups were compared with that in the CTR group using Fisher's exact test. The laboratory data are expressed as mean (SE). Differences in mean body weight and laboratory data between the CHOL and CTR groups were analyzed using a repeated-measures analysis of variance (MANOVA). If an interaction between 2 factors in a group at 1 time point was significant in the repeated MANOVA, a simple main effect test of the group difference at the same time point was applied. Temporal changes in the laboratory data were analyzed in each group using the Wilcoxon signed-rank test. Statistical analyses were performed using the JMP 9.0 software package (SAS Institute, Cary, NC). All p-values $< 0.05$ were considered to be statistically significant.

## Results

### Experiment 1

During the experimental period in Experiment 1, the mean loss in body weight in the CHOL group (707 (SD 133) g) was significantly greater than that observed in the CTR group (380 (SD 219) g) ($p < 0.001$).

### Incidence of ON

ON occurred in 15 of 20 rabbits in the CTR group and in 3 of 20 rabbits in the CHOL group ($p < 0.001$). The histological appearance of ON in the CHOL group was similar to that observed in the CTR group. In the metaphyses and diaphyses of the ON-positive rabbits in both groups, yellowish areas were observed in which accumulation of bone marrow cell

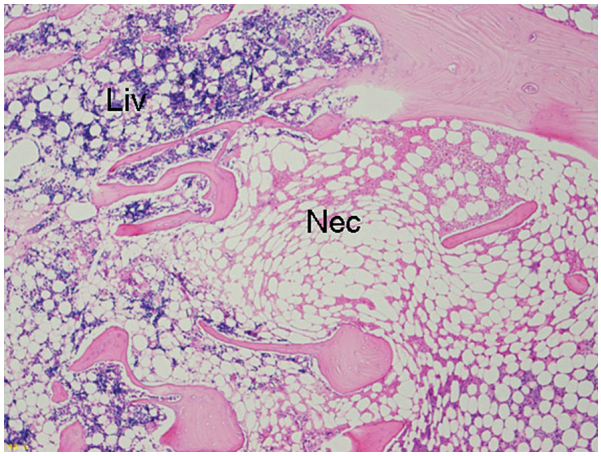


Figure 1. Histopathological features in the CHOL group (×40). Accumulation of bone marrow cell debris was observed, in addition to bone trabeculae showing empty lacunae in the necrotic areas (Nec). 2 weeks after steroid administration in the CHOL group, neither granulation tissue nor appositional bone formation was obvious between the necrotic areas and living bone marrow tissue (Liv).

debris was apparent, and the bone trabeculae had empty lacunae (Figure 1).

### *Laboratory data*

Following the administration of the cholesterol-rich diet, the mean level of TC in the CHOL group increased from 16 (SE 1.4) mg/dL at week 0 to 455 (SE 44) mg/dL at week 2 ($p < 0.001$) (Figure 2a). Following the administration of MPSL at week 2, the mean level of TC in the CHOL group increased to 629 (SE 56) at week 4 ($p < 0.001$). On the other hand, in the CTR group, the mean level of TC changed from only 28 (SE 2.5) mg/dL at week 2 to 70 (SE 8) mg/dL at week 4 ($p < 0.001$). However, the levels of TC in the CHOL group were higher than those observed in the CTR group throughout the experimental period (all $p < 0.001$).

Following administration of the cholesterol-rich diet, the mean LDL/HDL ratio in the CHOL group increased from 0.4 (SE 0.03) at week 0 to 2.8 (SE 0.15) at week 2 ($p < 0.001$) (Figure 2b). In addition, this parameter increased in the CHOL group, even after steroid administration at week 2 ($p < 0.001$). On the other hand, this parameter showed no statistically significant changes in the CTR group throughout the period of the experiment.

### *Experiment 2*

During the experimental period, the mean loss in body weight in the LA group was 430 (SD 122) g.

### *Incidence of ON*

ON in the LA group occurred in 2/20 rabbits, an incidence which was much lower than that in the CTR group (15 of 20; $p < 0.001$). The histological appearance of ON was similar to that observed in Experiment 1.

### *Laboratory data*

Following administration of the diet with 3% lanolin, the mean level of TC in the LA group increased from 22 (SE 1.4) mg/dL at week 0 to 303 (SE 29) mg/dL at week 2 ($p < 0.001$). Following administration of MPSL at week 2, although the

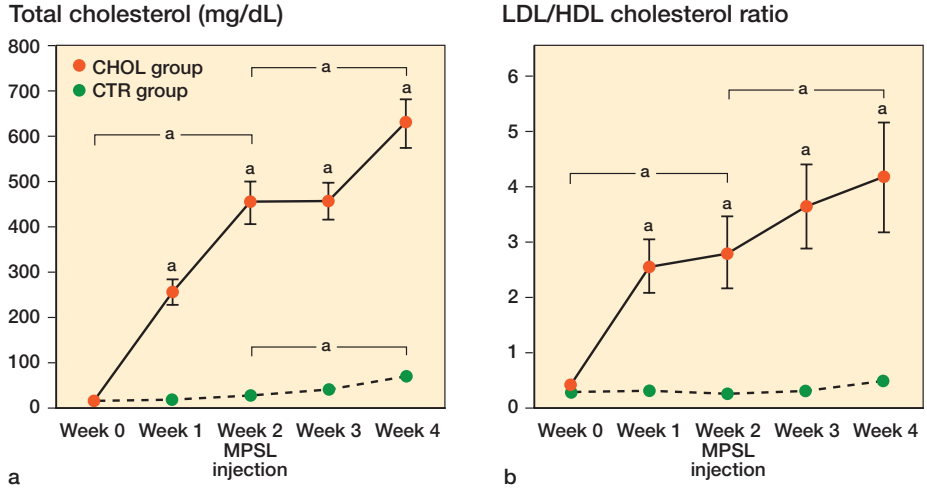


Figure 2. Sequential changes in serum lipid levels. The data are mean with standard error bars. The levels of total cholesterol (TC) and ratios of low-density lipoprotein to high-density lipoprotein (LDL/HDL ratios) were different between the CHOL group and the CTR group (both $p < 0.001$, repeated MANOVA). a. The mean level of TC in the CHOL group increased from week 0 to week 2 ($p < 0.001$, Wilcoxon signed-rank test). Following methylprednisolone acetate (MPSL) injection, the mean level of TC increased in both groups (both $p < 0.001$). However, the mean levels of TC in the 2 groups were different at weeks 1, 2, 3, and 4 ($p < 0.001$). b. The mean LDL/HDL ratio in the CHOL group increased from week 0 to week 2 and also increased from week 2 to week 4 (both $p < 0.001$, Wilcoxon signed-rank test). There were no statistically significant changes in the CTR group. Thus, the LDL/HDL ratios in the 2 groups were different at weeks 1, 2, 3, and 4 ($p < 0.001$).

mean level of TC in the LA group increased additionally to 366 (SE 32) mg/dL at week 4, this change was not statistically significant.

The mean LDL/HDL ratio in the LA group increased from 0.2 (SE 0.02) at week 0 to 3.3 (SE 0.2) at week 2 ($p < 0.001$). Following the administration of MPSL at week 2, although the mean ratio of LDL/HDL in the LA group increased additionally from 3.3 (SE 0.2) at week 2 to 3.8 (SE 0.3) at week 4, this change was not statistically significant.

## Discussion

Since hyperlipidemia is thought to be associated with the development of ON (Wang et al. 1978, Yamamoto et al. 1997, Miyanishi et al. 2001, Pritchett 2001, Motomura et al. 2004, Kang et al. 2008, Ikemura et al. 2011), we had expected that preexisting hypercholesterolemia would increase the incidence of ON in rabbits. However, in the current study, the rabbits maintained on the cholesterol-rich diet had a low incidence of ON in spite of the presence of hypercholesterolemia.

Although many studies have shown that hypercholesterolemia occurs after steroid administration (Wang et al. 1978, Yamamoto et al. 1997, Motomura et al. 2004), no positive relationships between the level of hypercholesterolemia and the development of ON have as yet been reported. Previous experimental studies have not shown any significant differences in the levels of TC between rabbits with ON and those without (Yamamoto et al. 1997, Kang et al. 2008). On the other hand, no studies have examined the effects of preexisting hypercholesterolemia on the development of ON. We fed the rabbits on diets with abnormally high cholesterol levels—1% of the total diet based on an established protocol (Ohara et al. 1993)—in order to give them evidence of hypercholesterolemia beyond physiological levels. Our results show that hypercholesterolemia itself occurring before the administration of steroids has rather negative effects on the development of steroid-induced ON in rabbits.

Regarding some possible factors for the low incidence of ON observed in the rabbits fed a cholesterol-rich diet, we speculate that some constituents of the cholesterol-rich diet affected the incidence of ON. The cholesterol-rich diet used in our study contained lanolin, a compound extracted from wool as a cholesterol source (Clark 1980). We also investigated the effects of lanolin itself, which similarly reduced the incidence of steroid-induced ON in the rabbits.

The primary ingredient of lanolin is lanosterol, a precursor of various cholesterols in animals and fungi (Corey et al. 1966). The analysis of lanosterol under enzyme catalysis reveals a core structure of steroids and eventually yields cholesterol via cytochrome P450 (Abe et al. 1993). Since lanosterol is a precursor of endogenous steroids (Fahy et al. 2005), the presence of lanosterol in the cholesterol-rich diet may have competitively influenced the metabolism and pharmaceutical effects of the steroids including methylprednisolone, resulting in a lower incidence of ON.

In addition, methylprednisolone is normally inactivated when the double bond between C4 and C5 in the A-ring is reduced by 5-α-reductases. The 5α-reduced metabolites of methylprednisolone acetate are considered to be largely inert. It could be that the 2-week-long cholesterol- or lanolin-rich diet induced hepatic 5-α-(and/or 5β-) reductases, which produce flat and rapidly secreted bile acids of cholesterol, and that the methylprednisolone acetate was therefore also rapidly inactivated (Monte et al. 2005, Nixon et al. 2012).

Our study has demonstrated the negative effects of hypercholesterolemia induced by a cholesterol-rich diet containing lanolin on the development of steroid-induced ON; however, further research into the role of lanosterol may eventually help in the development of prophylactic treatments for steroid-induced ON.

GZ and RY: data management, statistical analysis, and writing of the manuscript. TY, GM, and YI: study design and revision of the manuscript. SI and KI: study design and database preparation.

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No competing interests declared.

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