Gender Differences in Bone Loss Induced by Iron Overload in Rats

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Abstract

Excessive iron has been recognized as a risk factor for infections, carcinogenesis, arthropathy and osteoporosis. Genetic hemochromatosis is an iron overload disorder, resulting in an osteoporosis in males. The risk of osteoporosis is influenced by the degree of iron overload. Much of the toxicity of the superoxide radical and H_2O_2 to living organisms is due to the iron ion-dependent generation of the hydroxyl radical, and/or other powerful oxidants. We investigated the effects of a colloidal iron overload on femoral bone loss and gender-based differences therein in rats. The rate of increase of iron accumulation by iron overloading into liver and kidney appeared to be more elevated in male rats than female rats. There was little difference in the nephropathy induced by iron-overloading between the genders. The reduction in plasma levels of calcium and alkaline phosphatase activity were much greater in the male rats, and circulating levels of estradiol were much higher in the female rats. Although plasma estradiol levels were not affected by iron-overload in both sexes, testosterone levels were slightly reduced by iron-overload in males. Femoral bone mineral density in male, but not female, was markedly reduced by iron overload. It is suggested that iron overload induces bone loss associated with renal dysfunction and hypogonadism in male rats, and circulating estradiol can prevent the bone absorption in female rats. Either genetically or therapeutically, iron overload may give rise to osteoporosis combined with renal dysfunction, liver iron overload syndrome and in part hypogonadism in male mammals.

Key words —— Gender differences, iron overload, nephrotoxicity, bone loss, male rats

Introduction

Patients with thalassemia major require multiple blood transfusions leading to hemochromatosis. Genetic hemochromatosis is an iron overload disorder mainly caused by a mutation (C282Y) of the HFE gene. In 32 Italian patients (28 males), the risk of osteoporosis is increased by liver cirrhosis and influenced by the degree of iron overload[1]. Of 38 French male patients, osteopenia and osteoporosis were 76% and 34%, respectively, and bone mineral density (BMD) in the femoral neck appeared to decline with rising hepatic iron concentrations[2]. Evidence of the drinking of beer brewed in iron containers and ascorbic acid deficiency were found in 88 % of 50 black African patients with femoral neck fractures, i.e. the oxidative stress from iron overload might cause osteoporosis[3]. In 76 thalassaemic patients of Greek Cypriot origin, osteoporosis and/or osteopenia in males were more frequent and severe than in females[4]. However, hypogonadism led a greater impact on spine BMD in females, but not males.

Experiments with animals have shown that the intraperitoneal injection of ferric nitrilotriacetate into rodents induces free radical injury and cancer in the

kidneys[5]. Bleomycin-detectable iron is increased in in vivo and in vitro model of cisplatin-induced nephrotoxicity. Treatment with both iron chelators and hydroxyl radical scavengers prevented cytotoxicity and acute renal failure induced by cisplatin. A critical role for iron in mediating tissue injury is played via the formation of hydroxyl radicals in nephrotoxicity induced by cisplatin[6]. Male mice are more susceptible to nephrotoxicity and the carcinogenic effect of ferric nitrilotriacetate than female mice[7]. It has been proposed that much of the toxicity of the superoxide radical and H_2O_2 to living organisms is due to the iron ion-dependent generation of the hydroxyl radical, and/or other powerful oxidants[8]. Increased levels of the hydroxyl radical have been demonstrated in renal tubular cells subjected to hypoxia/re-oxygenation[9]. Iron overload-associated toxicity in liver and/or kidney seems to be greater in males than females.

In the present study, we investigated the gender differences in femoral bone loss induced by a colloidal iron overload in rats.

Materials and Methods

Animals, Experimental Design, and Tissue Preparation

Wistar male and female rats (Oriental Yeast Co., Ltd., Tokyo, Japan) at 6 weeks of age, were housed in stainless steel cages under controlled conditions (24 ± 0.5 ℃ and 12 h of light from 0600 to 1800 h) in accordance with the principles outlined in the Guide for Animal Care and Use of the Committee of Tokyo Medical and Dental University. All rats had free access to a commercial diet (Oriental Yeast, Tokyo, Japan) and tap water in the animal room of the University. Each gender was divided into two groups of 12 rats each at 24 weeks of age: intact male and female rats were intravenously injected with 2 mL of a 0.9 % NaCl solution into the tail as a control at 24 weeks of age (Group 1 and 3, respectively), while colloidal iron-treated male and female rats were intravenously injected with 40 mg of saccharated ferric oxide (2 mL solution) (Fesin[TM], Mitsubishi Pharma Corporation, Tokyo, Japan) (Groups 2 and 4, respectively). All animals were anesthesized with urethane (1.5 g/kg body weight; Merck, Darmstadt, Germany), weighed, bled by cardiac puncture, and

sacrificed at 28 weeks of age. The serum samples obtained were tested commercially afterwards (SRL, Inc., Tokyo, Japan). At autopsy, the anterior pituitary, kidney, spleen, and liver were each weighed, immediately fixed in a 10 % formalin solution (pH 7.2), embedded in paraffin, prepared as 5 μm serial sections, stained with Mayer's hematoxylin and eosin for histological examination, and moreover, exposed to 2 % potassium ferrocyanide and 2 % hydrochloric acid for ferric staining (Berlin blue method). Detected iron particles are recognized as blue grains. Six sections of each sample were randomly chosen and iron-associated blue grains were counted in 400 cells per section. The degree of iron-accumulation was rated on a scale from 0 to 5. The femur was fixed in 99.5 % ethanol and stored for the measurement of BMD and preparation of bone sections for histological examination. BMD was determined by dual energy absorptiometry (DXA). Total BMD (mg/cm^2) of the whole femur was measured by DXA (Aloka, DCS-600, Tokyo, Japan) as bone mineral content/bone area. The BMD of the distal metaphysis of the femur was also measured by DXA as bone mineral content/bone width (BMC/BW) (mg/cm^2), i.e. part of the trabecular bone. All experimental procedures conformed to the regulations described in the U.S. National Institutes of Health (NIH) Guide to the Care and Use of Laboratory Animals.

Statistical Analysis

Data were expressed as the mean ± SEM (standard error of the mean). Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the unpaired t-test. Differences between the groups were considered statistically significant at the $p < 0.05$ level.

Results

Body growth was markedly reduced in both iron-treated groups (Groups 2 and 4) compared with the corresponding control groups (Groups 1 and 3) ($p < 0.01$) (Table 1).

Although the wet weights of anterior pituitaries were greater in female rats (Groups 3 and 4) than male rats (Groups 1 and 2), they were not affected by the iron

Table 1 Body growth and organ weights

	Male		Female	
	Control	Iron	Control	Iron
Body weights				
Initial (g)	324 ± 2	318 ± 4	207 ± 2	203 ± 1
Final (g)	535 ± 6	473 ± 7**	306 ± 7	277 ± 3**
Growth (%)	161 ± 2	151 ± 2**	152 ± 3	136 ± 1**
Organ weights				
Anterior pituitary (mg/100 g B. Wt.)	2.35 ± 0.12	2.36 ± 0.08	6.00 ± 0.26	6.12 ± 0.61
Kidney (mg/g B. Wt.)	2.74 ± 0.07	2.82 ± 0.05	2.78 ± 0.06	3.04 ± 0.08*
Spleen (mg/g B. Wt.)	1.36 ± 0.04	1.53 ± 0.06*	1.53 ± 0.06	2.02 ± 0.06**
Liver (g/100 g B. Wt.)	3.67 ± 0.03	4.01 ± 0.05**	3.21 ± 0.07	3.80 ± 0.04**

Iron: iron-overloaded group
Data are the mean ± SEM.
** and * Significantly different from the corresponding control at $P < 0.01$ and 0.05, respectively.

treatment (Table 1). The iron-overloading increased the weights of the spleen ($p < 0.05$) and liver ($p < 0.01$) in male rats, and the weights of the kidney ($p < 0.05$), spleen, and liver ($p < 0.01$) in the females. In general, organ weights were influenced by the iron overload more in the females than males.

The numbers of erythrocytes (red blood cells) were markedly increased in both sexes ($p < 0.01$ and 0.05), though the values of hemoglobin and hematocrit were not affected by the iron overload (data not shown). The numbers of leukocytes (white blood cells) were smaller in female rats than male rats ($p < 0.01$), but were not affected by the iron overload in either sex.

There were few differences in albumin, glucose, total cholesterol (TCh), triglyceride concentrations, and the activity levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) between control and iron-overloaded groups in both sexes (Table 2). However, the colloidal iron-overloading markedly elevated the concentrations of blood urea nitrogen (BUN) ($p < 0.01$) and creatinine ($p < 0.01$) compared with the control in male rats alone, but

not in female rats. The concentrations of calcium (Ca), inorganic phosphorus (iP) and potassium, but not sodium, were also significantly lowered by iron-overloading in male rats alone. The concentrations of plasma iron were markedly higher in female rats than male rats ($p < 0.01$), and were reduced by iron overload in female rats alone, but not male rats ($p < 0.05$). Plasma levels of estradiol (E_2) were markedly higher in female rats than male rats ($p < 0.01$), but were not affected by iron overload in both sexes. Plasma testosterone levels were reduced to approximately 80 % of the control by iron overload in male rats, though there were no significant differences.

Rats treated with iron in both sexes (Groups 2 and 4) showed vacuolization, swelling and desquamation in the proximal tubular epithelial cells of the kidney (Fig.1B), compared to the corresponding control group (Groups 1 and 3) (Fig.1A).

Iron accumulated in the liver (Fig.2A) and kidney (Fig.2B) with iron overload in both sexes. The rates of iron-deposits induced by iron overload were much greater in the males more than females, i.e. approximately 5-fold that of the male control liver and 4-fold that of the female

Table 2　Plasma levels of biochemical markers and hormones

	Male		Female	
	Control	Iron	Control	Iron
Albumin (g/dL)	3.77 ± 0.16	3.77 ± 0.08	3.53 ± 0.12	3.60 ± 0.16
Glucose (mg/dL)	225 ± 16	212 ± 10	265 ± 21	258 ± 19
TCh (mg/dL)	66.1 ± 3.2	65.8 ± 2.1	72.7 ± 3.3	76.8 ± 5.5
Triglyceride (mg/dL)	39.0 ± 10.7	34.3 ± 4.1	68.4 ± 11.0	45.0 ± 8.9
AST (IU/L)	119 ± 10	128 ± 8	106 ± 6	99 ± 6
ALT (IU/L)	55.5 ± 3.6	53.8 ± 4.1	35.6 ± 2.9	33.3 ± 2.8
ALP (IU/L)	475 ± 50	402 ± 51	505 ± 44	476 ± 31
BUN (mg/dL)	22.5 ± 0.7	24.9 ± 0.5**	25.3 ± 1.6	25.6 ± 1.1
Creatinine (mg/dL)	0.31 ± 0.02	0.39 ± 0.02**	0.39 ± 0.02	0.37 ± 0.03
Calcium (mg/dL)	9.82 ± 0.22	9.37 ± 0.06*	8.97 ± 0.14	9.03 ± 0.24
iP (mg/dL)	7.03 ± 0.48	5.17 ± 0.26**	3.60 ± 0.19	3.30 ± 0.29
Potassium (mEq/L)	4.82 ± 0.47	3.84 ± 0.15*	3.16 ± 0.08	3.43 ± 0.32
Sodium (mEq/L)	144 ± 1	144 ± 1	138 ± 1	139 ± 1
Plasma iron (mg/dL)	178 ± 11	161 ± 5	286 ± 16	243 ± 13*
Estradiol (pg/mL)	5.15 ± 0.51	4.67 ± 0.29	11.5 ± 2.51	12.7 ± 1.28
Testosterone (ng/mL)	1.38 ± 0.52	1.12 ± 0.06		

Iron: iron-overloaded group　　　　　　　　　　　　　Data are the mean ± SEM.
** and * Significantly different from the corresponding control at $P < 0.01$ and 0.05.
TCh: total cholesterol, AST: aspartate aminotransferase, ALT: alanine aminotransferase,
ALP: alkaline phosphatase, BUN: blood urea nitrogen, iP: inorganic phosphorus.

control liver, and 3-fold that of the male control kidney and 2-fold that of the female control kidney, i.e. especially in the proximal tubular epithelial cells of the kidney compared with those of both sexes ($p < 0.01$).

The connectivity of cancellous bone in the epiphysis and of trabecular bone in the metaphysis of the distal femur exhibited a greater loss in the iron-overloaded male animals（Group 2）(Fig.3B) than the controls （Group 1）(Fig.3A). However, there were no differences between the female groups（Groups 3 and 4）(Fig.3C and 3D).

Bone mineral density（BMD）was significantly reduced to 96.4 % and 93.1 % of the control level by iron-overloading in the whole femur（upper left-side）（p < 0.05）and the distal metaphysis of the femur in male rats（upper right-side）（Group 2）（p < 0.01）(Fig.4). However, neither BMD in the whole femur（lower left-side）nor BMD in the distal metaphysis of the femur （lower right-side）differed between the female groups （Groups 3 and 4）.
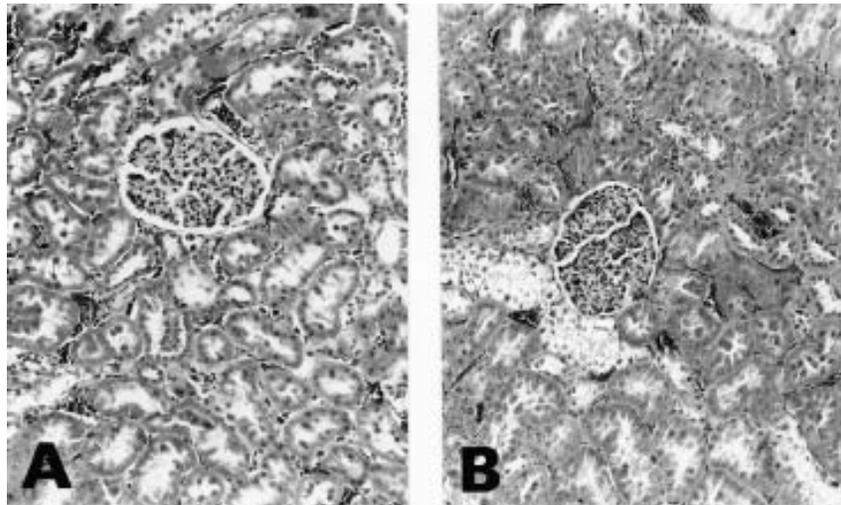
Fig.1 Histological structure of the kidney.
(HE staining, original magnification x 299).
A normal control group,
B iron-overloaded group; vacuolization, swelling, desquamation and necrosis in the proximal tubular epithelial cells of the kidney.
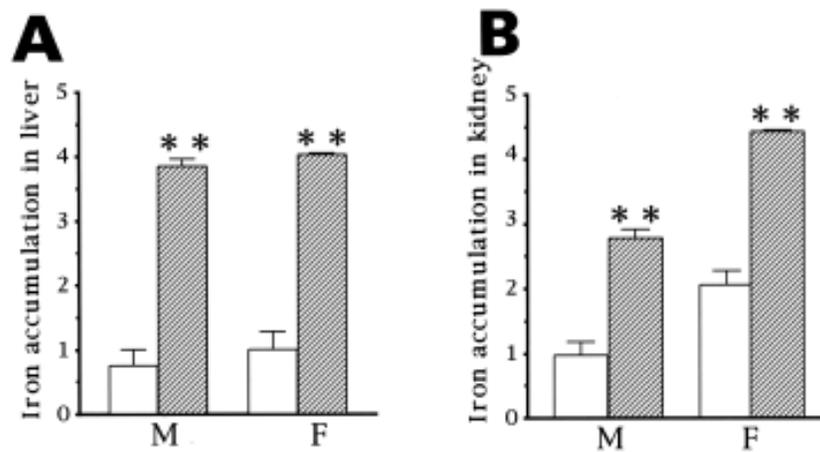

Fig.2 Extent of iron-accumulation in the liver (A) and kidney (B).
The degree to which iron was incorporated into the organs was rated from 0 to 5.
Gray-stripe pattern bars show the iron-overloaded organs.
M: male, F: female
** Significantly different from each corresponding control at p < 0.01.

Discussion

There has been considerable interest in the damage that can be done to living systems by the generation of reactive oxygen species, such as the superoxide radical, H_2O_2, and the hydroxyl radical. Excessive and misplaced iron has been recognized as a risk factor for infections, carcinogenesis, arthropathy, osteopenia, and osteoporosis[10]. Iron overload markedly reduced the extent to which osteoblasts formed and new bone was generated in pigs, whereas osteoclast resorption surfaces were unchanged[11]. Iron lactate induced osteomalacia in rats with high levels of osteocalcin in serum and low levels of PTH and iP. The bone lesion was similar to low turnover osteomalacia[12]. Genetic hemochromatosis is an iron overload disorder, resulting in an osteoporosis associated with the degree of iron accumulation in the liver in male patients[1, 2]. Bone mineral was increased in
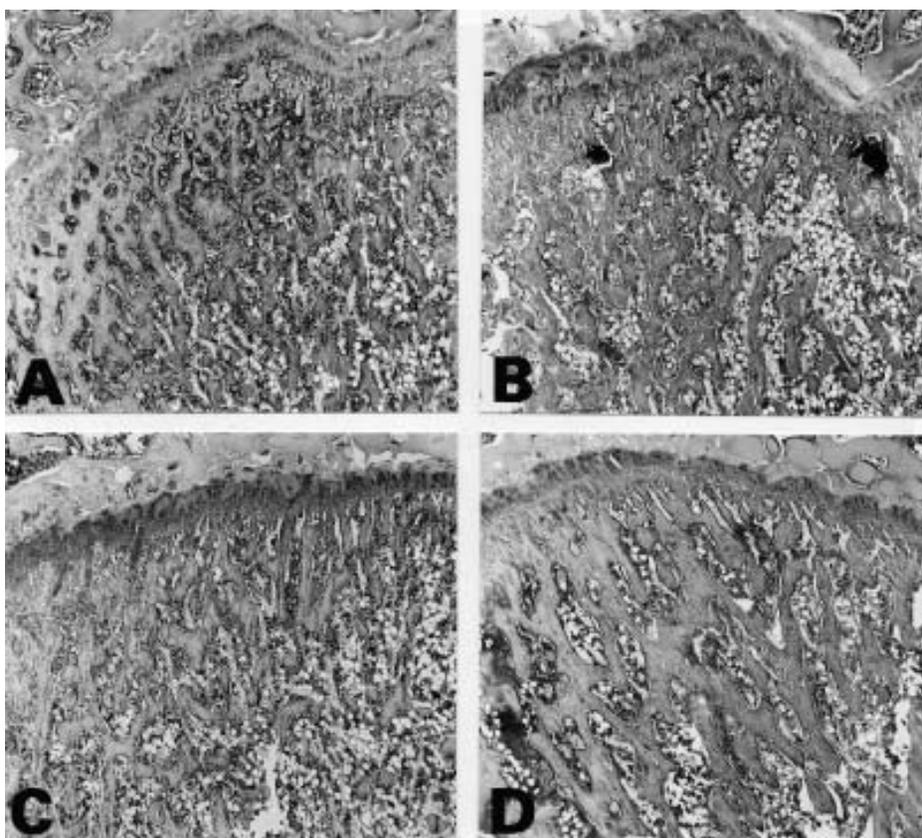
Fig.3　Histological structure of the femur.
　　　（HE staining, original magnification x 40）.
　　　A male normal control, B male iron-overloaded,
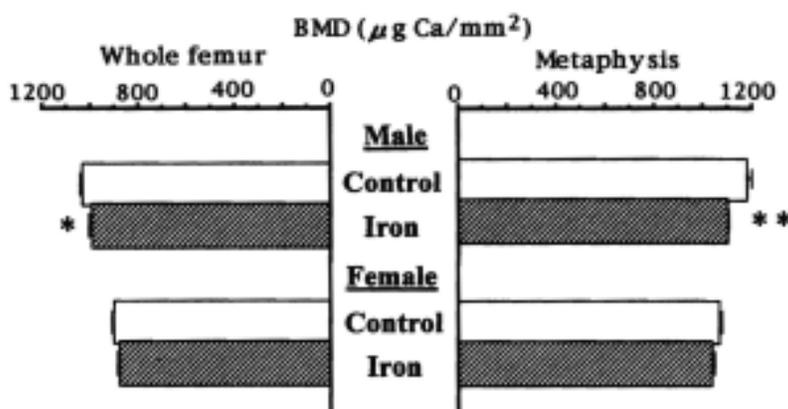　　　C female normal control, D female iron-overloaded.

Fig.4　Bone mineral density（BMD: μg Ca/mm^2）of the femur.
　　　Data are the mean ± SEM in whole femur（left-sided）and distal femur（right-sided）for 10 samples.
　　　A couple of upper bars for the male, and a couple of lower bars for the female.
　　　Gray-stripe pattern bars show the iron-overloaded organs.
　　　** and * Significantly different from each corresponding control at p < 0.01 and 0.05.

the lumbar spine and forearm in hypogonadal men with hemochromatosis treated by testosterone replacement therapy and venesection[13]. Replacement of testosterone may prevent the resorption of bone in hypogonadal men such as estradiol in postmenopausal women. Venesection and calcium supplementation led to the increase of BMD in a premenopausal woman with hemochromatosis[14]. An animal study has shown that male mice are more susceptible to the nephrotoxicity of ferric agents than female mice[7].

In the present study, iron overloading induced weight loss in both sexes, i.e. excessive iron has been recognized as a risk factor for body growth and infection[10]. The weights of the liver and kidney were increased more in female than male rats by iron overload. However, the rate of increase of iron deposition in the liver and kidney appeared to be greater in the male rats. Iron overload in the liver is known to reduce bone mass with liver cirrhotic change in liver iron overload syndromes. Reductions in plasma levels of Ca and ALP activity were much greater in the male rats. Vacuolization, swelling, desquama-tion and necrosis in the proximal tubular epithelial cells of the kidney were observed in iron-overloaded rats. The urinary discharge of Ca might be increased by the disturbance in reabsorption of urinary Ca from the proximal tubular epithelial cells affected by the hy-droxyl radical produced with iron overload. Although there was little difference in the nephropathy induced by iron-overloading between the genders, it was suggested that the male rats were more susceptible than the female rats. The connectivity of cancellous bone in the epiphysis and of trabecular bone in the metaphysis of the distal femur showed a marked loss in iron- overloaded rats. Each value of BMD in femur was markedly lowered in iron-overloaded rats than the controls. Plasma levels of testosterone were slightly lowered by iron overload in male rats. Markedly predominant levels of circulating estradiol in fe-male rats appeared to prevent bone absorption, resulting in inhibition of the reduction in femoral BMD.

In summary, it is suggested that iron overload induces bone loss associated with renal dysfunction and in part hypogonadism in male rats, and circulating estradiol can prevent the bone absorption in female rats. Taken together, either genetically or therapeutically, iron overload may give rise to osteoporosis combined with renal dysfunction, liver iron overload syndrome and in part hypogonadism in male mammals.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KUD made substantial contributions to the study's conception and the analysis of the data, and carried out the animal experiments under the guidance of SAS, and has involved in drafting the manuscript. OKA and NEM participated in drafting the manuscript. KIK and SAS performed the statistical analysis. SAK conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final draft.

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過剰鉄誘発ラット骨量減少症における性差

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要旨

　過剰鉄は感染，癌化，関節症，骨粗鬆症などの危険因子として知られている．遺伝的血色素症（遺伝性ヘモクロマトーシス）は鉄の異常蓄積で男性に骨粗鬆症を引き起こし，その病態は鉄の過剰状態によって影響される．スーパーオキサイドラジカルや H_2O_2 の生体へ与える毒性は水酸基ラジカルや他の強力な酸化剤の鉄イオン依存性物質による事が多い．今回，ラット大腿骨骨量減少に与える過剰コロイド鉄の影響とその性差について検討を加えた．

　肝臓および腎臓への鉄集積状態は雌よりも雄の方が強く認められた．過剰鉄による腎症では雌雄差が認められなかった．血中カルシウムとアルカリフォスファターゼ活性の減少は雄で多く，血中エストラジオール濃度は当然雌で多かった．血中エストラジオール濃度は雌雄ともに過剰鉄による影響を受けなかったが，男性ホルモン濃度は雄で過剰鉄によりわずかに減少が認められた．大腿骨骨密度は雄で過剰鉄により顕著な減少が認められた．鉄過剰状態は雄において腎機能低下に伴う骨量減少と性機能低下を引き起こし，また雌においてはエストロゲンが骨吸収を受動的に予防する可能性が示唆された．鉄過剰状態は腎機能低下に伴う骨粗鬆症，肝過剰鉄症候群および一部で性機能低下をもたらすことが推測された．

キーワード

性差，鉄過剰，腎毒性，骨量減少，雄ラット