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Original Article

Growing Rats Are Resistant to Bone Loss Associated With Short-Term Hypercholesterolemia: A Preliminary Experimental Study

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Abstract

We aimed to evaluate the potential of growing rats as a model of osteoporosis induced by short term hypercholesterolemia. Twelve growing female Sprague-Dawley rats were randomly allocated into two groups. Control rats received standard diet while rats in group 2 were fed with diet contained 20% sunflower oil, 2% cholesterol and 0.5% cholic acid for 2 weeks and then a diet contained 10% sunflower oil, 1% cholesterol and 0.25% cholic acid for the next 4 weeks. At the end of second week, serum total cholesterol level was assayed which was repeated at the end of the 6th week along with serum carboxy-terminal collagen crosslinks (CTX) and procollagen type 1 N propeptide (PINP) levels. Finally, rats were euthanized and right and left tibiae were dissected for histomorphometric study and determination of bone mineral density, respectively. Hypercholesterolemia was present in rats of group 2 in both sampling times. No significant difference was observed in body weight, epiphyseal and metaphyseal histomorphometric parameters and mineral density as well as serum CTX and PINP. Although this was a preliminary study with relatively low sample size, it seems that growing female Sprague-Dawley rats do not show bone loss due to short term hypercholesterolemia and may not be a proper animal model in this regard.

Key words: animal model, diet, hypercholesterolemia, osteoporosis, rats, short term.

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1. Introduction

For years, osteoporosis and cardiovascular disease were considered as independent chronic diseases that are more commonly associated with advanced age. In 1997, Parhami and Demer suggested that products of lipid and lipoprotein oxidation might have a

role in pathophysiology of osteoporosis [1]. Now, increasing evidence supports a direct association between cardiovascular disease and osteoporosis. For instance, previous studies have demonstrated that lipid-lowering agents from the statin group, which inhibit the rate-limiting step in cholesterol synthesis, increase bone mineral density and lower fracture risk [2-4]. Moreover, the available epidemiological evidence indicates that elevated serum cholesterol concentration is a risk factor in development of osteoporosis [5 and 6].

Experimental studies on animals that cover the subject of relationship between consumption of high fat diets and osteoporosis are relatively scares and mostly are performed on mice. In a pioneer study performed by Cao et al., (2009); mice showed a decrease in cancellous bone mass due to consumption of high fat diets for an extended time (14 weeks) [7]. Few years later, Patsch et al., (2011) reported that both short-term and extended term high fat diet can induce obesity and significant bone loss in mice [8]. Recently, Pelton *et al* concluded that hypercholesterolemia due to consumption of high fat/high cholesterol diet for 4 months, can directly affect bone health in mice and results in osteoporosis by promoting osteoclastogenesis. Based on this observation, they suggested that mice could be used as an animal model for testing intervention strategies for reducing negative effects of hypercholesterolemia on bone health [9].

Laboratory rats meet most of the criteria as an animal model. The availability of detailed knowledge of the rat skeleton and protocols for rapid induction of osteopenia, have increased this model's popularity for researchers working on subjects related to metabolic bone diseases including osteoporosis [10]. Rats reach their sexual maturity at the age of about 2.5 months, however their skeleton is considered mature after the age of 10 months [11]. Skeletally-immature rats have a low peak bone mass and are an appropriate animal model in the research of endocrine, nutritional and environmental factors [10]. Unfortunately, there is insufficient knowledge about the potential of rats as an animal model for study of the role of hypercholesterolemia in bone metabolism and architecture. As the only report that we found in this regard, You et al., (2011) declared that growing rats that have consumed a high fat/high cholesterol diet for 3 months show decreased bone mineral density (BMD) and serum osteocalcin accompanied by increased serum carboxy-terminal collagen crosslinks (CTX) level [12].

In the present study, we aimed to see whether consumption of diets with lower fat content for relatively short term is associated with bone changes in rats. This can help researchers for selecting the optimum period and lipid content of the diet which may be used for inducing bone changes in growing rats.

2. Materials and Methods

2.1. Animals and Experimental Design

Twelve growing female Sprague-Dawley rats, with a mean body weight of about 150 g, were purchased from animal house of Shiraz Medical University, Shiraz, Iran. Rats were acclimatized for one week before the beginning of the experiment to the ambient conditions (temperature about 23°C and a 12h/12h, light/dark cycle). Animals had free access to tap water and standard rat chow diet prepared by Razi Vaccine and Serum Research Institute, Shiraz, Iran. After adaptation, rats were randomly allocated into two equal groups (n = 6 each). Group 1 (control) rats received the standard diet during the experiment while rats in group 2 were fed with a diet contained 20% sunflower oil, 2% cholesterol (BDH, England) and 0.5% cholic acid (Sigma, USA) for 2 weeks and then a diet contained 10% sunflower oil, 1% cholesterol and 0.25% cholic acid for the next 4 weeks. At the end of the second and 6th week of the experiment fasting blood samples were collected from all rats by cardiocentesis under anesthesia. At the end of the experiment, rats were euthanized by deepening anesthesia and right and left tibial bones were dissected for histomorphometric and radiographic evaluation, respectively.

Procedures used in the present study are in accordance with institutional ethical guidelines of School of Veterinary Medicine, Shiraz University, for care and use of laboratory animals in experiments.

2.2. Determination of Serum Cholesterol Levels

After centrifugation at 3000 rpm for 10 min, harvested sera were stored in -80°C until use. Serum total cholesterol of samples collected at the end of the second and 6th week of the experiment were assayed by commercial colorimetric kits prepared by Ziest Chem® Diagnostics, Tehran, Iran.

2.3. Determination of Markers of Bone Metabolism

Sera harvested at the end of the experiment (6th week) were used for determination of biochemical bone markers. Procollagen type 1 N propeptide (PINP) and CTX were assayed by Rat PINP ELISA kit (Shanghai crystal day biotech Co., China) and Rat CTX-1 ELISA kit (Shanghai crystal day biotech Co., China) respectively.

2.4. Histomorphometric Study of Bone Samples

Bones were fixed in 4% formaldehyde solution and decalcified using formic acid–sodium citrate method [13]. Then 5-µm longitudinal sections of proximal epiphysis and metaphysis were made in the median plate. Sections were stained by using H&E method. Histomorphometric parameters were determined by a digital photo microscope connected to a personal computer with Ziess axio vision LE software. Trabecular width (Tb.Wi) was determined in epiphysis as well as in secondary spongiosa of metaphyseal side of growth plate; Bone area/tissue area (B.Ar/T.Ar) was measured in epiphysis. The

region of the cancellous bone marked for the measurements was the central zone of cancellous tissue. The nomenclature of parameters is in compliance with ASBMR histomorphometry nomenclature committee [14].

2.5. Determination of Bone Mineral Density

Lateral radiographs were prepared from left tibiae of rats. Density of the proximal epiphysis and metaphysis was quantitatively determined by using an aluminum step wedge and ImageJ software as described by Haidekker et al., 2004 [15].

2.6. Statistical Analysis

Data were presented as mean ± SD. Data analysis was carried out by using one-way ANOVA and Tukey's multiple comparison tests as the *post hoc* (SPSS 11.5 for windows software). Differences were considered significant at $p < 0.05$.

3. Results and Discussion

As shown in table 1, rats of both groups had statistically the same body weight at the end of the experiment. At the end of the second week of the experiment, serum cholesterol level of rats received a high cholesterol diet increased to more than 4 times the control group. At the end of the experiment, hypercholesterolemia was still present in rats of group 2, although it was milder than the second week (table 1). No significant differences were observed in serum PINP and CTX levels between two groups (table 2). Representative slides for determination of histomorphometric parameters are shown in figure 1.

Histomorphometric parameters as well as bone mineral density of proximal epiphysis and metaphysis of tibiae of rats in both groups were statistically the same (table 3).

In 2011 Patsch *et al.* reported that mice can properly show bone loss due to short term consumption of high fat diets [8]. In contrary, findings of the present study clearly demonstrate that growing female Sprague-Dawley rats are resistant to bone loss due to short term dietary induced hypercholesterolemia.

Table 1. Serum cholesterol levels (mean±SD) of rats at the end of the second and 6th week of the experiment.

	serum cholesterol (mg/dl)		**Body weight (g)**	
	end of second week	end of 6th week	end of second week	end of 6th week
Group1	61±14	70±13	140±11	183±15
Group 2	255±81*	112±22*	153±13	193±9

Rats in group 1served as control while rats in group 2 received high cholesterol diet.Asterisk sign is used to demonstrate significant difference with control group ($p<0.05$).

Table 2. Serum levels of biochemical markers of bone metabolism (mean±SD).

	PINP (ng/ml)	CTX (ng/ml)
Group1	290±53	295±48
Group 2	301±54	238±57

Rats in group 1served as control while rats in group 2 received high cholesterol diet.

No significant difference was observed between the two groups.

Within the same species, our results regarding the capability of rats to be used as an animal model for hypercholesterolemia-induced bone loss is inconsistent with the findings of You et al., 2011 [12], which to our knowledge was the only published record related to this area. These researchers have reported a reduction in BMD of femur (but not lumbar vertebrae) and serum osteocalcin accompanied by an increase in serum CTX level that none of them was observed in our study. To describe this controversy, it is very important to mention that the rate of bone loss in male and female rats is highly dependent on the method used to induce osteoporosis, the site evaluated and whether this loss concerns cancellous or cortical bone [16]. You et al., [12] have used skeletally immature female Sprague-Dawley rats which is common with our study; however, the cholesterol and fat content of the diet that has been used (3% cholesterol and 20% lard during the whole study period) is remarkably higher than what fed to rats in the present study. Another aspect is that You et al., [12] have evaluated bone mineral density of femur and lumbar vertebrae which was replaced by proximal tibiae in our study. Longer duration of the study performed by You et al., [12] (twice the period of our study), can have a critical role in acceleration of bone resorption and reduction of bone formation which was reflected in the serum

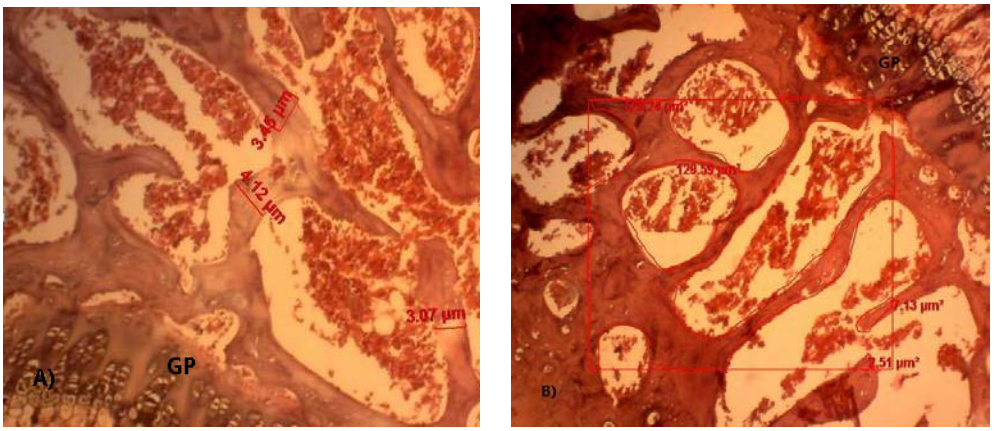

Figure 1. Photomicrographs of tibial epiphysis (mag. ×10). A) Determination of width of 3 trabeculae and B) determination of bone area/tissue area. The sum of the trabecular areas was divided to the area of a postulated rectangle. GP: growth plate.

Table 3. Histomorphometric parameters and bone mineral density of proximal epiphysis and metaphysis of tibiae (mean±SD).

	epiphyseal trabecular width (µm)	metaphyseal trabecular width (µm)	Epiphyseal bone area/tissue area	Epiphyseal mineral density (mm Al)	metaphyseal mineral density (mm Al)
Group 1	3 ±0.7	3.3±0.3	19±4	2.2±0.1	2.3±0.1
Group 2	3.2±0.6	2.6±0.6	23±9	1.9±0.4	2.2±0.4

Rats in group 1served as control while rats in group 2 received high cholesterol diet.

No significant difference was observed between the two groups.

levels of biochemical markers of bone metabolism. Therefore it seems that 6 weeks may not be a sufficient time for a change in bone metabolism of rats due to high cholesterol diets.

Despite the higher fat content of the diet used by You et al., [12] the severity of hypercholesterolemia at the end of the both studies is roughly the same (about 1.6 times the control level). This can be simply described by the lack of cholic acid in the diet used by You et al [12]. An interesting fact is that rats in the study performed by You et al., [12] had become obese, which was not true for our study. In 2012, Pelton et al., [9] reported that feeding a high fat/high cholesterol diet which was isocoloric with a low fat/no cholesterol diet (as control) to mice can result in hypercholesterolemia and osteoporosis without appreciably affecting body weight. They concluded that hypercholesterolemia itself (without accompanied obesity) can contribute to development of osteoporosis in mice, a fact that may not be applicable to rats with regard to the results of our study and the one performed by You et al., [12] although this needs to be further examined in future studies especially by considering the importance of duration of the experiment.

4. Conclusion

As a conclusion, although this was a preliminary study with relatively low sample size, it seems that in contrary to mice, growing female Sprague-Dawley rats do not show bone loss due to short term consumption of high cholesterol diets and therefore may not be an appropriate model in this regard.

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References

[1] Parhami F, Demer LL. Arterial calcification in face of osteoporosis in ageing: can we blame oxidized lipids? *Curr. Opin. Lipidol.* (1997) 8(5): 312-314.

[2] Lupattelli G, Scarponi AM, Vaudo G, Siepi D, Roscini AR, Gemelli F, Pirro M, et al. Simvastatin increases bone mineral density in hypercholesterolemic postmenopausal women. *Metabolism* (2004) 53(6):744–748.

[3] Schoofs MW, Sturkenboom MC, Van der Klift M, Hofman A, Pols HA, Stricker BH. HMG-CoA reductase inhibitors and the risk of vertebral fracture. *J. Bone Miner. Res.* (2004) 19(9):1525–1530.

[4] Bakhireva LN, Shainline MR, Carter S, Robinson S, Beaton SJ, Nawarskas JJ, Gunter MJ. Synergistic effect of statins and postmenopausal hormone therapy in the prevention of skeletal fractures in elderly women. *Pharmacotherapy* (2010) 30(9): 879–887.

[5] Nuzzo V, de Milita AM, Ferraro T, Monaco A, Florio E, Miano P, Montemarano E, et al. Analysis of skeletal status by quantitative ultrasonometry in a cohort of postmenopausal women with high blood cholesterol without documented osteoporosis. *Ultrasound Med. Biol.* (2009) 35(5): 717–722.

[6] Tarakida A, Iino K, Abe K, Taniguchi R, Higuchi T, Mizunuma H, Nakaji S. Hypercholesterolemia accelerates bone loss in postmenopausal women. *Climacteric* (2011) 14(1): 105–111.

[7] Cao JJ, Gregoire BR, Gao H. High-fat diet decreases cancellous bone mass but has no effect on cortical bone mass in the tibia in mice. *Bone* (2009) 44(6): 1097–1104.

[8] Patsch JM, Kiefer FW, Varga P, Pail P, Rauner M, Stupphann D, Resch H, et al. Increased bone resorption and impaired bone microarchitecture in short-term and extended high-fat diet-induced obesity. *Metabolism* (2011) 60(2): 243-249.

[9] Pelton K, Krieder J, Joiner D, Freeman MR, Goldstein SA, Solomon KR. Hypercholesterolemia promotes an osteoporotic phenotype. *Am. J. Pathol.* (2012) 181(3):928-936.

[10] Lelovas PP, Xanthos TT, Thoma SE, Lyritis GP, Dontas IA. The laboratory rat as an animal model for osteoporosis research. *Comp. Med.* (2008) 58(5):424-430.

[11] Jiang Y, Zhao J, Liao EY, Dai RC, Wu XP, Genant HK. Application of μCT-assessment of 3D bone microstructure in preclinical and clinical studies. *J. Bone Miner. Metab.* (2005) 23:122–131.

[12] You L, Sheng ZY, Tang CL, Chen L, Pan L, Chen JY. High cholesterol diet increases osteoporosis risk via inhibiting bone formation in rats. *Acta Pharmacol. Sin.* (2011) 32(12):1498-1504.

[13] Kiernan JA. *Histological and Histochemical Methods: Theory and Practice*, 4th ed., Scion Publishing Ltd: London (2008).

[14] Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J. Bone Miner. Res.* (1987) 2(6): 595–610.

[15] Haidekker MA, Stevens HY, Frangos JA. Computerized methods for X-ray-based small bone densitometry. *Comput. Methods Programs. Biomed.* (2004) 73(1): 35-42.

[16] Jee WSS, Yao W. Overview: animal models of osteopenia and osteoporosis. *J. Musculoskelet. Neuronal Interact.* (2001) 1(3):193–207.

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