Differential Bone Metabolism and Protein Expression in Mice Fed a High-Fat diet Versus Pre-Hibernation Fattening in Daurian Ground Squirrels: A Comparison Between Pathological and Healthy Obesity

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Research

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Abstract

Background

This study compared effects on bone metabolism and morphology of pathological obesity induced by excessive fat intake in a non-hibernator (mice) versus healthy obesity due to pre-hibernation fattening in a hibernator (ground squirrels).

Methods

Kunming mice were fed a high-fat diet for 3 months to provide a model of pathological obesity (OB group). Daurian ground squirrels fattened naturally in their pre-hibernation season (PRE group) were used as a healthy obesity model. Body weight and adipose tissue wet weight were measured. Micro-computed tomography and three-point bending tests were used to determine the microstructure and mechanical properties of bone. Western blots were used to analyze protein expression levels related to bone formation (RunX2, OCN, ALP), bone resorption (RANKL, Cathepsin K, MMP9) and Wnt signaling (P- β -catenin, GSK-3 β).

Results

Micro-CT showed that there was no obvious bone loss in the OB group, compared with controls, whereas bone formation in the PRE group was enhanced, compared to summer-active squirrels. Results of three-point bending tests showed that stiffness of the femur in OB mice was significantly enhanced, whereas the mechanical properties of bone in the PRE group did not change. Analysis of bone protein expression showed significantly increased expression levels of ALP, OCN, RANKL, MMP9, Cathepsin K, GSK-3 β and P- β -catenin in OB mice, but RunX2 expression did not change. By contrast, PRE ground squirrels, showed significantly increased expression of most proteins except for OCN that decreased significantly, and P- β -catenin that did not change.

Conclusion

For non-hibernating mice, moderate obesity had a certain protective effect on bones, demonstrating two-way regulation, enhancing both bone loss and bone formation, so that bone metabolism was at a higher level of metabolic balance. For pre-hibernating ground squirrels, the healthy obesity acquired before hibernation had a positive effect on the microstructure of bones and also enhanced the expression levels of proteins related to bone formation, bone resorption and Wnt signaling.

1. Background

Human obesity has become a major epidemic around the world[1]. Obesity caused by excessive fat intake causes a variety of disease states, including bone structure destruction and bone loss[2, 3]. Previous studies with model species have shown that nutritional obesity will lead to massive bone loss, decreased size-independent mechanical properties, destruction of bone microstructure, abnormal bone metabolism and so on[4–7]. For example, the hind limb bones of male 6-week-old C57BL/6J mice (*Mus musculus*) fed a high-fat diet for 17 weeks showed a decrease in bone mass, bone density and bone strength[8]. In both 3 week-old and 15-week-old male C57BL/6 mice fed a high-fat diet for 16 weeks, obesity was accompanied by massive bone loss and bone microstructure destruction[9]. In addition, nutritional obesity may also lead to a decrease of mechanical properties. Studies showed that male C57BL/6 mice fed a high-fat diet for 12 weeks had reduced femoral mineral density and bone ductility[10]. A similar study with male C57BL/6 mice fed a high-fat diet also showed a decrease in bone mineral density[11]. Other studies confirm that nutritional obesity induced by a high-fat diet usually inhibits bone formation, but bone resorption was enhanced or unchanged[12]. However, the specific mechanisms of this bone loss are not yet fully understood. Therefore, an in-depth understanding of the effects of obesity on the skeletal system may help prevent obesity-induced bone loss.

Different from nutritional obesity, a natural model of fattening is the seasonal acquisition of huge fat reserves by some mammals prior to winter hibernation. Fat storage begins weeks before hibernation, with a period of hyperphagia that greatly increases body mass[13]. For example, Arctic ground squirrels (*Spermophilus parryii*) and marmots (*Marmota flaviventris*) often gain approximately double their body mass[14, 15]. However, this kind of obesity is not accompanied by harmful diseases, such as type 2 diabetes, hyperglycemia and hyperlipidemia that are common in human obesity[16]. Therefore, the obesity caused by fattening before hibernation is called healthy obesity[17]. Although the weight gain of these hibernating animals is much greater than that of nutritionally obese animals, they do not experience osteoporosis, and their bone strength and bone microstructure do not change significantly[18].

Bone metabolism is regulated by bone formation and bone resorption[19]. Osteoblasts are the main cells involved in bone formation[20]. The differentiation of osteoblasts is regulated by a variety of factors, including Runt-related transcription factor 2 (RunX2), osteocalcin (OCN), and bone-derived alkaline phosphatase (ALP)[21]. RunX2 is a crucial transcription factor for osteoblast differentiation and plays a vital role in bone development[22, 23]. RunX2 not only induces the activation of the OCN promoter to accelerate bone mineralization, but also interacts with Wnt signaling to promote osteoblast differentiation[24, 25]. When the body is extremely obese, RunX2 is down-regulated. Studies have shown that the RunX2 mRNA levels decreased in 5-week-old male Sprague Dawley (SD) rats (*Rattus norvegicus*) fed with a high-fat diet for 12 weeks[26]. OCN is one of the signs of bone formation and can promote bone mineralization[27]. Studies have shown that the expression of OCN decreased in Col1a1^{Jrt/+} mice fed a high-fat diet for 26 weeks[28]. In addition, ALP is a key enzyme marker of bone formation due to its action in regulating the process of biomineralization[29]. Previous studies have shown that the expression level of ALP decreased in 6-week-old male C57BL/6 mice fed a high-fat diet for 6 months[30].

Therefore, the expression levels of bone formation marker proteins (RunX2, OCN and ALP) can be useful markers that can reflect the status of bone formation metabolism.

Osteoclasts play an important role in bone resorption[21]. Nuclear factor kappa B receptor activator ligand (RANKL), is a membrane-bound protein present in osteoclasts and bone cells and is a receptor activator necessary for the differentiation of osteoclast precursors into osteoclasts[31]. Studies have shown that the expression level of RANKL was up-regulated in the left femurs of 5-week-old male Wistar rats fed a high-fat diet for 6 weeks after 4 weeks of caloric restriction[32]. Cathepsin K is also necessary for normal bone resorption. It can degrade the tissue matrix and directly regulate the expression of osteoclast bone resorption factors, including cytokines, hormones and nuclear transcription factors[33]. Previous studies have shown that Cathepsin K was up-regulated in 3-week-old female Wistar rats fed a high-fat diet for 5 weeks[34]. Metalloproteinases (MMP) are a protein family composed of zinc-dependent endopeptidases that regulate tissue remodeling under physiological and pathological conditions[35]. Among them, MMP9, also known as gelatinase B or 92-kDa type IV collagenase, is responsible for degrading the extracellular matrix[35]. The up-regulation of MMP9 may trigger or aggravate the hydrolysis of proteins in the bone matrix and promote bone resorption. Under obesity and hyperglycemia, the expression level of MMP9 is significantly increased[36]. Therefore, the expression levels of bone loss-related proteins (RANKL, Cathepsin K and MMP9) reflect bone loss metabolism.

In recent years, more and more studies have shown that bone formation and bone resorption are regulated by Wnt signaling[37]. Wnt/ β -catenin signaling plays an important role in the development and functional regulation of osteoblasts[38], activation of the Wnt pathway promoting the proliferation and differentiation of osteoblasts[39]. When Wnt/ β -catenin signal transduction is disturbed, a variety of bone diseases such as osteoporosis can occur[40]. Studies have shown that decreased activity of Wnt/ β -catenin signaling leads to decreased bone formation in male rats fed a high-fat diet for 4 weeks[41]. When the Wnt ligand is missing, glycogen synthase kinase 3 β (GSK-3 β) phosphorylates β -catenin, leading to a rapid degradation of P- β -catenin by the proteasome[42]. After the Wnt receptor is activated, GSK-3 β is inhibited, leading to accumulation of non-phosphorylated β -catenin and activating target genes involved in regulating the proliferation and differentiation of bone marrow stromal cells[43]. It has been shown that β -catenin deletion inhibits osteoblast differentiation[44]. In addition, Wnt signaling can indirectly affect the function of osteoclasts by regulating the expression of bone resorption-related proteins[45]. Wnt signaling causes less bone resorption by down-regulating RANKL in the osteoclasts[46]. However, research on Wnt signaling in the fattening stage before hibernation is currently imperfect.

Based on our previous research, we chose to compare a healthy obesity model, the Daurian ground squirrel (*Spermophilus dauricus*) that naturally fattens before hibernation but does not show associated muscle atrophy, with an obesity mouse model induced by a high-fat diet that does show atrophy[13]. We aimed to determine if the skeletal system of pre-hibernation ground squirrels has a special mechanism to avoid the bone loss that occurs in the high-fat obesity model of mice. Little is known to date about the changes in bone of fattening animals before hibernation, and the related mechanisms are not clear. Therefore, an in-depth understanding of the bone state in the fattening stage before hibernation and a

comparison of the differential regulatory mechanisms of pathological obesity versus healthy obesity in bone formation and bone resorption are of significance for gaining a greater understanding of the mechanisms that can prevent bone loss. We propose the following hypothesis: there are differences in bone metabolism between the two types of obesity models, which are partly achieved by regulating the expression levels of proteins related to bone formation, bone resorption, and Wnt signaling. We used Kunming mice fed a high-fat diet for 3 months as a pathological model for nutritional obesity and Daurian ground squirrels fattened before hibernation as a healthy obesity model. Hind limb bones were used to compare bone microstructure, mechanical properties, expression levels of bone formation-related proteins (RunX2, OCN and ALP), bone resorption-related proteins (RANKL, Cathepsin K and MMP9) and Wnt signaling proteins (P- β -catenin and GSK-3 β). The data further clarifies the differential regulation and mechanisms of bone remodeling occurring between models of pathological obesity and healthy obesity.

2. Experimental Materials And Methods

2.1. Experimental animals

2.1.1 Animal model of pathological obesity

All animal experiments were approved by the Experimental Animal Protection Committee of the Ministry of Health of the People's Republic of China. Four-week-old male Kunming mice were purchased from Chengdu Dashuo Experimental Animal Company. The initial weight of the mice was 23 to 25 grams. The mice were kept in plastic cages in the animal room and provided with food and water *ad libitum*. The animal room was maintained at a temperature range of 18–25°C, and lighting was changed daily to coincide with local sunrise and sunset. After a week of normal diet feeding, the mice were randomly divided into two groups (n = 6): CON: Control mice fed a normal diet for 3 months; and OB: Obesity mice fed a high-fat diet for 3 months. The normal feed and high-fat diet feed were purchased from Chengdu Dashuo Experimental Animal Company and the compositions of both are shown in Table 1. After the 3 months dietary intervention, all mice were sacrificed.

Table 1
A. Composition of normal diet for mice

Components	Content
Corn	25.4 g
Wheat	30.6 g
Soybean	13 g
Fish meal	6 g
Rice bran	6 g
Wheat bran	10 g
Soybean meal	5 g
Other	4 g
Total	100g

Table 1
B. Composition of high fat diet for mice

Components	Content
Patterned animal base	50 g
Soy flour	5 g
Fish meal	5 g
Milk powder	10 g
Peanut	6 g
Egg yolk powder	5 g
Lard	12 g
Salt	2 g
Sucrose	5 g
Total	100 g
Fat calorie percentage	61%

2.1.2 Animal model of healthy obesity

Ground squirrels were obtained as previously described by our laboratory[47]. Briefly, twelve Daurian ground squirrels of both sexes were caught from the Weinan region in Shaanxi Province of China. Ground

squirrels were kept in plastic cages in the animal room and provided with food and water *ad libitum*. The animal room was maintained at a temperature range of 18–25°C, and lighting was changed daily to coincide with local sunrise and sunset. Ground squirrels were divided into two groups (n = 6): summer active (SA) controls that were captured and sacrificed at the end of June, and the pre-hibernation (PRE) group that were captured and sacrificed at the end of September, after natural fattening. Both groups of ground squirrels acquired natural foods before being caught. After returning to the lab, they were fed rat chow with the same composition as the normal diet in Table 1A. In order to ensure the nutritional requirements during the fattening period, appropriate amounts of high-fat and high-protein nuts (such as peanuts) were added to the PRE ground squirrels.

2.2. Sample collection

After body weight was recorded, mice were anesthetized with 2 mg/kg sodium pentobarbital intraperitoneally, and ground squirrels with a 90 mg/kg dose. Then, the femurs and tibias from both legs were carefully dissected free of associated connective tissue, weighed quickly, and immediately placed in sealed containers with lactated 70% absolute ethanol, followed by freezing in liquid nitrogen and storage at -70°C. We also sampled adipose tissues, including mesenteric adipose, perirenal adipose and back scapula subcutaneous adipose. Similarly, adipose tissues were weighed, then frozen in liquid nitrogen and storage at -70°C. Animals were euthanized by an overdose injection of sodium pentobarbital after sampling.

2.3. Biomechanical testing

Mechanical properties of the femurs were determined by loading the left femurs to failure in a 3-point bending test, exactly as described previously with female mice[48]. Three parameters – ultimate bearing capacity, stiffness and ultimate bending energy – were determined as described previously[49]. Stiffness was defined as the slope of the linear region of the pre-yield load displacement curve, and the yield point was defined as the point where the load-displacement curve intersected with a regression line that was 10% lower than that used to define stiffness. Maximum load was defined as the load at which the bone catastrophically failed.

2.4 Micro-computed tomography (micro-CT)

The right femurs and tibias of mice and ground squirrels were scanned using a micro-CT scanner (L-SP, GE, USA). Then the images were analyzed by GEHE Microview V2.1.2 (GE, USA), and images with voxel size of 43.305 µm were obtained. Next, a region of interest (ROI) at the longitudinal plane was then analyzed; this was the coronal central region (1-mm thick) of the distal femur and proximal tibia. A top-down tomographic scan was performed starting at 3% of the bone length, 0.5 mm at a time, and cycled three times. The chosen ROI of trabecular and cortical bones was analyzed for the following parameters: bone surface density (BS/BV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), bone volume fraction (BV/TV), average cortical thickness (Ct.Th), marrow area (Ma.Ar), cortical bone area (Ct.Ar), the periosteal envelope (Tt.Ar), tissue mineral density (TMD), tissue mineral content (TMC), bone mineral density (BMD) and bone mineral content (BMC)[50].

2.5 Western blots

Methods were those described previously by our lab[51]. Briefly, total protein was extracted from frozen femurs of mice and ground squirrels by homogenization and put into a sample buffer (pH 6.8, 100 mM Tris, 4% SDS, 5% glycerol, 5% 2- β -mercaptoethanol, and bromophenol blue). Then, the bone protein extracts were separated by SDS-PAGE (10% Laemmli gels with an acrylamide/bisacrylamide ratio of 29:1 for ALP, P- β -catenin, Cathepsin K, GSK-3 β , MMP9, OCN, RANKL and RunX2). After electrophoresis, total proteins bands were visualized by putting the gel on the UV transilluminator and irradiating the gel for 2 min, and a Syngene G:BOX system (Syngene, Frederick, MD) was used to take photographs of the gel. Proteins in the gels were then transferred to PVDF membranes (0.45 μ m) using a Bio-Rad semi-dry transfer apparatus. Membranes were blocked with 5% skim milk dissolved in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) for 2 h at room temperature and then incubated with a primary antibody. Antibodies (diluted 1:1000 v:v before use) were ALP (Abcam, 81283), P- β -catenin (Sigma, 4504476), Cathepsin K (Cell Signaling Technology (CST), 9205S), GSK-3 β (CST, 2855S), MMP9 (CST, 2556S), OCN (CST, 2539S), RANKL (CST, 4146S) and RunX2 (Sigma, T6277). Membranes were incubated at 4°C overnight in TBST containing 0.1% bovine serum albumin (BSA) and the chosen antibody. Then membranes were washed with TBST for 4 x 10 min each followed by incubation with HRP-conjugated anti-mouse secondary antibody (1:10000, Thermo Fisher Scientific, A28177) or HRP-conjugated anti-rabbit secondary antibody (1:5000, Thermo Fisher Scientific, A27036) for 2 h at room temperature. Then each PVDF membrane was washed for 3 x 10 min followed by visualizing bands using enhanced chemiluminescence reagents (Thermo Fisher Scientific, NCI5079). NIH Image J software was used to carry out quantification analysis. The density of the target immunoblot band in each lane was standardized against the summed densities from a group of total protein bands in the same lane that were well separated from the band of interest and present in all lanes.

2.6 Statistical analyses

An independent-samples *t*-test was used to determine the significant differences between the OB and the CON mice, or the PRE and the SA ground squirrels. All data were analyzed using SPSS 24 and expressed as means $\pm$ SD. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Body weight

The composition of the normal and high fat diets fed to mice is shown in Table 1. The fat calorie percentage of the high fat diet was 61%. Table 2 shows that after feeding with high-fat diet versus normal diet for 3 months, the OB group had gained 10.6% higher body weight than the CON group (OB: 52.0 ± 0.7 g vs. CON: 47.0 ± 0.8 g, $P < 0.05$). In ground squirrels, the body weight of the PRE group was also significantly higher by 62.5% as compared with the SA group (PRE: 347.7 ± 10.8 g, vs. SA: 214.0 ± 6.4 g, $P < 0.05$), a mean rise of 134 g per animal over the pre-hibernation fattening period.

Table 2
Body weight for all groups

	CON	OB	SA	PRE
Body weight before high fat-fed (g)	24.2 ± 0.3	24.5 ± 0.2	—	—
Body weight at experiment time (g)	47.0 ± 0.8	52.0 ± 0.7 *	214.0 ± 6.4	347.7 ± 10.8 #
CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30–40 d before hibernation). Values are mean ± SD, <i>n</i> = 6. * <i>P</i> < 0.05 compared with CON group. # <i>P</i> < 0.05 compared with SA group.				

3.2 Adipose tissue wet weight

As shown in Table 3, compared with the CON group of mice, the mesenteric adipose wet weight in the OB group was significantly increased by 2.3-fold from 0.89 g to 2.01 g (*P* < 0.05), whereas the perirenal adipose wet weight increased significantly by 2.3-fold from 0.28 g to 0.63 g (*P* < 0.05) in mice. Compared with the SA group of ground squirrels, the mesenteric adipose wet weight in the PRE group was significantly increased by 22.5-fold from 0.87 g to 19.54 g (*P* < 0.05), the perirenal adipose wet weight also increased significantly by 8.2-fold from 0.39 g to 3.21 g (*P* < 0.05), and the subcutaneous adipose wet weight rose significantly by 37.9-fold from 0.35 g to 13.25 g (*P* < 0.05) in ground squirrels.

Table 3
Adipose tissue wet weight

	CON	OB	SA	PRE
Mesenteric adipose wet weight (g)	0.89 ± 0.12	2.01 ± 0.12 *	0.87 ± 0.22	19.54 ± 2.57 #
Perirenal adipose wet weight (g)	0.28 ± 0.03	0.63 ± 0.08 *	0.39 ± 0.12	3.21 ± 0.40 #
Subcutaneous adipose wet weight (g)	—	—	0.35 ± 0.17	13.25 ± 0.92 #
CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30–40 d before hibernation). Values are mean ± SD, <i>n</i> = 6. * <i>P</i> < 0.05 compared with CON group. # <i>P</i> < 0.05 compared with SA group.				

3.3 Bone structure and function

3.3.1 Femoral bone structure and function

Representative micro-CT images of distal femur of mouse CON and OB groups and ground squirrel SA and PRE groups are shown in Fig. 1. The images indicate no differences in the structure of the mouse femur between the CON and OB groups and substantial differences in the structure of the ground squirrel femur between the SA and PRE groups. This is backed up by quantification of multiple femoral bone

parameters presented in Table 4. These quantified data show that for the trabecular region of interest (ROI) chosen, the mice showed no significant differences between the CON and the OB groups in any of the trabecular bone parameters measured. By contrast with mice, the PRE group of ground squirrels, as compared with the SA group, showed both greater bone surface density (BS/BV) and trabecular number (Tb.N) values that were significantly higher in the PRE group (by 36.4% and 28.6%, respectively, $P < 0.05$) as compared with SA animals. However, the trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) values were both reduced significantly in the PRE group (by -31.6% and - 24.3%, respectively, $P < 0.05$) as compared with SA squirrels. Finally, the bone volume fraction (BV/TV, the ratio of bone tissue volume to tissue volume) showed no significant differences between the SA and the PRE ground squirrels.

Table 4
Femoral bone structure and function

		CON	OB	SA	PRE
Trabecular bone	BS/BV (mm ² /mm)	22.70 ± 1.42	21.42 ± 2.04	11.03 ± 2.04	15.04 ± 1.45 [#]
	Tb.N (1/mm)	3.26 ± 0.43	3.42 ± 0.20	1.12 ± 0.24	1.44 ± 0.09 [#]
	Tb.Th (mm)	0.09 ± 0.01	0.09 ± 0.01	0.19 ± 0.03	0.13 ± 0.01 [#]
	Tb.Sp (mm)	0.22 ± 0.04	0.20 ± 0.02	0.74 ± 0.21	0.56 ± 0.06 [#]
	BV/TV (%)	28.79 ± 4.13	32.13 ± 3.48	20.56 ± 4.29	19.34 ± 3.03
Cortical bone	Ct.Th (mm)	0.07 ± 0.01	0.08 ± 0.01	0.23 ± 0.03	0.11 ± 0.03 [#]
	Ma.Ar (mm ²)	0.20 ± 0.11	0.20 ± 0.11	2.36 ± 0.48	0.27 ± 0.12 [#]
	Ct.Ar (mm ²)	0.12 ± 0.03	0.13 ± 0.05	1.59 ± 0.37	0.18 ± 0.07 [#]
	Tt.Ar (mm ²)	0.32 ± 0.13	0.34 ± 0.16	3.95 ± 0.69	0.45 ± 0.19 [#]
Bone mineral	TMD (mg/cc)	717.91 ± 16.51	804.86 ± 73.18	750.73 ± 51.83	692.35 ± 33.60
	TMC (mg)	1.03 ± 0.15	1.28 ± 0.16	13.06 ± 2.34	2.25 ± 0.42 [#]
	BMD (mg/cc)	275.11 ± 44.33	302.27 ± 14.45	218.62 ± 24.06	190.56 ± 22.39

CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30–40 d before hibernation). Values are mean ± SD, *n* = 6. **P* < 0.05 compared with CON group. [#]*P* < 0.05 compared with SA group.

	CON	OB	SA	PRE
BMC (mg)	1.26 ± 0.17	1.56 ± 0.14 *	17.95 ± 2.74	3.25 ± 0.59 #
CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30–40 d before hibernation). Values are mean ± SD, <i>n</i> = 6. * <i>P</i> < 0.05 compared with CON group. # <i>P</i> < 0.05 compared with SA group.				

Cortical bone quantitative parameters are also presented in Table 4. In mice, there were no significant differences in any of measured parameters between the CON and the OB groups. Among ground squirrels, as compared with the SA group, the PRE group showed significant reductions in average cortical thickness (Ct.Th), marrow area (Ma.Ar), cortical bone area (Ct.Ar) and total area (Tt.Ar) by -52.2%, -88.6%, -88.7% and -88.6%, respectively (*P* < 0.05).

Table 4 also shows an analysis of bone mineral-related data. Compared with the CON group, the tissue mineral density (TMD) and tissue mineral content (TMC) showed rising trends in the OB group (*P* = 0.095 and 0.070, respectively). The bone mineral density (BMD) of the OB group did not change significantly as compared with the CON mice. And bone mineral content (BMC) in the OB mice increased significantly by 1.2-fold. Ground squirrels showed a very different profile. No significant difference in tissue mineral density (TMD) was found between the PRE and SA ground squirrels. However, compared with the SA group, the tissue mineral content (TMC) in the PRE group bone was significantly and strongly reduced by 82.8% (*P* < 0.05). There was no significant difference in bone mineral density (BMD) between the PRE and SA ground squirrels but bone mineral content (BMC) of the PRE group was significantly reduced by 81.9% compared with the SA group (*P* < 0.05).

3.3.2 Tibial bone structure and function

Figure 1 also shows representative micro-CT images of the proximal tibia in the different groups and Table 5 shows quantified parameters for tibial bone. The mouse images (Fig. 1) show no visual differences between the CON and the OB groups. This is backed up by the lack of significant change by any of the five parameters assessed (Table 5). By contrast, in the ground squirrels, of the parameters measured for trabecular bone only bone volume fraction (BV/TV) increased significantly in the PRE group, by 40.4% (*P* < 0.05). However, the Tb.Sp was reduced significantly in the PRE group of ground squirrels (-33.0%, *P* < 0.05) whereas the Tb.N value showed an increasing trend but did not reach significance in the PRE group compared with the SA group (*P* = 0.064).

Table 5
Tibial bone structure and function

		CON	OB	SA	PRE
Trabecular bone	BS/BV (mm ² /mm)	17.72 ± 0.80	17.30 ± 0.31	8.70 ± 1.68	8.43 ± 0.68
	Tb.N (1/mm)	1.77 ± 0.30	1.69 ± 0.09	0.78 ± 0.11	1.07 ± 0.17
	Tb.Th (mm)	0.11 ± 0.004	0.12 ± 0.002	0.24 ± 0.05	0.24 ± 0.02
	Tb.Sp (mm)	0.46 ± 0.11	0.48 ± 0.03	1.06 ± 0.14	0.71 ± 0.13 [#]
	BV/TV (%)	19.93 ± 2.91	19.55 ± 0.7	18.06 ± 1.19	25.35 ± 2.89 [#]
Bone mineral	TMD (mg/cc)	795.79 ± 21.62	829.88 ± 32.51	750.79 ± 76.21	661.59 ± 50.78
	TMC (mg)	0.84 ± 0.04	0.91 ± 0.22	12.27 ± 4.44	13.44 ± 0.67
	BMD (mg/cc)	175.93 ± 34.64	184.72 ± 12.43	165.85 ± 18.03	199.21 ± 8.56 [#]
	BMC (mg)	0.97 ± 0.05	1.05 ± 0.25	15.01 ± 4.48	16.28 ± 0.59
CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30–40 d before hibernation). Values are mean ± SD, <i>n</i> = 6. [#] <i>P</i> < 0.05 compared with SA group.					

Mineral-related data for tibial bone were also analyzed and the results are shown in Table 5. In the OB mice, there were no significant differences in any of the four tibial bone mineral parameters as compared with the CON mice. There was no significant difference in tissue mineral density (TMD) or tissue mineral content (TMC) of the PRE group as compared with the SA group in ground squirrel as well as no change in bone mineral content (BMC) between the PRE and SA group. However, the bone mineral density (BMD) of the PRE group was significantly higher by 20.1% as compared with the SA group of ground squirrels (*P* < 0.05).

3.3.3 Three-point bending test

The results of the three-point bending test of the femurs are shown in **Table 6A**. In mice, neither the ultimate bearing capacity (N) nor the ultimate bending energy (J) differed between CON and OB mice but the stiffness parameter (N/mm) was significantly greater by 32.7% in the OB mice group ($P < 0.05$). Comparable analysis of bone from SA versus PRE ground squirrels found no significant differences in ultimate bearing capacity, stiffness or ultimate bending energy between the two ground squirrel groups.

The results of the three-point bending test for tibia are shown in **Table 6B**. The ultimate bearing capacity, stiffness and the ultimate bending energy were not significantly different between CON and OB groups of mice. The same was true of the comparison between SA and PRE ground squirrels although increasing trends were noted in the PRE group for the ultimate bearing capacity ($P = 0.051$) and stiffness ($P = 0.095$). Similarly, there was no significant difference in ultimate bearing capacity, stiffness and ultimate bending energy between the OB and the CON mice.

Table 6. Three-point bending test

(A) Three-point bending of the femur

	CON	OB	SA	PRE
Ultimate bearing capacity (N)	26.63 ± 0.93	29.00 ± 2.12	59.87 ± 18.21	58.71 ± 11.47
Stiffness (N/mm)	78.14 ± 12.91	103.67 ± 15.45 *	112.77 ± 42.38	82.47 ± 17.07
Ultimate bending energy (J)	0.01 ± 0.0004	0.01 ± 0.003	0.02 ± 0.01	0.02 ± 0.005

(B) Three-point bending of the tibia

	CON	OB	SA	PRE
Ultimate bearing capacity (N)	14.72 ± 1.95	14.96 ± 2.54	43.39 ± 5.87	59.48 ± 7.81
Stiffness (N/mm)	32.55 ± 5.54	37.58 ± 9.85	61.17 ± 17.68	108.27 ± 30.01
Ultimate bending energy (J)	0.004 ± 0.001	0.004 ± 0.0005	0.02 ± 0.007	0.02 ± 0.005

CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn

(end of September, 30-40 d before hibernation). Values are mean $\pm$ SD, $n = 6$. * $P < 0.05$ compared with CON group.

3.4 Relative protein expression levels

We used Western blotting to detect the expression levels of proteins related to bone formation, and the results are shown in Fig. 2. Compared with the CON mice, the expression level of RunX2 was not significantly different in the OB group (Fig. 2A, B). However, the expression levels of OCN and ALP showed small but significant increases between the OB and the CON mice (20.2% and 21.9%, respectively, $P < 0.05$) (Fig. 2A, C, D). By contrast, the expression level of RunX2 in PRE ground squirrels was significantly higher than in the SA group (27.2%, $P < 0.05$) (Fig. 2A, B), whereas the expression of OCN decreased in the PRE ground squirrel group (-38.4%, $P < 0.05$) (Fig. 2A, C). ALP expression level increased significantly between the SA and the PRE ground squirrels (26.0%, $P < 0.05$) (Fig. 2A, D).

The expression levels of proteins related to bone resorption are shown in Fig. 3. The expression levels of RANKL, Cathepsin K and MMP9 were significantly increased in OB mice as compared with the CON group by 45.3%, 27.7% and 44.4%, respectively ($P < 0.05$). For ground squirrels, the PRE group also showed significant increases in all three parameters as compared with SA group (37.9%, 2.0-fold and 40.0%, respectively, $P < 0.05$).

Proteins associated with Wnt signaling were also assessed by Western blots (Fig. 4). Compared with the CON group, the expression levels of P- β -catenin and GSK-3 β in the OB group were significantly increased by 20.2% and 38.8%, respectively ($P < 0.05$). The expression level of GSK-3 β was also significantly increased in the PRE group compared with the SA group (2.4-fold, $P < 0.05$), but P- β -catenin did not change between SA and PRE ground squirrels.

4. Discussion

In this study, we innovatively compared the differences in bone metabolism between pathological obesity (in mice) induced by a high-fat diet and healthy obesity (in ground squirrels) induced by natural fattening before hibernation. We measured body weight, adipose tissue wet weight, bone microstructure, bone mechanical properties, and protein expression levels related to bone formation, bone resorption and Wnt signaling. The results showed that obese mice (high-fat diet) showed no obvious alterations or abnormalities in bone microstructure as compared with controls, but bone strength increased and the expression levels of proteins related to bone formation and bone loss increased and maintained a dynamic balance. By contrast, bone formation was enhanced in pre-hibernation ground squirrels, which was manifested as an enhancement of bone microstructure, improvement of bone strength, increased expression levels of bone formation-related proteins (RunX2 and ALP), increased expression levels of bone resorption-related proteins, and enhanced Wnt signaling.

The body weight and adipose tissue wet weight of OB mice and PRE ground squirrels were significantly increased compared with CON mice and SA ground squirrels, respectively (Table 2 and Table 3). After being fed a high-fat diet, the body weight of mice in the OB group increased significantly by 10.6% compared with the CON group. However, after natural fattening, the body weight of ground squirrels in the PRE group increased much more with a significant 62.5% increase over the SA group (Table 2). This indicated that the degree of obesity in pre-hibernating ground squirrels was much greater than in mice. At the same time, our data showed that after fattening, the change in adipose tissue of mice occurred in perirenal and mesenteric adipose, whereas in ground squirrels, adipose accumulation was mainly in mesenteric and subcutaneous depots with lesser accumulation of perirenal adipose. There is so little subcutaneous adipose in mice that it cannot be easily separated by surgical procedures. In fact, we can't deny that subcutaneous adipose is always present and may be extracted by other methods. Previous studies have shown an inverse relationship between visceral fat and bone density[52]. Therefore, compared with mice, the characteristics of less visceral fat exhibited by ground squirrels may be related to the increase in bone density.

There was no significant change in the microstructure of the femur or tibia in the OB group, but the bone mineral parameters BMC of mice showed a significant increase, TMD and TMC showed an increasing trend ($P=0.095$ and 0.070 , respectively) in femur after fattening (Table 4). This indicated that the bone microstructure of mice was in a balanced state, but bone minerals had a tendency to increase in femur. By contrast, ground squirrels showed some substantial differences between SA and PRE states. The BS/BV and Tb.N parameters of the PRE group were significantly increased by 36.4% and 28.6%, respectively, whereas Tb.Th, Tb.Sp, Ma.Ar, TMC and BMC were all strongly reduced by 31.6%, 24.3%, 88.6%, 82.8% and 81.9%, respectively, indicating that the bone formation of the femur in the PRE group was enhanced (Table 4). The microstructural changes of the ground squirrel tibia was similar to those of the femur. The Tb.N showed an increasing trend ($P=0.064$), Tb.Sp was significantly decreased (-33.0%, $P<0.05$), and BV/TV and BMD were significantly increased (40.4% and 20.1%, respectively, $P<0.05$), which indicated that the bone formation was enhanced in the tibia (Table 5). In conclusion, there was no tissue specificity in bone formation on the femur and tibia between the mice and ground squirrels. This is consistent with another study, which shows that obese Wistar rats induced by high-fat diet have no difference in bone formation between femur and tibia[53]. Compared with the mice, the bone mass in the PRE group increased in both femur and tibia of ground squirrels, which showed that healthy obesity was not harmful to their bones. However, interestingly, the bone minerals of the femurs in the two models showed opposite changes, an increase in the OB mice and a decrease in the PRE ground squirrels. The mineral density is related to the mechanical properties of bones and therefore, we postulated that these two types of obesity have opposite effects on the mechanical properties of bones. Hence, we next determined the mechanical properties of bones from the two obesity models.

For this study, we recorded changes in the mechanical properties of the bones of mice fed the high-fat diet for 3 months and of ground squirrels fattened before hibernation. Using a three-point bending test, the stiffness of the femur in the OB group was significantly increased by 32.7%, but the mechanical properties of the tibia did not change significantly (**Table 6**). This indicated that the increased bending

resistance of the OB mice was mainly manifested in the femur, thereby reducing the risk of fracture. This may be an adaptation to the higher load caused by weight gain. Compared with the SA ground squirrels, the bones of the PRE group also showed different mechanical properties. The ultimate bearing capacity, stiffness and ultimate bending energy of the femur in the PRE group did not change significantly compared with SA group (**Table 6**), which indicated that the mechanical properties of the femur did not change during the approach to the hibernation season. However, the ultimate bearing capacity and stiffness of the tibia in the PRE group showed an increasing trend ($P = 0.051$ and 0.095 , respectively, **Table 6**), which suggests that an increase in bending resistance in the PRE group was mainly manifested in the tibia, which could also reduce the risk of fracture. The changes of bone microstructure and mechanical characteristics were related to changes in bone remodeling function. Therefore, we examined the expression levels of key proteins that regulate bone formation, bone resorption, and Wnt signaling pathways.

Bone metabolism is maintained by the dynamic balance of bone formation and bone resorption[19]. RunX2 is the main driving factor of bone formation and promotes the differentiation and maturation of osteoblasts[54]. The expression level of RunX2 in the OB mice did not change significantly as compared to CON group (Fig. 2B), which was different a previous study that showed a significant decrease in RunX2 mRNA levels in 4-week-old male rats fed with a high-fat diet for 22 weeks[55]. By contrast, the expression level of RunX2 in PRE ground squirrels was significantly up-regulated compared with SA group, indicating that bone formation in the PRE group was enhanced. The differential expression of RunX2 may be the reason for the different changes in bone microstructure between the PRE ground squirrels and the OB mice. OCN plays an important role in regulating calcium metabolism of the bone, mainly promoting bone mineralization[56]. In this study, the expression level of OCN in OB mice was up-regulated, whereas the expression level of OCN in PRE ground squirrels was down-regulated (Fig. 2C). This indicates that the OB mice had increased bone mineralization ability, whereas the PRE ground squirrels could be decreasing bone mineralization activity as the hibernation season approaches. In addition, OCN not only plays a role in bone formation, but also affects energy regulation[57] and, hence, the different changes in the expression of OCN in OB mice and PRE ground squirrels may also contribute to differential regulation of energy metabolism. This idea requires further experimental. ALP protein is one of the phenotypic markers of osteoblasts and can directly reflect the activity or function of osteoblasts[58]. The expression level of ALP in both the OB mice and the PRE ground squirrels was significantly increased (Fig. 2D), which indicated that the osteoblasts in both groups were in good activity and function. This is consistent with a previous study on 6-week-old male C57BL/6 mice fed a high-fat diet for 14 weeks, the results showing that the expression level of ALP in obese mice was significantly increased[59]. Studies have shown that ALP can promote the absorption of calcium ions by bones[60]. We speculate that due to insufficient obesity in the high-fat OB model in the present study, only a 10.6% weight gain was achieved compared with the controls. In order to adapt to the higher load caused by moderate obesity, the bones significantly increased the expression level of ALP, thereby promoting the absorption of calcium salts by the bones, increasing the bone minerals, and enhancing the mechanical strength of the bones. This was also in line with the increasing trend of TMD, TMC and BMC obtained by Micro CT in this study. Hence, we propose

that the difference in the expression level of RunX2 was a main reason for the difference in bone formation between the two models. Compared with the OB group, the bone formation in the PRE group was at a higher level.

In terms of bone resorption, the expression levels of RANKL, Cathepsin K and MMP9 increased significantly in both the OB mice and the PRE ground squirrels (Fig. 3). The enhancement of bone resorption in obese mice was consistent with previous studies[34, 61, 62]. We speculated that the reason why bone loss did not occur in the OB mice was that both bone formation and bone resorption were up-regulated to achieve a dynamic balance of high expression. Although there was no bone loss in the OB mice, the high expression levels of bone resorption proteins may be a potential risk for bone loss in mice. Studies have shown that bone loss occurred in mice when they were extremely obese[63]. The expression levels of bone resorption proteins were significantly increased in the PRE group of ground squirrels, but the bone substance was also increased, which may be caused by greater bone formation than bone resorption.

In addition, Wnt signaling also plays an important role in the regulation of bone remodeling[64]. In the present study, the expression levels of P- β -catenin and GSK-3 β in OB mice were significantly increased (38.8% and 20.2%, respectively, Fig. 4), and Wnt signaling was weakened, which could lead to an increase of bone resorption and a decrease of bone formation, which is consistent with a study that showed that obesity inhibited the Wnt signaling pathway[65]. Different responses were seen in ground squirrels, where the expression level of GSK-3 β in the PRE group was significantly increased (1.4-fold, Fig. 4C), but the expression level of P- β -catenin did not change (Fig. 4B), which indicated that the Wnt signal was strengthened and bone formation was enhanced before hibernation[47]. The differential expression of Wnt signals in the two models was also the cause of bone changes. Activation of typical Wnt signaling by inhibiting GSK-3 β has been shown to increase bone mass, which may involve many mechanisms[66]. However, although GSK-3 β inhibitors can promote osteogenesis, we should note that the activity of GSK-3 β is not only manifested in osteogenesis, but is also related to other intracellular biological processes, which has raised concerns about possible side effects of long-term treatment with these inhibitors in humans[67]. In addition, over-inhibition of GSK-3 β has the risk of tumorigenicity[68].

When comparing the two models, we found that weight gain will cause a significant increase in the expression of bone resorption proteins in both the OB mice and the PRE ground squirrels. The bone substance of the mice did not change significantly, which may be caused by an unchanged expression level of RunX2 and the significant increases in the expression levels of OCN and ALP. Although body weight of the OB mice only increased by 10.6% compared with the control group, the weight gain also brought a great risk of bone loss to the OB mice, which was manifested as a significant up-regulation of bone resorption proteins and weakened Wnt signaling. Different from mice, ground squirrels showed different regulatory mechanisms at work. Although the expression levels of bone resorption proteins in the PRE group also increased significantly, the protein expression levels of RunX2, ALP and GSK-3 β increased significantly, resulting in greater bone formation than bone resorption and a net increase in

bone mass. This mechanism, which is different from pathological obesity, suggests that ground squirrels fattened before hibernation can be studied as an anti-obesity bone loss model.

5. Conclusions

In summary, we compared the differences in bone metabolism between high-fat diet fattened mice and naturally fattened ground squirrels. Our study shows that the hind limb bones of mice in the OB group did not undergo bone loss, and the femurs of the ground squirrels in the PRE group developed bone formation. And the results of the three-point bending test showed that the skeletal mechanical properties of the OB mice were strengthened, while the PRE ground squirrels did not change significantly. Western Blot showed that the levels of proteins related to both bone formation and bone loss were up-regulated in the OB mice, and bone metabolism was at a higher level of metabolic balance. For pre-hibernating ground squirrels, the healthy obesity acquired before hibernation also enhanced the expression levels of proteins related to bone formation, bone resorption and Wnt signaling.

6. Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Experimental Animal Protection Committee of the Ministry of Health of the People's Republic of China.

Consent for publication

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HC conceived and designed the experiments. XG, SS, QN, WM, YH, ZH, NA, YY, YZ and HZ performed the experiments. XG, SS and QN analyzed the data. SS, QN and KS wrote the paper. All authors read and approved the final manuscript.

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7. References

1. Busutil R, Espallardo O, Torres A, Martínez-Galdeano L, Zozaya N: **The impact of obesity on health-related quality of life in Spain.** *Health Qual Life Out* 2017, **15**:197.
2. Yan L, Graef GL, Nielsen FH, Johnson LK, Cao J: **Soy protein is beneficial but high-fat diet and voluntary running are detrimental to bone structure in mice.** *Nutr Res* 2015, **35**:523-531.
3. Proietto J: **Obesity and Bone.** *F1000Res* 2020, **9**.
4. Halade GV, El Jamali A, Williams PJ, Fajardo RJ, Fernandes G: **Obesity-mediated inflammatory microenvironment stimulates osteoclastogenesis and bone loss in mice.** *Exp Gerontol* 2011, **46**:43-52.
5. Ionova-Martin SS, Do SH, Barth HD, Szadkowska M, Porter AE, Ager JW, 3rd, Ager JW, Jr., Alliston T, Vaisse C, Ritchie RO: **Reduced size-independent mechanical properties of cortical bone in high-fat diet-induced obesity.** *Bone* 2010, **46**:217-225.
6. Halade GV, Rahman MM, Williams PJ, Fernandes G: **High fat diet-induced animal model of age-associated obesity and osteoporosis.** *J Nutr Biochem* 2010, **21**:1162-1169.
7. Savvidis C, Tournis S, Dede AD: **Obesity and bone metabolism.** *Hormones (Athens)* 2018, **17**:205-217.
8. Cao JJ, Picklo MJ: **N-acetylcysteine supplementation decreases osteoclast differentiation and increases bone mass in mice fed a high-fat diet.** *J Nutr* 2014, **144**:289-296.
9. Ionova-Martin SS, Wade JM, Tang S, Shahnazari M, Ager JW, 3rd, Lane NE, Yao W, Alliston T, Vaisse C, Ritchie RO: **Changes in cortical bone response to high-fat diet from adolescence to adulthood in mice.** *Osteoporos Int* 2011, **22**:2283-2293.
10. Picke AK, Sylow L, Møller LLV, Kjøbsted R, Schmidt FN, Steejn MW, Salbach-Hirsch J, Hofbauer C, Blüher M, Saalbach A, et al: **Differential effects of high-fat diet and exercise training on bone and energy metabolism.** *Bone* 2018, **116**:120-134.
11. Gautam J, Choudhary D, Khedgikar V, Kushwaha P, Singh RS, Singh D, Tiwari S, Trivedi R: **Micro-architectural changes in cancellous bone differ in female and male C57BL/6 mice with high-fat diet-induced low bone mineral density.** *Br J Nutr* 2014, **111**:1811-1821.
12. Shapses SA, Sukumar D: **Bone metabolism in obesity and weight loss.** *Annu Rev Nutr* 2012, **32**:287-309.
13. Yan X, Niu Q, Gao X, Shen S, He N, Wang H, Fang R, Gao Y, Chang H: **Differential protein metabolism and regeneration in gastrocnemius muscles in high-fat diet fed mice and pre-hibernation daurian ground squirrels: a comparison between pathological and healthy obesity.** *Zool Stud* 2021, **60**:e6.

14. Galster W, Morrison P: **Seasonal changes in body composition of the arctic ground squirrel, *Citellus undulatus*.** *Can J Zool* 1976, **54**:74-78.
15. Klingenspor M: **Cold-induced recruitment of brown adipose tissue thermogenesis.** *Exp Physiol* 2003, **88**:141-148.
16. Kamine A, Shimozuru M, Shibata H, Tsubota T: **Changes in blood glucose and insulin responses to intravenous glucose tolerance tests and blood biochemical values in adult female Japanese black bears (*Ursus thibetanus japonicus*).** *Jpn J Vet Res* 2012, **60**:5-13.
17. Rigano KS, Gehring JL, Evans Hutzenbiler BD, Chen AV, Nelson OL, Vella CA, Robbins CT, Jansen HT: **Life in the fat lane: seasonal regulation of insulin sensitivity, food intake, and adipose biology in brown bears.** *J Comp Physiol B* 2017, **187**:649-676.
18. Pineda N, Owen M, Tucker C, Wojda S, Kitchen S, Black H, Donahue S: **Hibernating little pocket mice show few seasonal changes in bone properties.** *Anat Rec* 2017, **300**:2175-2183.
19. Datta HK, Ng WF, Walker JA, Tuck SP, Varanasi SS: **The cell biology of bone metabolism.** *J Clin Pathol* 2008, **61**:577-587.
20. Dallas SL, Prideaux M, Bonewald LF: **The osteocyte: an endocrine cell ... and more.** *Endocr Rev* 2013, **34**:658-690.
21. Lampropoulos CE, Papaioannou I, D'Cruz DP: **Osteoporosis—a risk factor for cardiovascular disease?** *Nat Rev Rheumatol* 2012, **8**:587-598.
22. Komori T: **Signaling networks in RUNX2-dependent bone development.** *J Cell Biochem* 2011, **112**:750-755.
23. Komori T: **Regulation of skeletal development by the Runx family of transcription factors.** *J Cell Biochem* 2005, **95**:445-453.
24. Koch FP, Merkel C, Al-Nawas B, Smeets R, Ziebart T, Walter C, Wagner W: **Zoledronate, ibandronate and clodronate enhance osteoblast differentiation in a dose dependent manner—a quantitative in vitro gene expression analysis of Dlx5, Runx2, OCN, MSX1 and MSX2.** *J Craniomaxillofac Surg* 2011, **39**:562-569.
25. Reinhold MI, Naski MC: **Direct interactions of Runx2 and canonical Wnt signaling induce FGF18.** *J Biol Chem* 2007, **282**:3653-3663.
26. Wang H, Chen F, Li J, Wang Y, Jiang C, Wang Y, Zhang M, Xu J: **Vaspin antagonizes high fat-induced bone loss in rats and promotes osteoblastic differentiation in primary rat osteoblasts through Smad-Runx2 signaling pathway.** *Nutr Metab (Lond)* 2020, **17**:9.
27. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, et al: **Increased bone formation in osteocalcin-deficient mice.** *Nature* 1996, **382**:448-452.
28. Tauer JT, Boraschi-Diaz I, Al Rifai O, Rauch F, Ferron M, Komarova SV: **Male but not female mice with severe osteogenesis imperfecta are partially protected from high-fat diet-induced obesity.** *Mol Genet Metab* 2021.

29. Chen H, Li J, Wang Q: **Associations between bone-alkaline phosphatase and bone mineral density in adults with and without diabetes.** *Medicine (Baltimore)* 2018, **97**:e0432.
30. Cao JJ, Gregoire BR, Michelsen KG, Picklo MJ: **Increasing dietary fish oil reduces adiposity and mitigates bone deterioration in growing C57BL/6 mice fed a high-fat diet.** *J Nutr* 2020, **150**:99-107.
31. Kim MS, Yang YM, Son A, Tian YS, Lee SI, Kang SW, Muallem S, Shin DM: **RANKL-mediated reactive oxygen species pathway that induces long lasting Ca²⁺ oscillations essential for osteoclastogenesis.** *J Biol Chem* 2010, **285**:6913-6921.
32. Liu X, Liang Y, Xia N: **Decrease in leptin mediates rat bone metabolism impairments during high-fat diet-induced catch-up growth by modulating the OPG/RANKL balance.** *3 Biotech* 2021, **11**:103.
33. Novinec M, Lenarčič B: **Cathepsin K: a unique collagenolytic cysteine peptidase.** *Biol Chem* 2013, **394**:1163-1179.
34. Rezende Yanagihara G, Carminati Shimano R, Atsuko Tida J, Suzuki Yamanaka J, Yasuyo Fukada S, Mardegan Issa JP, Shimano AC, Tavares JM: **Influence of high-fat diet on bone tissue: an experimental study in growing rats.** *J Nutr Health Aging* 2017, **21**:1337-1343.
35. Halade GV, Jin YF, Lindsey ML: **Matrix metalloproteinase (MMP)-9: a proximal biomarker for cardiac remodeling and a distal biomarker for inflammation.** *Pharmacol Ther* 2013, **139**:32-40.
36. Chung AW, Hsiang YN, Matzke LA, McManus BM, van Breemen C, Okon EB: **Reduced expression of vascular endothelial growth factor paralleled with the increased angiostatin expression resulting from the upregulated activities of matrix metalloproteinase-2 and -9 in human type 2 diabetic arterial vasculature.** *Circ Res* 2006, **99**:140-148.
37. Yang Y, Mallampati S, Sun B, Zhang J, Kim SB, Lee JS, Gong Y, Cai Z, Sun X: **Wnt pathway contributes to the protection by bone marrow stromal cells of acute lymphoblastic leukemia cells and is a potential therapeutic target.** *Cancer Lett* 2013, **333**:9-17.
38. Hwang JH, Cha PH, Han G, Bach TT, Min do S, Choi KY: **Euodia sutchuenensis Dode extract stimulates osteoblast differentiation via Wnt/ β -catenin pathway activation.** *Exp Mol Med* 2015, **47**:e152.
39. Hoepfner LH, Secreto FJ, Westendorf JJ: **Wnt signaling as a therapeutic target for bone diseases.** *Expert Opin Ther Targets* 2009, **13**:485-496.
40. Feng Y, Wan P, Yin L, Lou X: **The inhibition of microRNA-139-5p promoted osteoporosis of bone marrow-derived mesenchymal stem cells by targeting Wnt/Beta-Catenin signaling pathway by NOTCH1.** *J Microbiol Biotechnol* 2020, **30**:448-458.
41. Chen JR, Lazarenko OP, Wu X, Tong Y, Blackburn ML, Shankar K, Badger TM, Ronis MJ: **Obesity reduces bone density associated with activation of PPAR γ and suppression of Wnt/ β -catenin in rapidly growing male rats.** *PLoS One* 2010, **5**:e13704.
42. Lam AP, Gottardi CJ: **β -catenin signaling: a novel mediator of fibrosis and potential therapeutic target.** *Curr Opin Rheumatol* 2011, **23**:562-567.
43. Moorer MC, Riddle RC: **Regulation of osteoblast metabolism by Wnt signaling.** *Endocrinol Metab* 2018, **33**:318-330.

44. Hill TP, Später D, Taketo MM, Birchmeier W, Hartmann C: **Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes.** *Dev Cell* 2005, **8**:727-738.
45. Glass DA, 2nd, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA, Karsenty G: **Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation.** *Dev Cell* 2005, **8**:751-764.
46. Takahashi N, Maeda K, Ishihara A, Uehara S, Kobayashi Y: **Regulatory mechanism of osteoclastogenesis by RANKL and Wnt signals.** *Front Biosci (Landmark Ed)* 2011, **16**:21-30.
47. Gao X, Wang S, Zhang J, Wang S, Bai F, Liang J, Wu J, Wang H, Gao Y, Chang H: **Differential bone remodeling mechanism in hindlimb unloaded rats and hibernating Daurian ground squirrels: a comparison between artificial and natural disuse.** *J Comp Physiol B* 2021, **191**:793-814.
48. Lloyd SA, Bandstra ER, Willey JS, Riffle SE, Tirado-Lee L, Nelson GA, Pecaut MJ, Bateman TA: **Effect of proton irradiation followed by hindlimb unloading on bone in mature mice: a model of long-duration spaceflight.** *Bone* 2012, **51**:756-764.
49. Lloyd SA, Lang CH, Zhang Y, Paul EM, Laufenberg LJ, Lewis GS, Donahue HJ: **Interdependence of muscle atrophy and bone loss induced by mechanical unloading.** *J Bone Miner Res* 2014, **29**:1118-1130.
50. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR: **Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee.** *J Bone Miner Res* 1987, **2**:595-610.
51. Chang H, Peng X, Yan X, Zhang J, Xu S, Wang H, Wang Z, Ma X, Gao Y: **Autophagy and Akt-mTOR signaling display periodic oscillations during torpor-arousal cycles in oxidative skeletal muscle of Daurian ground squirrels (*Spermophilus dauricus*).** *J Comp Physiol B* 2020, **190**:113-123.
52. Liu CT, Broe KE, Zhou Y, Boyd SK, Cupples LA, Hannan MT, Lim E, McLean RR, Samelson EJ, Bouxsein ML, Kiel DP: **Visceral adipose tissue is associated with bone microarchitecture in the framingham osteoporosis study.** *J Bone Miner Res* 2017, **32**:143-150.
53. Minematsu A, Nishii Y, Sakata S: **High-fat/high-sucrose diet results in higher bone mass in aged rats.** *Bone Rep* 2018, **8**:18-24.
54. Kim JM, Yang YS, Park KH, Ge X, Xu R: **A RUNX2 stabilization pathway mediates physiologic and pathologic bone formation.** 2020, **11**:2289.
55. Chen F, Wang Y, Wang H, Dong Z, Wang Y, Zhang M, Li J, Shao S, Yu C, Huan Z, Xu J: **Flaxseed oil ameliorated high-fat-diet-induced bone loss in rats by promoting osteoblastic function in rat primary osteoblasts.** *Nutr Metab (Lond)* 2019, **16**:71.A
56. Koshihara Y, Hoshi K: **Vitamin K2 enhances osteocalcin accumulation in the extracellular matrix of human osteoblasts in vitro.** *J Bone Miner Res* 1997, **12**:431-438.
57. Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, Dacquin R, Mee PJ, McKee MD, Jung DY, et al: **Endocrine regulation of energy metabolism by the skeleton.** *Cell* 2007, **130**:456-469.
58. Buchet R, Millán JL, Magne D: **Multisystemic functions of alkaline phosphatases.** *Methods Mol Biol* 2013, **1053**:27-51.

59. Vimalraj S: **Alkaline phosphatase: Structure, expression and its function in bone mineralization.** *Gene* 2020, **754**:144855.
60. Bhattarai T, Bhattacharya K, Chaudhuri P, Sengupta P: **Correlation of common biochemical markers for bone turnover, serum calcium, and alkaline phosphatase in post-menopausal women.** *Malays J Med Sci* 2014, **21**:58-61.
61. Zhong L, Yuan J, Huang L, Li S, Deng L: **RANKL is involved in Runx2-triggered hepatic infiltration of macrophages in mice with NAFLD induced by a high-fat diet.** 2020, **2020**:6953421.
62. Fang ZY, Lin R, Yuan BX, Liu Y, Zhang H: **Tanshinone IIA inhibits atherosclerotic plaque formation by down-regulating MMP-2 and MMP-9 expression in rabbits fed a high-fat diet.** *Life Sci* 2007, **81**:1339-1345.
63. Núñez NP, Carpenter CL, Perkins SN, Berrigan D, Jaque SV, Ingles SA, Bernstein L, Forman MR, Barrett JC, Hursting SD: **Extreme obesity reduces bone mineral density: complementary evidence from mice and women.** *Obesity (Silver Spring)* 2007, **15**:1980-1987.
64. Baron R, Kneissel M: **WNT signaling in bone homeostasis and disease: from human mutations to treatments.** *Nat Med* 2013, **19**:179-192.
65. Penrose HM, Heller S, Cable C, Nakhoul H, Baddoo M, Flemington E, Crawford SE, Savkovic SD: **High-fat diet induced leptin and Wnt expression: RNA-sequencing and pathway analysis of mouse colonic tissue and tumors.** *Carcinogenesis* 2017, **38**:302-311.
66. Gu Y, Wang Z, Shi J, Wang L, Hou Z, Guo X, Tao Y, Wu X, Zhou W, Liu Y, et al: **Titanium particle-induced osteogenic inhibition and bone destruction are mediated by the GSK-3 β / β -catenin signal pathway.** *Cell Death Dis* 2017, **8**:e2878.
67. Martin SA, Souder DC, Miller KN, Clark JP, Sagar AK, Eliceiri KW, Puglielli L, Beasley TM, Anderson RM: **GSK3 β regulates brain energy metabolism.** *Cell Rep* 2018, **23**:1922-1931.e1924.
68. Vijay GV, Zhao N, Den Hollander P, Toneff MJ, Joseph R, Pietila M, Taube JH, Sarkar TR, Ramirez-Pena E, Werden SJ, et al: **GSK3 β regulates epithelial-mesenchymal transition and cancer stem cell properties in triple-negative breast cancer.** *Breast Cancer Res* 2019, **21**:37.

Figures

Figure 1

Representative micro-CT images of distal femur and proximal tibia in different groups. CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30-40 d before hibernation)

Figure 2

The protein expression levels related to bone formation (A) Representative immunoblots of RunX2, OCN and ALP in each group. (B) The relative protein expression level of RunX2 in mice and Daurian ground squirrels. (C) The relative protein expression level of OCN in mice and Daurian ground squirrels. (D) The relative protein expression level of ALP in mice and Daurian ground squirrels. CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30-40 d before hibernation). Values are mean $\pm$ SD, n = 6. *P < 0.05 compared with CON group. #P < 0.05 compared with SA group.

Figure 3

The protein expression levels related to bone resorption (A) Representative immunoblots of RANKL, Cathepsin K and MMP9 in each group. (B) The relative protein expression level of RANKL in mice and Daurian ground squirrels. (C) The relative protein expression level of Cathepsin K in mice and Daurian ground squirrels. (D) The relative protein expression level of MMP9 in mice and Daurian ground squirrels. CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30-40 d before hibernation). Values are mean $\pm$ SD, $n = 6$. * $P < 0.05$ compared with CON group. # $P < 0.05$ compared with SA group.

Figure 4

The protein expression levels of Wnt signal (A) Representative immunoblots of P- β -Catenin and GSK-3 β in each group. (B) The relative protein expression level of P- β -Catenin in mice and Daurian ground squirrels. (C) The relative protein expression level of GSK-3 β in mice and Daurian ground squirrels. CON: control mice, OB: obese mice induced by a high-fat diet for 3 months, SA: summer active Daurian ground squirrels, PRE: pre-hibernation, squirrels that finished natural fattening, sacrificed in late-autumn (end of September, 30-40 d before hibernation). Values are mean $\pm$ SD, n = 6. *P < 0.05 compared with CON group. #P < 0.05 compared with SA group.

Figure 5

Pathway diagrams of protein expression levels related to bone formation, bone resorption, and Wnt signaling in two obesity models (A) Changes in protein expression levels in the OB group compared with the CON mice. (B) Changes in protein expression levels in the PRE group compared with the SA ground squirrels. RunX2: Runt-related transcription factor 2; OCN: Osteocalcin; ALP: Alkaline phosphatase; RANKL: Nuclear factor kappa B receptor activator ligand; MMP9: Metalloproteinase-9; GSK-3 β : glycogen synthase kinase3 β .