Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 2022 23(12):1042-1056
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Research Article

https://doi.org/10.1631/jzus.B2100798

Differential bone metabolism and protein expression in mice fed a high-fat diet versus Daurian ground squirrels following natural pre-hibernation fattening

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Abstract: This study compared the 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). Kunming mice were fed a high-fat diet 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. Micro-computed tomography (micro-CT) 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 metabolism (Runt-related transcription factor 2 (RunX2), osteocalcin (OCN), alkaline phosphatase (ALP), osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL), cathepsin K, matrix metallopeptidase 9 (MMP9), patched protein homolog 1 (Ptch1), phosphorylated β-catenin (P-β-catenin), and glycogen synthase kinase-3β (GSK-3β)). Compared with controls, there was no obvious bone loss in the OB mice, and the stiffness of the femur was increased significantly. Compared with summer active squirrels, bone formation was enhanced but the mechanical properties did not change in the PRE group squirrels. In OB mice, western blots showed significantly increased expression levels of all proteins except RunX2, OPG, and Ptch1. PRE ground squirrels showed significantly increased expression of most proteins except OCN and Ptch1, which decreased significantly, and P-β-catenin and OPG, which did not change. In conclusion, for non-hibernating mice, moderate obesity had a certain protective effect on bones, demonstrating two-way regulation, increasing both bone loss and bone formation. 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.

Key words: High-fat diet; Pre-hibernation fattening; Bone formation; Bone loss; Wnt signaling

1 Introduction

Human obesity has become a major epidemic around the world (Busutil et al., 2017). Obesity caused by excessive fat intake causes a variety of disease states, including destruction of bone structure and bone loss (Yan et al., 2015; Proietto, 2020). In humans, some researchers believe that obesity is associated with lower bone mass (Petit et al., 2005; Wosje et al., 2009; Pollock et al., 2011). However, obesity in humans is complicated. Proietto (2020) concluded that obesity is linked to higher bone mass. Studies of obesity in teenagers showed that the obese children and teenagers had higher bone mass than their healthy peers (Leonard et al., 2004; Clark et al., 2006). 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, and abnormal bone metabolism (Halade et al., 2010, 2011; Ionova-Martin et al., 2010; Savvidis

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Received Sept. 15, 2021; Revision accepted May 20, 2022;
Crosschecked Nov. 18, 2022

et al., 2018). For example, the hind limb bones of male 6-week-old C57BL/6 mice (*Mus musculus*) fed a high-fat diet for 17 weeks showed a decrease in bone mass, bone density, and bone strength (Cao and Picklo, 2014). In both 3- 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 (Ionova-Martin et al., 2011). In addition, nutritional obesity may also lead to a decrease in mechanical properties. A previous study showed that male C57BL/6 mice fed a high-fat diet for 12 weeks had reduced femoral mineral density and bone ductility (Picke et al., 2018). A similar study with male C57BL/6 mice fed a high-fat diet also showed a decrease in bone mineral density (BMD) (Gautam et al., 2014). Previous research confirmed that nutritional obesity induced by a high-fat diet usually inhibited bone formation, but bone resorption was increased or unchanged (Shapses and Sukumar, 2012). However, the specific mechanisms of this bone loss are not yet fully understood. Therefore, an in-depth understanding of the effect of obesity on the skeletal system may help prevent obesity-induced bone loss.

Unlike 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 (Yan et al., 2021). 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 (Kamine et al., 2012). Therefore, the obesity caused by fattening before hibernation is called healthy obesity (Rigano et al., 2017). 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 (Pineda et al., 2017).

Bone metabolism is regulated by bone formation and bone resorption (Datta et al., 2008). Osteoblasts are the main cells involved in bone formation (Dallas et al., 2013). 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) (Lampropoulos et al., 2012). RunX2 is a crucial transcription factor for osteoblast differentiation and plays a vital role in bone development (Komori, 2005, 2011). RunX2 not only induces the activation of the OCN promoter to accelerate bone mineralization, but also interacts with Wnt signaling to promote osteoblast differentiation (Reinhold and Naski, 2007; Koch et al., 2011). When the body is extremely obese, RunX2 is down-regulated. *RunX2* message RNA (mRNA) levels decreased in 5-week-old male Sprague-Dawley rats (*Rattus norvegicus*) fed with a high-fat diet for 12 weeks (Wang et al., 2020). OCN expression is one of the signs of bone formation and can promote bone mineralization (Ducy et al., 1996). The expression of OCN decreased in $Col1a1^{Jrt/+}$ mice fed a high-fat diet for 26 weeks (Tauer et al., 2021). In addition, ALP is a key enzyme marker of bone formation due to its action in regulating the process of biomineralization (Chen et al., 2018). The expression level of ALP decreased in 6-week-old male C57BL/6 mice fed a high-fat diet for 6 months (Cao et al., 2020). Osteoprotegerin (OPG) has a dramatic effect on both osteoclast differentiation and activation. OPG is the decoy receptor activator of nuclear factor receptor-κB ligand (RANKL), which is the key activator of osteoclasts (Yun et al., 2001). The typical Wnt/β-catenin signaling pathway is a cascade that promotes osteoblasts to produce OPG (Gong et al., 2001; Kato et al., 2002; Glass et al., 2005). Therefore, the activation of the typical Wnt/β-catenin signal can lead to an increase in the ratio of OPG/RANKL in bone and shift the balance to osteogenesis rather than osteoclast formation, which is ultimately conducive to bone formation rather than bone resorption (Kurgan et al., 2019). A study of Wistar rats fed with a high-fat diet for 4 weeks showed an increase in OPG expression in obese rats (Liu et al., 2021). Therefore, the expression levels of bone formation marker proteins (RunX2, OCN, ALP, and OPG) can be useful markers that can reflect the status of bone formation metabolism.

Osteoclasts play an important role in bone resorption (Lampropoulos et al., 2012). 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 (Kim et al., 2010). 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 (Liu et al., 2021). 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 (Novinec and Lenarčič, 2013). Cathepsin K was up-regulated in 3-week-old female Wistar rats fed a high-fat diet for 5 weeks (Yanagihara et al., 2017). Matrix metallopeptidases (MMPs) are a protein family composed of zinc-dependent endopeptidases that regulate tissue remodeling under physiological and pathological conditions (Halade et al., 2013). MMP9, also known as gelatinase B or 92-kDa type IV collagenase, is responsible for degrading the extracellular matrix (Halade et al., 2013). 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 (Chung et al., 2006). Therefore, the expression levels of bone loss-related proteins (RANKL, cathepsin K, and MMP9) reflect bone loss metabolism.

In recent years, the study by Yang et al. (2013) has shown that bone formation and bone resorption are regulated by Wnt signaling. Wnt/β-catenin signaling plays an important role in the development and functional regulation of osteoblasts (Hwang et al., 2015) and activation of the Wnt pathway, promoting the proliferation and differentiation of osteoblasts (Hoeppner et al., 2009). When Wnt/β-catenin signal transduction is disturbed, a variety of bone diseases such as osteoporosis can occur (Feng et al., 2020). Decreased activity of Wnt/β-catenin signaling leads to decreased bone formation in male rats fed a high-fat diet for 4 weeks (Chen et al., 2010). When the Wnt ligand is missing, glycogen synthase kinase-3β (GSK-3β) phosphorylates β-catenin, leading to a rapid degradation of phosphorylated β-catenin (P-β-catenin) by the proteasome (Lam and Gottardi, 2011). After the Wnt receptor is activated, GSK-3β is inhibited, leading to accumulation of non-phosphorylated β-catenin and activation of target genes involved in regulating the proliferation and differentiation of bone marrow stromal cells (Moorer and Riddle, 2018). *β-Catenin* deletion inhibits osteoblast differentiation (Hill et al., 2005). In addition, Wnt signaling can indirectly affect the function of osteoclasts by regulating the expression of bone resorption-related proteins (Glass et al., 2005). Wnt signaling causes less bone resorption by down-regulating RANKL in the osteoclasts (Takahashi et al., 2011). However, little is known about the role of Wnt signaling in the fattening stage before hibernation.

In our previous research, we compared 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 (Yan et al., 2021). However, little is known about the changes in the bones of fattening animals before hibernation, and the related mechanisms are unclear. 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 significant 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 three 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, ALP, and OPG), bone resorption-related proteins (RANKL, cathepsin K, and MMP9), and Wnt signaling proteins (P-β-catenin, GSK-3β, and patched protein homolog 1 (Ptch1)). The data further clarified the differential regulation and mechanisms of bone remodeling occurring between models of pathological obesity and healthy obesity.

2 Results

2.1 Body weight

After the treatment with the high-fat diet for three months, the body weight of the pathological obesity (OB) group was 10.6% higher than that of the control (CON) group ((52.0±0.7) g vs. (47.0±0.8) g, $P<0.05$). In the ground squirrels, the mean body weight of the pre-hibernation (PRE) group was 62.5% higher than that of the summer active (SA) group ((347.7±10.8) g

vs. (214.0±6.4) g, P<0.05), with a mean rise of 134 g per animal over the pre-hibernation fattening period.

2.2 Adipose tissue wet weight

After high-fat diet treatment, the mesenteric adipose wet weight and perirenal adipose wet weight both increased by 1.3-fold in the OB mice when compared with the CON group (mesenteric adipose wet weight: (2.01±0.12) g vs. (0.89±0.12) g; perirenal adipose wet weight: (0.63±0.08) g vs. (0.28±0.03) g; P<0.05). After natural fattening, the mesenteric adipose wet weight, perirenal adipose wet weight, and subcutaneous adipose wet weight increased by 21.5-fold ((19.54±2.57) g vs. (0.87±0.22) g), 7.2-fold ((3.21±0.40) g vs. (0.39±0.12) g), and 36.9-fold ((13.25±0.92) g vs. (0.35±0.17) g), respectively, in the PRE ground squirrels when compared with the SA group (P<0.05).

2.3 Bone structure and function

2.3.1 Femoral bone structure and function

Representative micro-computed tomography (micro-CT) images of distal femurs of mice in the CON and OB groups and of ground squirrels in the SA and PRE groups are shown in Fig. 1. The images indicate no differences in the structure of the femur between the CON and OB mouse groups, but substantial differences between the SA and PRE ground squirrel groups. This was supported by quantification of multiple femoral bone parameters (Table 1). These quantified data showed 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. In contrast, the PRE group of ground squirrels showed both greater bone surface density (BS/BV (the ratio of bone tissue surface to bone tissue volume), increased by 36.4%) and trabecular number (Tb.N, increased by 28.6%) than the SA group (P<0.05). However, the

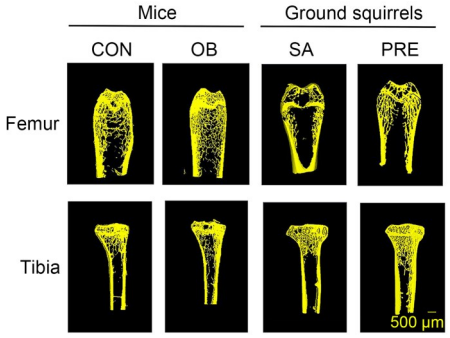

Fig. 1 Representative micro-CT images of the distal femur and proximal tibia in different groups. Micro-CT: micro-computed tomography; CON: control mice; OB: obese mice induced by a high-fat diet for three months; SA: summer active Daurian ground squirrels; PRE: pre-hibernation squirrels that had finished natural fattening and were sacrificed in late-autumn (end of September, 30–40 d before hibernation).

Table 1 Femoral bone structure and function

Structure	Parameter	CON	OB	SA	PRE
Trabecular bone	BS/BV (mm^{-1})	22.70±1.42	21.42±2.04	11.03±2.04	15.04±1.45$^{\#}$
	Tb.N (mm^{-1})	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/cm^3)	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/cm^3)	275.11±44.33	302.27±14.45	218.62±24.06	190.56±22.39
	BMC (mg)	1.26±0.17	1.56±0.14*	17.95±2.74	3.25±0.59$^{\#}$

The above data were obtained by micro-CT and expressed as mean±SD (n=6). $^{*}P$<0.05 compared with the CON group; $^{\#}P$<0.05 compared with the SA group. Micro-CT: micro-computed tomography; SD: standard deviation; BS/BV: bone surface density, the ratio of bone tissue surface to bone tissue volume; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; BV/TV: bone volume fraction, the ratio of bone tissue volume to tissue volume; Ct.Th: cortical thickness; Ma.Ar: marrow area; Ct.Ar: cortical bone area; Tt.Ar: total cortical bone area; TMD: tissue mineral density; TMC: tissue mineral content; BMD: bone mineral density; BMC: bone mineral content; CON: control mice; OB: obese mice induced by a high-fat diet for three months; SA: summer active Daurian ground squirrels; PRE: pre-hibernation squirrels that finished natural fattening and were sacrificed in late-autumn (end of September, 30–40 d before hibernation).

PRE group showed both reduced trabecular thickness (Tb.Th, reduced by 31.6%) and trabecular separation (Tb.Sp, reduced by 24.3%) compared with the SA group (P<0.05). Finally, the bone volume fraction (BV/TV, the ratio of bone tissue volume to tissue volume) showed no significant difference between the SA and PRE groups of ground squirrels.

Cortical bone quantitative parameters are also presented in Table 1. In mice, there were no significant differences in any of measured parameters between the CON and the OB groups. Among the ground squirrels, the PRE group showed significant reductions in average cortical thickness (Ct.Th, reduced by 52.2%), marrow area (Ma.Ar, reduced by 88.6%), cortical bone area (Ct.Ar, reduced by 88.7%), and total cortical bone area (Tt.Ar, reduced by 88.6%), compared with the SA group (P<0.05).

Table 1 also shows an analysis of bone mineral-related data. In mice, compared with the CON group, the tissue mineral density (TMD) and tissue mineral content (TMC) showed non-significant but rising trends in the OB group (P=0.095 and 0.070, respectively). The BMD of the OB group was not significantly different from that of the CON group. The bone mineral content (BMC) in the OB mice increased significantly by 20%. Ground squirrels showed a very different profile. There was no significant difference in TMD between the PRE and SA groups. However, the TMC in the PRE group bone was 82.8% lower than that in the SA group (P<0.05). There was no significant difference in BMD between the PRE and SA groups, but the BMC of the PRE group was 81.9% lower than that of the SA group (P<0.05).

2.3.2 Tibial bone structure and function

Fig. 1 also shows representative micro-CT images of the proximal tibia in the different groups and Table 2 shows quantified parameters for tibial bone. The mouse images (Fig. 1) show no visual differences between the CON and OB groups. This is supported by the lack of significant change in any of the five parameters assessed (Table 2). In the ground squirrels, among the parameters measured for trabecular bone, the BV/TV increased by 40.4% in the PRE group (P< 0.05). However, the Tb.Sp was reduced by 33.0% in the PRE group (P<0.05). The Tb.N value showed a non-significant increase in the PRE group compared with the SA group (P=0.064).

Mineral-related data for tibial bone were also analyzed and the results are shown in Table 2. In the mice, there were no significant differences in any of the four tibial bone mineral parameters between the OB and CON groups. In the ground squirrel, there were no significant differences in the TMD, TMC, or BMC between the PRE and SA groups. However, the BMD of the PRE group was 20.1% higher than that of the SA group (P<0.05).

2.3.3 Three-point bending test

The results of the three-point bending test of the femurs are shown in Table 3. In mice, neither the ultimate bearing capacity (N) nor the ultimate bending

Table 2 Tibial bone structure and function

Structure	Parameter	CON	OB	SA	PRE
Trabecular bone	BS/BV (mm^{-1})	17.72±0.80	17.30±0.31	8.70±1.68	8.43±0.68
	Tb.N (mm^{-1})	1.77±0.30	1.69±0.09	0.78±0.11	1.07±0.17
	Tb.Th ($\times10^{-1}$ mm)	1.10±0.04	1.20±0.02	2.40±0.50	2.40±0.20
	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±0.70	18.06±1.19	25.35±2.89$^{\#}$
Bone mineral	TMD (mg/cm^3)	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/cm^3)	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

The above data were obtained by micro-CT and expressed as mean±SD (n=6). $^{\#}$ P<0.05 compared with the SA group. Micro-CT: micro-computed tomography; SD: standard deviation; BS/BV: bone surface density, the ratio of bone tissue surface to bone tissue volume; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; BV/TV: bone volume fraction, the ratio of bone tissue volume to tissue volume; TMD: tissue mineral density; TMC: tissue mineral content; BMD: bone mineral density; BMC: bone mineral content; CON: control mice; OB: obese mice induced by a high-fat diet for three months; SA: summer active Daurian ground squirrels; PRE: pre-hibernation squirrels that finished natural fattening and were sacrificed in late-autumn (end of September, 30–40 d before hibernation).

Table 3 Three-point bending test of the femur and tibia

Bone	Group	Ultimate bearing capacity (N)	Stiffness (N/mm)	Ultimate bending energy ($\times10^{-2}$ J)
Femur	CON	26.63±0.93	78.14±12.91	1.00±0.04
	OB	29.00±2.12	103.67±15.45*	1.00±0.03
	SA	59.87±18.21	112.77±42.38	2.00±0.01
	PRE	58.71±11.47	82.47±17.07	2.00±0.50
Tibia	CON	14.72±1.95	32.55±5.54	0.40±0.10
	OB	14.96±2.54	37.58±9.85	0.40±0.05
	SA	43.39±5.87	61.17±17.68	2.00±0.70
	PRE	59.48±7.81	108.27±30.01	2.00±0.50

All values are expressed as mean±SD (n=6). * P<0.05 compared with the CON group. SD: standard deviation; CON: control mice; OB: obese mice induced by a high-fat diet for three months; SA: summer active Daurian ground squirrels; PRE: pre-hibernation squirrels that finished natural fattening and were sacrificed in late-autumn (end of September, 30–40 d before hibernation).

energy (J) differed between the CON and OB groups, but the stiffness parameter (N/mm) was 32.7% higher in the OB group (P<0.05). Comparable analysis of bone from ground squirrels found no significant differences in ultimate bearing capacity, stiffness, or ultimate bending energy between the PRE and SA groups.

The results of the three-point bending test for tibia are shown in Table 3. In mice, the ultimate bearing capacity, stiffness, or the ultimate bending energy was not significantly different between the CON and OB groups. 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, in the mice there were no significant differences in ultimate bearing capacity, stiffness, or ultimate bending energy between the OB and CON groups.

2.4 Relative protein expression

We used western blotting to detect the expression levels of proteins related to bone formation, and the results are shown in Fig. 2. In mice, there were no significant differences in the expression level of RunX2 between the OB and CON groups (Figs. 2a and 2b). The expression level of OPG was reduced by 67.6% in the OB group (P<0.05; Figs. 2a and 2e). However, in the OB group, the expression levels of OCN and ALP were 20.2% and 21.9% higher, respectively, than those in the CON group (P<0.05; Figs. 2a, 2c, and 2d). In contrast, in the ground squirrels, the expression level of RunX2 was 27.2% higher in the PRE group than in the SA group (P<0.05; Figs. 2a and 2b), whereas the expression of OCN decreased by 38.4% in the PRE group (P<0.05; Figs. 2a, and 2c). The ALP expression level was 26.0% higher in the PRE group squirrels (P<0.05; Figs. 2a and 2d). The expression level of OPG did not differ significantly between the two groups (Figs. 2a and 2e).

The expression levels of proteins related to bone resorption are shown in Fig. 3. In OB mice, the expression levels of RANKL, cathepsin K, and MMP9 were 45.3%, 27.7%, and 44.4% higher, respectively, than those in the CON group (P<0.05); also for the ground squirrels, the levels in the PRE group were 37.9%, 100.0%, and 40.0% higher, respectively, than those in the SA group (P<0.05).

Proteins associated with Wnt signaling were also assessed by western blots (Fig. 4). In mice, the expression levels of P-β-catenin and GSK-3β were 20.2% and 38.8% higher in the OB group than in the CON group, respectively (P<0.05), but Ptch1 expression did not change in the OB mice. In the ground squirrels, the expression level of GSK-3β was 1.4-fold higher in the PRE group than in the SA group (P<0.05), but there were no differences in P-β-catenin expression between the two groups. By contrast, Ptch1 expression was 66.6% lower in the PRE group squirrels (P<0.05).

3 Discussion

In this study, we compared the bone metabolism of mice with pathological obesity induced by a high-fat diet with that of ground squirrels with healthy obesity 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 (Fig. 5). The results showed that obese mice (high-fat diet) showed no

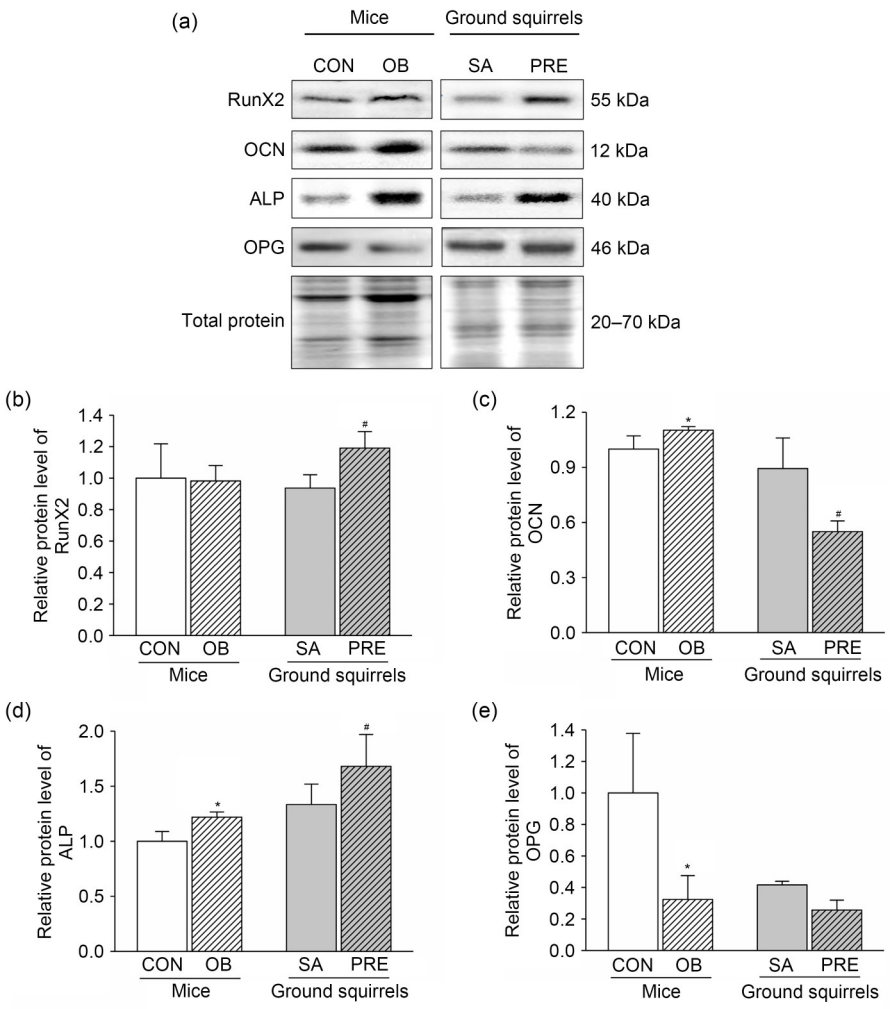

Fig. 2 Expression levels of proteins related to bone formation. (a) Representative immunoblots of RunX2, OCN, ALP, and OPG in each group; (b–e) The relative protein expression levels of RunX2 (b), OCN (c), ALP (d), and OPG (e) in mice and Daurian ground squirrels. Values are expressed as mean±SD (n=6). $^{*}P$<0.05 compared with the CON group; $^{\#}P$<0.05 compared with the SA group. RunX2: Runt-related transcription factor 2; OCN: osteocalcin; ALP: alkaline phosphatase; OPG: osteoprotegerin; CON: control mice; OB: obese mice induced by a high-fat diet for three months; SA: summer active Daurian ground squirrels; PRE: pre-hibernation squirrels that had finished natural fattening and were sacrificed in late-autumn (end of September, 30–40 d before hibernation); SD: standard deviation.

obvious alterations or abnormalities in bone microstructure 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 increased in pre-hibernation ground squirrels, as shown by an enhancement of bone microstructure and strength, increased expression levels of proteins related to bone formation (RunX2 and ALP) and bone resorption, and enhanced Wnt signaling.

Similarly to our previous results (Yan et al., 2021), 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, though the degree of obesity in pre-hibernating ground squirrels was much greater than that in OB mice. Another difference is that the change in adipose tissue of mice occurred in perirenal and mesenteric areas, whereas in ground squirrels, adipose accumulated mainly in mesenteric and subcutaneous depots with less accumulation of perirenal adipose. Previous research has shown an inverse relationship between visceral fat and bone density (Liu et al., 2017). Therefore, compared with OB mice, the lower amount of visceral fat in PRE ground squirrels may be related to the increase in bone density.

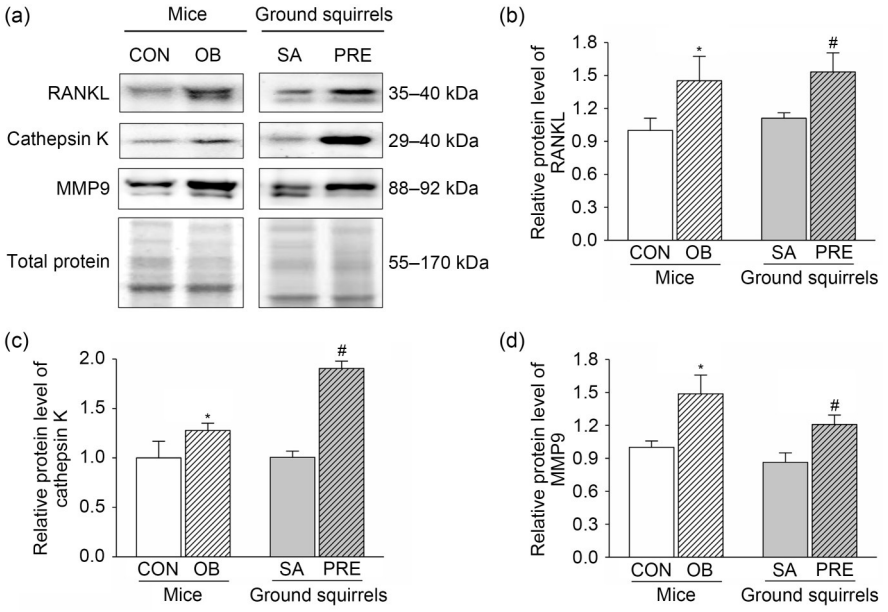

Fig. 3 Expression levels of proteins related to bone resorption. (a) Representative immunoblots of RANKL, cathepsin K, and MMP9 in each group; (b–d) The relative protein expression levels of RANKL (b), cathepsin K (c), and MMP9 (d) in mice and Daurian ground squirrels. Values are expressed as mean±SD (n=6). $^{*}P$<0.05 compared with the CON group; $^{\#}P$<0.05 compared with the SA group. RANKL: receptor activator of nuclear factor-κB ligand; MMP9: matrix metallopeptidase 9; CON: control mice; OB: obese mice induced by a high-fat diet for three months; SA: summer active Daurian ground squirrels; PRE: pre-hibernation squirrels that had finished natural fattening and were sacrificed in late-autumn (end of September, 30–40 d before hibernation); SD: standard deviation.

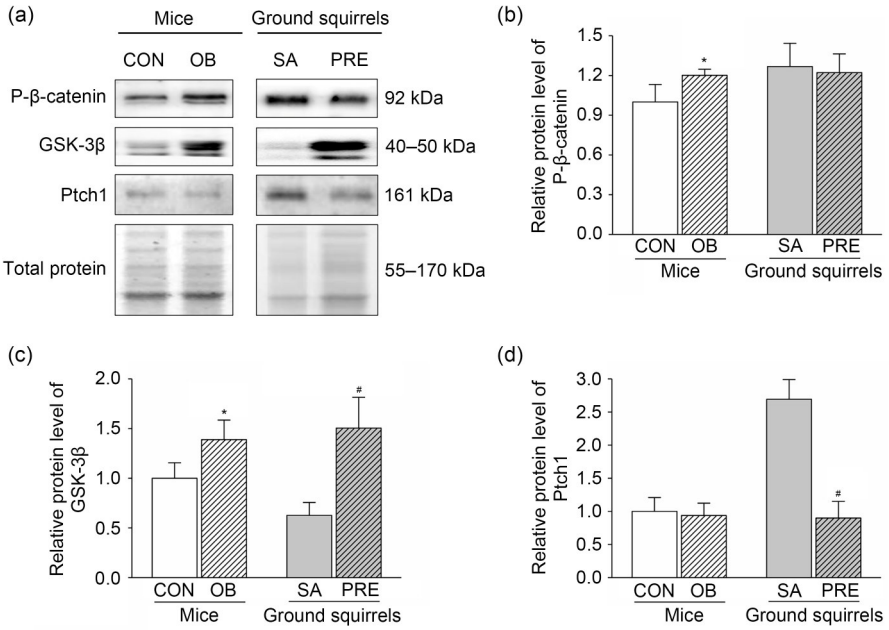

Fig. 4 Protein expression levels of Wnt signal. (a) Representative immunoblots of P-β-catenin, GSK-3β, and Ptch1 in each group; (b–d) The relative protein expression levels of P-β-catenin (b), GSK-3β (c), and Ptch1 (d) in mice and Daurian ground squirrels. Values are expressed as mean±SD (n=6). $^{*}P$<0.05 compared with the CON group; $^{\#}P$<0.05 compared with the SA group. P-β-catenin: phosphorylated β-catenin; GSK-3β: glycogen synthase kinase-3β; Ptch1: patched protein homolog 1; CON: control mice; OB: obese mice induced by a high-fat diet for three months; SA: summer active Daurian ground squirrels; PRE: pre-hibernation squirrels that had finished natural fattening and were sacrificed in late-autumn (end of September, 30–40 d before hibernation); SD: standard deviation.

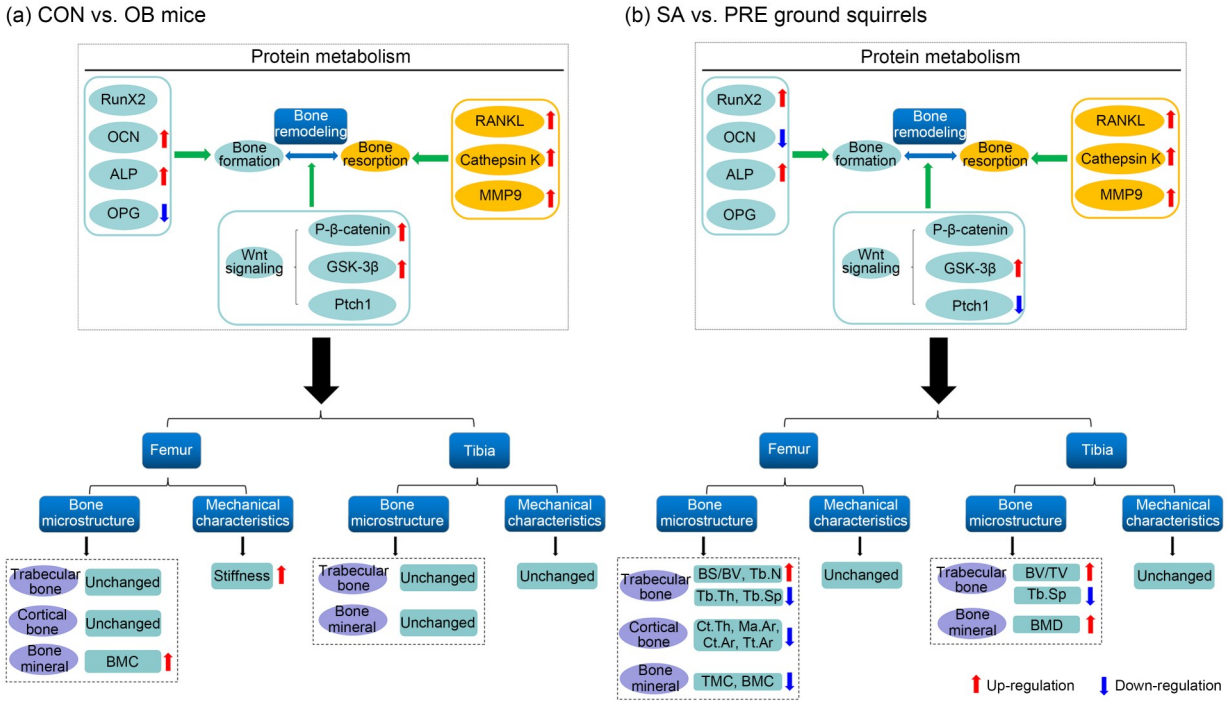

Fig. 5 Pathway diagrams of expression levels of proteins related to bone formation, bone resorption, and Wnt signaling in two obesity models. (a) Changes in protein expression levels and bone metabolism in mice in the OB group compared with those in the CON group; (b) Changes in protein expression levels and bone metabolism in ground squirrels in the PRE group compared with those in the SA group. OB: obese mice induced by a high-fat diet for three months; CON: control mice; PRE: pre-hibernation squirrels that had finished natural fattening and were sacrificed in late-autumn (end of September, 30–40 d before hibernation); SA: summer active Daurian ground squirrels; RunX2: Runt-related transcription factor 2; OCN: osteocalcin; ALP: alkaline phosphatase; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor-κB ligand; MMP9: matrix metallopeptidase 9; GSK-3β: glycogen synthase kinase-3β; Ptch1: patched protein homolog 1; BMC: bone mineral content; BS/BV: bone surface density, the ratio of bone tissue surface to bone tissue volume; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; Ct.Th: cortical thickness; Ma.Ar: marrow area; Ct.Ar: cortical bone area; Tt.Ar: total cortical bone area; TMC: tissue mineral content; BV/TV: bone volume fraction, the ratio of bone tissue volume to tissue volume; BMD: bone mineral density.

There was no significant change in the microstructure of the femur or tibia in the OB group, but the BMC showed a significant increase, and TMD and TMC showed an increasing trend (P=0.095 and 0.070, respectively) in femurs after fattening (Table 1). This indicated that the bone microstructure of mice was in a balanced state, but bone minerals had a tendency to increase in the femur. By contrast, ground squirrels showed some substantial differences between the SA and PRE states. The BS/BV and Tb.N parameters of the PRE group were significantly increased, whereas Tb. Th, Tb. Sp, Ma. Ar, TMC, and BMC were all strongly reduced, indicating that bone formation of the femur in the PRE group was enhanced (Table 1). The microstructural changes of the ground squirrel tibia were similar to those of the femur. The Tb.N showed an increasing trend, Tb. Sp was significantly reduced, and BV/TV and BMD were significantly increased, indicating that bone formation was enhanced in the tibia (Table 2). In conclusion, there was no difference in tissue specificity in bone formation of the femur and tibia between the mice and ground squirrels. This is consistent with another study by Minematsu et al. (2018), which showed no differences in bone formation between the femur and tibia in obese Wistar rats induced by a high-fat diet. Compared with the mice, the bone mass in the PRE group of ground squirrels increased in both the femur and tibia, which showed that healthy obesity was not harmful to their bones. However, 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 postulate 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 three 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, but the mechanical properties of the tibia did not change significantly (Table 3). This indicated that the increased bending resistance of the OB mice occurred mainly in the femur, thereby reducing the risk of fracture. This may be an adaptation to the higher load caused by weight gain. In the ground squirrels, the mechanical properties of the bones of the PRE group differed from those of the SA group. The ultimate bearing capacity, stiffness, or ultimate bending energy of the femur in the PRE group did not change significantly compared with the SA group (Table 3), 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 (Table 3), suggesting that an increase in bending resistance in the PRE group occurred mainly 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 pathway.

Bone metabolism is maintained by a dynamic balance of bone formation and bone resorption (Datta et al., 2008). RunX2 is the main driving factor of bone formation and promotes the differentiation and maturation of osteoblasts (Kim et al., 2020). The expression level of RunX2 in the OB mice was not significantly different from that of those in the CON group (Fig. 2b). This result differed from that of 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 (Chen et al., 2019). By contrast, the expression level of RunX2 in PRE ground squirrels was significantly up-regulated compared with that in the 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 the calcium metabolism of bone, mainly by promoting bone mineralization (Koshihara and Hoshi, 1997). In this study, the expression level of OCN was up-regulated in OB mice, but down-regulated in PRE ground squirrels (Fig. 2c). This indicated that the OB mice had increased bone mineralization ability, whereas the PRE ground squirrels may have been reducing bone mineralization activity as the hibernation season approached. In addition, OCN not only plays a role in bone formation, but also affects energy regulation (Lee et al., 2007). 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 investigation. ALP protein is one of the phenotypic markers of osteoblasts and can directly reflect the activity or function of osteoblasts (Buchet et al., 2013). The expression level of ALP in both the OB mice and the PRE ground squirrels was significantly increased (Fig. 2d), indicating that the osteoblasts in both groups had 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, which showed that the expression level of ALP in obese mice was significantly increased (Vimalraj, 2020). The study by Bhattarai et al. (2014) has shown that ALP can promote the absorption of calcium ions by bones. We speculated 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. 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 their mineral content and mechanical strength. This is in line with the increasing trends of TMD, TMC, and BMC shown by micro-CT in this study. The expression level of OPG was significantly decreased in the OB mice compared with the CON mice, which may have attenuated osteoclast differentiation and activity due to the reduced inhibitory effect of OPG (Yun et al., 2001). However, the expression level of OPG did not differ significantly between the SA and PRE ground squirrels. Hence, we proposed that the difference in the expression level of RunX2 was the main reason for the difference in bone formation

between the two models. Compared with the OB group, 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). OPG is a decoy receptor for RANKL, and changes in the ratio of OPG to RANKL often indicate a shift between osteoblastogenesis and osteoclastogenesis (Kurgan et al., 2019). The bone balance shifts towards bone formation as the ratio increases, and towards bone loss as the ratio decreases. Our results showed that RANKL expression had increased while OPG had decreased in the OB mice, implying that the OPG/RANKL ratio was decreased. That is, compared with the CON group, the bone balance of the OB mice shifted towards bone loss. In the ground squirrels, there was no significant difference between the OPG/RANKL ratios of the PRE group and the SA group, suggesting that bone metabolism in the PRE ground squirrels was in a balanced state. The increase in bone resorption in obese mice was consistent with previous studies (Fang et al., 2007; Yanagihara et al., 2017; Zhong et al., 2020). 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 represent a potential risk for bone loss in mice. Previous reseach has shown that bone loss occurred in mice when they were extremely obese (Núñez et al., 2007). In the ground squirrels, the expression levels of bone resorption proteins were significantly increased in the PRE group, but the bone substance was also increased, which may reflect greater bone formation than resorption.

Wnt signaling also plays an important role in the regulation of bone remodeling (Baron and Kneissel, 2013). In the present study, the expression levels of P-β-catenin and GSK-3β in OB mice were significantly increased while the expression level of Ptch1 did not change significantly (Fig. 4) and Wnt signaling was weakened. This could lead to an increase of bone resorption and a decrease of bone formation, which is consistent with a study reported by Penrose et al. (2017) showing that obesity inhibited the Wnt signaling pathway. The ground squirrels showed a different response. The expression level of GSK-3β in the PRE group was significantly increased (Fig. 4c), but the expression level of P-β-catenin did not change (Fig. 4b), and the expression level of Ptch1 was significantly reduced (Fig. 4d). This indicated that the Wnt signal was strengthened and bone formation was enhanced before hibernation, which is consistent with the results reported by Gao et al. (2021). 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-3β has been shown to increase bone mass, which may involve many mechanisms (Gu et al., 2017). However, although GSK-3β inhibitors can promote osteogenesis, the activity of GSK-3β is not only manifested in osteogenesis, but also related to other intracellular biological processes. This has raised concerns about possible side effects of long-term treatment with these inhibitors in humans (Martin et al., 2018). In addition, over-inhibition of GSK-3β carries a risk of tumorigenicity (Vijay et al., 2019). High expression of Wnt signaling also causes an increase in the expression level of RunX2 (Day and Yang, 2008). In our study, the Wnt signal in the OB mice was weak, while that in the PRE ground squirrels was significantly increased, which may partly explain the difference in bone mass between obese mice and PRE ground squirrels.

When comparing the two models, we found that weight gain caused 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 reflect an unchanged expression level of RunX2 and significant increases in the expression levels of OCN and ALP. Although the body weight of the OB mice increased by only 10.6% compared with those in the control group, the weight gain increased the risk of bone loss in the OB mice, as shown by the significant up-regulation of bone resorption proteins and weakened Wnt signaling. Results for the mice and 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-3β 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.

Bettis et al. (2018) have shown that muscle atrophy has a significant effect on bone metabolism and muscle atrophy may promote osteoclast formation through myostatin. Our previous study (Yan et al., 2021) has shown that a high-fat diet caused gastrocnemius atrophy in obese mice and that the expression level of myostatin in OB mice was increased, which promoted the bone loss of mice, consistent with the results reported in the present study. In contrast, the ground squirrels did not have such effect through myostatin.

Kunming mice are considered a high BMD strain, with a higher bone mass than another common strain, C57BL/6J (Zhou et al., 2018). This may be one reason why the mice in this study did not experience significant bone loss. In addition, a high-salt diet attenuates obesity in mice. Studies have shown that high-salt intake attenuates hyperglycemia and insulin resistance in WBN/Kob diabetic fatty (WBKDF) rats, and inhibits obesity in female Sprague-Dawley rats (Pitynski-Miller et al., 2017; Takagi et al., 2018). The high-fat diet used in this study had a high salt content with a mass fraction of 2%, which may be another reason for the lack of significant changes in bone loss in OB mice. However, Lanaspa et al. (2018) showed that long-term high-salt diets could lead to bone loss, but this effect takes four months or more to manifest. The mice in this study were fed a high-fat diet for only three months, and the degree of bone damage from this high-salt diet was not measured.

4 Conclusions

In summary, we compared the differences in bone metabolism between high-fat diet fattened mice (OB group) and naturally fattened ground squirrels (PRE group). Our results showed 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 underwent bone formation. The results of the three-point bending test showed that the skeletal mechanical properties of the OB mice were strengthened, while those of the PRE ground squirrels did not change significantly. Western blots 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 increased the expression levels of proteins related to bone formation, bone resorption, and Wnt signaling.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 31640072 and 31900338) and the Natural Science Basic Research Program of Shaanxi (No. 2020JM-428), China.

Author contributions

Hui CHANG conceived and designed the experiments. Xuli GAO, Shenyang SHEN, Qiaohua NIU, Weilan MIAO, Yuting HAN, Ziwei HAO, Ning AN, Yingyu YANG, Yu ZHANG, and Han ZHANG performed the experiments. Xuli GAO, Shenyang SHEN, and Qiaohua NIU analyzed the data. Shenyang SHEN, Qiaohua NIU, and Kenneth B STOREY wrote the paper. All authors have read and approved the final manuscript, and therefore, have full access to all data relevant to the study and take responsibility for the integrity and security of such data.

Compliance with ethics guidelines

Xuli GAO, Shenyang SHEN, Qiaohua NIU, Weilan MIAO, Yuting HAN, Ziwei HAO, Ning AN, Yingyu YANG, Yu ZHANG, Han ZHANG, Kenneth B STOREY, and Hui CHANG declare that they have no conflict of interest.

All animal experiments were approved by the Experimental Animal Protection Committee of the Ministry of Health of the People's Republic of China (Approval Number: SL-2012-42).

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Supplementary information

Materials and methods