Bone microstructure and metabolism changes under the combined intervention of ketogenic diet with intermittent fasting: an in vivo study of rats

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Abstract: Ketogenic diet (KD) has been used in epilepsy for decades, but previous studies found it may cause severe bone loss. Every-other-day ketogenic diet (EODKD), the combination of KD with intermittent fasting, showed better potential for seizure control recently, while its effects on bone remain unknown. This study aims to establish different ketogenic rat models and compare the influence of EODKD with KD on bone microstructure and metabolism. Thirty male Sprague-Dawley rats were divided into Control, KD and EODKD groups, fed with standard diet, continuous and intermittent ketogenic diet respectively. After 12 weeks, bone mineral density (BMD) and body fat percentage were obtained by dual energy X-ray absorptiometry. Micro-CT and three-point bending test were used to evaluate the bone microstructure and mechanical properties. Activities of serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) were measured, together with the osteogenic capabilities of bone marrow stromal cells (BMSCs) tested by ALP activities and alizarin red stain in different osteogenic stage. Both EODKD and KD induced higher ketone and more fat percentage, but led to lower body weight compared with Control group. They both compromised bone mass and mechanical properties. Compared with KD, EODKD demonstrated higher ketone levels, but it also inhibited osteoclastic process as well as early osteogenic differentiation. In general, EODKD accelerated ketosis, but may not deteriorate bone microstructure and strength than KD.

Key words: bone loss, bone metabolism, every-other-day ketogenic diet, micro-CT, microstructure

Introduction

Ketogenic diet (KD) is defined as a high fat and relatively low carbohydrates diet with adequate protein for development [20, 31]. It was designed to mimic the fasting state by utilizing fat for 90% of the required calories [34]. The clinical history of KD can be tracked back into the 1920s when it was introduced as an effective therapy for epilepsy for the first time [16]. KD became even more popular for its role in weight loss in 1970s. As it was also found curative and preventative in diseases like cardiovascular events and type 2 diabetes [22], there were studies indicating KD may also be a choice for brain tumor [35], autism spectrum disorder [11] and epilepsy during pregnancy [29].

The most acknowledged role of KD was in refractory epilepsy. In fact, fasting, as well as KD, has also been used to control seizures for decades since they both in-
duce a state called ketosis [6]. At this background, every-
other-day ketogenic diet (EODKD) was introduced re-
cently by Johns Hopkins University. It combined KD with intermittent fasting as a new regimen for epilepsy therapy. Our previous study discovered that EODKD led to a higher ketone level than KD in both serum and cerebrospinal fluid of rats [33], and researchers also found improved seizure control in pediatric patients by additional intermittent fasting during clinical KD treatment [13].

However, the side effects of KD on bones cannot be underestimated. KD related calcium and bone mass deficiency was first reported by Hahn [12], and soon, low bone mineral density and compromised bone mass resulted from KD treatment have attracted attention [36]. On the other hand, with higher ketogenic potential, it is ambiguous whether EODKD causes aggravated or alleviated bone loss compared with KD. The present study aims to establish rat models of EODKD as well as KD to compare the difference in metabolism under different ketogenic state, also to clarify the integrated effects of EODKD and KD in long bones of rats using dual energy X-ray absorptiometry (DEXA), micro-CT, three-point bending test and serum analysis, together with osteogenic capability evaluation of bone marrow stromal cells (BMSCs).

**Materials and Methods**

**Experimental animals**

Thirty male 6-week-old Sprague-Dawley rats were purchased from the Laboratory Animals Center of Southern Medical University. After acclimatized for two weeks, the 8-week-old rats with total mean body weight around 280–300g were randomly divided into the Control group (laboratory standard diet feeding), the KD group (ketogenic diet feeding) and the EODKD group (ketogenic diet feeding with every-other-day fasting). All rats were housed individually in wire hanging cages in an animal facility maintained at 22 ± 1°C and on a 12-h light-dark cycle. Rats in the Control group were fed with a standard diet (provided by Laboratory Animals Center of Southern Medical University, Guangzhou, China). The KD and EODKD groups were fed with the ketogenic diet, a formula diet with a 3:1 ratio of fat to carbohydrate and protein (Jielikang Inc., Shenzhen, China) while the rest nutrients meet for a criterion of AIN-93 [24]. The KD group experienced a continuous feeding while the EODKD group was fed and fasted every other day (ketogenic diet was removed every 8 a.m. and put back the next 8 a.m.). All rats had free access to tap water throughout the study. The detailed description of both diets, including energetic density, percentage of protein, fat and carbohydrates, dietary fiber, calcium, phosphorus and vitamin D content are shown in Table 1.

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<th>Table 1. Basic nutrient content comparison</th>
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*The calcium, phosphorus and vitamin D of ketogenic diet meet the criterion of AIN-93.

**Body weight, blood ketone and glucose levels**

Body weights of rats in each group were measured weekly for 12 weeks with a CS 200 (Ohaus, Pine Brook, NJ, USA) balance. Blood ketone and glucose levels were tested fortnightly. Tail vein puncture was conducted every-other-week using scalp acupuncture, and blood ketone levels were tested with Yicheng Blood Ketone Meter T-1 (Sentest Inc., Shenzhen, China) and Medisense Precision Xtra monitor (Abbott Laboratories, Montreal, Canada), while the blood glucose levels were tested with monitor JPS-5 (Leapon Inc., Beijing, China). For the EODKD group, blood samples were all obtained on the end of the fasting day, the morning before ketogenic diet was put back.

**Body fat percentage and bone mineral density (BMD)**

On the final day of respective diet feeding, all rats were given access to food with lights out for 1 h in the morning and then fasted for the next 6 h (to standardize gastrointestinal filling). After anesthetized with 0.3% pentobarbital, rats were fixed on the testing bed facing down. The whole body fat content and total BMD were analyzed with Dual Energy X-ray Absorptiometry (DEXA, GE-Lunar iDXA, GE Healthcare, Antwerp Area, Belgium).
Specimen preparation
After DEXA analysis, laparotomy were performed on rats under anesthesia, after which the blood samples were obtained from inferior vena cava and stored in 4°C overnight. After centrifuged 3,000 rpm for 12 min in the next morning, serum from supernatant were collected and stored in −80°C before testing. After decapitation, bilateral tibias and femurs were excised with adherent soft tissue clearly removed, and bone lengths were measured with a vernier caliper. Samples were then fixed in 4% paraformaldehyde for 48 h and stored in 70% ethanol at 4°C before analysis.

Microstructure of proximal tibia
Twenty-four tibias (eight from each group, all left side) were randomly selected for Microcomputed tomography (micro-CT) scan. For sample fixation, a cystosepiment were used to keep it straight in a rigid plastic tube. The metaphyseal region of proximal tibia within a rectangular area 2mm away from the central point of the growth plate-metaphyseal junction were scanned with a high-resolution micro-CT system (µCT 80, Scanco Medical, AG, Switzerland) for totally 180 slices, and an isotropic voxel size of 12 µm was set (55 kv, 145 µA, integration time 300 ms, averaged 2 times).

The following parameters were evaluated using the software provided by the manufacturer, bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), connectivity density (Conn.D) and tissue mineral density (TMD) for cancellous bone, while total cross-sectional area inside the periosteal envelope (Tarea), bone area (Barea) and thickness (Ct.Th) for cortical bone.

Biomechanical strength of femur
With eight samples in each group, the femurs were subjected to the three-point bending test using a mechanical testing machine (Electroplus E1000, Instron Inc., Norwood, MA, USA). The span length was set to 18 mm and a compressive force was applied to the middle femur at constant speed of 2 mm/min. Before testing, all samples were thawed at room temperature for 1h and the test was automatically stopped once failure. The maximum bending force and stiffness were calculated based on the force-displacement curve.

Serum analysis
All serum samples were processed as described above.

Circulating concentrations of calcium (Ca) and phosphate (P) levels (Beckman Coulter, Brea, CA, USA), together with total 25-OH vitamin D3 content (Siemens Healthcare Diagnostics, 10699533, Mishawaka, IN, USA) were measured by commercially available kits following the manufactures instructions. Serum enzymatic activities of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) were also tested (Beyotime, P0321 and P0332, Shanghai, China).

Osteogenic capability
Soon after decapitation, right tibia with femur from each rat were immediately separated and shortly immersed in 70% ethanol, and stored in sterile PBS (1% penicillin-streptomycin, and 0.1% fungizone) afterwards. The epiphyses of each bone were removed with a tissue scissor and discarded, bone marrow was then flushed out from the diaphysis with a syringe, and collected in culture medium (α-MEM containing L-glutamine, nucleosides, supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.1% fungizone). After filtration and centrifugation (800 rpm/min for 3min), the cell sedimentation was mixed with primary culture medium and plated at 5 × 10⁶ cells/6 cm tissue culture dish. On day 7, nonadherent cells were removed by aspiration, and adherent cells were replenished with secondary medium (primary medium supplemented with 50 µg/ml L-ascorbic acid, 10 mM β-glycerophosphate and 100 nm dexamethasone) to induce mesenchymal cells to form osteoblast. Subsequent media changes were performed every other day for up to 2 weeks. ALP activities of cells were measured after 7 days’ induction while calcium nodules were stained by alizarin red after 14 days’ induction.

Statistical analysis
Statistical analysis was performed using the SPSS v.20 software (SPSS Inc., IL, USA), and all data were expressed as means ± SD. Repeated measurement of one-way ANOVA with subsequent Tukey post hoc test was used to analyze body weight, blood ketone and glucose levels, while one-way ANOVA with Tukey post hoc test was selected to compare the rest statistics between dietary groups. P values of less than 0.05 were considered significant.
Results

Body weight, blood ketone and glucose levels

Before differential feeding, body weight, blood ketone and blood glucose levels of the three groups showed no difference.

All groups constantly gained weight throughout the experimental period with initial body weight all around 280g to 300g. During the entire experiment, the weight of the KD and EODKD groups were both significantly lower than the Control group ($P=0.042$ and $P=0.013$ respectively), however, the KD and EODKD showed similar level throughout the experiment ($P=0.987$) (Fig. 1a).

Both KD and EODKD group reached a peak ketone level within the first 3 weeks, and they were significantly higher than the Control group ($P<0.001$). EODKD kept a higher though fluctuant level than KD ($P=0.016$) (Fig. 1b).

On the contrary, the KD and EODKD groups showed decreased glucose levels than the Control group ($P=0.005$ and $P=0.011$), and no difference was found between the KD and EODKD ($P=0.232$) (Fig. 1c).

Body fat percentage, BMD and bone length

The KD group had significantly higher body fat percentage compared with the Control group, (Control: 33.42 ± 3.27%; KD: 40.52 ± 3.56%, $P=0.034$), but was similar with EODKD (45.03 ± 5.17%, $P=0.179$). Correspondingly, the BMD of KD were lower than the Control group (Control: 0.164 ± 0.007 g/cm$^2$; KD: 0.151 ± 0.005 g/cm$^2$, $P=0.006$), and no significance was found between KD with EODKD (0.149 ± 0.006 g/cm$^2$, $P=0.858$).

All groups had similar length of tibia (Control: 45.27 ± 1.60 mm; KD: 45.00 ± 1.35 mm; EODKD: 45.37 ± 1.46 mm) and femur (Control: 41.31 ± 2.01 mm; KD: 41.00 ± 0.68 mm; EODKD: 40.00 ± 1.66 mm).

Microstructure of proximal tibiae

Three-dimensional reconstruction and micro-CT images were shown in Fig. 2, and detailed micro-CT parameters were shown in Table 2. In cancellous bone, the KD and EODKD groups showed lower TMD, BV/TV,
They also both showed a statistically decrease in Tarea, Barea and Ct.Th in cortical bone. Compared with KD, EODKD showed lower mean level of TMD, but no statistical significance was found as it had a high standard deviation. And no difference was found between the KD and EODKD groups in all the other micro-CT parameters as well ($P>0.05$).

**Three-point bending tests**

The Fmax of femur in the KD group was $144.3 \pm 38.2$ N, the Stiffness was $289.2 \pm 40.4$ N/mm, lower than the Control group ($203.6 \pm 37.7$ N, $P=0.012$ and $395.6 \pm 75.6$ N/mm, $P=0.011$). The EODKD showed similar level of Fmax ($144.8 \pm 13.0$ N, $P=1.00$) and Stiffness ($308.0 \pm 57.9$ N/mm, $P=0.856$) with the KD.
Serum calcium, phosphate, vitamin D level and ALP, TRAP activities

All three groups had same levels of calcium (Control: 2.52 ± 0.06 mmol/l; KD: 2.51 ± 0.09 mmol/l; EODKD: 2.40 ± 0.14 mmol/l), phosphate (Control: 2.30 ± 0.21 mmol/l; KD: 2.31 ± 0.24 mmol/l; EODKD: 2.24 ± 0.25 mmol/l) and 1,25-(OH)₂D₃ (Control: 35.95 ± 3.51 ng/ml; KD: 35.00 ± 2.64 ng/ml; EODKD: 36.25 ± 1.35 mmol/l) (P>0.05). The KD group had significantly lower ALP activities and higher TRAP activities when compared with the Control group (Fig. 3a). The EODKD demonstrated similar ALP level with the KD, but the TRAP level was lower than the KD and higher than the Control (Fig. 3b).
BMSCs osteogenic capability

After 7-day osteogenic induction, the ALP activities of cells were lower in the KD group compared with the Control group ($P<0.001$), but were significantly higher when compared with the EODKD group ($P<0.001$) (Fig. 4a). After 14-days induction, the AR stain of calcium nodules in three groups displayed in Fig. 4b, and the tendency was consistent with the ALP activities.

Discussion

The present study demonstrated that by feeding for 12 weeks, both KD and EODKD led to compromised cancellous and cortical bone mass in the hind limb of rats, with degraded mechanical strength as well. The potential mechanisms may include both inhibited osteogenic and enhanced osteoclastic process. EODKD demonstrated both lower bone absorption activities and osteogenic differentiation compared with KD, however, it may not induce exaggerated bone loss in general.

The synthesis of higher-than-normal levels of ketone bodies usually derives from prolonged fasting, which in fact is a state of absence or scarcity of dietary carbohydrates [22]. KD limits daily carbohydrates intake, simulating the fasting process, meanwhile, it provides enough fat for ketone production, thus may lead to a more efficient ketogenic state. In the ketogenic model of our study, the KD group led to significantly higher ketone levels but lower glucose levels than the Control group during the whole experiment, indicating the success of the model establishment. Meanwhile, the EODKD resulted in an even higher ketone level than the KD, suggesting synergistic effects between KD and fasting in ketogenic process, but the discontinuous feeding pattern may cause fluctuation in serum ketone at the same time. The relationship of both KD and EODKD with bone mass could be explained from the change of body weight and fat content. Increased body weight has been considered a protective factor against osteoporosis as the mechanical stimulating and related hypertrophy of muscles increase bone mass [1], while a low body weight is a well-documented risk factor for fracture [7, 10, 14, 28, 30]. However, in patients with obesity, a high body fat percentage may lead to declined BMD since increased fat mass may interfere with bone metabolism through mechanical, hormonal and inflammatory factors [17, 18]. Clinically, there is strong supportive evidence that the use of KD in weight-loss is effective [3, 15, 32]. In our study, the KD and EODKD both had higher fat percentage than the Control, but body weight were also lower in these two groups, suggesting they not only suffered from the negative influence of accumulated fat mass but also were short of the stimulating effects from body weight compared with the Control group. Thus theoretically, they should have lower BMD, which matches with the results from DEXA, a golden standard for diagnosis of osteoporosis [23].

Generally, DEXA could only provide a macro data when used in rats. In order to compare the microstructure of bones between groups, we performed micro-CT analysis. As in the results of our study, both KD and EODKD resulted in reduced trabecular number and thickness, but larger separation. Besides, they led to decreased cortical bone thickness as well. Further three point bending test compared the overall mechanical properties between three groups, and the results were consistent with the that of micro-CT. These indicated that EODKD could also lead to osteoporotic effects in the long bones of rats. However, it caused the same level bone loss and decline in mechanical properties as KD, which was out of our expectations. The explanation may include that EODKD and KD showed same level body weight as well as body fat percentage during the whole experiment, indicating they influenced bone mass in a same macro level. On the other hand, as in the cancellous bone, after 12 weeks of feeding, EODKD demonstrated lower mean level of TMD than KD, but the high standard deviation eliminated the statistical significance. This suggested that although they showed similar level of BMD from the whole body, EODKD may somehow lead to lower bone density in an isolated bone compared with KD, but the effects on isolated bones would be fluctuant just like the ketone levels, and undeniable, a larger number of samples would be better to confirm the effects in future studies, which was a limitation of our study. Besides, the KD group has shown severe bone microstructure destruction, in which only 25% BV/TV, 19% TMD and 39% Tb.N remained compared with the Control, thus EODKD may not be able to cause more serious cancellous bone changes on this basis.

We further compared the potential difference in bone metabolism between three groups. The maldevelopment of bone microstructure induced by diets may result from lack of mineral absorption, as low-carbohydrate high-fat diets may affect digestibility of minerals and trace elements [9]. The calcium and phosphate in the ketogenic
diet of our study was relatively lower than the standard diet. However, these nutrients have already met the criterion of AIN-93 [24], which could fulfill the normal development of rats. Meanwhile, the serum calcium, phosphate and total 25-OH vitamin D₃ levels were actually the same among the three groups, suggesting mineral content in the diet was not the key factor of bone loss under ketogenic state.

In fact, the change in bone mass reflects the balance of bone turnover. Serum ALP is closely related with osteoblasts while TRAP is secreted by osteoclasts. The activities of these two enzymes under different diets suggested that KD and EODKD may inhibit bone formation and enhance bone resorption at the same time. But the expression of TRAP was lower in EODKD than KD in the serological test, demonstrating that EODKD and KD may have different effects on osteoclastic activities in bone resorption process. Previous studies found that semistarvation may lead to bone loss and inhibit skeletal turnover, but won’t change serum bone-specific alkaline phosphatase (BALP) level [27]. Moreover, fasting may also reduce the duration of increase in β-CTX, a bone resorption marker, but have no alterations on bone formation markers [26]. These may help explain why EODKD led to the same level bone formation markers but lower bone absorption markers compared with KD in serum.

On the other hand, we put the study further into early cellular level. BMSCs can be differentiated into osteoblast-like cells when cultured in α-MEM media supplemented with vitamin C and β-glycerophosphate. During the process, ALP is an early differentiation marker and is associated with organic bone matrix synthesis before its mineralization [4]. In the present study, the ALP activities and the formation of mineralizing nodules decreased in the BMSCs derived from rats fed by KD and EODKD, indicating negative effects of KD and EODKD on the osteogenic differentiation of BMSCs. More importantly, both the ALP activities and calcium nodules were significantly lower in EODKD than that of KD.

There were studies reported that bone marrow adipose tissue (BMAT) could increase markedly during starvation or calorie restriction due to survival adaptation [8], and it may elevate in patients with anorexia nervosa [2]. Combined with the results of our study, EODKD may cause serum fatty acids conversion to ketone bodies rather than lipid mobilization during fasting days. On the contrary, the adaptive mechanism of increased BMAT and the gluttony after intermittent fasting may contribute to fat deposition. Thus we speculated a shift away from the osteoblast lineage would send more cells down the adipocyte lineage [19, 21] in the differentiation of BMSCs during intermittent fasting, leading to the lower osteogenic differentiation of EODKD than KD.

In fact, previous research has revealed the direct relationship between 3-hydroxybutyrate as well as its derivatives (3-hydroxybutyrate methyl ester) and osteoclast, they found that they could inhibit the development of osteoporosis in mice maintained under simulated microgravity, helping preserve bone microstructure and mechanical property. They found 3-hydroxybutyrate and the derivatives could down-regulated the nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), which is the transcription factor of pre-osteoclast differentiation, leading to the prevention of bone absorption [5]. Meanwhile, Zhao et al. revealed 3-hydroxybutyrate could stimulate osteoblast differentiation as well as increase serum ALP activity and calcium deposition, and prevent BMD reduction resulting from ovariectomy [37]. However, a controversial result was found by Saito et al., in which acetocetate potentiated alkaline phosphatase activity in mouse primary osteoblasts in a concentration-dependent manner, while β-hydroxybutyrate lowered it in the same experimental settings [25]. In our study, we thought the influence of KD as well as EODKD on bone mass would not only depend on the ketone level but also on the macroscopic change of rats such as body weight and fat percentage since the ketogenic diet used in the experiment was essentially a mixture. EODKD and KD demonstrated same level body weight and body fat percentage, resulting in the same change in bone mass and microstructure. But the different ketone level between EODKD and KD indicated they may have difference in bone metabolism at serological and cellular level, which has been proved in our study. However, the direct effects of ketone bodies, including β-hydroxybutyrate, acetocetate and even acetone are of potential values to be explored to better reveal its osteoporotic or anti-osteoporotic effects.

Several limitations of the study should be mentioned. First, a continuous bone change should be observed at multiple time points under different diets. Second, more markers of bone turnover in both serological and molecular level during different stages as well as histological samples should be monitored as well as histological tests. Moreover, detection in the changes of sex
hormone and serum lipid level may help further explain the difference of KD and EODKD on bone metabolism.

In conclusion, the present study suggested that EODKD, the combination of KD and every-other-day fasting which has been developed for improvement of the ketosis caused by KD, could accelerate ketosis, indicating its stronger ketogenic ability and better seizure-control potential. However, it may not induce exaggerated bone loss than KD. It resulted in both suppressed bone resorption activity and osteogenic differentiation compared with KD, but the general effects on bone microstructure and mechanical strength were similar between them. This suggested that similar to the ketone level, bone has any better effects in intermittent ketogenic diet than in KD, thus EODKD could be a preferable clinical choice in the future.

Conflict of Interest

All authors declare that they have no conflict of interest.

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