Life-long caloric restriction does not alter the severity of age-related osteoarthritis

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Abstract Chronic adipose tissue inflammation and its associated adipokines have been linked to the development of osteoarthritis (OA). It has been shown that caloric restriction may decrease body mass index and adiposity. The objectives of this study were to investigate the effect of lifelong caloric restriction on bone morphology, joint inflammation, and spontaneously occurring OA development in aged mice. C57BL/NIA mice were fed either a calorie-restricted (CR) or ad libitum (AL) diet starting at 14 weeks of age. All mice were sacrificed at 24 months of age. Adipose tissue and knee joints were then harvested. Bone parameters of the joints were analyzed by micro-CT. OA and joint synovitis were determined using histology and semiquantitative analysis. Lifelong caloric restriction did not alter the severity of OA development in C57BL/NIA aged mice, and there was no difference in the total joint Mankin score between CR and AL groups ($p=0.99$).

Mice also exhibited similar levels of synovitis ($p=0.54$). The bone mineral density of the femur and the tibia was comparable between the groups with a small increase in cancellous bone volume fraction in the lateral femoral condyle of the CR group compared with the AL group. Lifelong caloric restriction did not alter the incidence of OA or joint synovitis in C57BL/NIA mice, indicating that a reduction of caloric intake alone was not sufficient to prevent spontaneous age-related OA. Nonetheless, early initiation of CR continued throughout a life span did not negatively impact bone structural properties.

Keywords Osteoarthritis · Bone density · Synovitis · Inflammation · Osteoporosis

Introduction

Osteoarthritis (OA) is the most prevalent form of arthritis, affecting approximately 27 million people in the USA (Felson et al. 2000). OA is characterized by degeneration of the articular cartilage, synovial inflammation, osteophyte formation, and remodeling of the subchondral bone (Goldring 2012). The primary risk factors for OA include joint trauma, obesity, and aging. In particular, individuals with lifelong obesity have a two out three risks for OA development (Hunter et al. 2007b). However, the mechanisms linking obesity or adiposity with OA are not fully understood and may involve a complex interaction of genetic, biomechanical, and metabolic factors (Aspden 2011; Guilak 2011; Wu et al. 2013). In animal models, higher body weight

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has been associated with spontaneous OA in guinea pigs (Kalichman et al. 2007b) as well as mice (Kalichman et al. 2007a).

In addition to the increased mechanical loading and/or altered joint alignment due to weight gain, obesity has been associated with chronic inflammation in adipose tissues (Hugle et al. 2012). Adipose tissue has the ability to secrete cytokines (i.e., “adipokines”), such as interleukin (IL)-1, IL-6, and tumor necrosis factor- α (TNF- α) that have been associated with development of OA (Pou et al. 2007). These pro-inflammatory cytokines have the ability to upregulate metalloproteinases and disrupt the synthesis of the extracellular matrix, leading to degradation of cartilage (Fernandes et al. 2002). Our previous studies and those of others have shown that diet-induced obesity significantly increased OA severity, as well as serum concentrations of IL-6 and IL-12 in obese mice following joint injury (Louer et al. 2012). Additionally, the severity of spontaneous OA in the mouse has been associated with higher serum levels of leptin, adiponectin, and IL-1 α (Griffin et al. 2010). Furthermore, mice lacking leptin or leptin receptors exhibit morbid obesity but no OA (Griffin et al. 2009). The results from these studies suggest that factors other than body weight, such as adipose tissue inflammation associated with obesity, may play a significant role in OA, particularly with age.

Caloric restriction (CR) in animals decreases energy intake to a relatively low level while still maintaining proper nutritional status (Fontana and Klein 2007), and a number of studies have shown that reduced caloric intake can moderate adverse health outcomes, especially those associated with obesity and aging. McCay et al. (1935) reported that lifelong CR extended the median and maximum lifespan of rats (Fontana and Klein 2007). Since that initial study, further experiments have demonstrated that CR decreases the likelihood of developing chronic diseases such as type 2 diabetes, neurodegeneration, and atherosclerosis (Fontana and Klein 2007; Masoro 2005; Weiss and Fontana 2011). The mechanisms by which CR is capable of disease prevention may involve an increase in DNA repair, reduction in reactive oxygen species, and inhibition of production of inflammatory cytokines such as IL-1, IL-6, and TNF- α (Paolisso et al. 1998; Weiss and Fontana 2011; You et al. 2007). These factors, together with increased body weight, have been implicated in the pathogenesis of spontaneous OA with age (Amin et al. 2007). Lifelong CR has been shown to decrease body mass index

(BMI) and adipose tissue percentage of total body weight (Fontana and Klein 2007). The reduction of adipose tissue, and thereby its associated inflammation, may decrease the possibility of developing a metabolic syndrome, which is typically characterized by insulin insensitivity, dyslipidemia, and hypertension (Weiss and Fontana 2011). Previous studies have demonstrated that dogs with CR have reduced shoulder OA, 6-year postdiet intervention, and also show decreased incidence of a combination of shoulder and hip OA (Kealy et al. 2000; Runge et al. 2008). Additionally, the implementation of CR has been linked to greater joint mobility as well as lower triglycerides in a canine OA model (Kealy et al. 2000; Lawler et al. 2008). A more detailed analysis of the effects of CR on arthritic bone and cartilage changes, and joint inflammation could provide further insight into the mechanisms by which diet and body weight influence OA development with age. In the present study, we investigated the effect of CR on naturally occurring knee OA, bone remodeling, and joint inflammation using an aged (24 months) murine model of lifelong CR.

Materials and methods

Animals All aspects of this study involving procedure and animal models were approved by the Duke University Institutional Animal Care and Use Committee. Seven male mice (C57BL/NIA) were obtained from the CR colony at the National Institution of Aging (NIA, NIH). Food feeding was based on the instruction provided by NIA (Turturro et al. 1999). Briefly, CR was initiated at 14 weeks of age at 10 % restriction of a NIH-31-fortified diet, 25 % restriction at 15 weeks, and 40 % restriction at 16 weeks where it was then maintained until sacrifice. Ten aged control male mice (C57BL) were fed *ad libitum* (AL). AL mice were fed NIH-31-fortified diet supplied as a constant food source. All mice were solitary housed in filter-top cages and received a constant water source. Each group remained on their respective diets until the sacrifice time point of 24 months of age.

Body weight and visceral adipose tissue Body mass was recorded throughout the 24 months and at sacrifice. A total body weight was averaged from these measurements. Upon sacrifice, the visceral fat (epididymal fat pad) was harvested and weighed. The weights of the

visceral fat pads were then normalized to the total body weight to obtain the percentage of visceral adipose tissue of total body weight.

Evaluation of joint bone morphology Bone morphology was assessed using desktop micro-computed tomography (micro-CT) system (Micro-CT 40 System; Scanco Medical) from the harvested hind limbs prior to histologic preparation. The bone morphometric parameters evaluated were the distal medial and lateral femoral condyles, the tibial epiphysis, and the tibial metaphysis. The parameters analyzed at the femoral condyles were cancellous bone fraction (bone volume/total volume (BV/TV)), bone mineral density (BMD; mg/cm³), cancellous bone thickness, and cancellous bone space. For the tibial epiphysis analyses, the parameters were cortical and cancellous bone fraction, BV (cm³), and BMD. For the tibial metaphysis, the parameters analyzed were BV and BMD.

Evaluation of OA The mice did not experience any additional manipulation or treatment, other than the dietary restriction, that would have impacted their hind limb joints. Only the right hind limb was analyzed. Upon sacrifice, hind limbs were removed and placed into 10 % buffered formalin at neutral limb alignment. The limbs were then processed through standard histological preparation, which involved decalcification in Cal-ex (Fisher Scientific), dehydration, and paraffin embedding in a tissue processor. Joints were then coronally sectioned at a thickness of 8 µm. The sections were stained with fast green and Safranin O, and then evaluated with the modified Mankin grading scheme by three independent graders who were blinded with regard to the diet group. The scores were compiled through the evaluation of the mice articular cartilage, which is assessed through the following categories of articular structure: tidemark duplication, loss of Safranin O staining, fibrocartilage formation, chondrocyte cloning above the tidemark, hypertrophic chondrocytes below the tidemark, and overall subchondral bone thickness. Each grader provided a total joint score as well as scores for each individual quadrant (lateral femur, lateral tibia, medial femur, and medial tibia). The maximum total score ranged from 0 to 120.

Evaluation of synovitis Separate joint sections were stained with hematoxylin and eosin. Three blinded graders assessed the thickness of synovial lining layer

and cell density in the synovium. The graders provided a score for each joint, as well as each quadrant (lateral femur, lateral tibia, medial femur, and medial tibia). For synovial lining thickness and synovial stroma density, scores ranged from 0 to 3. Each score was averaged for the individual quadrant and for the total joint (maximum score of 24 per joint).

Evaluation of behavior Velocity and force data were collected on all mice three times during the last 5 months prior to sacrifice using a pressure platform according to our previous study (Schmitt et al. 2010). Briefly, the pressure platform collected pressure at 50 Hz with four sensors/cm² over a 26×40 cm area. Mice were allowed to move freely along an open runway (1.5 m) with a pressure plate modified to collect single contacts embedded flush with the runway. Videos were recorded at 240 images/s.

Statistical analysis All statistical analyses were performed using one-way analysis of variance (ANOVA). Results were considered statistically significant if *p* value was less than 0.05. All data were reported as the mean ± SEM. JMP 8 (SAS Institute) was used to perform the analyses.

Results

Body mass and visceral adipose tissue Aged mice with free access to food had significantly higher body weights compared with the aged CR mice. Body mass was 31.2±0.94 g for AL and 26.9±1.12 g for CR (Fig. 1a, *p*=0.01). However, visceral fat mass was not significantly different between the two groups. AL had 0.29±0.05 g of visceral fat, and CR had 0.33±0.05 g (*p*=0.54). Visceral fat as a percentage of total body weight was 0.009±0.001 for AL and 0.011±0.002 for CR (Fig. 1b, *p*=0.19).

Morphologic assessment of bone 3D images of the joints are presented in Fig. 2. Joint BMD of the femur and tibia was not significantly different between the two diets (Table 1). The cancellous BV/TV at the lateral femoral condyle was significantly higher in the CR group in comparison to the AL (*p*=0.02); however, the cancellous bone space, thickness, and BMD at this location did not differ between the two groups.

Fig. 1 **a** AL mice were significantly heavier than CR mice at time of sacrifice (* $p=0.01$). **b** Visceral fat percentage of total body weight was not significant between the AL and CR groups ($p=0.19$)

Furthermore, none of the bone morphologic parameters were different at the medial femoral condyle between the two diet groups. The BV at the tibial epiphysis and metaphysis were similar in both diet groups.

Histologic evaluation of knee joint OA Joints from CR and AL mice exhibited similar proteoglycan content and cartilage surface (Fig. 3a). The modified Mankin scores for the total joint of the right limb demonstrated comparable levels of OA (Fig. 3b, $p=0.99$) for both groups. The lateral ($p=0.37$) and medial ($p=0.46$) sides of the joints were analyzed separately and were not found to be significantly different. This trend continued for each quadrant: Mankin scores did not demonstrate significant differences at the lateral femoral ($p=0.88$), lateral tibia ($p=0.16$), medial femoral ($p=0.28$), or medial tibia ($p=0.96$). The articular cartilage structure scores ($p=0.76$) and the hypertrophic chondrocyte scores ($p=0.64$) were also comparable between the two diet groups. CR did not change OA severity or alter articular cartilage structures in comparison to AL mice.

Histologic evaluation of knee joint synovitis No differences in joint synovitis were observed in the joints of CR and AL mice (Fig. 4a). Quantitative assessment of the synovium of the right knee joint demonstrated no differences between the AL and CR group (Fig. 4b, $p=0.54$). Further analysis was separately performed on the stromal cell density and synovial lining-layer thickness. The AL mice had a greater stromal cell density though statistically not significant ($p=0.29$). The lining layer thickness was also similar between the two groups ($p=0.99$). Quadrant analysis showed that the joint inflammatory level was similar in the diet groups. CR did not reduce the degree of synovitis in the knee joint.

Activity levels No significant differences were found between velocities (AL = 0.64 ± 0.25 m/s; CR = $0.67 \pm$

0.24 m/s; $p=0.58$) or the ground reaction forces (AL = 0.73 ± 0.14 N; CR = 0.79 ± 0.19 N; $p=0.1$).

Discussion

This study demonstrated that lifelong calorie-restriction did not alter the severity of OA in C57BL/NIA-aged mice. The CR and AL mice also exhibited similar levels of synovitis. While some changes in bone density were observed between the two groups, these results imply that decreased caloric intake alone was not sufficient to prevent age-associated joint degeneration in mice, and further suggest that spontaneous OA is not associated with the caloric intake of a standard diet.

These findings are consistent with previous studies examining radiographic OA in large animals showing that CR, when initiated early and continued throughout life, has little or no effect on OA incidence or severity. For example, dogs fed a regular diet demonstrated greater radiographic severity of OA in the elbow in comparison to CR dogs after 6 years of diet treatment, but at year 8 (end of life mark), OA severity of both groups was not statistically different (Huck et al. 2009). Similarly, a study that examined dogs with and without diet intervention for an entire lifespan found that there was only a 9 % decrease in OA incidence in the CR group (Kealy et al. 2002). These findings indicate that CR may delay the onset of symptoms or radiographic evidence of OA but may not ultimately prevent the development of OA (Kealy et al. 2002).

Additionally, the lack of difference in velocities and loading of the limbs between the AL and CR mice suggests that all mice were capable of similar levels of activity and limbs were loaded to similar levels during spontaneous locomotion. These results imply that long-term CR did not alter activity in aged mice. However,

Fig. 2 3D micro-CT reconstruction of knee joints ate 24 months of age. *AL* ad libitum, *CR* caloric restriction, *F* femur, *T* tibia, *P* patella

Table 1 Bone morphology measures in each group at 24 months

Location and measurement	Ad libitum (N=10)	Caloric restricted (N=7)	P value
Medial femoral condyle			
BV/TV	0.65±0.02	0.66±0.02	0.76
Cancellous bone thickness	0.06±0.003	0.06±0.004	0.96
Cancellous bone space	0.05±0.003	0.05±0.003	0.84
BMD (mg/cm ³)	1,159±12	1,159±14	0.97
Lateral femoral condyle			
BV/TV	0.59±0.03	0.71±0.04	0.02*
Cancellous bone thickness	0.05±0.003	0.05±0.004	0.88
Cancellous bone space	0.05±0.004	0.05±0.006	0.94
BMD (mg/cm ³)	1,132±26	1,131±31	0.98
Tibial epiphysis			
BV	0.52±0.04	0.56±0.05	0.55
BV/TV	0.36±0.02	0.42±0.02	0.06
BMD (mg/cm ³)	1,124±11	1,089±13	0.06
Tibial metaphysis			
BV	0.65±0.06	0.64±0.07	0.9
BMD (mg/cm ³)	1,060±16	1,010±19	0.06

BMD bone mineral density, BV bone volume, TV total volume

our findings differ from those of the study conducted by Means and co-workers, in which they demonstrated that mid-life onset of CR increased spontaneous alternation behavior (Means et al. 1993). The discrepancy may result from the age when the CR was initiated. Means and colleagues initiated CR at mid-life onset (12 months old), while we initiated at 14 weeks (3.5 months of age). Previous studies have demonstrated that short-term effects of CR, such as change in arousal and exploratory behavior, may be seen longitudinally throughout life (Forster and Lal 1999). In addition, our previous animal models showed that spontaneous locomotion is not altered by diet-induced obesity or OA severity in obese mice (Griffin et al. 2010, 2012), although severe OA induced by genetic collagenopathy may significantly alter locomotor activity (Costello et al. 2010). In humans, it also has been reported that recreational activities do not contribute to knee OA in normal and overweight individuals (Ageberg et al. 2012; Felson et al. 2007).

In addition to aging, obesity is another primary contributing risk factor for OA due to increased levels of adipokines and inflammatory cytokines (Fernandes et al. 2002; Griffin and Guilak 2008; Louer et al. 2012). Several studies have reported that animals allowed free access to food demonstrated increased OA severity and body adiposity. For example, guinea pigs placed on a restricted diet for 18 months had significantly decreased abdominal fat depot and less joint

Fig. 3 **a** Safranin O (glycosaminoglycans) and fast green (bone and tendon) histology in the AL and CR knee joint (*F* femur, *M* meniscus, *T* tibia). *Top*, lateral side; *bottom*, medial side. No significant difference in articular cartilage between AL and CR.

Bar=100 μ m. **b** Mankin scores representative of OA severity. The joints from AL and CR had comparable total joint Mankin scores. Mean $\pm$ SEM

degeneration compared with the AL group (Bendele and Hulman 1991). These findings imply that tissue adiposity and/or the levels of adipose tissue inflammation may have a potential association with OA severity. In the present study, although AL mice were significantly heavier than CR mice at the time of sacrifice, AL mice did not develop obesity. Furthermore, the percentage of visceral fat in the mice with continuous food access was not significantly different from the CR mice. Nonetheless, the comparable OA scores between the two diet groups in this study suggest the possibility that any differences in systemic inflammation between AL and CR mice were not great enough to alter OA severity.

It is also hypothesized that mechanical factors such as weight play an important role in obesity-associated OA (Hunter et al. 2007a; O'Connor et al. 2013). However, recent studies have emphasized the significance of metabolic factors in arthritic changes of the joints, as body mass alone may not be the only contributor in musculoskeletal diseases (O'Connor et al. 2013). For example, previous studies have shown that extremely obese mice (*ob/ob* and *db/db* strains) did not develop OA when fed a regular chow (Griffin et al. 2009). Furthermore, exercising obese mice that were fed a high-fat diet reduced the severity of OA in the absence of weight loss (Griffin et al. 2012). Numerous studies also demonstrated that

Fig. 4 **a** H&E histology of the lateral femoral and tibial condyle of AL and CR joints (*S* synovium). The synovium from AL and CR had similar thickness as well as density. *Bar*=100 μ m. **b**

Synovitis scores are representative of synovial inflammation. The joints from AL and CR synovial inflammation. Mean $\pm$ SEM

increasing body mass through an increase in standard chow consumption was not associated with an increase in OA development (Griffin and Guilak 2008; Silberberg and Silberberg 1950; Silberberg 1976). These results indicate that the amount of increase or decrease in body weight or caloric intake cannot solely explain OA development in obese animals. In this current study, AL mice were 16 % heavier than CR mice but did not exhibit more OA, further supporting this growing evidence. This finding supports a previous report showing that lifetime CR does not affect biomechanical properties of articular cartilage in rats (Athanasίου et al. 2000).

Reduced body mass due to CR has been shown to decrease BMD (Hawkins et al. 2010), which may be explained by mechanisms such as a decreased loading of the bones, less calcium intake from food, or changes in hormonal levels (Hawkins et al. 2010). Interestingly, we did not observe any alterations of BMD in the femoral condyles in our CR model. We did observe a trend toward increasing BMD in the epiphyseal and metaphyseal region of the tibia in the CR mice. This finding suggests that CR did not negatively affect bone structural properties when initiated early and continued through the entire lifespan. Previous studies have demonstrated that CR enhances bone structural properties in comparison to AL in rat models (Talbot et al. 2001; Westerbeek et al. 2008). The beneficial effect of CR on bone properties could be potentially due to the downregulation of reactive oxygen species, particularly the ones generated by osteoclasts (Brochmann et al. 2003). These oxygen-related free radicals have been shown to stimulate osteoclast differentiation and bone resorption (Garrett et al. 1990).

To assess the effect of lifetime CR on joint degenerative disease, we used C57BL/NIA-aged mice as our model, which have been bred for longevity. However, it is unknown whether these aged mouse colonies may be less prone to metabolic diseases and OA in comparison to other mouse models. Furthermore, we only evaluated OA at one time point in the current study, and future longitudinal investigations could shed light on the influence of CR on the onset and development of OA at various time points.

In summary, our results indicate that lifelong CR in C57BL/NIA-aged mice did not alter the incidence of OA development and joint inflammation. Animals with a normal diet controlled their body weight through natural signals for satiety as well as normal activity levels did not show higher levels of OA than animals whose weight was controlled by a low calorie diet.

These results and those of other studies suggest that obesity or dietary composition may play a more significant role than tissue adiposity in joint degeneration. This study extends our understanding of the links between CR diet and OA as well as can provide new insights into the development of diet intervention strategy for individuals with OA.

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