INTRODUCTION

Age-related osteoporosis adversely affects quality of life and the healthcare system. As of 1995, it was estimated that osteoporosis-related fractures cost the United States healthcare system $13.8 billion annually (Ray et al., 1997). With an increase in global life expectancy, osteoporosis-related fractures have the potential to become an even larger problem in the future (Hurley and Khosla, 1997) and strongly justify gerontological research investigating aging and bone health. Human studies are complicated by the difficulty of obtaining age-specific tissue samples, and longitudinal studies are complicated by the long lifespan of humans. Thus, surrogate animals are commonly used. However, age-associated disease, neoplastic lesions, and degenerative lesions may confound results obtained from inbred animal studies. For example, numerous physiologically significant lesions, such as chronic nephropathy, are more common in aged F344 rats than in other strains (Canalis and Delany, 2002). In contrast, the Fischer 344 x Brown Norway F-1 hybrid rat, developed by the National Institute on Aging (NIA) for aging research, lives considerably longer (Turturro et al., 1999), and has fewer pathologies for any given age versus inbred strains.

Caloric restriction (CR) is one paradigm that consistently extends the lifespan of animals and significantly reduces the incidence of age-related diseases and lesions regardless of species or sex (Lipman et al., 1999). Despite the beneficial aspects of CR on mortality and disease, diet affects bone health (Anderson et al., 1996), and thus CR paradigms may adversely affect bone mechanics.

The NIA Biomarkers of Aging Program has primary objectives to identify rodent biomarkers associated with aging to determine the efficacy of aging interventions (Turturro et al. 1999). NIA studies use Brown Norway (BN), Fischer (F344) and F1 F344 X BN hybrid rats (F344BN) fed ad libitum diets rats. An additional group of rats was calorically restricted (CR) as defined by the methods of Turturro et al. (1999) and assessed at 28 mo (n=6). Beginning at 14 wk of age, CR animals were introduced to a reduced caloric intake (60% of the calories of the mean food intake of the ad libitum group), while maintaining nutrition equivalent to the ad libitum group, incrementally over 3 wk at the NIA. The diet continued at the University of Calgary until euthanization.

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Bone Preparation- Immediately after euthanization via pentobarbital overdose, tibiae and L6 vertebrae were carefully dissected and cleaned of adherent non-osseous tissues. Bones were individually wrapped in saline soaked gauze (50 mM potassium phosphate buffer solution, pH = 7.4) and sealed hermetically in plastic bags that were frozen (–30°C) until biomechanical testing. Previous studies suggest that freezing and thawing does not adversely affect rat bone mechanical properties (Felker et al., 1984).

Bone Geometry- After fracture in three-point bending (see Bone Mechanics), fractured tibial surfaces were fixed together with cyanoacrylate adhesive. Fractured regions (cross head contact point) were subjected to µCT scanning (Skyscan 1073, Aartselaar, Belgium) at a magnification of 4X (resolution of 19 µm). Bitmap images generated from scanning were input to custom software (Matlab, Natick, MA) that thresholded images and calculated geometric parameters including total cross sectional area, cortical bone area, trabecular area, distances from centroid to the desired edge of the cross section, and cross sectional moment of inertia. To normalize for geometrical changes associated with total body mass, tibial length, total cross sectional area, and cortical bone area were divided by body mass.

Wafers of bone cut from the caudal surface of the L6 centra were scanned with µCT (Skyscan 1073, Aartselaar, Belgium) at a magnification of 23.87X (resolution of 11 µm). Bitmap images generated from scanning of the region that was in contact with the body of the centrum were thresholded and subjected to pixel counting routines to determine cross sectional area (Scion Image, Frederick, MD). Centrum height was measured with callipers and the
product of cross sectional area and centrum height defined $L_6$ volume.

**Bone Mechanics**- On the day of testing, tibiae and $L_6$ were thawed in 22°C buffer (50 mM potassium phosphate buffer solution, pH = 7.4) for at least 1 hr. When thawed, the round surfaced cross head probe of a servocontrolled electromechanical testing system (Model 1122, Instron Corp., Canton, MA) contacted the medial tibial surface at its longitudinal midpoint, between a 13.3 mm loading span and applied a preload of 5 N; the medial surface was in compression and the lateral surface was in tension. Testing order was stratified based on group, and load was applied at 25.4 mm/min until failure. Load-deformation curves were generated (RC Computerscope A/D Board, RC Electronics, Santa Barbara, CA). From the load-deformation curves, the following structural properties were determined: load at proportional limit, maximal load, and stiffness. As bone mineral density (BMD) and bone size are influenced by total body mass (Blain et al., 2002), and as BMD influences bone strength (Gatti et al., 2001), structural properties were normalized (divided by body mass). Material properties were calculated and included stress and strain at proportional limit, stress and strain at maximal load, and flexural rigidity (Zernicke et al., 1990).

Using established methods (Zernicke et al., 1990), caudal and rostral surfaces were cleaned of intervertebral discs and zygapophyses. To isolate the vertebral centrum, the neural spine and transverse processes were removed with a diamond wafer saw (Buehler Isomet, Lake Bluff, IL). To ensure parallel surfaces, a wafer was cut from the caudal surface bringing the total length of the tested centrum to 25.4 mm/min until failure. Load-deformation curves were generated (RC Computerscope A/D Board, RC Electronics, Santa Barbara, CA). From the load-deformation curves, the following structural properties were determined: load at proportional limit, maximal load, and stiffness. As bone mineral density (BMD) and bone size are influenced by total body mass (Blain et al., 2002), and as BMD influences bone strength (Gatti et al., 2001), structural properties were normalized (divided by body mass). Material properties were calculated and included stress and strain at proportional limit, stress and strain at maximal load, and flexural rigidity (Zernicke et al., 1990).

RESULTS AND DISCUSSION

Twenty-eight month-old and 36-month-old rats (490g ± 51) were significantly heavier than 8-month-old rats (441g ± 26). Body mass in 28-month-old CR animals (320g ± 12) was significantly lower than in the age-matched ad libitum fed animals (502g ± 36).

Tibial geometry did not change with age, with one exception—tibial cross sectional moment of inertia increased (>75% in 36 mo compared to 8 mo) possibly explaining the significant age-associated increase in tibial stiffness (>12% in 36 mo compared to 8 mo). The observed increase in cross sectional moment of inertia corresponded to age-related radial expansion of cortical bone seen in humans (Ruff and Hayes, 1988). In older human males, endosteal expansion was accompanied by periosteal bone apposition thereby maintaining cross-sectional area (Ruff and Hayes, 1988). By redistributing bone material farther from the cross-sectional centroid, bone strength can be maintained under conditions of decreased material strength (Ruff and Hayes, 1988).

As with the tibia, $L_6$ geometry did not change between 8 and 36 months, indicating that F344BN vertebral growth plateaued by 8 months of age. $L_6$ mechanics decreased with age (>10% reduction in all structural properties from 8 mo to 36 mo). Thus lower material properties accounted for the age-related decline in bone mechanical properties. Similar to our observed decreases in stiffness and initial maximum load in $L_6$, Sato et al. reported a decrease in stiffness and maximal load between 6 and 17 months in F344 rats (Sato et al., 2002).

![Exemplar tibial mid-diaphaseal cross sections for 28 mo and 28 mo CR rats](image)

Figure 1: Exemplar tibial mid-diaphaseal cross sections for 28 mo and 28 mo CR rats

Caloric restriction has been the one intervention that consistently increased mean and maximal male and female lifespan in nearly all species tested to date (Weindruch and Walford, 1988). In addition to the life-extending effect of CR, McCay and colleagues’ seminal studies chronicled the effect of long-term dietary restriction on body growth retardation (McCay et al., 1935). As bone growth is influenced by diet and body mass (Anderson et al., 1996), bone mechanics are altered with CR. The severe dietary restriction in those studies, however, likely induced calcium insufficiency (Lane et al., 1995) that adversely affected bone (Jiang et al., 1997). Subsequent studies, such as the current

**Statistics**- Means between groups were compared with Kruskal-Wallis nonparametric ANOVA. Mann-Whitney post hoc comparisons determined where inter-group significant differences were present. A significance level of $p \leq 0.05$ was used for all statistical tests.
study, investigated the effects of food restriction while maintaining nutritional sufficiency.

Distal femoral bone mineral content (BMC) decreased when restricted to 65% and 50% of ad libitum fed calories, but changes in BMC were entirely accounted by CR-associated body mass reduction (Sanderson et al., 1997). Lₘₖ structural properties in the current study were consistent with those earlier findings for the distal femur. Lₘₖ load at proportional limit and maximal load declined significantly with CR, but when normalized for body mass, significant differences disappeared (Figure 2). Both the distal femur and Lₘₖ are replete with trabecular bone. Most studies have found body mass a most significant predictor of BMD when assessing trabecular bone sites such as the vertebrae and femoral neck (Blain et al., 2002). Thus, body-mass-related changes in highly trabecular sites (e.g., distal femur and Lₘₖ) should be similar.

In contrast to Lₘₖ properties, changes in tibial diaphyseal shaft structural properties were not fully accounted for changes in body mass (Figure 2). That tibial material properties did not significantly decline with CR indicated that geometrical changes accounted for the majority of the changes in structural properties observed. When older (57 weeks) Sprague Dawley rats were fed 60% of the calories ad libitum fed rats received, a slight decrease in tibial density was observed (Talbott et al., 2001). With lower mineral, bone would not be as stiff and that could partially explain the decreased tibial flexural rigidity.

The mechanism by which CR affects bone is unknown. Concentrations of active glucocorticoids increase with CR (Leakey et al., 1995), and glucocorticoid treatment can disturb long bone growth in children (Blodgett et al., 1956). Alternatively, sex steroids decrease with CR and may stunt bone growth (Leakey et al, 1995).

**SUMMARY**

From 8 to 36 mo, there were no significant changes in Lₘₖ morphometry, and only the cross sectional moment of inertia changed (increased) with the tibia. CR-induced body mass reductions were consistent with changes in Lₘₖ, mechanics, but altered tibial mechanics were independent of body mass. In tibiae, geometrical changes dominated alterations in structural properties. These data demonstrated that whereas aging in ad libitum fed animals induced minor changes in bone mechanics, axial and appendicular bones are adversely and differentially influenced by CR.

**REFERENCES**

Lane et al. (1995) J Nutr 125, 1600-1610.
McCay et al. (1935) J Nutr 10, 63-79.
Sato et al. (2002) Endocrinology 143(9), 3230-3242.
Turturro et al. (1999) J Gerontol 54A(11), B492-B501.

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