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# The effects of knee immobilization on marrow adipocyte hyperplasia and hypertrophy at the proximal rat tibia epiphysis

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ABSTRACT

Marrow adipose deposition is observed during aging and in association with extended periods of immobility. The objective of this study was to determine the contribution of adipocyte hypertrophy and hyperplasia to bone marrow fat deposition induced by immobilization of the rat knee joint for 2, 4, 16 or 32 weeks. Histomorphometric analyses compared immobilized to sham-operated proximal tibia from age and gender matched rats to assess the contribution of aging and duration of immobilization on the number and size of marrow adipocytes. Results indicated that marrow adipose tissue increased with the duration of immobilization and was significant larger at 16 weeks compared to the sham-operated group (0.09956 ± 0.13276 $mm^2$ vs 0.01990 ± 0.01100 $mm^2$, p = 0.047). The marrow adipose tissue was characterized by hyperplasia of adipocytes with a smaller average size after 2 and 4 weeks of immobilization (at 2 weeks hyperplasia: 68.86 ± 33.62 vs 43.57 ± 24.47 adipocytes/$mm^2$, p = 0.048; at 4 weeks hypotrophy: 0.00036 ± 0.00019 vs 0.00046 ± 0.00023 $mm^2$, p = 0.027), and by adipocyte hypertrophy after 16 weeks of immobilization (0.00083 ± 0.00049 vs 0.00046 ± 0.00028 $mm^2$, p = 0.027) compared to sham-operated. Both immobilized and sham-operated groups showed marrow adipose conversion with age; immobilized (p = 0.008; sham: p = 0.003). Overall, fat deposition in the bone marrow of the proximal rat tibia epiphysis and induced by knee joint immobilization was characterized by hyperplasia of small adipocytes in the early phase and by adipocyte hypertrophy in the later phase. Mediators of marrow fat deposition after immobilization and preventive countermeasures need to be investigated.

## 1. Introduction

The bone marrow has been described as containing red hematopoietic cells and yellow fat cells: the adipocytes. Approximately 70% of all marrow spaces are filled with adipose tissue (Kirkland et al., 2002). And this adipose depot in the bone marrow represents up to 5% of the total fat mass in adults. During growth, red marrow is gradually converted to yellow marrow, a process that begins in the toes (Follett, 1964). The conversion of red into yellow marrow is described as a natural process that gradually continues over the human lifespan (Ricci et al., 1990; Gurevitch et al., 2007; Justesen et al., 2001). By the age of 25, red marrow is found mostly in lumbar vertebrae, pelvic bones and proximal femur. Bone marrow adipose conversion has been shown to be age, bone site, and gender dependent (Rozman et al., 1989; Moore and Dawson, 1990). Histological analysis of the rat femur or tibia has confirmed the increase of bone marrow adipocytes number with age in this animal model (Perrien et al., 2007). The pace of marrow adipose conversion is also subject to pathophysiological changes and accelerates in states such as osteoporosis, anorexia nervosa, and with anti-diabetes therapies. Recent work indicates that marrow adipose tissue can be exploited as a source of mesenchymal stem cells (MSC) in the treatment of degenerative diseases. MSC-based therapy combining bone marrow fat as a source of stem cells and tissue-engineering strategies have been tested to regenerate articular cartilage in experimental models with promising results (Musumeci et al., 2011, 2014a, 2014b; Szychlinska et al., 2017).

Skeletal unloading also accrues marrow adipose tissue (Hamrick et al., 2016). A previous study in paraplegic patients found a 54% increase in marrow adipose tissue between 3 and 15 weeks after their spinal cord injury (Minaire et al., 1984). Similar results were observed when looking at iliac crests where marrow adipose tissue volume increased two-fold by the 20th week of enforced immobility (Minaire et al., 1974). Consistent with these findings, immobility induced by bed rest may lead to long lasting and potentially irreversible increases of

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marrow adipose tissue (Payne et al., 2007). Female participants in a 60-day bed rest study showed a 9% increase in vertebral fat fraction that failed to return to baseline values 1 year after normal activities were resumed (Trudel et al., 2009). The detrimental effects of whole body immobility on marrow adipose tissue conversion and potentially irreversible effects remain to be investigated.

Animal models have shed light on important aspects of marrow adipose tissue conversion associated with skeletal unloading. Seven days of hind limb suspension increased the number and volume of adipocytes in the bone marrow of rats' long bones metaphysis (Ahdjoudj et al., 2002). The combined effects of aging and skeletal disuse were described in a different study. Six and 32 months old rats were hind limb suspended for 2 weeks and marrow fat compared by visual examination of stained sagittal sections of the proximal tibia (Perrien et al., 2007). The immobilization-related increase in marrow fat tissue was qualitatively reported as more important in aged animals. The marrow adipose tissue analysis was qualitative and the question whether the increase of marrow adiposity is due to adipocyte hyperplasia or adipocyte hypertrophy was not addressed. To our knowledge, histomorphometric methods to assess adipocyte number and size as well as percentage of adipocyte volume per tissue volume have not been applied to quantify marrow adipocytes changes induced by immobilization. In addition, the effect of skeletal unloading was studied for relatively short periods, 7–14 days, with no information on the effect of long term immobilization. The current study focused on the histomorphometric characterization of bone marrow adipocytes in the proximal tibial epiphysis of rat knees immobilized over increased durations up to 32 weeks. Our hypotheses were that 1) bone marrow conversion will be accelerated by knee immobilization compared to sham operated knees and 2) adipocyte hyperplasia will contribute more significantly to fat accumulation than adipocyte hypertrophy.

## 2. Materials and methods

### 2.1. Animals and surgical procedure

Animal procedures and protocol (#ME-2461) were approved by the University of Ottawa Animal Care Committee and animal care, handling and procedures complied with the regulations of the Ontario Ministry of Agriculture and Rural Affairs under the Animals for Research Act and the Canadian Council on Animal Care standards overviewed by the Animal Care and Veterinary Service at the University of Ottawa. Forty four adult male Sprague Dawley rats, 11 weeks old and averaging 340 g in weight, were used (Charles River Laboratories, St-Constant, Quebec, Canada). In the experimental group (n = 23), one knee was immobilized in flexion at 145 ° using screws to fix a Delrin® plate to the proximal femur and distal tibia as previously described (DuPont Engineering Polymers, Wilmington, DE, USA) (Trudel et al., 1999). For sham operated rats (n = 21), only screws were inserted into the bones. Alternated left and right knees were operated. Animals were housed individually and received postoperative analgesia (0.05 mg/kg Buprenorphine) as well as continuous veterinarian care, unlimited activity and free access to water and food. The duration of immobilization was 2, 4, 16 or 32 weeks. Sham operated rats were harvested at the same time points. Durations of immobilization were selected based on previous data from our group reporting effects of knee flexion contractures as early as 2 weeks (Trudel et al., 1999). The numbers of animals sacrificed at the end of each time point were: 2 weeks (n = 14, 7 sham and 7 immobilized), 4 weeks (n = 9, 4 sham and 5 immobilized), 16 weeks (n = 10, 5 sham and 5 immobilized) and 32 weeks (n = 11, 5 sham and 6 immobilized).

### 2.2. Preparation of knee sections and staining

Range of motion and knee capsule cellularity data on this group of animals has already been reported (Matsumoto et al., 2002). The current histological analysis focused on bone marrow changes in the proximal epiphysis of the tibia of immobilized and sham-operated rat knees. Operated legs (experimental and sham-operated) were harvested, the skin and the paw were removed, and knees were fixed in 10% formalin (Sigma Aldrich Canada, Ontario, Canada) and decalcified in EDTA 0.49 M at pH 7.0 (Fisher Scientific Limited, Ontario, Canada) for approximately 2 months including a change of EDTA solution every 3 days. Decalcified knees were processed for histology analysis including steps of fixation and embedding in paraffin (Oxford Labware, St. Louis, MO, USA) using an automated system (PALM Histology Core Facility at the University of Ottawa). Embedded knees were cut into 7 µm sagittal sections at the mid-medial condyle as previously described (Matsumoto et al., 2002). Specimens were then stained with hematoxylin & eosin (H & E) with an automated system (PALM Histology Core Facility at the University of Ottawa) (Feldman and Wolfe, 2014).

### 2.3. Placement of fields of view (FOV) in the proximal tibial epiphysis

Marrow fat characterization was conducted in the proximal tibial epiphysis. Digital images from the knee sections limited to the proximal tibial epiphysis were taken using an Olympus CX41 microscope and a Luminera Infinity 2 camera at a magnification of 33X. Color images were then uploaded into the image analysis software ImageJ (v.2.0.0-rc-49/1.51d) (Rasband, 2016). Adipocytes were characterized in 6 FOVs of equal and fixed areas (0.136 $mm^2$ each) that were positioned every 15% of the length of a straight anteroposterior line proximal to the epiphyseal growth plate. Next, the FOVs were inspected and data from incomplete adipocytes found at the edge of FOV, non-adipocyte cells and acellular interstitial tissue were removed manually.

### 2.4. Measurements of marrow adipose tissue, adipocyte number and adipocyte cross-sectional area

Processed images were then analyzed with ImageJ's particle analyzer function which was set with the following parameters: Circularity: 0.35–1.00 and Size ($mm^2$): 0.00001-Infinity. The cross-sectional area of each particle was measured to evaluate adipocyte hypertrophy, and adipocyte count was used to assess hyperplasia. For each FOV, the number of adipocytes per $mm^2$, the cross-section area of individual adipocytes and the trabecular bone area, were recorded. Trabecular bone area was measured using the freehand selection tool and measure function of ImageJ. All data were saved in excel sheets. When measuring adipose tissue area and the number of adipocytes per $mm^2$ of marrow area, incomplete adipocytes found on the edges of a FOV were included. However, when measuring adipocyte cross sectional area, incomplete adipocytes were excluded.

### 2.5. Calculations

The adipose tissue area was calculated by summating the cross-sectional areas of individual adipocytes. The marrow area was calculated as FOV area minus trabecular bone area. The hemopoietic area of each FOV could have been calculated by the FOV area minus both the trabecular bone and the adipose area, but this calculation has not used in this report.

#### 2.5.1. The following calculations were performed

1) To determine marrow adipose area in each FOV, the cross-sectional areas of all adipocytes were summated and the total was divided by the Marrow area. Marrow area was calculated as the FOV area minus the trabecular bone area.

$$\frac{Total\ Adipocyte\ Area}{Marrow\ Area} = \frac{Total\ Adipocyte\ Area}{FOV\ Area - Trabecular\ Bone\ Area}$$

2) A similar calculation was performed to measure the number of