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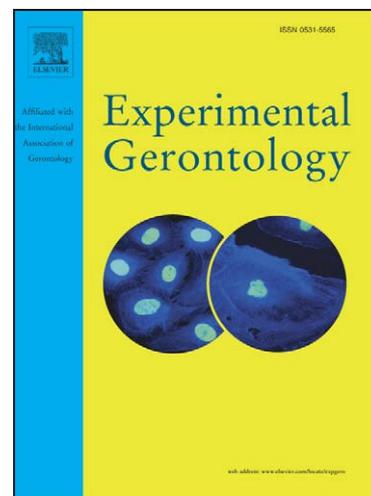
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Running has a negative effect on bone metabolism and proinflammatory status in male aged rats

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Short running title: Running has a negative effect on bone metabolism

Abstract

Animal models for male osteoporosis are scarce. This study aimed at identifying the impact of different living conditions on bone structure and metabolism as well as the inflammatory status in a rat model of age-related male osteoporosis. Bone mineral density, bone histomorphometric data, *ex vivo* osteoclast generation, and bone metabolism serum marker as well as intracellular cytokine expressions were evaluated in 23-month-old male Sprague-Dawley rats subjected to different housing conditions from the age of 5 months. Running rats were housed individually and were exercised voluntarily in running wheels attached to their cages. Dieting rats were housed individually, too, but were fed to pair weight with the running rats. Walking rats were exercised mildly by use of a treadmill (800 m/day, five days a week) and social rats were kept as four in a cage and fed ad libitum. Whereas no marked differences could be found for bone mineral density, trabecular bone volume as well as trabecular bone surface were diminished in walking rats. The *ex vivo* osteoclast generation assay revealed no significant differences between groups. Osteoblasts of running rats were not only decreased in number, but displayed also a lower activity as indicated by decreased serum osteocalcin levels. Osteoclast activity was increased in the same group as indicated by elevated CTX (c-terminal telopeptide of type I collagen) levels. Additionally, production of tumor necrosis factor (TNF)- α and interferone (IFN)- γ by CD8⁺ T cells was elevated in running rats. In conclusion, running has a negative effect on bone metabolism and proinflammatory status in male aged rats.

Introduction

Osteoporosis, a classic age-related disease, is a skeletal disorder characterized by a compromised bone strength predisposing a person to an increased risk of fracture. The pathogenesis of osteoporotic fractures in men is multifactorial, including genetic, endocrine, and life style factors (Obermayer-Pietsch, 2006; Patsch et al., 2007). Osteoimmunology has emerged as a major player in the understanding of the pathophysiological processes of a range of bone diseases. The key cytokines connecting the immune and the bone system are receptor activator of nuclear factor- κ B (RANK) and its ligand (RANKL) as well as osteoprotegerin (OPG). The prominent function of RANKL concerning bone metabolism is the promotion of osteoclastogenesis through its receptor RANK, which is expressed on osteoclasts and their progenitors, whereas OPG serves as a decoy receptor for RANKL thus acting as a natural antagonist (Rauner et al., 2007). However, whereas postmenopausal osteoporosis has already been a matter of intense research for many years, male osteoporosis has been considered as a clinically relevant problem only recently. Hence, there is still a great demand for basic and applied research to investigate mechanisms for bone loss in men.

Rat models of male osteoporosis are scarce. Wang et al. (2001) demonstrated an age-related decline of bone mass in the axial and appendicular skeleton and a decrease in bone formation in the spine in aged male Sprague-Dawley rats. Recently, Pietschmann et al. (2007) described similar changes, i.e. markedly reduced cancellous bone mineral density, bone volume, and trabecular number, in the proximal tibiae of aged male rats. Additionally, bone formation as well as osteoclastogenesis appeared decreased, which were substantiated by decreased insulin-like growth factor 1 (IGF-1), osteocalcin, and RANKL serum levels. The IGF-system comprises IGF-1, IGF-2, and six high-affinity IGF-binding proteins (IGFBPs) with IGF-1 being involved in differentiation, proliferation, and matrix formation in osteoblasts (Kim et al., 2007). In consequence of the bone formation-promoting effects of IGF-1, reduced bone mineral density has been observed in men with decreased IGF-1 levels (Patel et al., 2005). Other regulators of bone formation include the adipokines leptin and adiponectin, whose effects on bone metabolism have been reviewed by Reid (2007). Both adipocyte-derived hormones have been recognized to increase proliferation and differentiation of osteoblasts and to interfere in osteoclastogenesis by reducing production of RANK and its ligand while at the same time increasing OPG (Reid et al., 2007). Interestingly, the effects of leptin on the

hypothalamic arcuate nucleus are reinforced by insulin and vice versa. Thus, a connection between body weight on the one hand and bone mass and quality on the other hand is evident.

Effects of physical exercise on bone quality depend on age and, to some extent, on gender. Additionally, bone-loading activities, such as running, are reported to have a more beneficial impact on bone mineral density (BMD) than non-weight-bearing exercise, such as road cycling, in men aged 20 to 59 years (Rector et al., 2008). From these data it is concluded, that intervention strategies aimed at reducing the incidence of osteoporosis should be sustained during adulthood to have maximal effect. Notably, endurance training has been reported several times to have a negative impact on BMD. Hind and colleagues (2006) described low lumbar (L2-L4) and hip BMD in male endurance runners. Notably, despite these morphologic changes of the skeletal system of endurance runners described above, a significant decrease of sRANKL and an increase of OPG could be found in long-distance runners (Ziegler et al., 2005), which would point towards decreased osteoclast activity and a presumably higher bone mass in these subjects.

It has been demonstrated that voluntary running and dietary restriction improve survival as well as body composition of ageing Sprague-Dawley rats (Narath et al., 2001; Skalicky et al., 2001; Viidik and Skalicky, 2003; Loupal et al., 2005). Therefore, this study aimed at examining the effects of different living conditions with regard to exercise and diet on bone structure and serum parameters of bone turnover, as well as cytokines and adipokines in male aged rats.

Animals, Materials and Methods

Animals

Male Sprague-Dawley rats were subjected to a 12 h light and dark cycle with a temperature of $22 \pm 1^\circ\text{C}$ and a humidity of 40 – 50%. At an age of 5 months and a mean body weight of 452.6 ± 3.1 g the rats were divided into four groups. Animals were housed and fed as described by Hansalik et al. (2006) for further 18 months with running rats corresponding to the RW (running wheel) group ($n_{\text{initial}} = 32$, $n_{\text{final}} = 29$), dieting rats to the PW (pair-weight) group ($n_{\text{initial}} = 32$, $n_{\text{final}} = 24$), walking rats to the TM (treadmill) group ($n_{\text{initial}} = 40$, $n_{\text{final}} = 29$), and social rats to the S4 (sedentary animals housed four animals per cage) group ($n_{\text{initial}} =$

48, $n_{\text{final}} = 41$). To mark the characteristics of each group, these are presented in short: running rats were housed individually and were exercised by providing a running wheel. At an age of 7 months they reached their maximum daily running distance, in average 1844 m, which thereafter stepwise declined to about 700 m. Terminal body weight at an age of 23 months was 447.9 ± 19.6 g. Contrary, dieting rats were housed individually, too, but were fed restrictively to pair weight with running rats. Walking rats were exercised mildly by use of a treadmill (800 m/day, five days a week). Social rats were kept as four in a cage and fed ad libitum. Whereas walking and social rats consumed about 24 g/day of Altromin[®] 1324 FORTI rat pellets, food intake of dieting rats was restricted to 16 g/day in average. Running rats received 90 % of the food of sedentary rats housed individually and fed ad libitum, which were part of another study (Pietschmann et al., 2007). Terminal body weight of dieting rats was 438.8 ± 23.5 g, that of walking rats 517.2 ± 52.4 g, and that of social rats 629.0 ± 75.2 g, respectively. At the end of the experiment, blood was collected by heart puncture from overnight-fasted anaesthetized rats. Following sacrifice by isoflurane anaesthesia the fore- and hindlimbs were collected. The experiments were conducted according to the Austrian federal law; the study protocol was approved by the Institutional Committee of Animal Experimentation and the Austrian Ministry of Science and Education.

DXA, histomorphometry

Areal BMD of femora and tibiae averaged for whole bone was measured using a Delphi A (S/N 45313) DXA bone densitometer (Hologic, Bedford, MA, USA). For histomorphometric analyses the fourth lumbar vertebral bodies (L4) were fixed in formalin, dehydrated in an ascending ethanol series, embedded in methylmethacrylate, and finally cut into 4 μm sections. After mounting, the sections were stained with Goldner's trichrome stain. Trabecular bone volume (BV/TV), bone surface over trabecular volume (BS/TV), trabecular osteoblast surface (Ob.S./BS), trabecular osteoclast surface (Oc.S./BS), and trabecular thickness (Tb.Th) were measured with the Osteomeasure system (OsteoMetrics, Decatur, GA, USA). Measurements, terminology, and units used for histomorphometric analysis were those recommended by the Nomenclature Committee of the American Society of Bone and Mineral Research (Parfitt et al., 1987).

Bone metabolism markers and ex vivo osteoclast generation

Serum concentrations of osteocalcin (EIA-2095, DRG, USA; detection limit: 0.5 ng/ml), c-terminal telopeptide of type I collagen (RatLaps[™], E04-022-96, IASON, Graz, Austria;

detection limit: 2 ng/ml), tartrate-resistant acid phosphatase isoform 5b (TRAP 5b, RatTRAP™, TR102, SBA Science, Turku, Finland; standard range: 0-9 U/l), soluble RANKL (BI-20522, Biomedica, Vienna, Austria; standard range: 0-100 pmol/l), osteoprotegerin (BI-20602, Biomedica, Vienna, Austria; standard range: 0-100 pmol/l), cathepsin K (BI-20432, Biomedica, Vienna, Austria; standard range: 0-300 pmol/l), PTH (60-2500, Immunotopics, USA; standard range: 0-3000 pg/l), testosterone (DE2300, R&D Systems, Wiesbaden, Germany; standard range: 0-2000 pmol/l), IGF-1 (E04-041-96, IASON, Graz, Austria; detection limit: 2 ng/ml), leptin (MOB00, R&D Systems, Wiesbaden, Germany; standard range: 0-4000 pmol/l) and adiponectin (ZZ-39-K1002-1, Biomedica, Vienna, Austria; standard range: 0-100 pmol/l) were determined by commercially available ELISA kits according to the manufacturers' protocols. Femoral bone marrow cultures and osteoclast generation assays were performed as described by Pietschmann et al. (2007). In brief, bone marrow cells were flushed out from femura and cultured for 7 days in VitD₃-supplemented α MEM. At the end of the culture period, adherent cells were washed, fixed, and stained for tartrate-resistant acid phosphatase (TRAP) by a commercially available kit from Sigma-Aldrich (Steinheim, Germany). TRAP-positive, multinucleated cells were counted as osteoclasts.

Flow cytometry

Flow cytometric assessment of T cell cytokine production was performed essentially as described previously (Willheim et al., 1999; Sipos et al., 2005). Mononuclear cells were isolated from heparinized blood obtained by cardiac puncture by density gradient centrifugation with Ficoll-Paque. After washing in PBS, cells were resuspended (2×10^6 /ml) in Ultra Culture Medium (BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo, USA), 170 mg/l gentamycin sulfate (Sigma Chemical Co, St. Louis, Mo, USA) and 5 ml/l mercaptoethanol (Merck, Darmstadt, Germany). For the determination of stimulated intracellular cytokine production, cells were incubated for 4 h at 37°C in culture medium (as described above) containing phorbol 12-myristate 13-acetate (10 ng/ml), ionomycin ($1.25 \mu\text{M}$) and brefeldin A (10 $\mu\text{g/ml}$; all from Sigma Chemical Co, St. Louis, Mo, USA). Thereafter cells were harvested, washed twice with PBS and fixed with 2 % formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. Subsequently cells were washed, resuspended in HBSS containing 0.3 % BSA (Biomol Feinchemikalien, Hamburg, Germany) and 0.1 % sodium azide (Merck, Darmstadt, Germany) and stored at 4°C. Thereafter, cells were washed once with PBS and

permeabilized by washing twice with saponin (0.1 %, Sigma Chemical Co.) in PBS. Subsequently, cells were incubated in the presence of saponin with saturating concentrations of the antibodies for 25 min at room temperature. After washing once with saponin (0.1 %) in PBS, the cells were resuspended and analyzed by four color flow cytometry. Lymphocytes were gated by their light scatter characteristics and subsequently defined as CD4⁺ or CD8⁺ cells. Cells fulfilling both criteria (lymphocyte and CD4⁺ and/or CD8⁺) were further analyzed for their coexpression of cytokines. Monoclonals (BD Biosciences Pharmingen, San Jose, CA, USA) in use were as follows: OX-35 (mouse anti-rat) directed against CD4, OX-8 (mouse anti-rat) directed against CD8 α , TN3-19.12 (hamster anti mouse/rat) directed against TNF- α , OX-81 (mouse anti-rat) directed against IL-4 and DB-1 (mouse anti-rat) directed against IFN- γ .

Statistics

Data are presented as means and standard deviations. First, data were tested for normal distribution by Kolmogorov-Smirnov test. This was the case for all data sets except sRANKL, OPG, and IL-4. Normally distributed data were further analyzed by ANOVA with the Duncan test serving as follow-up test. Nonparametrical data sets were analyzed by Kruskal-Wallis test and Mann-Whitney test. All statistical analyses were performed using SPSS, version 14.0. P values less than 0.05 were considered statistically significant.

Results

Mean total BMD of the femur was identical in all 4 groups with 0.19 ± 0.02 g/cm² as measured by DXA. Also, tibial BMD did not differ notably among groups with mean values ranging between 0.15 and 0.16 g/cm². Histomorphometric analysis of the lumbar spine (Fig. 1) revealed that trabecular bone volume (BV/TV) was lowest in walking rats, whereas no significant differences between the other groups were seen. Trabecular bone surface (BS/TV) was higher in running and dieting rats than in walking or social rats. Mean trabecular osteoblast surfaces (Ob.S/BS) ranged from 0.38 to 0.60 % in running, dieting, and walking rats, but were highest in social rats with a mean of 1.09 %. Mean trabecular osteoclast surfaces (Oc.S/BS) did not differ significantly between groups, but tended to be higher in social rats. Mean trabecular thickness (Tb.Th) was significantly higher in social rats when compared to other groups.

The *ex vivo* osteoclast generation assay revealed that walking rats tended to have the highest capability to form osteoclasts, however, osteoclast-forming capacities did not differ significantly between groups (data not shown). Figure 2 shows osteoclastogenesis-related markers and markers of osteoclast function. Running rats exhibited highest CTX levels. TRAP5b levels were significantly higher in walking than in dieting rats. Systemic sRANKL and OPG were expressed quite uniformly in all groups with social rats tending to produce more OPG. RANKL:OPG ratios were 0.21, 0.19, 0.24, and 0.17 for the four groups. Cathepsin K levels tended to be higher in running and dieting rats. PTH levels did not differ significantly between the groups.

Osteoblast and osteoblastogenesis-related markers are shown in figure 3. Osteocalcin levels were lowest in running and dieting rats, reaching significance when compared to osteocalcin expressions of social rats. A comparable serum concentration could be observed for IGF-1. The expression of leptin in social rats was significantly higher in comparison to all other groups, whereas adiponectin levels were higher in running and dieting rats.

Intracellular expressions of IL-4, IFN- γ , and TNF- α were determined for CD4⁺ and CD8⁺ T cells (Fig. 4). Whereas no significant differences in cytokine production by CD4⁺ T cells could be found between groups, prominent differences could be found for cytokine production by CD8⁺ T cells with running and dieting rats showing significantly higher IFN- γ and TNF- α expressions.

Discussion

Aim of this study was to compare changes in bone structure and metabolism in aged male rats based in different housing conditions imitating different life-styles concerning physical exercise and nutrition. Changes in bone density and structure are a well known feature of the elderly. The aged male Sprague-Dawley rat represents a suitable model to study age-related changes in bone metabolism. Wang et al. (2001) found a loss of trabecular elements in the spine of aging rats and Pietschmann et al. (2007) described a decrease of cancellous bone mass of the tibiae due to loss of trabecular elements. Using this model, our study demonstrates an influence of living conditions on bone density and structure.

Bone structure and metabolism can be assessed by several parameters (Seibel, 2007). Beside densitometry, which is a valuable tool, but gives only little information about bone microstructure, histomorphometry was applied to compensate for this limitation. Whereas bone density of femur and tibia as measured by DXA was nearly the same in all four groups, bone histomorphometric analysis of the spine revealed a diminished trabecular bone volume in mildly exercised, i.e. walking, rats and a significantly decreased bone surface in walking as well as social rats, suggesting a lower trabecular number especially in these groups. Voluntary running had an opposite impact on vertebral bone structure. As mentioned above, trabecular surface, giving evidence of the shape of the trabecular microarchitecture, was diminished social rats. This feature of trabecular structure was accompanied by a higher trabecular thickness of remaining trabecula, most reasonably due to the increased osteoblast number as suggested by the high Ob.S/BS ratio. Osteoblasts of social rats also exhibited a significantly higher activity as indicated by enhanced serum osteocalcin. Seemingly, also elevated IGF-1 and leptin levels contributed to the higher osteoblast activity in this group. Bone turnover markers of social rats were similar to those of previously described aged rats housed in individual cages (Pietschmann et al., 2007). Nevertheless, the aforementioned relative increase of osteoblast markers and modulators in social rats appears to be beneficial when compared to the running rats.

Whereas osteoblast activity thus was decreased in particular in running rats, osteoclast activity was found significantly elevated in this group. This finding is in line with the increased osteoclast activity and thus diminished BMD of the appendicular skeleton in subjects doing hard exercise, who are mimicked by the running rats in our experiment to some extent (Hind et al., 2006). Despite the comparably high osteoclast activity in aged running rats it has to be kept in mind, that there is an age-related decrease of sRANKL, which is accompanied by a decline in osteoclast formation (Pietschmann et al., 2007). RANKL:OPG ratios are often used as a tool for the characterization of osteoclast recruitment or activity, although this parameter is not without controversy, as reviewed by Rauner et al. (2007). In this study, RANKL:OPG ratios appeared to be an interesting instrument for estimating osteoclast activity. Consequently, walking rats showing the highest ratio had the lowest BV/TV and social rats with the lowest ratio had a significantly higher BV/TV than the walking rats and also a low osteoclast activity. In summary, age and physical exercise seem to activate osteoclasts irrespective of their absolute number. However, from a biomechanical point of view, physical

exercise and food restriction are somewhat effective in counteracting the age-related decrease of the biomechanical strength of the distal metaphysis of the male rat femur (Thomsen et al., 2008). In this context it should be borne in mind, that the femur is subjected to higher mechanical loads than the lumbar spine in quadruped animals and that markers of bone metabolism measured in blood reflect the whole skeleton's metabolism. In aged male rats, exercise thus appears to have different local and systemic skeletal effects.

Aging is characterized by various physiological and pathophysiological processes. Amongst these, a continuous increase of proinflammatory cytokines causes a state termed "inflammaging" (Franceschi et al., 2000a,b; Kovacs, 2005). This proinflammatory status may contribute to age-related alterations including those of the skeletal system. This hypothesis can be inferred from the known pro-osteoclastogenic effects of inflammatory cytokines, as reviewed by Sipos et al. (2008). Therapeutical concepts in gerontology should involve a pharmacological/immunological antagonization of proinflammation and a promotion of a lifestyle that is related with a decreased production of mediators of inflammation. CD8⁺ lymphocytes of running and dieting rats produced significantly higher amounts of TNF- α and IFN- γ . Thus, living conditions with moderate walking and social interactions seem to have a beneficial effect on the immune system. However, although in this study no pronounced effect of any housing condition on survival could be observed, social rats were shown to have the highest value of the collagen biomarker of aging as well as the highest scores of kidney lesions in other investigations (Viidik and Skalicky, 2003; Loupal et al., 2005). These data give evidence of the very complex pathophysiological phenomenon of aging, thus making interpretation of results a demanding task.

The presented model assesses different factors relevant for bone structure and metabolism in elderly men. These factors are physical exercise, food consumption, and social interactions. Living conditions observed in the social rats had the most beneficial impact on osteoblast parameters and proinflammatory status. Moreover, bone resorption and proinflammation were most pronounced in aged rats allowed voluntary running also when compared to young rats aged 5 months described previously (Pietschmann et al., 2007).

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Legend to Figures

Figure 1: Bone histomorphometric parameters BV/TV, BS/TV, Ob.S/BS, Oc.S/BS, and Tb.Th were evaluated by analyzing sections of the L4. Group A: running rats, group B: dieting rats, group C: walking rats, group D: social rats. Data are presented as mean \pm SD.

Figure 2: Markers of osteoclastogenesis and osteoclast function were measured by means of commercially available ELISA systems. Group A: running rats, group B: dieting rats, group C: walking rats, group D: social rats. Data are presented as mean \pm SD.

Figure 3: Markers of osteoblastogenesis and osteoblast function were measured using commercially available ELISA systems. Group A: running rats, group B: dieting rats, group C: walking rats, group D: social rats. Data are presented as mean \pm SD.

Figure 4: Cytokine production of CD4⁺ and CD8⁺ T cells was measured at protein level by intracellular cytokine staining and subsequent flow cytometric analysis. Data are presented as mean \pm SD.

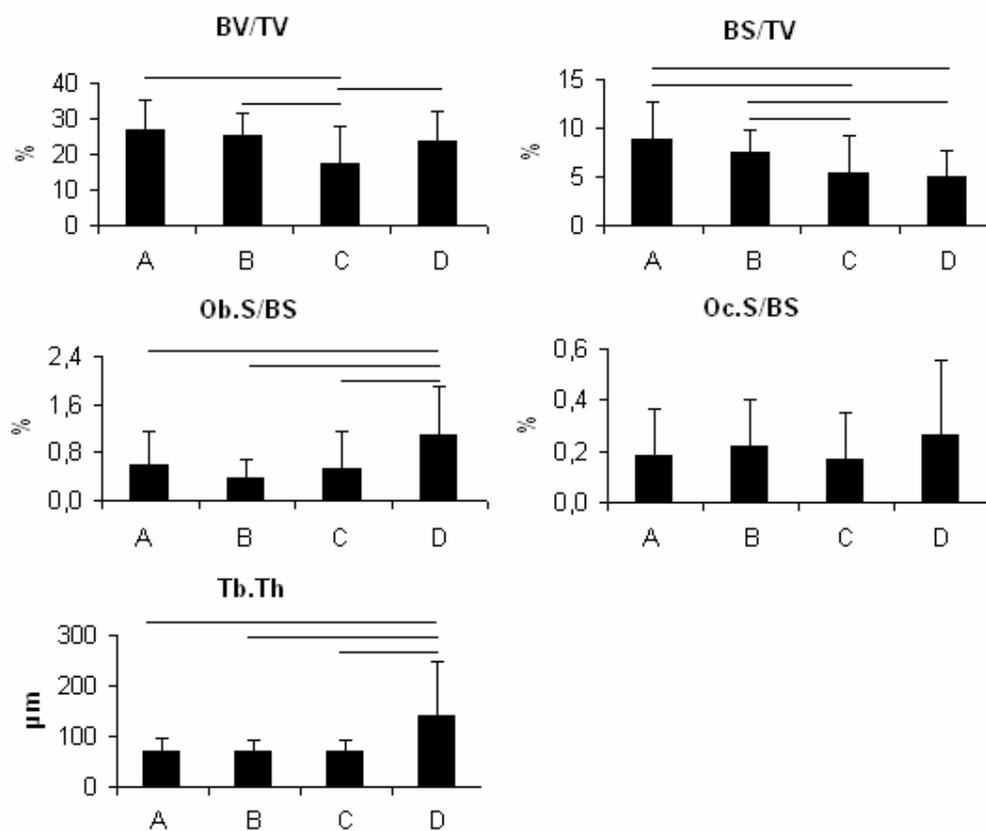


Figure 1.

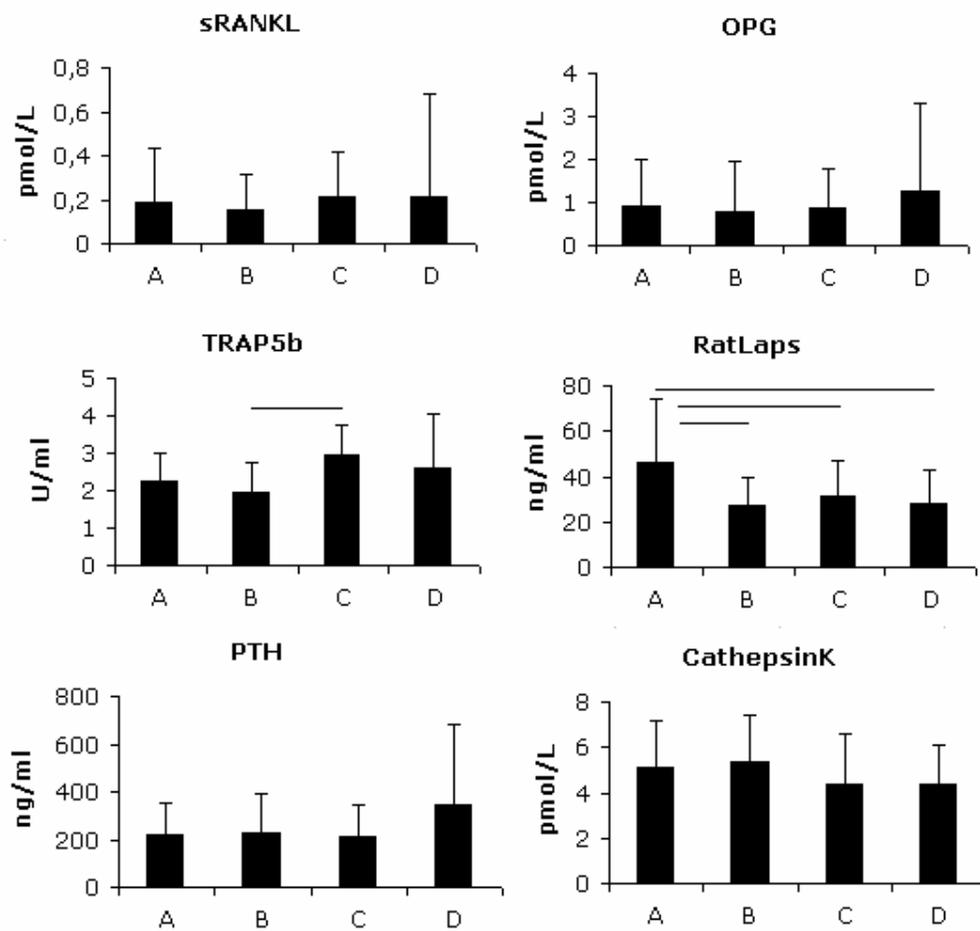


Figure 2.

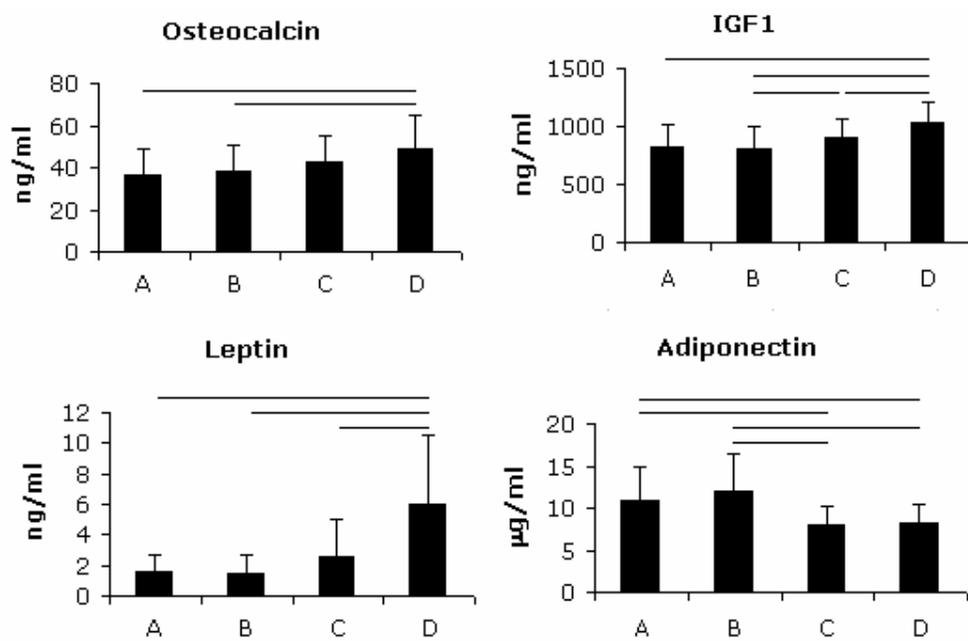


Figure 3.

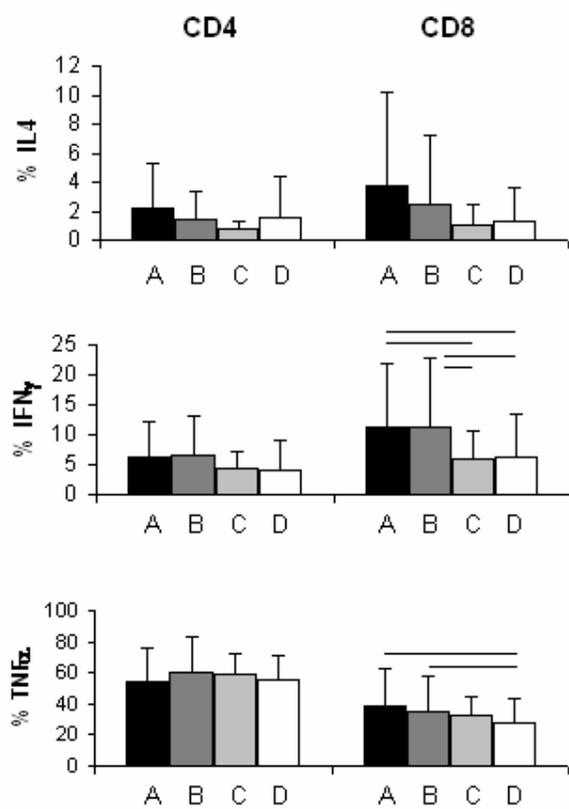


Figure 4.