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Original Article

Markers of bone metabolism during 14 days of bed rest in young and older men

J. Buehlmeier[1,2], P. Frings-Meuthen[2], N. Mohorko[3], P. Lau[2], S. Mazzucco[4], J.L. Ferretti[5], G. Biolo[4], R. Pisot[3], B. Simunic[3], J. Rittweger[2]

[1]Department of Nutrition and Food Science, University of Bonn, Germany; [2]Institute of Aerospace Medicine, German Aerospace Center (DLR), Germany; [3]University of Primorska, Science and Research Centre, Institute for Kinesiology Research, Slovenia; [4]Clinica Medica, Department of Medical, Surgical and Health Sciences, University Trieste, Italy; [5]National University of Rosario, Center of P-Ca metabolism studies (CEMFoC), Argentina

Abstract

Objective: We aimed at comparing markers of bone metabolism during unloading in young and older men, and to assess countermeasure effectiveness. **Methods:** 16 older (60±2 years) and 8 younger men (23±3 years) underwent bed rest (BR) for 14 days. A subgroup of the Older performed cognitive training during BR and supplemented protein and potassium bicarbonate afterwards. Biochemical markers of bone and calcium/phosphate metabolism were assessed. **Results:** At baseline urinary NTX and CTX were greater in younger than in older subjects (P<0.001), but increased during BR (P<0.001) by a similar amount (P>0.17). P1NP was greater in young than in older subjects (P<0.001) and decreased during BR in the Young (P<0.001). Sclerostin increased during BR across groups (P=0.016). No systematic effects of the countermeasure were observed. **Conclusion:** In men, older age did not affect control of bone metabolism, but bone turnover was reduced. During BR formation markers were reduced only in younger men whereas resorption markers increased to a comparable extent. Thus, we assume that older men are not at an elevated, and possibly even at a reduced risk to lose bone when immobilized.

Keywords: Mechano-adaptation, Age effects, Immobilization, Wnt-signalling, Countermeasure

Introduction

Mechanical loading of bone is of primordial importance for maintenance of bone. Muscle contractions, e.g. during locomotion enforce skeletal adaptation to the mechanical stimulus and thus constitute a major determinant of bone metabolism. Consequently disuse of the musculoskeletal unit during prolonged bed rest induces substantial loss in bone mass especially of the lower extremity[1,2] that is mostly pronounced in the cortical section of the epiphyses[3]. At the metabolic level this seems to result from an imbalance of bone resorption and formation, described by a rapid increase in collagen breakdown products[4-7] without any[4,5,7,8] or with marginal changes[9,10] in markers of bone formation. Current view suggests Wnt signalling as potential link between mechanical unloading and related bone loss[11]. In keeping with this view, unloading elevates levels of circulating sclerostin[12,13], the latter being a glycoprotein that has inhibitory effects on Wnt signaling in osteoblasts[14].

Whilst the mechanisms of disuse-induced bone loss have been intensively studied in younger adults, little is known about adaptation of bone metabolism to disuse in older people. Established evidence demonstrates a decline in bone mass and strength, an increase in fracture risk as well as a decrease in markers of bone turnover at old age in both sexes with similar patterns but differing progression[15-19]. Studies in rodents have shown that these age-related bone losses are associated with less osteoblastic differentiation potential and viability[20]. There may be more than one reason for this observation, such as the decline in sex steroids, secondary hy-

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Corresponding author: Jörn Rittweger, German Aerospace Center (DLR), Institute of Aerospace Medicine, Linder Höhe, 51147 Cologne, Germany
E-mail: joern.rittweger@dlr.de

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perparathyroidism, vitamin D deficiency, somatopause and sarcopenia[15]. As muscle force and power, as well as physical activity decline with age, the question arises of whether the age-related decline in bone mass and strength is primarily related to reductions of the mechanical demands. If so, then one should expect that bone loss in response to bed rest is mitigated at old age, simply because the transition to bed rest should cause less disruption in people with lower levels of mechanical demands. Moreover disuse studies in rodents suggest that the osteogenic potential during disuse may be reduced with age as a consequence of less responsiveness to bone morphogenetic protein and IGF-1[21]. However, to the best of our knowledge none of the very few immobilization studies in older people investigated bone metabolism[22-24].

Current multi-disciplinary countermeasure research in the field of disuse-induced physiological phenomena's, besides exercise, focusses on nutrition and lately also involves stimulation of the central nervous system. Therefore a high-protein diet was tested in the presented study in its potential to boost musculoskeletal recovery[25]. To lower the associated acidogenicity[26] and maximize anabolic effects, the diet was alkalinized with potassium bicarbonate. During bed rest the study has included a cognitive training program as the possibility of a relatively direct interaction of the central nervous system with the musculoskeletal system has been raised[27]. For bone, specifically, there is a sound rationale for such direct bone-brain interactions[28,29].

Thus the main hypotheses of the presented study were that older people have reduced bone metabolism (H1), and that bone metabolism in older people would be less affected by experimental bed rest than in young (H2). Moreover, it was hypothesized that the countermeasure protocol affects bed-rest induced changes in bone metabolism in older people (H3).

Materials and methods

Study design and setting

Eight healthy young males and 16 healthy older males participated in a 14-day horizontal bed rest study (Table 1). All men were Caucasians. The study was divided into three 3 stationary study phases and an ambulatory follow-up phase: The stationary phases consisted of a 3-day baseline data collection phase (BDC), 14 days of bed rest (BR) and 2 days of stationary recovery (Rec). Throughout these periods the subjects were confined to the Valdoltra Orthopaedic Hospital of Ankaran, Slovenia. During and after BR a combined countermeasure was assessed in the older subgroup in a randomized parallel design. Therefore after BDC the group of older subjects was randomly divided into one group that did BR only (N=8; O_BR), and one that did a combined countermeasure (CMS) during and after BR (N=8; O_CMS). The CMS consisted of a daily cognitive training during BR and dietary protein plus alkaline supplementation during the first 28 days of recovery. The group of young subjects did BR only (Y_BR). After 2 days of stationary recovery subjects were discharged from the clinic, but they returned at days 7, 14 and 28 of recovery for follow-up measurements (Rec 7, 14 and 28). During the whole period of 28 days of recovery, all subjects followed a physical rehabilitation programme with three weekly sessions of 60 minutes each.

Biological endpoints of the presented study were biomarkers and regulators of bone metabolism as well as calcium and phosphate homeostasis. The study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki and it was approved by the Republic of Slovenia National Medical Ethics Committee. Written informed consent was obtained from all volunteers prior to study start.

Subjects

Study participants were recruited by advertisements in local newspapers and all applicants were examined prior the study inclusion with an interview, routine blood and urine analysis, and a fitness battery test. Exclusion criteria were: smoking, regular alcohol consuming, history of deep vein thrombosis with D-dimer >500 $\mu g \cdot L^{-1}$; acute or chronic skeletal, neuromuscular, metabolic and cardiovascular disease condition; pulmonary embolism; a Short Physical Performance Battery score<9[30]. Body mass index at BDC was matched between young and old (P=0.861). After study completion the participants received a financial allowance.

Bed rest protocol

During BR subjects were constrained to bed 24h/day. Therefore all activities of daily living took place in bed. The protocol was performed in conformity with the European Space Agency's bed rest standardization plan[31], with the exception that subjects lay in a horizontal bed, rather than at -6° head down tilt. A maximum of one pillow was allowed for head elevation in any time of the BR period. Study participants were under constant surveillance and provided with 24-hour medical care and regular daily ward rounds. The bedrooms were air-conditioned and the room temperature was kept below 25°C. Every second day a physiotherapist performed a passive stretching.

Interventions

Eight of the 16 older subjects did a combined countermeasure (CMS) protocol consisting of a cognitive training and a nutritional intervention (O_CMS). The computerized cognitive training was applied daily from 2nd to 13th day of BR with 50 minutes of spatial navigation. Participants moved through the virtual environment using a joystick (Trust predator GM-2550) presented on a 17-inch LCD monitor situated approximately 60 cm in front of them. The training program consisted of virtual mazes representing a series of interconnected corridors, with three available paths at each intersection. For each intersection, a pair of verbal or pictorial cues were displayed, placed at either opposite corner of the intersection and in corridors at various non-decision-making points. After familiarization, participants were instructed to select the quickest (correct) path toward the finish line. The

training was performed using several virtual maze environments, each of increasing difficulty and length.

The nutritional intervention was applied during stationary and ambulatory recovery up to 28 days after BR. It was composed of a hyperprotein bolus of 0.4 g whey protein/kg body weight/day for breakfast and 3 x 30 mmol potassium bicarbonate (KHCO3)/day.

Diet

Dietary intake was individually tailored and controlled during the entire stationary study period. Resting energy expenditure (REE) was calculated according to Harris and Benedict[32] and multiplied by 1.4/1.2 (before/during BR) to account for physical activity and diet-induced thermogenesis. Macronutrient intake was kept constant with 55% of total energy intake being carbohydrates, 10-15% protein and 25-30% fat. Individual menus for each subject were provided in form of standard hospital meals, individually weighted to meet the required energy and macronutrient intake. The diet excluded alcohol or methylxanthine derivates. Dietary intake was calculated using the Open Platform for Clinical Nutrition (OPEN) online recipe calculation method[33], accessible through the web site http://www.opkp.si/en_GB/cms/vstopna-stran. The OPEN method relies on the procedure that was originally recommended by INFOODS[34].

Biological samples

24h urine collections and fasting blood samples were obtained on the last days of BDC (BDC-2, BDC-1), and on days 2, 5, 10 and 14 of BR, as well as on days 7 and 14 of Rec. After an overnight fast, blood samples were collected shortly after awakening from an antecubital vein through a short catheter into serum vacutainers® (BD, USA). Whole blood was centrifuged after coagulation (3000 rpm, 4°C) then serum was distributed into aliquots and immediately frozen at -20°C or -80°C until analysis. Urine aliquots were stored at -20°C until analysis.

Biochemical analysis

Serum and urinary calcium and phosphate were analyzed by an automated analyzer (COBAS INTEGRA 400, Roche Diagnostics, Germany). All biomarkers and regulators of bone metabolism were analyzed by commercially available immunoassays in the laboratory of the Institute of Aerospace Medicine, Germany. Biomarkers of bone formation (Procollagen type I N-terminal propeptide (P1NP), bone alkaline phosphatase (bAP)) as well as Parathyroid hormone (PTH) and 25-hydroxyvitamin d (25-OHD) were analyzed by radioimmunoassay (P1NP: RIA Orion Diagnostica, Finland; bAP: Immunotech, Czech Republic; PTH: Immunotech, Czech Republic; 25-OHD: Diasorin, USA). Inter- and intraassay variations were as follows: P1NP: 2.1%, 2.3%; bAP: 8.7%, 4.8%; PTH: 5.3%, 6.2%; 25-OHD: 17%, 20.3%. Markers of bone resorption (Urinary C/N-telopeptide (UCTX/UNTX)) and further regulators of bone metabolism (Osteoprotegerin (OPG), soluble RANK ligand (sRANKL), Dickkopf-related protein 1 (DKK1), Sclerostin) were analyzed by enzyme-linked immunosorbent assay (UCTX/UNTX: Immunodiagnostic Systems, UK; OPG, sRANKL, DKK1: Biomedica, Vienna, Austria; Sclerostin: Quidel, San Diego, USA). Inter- and intraassay variations were as follows: UCTX: 6.8%, 5.9%; UNTX: 2.7%, 1.2%; OPG: 3.7%, 2.9%; sRANKL: 6.2%, 1.0%; DKK1: 3.3%, 3.4%; Sclerostin: both 2.1%. Samples of each subject were analyzed in one batch to avoid inter-assay variation within one subject.

Statistical analyses

Statistical analyses were carried out using the R-environment in its version 3.1.1 for the 64-bit Windows platform (www.r-project.org). Data were analysed following the per-protocol principle and are given as means and standard errors (SE) in the figures and tables and as best linear unbiased predictors in the text. The level for statistical significance was set to α=0.05 and β was set to 0.2.

Linear mixed effect (LME) models with time and intervention as fixed effects and subject as random effect were constructed in order to assess intervention effects. As the primary hypotheses of this study addressed age-related differences in BR-induced responses, potential CMS-effects were of secondary priority in the statistical approach. Variances were allowed to differ between participants and intervention, and LME models were optimized according to Akaike's information criterion (see p.353 and p.652 in[35]). Data were box-cox transformed where warranted by non-linear quantile-quantile plots or in case of heteroscedacity. Data from the baseline data collection phase (BDC) were lumped together, and so were data from the BR phase and from the recovery phase (Rec). Models were simplified in a step-wise manner, as long as $P>\beta$. Firstly, data from O_BR and O_CMS Groups were pooled to compare older versus young subjects (AgeGroup). Next, the Phase*Group interaction term, and then the Phase*AgeGroup term was deleted from the statistical model. The last step of model simplification was to delete AgeGroup from the model altogether, so that pure Phase effects were analyzed as the simplest model. Any significant effects were followed up with treatment contrasts, using BDC and O_BR as reference, or the older group where AgeGroup effects were assessed.

Results

Study conduct and subjects

23 subjects finished the protocol; one young subject dropped out on study day 1 due to second D-dimer values above 1500 $\mu g \cdot L^{-1}$. Overall, the 14 days of immobilization were well tolerated and none of the study finishers had major difficulties in re-ambulation. The mean age-difference between the age groups was 37 years but body weight was nonetheless comparable (Table 1). According to reduced energy expenditure and intake with aging, calcium intake was approximately 150 mg lower in the older subjects compared

Table 1. Anthropometrics of test subjects at study entrance.

	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m²)
Y_BR (n=7)	23 (3)	176.7 (6.6)	74.8 (8.8)	24.0 (2.4)
O_BR+O_CMS (n=16)	60 (2)	173.4 (4.9)	79.9 (12.3)	26.6 (4.4)
O_BR (n=8)	59 (3)	172.5 (4.0)	79.6 (10.5)	26.2 (4.8)
O_CMS (n=8)	60 (2)	174.3 (5.8)	80.3 (14.7)	26.4 (4.8)

Data are presented as mean (sd) for young (Y_BR) and older age groups and subgroups of the Older (O_BR, O_CMS).

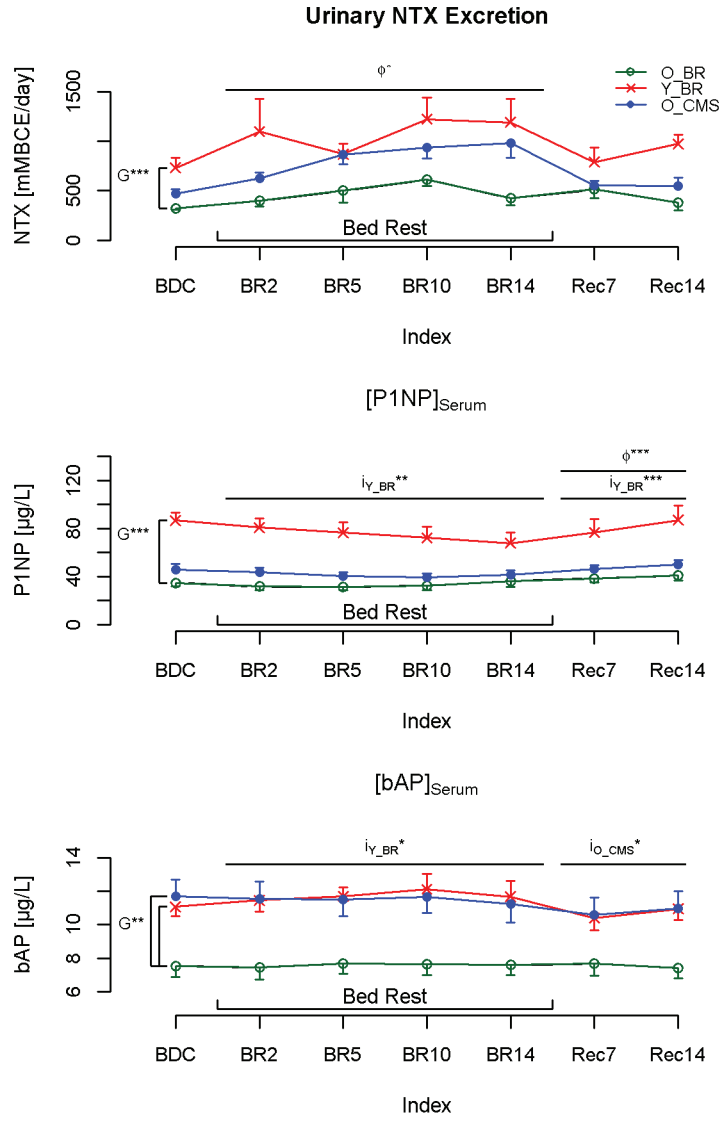

Figure 1. Urinary NTX excretion, serum concentrations of bone formation markers P1NP and bAP. Φ denotes significant main effects for phase, the reference level for contrasts being baseline (BDC); G denotes main effects for group with O_BR as reference for contrasts; iY_BR and iO_CMS denote phase*group interactions that were significant for the respective subgroups with BDC*O_BR used as contrast (***P<0.001, **P<0.01 and *P<0.05, respectively).

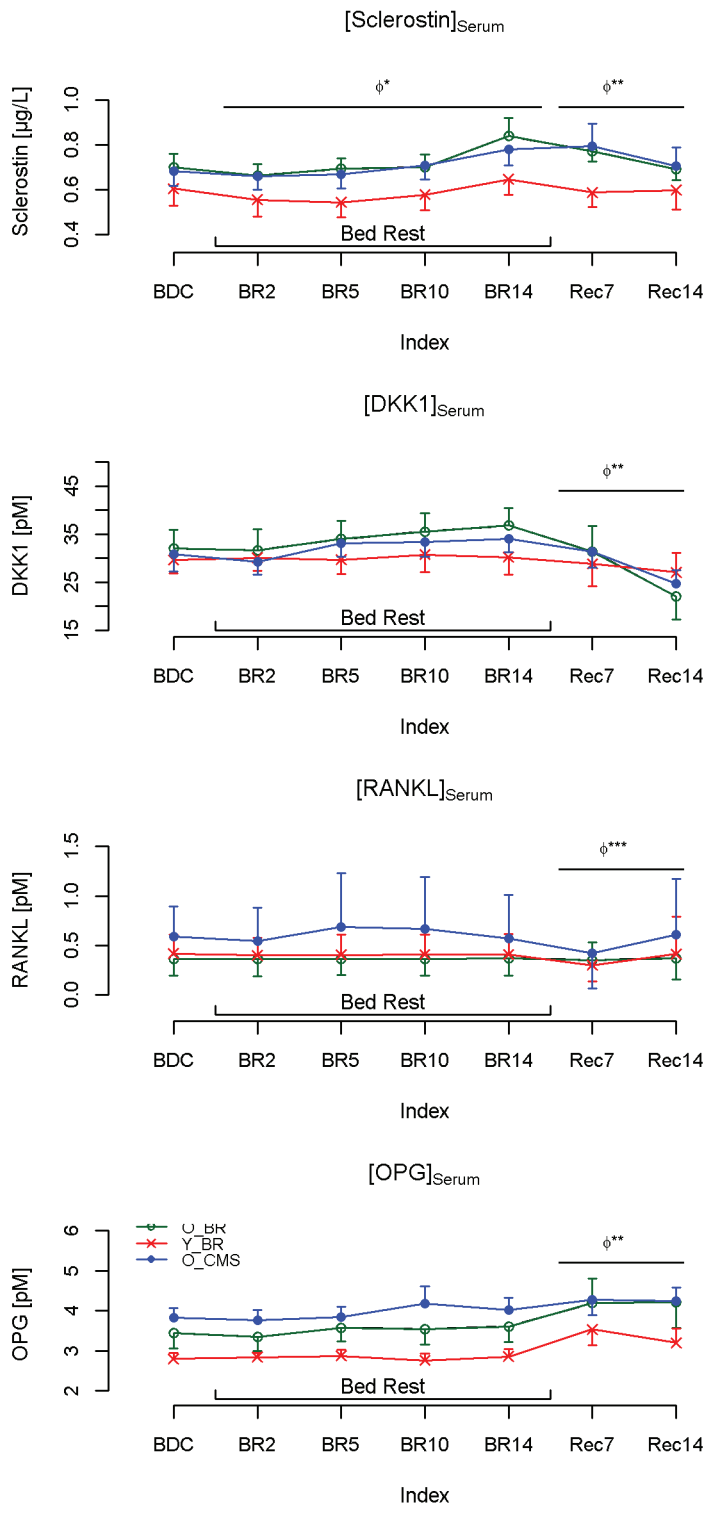

Figure 2. Serum concentrations of regulators of bone metabolism. Φ denotes significant main effect for phase with baseline (BDC) as reference for contrasts (***P<0.001 and *P<0.05, respectively).

to the young subjects (Young: 790 mg (SD 79), Older: 655 mg (SD 44)) and less than the recommended dietary allowance of 800 mg/d[36]. Data collection was completed with the exception of missing voids for the 24h urine collections on days BDC-2, Rec+7, as well as for two subjects on day Rec+14. Data from these 24-hour collections were discarded from further analysis.

Biochemical markers of bone metabolism (Figure 1)

At baseline, urinary bone resorption markers and serum bone formation markers where higher in young than in older subjects (CTX not shown). In both age groups bone resorption markers were elevated during BR. Bone formation marker P1NP increased during recovery across age groups. However, both bone formation markers were affected by BR only in the Young (lower P1NP/higher bAP levels). The older subgroups depicted differing levels in CTX (O_CMS>O_BR) and bAP at baseline.

Regulators of bone metabolism (Figure 2)

Sclerostin increased during BR and remained elevated during recovery across age groups. Besides, recovery was characterized by reduced levels of DKK1 and RANKL as well as elevated OPG levels (Note: statistical analysis was conducted with n=6 for Y_BR, n=7 for O_BR and n=5 for O_CMS due to RANKL values beneath the biochemical detection limit). Consequently, the RANKL/OPG ratio was elevated during recovery (not shown). No differences according to age groups were found.

Calcium and phosphate homeostasis (Figure 3)

At baseline, the young subjects started with higher 25-OHD levels. Only their serum calcium levels increased during BR and did not return to baseline levels within recovery. Serum phosphate and the serum calcium phosphate product (not shown) increased in both age groups during BR and recovery, PTH levels declined during BR. Urinary calcium excretion was only higher in O_CMS during BR (P<0.001, not shown) and no significant effects in urinary phosphate excretion were found.

Discussion

The main aim of the present study was to compare biochemical markers expression of bone metabolic responses to immobilization by BR in an older and younger group of men, as well as to study possible effects of a countermeasure in the older people. The main findings were: a) that older men had lower baseline levels of bone resorption markers, bone formation markers and 25-OHD; b) that despite the lower baseline levels in the older men, the increase in markers of bone resorption during BR was comparable among the age groups; c) that markers of bone formation were affected by BR in the young men, but not in the older. Across the age groups we found an activation of P1NP in the recovery period, reflecting an increase in bone collagen synthesis, which was paralleled by reductions in circulating levels of the Wnt-signaling marker DKK1 and the RANK/RANKL/OPG pathway. Notably, there was no systematic effect of a cognitive training and nutritional countermeasure on bone metabolism in a subgroup of the older people assessed.

General effects of age

The results of the presented study indicate a general reduction in overall bone turnover in healthy men around the age of 60. This seems to be independent from vitamin D deficiency as 25OH-D levels of the older subgroup were well above the threshold for bone calcification processes of 40 ng/mL[37]. These findings are in direct agreement with epidemiologic studies in Caucasian men[16,17,19,38,39] where reduced bone turnover with increasing age has been linked to falling levels in androgens and IGF-1. Only Szulc et al. observed higher levels of bone resorption in a subgroup of some men older than 60[40]. This imbalance in bone turnover was associated with lower BMD and may: a) be linked to general health status and b) constitute a relevant turning point in the progression of age-related bone loss and increasing fracture risk, respectively.

Effects of un- and reloading on markers of bone metabolism and their control

Unaffected by differing baseline levels, markers of bone resorption increased in both age groups during BR. Markers of bone formation, however, were affected by disuse conditions only in young men. As previously described in healthy young men during 60 days of BR[41] we observed an increase in bAP and Sclerostin in the young subgroup within 14 days of immobilization. As apparent in Figure 2 and shown in former studies[12,41], Sclerostin levels increased between day 11 and 14, leading to respective reductions in osteoblast differentiation (and circulating bAP levels) according to the long-term disuse data presented by Belavy et al.[41]. Hence differentiation of osteoblasts, regulated by osteocytic Sost expression in healthy young men seems to adapt to mechanical unloading and lower PTH levels within a couple of days, whereas according to our data P1NP, reflecting bone collagen synthesis in mature osteoblasts, seems to be diminished by unloading rapidly in young people.

Previous findings of elevated DKK1 levels during BR with -6° head-down tilt[12] were not reproduced in this horizontal BR study. Furthermore there were no significant changes of the RANKL/OPG system observed during BR, and its crucial involvement in disuse-induced bone losses in man with the age of 20-60 years therefore seems questionable. Nevertheless the present data are well compatible with an involvement of OPG and of the RANKL/OPG ratio in modulating bone resorption after reloading. Additionally DKK1 levels were reduced below baseline levels during the 14 days of re-ambulation, and bone collagen synthesis was simultaneously intensified compared to baseline.

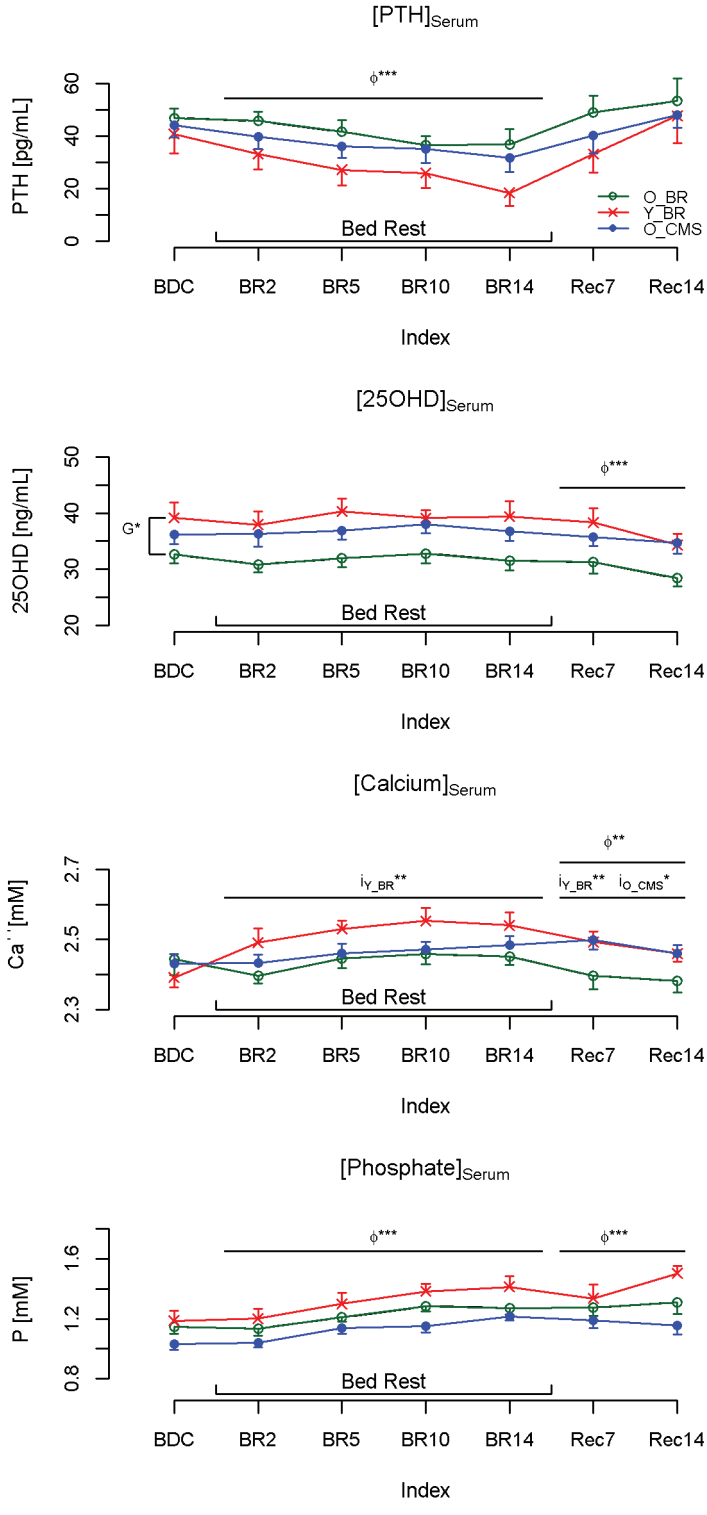

Figure 3. Parameters of calcium and phosphate homeostasis. Φ denotes significant main effect for phase (reference level: BDC); G denotes a main effects for group (reference level: O_BR); and iO_CMS denotes a phase*group interaction that was significant the respective subgroups with BDC*O_BR used as contrast (***P<0.001, **P<0.01 and *P<0.05, respectively).

Age-related effects of immobilization

BR elicited substantial increases in bone resorption markers which were comparable across age groups in absolute terms. These are well-known adaptations of the mature human skeleton to mechanical unloading[4-6,9,10,12], which are confirmed for the first time for the male senile human skeleton. On the other hand neither P1NP nor bAP were affected by BR in the older men, although the increase in Sclerostin was apparent across both age groups. This may indicate an altered antagonistic influence of Sclerostin to the Wnt pathway and thus, a reduced mechano-sensitivity of bone formating processes with ageing. Re-ambulation, however, appeared to affect DKK1, the RANKL/OPG system as well as P1NP in the older just as in the young men. Thus, the imbalance of bone resorption and formation during BR appears to be diminished in the older men, and all groups seemed to readily respond to the mechanical stimuli associated with the re-ambulation. Taking bone resorption and bone formation responses together, the present data suggest a small 'sparing' effect for immobilization-induced bone losses at old age, which would depend on sparing of the bone formation depression. In consequence, the present bone metabolism data do not support the often-heard notion that immobilization would be more deleterious in older people than in younger people.

Calcium and phosphorus homeostasis

As previously observed in other studies, BR elicited modest increases in serum levels of calcium and phosphate as well as decreases in PTH[12,42]. It seems evident that bone resorption challenges calcium homeostasis in the serum and other body fluids, and serum PTH is a key player in the control of serum Ca^{++} concentration, at least within the physiological range[43]. Thus, serum PTH should decrease as a secondary response to the primary elevation of Ca^{++} levels resulting from the disuse-induced increase in bone resorption. In addition, the younger subjects had higher 25-OHD serum levels at baseline, and this is the likely explanation for the greater increase in serum calcium levels during BR than in the older groups. As expected serum phosphate levels were concomitantly increased during BR, which was observed in all three groups to a very similar extent. However, the continued increase of phosphate during the entire 14 days of follow-up is very surprising, even more so as PTH is not decreased during the follow-up phase, and since it is therefore unlikely that the effect is engendered by renal conservation of phosphate alone.

Effects of cognitive training and nutritional countermeasure

As a combined countermeasure protocol was foreseen for the study, we additionally analyzed the impact of cognitive training and nutrition on bed-rest induced changes in bone metabolism in older people. There were a few salient differences between the two groups of older subjects. These encompassed a greater urinary CTX excretion in the countermeasure group (O_CMS) at baseline, a greater calcium excretion during BR, levated bAP at baseline and reduced bAP levels during recovery. These differences between the subgroups may suggest that the countermeasure not only failed to protect bone during BR, but that it might actually be rather counterproductive. However, the fact that CTX values were greater already at baseline, i.e. when the countermeasure protocol had not even started, likely suggests that the differences between O_BR and O_CMS groups in this study are caused by random variation between subjects.

Limitations

'The elderly' represents a very heterogeneous group concerning morbidity and functionality, which makes it difficult to study pure aging processes in humans. We studied a small subgroup of older men who were at a good state of health and sufficient in vitamin D in order to reduce side effects in addition to the ageing process on bone turnover. However, the subjects were rather 'young' older people (55-65 years) whose ageing processes may be less pronounced than in people advanced in years. Furthermore the BR period of 2 weeks was too short to study morphologic changes and deflect bone mass changes or fracture risk, so that the present conclusions are limited to assessment of circulating markers of bone metabolism. The former should be addressed in further long-term observations.

Conclusion

From the obtained results we conclude that in men ageing is characterized by a reduction in bone turnover independently from vitamin D deficiency. Unlike bone resorption, bone formation appeared to be less responsive to mechanical unloading in men with older age, suggesting that bone loss in the long run would be less pronounced than in younger people. This however needs to be confirmed by morphological analyses after longer periods of disuse.

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