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Induction of osteoporosis with its influence on osteoporotic determinants and their interrelationships in rats by DEXA

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Christian Heiss*[1,2ABDEG], Parameswari Govindarajan[1BCDEF], Gudrun Schlewitz[2ABFG], Nasr Y.A. Hemdan[1,3CDEF], Nathalie Schliefke[2ABCD], Volker Alt[1,2AG], Ulrich Thormann[2AB], Katrin Susanne Lips[1AB], Sabine Wenisch[4AB], Alexander C. Langheinrich[5AB], Daniel Zahner[6AB], Reinhard Schnettler[1,2AG]

[1] Laboratory of Experimental Trauma Surgery, Justus-Liebig University, Giessen, Germany
[2] Department of Trauma Surgery, University Hospital of Giessen-Marburg, Giessen, Germany
[3] Department of Zoology, Faculty of Science, University of Alexandria, Epypt
[4] Institute of Veterinary-Anatomy, Justus-Liebig University, Giessen, Germany
[5] Department of Radiology, University Hospital of Giessen-Marburg, Giessen, Germany
[6] Animal Laboratory, Justus-Liebig University of Giessen, Germany

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Summary

Background: As women are the population most affected by multifactorial osteoporosis, research is focused on unraveling the underlying mechanism of osteoporosis induction in rats by combining ovariectomy (OVX) either with calcium, phosphorus, vitamin C and vitamin D2/D3 deficiency, or by administration of glucocorticoid (dexamethasone).

Material/Methods: Different skeletal sites of sham, OVX-Diet and OVX-Steroid rats were analyzed by Dual Energy X-ray Absorptiometry (DEXA) at varied time points of 0, 4 and 12 weeks to determine and compare the osteoporotic factors such as bone mineral density (BMD), bone mineral content (BMC), area, body weight and percent fat among different groups and time points. Comparative analysis and interrelationships among osteoporotic determinants by regression analysis were also determined.

Results: T scores were below-2.5 in OVX-Diet rats at 4 and 12 weeks post-OVX. OVX-diet rats revealed pronounced osteoporotic status with reduced BMD and BMC than the steroid counterparts, with the spine and pelvis as the most affected skeletal sites. Increase in percent fat was observed irrespective of the osteoporosis inducers applied. Comparative analysis and interrelationships between osteoporotic determinants that are rarely studied in animals indicate the necessity to analyze BMC and area along with BMD in obtaining meaningful information leading to proper prediction of probability of osteoporotic fractures.

Conclusions: Enhanced osteoporotic effect observed in OVX-Diet rats indicates that estrogen dysregulation combined with diet treatment induces and enhances osteoporosis with time when compared to the steroid group. Comparative and regression analysis indicates the need to determine BMC along with BMD and area in osteoporotic determination.

key words: **osteoporosis • animal model • ovariectomy • skeletal site • Dual Energy X-ray Absorptiometry**

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Author's address: Christian Heiss, Department of Trauma Surgery, University Hospital of Giessen-Marburg, Rudolf-Buchheim-Strasse 7, 35385 Giessen, Germany, e-mail: christian.heiss@chiru.med.uni-giessen.de

Background

Osteoporosis is a multifactorial, age-related metabolic bone disease characterized by low bone mineral density and bone deterioration leading to enhanced fracture risk with high costs [1–5]. Postmenopausal women present, in a few years, the first outbreak of osteoporosis, which is more common in menopausal women than in men of similar age [5].

The average lifetime risk in a 50-year-old person of experiencing an osteoporotic fracture has been estimated at 40–50% for women and at 13–22% for men [6] and is expected to increase by more than 3-fold over the next 50 years of life [7]. Many fracture types are associated with osteoporosis, but the hip, spine, forearm and shoulder are the most common sites [8]. Hip fractures cause prolonged hospital care, and higher morbidity and mortality rates; therefore, there is a great need to improve fracture treatment and accelerate fracture healing in elderly patients [9]. There is also a need for a distinction to be made between diagnosis of osteoporosis and the assessment of fracture risk, which in turn implies a distinction between diagnostic and intervention thresholds [10].

The measurement of bone mass is essential in diagnosing and monitoring the treatment of bone loss [11]. Among the current methods available for bone mineral measurements, dual energy X-ray absorptiometry (DEXA) has become the method of choice [12], mostly due to its flexibility, excellent precision, high reproducibility, lower cost and low radiation exposure [13]. DEXA measurements are advantageous not only in diagnosis of osteoporosis but also for assessing fracture risk and monitoring the patient's response to osteoporotic treatment [11,13–15].

A suitable animal model minimizes the limitations associated with studying the disease in humans, namely time and behavioural variability among test subjects [16,17]. Laboratory rats meet most of these criteria, and accumulating data supports the utility of ovariectomized, aged rats in assessing the therapeutic potential of compounds to prevent or treat postmenopausal osteoporosis [2,18].

Postmenopausal osteoporosis is the rapid decrease in bone mineral density (BMD) due to estrogen deficiency after menopause and is a serious public health problem [19]. Osteoporosis is also recognized as a major complication of corticosteroid (CS) therapy, and is mediated by direct actions of the drug on bone cells [20,21]. Apart from this, BMD, BMC and bone size play a major role in determining the osteoporotic disposition [22]. Since the disease mechanism is multifactorial, a thorough knowledge should be obtained by analyzing osteoporosis by various methods, in various skeletal sites, ages and animal models so as to minimize the risk of fracture.

Thus, in the current study, rats were used as a small animal model for induction of osteoporosis. Two different approaches were explored, either by applying diet (calcium/vitamin D3 deficiency) or steroid (dexamethasone) injection, with OVX (ovariectomy) being the base in both methods, to enhance the osteoporotic effect. Further, considering the multifactorial aspect, attempts were made to assess interrelations between the skeletal sites, treatment regimens and the influence of these on the BMD, BMC, area, fat content and weight of the animals. Other investigations were also conducted to determine whether the induced osteoporotic nature influences the standard model of BMD calculations. These sets of experiment will be further used by us for long-term study in this rat model and subsequently in a larger animal model. These models will hopefully facilitate the development of new therapies for osteoporosis, implants and bone replacements for osteoporotic bone and fractures.

Material and Methods

Maintenance of animals

Fifty female Sprague-Dawley rats aged 10 weeks were purchased from Charles River (Sulzfeld, Germany). The average weights of the animals ranged between 250–290 g. Animals were maintained under standard laboratory conditions and underwent an acclimatization period of 4 weeks. The experimental procedures were approved by the German animal protection laws of district government "RP" Giessen (89/2009).

Grouping of animals

The animals divided into 3 groups: Group 1 (Sham-operated), Group 2 (OVX and diet) and Group 3 (OVX and steroid). The number of animals in each group was in the range of 6 to 25, with Group 1 and Group 3 containing 6 and 9 animals, respectively and Group 2 containing 25 animals. At the age of 14 weeks, the animals of Group 1 underwent a surgical procedure of laparotomy after being anaesthetized with intraperitoneal injection of 62.5 mg/kg body weight ketamine (Hostaket®, Hoechst) and 7.5 mg/kg body weight xylazine (Rompun®, Bayer) and were fed with normal feed. Group 2 animals were ovariectomized and were fed 2 weeks post-surgery with deficient diet (deficient in vitamin D2/D3, vitamin C, calcium, soy-free, phytoestrogen-free and scarce phosphorus supply, purchased from Altromin (Altromin-C1034, Altromin Spezialfutter GmbH, Lage, Germany). Group 3 rats were ovariectomized, then received glucocorticoid injection of dexamethasone-21-isonicotinate (Voren-Depot®, Boehringer Ingelheim, Germany) at a dose of 0.3 mg/kg body weight applied once every 2 weeks. The steroid therapy was started 2 weeks after OVX.

Measurement by Dual Energy X-ray Absorptiometry (DEXA)

Animals were scanned using DEXA (Lunar Prodigy, GE Healthcare, Germany). The rats were anaesthetized, ventrally positioned and scanned, with spine, pelvis, femur and tibia being the regions of interest (ROI) to determine the parameters of bone mineral density = BMD (g/cm^2), bone mineral content = BMC (g), lean mass (g) and fat (%). Animals were scanned immediately after OVX and laparotomy to obtain the baseline measurements followed by measurements at 1 and 3 months post-operative. Analysis was performed using the small-animal mode of the enCORE software (GE Healthcare, v. 13.40); the instrument was calibrated at each start.

Statistical analysis

Statistical analysis was done to determine the variation of various parameters (body weight, BMD, BMC, percent fat) across time points in each group and among groups at particular

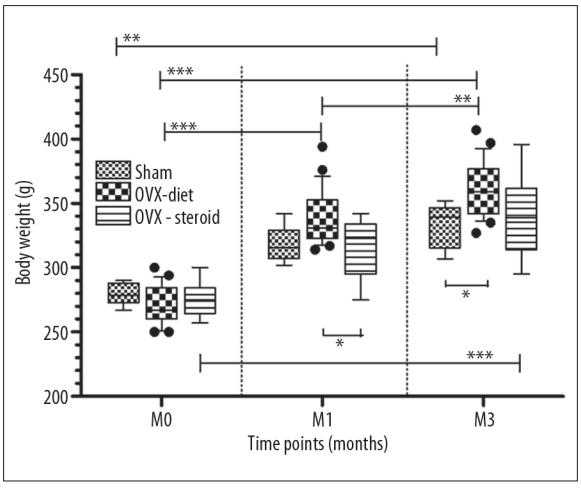

Figure 1. Body weight of different rat groups at different time points (M being months). Asterisks indicate the significance level.

times. Two-way ANOVA test accompanied by Bonferroni multiple comparison was done to determine the variation between groups at particular time points. Repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's multiple comparison test was done to determine variation in a particular group across time points. Calculation of T-scores was done using the formula T- score=(BMD-YN)/SD[14] where YN is the "young normal mean, which is the mean baseline of all groups", and SD is the standard deviation. Wilcoxon matched-pairs signed rank two-tailed test was undertaken to test variations of T-scores over time. Interrelationships between bone parameters were done by linear regression analysis. Unless otherwise mentioned, the asterisks indicate the significance level (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$). Statistical analysis was done by using GraphPad Prism version 5.

Results

Body weight

Body weights of rats were determined at baseline and at 1 and 3 months post-OVX. Irrespective of the treatments, growth of rats was normal and an increase in body weights was observed over time. Gradual and significant increase at 12 weeks was observed in sham rats, whereas in the Diet group significant increase was observed at both time points, with greater weight, though not significant, than other rats. Steroid rats exhibited significant increase at their third month post-OVX comparable to the baseline (Figure 1).

T-scores

In the current study, 14-week-old rats prior to any designated treatment were considered as the "Young Normal" condition. The Diet group showed a significant decrease in T-scores, which were well below -2.5 at all the skeletal sites in comparison to sham control at both time points. Significant decrease in T-scores between time points of Diet rats was seen in tibia and pelvis. Conversely, the control group showed an increase in positive scale in femur, spine and pelvis with time. In case of the steroid group, T-scores were not much lower than the threshold level but showed skeletal site- and time-dependent variation. Significant differences between the steroid and the Diet group were observed at all skeletal sites and time points (Figure 2).

Bone mineral density, bone mineral content and percent fat

The rats of all the groups were scanned at 14 weeks to obtain the baseline BMD values of various skeletal sites. The variation in BMD was more pronounced in the spine, which was nearly comparable to the pelvis, followed by tibia and femur.

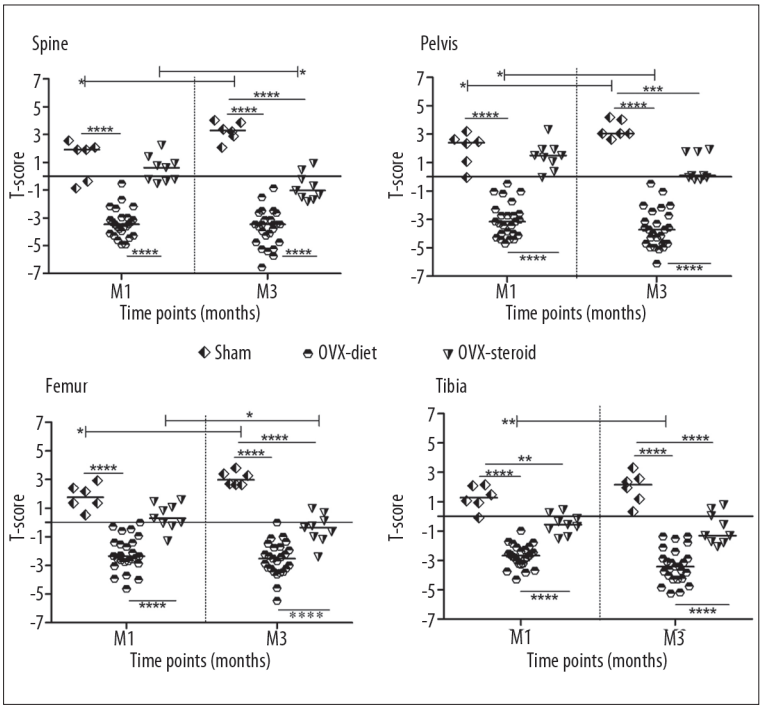

Figure 2. Variation in T score of rat groups at months 1(M1) and 3(M3) post-OVX with the significance (two way ANOVA, along with Wilcoxon matched-pairs signed rank test) indicated as asterisks.

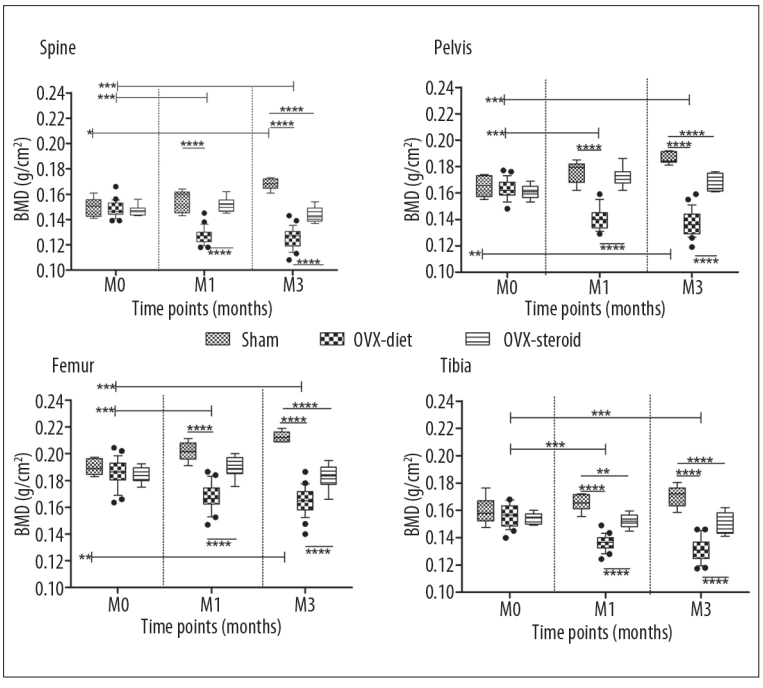

Figure 3. Variation in BMD of various rat groups (two-way ANOVA and Bonferroni's multiple comparison test) and longitudinal variation across time points (Friedman's test and Dunn's multiple comparison test).

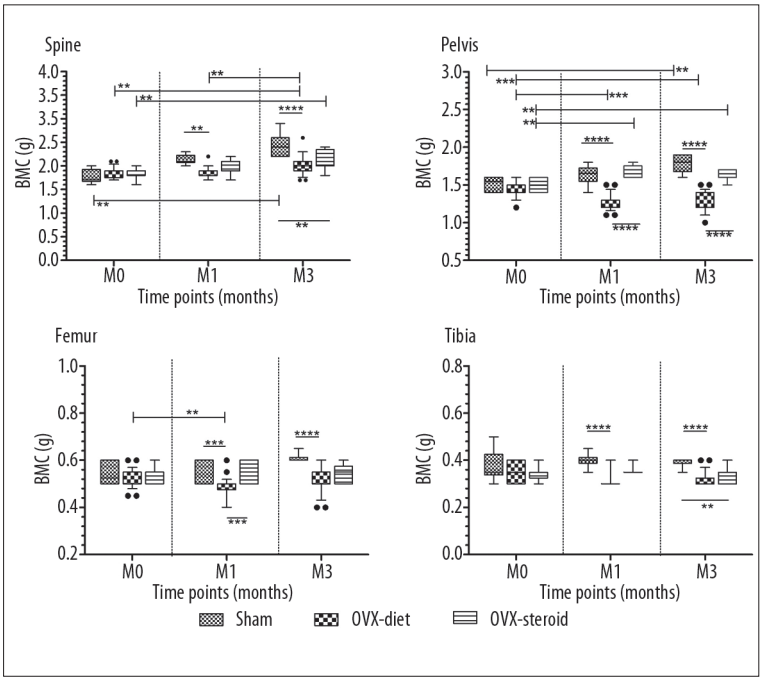

Figure 4. Variation in BMC of various rat groups at different time points and skeletal sites (two-way ANOVA and Bonferroni's multiple comparison test along with Friedman's test and Dunn's multiple comparison test).

Figure 3 demonstrates the variation in BMD of different rat groups at various skeletal sites and time points. Sham rats showed a gradual significant increase at 3 months post-OVX when compared to the 0 time point at all skeletal sites except for the tibia. OVX-Diet rats showed a significant decrease at all skeletal sites when compared to its respective baseline or age-matched sham rats. In OVX-Steroid rats, though no significant difference over time was seen, significant differences resulted on comparing different skeletal sites.

Pelvis and spine of sham rats showed a significant increase in BMC at 3 months, unlike other sites. Diet rats showed sham group-dependent significant decrease in all skeletal sites and time points, whereas when observing over time, pelvis showed significant decrease at both time points. In the femur, BMC decreased at 1 month, but the spine showed a slight significant increase at 3 months. In steroid rats, the tibia and spine showed significant decrease compared to sham rats, and a time-dependent increase in BMC was observed in the spine and pelvis compared to its baseline (Figure 4).

Generally, no significant differences were found among groups concerning% fat. Unlike in tibia of sham rats, where%fat increased at 1 month and decreased again at

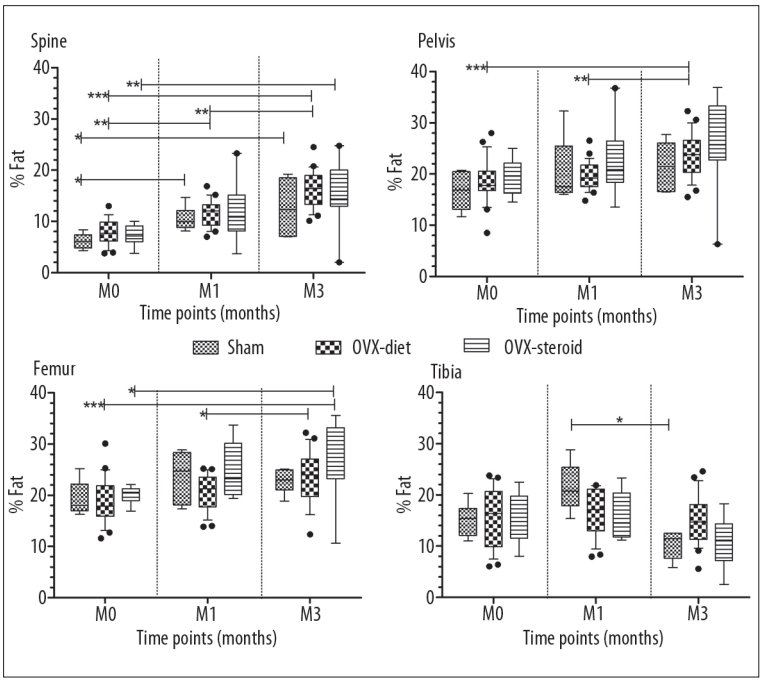

Figure 5. Fat percent of various skeletal sites of between groups (two-way ANOVA and Bonferroni's multiple comparison test) and across time points (Friedman's test and Dunn's multiple comparison test).

3 months, the spine showed an increase over time. In the Diet group, femur, pelvis and spine showed a significant increase at both time points; whereas in the steroid group, significant increase was observed only in the femur and spine comparable to baseline (Figure 5).

Comparison of BMD, BMC and area in osteoporotic and non-osteoporotic rats

The OVX-Diet group that was more osteoporotic was considered alone and comparisons were made with sham rats at respective time points (Tables 1, 2). The spine of osteoporotic rats showed a significant decrease in BMD and BMC when compared to sham rats, even though the area increased significantly at both time points. However, BMC of Diet rats gradually increased with time but was always significantly lower than sham rats.

The pelvis showed a significant decrease in BMD, BMC and area too, but a gradual increase in area lower than sham across the time points was observed in diet rats compared to its baseline. In the tibia and femur, BMD and BMC were significantly lower when compared to the controls at both time points, with no decrease in the area.

Interrelationships between BMC, BMD and area

Regression analysis was done to determine the linear relationships between the determining parameters of osteoporosis. Spine and pelvis of OVX-Diet group at the third month were selected for analysis as these sites showed prominent osteoporosis. Each of the parameters BMD, BMC and area were compared to each other (Figure 6).

In the pelvis (Figure 6A) gradual increase in BMD was found with an increase in BMC, linear relationship with correlation coefficient of 0.90 and R^2 of 0.82. It also shows that 82% of variability in BMD could be explained by BMC, but the remaining 18% remained unexplained. The correlation coefficient between BMC and area was 0.74 with R^2 of 0.56, whereas R^2 of 0.29 was observed between BMD and area. In the spine (Figure 6B) the correlation coefficient between BMC and BMD was 0.75, indicating 57% of the variation in BMD could be explained by BMC. Linear relationship with R of 0.72 and R^2 of 0.53 was observed between BMC and area, with low R^2 being 0.02 and R of 0.14 between BMD and area. In all analyses, the deviation from zero was significant, with t statistic of the slope being $p<0.0001$.

Discussion

Fragility fractures are the main public health consequence of osteoporosis [23]. Rats were chosen for the study because of their similarity to humans, low costs, and convenience [24]. Several studies indicate rats to be an almost analogous small animal model to humans, especially for the medical treatment of osteoporosis [25–30].

A lack of estrogen in postmenopausal women prevents the absorption and utilization of calcium, leading to osteoporosis development in older women [31]. Estrogens and perhaps also progestins are skeletally active steroids that markedly influence bone turnover, the withdrawal of which accelerates bone loss [32]. The most important effect of glucocorticoids is suppression of bone formation, affecting the differentiation, activity and lifespan of osteoblasts and osteocytes, and increasing osteoclast survival [33,34]. Postmenopausal women taking oral corticosteroids have the highest risk of bone loss and vertebral fracture, yet to date there has been no study of steroid-induced postmenopausal osteoporosis in small animal species [26,28]. Diet also plays a major role and osteoporosis in humans may also result from long-term negative calcium balance [35]. Decreased availability of calcium and phosphorous along with vitamin D deficiency affects mineralization, leading to low bone mineral density and osteoporosis [36–38].

Table 1. Comparison of BMC, BMD and area in spine and pelvis.

Animal groups/skeletal site	Spine			Pelvis		
	BMD (g/cm²)	BMC (g)	AREA (cm²)	BMD (g/cm²)	BMC (g)	AREA (cm²)
Sham 0 month	0.1506 (0.01)	1.8300 (0.17)	12.1000 (0.5676)	0.1650 (0.00)	1.5500 (0.1269)	9.3000 (0.48)
OVX-Diet 0 month	0.1478 (0.01)	1.8500 (0.11)	12.5000 (0.6823)	0.1619 (0.01)	1.4600) (0.10)	9.0333 (0.41)
t test P value	0.3313	0.6856	0.0870	0.3164	0.0502	0.1016
Sham 1 month	0.1560 (0.01)	2.1286 (0.10)	13.7143 (0.76)	0.1757 (0.01)	1.6429 (0.1272)	9.5714 (0.53)
OVX-Diet 1 month	0.1274 (0.01)	1.8520 (0.12)	14.6000 (0.76)	0.1407 (0.01)	1.2720 (0.11)	9.0400 (0.35)
t test P value	<0.0001	0.0002	0.0169	<0.0001	<0.0001	0.0053
Sham 3 months	0.1682 (0.00)	2.4333 (0.26)	14.5000 (1.38)	0.1860 (0.00)	1.7833 (0.12)	9.5000 (0.55)
OVX-Diet 3 months	0.1252 (0.01)	2.0240 (0.20)	16.1600 (0.98)	0.1366 (0.01)	1.2600 (0.13)	9.3600 (0.49)
t test P value	0.0002	0.0020	0.0080	0.0002	0.0002	0.3350

Comparison between BMD, BMC and area in spine and pelvis of osteoporotic and control rats. Values indicate mean and standard deviation within brackets and t test p value determines the significance.

Table 2. Comparison of BMC, BMD and area in femur and tibia.

Animal groups / Skeletal site	Femur			Tibia		
	BMD (g/cm²)	BMC (g)	AREA (cm²)	BMD (g/cm²)	BMC (g)	AREA (cm²)
Sham 0 month	0.1899 (0.01)	0.5500 (0.05)	3.0000 (0.00)	0.1591 (0.01)	0.3600 (0.07)	2.1500 (0.37)
OVX-Diet 0 month	0.1856 (0.01)	0.5200 (0.05)	2.9667 (0.13)	0.1557 (0.01)	0.3433 (0.05)	2.3833 (2.00)
t test P value	0.0640	0.0210	0.4230	0.2473	0.3751	0.1460
Sham 1 month	0.2021 (0.01)	0.5714 (0.05)	3.0000 (0.00)	0.1639 (0.01)	0.4000 (0.04)	2.4286 (0.53)
OVX-Diet 1 month	0.1677 (0.01)	0.4840 (0.05)	3.0000 (0.00)	0.1363 (0.01)	0.3160 (0.04)	2.1000 (0.30)
t test P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0044
Sham 3 months	0.2128 (0.01)	0.6083 (0.02)	3.0000 (0.00)	0.1705 (0.01)	0.3917 (0.02)	2.0000 (0.00)
OVX-Diet 3 months	0.1646 (0.01)	0.5100 (0.05)	3.0000 (0.00)	0.1313 (0.01))	0.3160 (0.04)	2.2000 (0.39)
t test P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0961

Comparison between BMD, BMC and area in femur and tibia of osteoporotic and control rats. Values indicate mean and standard deviation within brackets and t test p value determines the significance.

Fracture healing and treatment of the osteoporosis is of extreme importance, and this requires establishment of a standardized animal model in which the newly developed biomaterials, new osteosynthesis materials and bone substitutes for osteoporotic bone can be studied *in vivo*. This in turn would help to elucidate new pathological and pharmacological mechanisms in osteoporotic bone.

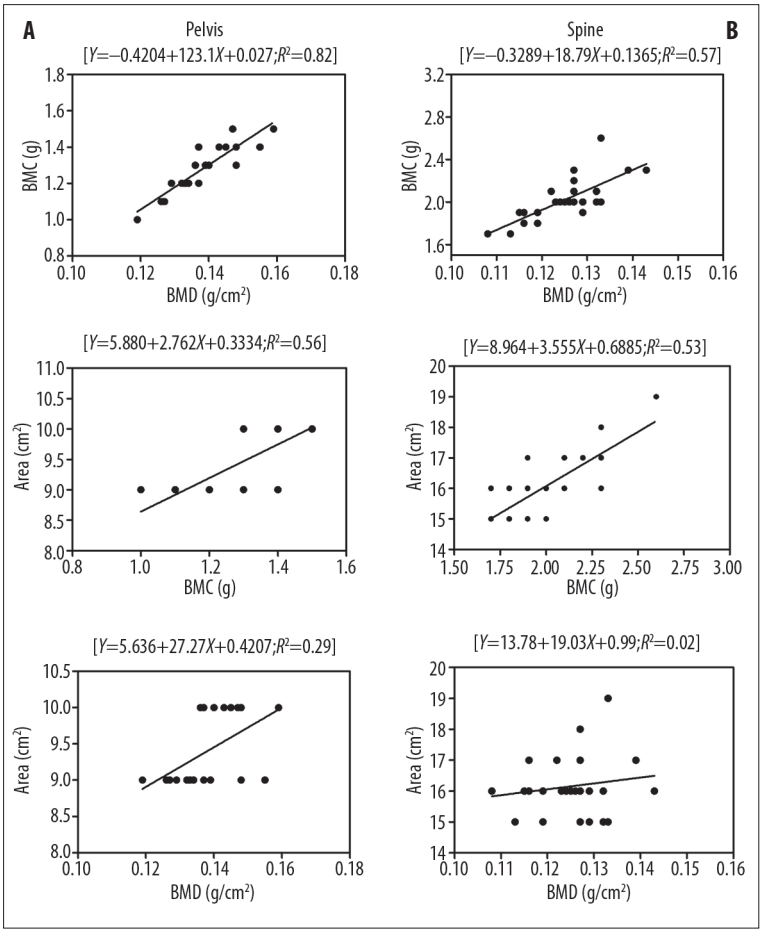

Figure 6. (**A**) Relationships between BMC, BMD and area of pelvis. (**B**) Relationships between BMC, BMD and area of spine.

In this study efforts were made to induce osteoporosis in rats, and 2 different methods of either OVX-Diet or OVX-Steroid were established to obtain rats with pronounced osteoporotic effect. In-depth detailed analysis was done at various skeletal sites to understand the effect of the treatment methods on body weight, BMD, BMC, area, and fat percent. The rats were also monitored at different time periods after a specific treatment to determine the advancement of the disease.

In our study the, T-scores were much below the SD-2.5, analogous to WHO definition for human, in case of Diet rats at all skeletal sites, in contrast to steroid rats where the effect was less pronounced but seemed to increase with time comparable to sham rats which showed an increase in T-score. The study will be continued to monitor the effect for a prolonged period to determine long-term effects of the treatment and disease progression with time.

There was a huge variation in the BMD between the groups and the time points. Sham rats showed significant gradual increase in the BMD compared to its baseline, whereas a significant decrease in the BMD was observed in Diet rats in comparison with the age-matched sham rats or its with its baseline. OVX-Steroid rats also exhibited reduced BMD, but with a lower extent than in Diet rats.

While the BMC gradually increased in sham rats over time, there was a gradual decrease in BMC in steroid and diet rats based on skeletal sites and time points. Significant increase in percent fat fraction was seen irrespective of the treatment methods based on the skeletal site.

BMD is the mineral content of the bone normalized to an apparent cross-sectional area. Interpretation of BMD changes in growing animals is especially difficult because time and treatment influence the cross-sectional area and mineral content. In contrast, densitometry provides quantitative measurement of material (BMC), which is directly related to bone mass and is the more informative endpoint and should always be reported [39]. Also, the mathematical definition indicates that the larger the bone size, the smaller the BMD, which seems to create an apparent conflict with previous findings that larger bone sizes and higher BMD values are both associated with stronger bone [22]. Thus, determining BMD alone will not be very helpful and other parameters should also be considered. Reports also suggest that the cross-sectional size of the bones must be taken into account when establishing the relationship between the mechanical characteristics of the bone and its morphology. Thus, in our study comparisons were made between the parameters (BMD, BMC and area) that determine the osteoporotic nature of the bone and their interrelationships determined by regression analysis.

Larger area, lower BMC and BMD in spine against smaller area, BMD and BMC was observed in pelvis compared to sham rats. Femur and tibia showed decreases in BMD and

BMC, with no change or increase in area. This shows that the behaviour of the attributes is altered in case of osteoporotic bone and based on the skeletal sites and requires the analysis of BMD along with BMC and area in osteoporotic patients.

Linear regression was done to determine the relationship between 2 variables and to determine how much change is observed in a variable induced by another variable. Good correlation was observed in pelvis (R 0.90), whereas in the spine correlation was slightly lower since BMC gradually increased with time when BMD decreased in osteoporotic rats. In case of relation between area and BMD, the correlation was very low, especially in the spine (R approximately 0.14) due to the decrease in BMD with increase in area. Between area and BMC, the correlation was more or less the same for both pelvis and spine. These results are somewhat similar to the human case studies related to fracturing and non-fracturing women [22]. This also leads to the supportive information that the osteoporotic nature in rats, along with the behaviour of the osteoporotic attributes, is comparable with the human case studies. Further, careful analysis of the parameters such as BMC, BMD and area in various skeletal sites is prerequisite in the diagnosis of the fracture and for the appropriate timely fracture treatment. Since the rat is the recommended model for the study related to establishment of peak bone mass, sexual dimorphism of skeleton, fracture repair, disuse, steroid induced bone loss, alcohol abuse-induced osteoporosis and senile-related bone loss [39], these in-depth studies will also help us to develop studies in animal models that simulate humans, which would be useful in establishing fracture healing and for studies related to new implants and bone substitutes.

Conclusions

This study was designed to obtain a pronounced osteoporotic rat model by combining the effect of ovariectomy either with steroid or nutrient deficiency in inducing osteoporosis. Skeletal site- and time-dependent significant increase in BMD and BMC was observed in sham rats, whereas gradual decrease of the same was observed both in OVX-Diet and in OVX-Steroid, with the Diet group showing enhanced body weight and higher effect in osteoporotic induction than the steroid group, with T-scores below -2.5. This results in establishing a standard small animal model by two different methods; the continuity and the establishment of which will be carried over to larger animal models, combined with varied analysis including imaging, biomechanical and molecular level in both models. The study also focused on the interrelationships between the bone-determining parameters and implies the significance of evaluating bone size, BMD and BMC in predicting the probability of osteoporotic fractures. All these analyses should help provide new insights into the appropriate design of new implants for osteoporotic bone and also in osteoporotic fracture healing.

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