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Skeletal effects of plyometric exercise and metformin in ovariectomized rats☆

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ABSTRACT

Estrogen deficiency causes bone loss and skeletal muscle dysfunction, and attenuates the musculoskeletal effects of exercise. The anti-diabetic drug metformin has been suggested to promote beneficial skeletal effects. To explore whether metformin can improve musculoskeletal training response during estrogen deficiency, we investigated the skeletal effects of plyometric exercise and metformin, in an ovarectomized (OVX) rat model of osteoporosis. Female Sprague Dawley rats, 12 weeks of age, rats were allocated to a sham-operated group (Sham), and four OVX groups; metformin (OVX-Met), exercise (OVX-Ex), combined metformin and exercise (OVX-MetEx) and a control group (OVX-Ctr), $n = 12$/group. Dual X-ray absorptiometry, micro computed tomography, fracture toughness testing, histomorphometry and plasma analyses were performed to explore skeletal effects. All intervention groups exhibited a higher gain in femoral bone mineral density (BMD) than OVX-Ctr ($p < .01$). The combined intervention also resulted in a higher gain in femoral and spine BMD compared to OVX-Met ($p < .01$). Both exercise groups displayed improved microarchitecture, including both cortical and trabecular parameters ($p < .05$). This was most evident in the OVX-MetEx group where several indices were at sham level or superior to OVX-Ctr ($p < .05$). The OVX-MetEx group also exhibited an enhanced toughening effect compared to the other OVX groups ($p < .05$). The beneficial skeletal effects seemed to be mediated by inhibition of bone resorption and stimulation of bone formation. The training response (i.e. jumping height) was also greater in the metformin treated rats compared to OVX-Ex ($p < .01$), indicating a performance-enhancing effect of metformin. Both exercise groups displayed higher lean mass than OVX-Ctr ($p < .05$). In conclusion, the combination of plyometric exercise and metformin improved trabecular microarchitecture and bone material properties relative to OVX controls. However, no additive effect of the combined intervention was observed compared to exercise alone.

1. Introduction

Osteoporosis and sarcopenia are major health problems in postmenopausal women, both contributing to increased fracture risk [1,2]. Estrogen deficiency plays a critical role in the pathophysiology of these conditions [2,3]. Exercise is advocated as an important countermeasure for age-related loss of bone and muscle [4]. In this context, strength training and plyometric exercises are recognized as highly effective for skeletal adaptations in both humans and rodents [5–8]. Although exercise can counteract some of the negative skeletal effects of ageing, these effects seem to be mitigated by estrogen deficiency [6,9]. We have previously shown that maximal strength training induced a significant increase in bone mineral density (BMD) in young women, whereas the skeletal effects were modest in postmenopausal women with osteopenia or osteoporosis [10,11]. In line with this, plyometric exercise has been reported to improve femoral BMD in premenopausal, but not in postmenopausal women [6]. Moreover, Lee et al. demonstrated that disturbance of estrogen signaling by deletion of estrogen receptor alpha

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(ER-α) caused impaired skeletal response to mechanical strain in female mice [12]. Another study in female rats showed that OVX inhibited exercise-induced bone adaptation during recovery from disuse [13]. Taken together, these data indicate that strength training and plyometric exercise induce skeletal adaptations that seem to be mitigated by estrogen deficiency.

Metformin is the most widely used drug for treatment of type 2 diabetes. Studies have revealed that metformin may also be beneficial for bone [14–18]. Although no clinical trials have been conducted, cross-sectional studies have reported an inverse association between metformin use and fracture risk in patients with diabetes, an effect that was most evident in women [14,15], whereas other studies could not find such an association [19,20]. Preclinical studies have shown that metformin promotes osteoprotegerin (OPG) release from osteoblasts, inhibits osteoclast differentiation in vitro, and reduces ovariectomy (OVX) induced bone loss in female rats [16,18]. In these studies metformin was administered with oral gavage. However, Jeyabalan et al. could not find any skeletal effects following 8 weeks of metformin administration, ad libitum in the drinking water, in female rats [21].

Physiological stress such as skeletal muscle contractions and exercise, induces homeostatic disruptions that activate several cellular processes, including 5′ adenosine monophosphate-activated protein kinase (AMPK) [22]. Activation of AMPK is also recognized as being one of metformin's main mechanisms of action [23]. Thus, metformin and exercise might share AMPK as a pathway for mediating their effects. Estradiol has been shown to activate AMPK via ER-beta and alpha, indicating that some of the adverse effects of estrogen deficiency could be due to impaired AMPK-signaling [24–26]. Several studies suggest that AMPK signaling may play a role in bone metabolism [17,18,27]. Accordingly, Jeyabalan et al. reported accelerated bone turnover rate, favoring bone resorption in AMPKα1$^{-/-}$ mice [28].

So far, no studies have compared the effects of metformin and exercise. We hypothesized that metformin has the ability to interact with exercise to improve the skeletal effects during estrogen deficiency. The purpose of this study was therefore to investigate the effect of metformin and plyometric jumping exercise, alone or in combination, on bone metabolism and skeletal properties in OVX rats, as a model of postmenopausal osteoporosis.

2. Methods

2.1. Animals

This study was approved by the Animal Welfare Committee at the Norwegian University of Science and Technology (NTNU), Trondheim. Sixty female Sprague Dawley rats (12 weeks of age, 231 ± 19 g) were housed in wire-top cages with aspen woodchip bedding (B&K Universal Ltd., UK). Room temperature was 24 ± 1 °C with a relative humidity of 40–50% and a 12 h light/day cycle. Rat and mouse diet (B&K Universal Ltd., UK), along with tap water were provided ad libitum. After 5 days of acclimatization, the animals were randomly allocated to five groups of 12 animals. One group was sham-operated (Sham), while the other four groups underwent OVX to induce osteoporosis as previously described [29]. The intervention started 4 weeks after surgery and lasted for 8 weeks. Two of the OVX groups were given metformin 100 mg/kg/day (Chiron, Norway) (OVX-Met) or metformin was combined with jumping exercise (OVX-MetEx), using methylcellulose (M7140, Sigma-Aldrich, Norway) as vehicle. The dosage of metformin was based on previous studies showing that ~100 mg/kg/day was sufficient to induce skeletal adaptations in OVX rats [16,18]. A third group underwent jumping exercise and vehicle administration (OVX-Ex). The Sham and OVX control groups (OVX-Ctr) were given vehicle only.

Substances were administered by daily intragastric gavage. The day before sacrifice, animals were fasted, with free access to water. Animals were anesthetized with isoflurane by inhalation (1% isoflurane mixed with 30% O_2/70% N_2O) during surgery and DXA scanning. Buprenorphine (Temgesic) was administered immediately after surgery for pain relief. At sacrifice, blood samples were collected by cardiac puncture. Both hind limbs were dissected; femurs and the right tibia were stored at −80 °C, while the left tibia was stored in 70% ethanol. All procedures and analyses were performed by blinded staff.

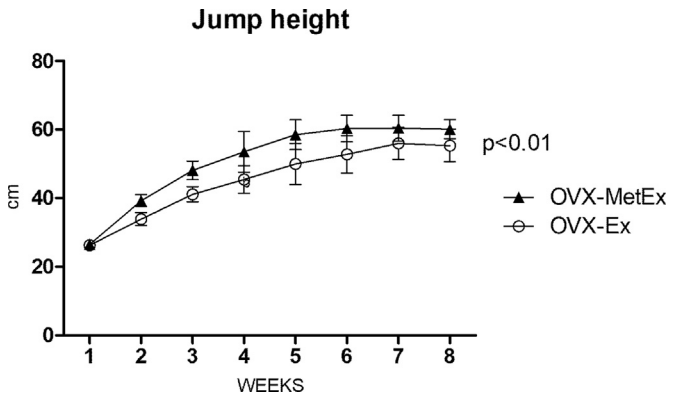

Fig. 1. Weekly progression in jump height for the ovariectomized exercise groups. OVX-MetEx = concurrent metformin administration and jumping exercise, OVX-Ex = jumping exercise only. Data are presented as mean ± SD.

2.2. Training program

The rats in the two exercise groups underwent 8 weeks of high-intensity jump training, 3 days a week, for a total of 24 sessions. The jumping exercise was based on a model described by Umemura et al. [30], emphasizing progressive jumping height throughout the training period. The animals were placed in a customized jump-cage where they were stimulated to jump on to a wooden box by a short burst of high pressure air. After each jump, the rats were carefully lifted back to the bottom of the jump-cage to perform the next jump. The jumping height was related to each animal's jumping capacity. The training sessions included 3 sets of 6 jumps. Each animal's jumping height was evaluated every session. If the rats could comfortably perform 6 jumps, and were able to carry out a seventh jump, the jumping height was increased by 4 cm (i.e. one increment in our jump-cage system). Each set was separated by 2–3 min of rest. Sessions lasted for approximately 15 min for each animal. This training intervention has also been described earlier [31].

2.3. Dual X-ray absorptiometry (DXA)

Body weight (g), fat mass (g) and lean mass (g), along with BMD (g/cm^2) at the whole body, femur and spine, were measured using a Hologic QDR 4500A, and small animal software (Hologic, Bedford, MA, USA). The coefficient of variation (CV) was 2.4% for body weight, 2.2% for fat mass, 0.28% for lean mass, 0.60% for whole body BMD, 0.71% for femur BMD and 1.0% for spine BMD. Measurements were performed at all sites before the OVX surgery, at baseline (1 month) and after the intervention (3 months).

2.4. Microcomputed tomography

Left tibias were scanned with a desktop 1176 microcomputed tomography (μCT) imaging system (SkyScan, Kontich, Belgium) at 18-μm voxel resolution using a source voltage of 65 kV and a current of 385 μA with a 1.0 mm aluminum filter to optimize contrast. The x-ray source was rotated around the samples with a rotation step of 0.5°. Raw images were then reconstructed with standard SkyScan reconstruction software (NRecon) to 3-dimensional cross-sectional image data sets using a 3-dimensional cone beam algorithm. For the reconstruction, beam

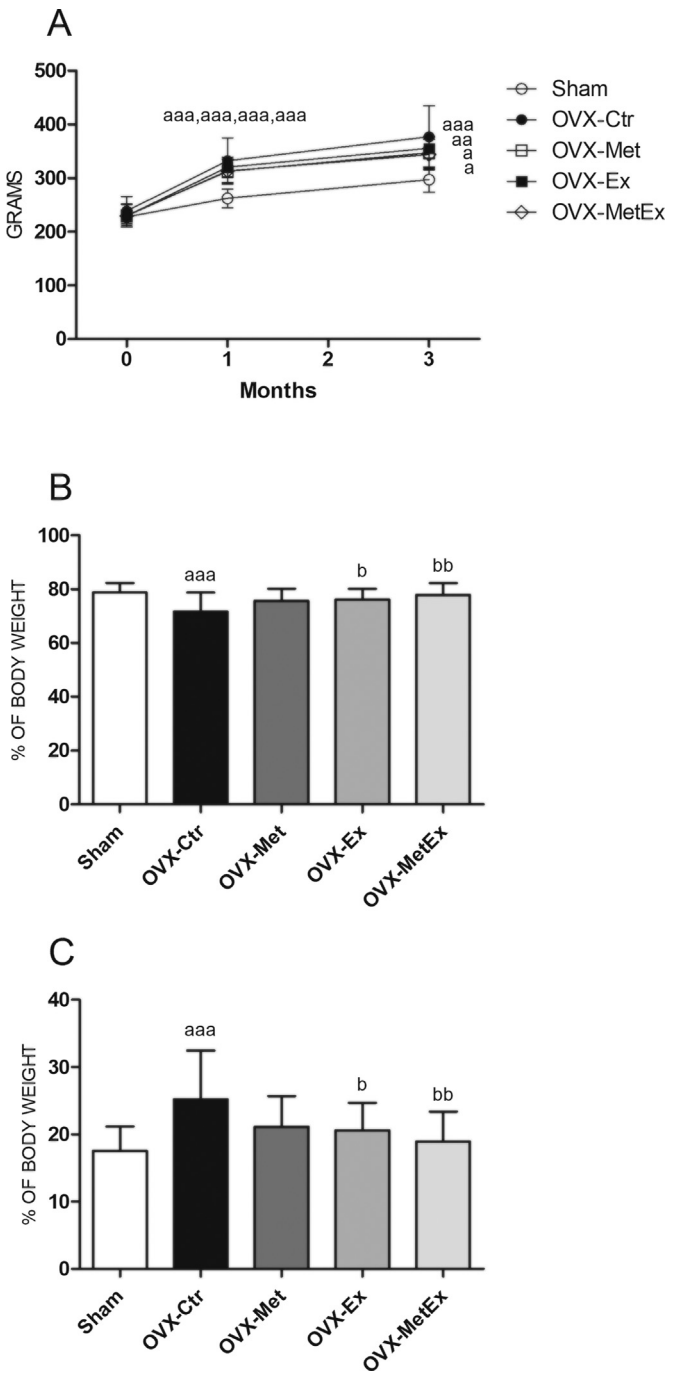

Fig. 2. Body weight (A), lean mass (B) and fat mass (C). Groups are sham-operated animals given vehicle (Sham), and ovariectomized animals given; vehicle (OVX-Ctr), metformin (OVX-Met), exercise and vehicle (OVX-Ex) and exercise and metformin (OVX-MetEx) a,aa,aaa = $p < .05, p < .01, p < .001$ compared to Sham; b, bb = $p < .05$, $p < .01$ compared to OVX-Ctr. Data are presented as mean ± SD.

hardening was set to 30%, smoothing to 2, and ring artifact reduction to 7. Analyses of reconstructed images were performed applying the standard SkyScan software (CTan). Trabecular bone was analyzed in the proximal metaphysis region starting just distal to the metaphysis and continued distally for 150 slices. Cortical bone was analyzed by a region of 100 slices, starting 9 mm distal to the metaphysis. Cortical area (Ct.Ar), cortical thickness (Ct.Th), cortical BMD (Ct.BMD), bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), structure model index (SMI) and trabecular BMD (Tb.BMD) were determined according to guidelines [32]. All parameters were assessed using standardized algorithms from BatchManager (BatMan) in the CTan software (SkyScan).

2.5. Fracture toughness testing

Right femur from each rat were notched in the anterior mid-diaphyseal region using a slow speed diamond blade (Buehler) to conduct fracture mechanics tests that provide a measure of the bone material's resistance to fracture [33]. Notching was performed on the anterior side to mimic natural loading conditions during three-point bending tests [34]. Following notching, microcomputed tomography (Viva CT40, Scanco) was performed to assess bone cross-sectional parameters (cortical area, radii) as well as notch angle. Notched bones were soaked in saline for 1 h and tested in wet conditions. The bending tests were done on a custom made fixture in the displacement feedback mode (Elf 3200, EnduraTEC) at cross-head rate of 0.001 mm/s until fracture. Fracture toughness, or critical stress intensity factor (K), was calculated using a solution for a circumferential through-wall flaw in a cylinder at initiation and maximum loads [34,35]. The initiation point was calculated based on 5% secant method while the maximum load point corresponds to the maximum load value measured during the test [35]. Toughening effect was defined as the difference between initiation and propagation toughness and it provides a measure of energy dissipated during fracture.

2.6. Bone histology and histomorphometry

Proximal tibias (right) were fixed in 70% ethanol, and processed and embedded in methylmethacrylate as previously described [36]. Five-μm-thick midsagittal sections of methylmethacrylate-embedded bones were prepared using a HM 355S microtome (Microm, Walldorf, Germany), and were stained with toluidine blue at acid pH as described [36]. Histomorphometric measurements were made using a semiautomatic system (OsteoMeasure, OsteoMetrics, Decatur, GA) and a Zeiss Axioskop microscope with a drawing attachment [36]. The area within 0.5 mm from the growth plate was excluded from the measurements.

2.7. Immunoassays

The bone formation marker type 1 collagen amino propeptide (P1NP) in plasma was determined by a Rat/Mouse P1NP enzyme-linked immunosorbent assay (ELISA) kit (Immunodiagnostic Systems Nordic A/S, Denmark). The detection limit was 0.7 ng/ml, while intra- and inter-assay variations were 6.4% and 9.2%. Bone resorption marker in plasma, C-terminal telopeptides of type I collagen (CTX-1), was analyzed by a RatLaps ELISA kit (Immunodiagnostic Systems Nordic A/S, Denmark). The detection limit was 3.0 ng/ml, and intra/inter-assay variations were 5.6% and 11%, respectively. Receptor activator of nuclear factor κ-B ligand (RANKL) and myostatin (GDF-8) in plasma were measured by Quantikine ELISA kits (R&D Systems, USA). Detection limits were 5.0 pg/ml and 2.3 pg/ml, intra-assay variations were 4.3% and 2.5%, and inter-assay variations were 6.9% and 3.6%, respectively. OPG, sclerostin, dickkopf-related protein 1 (DKK1) and leptin were analyzed with a Milliplex Rat Bone Magnetic Bead Panel 2 kit (Millipore, USA). Detection limits were 1.0, 1.9, 7.2 and 1.5 pg/ml, respectively. Intra- and inter-assay variation coefficients were < 15%. All analyses were performed according to the manufacturers' protocol.

2.7.1. Statistics

Determination of sample size was based on previous studies with OVX rats showing improved skeletal properties from metformin treatment applying 15 and 8 animals, respectively [16,18], and a study from our group showing improved femoral BMD after the same jump exercise-intervention as applied in the present study [31]. Collectively, these studies suggested that 12 rats/group would be sufficient. All data

Table 1
BMD (g/cm^2) at the whole body, spine and femur.

	Sham (n = 12)	OVX-Ctr (n = 11)	OVX-Met (n = 11)	OVX-Ex (n = 11)	OVX-MetEx (n = 9)
Whole body					
Baseline	0.143 ± 0.007	0.143 ± 0.007	0.140 ± 0.007	0.141 ± 0.009	0.141 ± 0.007
1 month	0.160 ± 0.006	0.149 ± 0.006^{aaa}	0.148 ± 0.007^{aaa}	0.151 ± 0.004^{aaa}	0.150 ± 0.006^{aaa}
3 months	0.171 ± 0.003	0.161 ± 0.007^{aaa}	0.162 ± 0.006^{aaa}	0.166 ± 0.005^{a}	0.162 ± 0.005^{aaa}
Spine					
Baseline	0.185 ± 0.010	0.191 ± 0.012	0.189 ± 0.011	0.190 ± 0.010	0.186 ± 0.008
1 month	0.225 ± 0.016	0.196 ± 0.015^{aaa}	0.199 ± 0.017^{aa}	0.204 ± 0.024^{aa}	0.195 ± 0.011^{aaa}
3 months	0.246 ± 0.016	0.206 ± 0.019^{aaa}	0.206 ± 0.021^{aaa}	0.215 ± 0.016^{aaa}	0.221 ± 0.019^{aa}
Femur					
Baseline	0.240 ± 0.010	0.245 ± 0.009	0.241 ± 0.011	0.238 ± 0.011	0.239 ± 0.010
1 month	0.290 ± 0.011	0.268 ± 0.017^{aaa}	0.262 ± 0.014^{aaa}	0.266 ± 0.010^{aaa}	0.258 ± 0.013^{aaa}
3 months	0.312 ± 0.014	0.285 ± 0.017^{aaa}	0.289 ± 0.017^{aa}	0.299 ± 0.014^{a}	0.296 ± 0.012^{a}

Data are presented as mean ± SD. BMD at baseline, pre-treatment (1 month) and post-treatment (3 months). Groups represent sham-operated animals given vehicle (Sham), and ovariectomized animals given; vehicle (OVX-Ctr), metformin (OVX-Met), exercise and vehicle (OVX-Ex) and exercise and metformin (OVX-MetEx). a,aa,aaa = p < .05, p < .01, p < .001 compared to Sham.

were tested for normal distribution with Shapiro-Wilk normality test. Normally distributed data were analyzed applying one-way ANOVA with LSD post-test. Kruskal Wallis with Dunn's post-test was used for data that were not normally distributed. Differences between the two exercise groups (OVX-Ex and OVX-MetEx) in jump-height progression throughout the intervention were analyzed with repeated measures ANOVA. Fat and lean mass are presented in % of body weight. Elsewhere, data are presented as absolute values. All statistical analyses were made using software program SPSS (version 19.0) and GraphPad Prism (version 5.0). *P*-values lower than 0.05 were regarded as significant. Data are presented as mean ± standard deviation (SD).

3. Results

3.1. General observations and training response

At the beginning of the study, four animals died due to undoing the suture by chewing (overnight). These animals were equally distributed between the four intervention groups. Two animals in the OVX-MetEx group died following complications with the oral gavage. Otherwise, no differences were observed in the animals' wellbeing throughout the study. Jumping height improved significantly from baseline in both the OVX-MetEx and OVX-Ex group, with 33.2 ± 3.1 cm and 29.0 ± 4.5 cm, respectively ($p < .01$). Jumping capacity improved significantly more in the OVX-MetEx group relative to OVX-Ex ($p < .05$, Fig. 1).

3.2. Body composition

Body weight and body composition did not differ between the groups at baseline (Fig. 2). A significant group effect was observed for body weight ($p < .001$), lean mass (percent of body weight, $p = .014$) and fat mass (percent of body weight, $p < .01$). Body weight was higher in all OVX groups compared to Sham, both before and after the intervention, while no differences occurred between the OVX groups (Fig. 2). Lean mass was lower in the OVX-Ctr group compared to Sham after the intervention ($p < .01$). Furthermore, OVX-Ex and OVX-MetEX displayed higher lean mass than OVX-Ctr (76 ± 4% and 78 ± 4% vs 72 ± 2% respectively, $p < .05$), while OVX-Met had lean mass similar to Sham (Fig. 2). At the end of the study, fat mass was higher in OVX-Ctr relative to Sham (p < .01), and compared to OVX-MetEx and OVX-Ex (25 ± 7% vs 19 ± 4% and 21 ± 4% respectively, p < .05). Fat mass was also similar to Sham in the OVX-Met group (Fig. 2).

3.3. Whole body, spine and femur BMD

At baseline, there were no differences in whole body or regional BMD. Whole body BMD was lower in all OVX groups compared to Sham at the start of the intervention and at the end of the study (Table 1). Similarly, BMD at the spine and femur was lower in all OVX groups compared to Sham, and no differences were observed between the OVX groups at pre- and post-intervention (Table 1). However, a significant group effect was observed in pre- to post-intervention changes in femoral BMD ($p < .001$). The gain in femoral BMD throughout the intervention was significantly higher in the OVX-MetEx group compared to Sham, OVX-Ctr and OVX-Met (15 ± 4% vs 6 ± 3%, 10 ± 2% and 2.7 ± 2.2% respectively, $p < .01$, Fig. 3), along with a trend towards higher femoral BMD gain than OVX-Ex ($p = .058$). The OVX-Ex and OVX-Met groups also improved femoral BMD more than the OVX-Ctr group (p < .01, Fig. 3). The effects were less prominent at the spine, although a between group effect was observed in pre- to post-intervention changes in BMD ($p = .013$). The spine BMD gain was higher in the OVX-MetEx group compared to OVX-Ctr, OVX-Met and OVX-Ex (13 ± 8% vs 5 ± 6%, 3 ± 6% and 6 ± 9% respectively, $p < .05$).

3.4. Proximal tibia μCT parameters

An overall group effect was observed for all μCT parameters ($p < .001$). Cortical area was higher in the exercise groups (OVX-Ex and OVX-MetEx) relative to Sham, OVX-Ctr and OVX-Met ($p < .05$, Fig. 4B). OVX-Ex and OVX-MetEx also had greater cortical thickness than both OVX-Ctr and Sham (p < .05, Fig. 4C). Trabecular BV/TV was higher in the OVX-MetEx group compared to OVX-Ctr (p < .05). All groups except OVX-MetEx exhibited significantly lower BV/TV relative to Sham (Fig. 4E). All groups except OVX-MetEx had lower Tb.N and Tb.BMD compared to Sham (Fig. 4F and J). Tb.Th was significantly higher in the OVX-MetEx group relative to OVX-Ctr and OVX-Met ($p < .05$, Fig. 4G). Both exercise groups (OVX-Ex and OVX-MetEx) also displayed Tb.Th similar to Sham. Compared to Sham, Tb.Sp was higher in all groups, except OVX-MetEx (Fig. 4H). SMI was significantly lower in the OVX-MetEx group relative to OVX-Ctr (Fig. 4I).

3.5. Fracture properties of bone

A significant group effect was observed for toughening effect ($p = .042$). The OVX-MetEx group demonstrated a superior toughening effect, compared to the other OVX ($p < .05$) groups, and by trend compared to Sham ($p = .058$, Table 2). Initiation and propagation toughnesses values demonstrated the same trend as toughening effect but were not significantly different between groups (Table 2).

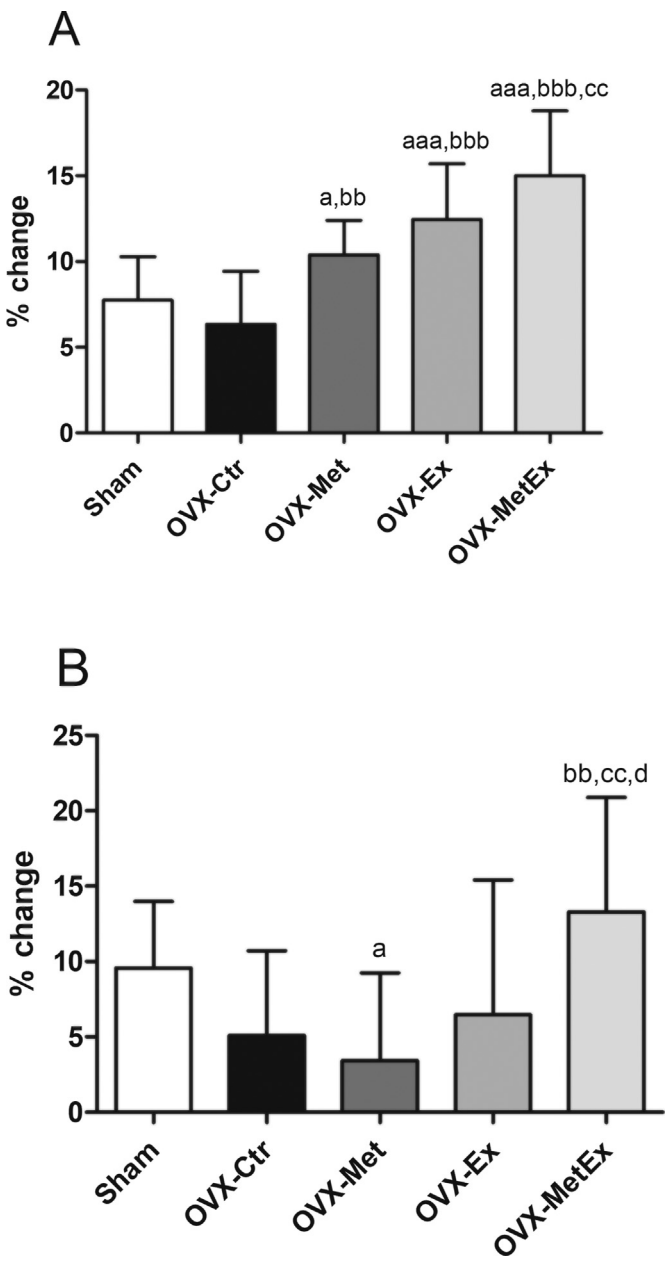

Fig. 3. Changes in BMD at the femur (A) and spine (B). Groups are sham-operated animals given vehicle (Sham), and ovariectomized animals given; vehicle (OVX-Ctr), metformin (OVX-Met), exercise and vehicle (OVX-Ex) and exercise and metformin (OVX-MetEx) a, aaa = $p < .05$, $p < .001$ compared to Sham; bb, bbb = $p < .01$, $p < .001$ compared to OVX-Ctr; cc = $p < .01$ compared to OVX-Met; d = $p < .05$ compared to OVX-Ex. Data are presented as mean ± SD.

3.6. Bone histomorphometry

A significant group effect was evident for all histomorphometric parameters ($p < .01$). Mineralized surface (MS/BS) was significantly higher in the OVX-Ctr and OVX-Met groups compared to Sham $p < .01$), whereas OVX-Ex and OVX-MetEx did not differ from Sham (Fig. 5B). Surface-based bone formation rate (BFR/BS) was significantly higher than Sham in OVX-Ctr, OVX-Ex and OVX-MetEx ($p < .05$), while the OVX-Met group scored similar to Sham (Fig. 5C). Mineral apposition rate (MAR) was higher than Sham in the OVX-Ctr group and lower than OVX-Ctr in the OVX-Met group ($p < .05$ and 0.01, respectively, Fig. 5D). Osteoblast surface (Ob.S/BS) and osteoid surface (OS/BS) were similar to Sham in the OVX-Met and OVX-MetEx groups (Fig. 5E and F). Osteoclast surface (Oc.S/BS) was significantly lower in the OVX-MetEx group compared to OVX-Ctr. All of the intervention groups (OVX-Met, OVX-Ex and OVX-MetEx) displayed osteoclast surface similar to Sham (Fig. 5G).

3.7. Plasma analyses

A significant group effect was seen for the bone formation marker P1NP ($p < .01$), the resorption marker CTX-1 ($p < .01$), sclerostin ($p < .01$), and leptin ($p = .018$). P1NP was lower in the OVX-MetEx and OVX-Ex group compared to OVX-Ctr, and also relative to Sham in the latter ($p < .05$, Table 3). CTX-1 was higher than Sham in all the OVX groups ($p < .05$, Table 3), whereas OVX-Ex displayed lower levels than OVX-Ctr ($p < .05$, Table 3). None of the groups differed in OPG, RANKL or OPG/RANKL ratio. Sclerostin was lower in all the OVX groups compared to Sham. Plasma leptin level was lower than OVX-Ctr in the OVX-Ex and OVX-MetEx groups. The Sham group also had lower leptin levels than OVX-Ctr ($p < .05$, Table 3).

4. Discussion

This study is the first to investigate the combined effect of metformin and exercise on skeletal properties in estrogen-deficient rats. A higher gain in femoral BMD was observed in all intervention groups compared to OVX controls. The combined intervention generally improved trabecular microarchitecture and bone material properties compared to OVX controls, however, no significant differences between the intervention groups were observed. The beneficial skeletal effects seemed to be mediated by inhibition of bone resorption and stimulation of bone formation. The training response (i.e. jumping height) was greater in the metformin treated rats compared to OVX-Ex, indicating a performance-enhancing effect of metformin. Moreover, the training intervention induced higher lean mass in both of the exercise groups compared to OVX controls.

In line with previous studies [16,18,31], jumping exercise and metformin each influenced femoral BMD positively. The combined intervention induced a 15% gain in femoral BMD, versus 12 and 10% in the OVX-Ex and OVX-Met, respectively. The gains in femoral BMD in the OVX-Ex and OVX-MetEx groups were also superior to Sham. As expected from this type of exercise intervention, the increase in BMD was most pronounced at femur. The combination group also exhibited a higher gain in spine BMD than the other OVX groups. Although comparable studies are few, the lack of effect from exercise alone is in contrast to a previous study, reporting increased BMD at the spine following 12 weeks of jumping exercise (20 jumps, 5 days/week) in OVX rats [37]. This discrepancy might be due to differences in training volume and duration of the intervention period. Nevertheless, the combination of metformin and exercise seemed to induce an additive effect on spine BMD. In this regard, histomorphometric analyses of vertebras would have been useful to explore the cellular mechanisms involved, which warrant further research.

The μCT data reflected the BMD findings, showing that jumping exercise partly restored the OVX-induced deterioration in microarchitecture, whereas metformin alone did not affect any of the μCT-derived parameters. Both exercise groups displayed trabecular thickness at sham level, whereas cortical thickness was superior to Sham and OVX controls. These findings are compatible with jumping exercise exerting a stimulatory effect on bone formation. Trabecular number and spacing were restored to sham level in the OVX-MetEx group, indicating that metformin enhanced the positive effect of exercise on bone architecture. A previous study in OVX rats reported that metformin given in a similar dosage as in our study, improved most of the trabecular parameters after 8 weeks of intervention [18]. This disparity may be attributed to differences in study design, as the intervention was initiated 4 weeks after the OVX procedure in the present study, while immediately after surgery in the previous study [18].

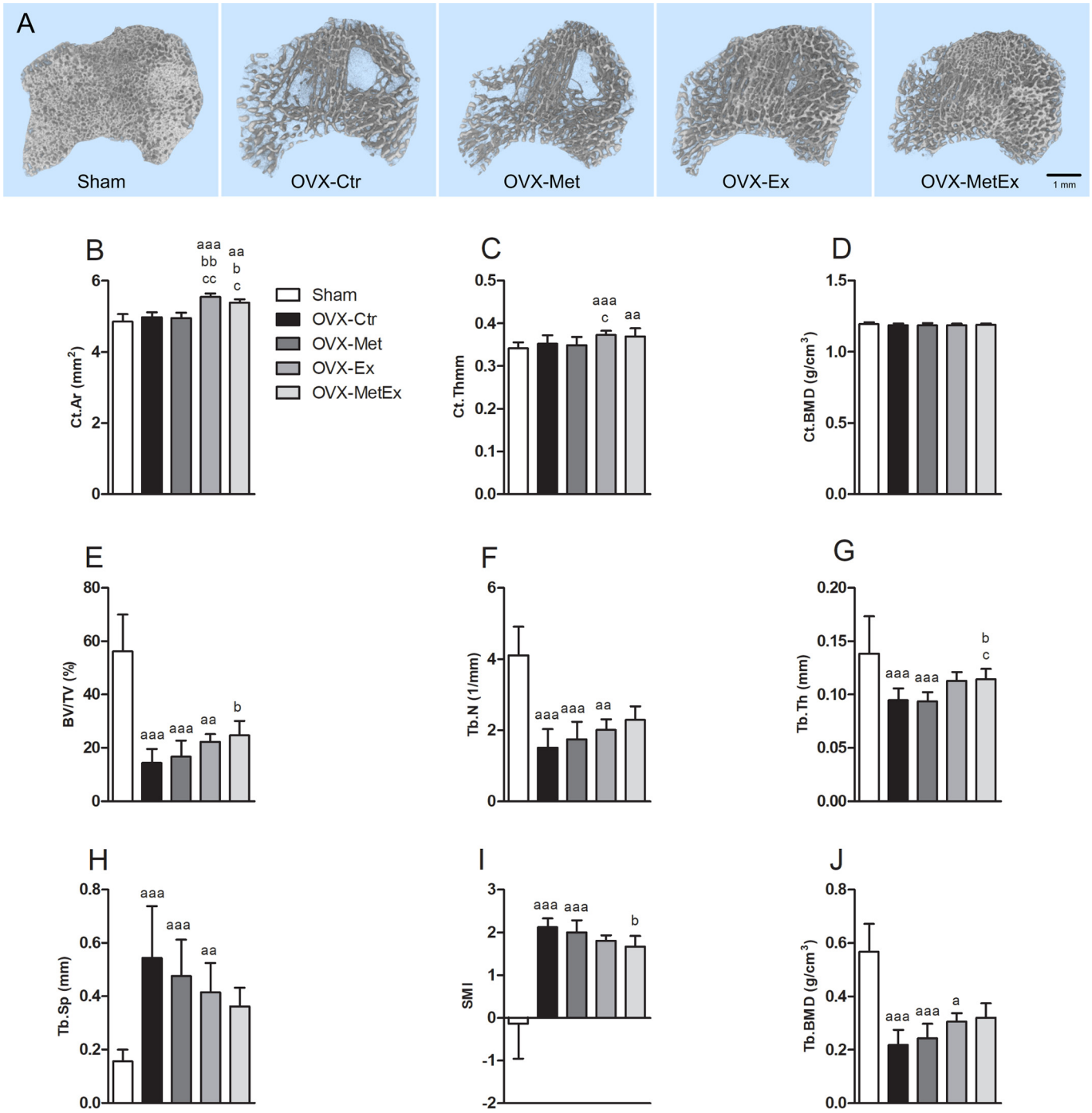

Fig. 4. Representative images of trabecular bone structure (A). Ct.Ar = cortical area (B); Ct.Th = cortical thickness (C); Ct.BMD = cortical bone mineral density (D); BV/TV = bone volume fraction (E); Tb.N = trabecular number (F); Tb.Th = trabecular thickness (G); Tb.Sp = trabecular separation (H); SMI = structure model index (I); Tb.BMD = trabecular BMD (J). Groups represent sham-operated animals given vehicle (Sham), and ovariectomized animals given; vehicle (OVX-Ctr), metformin (OVX-Met), exercise and vehicle (OVX-Ex) and exercise and metformin (OVX-MetEx). a, aa, aaa = $p < .05$, $p < .01$, $p < .001$ relative to Sham. b, bb = $p < .05$, $p < .01$ relative to OVX-Ctr. c, cc = $p < .05$, $p < .01$ relative to OVX-Met. Data are presented as mean ± SD.

In addition to bone mass and microarchitecture, bone material properties are essential for resistance against fracture. In the present study, we assessed toughness, which is a critical property for prevention of fracture [38]. Toughening effects reflect the ability of bone to absorb impact energy during the propagation of fracture from e.g. a microcrack [39]. To our knowledge, this is the first study to investigate how toughness is affected by metformin and exercise. The metformin + exercise group exhibited a distinctive toughening effect compared to the other OVX groups, which may translate into reduced fracture risk. Increased fracture toughness has previously been described in mice after treadmill exercise [40]. Our results suggest that this effect may partly be attributed to the improved bone material properties caused by enhanced cortical bone formation. For example, bone formation causes deposition of bone that is rich in non-collagenous matrix proteins, such as osteopontin and osteocalcin, as well as collagen that is not modified by accumulation of advanced glycation end products (AGEs). Toughness is shown to be reduced by the age-related accumulation of AGEs in collagen and loss of non-collagenous proteins [41,42]. Metformin and

Table 2
Biomechanical parameters.

	Sham	OVX-Ctr	OVX-Met	OVX-Ex	OVX-MetEx
Initiation toughness [MNmˆ(−3/2)]	1.61 ± 0.47	1.03 ± 0.27	1.26 ± 0.23	1.51 ± 0.61	1.22 ± 0.28
Propagation toughness [MNmˆ(−3/2)]	6.06 ± 1.30	5.55 ± 1.02	5.90 ± 0.57	6.04 ± 1.40	6.86 ± 0.81
Toughening effect [MNmˆ(−3/2)]	4.72 ± 1.34	4.52 ± 1.02	4.50 ± 0.76	4.36 ± 1.02	5.64 ± 0.70[b,c,d]

Data are presented as mean ± SD. Groups represent sham-operated animals given vehicle (Sham), and ovariectomized animals given; vehicle (OVX-Ctr), metformin (OVX-Met), exercise and vehicle (OVX-Ex) and exercise and metformin (OVX-MetEx). b = p < .05 compared to OVX-Ctr; c = p < .05 compared to OVX-Met; d = p < .05 compared to OVX-Ex.

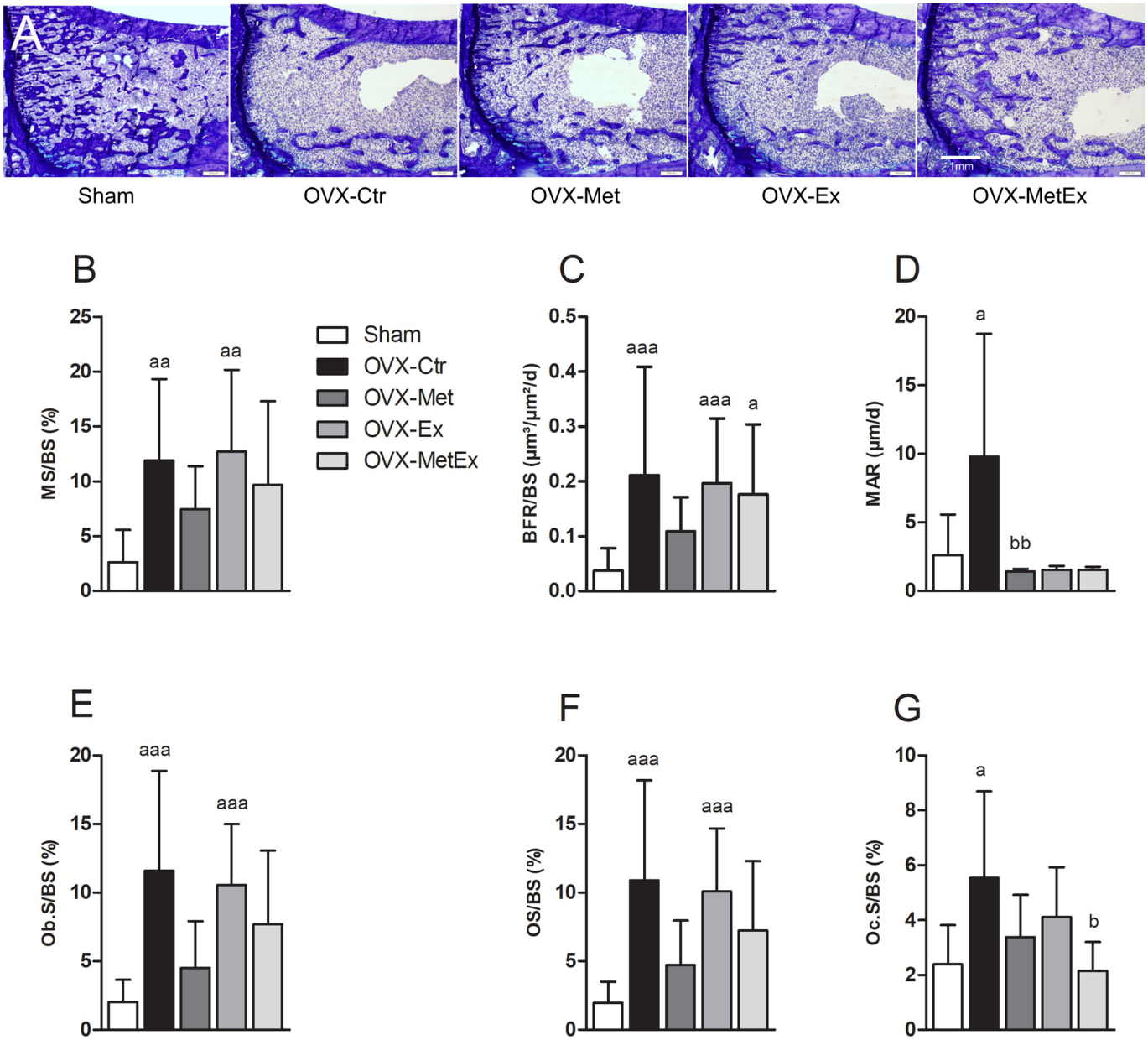

Fig. 5. Representative images of histological cross sections. MS = mineralized surface; BS = bone surface; BFR = bone formation rate; MAR = mineral apposition rate; Ob.S = osteoblast surface; OS = Osteoid surface; Oc.S = osteoclast surface. Groups represent sham-operated animals given vehicle (Sham), and ovariectomized animals given; vehicle (OVX-Ctr), metformin (OVX-Met), exercise and vehicle (OVX-Ex) and exercise and metformin (OVX-MetEx). a,aa,aaa = $p < .05$, $p < .01$, $p < .001$ compared to Sham. b, bb = p < .05, p < .01 relative to OVX-Ctr. Data are presented as mean ± SD.

exercise have both been associated with reduced content of AGEs [43,44], and the improved fracture toughness in the OVX-MetEx group may be attributed to a synergistic effect.

The beneficial skeletal effects observed in the combination group and OVX-Ex group appeared to be mainly mediated by a decrease in OVX-induced bone resorption, combined with a maintenance of the OVX-induced increase in bone formation, as reflected in histomorphometric analyses. Osteoclast surface was at sham level in the OVX-MetEx group and significantly lower than in the OVX-Ctr. The other intervention groups exhibited a similar pattern, implying that both exercise

Table 3
Serum analyses.

	Sham	OVX-Ctr	OVX-Met	OVX-Ex	OVX-MetEx
P1NP (ng/ml)	6.2 ± 1.2	6.8 ± 1.2	5.8 ± 1.2	4.8 ± 0.9^{a,bb}	5.3 ± 1.8^{b}
CTX-1 (ng/ml)	7.6 ± 2.4	12.2 ± 3.4^{aaa}	10.7 ± 3.0^{aa}	9.9 ± 1.9^{a,b}	10.5 ± 1.8^{a}
OPG (pg/ml)	220 ± 119	317 ± 247	210 ± 108	257 ± 160	314 ± 132
RANKL (pg/ml)	86 ± 28	127 ± 61	101 ± 16	113 ± 17	119 ± 31
OPG/RANKL (ratio)	2.6 ± 1.8	3.2 ± 2.5	2.2 ± 1.3	2.3 ± 1.5	2.9 ± 1.6
Sclerostin (pg/ml)	292 ± 50	237 ± 47^{aa}	221 ± 42^{aaa}	226 ± 32^{aa}	252 ± 36^{a}
DKK1 (pg/ml)	734 ± 147	717 ± 363	571 ± 141	591 ± 160	661 ± 188
Leptin (ng/ml)	3.5 ± 1.5	7.0 ± 4.2^{a}	5.0 ± 1.9	4.4 ± 1.9^{b}	4.3 ± 1.2^{b}

Data are presented as mean ± SD. P1NP = type 1 collagen amino propeptide, CTX-1 = C-terminal telopeptides of type I collagen, OPG = osteoprotegerin, RANKL = receptor activator of nuclear factor κB ligand, DKK1 = dickkopf-related protein 1. Groups represent sham-operated animals given vehicle (Sham), and ovariectomized animals given; vehicle (OVX-Ctr), metformin (OVX-Met), exercise and vehicle (OVX-Ex) and exercise and metformin (OVX-MetEx). a,aa,aaa = $p < .05$, $p < .01$, $p < .001$ compared to Sham; b, bb = $p < .05$, $p < .01$ compared to OVX-CTR.

and metformin decreased bone resorption. This is in line with previous studies, showing that metformin and exercise reduce osteoclastogenesis through downregulation of RANKL and upregulation of OPG [16,45,46]. In contrast to osteoclast number, indices of osteoblast activity, MS/BS and BFR/BS, remained elevated in the OVX-Ex and OVX-MetEx groups compared to Sham, at levels similar to OVX-Ctr rats. Taken together, these findings denote a high turnover state in the OVX-Ctr group, with bone resorption exceeding bone formation. Whereas exercise seemed to have a dual action, inhibiting bone resorption and stimulating bone formation, the effect of metformin was mainly anti-resorptive. Li et al. showed a similar pattern in OVX rats exercising in a running wheel [46]. However, bone formation was mainly increased in trabecular bone, and the authors proposed that exercise might differentially affect bone formation in trabecular and cortical bone [46]. This is in contrast to our finding of increased exercise-induced bone mass in cortical and trabecular bone, illustrating that the response is dependent on the type of exercise.

The plasma levels of the bone turnover markers CTX and P1NP did not comply with histomorphometry and micro-CT data. All OVX-rats displayed higher CTX levels than Sham, whereas P1NP levels were similar to Sham. The lack of P1NP response from exercise alone is in line with previous studies applying jumping and treadmill exercise, respectively [31,47]. However, other studies have shown an increase in the bone formation marker osteocalcin after jumping exercise in rats [7,43]. Although previous rat studies with metformin did not assess bone turnover markers in serum, human studies seem to consistently report lower P1NP levels in patients using metformin [48,49]. The relatively short the half-life of metformin (6–7 h) may have contributed to attenuate the measurable effect on serum P1NP in our study. This is presumably because blood was collected 48 h after cessation of the intervention. Thus, the effect on bone markers may have been blunted and is more likely to reflect the estrogen deficiency state in these rats.

At the cellular level, the anabolic response of exercise may be mediated through several mechanisms. Suppression of the Wnt-inhibitors sclerostin and DKK1, as a response to mechanical stimuli is one potential mechanism. We observed lower levels of these substances in all OVX groups, compared to Sham, although not significant for DKK1. The low sclerostin levels observed in the intervention groups in our study may reflect the lower bone mass or could be an effect of exercise and metformin. Studies in young animals, and also clinical studies have shown an exercise-induced decline in sclerostin [50–52]. In one study, metformin was found to reduce sclerostin levels in men with type 2 diabetes [53]. It is, however, reasonable that the effect of the intervention on sclerostin had leveled off after 48 h when blood samples were drawn. Finally, leptin levels were lower in both exercise groups than in the OVX controls, reflecting the lower fat mass.

We observed a performance-enhancing effect of metformin during plyometric exercise. This is in line with a previous study showing that metformin improved aerobic cycling performance in healthy subjects [54]. Moreover, high-intensity jump training seems to be a good model mimicking plyometric exercise in humans, as the improved jumping capacity in the exercise groups coincided with substantial effects on skeletal properties and lean mass. This complies with clinical studies showing that plyometric exercise such as jump training promotes gain in bone mass [55]. Official guidelines also recommend plyometric exercise, along with resistance training, as a countermeasure for age-related bone loss [56]. Our study suggests that metformin could enhance the skeletal response to exercise in postmenopausal osteoporosis.

In summary, plyometric exercise and metformin, alone or in combination, induced higher femoral BMD gains than OVX controls. The combined intervention generally improved trabecular microarchitecture and bone material properties compared to OVX controls, but did not give any distinctive effects over exercise alone. Whereas exercise caused a dissociation of bone turnover, inhibiting bone resorption and stimulating bone formation, metformin exerted an anti-resorptive effect. Both exercise groups exhibited higher lean mass than OVX-Ctr. Moreover, metformin seemed to have a performance-enhancing effect. More research is needed to further unravel the combined effect of metformin and exercise on bone and muscle.

Declaration of competing interest

All authors state that they have no conflicts of interest

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Authors' contributions

Study design: MPM, KS and US. Animal handling and exercise: MPM and KS. DXA measurements and analyses: MPM and KS. μCT: MPM. Fracture toughness testing: MT and DV. Tissue preparation and histomorphometric analyses: ERG, CS. Immunoassays: KS and MPM. Data interpretation: MPM, KS, US and EFE. Preparation of manuscript: MPM, KS, EFE and US.

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