

Original Article

Effect of (-)-epigallocatechin-3-gallate in preventing bone loss in ovariectomized rats and possible mechanisms

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Abstract: Despite recent developments reported in studies of (-)-epigallocatechin-3-gallate (EGCG), its early preventive effect of mitigating bone loss is not well understood. We investigated the effect of EGCG in preventing bone loss in ovariectomized (OVX) female rats, and explored the possible underlying mechanisms. Twelve-week-old female Sprague-Dawley rats, were divided into 3 groups: group A received intraperitoneal EGCG for 12 consecutive weeks, begun 3 days after ovariectomy; group B received ovariectomy alone; group C, received a sham operation. At the end of the experiment, tibias and femurs were harvested for: (1) micro-CT scanning and measurement of bone mineral density (BMD) and bone morphological parameters; (2) a 3-point bending test; (3) HE staining and an immunohistological study investigating Sema4D expression. Results: The BMD and BV/TV of group A were significantly higher than for the OVX group. The trabecular separation (Tb.Sp) of group A was significantly lower than for group B. Results from the 3-point bending test showed no statistical significance among all the groups. Bone histological studies indicated that trabecular bone was denser in group C, while group B had less dense trabecular bone, and the bone morphological status of group A was intermediate between groups A and C. The immunohistological study demonstrated that Sema4D was more highly expressed as a percentage of the brown-stained area in group B than in the other 2 groups. Conclusion: EGCG had a positive effect on mitigating bone loss in ovariectomized rats, and it inhibited Sema4D expression in bone tissue. Early stage supplementation of EGCG at a dose of 10 mg/kg/day after the onset of ovariectomy did not entirely eliminate bone loss.

Keywords: EGCG, osteoporosis, bone loss, microarchitecture, BMD, Sema4D

Introduction

Osteoporosis has become a global public health issue as the aging of the population increases. It is a degenerative disease characterized by low bone mass, an increased rate of bone fragility, and susceptibility to fractures, especially of the hip, spine and wrist [1]. Postmenopausal women have a high risk of osteoporosis due to lack of estrogen [2]. Although various drugs for the treatment and prevention of osteopenia and osteoporosis are available, the development of new, effective and economical medicines that cause fewer complications is still urgent.

Green tea, produced from the dried leaves of *Camellia sinensis*, is a popular beverage world-

wide [3]. Antioxidant effects are a widely recognized peculiarity of tea polyphenols [4, 5], which might also regulate estrogen deficiency-induced bone loss in postmenopausal women [4, 5]. Catechins comprise more than 80% of green tea polyphenols, mainly consisting of (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin-3-gallate (EGCG), which accounted for more than 50% of the total catechins [6]. Supported by *in vitro* studies [3, 7], an *in vivo* study that focused on the therapeutic effects of EGCG on the bone microarchitecture of osteoporosis in a rodent model has been carried out, and it demonstrated that EGCG could mitigate bone loss in ovariectomized (OVX) rats, but total recovery of the bone structure was not observed [8]. Whether early preventive supplementation of

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EGCG could achieve an ideal recovery of bone structure in rodent models has not been demonstrated.

Semaphorins were reported to be involved in the cell-to-cell communication between osteoclasts and osteoblasts [9, 10]. Evidence suggested that osteoclasts express semaphorin4D (Sema4D or CD100), which potently inhibits bone formation [9]. Consequently, Sema4D provides a new therapeutic target for anti-osteoporosis treatment. However, whether EGCG regulates bone metabolism via this signal pathway is unknown, and related *in vivo* studies are rare. Therefore, we conducted this study in female ovariectomized (OVX) rats and intervened with EGCG in the early stages after ovariectomy to assess the preventive effects on bone microstructure and their possible mechanisms. In addition, we also studied the expression of Sema4D in bone tissue.

Materials and methods

Experimental characteristics and group design

Soochow University permits and approvals this work and study. Virgin female OVX Sprague-Dawley (SD) rats were used because (a) this study was focused on simulating the condition of postmenopausal women, for whom pregnancy and lactation might influence bone structure [11] and (b) OVX rats that simulate estrogen deficiency are currently a widely used animal model for postmenopausal osteoporosis [12]. Rats were purchased from Shanghai Laboratory Center, China. This animal study was approved by the Institutional Animal Care and Use Committee. EGCG (99% purity) was purchased from Hangzhou Ebeikar Tea Development CO., LTD, Hangzhou, China. All rats were raised in a temperature-controlled room at approximately $23\pm 1^{\circ}\text{C}$ and maintained on a regular rodent chow diet with distilled water given *ad libitum* throughout the experimental period. After a 1-week acclimation phase, 35 rats were weighed and assigned into 3 groups with no statistically significant differences among the pre-operative weights: Group A (n=15) was ovariectomized and EGCG was given intraperitoneally at 10 mg/kg/day, beginning 3 days after ovariectomy and continuing for 12 consecutive weeks; this group was labeled as OVX+EGCG/IP; Group B consisted of ovariectomized controls (n=10), labeled as OVX, and Group C

consisted of sham-operated controls (n=10), labeled as SHAM.

Surgical procedures

Ovariectomy was performed under intraperitoneal anesthesia. A ventral middle incision approximately 1.5-2.5 centimeters was made in the lower abdomen. After separation of muscles and exposure of the retroperitoneal fat tissues, a Y-shaped uterus appeared. Two ovaries were present, resembling two irregular-shaped masses distal to the fallopian tubes bilaterally. The fallopian tubes were ligated at the junction with the ovary and the ovaries were excised bilaterally. For sham-operated rats, similar sized retroperitoneal fat tissues were removed instead. Finally, the incision was closed layer by layer, and 0.1 ml penicillin (4000 units per rat) was injected to prevent inflammation.

Sample preparations

At the end of the experiment, the rats were anesthetized and euthanized, and the final body weight was recorded. The distal femur and proximal tibiae were harvested and adhering soft tissues were removed. The right femur and tibia samples were preserved in 4% paraformaldehyde for histology and immunohistochemistry testing. The left femur samples were kept in a 10% cold phosphate buffer solution (PBS) for micro-CT examination and the 3 point-bending test.

Micro-CT analysis

The bone microarchitecture of the distal left femur samples was evaluated by μCT (Skyscan 1176; Skyscan, Antwerp, Belgium) and related software (CT-analyser, Version: 1.10.11.0). All scans were performed in a fixed matrix resulting in an isotropic voxel resolution of 18 μm . Data were collected every 0.5° rotation step through 180° . The volume of interest (VOI) of the trabecular bone of the femur included the secondary spongiosa in 100 cross-sectional slices of the distal femur beginning 50 slices up from the growth plate region, and the VOI of the cortical bone of the femur was composed of 100 cross-sectional slices of the distal femur beginning 350 slices up from the growth plate region [13]. Trabecular parameters for the femur included bone mineral density (BMD), trabecular bone volume (BV), total bone volume

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(TV), the fraction (BV/TV, %), trabecular number (Tb.N, n/mm), trabecular thickness (Tb.Th, μm) and trabecular separation (Tb.Sp, μm) calculated by Skyscan software CTscan. Cortical thicknesses were also calculated by measuring the average values of 4 quartile points at a single cross-section 340 slices up from the femoral growth plate and repeating the measurement twice, at intervals of 100 slices.

Histological presentations

The distal right femur and proximal right tibiae were harvested and assessed for the effect of EGCG on trabecular bone tissues. Decalcified bone tissue histological sections embedded in paraplast were made and observed. Each section was 5 μm thick and was stained with hematoxylin and eosin.

Immunohistochemistry study

Right distal femur and proximal tibiae were fixed overnight in 4% paraformaldehyde in PBS and decalcified for 5 weeks in 0.5 M EDTA, pH 7.4. Sections of right distal femur and proximal tibiae samples 5- μm thick were made. The sections were deparaffinized in xylene and washed twice with a graded series of ethanol solutions. Blocking solutions were applied. Sections were incubated in 0.01 M sodium citrate buffer for 10 minutes at 100°C for antigen retrieval. Purified mouse anti-CD100 (BD Bioscience) was used. After incubation with anti-CD100 overnight at 4°C in a humid chamber, a phosphate buffer was used for washing, and a secondary antibody (Gene Tech) was applied before incubation with horseradish peroxidase-conjugated streptavidin. HRP activity was measured using diaminobenzidine (DAB) as a substrate. Stained samples were observed under an Axio imager M1 microscope (Zeiss, Germany) equipped with a camera and software. The relative density of immunostaining (density/area) and the positive area percentage was measured using Image-Pro plus 5, Media Cybernetics, Silver Spring, Maryland, USA.

Biomechanical test

A three-point bending test was applied to test the mechanical strength of the intact femur. The bones were wrapped in gauze soaked in isotonic saline and placed in a moisture box at room temperature 4 hours before mechanical testing [14]. A biomechanical test machine (Instron E10000, USA) and associated soft-

ware (Bluehill 2, USA) were employed. The femurs were placed between two metal supports with a distance of 18 mm between them, while the loading pin was compressed at the anterior surface in the middle of the femoral shaft with a blunt metal edge. A load was applied at a rate of 5 mm/min and directed to the mid-diaphyseal region. Compression of the loading pin continued until a fracture occurred. The biomechanical parameters bending displacement (mm), ultimate bending stress (N) and elastic modulus (MPa) to break bones, and modulus of elasticity were recorded and evaluated.

Statistical analysis

Data are presented as the mean \pm standard deviation and were analyzed with SPSS software, version 19.0 (SPSS Inc., Chicago, IL). The Kolmogorov-Smirnov normality test for each continuous variable was performed. A 95% confidence interval was applied, and a p value less than 0.05 determined statistical significance. A one-way analysis of variance (ANOVA) was applied to compare different groups for BMD, bone morphological parameters (trabecular BV, TV, BV/TV, Tb.N, Tb.Th, Tb.Sp and cortical thickness), biomechanical test parameters (bending displacement, ultimate bending stress and elastic modulus), and immunostaining parameters in the immunohistochemistry test (density/area and the positive area percentage). Post hoc multiple comparisons were made if statistical significance was indicated by the one-way ANOVA ($P < 0.05$).

Results

Body weight

Before ovariectomy, there was no statistical significance among any of the treatment groups. At the end of the experiment, the body weight was significantly higher in the OVX group compared with the SHAM group. EGCG supplementation did not reduce body weight as the body weight in the OVX+ EGCG/IP group at the end of the experiment did not show any significant differences with the OVX group (**Table 1**).

Bone morphological parameters

Measured from the trabecular bone of the distal femur of the female SD rats, the BMD of the SHAM group was significantly higher than in the other 2 groups ($P < 0.01$), while the BMD of OVX+

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Table 1. Changes of body weight before and after treatment

| Group | OVX+ EGCG/IP (Group A) | OVX (Group B) | SHAM (Group C) |
|---------------------|-----------------------------|----------------------------|----------------------------|
| Before treatment, g | 241.83±10.43 | 237.20±9.51 | 239.20±9.74 |
| After treatment, g | 352.92±14.69 ^{##} | 361.67±16.39 ^{##} | 311.10±11.49 ^{**} |
| Difference value, g | 111.08±9.89 ^{**##} | 124.44±8.71 ^{##} | 71.90±9.48 ^{**} |

Abbreviations: OVX for ovariectomy; SHAM for sham operation. All data are presented as the mean ± standard deviation. ^{##}P<0.01 vs. SHAM group; ^{**}P<0.01 vs. OVX group.

Table 2. Bone morphological parameters in the trabecular bone of the distal femur in female SDrats

| Group | OVX+ EGCG/IP (Group A) | OVX (Group B) | SHAM (Group C) |
|-----------------------|-----------------------------|---------------------------|---------------------------|
| BMD | 0.081±0.030 ^{*,##} | 0.046±0.026 ^{##} | 0.192±0.034 ^{**} |
| BV/TV, % | 40.11±10.01 ^{*,##} | 28.93±7.60 ^{##} | 81.53±9.17 ^{**} |
| TbTh, mm | 0.19±0.02 ^{##} | 0.17±0.01 ^{##} | 0.27±0.05 ^{**} |
| TbN, mm ⁻¹ | 2.14±0.39 | 1.70±0.41 | 2.08±1.63 |
| TbSp, mm | 0.41±0.12 ^{*,##} | 0.57±0.18 ^{##} | 0.13±0.02 ^{**} |

Abbreviations: OVX for ovariectomy; SHAM for sham operation; BV for bone volume; TV for total volume; TbTh for trabecular thickness; TbN for trabecular number; TbSp for trabecular separation. All data are presented as the mean ± standard deviation. ^{##}P<0.01 vs. SHAM group; ^{*}P<0.05, ^{**}P<0.01 vs. OVX group.

Table 3. Bone morphological parameters in the cortical bone of the distal femur in female SD rats

| Group | OVX+ EGCG/IP (Group A) | OVX (Group B) | SHAM (Group C) |
|------------------------|--------------------------|-------------------------|-------------------------|
| BV/TV, % | 55.39±3.50 ^{##} | 57.51±1.89 [#] | 62.86±3.46 [*] |
| Cortical thickness, mm | 0.629±0.023 | 0.626±0.034 | 0.634±0.010 |

Abbreviations: OVX for ovariectomy; SHAM for sham operation; BV for bone volume; TV for total volume. All data are presented as the mean ± standard deviation. [#]P<0.05, ^{##}P<0.01 vs. SHAM group; ^{*}P<0.05, vs. OVX group.

EGCG/IP group was significantly higher than in the OVX group (P<0.05) (**Table 2**). Regarding the measurements of the trabecular bone morphological parameters of the distal femur in female SD rats, the SHAM group had a higher BV/TV, TbTh, and a lower TbSp than the OVX group (P<0.01), while the OVX+ EGCG/IP group had a higher BV/TV and a lower TbSp than the OVX group (P<0.05). There was no significant difference in TbN among the 3 groups. Regarding the measurement of the cortical bone morphological parameters of the distal femur in female SD rats (**Table 3**), the SHAM group had a higher BV/TV than the OVX+ EGCG/IP group (P<0.01) and the OVX group (P<0.05), while there was no significant difference between the OVX+ EGCG/IP group and the OVX group (P>0.05) (**Figure 1**).

Three-point bending test

Although the SHAM group had higher average values of bending displacement, ultimate bend-

ing stress, and elastic modulus than the other 2 groups, there was no significant difference among the 3 groups (**Table 4**).

Histological presentations

In the histological study as indicated by to the HE staining, the trabecular bone distribution was denser and the trabecular number was higher in the SHAM group and the OVX+ EGCG/IP group than in the OVX group (**Figure 2**).

Immunohistochemistry study

The results of immunohistochemistry study are illustrated in **Table 5**. The brown-stained area is the positive area, and it was expressed mainly along the bone surface. The Brown/Total area ratio

was significantly higher in the OVX group than in the other 2 groups (P<0.01), but there was no statistical difference of the Brown/Total area ratio between the OVX+ EGCG/IP group and the SHAM group. Similar results were observed for the mean IOD (**Figure 3**).

Discussion

In our study, a model of OVX estrogen deficiency-induced female Sprague-Dawley rats was successfully applied to assess the effect of EGCG supplementation on bone microarchitecture and to investigate possible mechanisms. The conventional model of estrogen deficiency-induced female rats in researching osteoporosis or osteopenia was established in 12-week-old female rats [8, 15], but some OVX models used rats less than 12 weeks old [16, 17] and other models used rats as old as 8 months old [18]. It should be taken into consideration that the rats were still in the growing phase [17] in

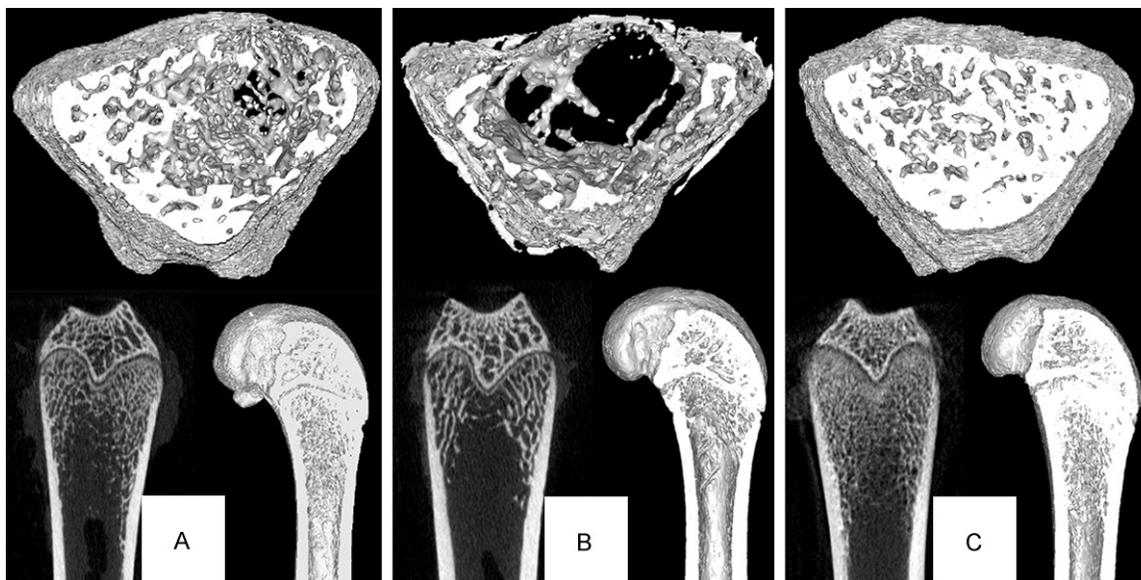


Figure 1. Micro-CT analysis of the distal femur of female SD rats. Each group contains 3 images. The top is the cross-sectional image of trabecular bone, the left bottom is the original coronal image of CT scanning, and the right bottom is the 3D reconstruction image. The images clearly show that group A and group C have a more dense distribution of trabecular bone than group B.

Table 4. Biomechanical test parameters of the femur shaft in female SD rats

| Group | OVX+ EGCG/IP (Group A) | OVX (Group B) | SHAM (Group C) |
|----------------------------|------------------------|----------------|----------------|
| Bending displacement, mm | 1.03±0.31 | 1.01±0.27 | 1.03±0.36 |
| Ultimate bending stress, N | 158.35±17.94 | 125.84±6.10 | 145.73±18.70 |
| Elastic modulus, MPa | 4079.91±1004.41 | 3248.77±391.94 | 3846.10±600.47 |

Abbreviations: OVX for ovariectomy; SHAM for sham operation. All data are presented as the mean ± standard deviation.

these models. In our study, we used the OVX group and the SHAM group as 2 control groups for comparisons.

A study by Shen et al. [19] found that, although GTP supplementation for 16 weeks markedly improved both femoral BMD and the microarchitecture of trabecular and cortical bone in the tibia and femur in middle-aged 14-month-old female rats, GTP did not completely prevent bone loss due to aging plus estrogen deficiency in OVX rats. Similar results were observed in a study by Chen et al. [8] who studied a 3-month-old female OVX rat model. EGCG supplementation was begun at 12 weeks post-operatively and maintained for 12 weeks. Chen et al. that found that EGCG supplementation could mitigate bone loss in OVX rats, but the OVX rats still had a relative low BMD, BV/TV, TbTh, and TbN compared to sham-operated rats.

A study reported that major changes in the bone structure of estrogen deficiency-induced rats were detected within the first 3 months after ovariectomy [20] demonstrated that early

intervention is important. In our study, we decided to intervene in 12-week-old female OVX rats with EGCG 3 days after ovariectomy to investigate the magnitude of any preventive effects of EGCG on the bone microarchitecture as a simulation of the early stages of post-menopause without the influence of senility [17, 21].

As illustrated by our results, decreases of BMD, BV/TV, TbTh, and an increase of TbSp in the distal femur of trabecular bone were detected in the OVX group (Table 2). The results in the EGCG-intervened estrogen deficiency-induced group suggested an attenuation of bone loss in female OVX rats. However, in comparison with the SHAM group, the OVX+ EGCG/IP group had relatively lower parameters for BMD, BV/TV, and higher parameters of TbSp. Although the average value of parameters of TbTh and TbN

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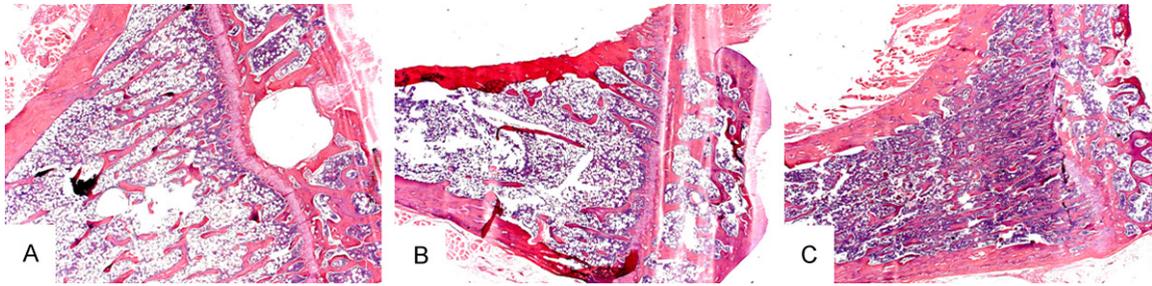


Figure 2. The HE staining of the proximal tibia of female SD rats indicates that group (B) had a sparser trabecular distribution, while group (A) and (C) had a more dense distribution ($\times 25$).

Table 5. Results of immunohistochemistry study of Sema4D

| Group | OVX+ EGCG/IP (Group A) | OVX (Group B) | SHAM (Group C) |
|---------------------|------------------------|---------------------|---------------------|
| Brown/Total area, % | 15.08 \pm 12.51** | 45.29 \pm 4.63## | 21.32 \pm 14.80** |
| Mean IOD | 0.026 \pm 0.019** | 0.065 \pm 0.010## | 0.029 \pm 0.010** |

Abbreviations: OVX for ovariectomy; SHAM for sham operation; IOD for integrated option density. All data are presented as the mean \pm standard deviation. ## P <0.01 statistical significance achieved in comparison with the SHAM group; ** P <0.01 statistical significance achieved in comparison with the OVX group.

were higher in the OVX+ EGCG/IP group than in the OVX group, no statistical significance was observed. The results were similar to those from an experiment conducted by Shen et al. [19] who used GTP for early intervention 2 weeks after ovariectomy in middle-aged female rats and did not observe complete prevention of bone loss due to aging plus estrogen deficiency.

Cellular studies support the beneficial effect of EGCG in preventing bone loss via its effects on osteoblasts and osteoclasts. EGCG could stimulate osteoblastogenesis via: 1) enhancing the mRNA expression of osteogenic genes, such as alkaline phosphatase activity, osteocalcin, and core binding factor $\alpha 1$, eventually stimulating mineralization [3, 22]; 2) increasing proliferation and differentiation of osteoblasts via the Wnt signaling pathway [23]; 3) improving the survival of osteoblasts through the inhibition of TNF- α and interleukin-6 production [24]; 4) suppressing endothelin-1-induced interleukin-6 synthesis in osteoblasts through inhibition of p44/p42 mitogen-activated protein kinase [25]; 5) enhancing prostaglandin $F_{2\alpha}$ -induced vascular endothelial growth factor via the up-regulation of SAPK/JNK activation in osteoblastic-like MC3T3-E1 cells [26]. Meanwhile, EGCG could influence osteoclasts via: 1) inhibiting the survival of differentiated osteoclasts and

increasing apoptosis in osteoclasts [27, 28]; 2) stimulating osteoclastic cell death via the Fenton reaction and caspase activation [28, 29]; 3) suppressing the formation of osteo-

clastic cells via inhibiting matrix and metalloproteinase-9 expression [27].

As previously reported by Hirschberg et al., Sema4D positively regulates neuron migration during cortical development [30]. As indicated by Negishi-Koga et al., Sema4D, is an axon guidance molecule and is expressed in osteoclasts, and could potentially inhibit bone formation. Through the binding of Sema4D to its receptor Plexin-B1 in osteoblasts, the activation of the small GTPase RhoA resulted, and bone formation was inhibited by suppression of IGF-1 signaling and by modulation of osteoblast motility [9]. Negishi-Koga et al. found that bone volume, trabecular thickness, and bone strength was significantly greater in Sema4D $^{-/-}$ mice and Plxn1 $^{-/-}$ mice compared to wild-type mice, suggesting an osteosclerotic phenotype in Sema4D $^{-/-}$ mice and Plxn1 $^{-/-}$ mice. Meanwhile, blocking the Sema4D-Plexin-B1 interaction could be a new and potentially effective strategy for increasing bone formation.

According to our immunohistochemistry study, the OVX group had relatively higher positive area percentage of Sema4D expression and mean IOD than the other 2 groups, and EGCG supplementation decreased the expression of

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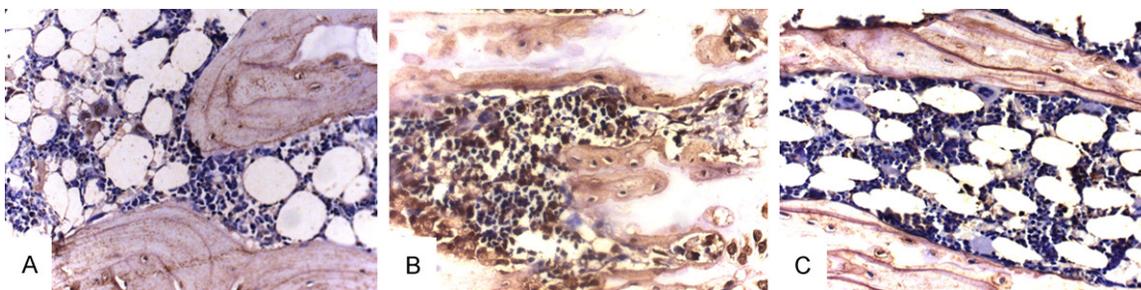


Figure 3. The Immunohistochemistry study of Sema4D expression in bone tissue in female SD rats demonstrated that group (B) has a higher expression shown by an increase in the size of the brown stained area, while groups (A) and (C) have a relatively lower positive area ($\times 400$).

Sema4D mainly along the osteoclast and osteoblast surface. These results indicated that in female estrogen-deficient rats, the possible mechanism of bone loss is likely to be an increase in the expression of Sema4D by osteoclasts, which act on osteoblasts to further suppress osteogenesis; moreover, the intervention of EGCG can reduce the expression of Sema4D to a certain degree. Although Plexin-B1 is responsible for recognizing Sema4D, some recognition is mediated by other receptors, such as Plexin-B2 [9]. Thus the expression of Plexin-B1 has a limited role in explaining the observed effects.

EGCG has positive effect on mitigation of bone loss in ovariectomized rats, and inhibits the expression of Sema4D in bone tissue. Early supplementation of EGCG at a dose of 10 mg/kg/day after ovariectomy does not entirely prevent bone loss. Further studies exploring the underlying mechanisms, different dose supplementations of EGCG, and a middle-aged rat model should be conducted.

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Disclosure of conflict of interest

None.

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