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Decrease in the number and apoptosis of alveolar bone osteoclasts in estrogen-treated rats

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Background and Objective: Bone is a mineralized tissue that is under the influence of several systemic, local and environmental factors. Among systemic factors, estrogen is a hormone well known for its inhibitory function on bone resorption. As alveolar bone of young rats undergoes continuous and intense remodeling to accommodate the growing and erupting tooth, it is a suitable *in vivo* model for using to study the possible action of estrogen on bone. Thus, in an attempt to investigate the possibility that estrogen may induce the death of osteoclasts, we examined the alveolar bone of estrogen-treated rats.

Material and Methods: Fifteen, 22-d-old female rats were divided into estrogen, sham and control groups. The estrogen group received estrogen and the sham group received corn oil used as the dilution vehicle. After 8 d, fragments containing alveolar bone were removed and processed for light microscopy and transmission electron microscopy. Sections were stained with hematoxylin and eosin and tartrate-resistant acid phosphatase (TRAP)–an osteoclast marker. Quantitative analysis of the number of TRAP-positive osteoclasts per mm of bone surface was carried out. For detecting apoptosis, sections were analyzed by the Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) method; TUNEL/TRAP combined methods were also used.

Results: The number of TRAP-positive osteoclasts per mm of bone surface was significantly reduced in the estrogen group compared with the sham and control groups. TRAP-positive osteoclasts exhibiting TUNEL-positive nuclei were observed only in the estrogen group. In addition, in the estrogen group the ultrastructural images revealed shrunken osteoclasts exhibiting nuclei with conspicuous and tortuous masses of condensed chromatin, typical of apoptosis.

Conclusion: Our results reinforce the idea that estrogen inhibits bone resorption by promoting a reduction in the number of osteoclasts, thus indicating that this reduction may be, at least in part, a consequence of osteoclast apoptosis.

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Bone is a mineralized tissue that undergoes continuous remodeling by the combined action of osteoclasts, osteoblasts and osteocytes (1), which are influenced by several systemic, local and environmental factors (2). These factors regulate the proliferation, differentiation, function and survival of bone cells (3). Among systemic factors, estrogen is a hormone well known for its inhibitory function on bone resorption (4–6). More recently, it has been shown that estrogen depletion promotes intense resorptive activity in the alveolar bone of rats (7,8). There is evidence that estrogen promotes an increase in the levels of osteoprotegerin, a protein that inhibits osteoclast formation

(9–12). In the tibia of estrogen-treated rats, a decrease in the number of osteoclasts was shown, and osteoclast disintegration (6) or death was suggested (13). Therefore, the mechanism by which estrogen acts upon skeletal tissues remains unclear (14).

Some systemic and local factors are capable of switching on the chain of molecular events that leads to the programmed cell death (apoptosis) of bone cells (15–18). Apoptosis contributes to the homeostatic regulation of bone by controlling the life span and the number of bone cells (19). Osteoblast and osteocyte apoptosis has been observed under physiological conditions (20–22) and in estrogen-deficient rats (16,17). Moreover, it has been suggested that apoptotic osteocytes may attract osteoclasts to specific bone sites and thereby contribute to the control of bone remodeling (23).

The chances of observing images of the extremely rapid events of apoptosis are rare. Apoptosis of osteoclasts has not been observed *in vivo*, i.e. under physiological conditions (20,24). The alveolar bone of young rats undergoes continuous and intense remodeling to accommodate the growing and erupting tooth (25) and it is therefore a suitable *in vivo* model for using to study the action of estrogen on bone cells.

Thus, in an attempt to investigate the possibility that estrogen may induce the death of osteoclasts, we examined the alveolar bone of estrogen-treated young rats using quantitative analysis of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts on the bone surface. In addition to hematoxylin and eosin-stained sections, the TUNEL method, TRAP and TUNEL combined methods, and transmission electron microscopy were used to detect apoptosis.

Material and methods

The principles of laboratory animal care (NIH publication 85–23, 1985) and national laws on animal use were observed in the present study, which was authorized by Ethical Committee for Animal Research of the São Paulo State University, Brazil (Araraquara Dental School-UNESP).

Fifteen, 22-d-old female Holtzman rats (*Rattus norvegicus albinus*) from the São Paulo State University animal house were placed into estrogen, sham or control groups (five rats in each group). Rats in the estrogen group received estrogen (estradiol hexahydrobenzoate, Benzoginoestril ap®; Hoechst Marion Roussel, São Paulo, Brazil) diluted in corn oil. The animals received an intramuscular injection of 0.125 mg/100 g body weight estrogen [based on Silberberg & Silberberg (26) and Chow *et al.* (27)], daily, for 7 d. The rats of the sham group received the same dose of the corn oil used to dilute the estrogen. Twenty-four hours after the last injection, the rats from the sham group and the estrogen group were killed. In the control group, the rats did not receive any treatment.

The rats were killed with chloral hydrate (600 mg/kg), and fragments of the maxilla containing alveolar bone surrounding the upper molars were removed and immediately immersed in the fixative solution.

Light microscopy

The specimens containing alveolar bone were fixed in 4% formaldehyde (freshly derived from paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium phosphate, at room temperature, for 48 h. After decalcification for 40 days in 7% disodium ethylene-diaminetetracetic acid (EDTA) solution containing 0.5% formaldehyde, in sodium phosphate 0.1 M, at pH 7.2, the specimens were dehydrated in graded concentrations of ethanol and embedded in paraffin. Sections 6 μm thick were stained by hematoxylin and eosin, submitted to TUNEL method, the TRAP reaction, and TUNEL/TRAP combined methods.

Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) method

For detection of DNA breaks, we used the TUNEL method (28). The Apop Tag® Peroxidase *In Situ* Apoptosis Detection kit (Chemicon International, Chemicula, CA, USA) was used, as previously described (29).

Deparaffinized sections adhered to silanized (3-aminopropyltrithoxysylane; Sigma Chemical Co., St Louis, MO, USA) slices were washed in phosphate-buffered saline (50 mM sodium phosphate, 200 mM NaCl, pH 7.2) and treated with 20 μg/ml proteinase K (Oncor-Protein Digesting Enzyme) in phosphate-buffered saline, at room temperature. The sections were subsequently treated with 3% hydrogen peroxide to block endogenous peroxidase and then immersed in an equilibration buffer. The reaction was followed by incubation in solution containing terminal deoxynucleotidyl transferase (TdT), in a humid chamber at 37°C, for 1 h. The sections were immersed in the stop/wash solution and were then incubated in the anti-digoxigenin-peroxidase. After washing in phosphate-buffered saline, they were treated with 0.06% 3.3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in phosphate-buffered saline and then counterstained with Carazzi's hematoxylin. Sections of mammary gland provided by the manufacturer of the kit were used as positive controls for the TUNEL method. Negative controls were incubated in a TdT enzyme-free solution.

TRAP

The TRAP method was used as an osteoclast marker (30). Deparaffinized sections were incubated in a solution prepared by dissolving 8 mg of naphtol AS-BI (Sigma Chemical Co.) in 500 μl of *N*-*N*-dimethylformamide followed by the addition of 50 ml of 0.2 M sodium acetate buffer (pH 5.0) and 70 mg of Fast Red Salt TR (Sigma Chemical Co.). Sodium tartrate dihydrate (50 mM) was added to the solution. After incubation at 37°C, the sections were washed in distilled water and stained with hematoxylin. As a control of specificity for TRAP activity, some sections were incubated in substrate-free medium. For the simultaneous demonstration of apoptosis and TRAP-activity, some sections were submitted to the TUNEL method followed by the TRAP reaction (21).

The hematoxylin and eosin-stained sections and the sections submitted to

the TRAP reaction, TUNEL method and TUNEL/TRAP combined methods were examined and photographed in a light microscope (Olympus BX-50; Olympus America Inc., Miami, FL, USA).

Number of osteoclasts on the alveolar bone surface

Quantitative analysis of the number of TRAP-positive osteoclasts per mm of linear surface of the alveolar bone around the first molar was carried out in the estrogen, sham and control groups. Four sections of the first molar from each animal were used. The shortest distance between the sections was 60 μm.

The surface of the alveolar bone was measured using an image analysis system (Leica Qwin Professional, Leica Microsystems, Wetzlar, Hesse, Germany). Subsequently, the multinucleated TRAP-positive osteoclasts on the alveolar bone surface were counted on a light microscope (Carl Zeiss, Inc., Jena, Germany), at ×500 magnification.

Statistical analysis

Statistical analysis was performed using SIGMA STAT 2.0 (Jandel Scientific, Sausalito, CA, USA). The data were submitted to multiple comparisons using the nonparametric Kruskal–Wallis test. The significance level accepted was $p \leq 0.05$.

Transmission electron microscopy

Specimens containing alveolar bone of the first molar were fixed in a mixture of 4% of glutaraldehyde and formaldehyde (freshly derived from paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium cacodylate, at room temperature. After decalcification in a 7% solution of EDTA containing 0.5% formaldehyde in 0.1 M sodium cacodylate buffer, at pH 7.2, the specimens were washed in 0.1 M sodium cacodylate, pH 7.2. They were then transferred to 0.1 M sodium cacodylate-buffered 1% osmium tetroxide solution for 1 h, at room temperature. Subsequently, the specimens were washed in distilled water and treated with aqueous 2% uranyl acetate for 2 h. The specimens were dehydrated in graded concentrations of ethanol, treated with propylene oxide and then embedded in Araldite.

Semithin sections were stained with 1% toluidine blue and examined in a light microscope. Suitable regions were carefully selected for trimming of the blocks. Ultrathin sections from selected regions were collected on grids and stained in alcoholic 1% uranyl acetate and in lead citrate solution before examination in a Philips CM 200 transmission electron microscope.

Results

The alveolar bone from the upper first molar of 29-d-old control rats exhibited several TRAP-positive osteoclasts, typical of bone undergoing rapid remodeling/turnover (Fig. 1). Numerous multinucleated osteoclasts, exhibiting conspicuous TRAP-positive activity in their cytoplasm (red staining), were often observed apposed to resorption bone surfaces in the control and sham groups (Fig. 2A). On the other hand, only a few TRAP-positive osteoclasts were found on the alveolar bone surfaces of estrogen-treated rats (Fig. 2B). Quantitative analysis revealed a significant reduction ($p \leq 0.05$) in the number of TRAP-positive osteoclasts per mm of linear surface of the alveolar bone in the estrogen group compared with the control and sham groups. No difference was found between the control group and the sham group (Table 1).

Hematoxylin and eosin-stained sections of alveolar bone from the estrogen group revealed large giant cells exhibiting round/ovoid bodies in their interior. Frequently, dense round/ovoid bodies containing basophilic masses were in close juxtaposition to these giant cells. Several smaller structures were observed surrounding the giant cell (Fig. 3A). When the TRAP method was applied, osteoclasts with conspicuous cytoplasmic activity (red staining) exhibited nuclei strongly stained by hematoxylin, typical of condensed chromatin (Fig. 3B). The TUNEL method showed that osteoclasts apposed to resorption surfaces of alveolar bone of the estrogen group

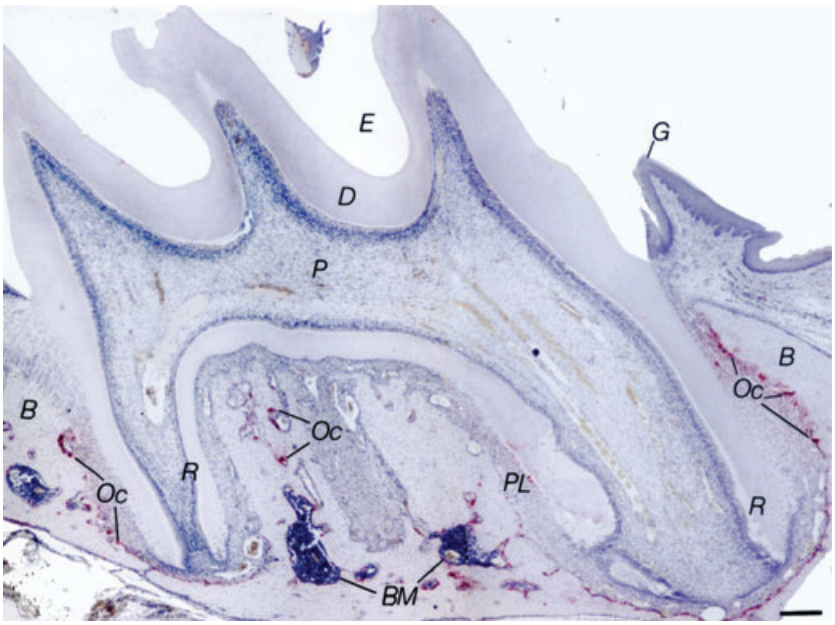

Fig. 1. Light micrograph of a sagittal section of the first upper molar of a 29-d-old control rat. The alveolar bone (B) surrounding the molar roots (R) exhibits numerous tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts (Oc) – red color. BM, bone marrow; D, dentine; E, enamel space; G, gingiva; P, dental pulp; PL, periodontal ligament. Counterstained with hematoxylin. Bar, 150 μm.

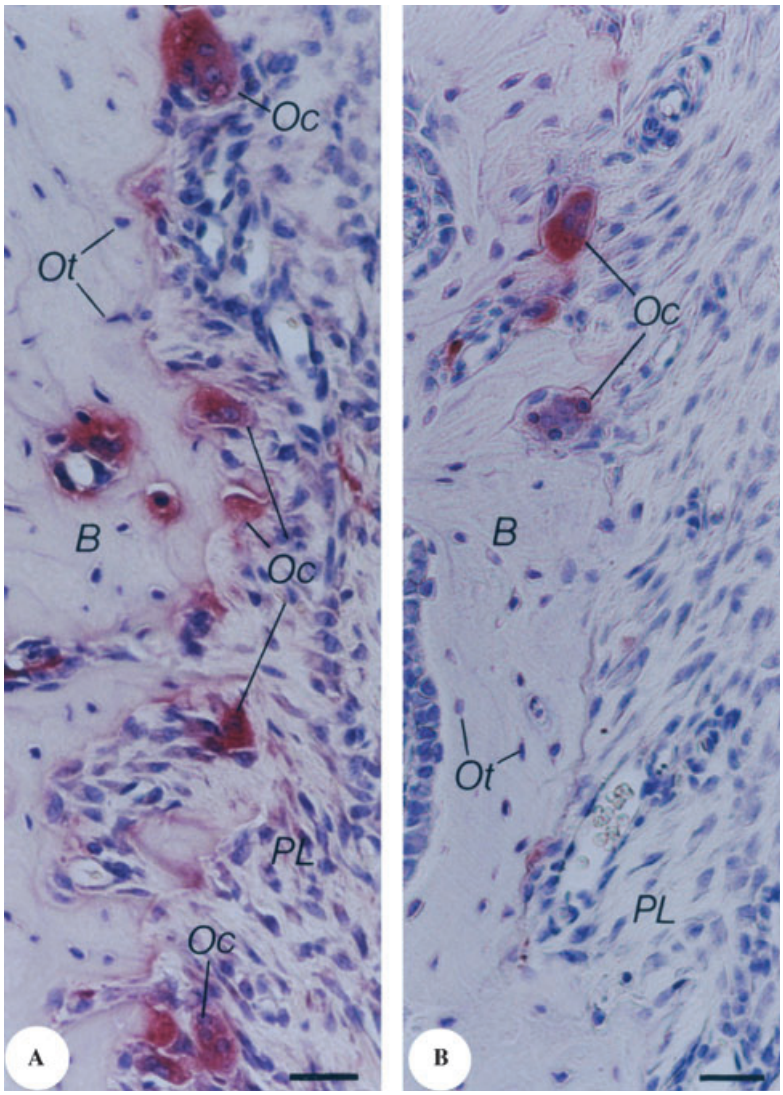

Fig. 2. Light micrographs of portions of the alveolar bone of sham (A) and estrogen-treated rats (B) submitted to the tartrate-resistant acid phosphatase (TRAP) reaction and counterstained with hematoxylin. (A) Numerous TRAP-positive (red color) osteoclasts (Oc) are apposed on the alveolar bone surface. (B) Alveolar bone exhibiting only two red-stained osteoclasts (Oc). B, alveolar bone; Oc, osteoclasts; Ot, osteocytes; PL, periodontal ligament. Bar, 30 μm.

occasionally exhibited brown–yellow nuclei, characteristic of TUNEL-positivity (Fig. 3C). The TUNEL method combined with the TRAP reaction, in the same section, revealed that red-stained osteoclasts exhibited brown–yellow TUNEL-positive nuclei (Fig. 3D,E). Portions of TRAP-positive osteoclasts apposed to bone surfaces exhibited strongly TUNEL-positive nuclei (Fig. 3D). TRAP-positive osteoclasts with apparent intact nuclei were also observed on the alveolar bone of the estrogen group. Sometimes, these osteoclasts showed round/ovoid bodies with irregular masses of condensed chromatin inside large vacuoles in their TRAP-positive cytoplasm (Fig. 3F). The osteoclasts exhibiting condensed chromatin and/or TUNEL-positive nuclei were not present in all sections examined in the alveolar bone of the estrogen group. Moreover, TRAP-positive osteoclasts exhibiting TUNEL-positive nuclei and/or condensed chromatin were not observed on the alveolar bone surface of the sham group or the control group.

Controls for the TUNEL method, using involuting mammary gland sections, revealed positive structures. Sections incubated in medium lacking the TdT enzyme were negative. No reaction product for TRAP activity was observed when the sections were incubated in substrate-free medium (data not shown).

Ultrastructural examination of alveolar bone from the estrogen group revealed osteoclasts with a grossly changed ultrastructure intermingled with normal osteoclasts (Fig. 4A,B). The altered osteoclasts showed irregularly shaped nuclei with conspicuous and tortuous masses of condensed chromatin. In addition, they exhibited shrunken and convoluted cytoplasm containing numerous vesicles and vacuoles, which occupied most of the cytoplasm. Images of organelles, such as mitochondria, endoplasmic reticulum and Golgi elements, were rarely observed between vesicles and vacuoles. Moreover, the ruffled border and clear zone were not observed in these osteoclasts (Fig. 4A). The normal multinucleated osteoclasts exhibiting

Table 1. Number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts per mm of alveolar bone surface in animals from the control, sham-treated and estrogen-treated groups

	Groups		
Animal no.	CG	SG	EG*
1	1.72	1.76	0.81
2	1.89	1.80	0.88
3	1.91	1.85	1.03
4	1.99	2.20	1.24
5	2.62	2.33	1.27
Mean ± SD	2.05 ± 0.34	1.99 ± 0.25	1.04 ± 0.88

CG, control group; EG, estrogen-treated group; SG, sham-treated group; SD, standard deviation.
*statistically significant ($p \leq 0.05$) from CG and SG.

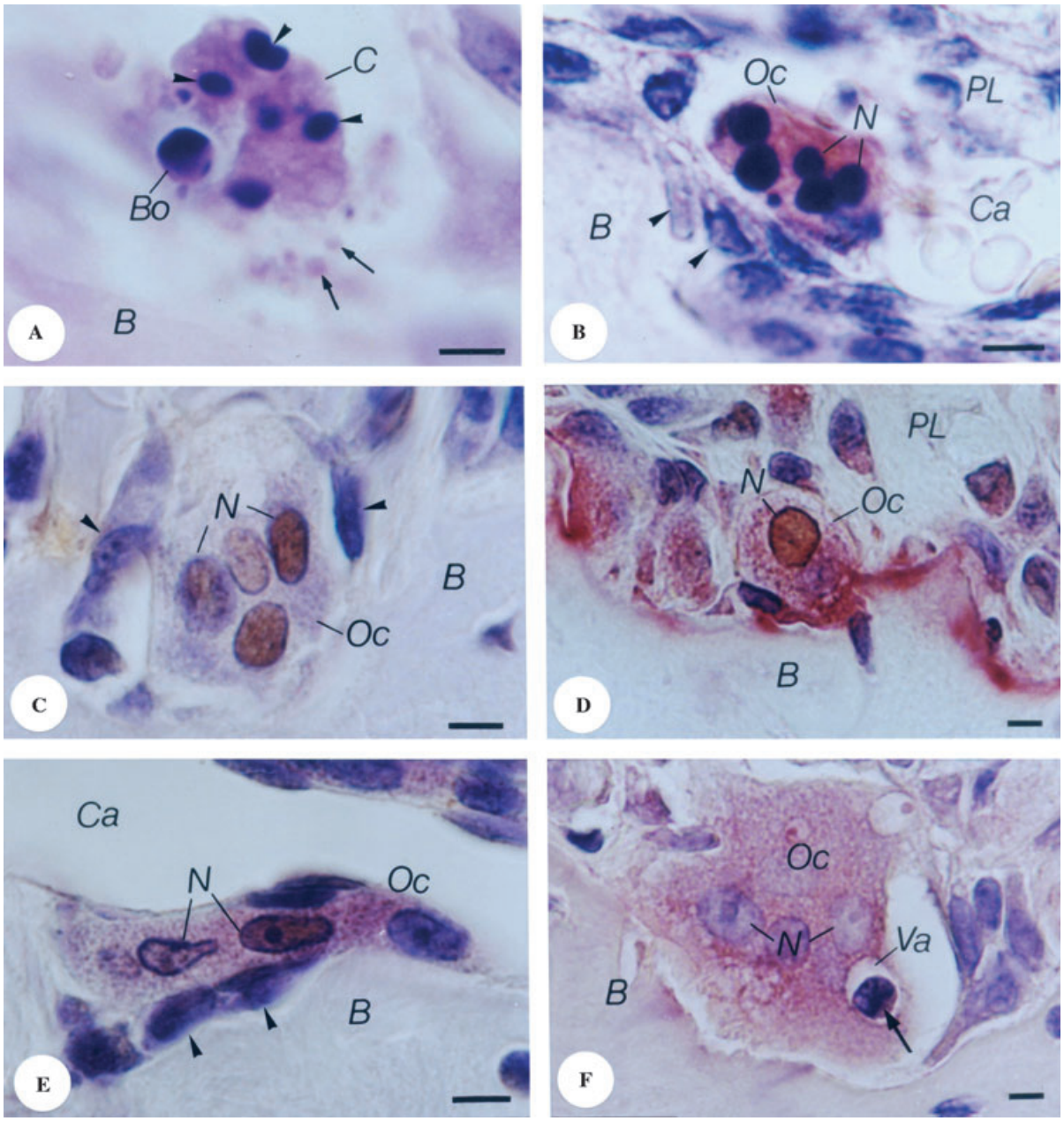

Fig. 3. Light micrographs of portions of the alveolar bone of estrogen-treated rats. (A) A large giant cell (C), adjacent to the bone surface (B), exhibits several round-ovoid dense bodies (arrowheads) in its interior. A round-ovoid body (Bo) containing basophilic masses is in close juxtaposition to the giant cell (C). Several smaller structures (arrows) are observed surrounding the giant cell (C). Hematoxylin and eosin staining. Bar, 5 μm. (B) A portion of alveolar bone submitted to the tartrate-resistant acid phosphatase (TRAP) reaction and counterstained with hematoxylin. A TRAP-positive osteoclast (Oc) exhibits several nuclei (N) strongly stained by hematoxylin, typical of condensed chromatin. Arrowheads, bone-lining cells; B, bone matrix; Ca, capillary; PL, periodontal ligament. Bar, 6 μm. (C) A portion of alveolar bone submitted to the TUNEL method (brown–yellow) and counterstained with hematoxylin. The osteoclast (Oc) located in an excavation of the bone surface (B) exhibits TUNEL-positivity in all nuclei (N) observed. Arrowheads, bone-lining cells. Bar, 6 μm. (D,E) The TUNEL method (brown–yellow) was combined with the TRAP reaction (red). (D) A portion of a TRAP-positive osteoclast (Oc) apposed to the bone surface (B) exhibits a TUNEL-positive nucleus (N). PL, periodontal ligament. Bar, 5 μm. (E) An elongated TRAP-positive osteoclast (Oc) shows some TUNEL-positive nuclei (N). Arrowheads, bone-lining cells; B, bone matrix; Ca, capillary. Bar, 6 μm. (F) A portion of alveolar bone submitted to the TRAP reaction and counterstained with hematoxylin. A TRAP-positive osteoclast (Oc) containing normal nuclei (N) is located in an excavation of the bone surface (B). The osteoclast (Oc) exhibits a round/ovoid body (arrow) with irregular masses of condensed chromatin inside a large vacuole (Va). Bar, 5 μm.

a ruffled border, numerous mitochondria, vesicles and vacuoles of varied sizes, were located on excavations of the bone surface (Fig. 4B).

Discussion

Our quantitative results showed a marked decrease in the number of alveolar bone osteoclasts (TRAP-positive) in estrogen-treated rats, when compared with the control group and sham-treated rats. The mechanisms involved in the reduction of osteoclast number is not yet understood. However, this reduction may be caused either by estrogen-induced cell death (13,31) or by interference of estrogen on the receptor activator of NF-κB (RANK)/RANK ligand/osteoprotegerin system, a pathway involved in the mechanism of osteoclast formation (9–12,32).

Our morphological results, i.e. those obtained by the hematoxylin and eosin staining, the TRAP reaction, the TUNEL method and the TUNEL/TRAP combined methods, suggest that alveolar bone osteoclasts undergo cell death in the estrogen group, and hence the decrease in numbers observed. Hematoxylin and eosin- and TRAP-stained sections of estrogen-treated rats revealed shrunken osteoclasts exhibiting condensed chromatin. These osteoclasts were occasionally surrounded by small round/ovoid structures containing dense material, similar to apoptotic bodies, as also previously described (20,33–35). Furthermore, TRAP-positive osteoclasts also exhibited TUNEL-positivity. The TUNEL and TRAP methods in the same section (21) allowed simultaneous demonstration of DNA breaks (28) and tartrate-resistant acid phosphatase, a characteristic marker of osteoclasts (30). DNA breaks constitute part of the cascade of molecular events observed during apoptosis (36,37). However, it should be noted that the TUNEL method alone is not specific for apoptosis (38). Although several markers for apoptosis are available, it is generally accepted that ultrastructural images are most relevant for the identification of classical apoptosis of mononucleated cells (35,39). It must also be taken into account that

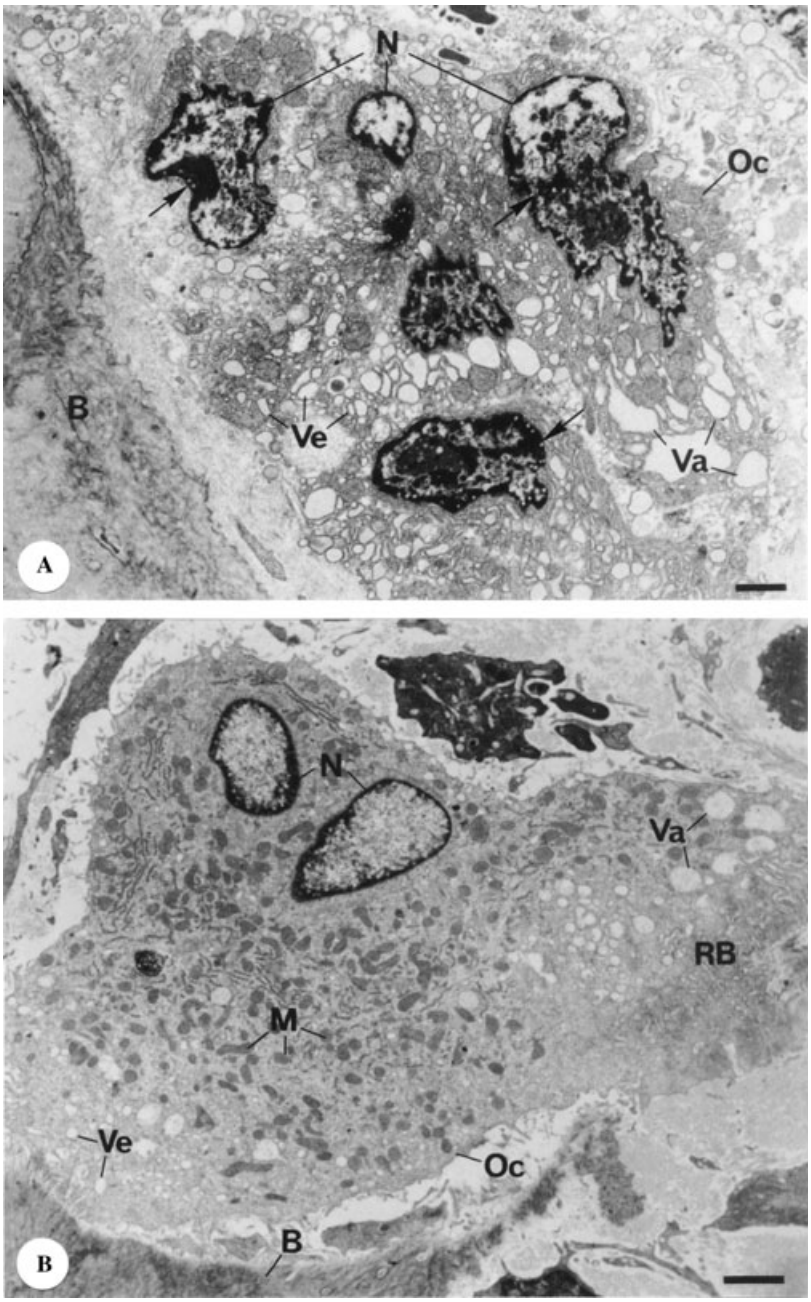

Fig. 4. Electron micrographs of portions of the alveolar bone of estrogen-treated rats. (A) A multinucleated osteoclast (Oc) located next to the bone surface (B) shows several irregularly shaped nuclei (N) exhibiting conspicuous and tortuous masses of condensed chromatin (arrows); the shrunken and convoluted cytoplasm contains numerous vesicles (Ve) and vacuoles (Va), which occupy most of the cytoplasm. Bar, 1.5 μm. (B) A typical osteoclast (Oc), exhibiting a ruffled border (RB), numerous mitochondria (M), vesicles (Ve) and vacuoles (Va) of varied sizes, is located on an excavation of the bone surface (B). N, nuclei. Bar, 2.5 μm.

osteocytes (40) and apoptotic bone cells (20,21,41) are engulfed by osteoclasts during bone resorption. So, it is important to note that some bodies found in osteoclasts may be apoptotic osteoblasts/osteocytes internalized by osteoclasts. These internalized bodies can be distinguished from apoptotic osteoclast nuclei because they are usually surrounded by a clear halo.

Our ultrastructural images revealed that some osteoclasts apposed to bone

surface of the estrogen group exhibited conspicuous and tortuous masses of condensed chromatin within their nuclei. These nuclei are typical of dying cells that may be undergoing apoptosis (20,29,33,35). However, the cytoplasm of osteoclasts, unlike that observed in mononuclear apoptotic cells (22,33), was packed with vesicles and vacuoles and was poor in mitochondria, endoplasmic reticulum and Golgi elements. Moreover, osteoclasts did not exhibit a ruffled border or a clear zone. The absence of both structures was also observed in apoptotic osteoclasts of rats treated with bisphosphonate (42,43), possibly as a result of degradation of cytoskeleton proteins, which occurs during the proteolytic cascade of apoptosis (37,44).

Although some features observed were characteristic of cell death by apoptosis, the presence of numerous vesicles and vacuoles in the cytoplasm of osteoclasts in the estrogen group differs from classical apoptosis. Moreover, dying osteoclasts exhibited few organelles, thus differing from classical apoptosis of mononucleated cells in which organelles remain intact (33,34). It is important to mention that the presence of numerous vesicles and vacuoles in dying osteoclasts may be a result of the extremely convoluted surface of the osteoclast. Thus, in ultrathin sections, these vesicles and vacuoles may represent cross and oblique sections of the convoluted surface and/or the ruffled border membrane that may have been internalized. However, it should be noted that osteoclasts possess numerous vesicles and vacuoles of a lysosomal nature (2,41).

Taken together, our results indicate that osteoclasts undergo a process of apoptosis that differs, in some aspects, from classical apoptosis (45). Cell death of chondrocytes also seems to differ from classical apoptosis (46).

Although apoptotic bodies adjacent to the bone surface were observed, we were unable to discern images of apoptotic bodies arising from osteoclasts or from other dying cells. Apoptotic bodies located next to bone and next to dying osteoclasts do not differ from apoptotic structures derived from mononucleated cells (20,29,33,35). In addition, as apoptosis is an extremely rapid process, it is possible that neighboring cells, such as fibroblasts, osteoblasts or other osteoclasts may have engulfed apoptotic bodies deriving from apoptotic osteoclasts. In most other tissues, apoptotic bodies are removed by the combined action of neighboring, same-type cells and macrophages (22,47).

As the majority of studies involving osteoclast death have been carried out *in vitro* (31,48) and/or were restricted to light microscopy (13,49), we believe that our results show, for the first time, *in vivo* ultrastructural images of dying alveolar bone osteoclasts in estrogen-treated rats. The absence of images of dying osteoclasts in the control and sham groups agrees with the results of other authors (20,24). In the estrogen group, it was difficult to find dying osteoclasts and there was no observable pattern in the distribution of these dying cells through the alveolar bone surface. Thus, dying osteoclasts co-existed with normal osteoclasts. However, we have no explanation for the fact that some osteoclasts were affected by estrogen but others were not.

Estrogen exerts an inhibitory action on bone resorption and therefore it is used to prevent and treat osteoporosis. However, the cellular and molecular mechanisms of estrogen action on bone remain unclear. It has been suggested that estrogen promotes the down-regulation of osteoclast formation, activity and survival (3). The action of estrogen on osteoclasts may be mediated by T cells (50), monocytes and osteoblasts (51), as well as occuring directly on osteoclasts via the estrogen receptor (52,53). There is evidence that estrogen stimulates the secretion of osteoprotegerin, a decoy receptor of the RANK ligand, and thereby inhibits osteoclastogenesis (9–12). Moreover, it has also been suggested that estrogen inhibits the production of cytokines such as interleukin-1, tumor necrosis factor, interleukin-6, macrophage colony-stimulating factor (51) and RANK ligand (32). Because some of these cytokines stimulate the formation, activity and survival of osteoclasts, it has been suggested that estrogen decreases bone resorption via the action of cytokines, by reducing the number of osteoclasts. Our results reinforce the idea that estrogen inhibits bone resorption by promoting a reduction in the number of osteoclasts, indicating therefore that this reduction may be, at least in part, a consequence of osteoclast apoptosis. However, we cannot exclude the possibility that this reduction may also be associated with the inhibitory activity of estrogen on osteoclast formation (32,50,51).

Our quantitative results, when combined with TUNEL/TRAP methods and transmission electron microscopy, support the interpretation that estrogen promotes – directly and/or indirectly – the apoptosis of alveolar bone osteoclasts. However, further studies are required to clarify the underlying molecular events involved.

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