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Effects of estrogen status in osteocyte autophagy and its relation to osteocyte viability in alveolar process of ovariectomized rats

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ABSTRACT

Estrogen maintains osteocyte viability, whereas its deficiency induces osteocyte apoptosis. As autophagy is important for osteocyte viability, we hypothesized whether the anti-apoptotic effect of estrogen is related to autophagy in osteocytes. Thirty adult female rats were sham-operated (SHAM) or ovariectomized (OVX). After three weeks, twelve rats of SHAM and OVX groups were killed before treatment (basal period), whereas the remaining rats received estrogen (OVXE) or vehicle (OVX) for 45 days. Fragments of maxilla containing alveolar process of the first molars were embedded in paraffin or Araldite. Paraffin-sections were stained with hematoxylin/eosin for histomorphometry, or subjected to the silver impregnation method for morphological analysis of osteocyte cytoplasmic processes. Autophagy was analyzed by immunohistochemical detections of beclin-1, MAP-LC3α and p62, whereas apoptosis was evaluated by immunohistochemical detections of cleaved caspase-3 and BAX, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) method and by ultrastructural analysis. Araldite-semithin sections were subjected to the Sudan-black method for detection of lipids. OVX-basal group showed high frequency of caspase-3-, TUNEL- and p62-positive osteocytes accompanied with low frequency of beclin-1- and MAP-LC3α-positive osteocytes. At 45 days, OVXE group exhibited higher number of osteocytes, higher frequency of beclin-1- and MAP-LC3α-positive osteocytes, and lower frequency of caspase-3, BAX-, TUNEL- and p62-positive osteocytes than OVX group. Significant reduction in bone area was observed in the OVX compared to OVXE and SHAM groups. The highest frequency of Sudan-Black-positive osteocytes and osteocytes with scarce cytoplasmic processes, or showing apoptotic features were mainly observed in OVX groups. Our results indicate that estrogen deficiency decreases autophagy and increases apoptosis, whereas estrogen replacement enhances osteocyte viability by inhibiting apoptosis and maintaining autophagy in alveolar process osteocytes. These results suggest that the anti-apoptotic effect of estrogen may be, at least in part, related to autophagy regulation in osteocytes.

1. Introduction

Osteocytes, the most abundant bone cell type, play essential roles for the maintenance of bone homeostasis by acting as mechanosensors and orchestrators of the bone remodeling process [1–3]. As osteocytes are long-living cells encased in mineralized bone matrix, they are located in an environment more susceptible to hypoxia, nutrient deprivation and to the accumulation of oxidative stress [4]. In addition, increasing number of studies has shown that autophagy plays essential role for osteocyte survival [5–8].

Autophagy is a programmed cell survival mechanism whereby unnecessary cellular components such as malfunctioning proteins and organelles are targeted to the lysosomes for degradation. The molecules resulting from this degradation are converted into substrates for energy production when nutrients are limiting [9]. Basal autophagy occurs in all cell types, but it can be induced by factors that cause cell stress including nutrient deprivation, hypoxia and increased oxidative stress [10,11]. The process of autophagy begins with the formation and nucleation of the phagophore, a membrane that elongate engulfing non-functional cytoplasmic material to form the autophagosomes [9].

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Beclin-1 and the microtubule-associated protein 1A/1B-light chain (MAP LC3) are autophagic proteins essential for phagophore nucleation and elongation, respectively [11].

There are three human genes that encode highly homologous MAP LC3 proteins (MAP LC3α, MAP LC3β, and MAP LC3γ), two of which (MAP LC3α and MAP LC3β) are conserved in rodents [12]. MAP LC3α, MAP LC3β, and MAP LC3γ are either cytosolic (MAP LC3I) or membrane associated (MAP LC3II) proteins. MAP LC3α and MAP LC3β are essential for autophagosome formation; during phagophore elongation, the cytosolic form of LC3 (MAP LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (MAP LC3-II), which is recruited to the autophagosomal membrane. Specific malfunctioning proteins can also be selectively directed to autophagosomes by the sequestosome 1 (SQSTM1), also known as p62 protein, which binds to ubiquitinated non-functional proteins and targeting them to autophagosomes for lysosome degradation. p62 is also degraded in this process, then acting as a specific autophagosome substrate that indicates the levels of autophagic flux in cells [13].

In recent years, studies have shown that autophagy plays important roles for the maintenance of bone homeostasis, and its dysregulation has been related to bone loss and osteoporosis [18],14–17]. It has been demonstrated that suppression of ATG7 (autophagy related 7), an essential gene for autophagy, in mice osteocytes promotes a marked increase in oxidative stress and bone loss [18]. Also, an age-related reduction in the autophagic flux in osteocytes was correlated to bone loss and osteoporosis in senile rats [19]. More recently, it has been demonstrated that rapamycin, an inductor of autophagy, reduces the severity of age-related trabecular bone loss by activating autophagy in osteocytes of old male rats [20]. All these studies strongly indicate that autophagy is essential for osteocyte viability and bone homeostasis.

On the other hand, estrogen plays essential role for the maintenance of bone homeostasis by inhibiting both excessive bone resorption and osteoblast/osteocyte apoptosis [21–23]. It has been shown that the pro-survival effect of estrogen is in part related to autophagy levels in bone cells [24,25]. Estrogen added to osteoblasts in culture induces autophagy and reduces apoptosis, suggesting that this hormone increases osteoblast survival by autophagy induction [24]. In tibias of ovariectomized rats, estrogen deficiency increased oxidative stress, which then induced autophagy in osteoblasts and osteocytes, whereas estrogen replacement counteracted these effects [25].

It has widely been demonstrated that estrogen deficiency induces bone loss not only in the vertebrae and long bones, but in bone surrounding teeth as well [26–29]. However, despite similarities, it is known that long and jaw bones display different osteogenic and osteoclastogenic potentials, and can also respond differently to mechanical loading, homeostatic regulatory signals and estrogen deficiency [30–33]. Despite it has been reported that estrogen status regulates bone cell autophagy in vitro and in long bones of OVX rats, its effects on osteocyte autophagy in jaw bones is poorly understood.

Thus, in this study we hypothesized whether the protective effect of estrogen is related to autophagy incidence in alveolar process osteocytes, using ovariectomized rats as an estrogen-deficient in vivo model.

2. Material and methods

2.1. Animals and experimental protocol

Thirty female Wistar rats (*Rattus norvegicus albinus*) aged 4 months were maintained in a room with controlled temperature (23 ± 2 °C) and standard 12-h light/dark cycle, with food and water *ad libitum*. The protocol of this study followed the national guidelines for laboratory animal care and was approved by the Ethical Committee for Animal Research of Federal University of São Paulo (UNIFESP/EPM).

After one week of adaptation period, the animals were anaesthetized with an intraperitoneal injection of 10% ketamine hydrochloride (0.08 mL/100 g b.w.) combined with 2% xylazine hydrochloride (0.04 mL/100 g b.w.) and then SHAM-operated (SHAM) or ovariectomized (OVX). Ovariectomy was followed by a resting period of twenty-one days to ensure estrogen depletion and recovery from surgery stress, as previously reported [29,34].

Twenty-one days after surgery, vaginal smears were obtained once a day during five consecutive days in all animals; only the SHAM rats that showed regular estrous cycle and the OVX rats that were in permanent anestrous followed in the experiment. At this point, 6 SHAM and 6 OVX rats were euthanized (as described below) and used as basal control groups (basal period). The other animals received subcutaneous injections of estrogen (OVXE) (diethylstilbestrol, Sigma-Aldrich Co. LLC, Brazil), dissolved in corn oil, at the dose of 30 μg/kg body weight [35], or received only corn oil as vehicle solution (SHAM and OVX groups), for 45 consecutive days (n = 6 rats for each group). The analysis was performed after 45 days to ensure significant bone loss in the alveolar process in the OVX group, as previously described in this animal model [30].

After treatment, the animals were anaesthetized as previously described and blood samples (2 mL each) were collected by cardiac puncture (BD Vacutainer® Blood Collection Tubes, SST II Plus, BD Biosciences). The blood samples were centrifuged and the serum stored at -80 °C for serum estradiol measurement. The animals were euthanized by whole body fixation via transcardial perfusion with saline followed by a fixative solution of 4% formaldehyde (prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer solution, pH 7.2. Subsequently, the fragments of maxilla containing alveolar process surrounding the first molars were removed and immediately immersed in the fixative solution.

2.2. Estradiol measurement

Serum estradiol concentration was measured by an electrochemiluminescence immunoassay using a calibrated automatic counter (Cobas e601/Roche Diagnostic, Mannheim Germany). An estradiol kit containing rabbit polyclonal biotinylated antibody specific for estradiol detection in rats and humans (Roche Cobas Estradiol II assay/03000079, Mannheim, Germany) was used. The linear range of the assay was 5 pg/mL to 3000 pg/mL and the coefficient of variation intra-assay was 3.3%. The samples were dosed in duplicate and values below the limit of detection (< 5 pg/mL) were not detected by the assay. The analyses were performed in the Central Laboratory of São Paulo Hospital (HSP), SP, Brazil.

2.3. Histological processing

Fragments of the right maxilla were immersed for 48 h at room temperature in 4% formaldehyde buffered pH 7.2 with 0.1 M sodium phosphate. After decalcification for 60 days in a solution of 7% EDTA (ethylenediaminetetraacetic acid) that contained 0.5% formaldehyde in 0.1 M sodium phosphate buffer at pH 7.2, the fragments were dehydrated and embedded in paraffin. Sagittal sections (6 μm thick) were stained with hematoxylin and eosin (HE), and bone area of the alveolar process situated between the first molar roots was measured. The number of osteocytes in the alveolar process was also estimated. Some sections were subjected to the silver impregnation method for morphological analysis of osteocyte cytoplasmic processes. Sections were also adhered to silanized slides and subjected to TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and immunohistochemical reactions.

2.4. Interradicular bone area

The bone area of the alveolar process situated between the first molar roots was estimated using a software of image analysis (Axionvision 4.2 REL, Carl Zeiss) coupled with a light microscope (Axiolab 2.0, Carl Zeiss). At a magnification of ×40, two images/