Original Article

The Short- and Long- term Effects of Estrogen Deficiency on Apoptosis in Musculoskeletal Tissues: An Experimental Animal Model Study

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Abstract

Background: Estrogen is the major sex steroid affecting the growth, remodeling, and homeostasis of the female skeleton. Estrogen loss in postmenopausal women leads to osteoporosis. The aim of this study was to evaluate the early and long- term effects of estrogen loss on bones, tendons, muscles, and menisci in ovariectomized rats.

Methods: Fifteen rats were randomized into three groups of five animals each. The first group was the control group with no additional surgical procedure, but the rest (groups 2 and 3) were bilaterally ovariectomized. All animals in the group 2 were sacrificed at 14th week to evaluate the short- term effect, and all of other animals in the groups 1 and 3 were sacrificed at 28th week to analyze the long- term effect of estrogen loss in the ovariectomized group and to control with the group 1. Quadriceps muscles, Achilles tendons, menisci, and femur cortical bones from both lower extremities were taken. The amount of apoptosis was measured.

Results: There was a significant increase in cell apoptosis in bones, muscles, and tendons with insignificant increase in cell apoptosis in menisci at early and late periods in rats with ovariectomies than the control.

Conclusion: The results indicated that estrogen loss after ovariectomy does not only affect bones; it may also increase cell apoptosis in different tissues such as muscles, tendons, and menisci.

Keywords: Apoptosis, estrogen, estrogen deficiency, osteoporosis.

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Introduction

Osteoporosis is characterized by a low bone mass and microarchitectural deterioration of the skeleton leading to an increased risk of fracture after minimal trauma. Postmenopausal osteoporosis is the most common form of osteoporosis and the most common of all systemic osteopathies.[1] Estrogen is the major sex steroid affecting the growth, remodeling, and homeostasis of the female skeleton. Estrogen loss in postmenopausal women leads to osteoporosis. The main actions of estrogen at the cellular level are to inhibit activation frequency of the basic multicellular units (BMUs) and to maintain remodeling balance by inhibiting osteoclast function and, perhaps, by stimulating osteoblast function. At the tissue level, these cellular effects maintain equivalent levels of bone resorption and bone formation, and, at the organ level, this conserves bone mass.[2]

Previous studies indicate that osteocyte apoptosis may play a central role in signaling the activation and maintaining the progress of bone remodeling.[3–6] Apoptosis is the process of programmed cell death that may occur in multicellular organisms[7] and occurs as a part of normal development as well as in pathological processes associated with some diseases. Programmed cell death, or apoptosis, is the process whereby certain cells are induced to activate their own death or cell suicide. We hypothesized that osteoporosis due to estrogen deficiency is not related only to bone loss in the postmenopausal period; it also may affect other musculoskeletal tissues such as tendons, menisci, and muscles. Based on this hypothesis, we evaluated the early and long- term effects of estrogen loss on bone, tendon, muscle, and meniscus as well as measured the amount of apoptosis by Annexin V-FITC in ovariectomized (OVX) rats.

Materials and Methods

Fifteen 3.0 – 3.5-month-old female Wistar- Albino rats weighing between 250 and 300 g were randomized into three groups of five animals each. The first group was the control group with no additional surgical procedure but the rest of the groups (groups 2 and 3) were bilaterally OVX. The rats in OVX groups (2 and 3) were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg- Ketalar®); 500 mg/kg ampicillin-sulbactam (Ampisid®) was injected intramuscularly to the rats. The rats were shaved and aseptically prepared, and then were operated by the same surgeon. OVX was performed by a transverse ventral incision. The rats were acclimatized to caged laboratory conditions and were allowed to feed with a standard diet and water ad libitum. The room temperature and humidity were maintained at 20 – 24°C

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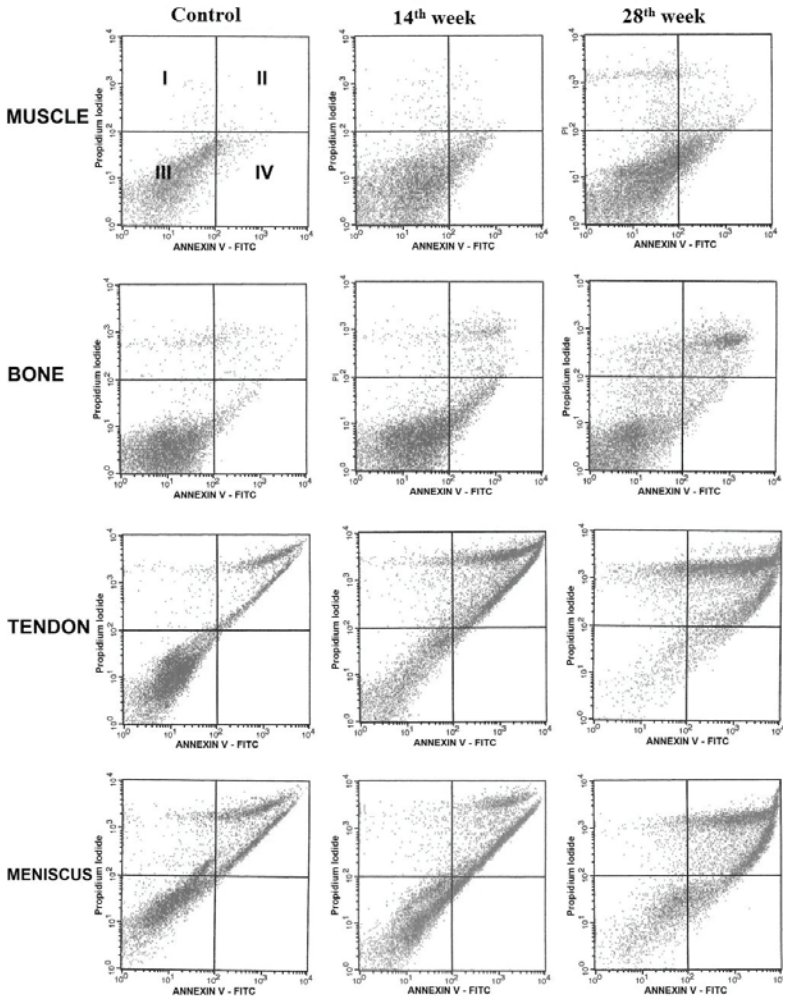

Figure 1. The dot plots of Annexin V-PI -stained cells of muscle, bone, tendon, and meniscus.

and at 50% – 60%, respectively. The light cycle was fixed at 12 h. All animals in the group 2 were sacrificed at 14th week to evaluate the short- term effect, and all of other animals in the groups 1 and 3 were sacrificed by using high- dose ketamine at 28th week to analyze the long- term effect of estrogen loss in the OVX group and to control with the group 1. Quadriceps muscles, Achilles tendons, menisci, and femur cortical bones from both lower extremities were taken and were collected in a medium solution. The amounts of apoptosis of the same tissue in the left and right extremities were measured and the mean values were accepted as the results of the study per each tissue. All animals formed part of a larger experiment for which approval was obtained from the Kocaeli University Ethics Committee in Animal Experiments.

Cell isolation

All tissues were collected in pH 7.2 Hanks buffered salts solution (HBSS) containing 20 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) and 5% antibiotics (Pen-Strep).

Tendons were gently pulled free of sheath, placed in a sterile dish with HBSS, and minced into five to six pieces that were incubated in a 37 °C water bath in 0.5% collagenase (from Clostridium histolyticum, Gibco® Paisley,United Kingdom) with constant, mild agitation for 19 h. The sample was vortexed from time to time to free the cells and filtered through a 70 μm cell strainer. The cells were sedimented at 1500 rpm for 5 min and washed with phosphate buffered saline (PBS) before the flow cytometric analysis.

Muscle tissue was minced into 10 – 12 pieces of 3 mm^3 and enzymatically dissociated by a mixture of 2.4 U/mL dispase and 0.1% collagenase (Gibco® Paisley,United Kingdom) in HBSS for 2 h at 37 °C in a shaking water bath. The slurry was vortexed from time to time to free the cells and filtered through a 70 μm cell strainer. The cells were sedimented at 1500 rpm for 5 min and washed with PBS before the flow cytometric analysis.

Menisci were cut into four pieces and enzymatically digested in 0.1% collagenase (Gibco® Paisley,United Kingdom) in HBSS for 18 h at 37 °C in a shaking water bath. The sample was filtered through a 70 μm cell strainer. The cells were sedimented at 1500 rpm for 5 min and washed with PBS before the flow cytometric analysis.

The cortical parts of the femur were cut into two and digested in 0.1% collagenase (Gibco® Paisley,United Kingdom) in HBSS for 2.5 h at 37 °C in a shaking water bath. The sample was filtered through a 70 μm cell strainer and the cells were sedimented at 1500 rpm for 5 min and finally washed with PBS before the flow-cytometric analysis.

Table 1. The percentages (mean± SD) of total apoptotic cells of muscle, bone, tendon, and meniscus.

Tissue	Control	14th week	28th week
Muscle	6.02 ± 1.90	8.23 ± 2.23	14.92 ± 4.67
Bone	5.01 ± 2.26	16.10 ± 2.42	20.30 ± 4.76
Tendon	36.03 ± 12.76	53.04 ± 12.02	70.98 ± 12.61
Meniscus	43.84 ± 5.69	59.62 ± 5.22	66.02 ± 22.27

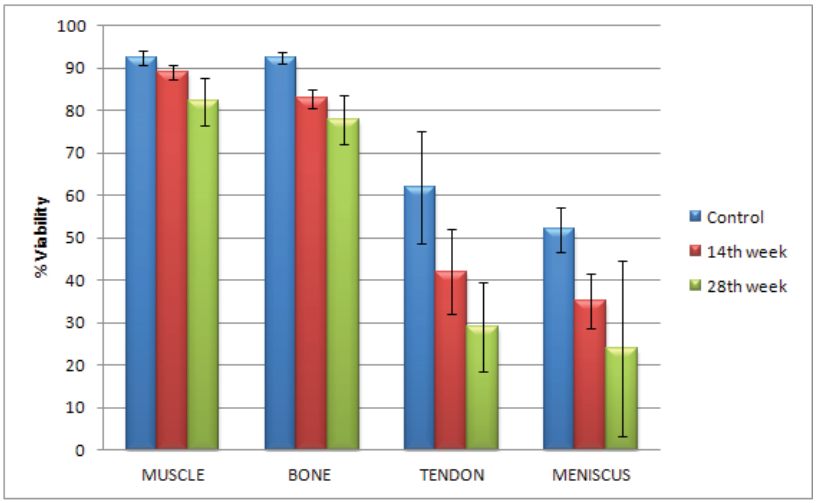

Figure 2. Percent cell viability of muscle, bone, tendon, and meniscus in all groups.

Flow cytometry

Apoptotic cells were detected by Annexin V staining using Annexin Apoptosis Detection Kits (BD PharMingen, Heidelberg, Germany). Briefly, the cells were washed twice and resuspended in binding buffer (10 nM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 nM CaCl2). The cells were incubated with fluoresceine isothiocyanate (FITC)-conjugated Annexin V and propidium iodide for 15 min at room temperature in dark, washed, and then analyzed on a FACS can flow cytometer (BD Biosciences). All types of cells were evaluated and the total apoptosis of the tissue was noted.

Statistical analysis

The mean and standard deviation (SD) were calculated for descriptive statistics of continuous variables and the median values for discrete variables. Kolmogorov-Smirnov test was used to analyze the normality of data. The means of groups were analyzed by using Kruskal- Wallis and then Post-Hoc test Bonferroni. Two-tailed hypothesis was considered in the analyses and a significant difference was accepted while $P \leq 0.05$. SPSS 15.0 Software for Windows (SPSS Inc., Chicago, IL, USA) was used in the evaluation of statistical analyses.

Results

The mean cell apoptosis of muscle in the group 1 was 6.02 ± 1.90%. It was obtained as 8.23 ± 2.23% in the group 2, while the value of 14.92 ± 4.67% of muscle apoptosis was significantly higher in the group 3 (P = 0.012). The mean values of bone apoptosis in groups 1, 2, and 3 were 5.01 ± 2.26, 16.10 ± 2.42, and 20.30 ± 4.76, respectively (P = 0.008), although the values were higher for tendon (36.03 ± 12.76, 53.04 ± 12.02, and 70.98 ± 12.61, respectively; P = 0.039). According to the values obtained for meniscus apoptosis, there were no significant differences between the control and either group 2 or group 3 in order of 43.84 ± 5.69, 59.62 ± 5.22, and 66.02 ± 22.27, P = 0.141 (Table 1). The dot plots of Annexin V-PI -stained cells of muscle, bone, tendon, and meniscus are given in Figure 1. With respect to percent viability, cell viability decreased for all tissues in the group 2 and group 3 when compared with the control group (Figure 2).

Discussion

Osteoporosis is a pathological condition that is characterized by skeletal fragility and increased fracture incidence. Osteoporotic fractures are due, in part, to a suboptimal bone architecture resulting from underlying severe bone loss. Pathological bone loss can result from numerous causes, but postmenopausal osteoporosis is its most common form and the most common of all systemic osteopathies.[7,8]

Estrogens are important regulators of skeletal development and homeostasis.[9] This is demonstrated by the dramatic loss of bone that occurs after menopause.[10,11] Estrogen loss disrupts local regulatory control of cytokines in the bone marrow, provoking an inappropriate increase in bone remodeling far in excess of that necessary to repair microdamage and replace old BMUs. Increased production of cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor (TNF) after estrogen deficiency mediates the increase in osteoblast and osteoclast numbers and, consequently, the increased frequency of activation.[12] In addition to changes in the number of bone cells, a qualitative abnormality also occurs; osteoclasts erode deeper-than-normal cavities. Increased remodeling alone causes a transient loss of bone mineral density; because bone resorption is faster than bone formation and new BMUs are less dense than older ones. Additionally, if resorption penetrates through a trabecular structure, the substrate

for the coupled bone formation is lost forever. In this manner, estrogen deficiency removes some cancellous elements entirely; the remainder are more widely separated and less well connected. An equivalent amount of cancellous bone distributed as widely separated, disconnected, thick trabeculae is biomechanically less competent than when arranged as more numerous, connected, thin trabeculae.[13,14]

To determine the role of estrogen deficiency in osteoblast and osteocyte apoptosis, Weinstein, et al. assessed the prevalence of these cells in murine vertebrae removed 28 days after OVX. When compared with sham-operated control animals, apoptosis of osteoblasts increased 10-fold, whereas apoptosis of osteocytes increased 4-fold. These results indicate that the accelerated loss of bone that occurs after estrogen deficiency is not only from an increase in osteoclast number and lifespan, but also from a premature reduction in the work of the osteoblasts. Furthermore, the increase in osteocyte apoptosis could further weaken the skeleton by impairment of the osteocyte-canalicular mechanosensory network.[14]

There are two species of estrogen receptors (ER)—ERα and ERβ. Both ERs are found in osteoblasts: ERα is mainly found in cortical bone, and ERβ is mainly found in trabecular bone. ER α also has been demonstrated to be present in osteoclasts and osteocytes.[15] Consistent with the expression of ERs by many bone cell types, there are also many regulatory roles of estrogen in these cells that can be classified into six different categories: regulation of osteoblast number, regulation of matrix production and mineralization, regulation of growth factor expression and responsiveness, regulation of factors that modulate bone resorption, regulation of receptor expression and signal transduction, and miscellaneous responses.[16] It has been published that human skeletal muscle also contains ER α and ERβ.[17,18] Vasconsuelo, et al. showed that 17b-estradiol exerts antiapoptotic effects in skeletal muscle cells which are mediated by ER α and ER β and involve the phosphatidylinositol 3-kinase /Akt pathway.[19]

There are many studies about relation of either estrogen loss or OVX to osteoporosis and apoptosis of bone cells in the literature.[9,10,14–16] However, the effect of estrogen loss on tendons and menisci is not clear yet. In our experimental study, we found a statistically significant increase in cell apoptosis in bones, muscles, and tendons but an insignificant increase in cell apoptosis in menisci at early and late periods in rats with OVX. This result indicates that estrogen loss after OVX and osteoporosis does not only affect bones. It may also increase cell apoptosis in different tissues such as muscles, tendons, and menisci.

In the control group, the amount of cell apoptosis in menisci and tendons were higher than bones and muscles. Apoptosis is part of the tissue regeneration process. Therefore, cell turnover and also cell apoptosis is greater in these tissues because of continuous exposure to cyclic loads. It was demonstrated that abnormal biomechanical loading on meniscal tissue resulted in apoptosis.[20] Uysal, et al. reported that increased numbers of apoptotic cells were present in the biomechanically failed meniscal tissues but the relation between torn type and apoptosis index was not clearly specified.[21] In the present study, although there was an increase of apoptosis index of meniscus in the group 2 and 3 when compared to the control group, the amount of increase was not statistically significant; therefore, this study could not explain clearly the direct or indirect relationship between estrogen loss and apoptosis in menisci. Indeed, menisci are active tissues although greater parts of them are avascular. Fibrochondrocytes respond to load changes by changing the synthesis of proteoglycan[22] and the effect of estrogen on this synthesis is still questionable.

Previous studies have revealed that tendon healing can be stimulated by several growth factors (e.g. platelet-derived growth factor (PDGF), transforming growth factor (TGF)- beta, insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), bone morphogenetic proteins (BMPs) like growth differentiation factor (GDF)-5, -6, -7), or by a thrombocyte concentrate (PRP).[23] Estrogen has been, to date, the primary hormone investigated to play a potential role in musculoskeletal disease and injury. Research has identified estrogen as being important to the homeostasis of many musculoskeletal tissues, often with an incomplete understanding of the role this hormone plays on tissue structure and function, since sex and collagen content are known to affect mechanical properties of some connective tissues.[24] It is reasonable to believe that these factors might be causing variability in the properties of tendons. In vitro studies revealed that estradiol has an inhibiting effect on collagen formation in ligaments.[25,26] Collagen contents were associated with estrogen status, although tendon mechanical properties were not related to estrogen deficient status.[27] Circi, et al. concluded that estrogen may improve tendon healing.[28] In the early apoptosis group the percent increase of bone cell apoptosis was greater than the other tissues when compared to the control group. This result clearly identified the early effect of estrogen loss in bones. Nevertheless, the increasing percent of apoptosis in muscles and tendons was higher than in bones at the 28th week. This outcome is in accordance with expectations with respect to late period effect of estrogen loss on muscles and tendons as well as bones.

Three different models were defined as mechanisms leading to apoptosis.[29] According to the first model, cell killer signals cause the activation of either caspases or Bcl-2 preapoptotic members. In the second model, preapoptotic member of Bcl-2 family constitutes ion channels on endoplasmic reticulum, nucleus membrane, and mitochondria.[30] Separation of mitochondrial membrane leads to induction of apoptotic factors from mitochondria and these factors activate caspase to provide cell apoptosis. As a third model, the ligation of receptors belonging to the TNF family represents a pathway for inducing apoptosis. Ligand-induced clustering of members of this receptor family that contains a cytosolic "death domain" elicits recruitment of various adapter proteins to the receptor complex. These adapter proteins in turn bind caspase-8, activating it by the "induced proximity" mechanism and thus initiating the proteolytic cascade.[29,31] In the current study, cell apoptosis was measured by Annexin V-FITC, which binds to phosphatidylserine residues that are redistributed from inner to the outer leaflet of the cell membrane as an early event in apoptosis. After loss of membrane integrity, PI can enter the cell and intercalate into DNA.[32]

Bcl-2 gene which is localized on chromosome 18 regulates whether the conditioned cell apoptosis is irreversible.[33] Bcl-2 protein is the most well characterized of the molecules related to cell apoptosis. It was demonstrated that Bcl-2 protein has a certain role on suppression of cell apoptosis. Studies on endometrial cell proliferation showed an increase in Bcl-2 protein activation with the presence of estrogen while this positive effect might be suppressed by the existence of progesterone.[34–37] We think that the amount of cell apoptosis in musculoskeletal tissues may be affected by the Bcl-2 protein which is influenced by estrogen. There-

fore, postmenopausal aging in muscles, tendons, menisci as well as in bones may be caused by the same way.

There is a link between the way of undergoing apoptosis and the signal transduction mechanism.[37] According to Raff, all cells are programmed to die and should receive signal from other cells continuously to resume their lives.[38] If this signal is interrupted in any way, the cell commits suicide. The Ca^{++} ion which is an important part of the mechanism of signal transmission activates apoptosis in some cells. It was well documented that apoptosis does not occur if Ca^{++} ions can be blocked.[39] The mechanism of this effect is still unknown but the role of Ca^{++} / Mg^{++} -related DNA endonuclease enzymes has been proposed.[37,38] Estrogens have also indirect effects on the skeleton.[9,40,41] The extra- skeletal actions of these steroids on calcium homeostasis include the regulation of intestinal calcium absorption or secretion.[42–44] These also include the modulation of serum 1,25-dihydroxy-vitamin D3 levels, renal calcium excretion, and the secretion of parathyroid hormone.[40,41] In addition, the disruption of intracellular signaling due to the lack of estrogen effect on regulation of calcium metabolism may cause an increase in cell apoptosis in muscles, tendons, bones, and menisci as this may have occurred in our study.

The current study mainly suffers from relatively low number of animals and detailed analyses that would help better understand the mechanism leading to this outcome. Total cell apoptosis was investigated in the current study; therefore, the amount of different cell apoptosis (osteocytes, osteoclasts, osteoblasts, myocytes, tenocytes, satellite cells, ect.) is still questionable.

Although our study obviously showed that estrogen loss leads to an increase in cell apoptosis in musculoskeletal tissues such as bones, muscles, tendons, and menisci, further analyses using larger experimental groups, measurement of blood electrolyte and hormone levels, and determination of estrogen receptors in each tissue are needed to determine the direct or indirect effect of estrogen on the musculoskeletal system. Future studies should consider evaluating the relationship between osteoporosis and exercise, mobilization, TENS (transcutaneous electrical nerve stimulation) as well as rehabilitation or the effect of new drugs on decreasing apoptosis in musculoskeletal tissues.

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