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The protective effect of the proteasome inhibitor bortezomib on the uterus of ovariectomized rats

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Summary

Bortezomib (BORT) is an anti-tumour agent that inhibits proteasome, which is responsible for the degradation of many intracellular proteins. Although some side-effects and chemotherapeutic effects of BORT are known, there has not been enough research regarding its effects on different tissues of proteasome inhibition in the senile period (post-menopausal). The aim of this study was to investigate the safety of using BORT during the post-menopausal period. The post-menopausal effects of BORT were investigated on ovariectomized (OVX) Spraque-Dawley rats. The female rats were separated into three groups: control, ovariectomized (OVX), and OVX + BORT. OVX and OVX + BORT groups consisted of six rats in each. BORT was administered intraperitoneally in a dosage of 0.2 mg/kg two days a week for four weeks after OVX. The uteri of the rats were investigated using morphometrical, histopathological, and immunohistopathological methods. A striking atrophy in the endometrium and myometrium was observed due to an estrogen deficiency in the OVX group. The partial protective effect of BORT administration was observed morphometrically and histopathologically. In immunohistochemical research, cytoplasmic NF-κB activity was observed in the presence of proteasome inhibition in the endometrium. In light of these findings, the limited protective effects of post-menopausal BORT administration are worth mentioning.

Key words: Bortezomib; Proteasome; Protective effects; Post-menopause.

Introduction

Menopause is a natural process that occurs in women as a normal part of aging [1]. The average age of menopause is 51 years. Menopause causes dramatic hormonal changes, such as the ablation of estrogen, which can affect the immune-regulatory system. Estrogens, which are female sex hormones, regulate growth, differentiation, and the function of many reproductive tissues. During the menopausal period, estrogen levels are chronically reduced. As a result, impairment of immune functions [2], osteoporosis [3], cardiovascular diseases [4], cognition, learning and memorization [5], and many neurodegenerative problems can occur [6].

Bortezomib (BORT) is a novel proteasome inhibitor that has been used in preclinical and phase 1 studies and has been shown to induce anti-myeloma activity. Normally, proteasome activity is important for healthy cell life and cycles. It is known that proteasome activity is effective in the regulation of some protein groups which play an important role in cell cycle control, the beginning of the transcription process, apoptosis, and intracellular signalling, as well as not-required (incorrectly synthesized, damaged, or oxidized) proteins [7]. Actually, proteasome inhibition leads to the suppression of nuclear factor kappa B (NF-κB), which is one of the most important intracellular pathways [8]. On the other hand, the suppression of NF-κB can inhibit regulation of the transcription of various genes which code the pro-apoptotic and anti-apoptotic proteins of apoptosis, cell proliferation, growth factors, cytokines, and cell adhesion molecules [9].

Proteasomal pathway inhibition may lead to a variety of effects at the cellular and tissue levels, such as aging, geriatric diseases, BORT resistance, deficiency of estrogen, and some transcription factors. In the current literature, there are few studies that include the effects of BORT on uterine tissue. One of these studies demonstrated that BORT treatment had positive effects on a patient who was suffering from uterine cancer [10]. In addition, the authors showed that it led to an increase in cell death in the endometrial carcinoma cell line under in-vitro conditions [11]. The present study is the first experimental study that aimed to show the possible effects of BORT on uterine tissue. The possible effects of BORT treatment on uterine tissue were investigated by using morphometric, histopathological, and immunohistochemical methods.

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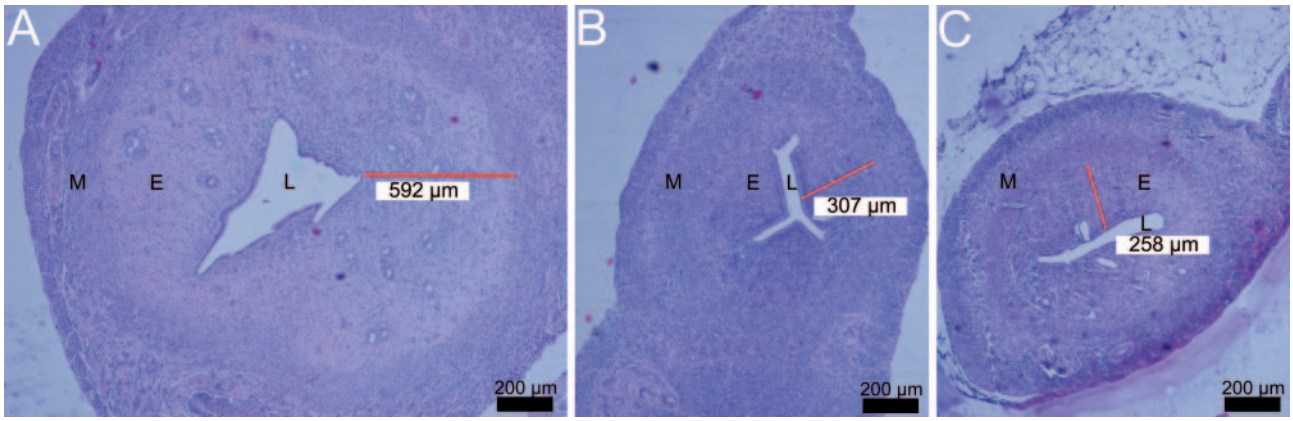

Figure 1. — The appearance of morphometric features in the uterine endometrium, including both decreasing the number of endometrial glands and endometrial thickness. (Control groups = A, OVX groups = B, OVX+BORT groups = C; L: uterine lumen, E: endometrium, M: myometrium) Dye: H&E, Bar: 200 µm

Materials and Methods

Animals and experimental groups

The animals used in this study were kept in facilities accredited by international guidelines and the studies were approved and conducted in accordance with the Institutional Animal Care and Use Committee of Ataturk University. Eighteen adult (12 week-old) female Sprague-Dawley rats were used from the Ataturk University Experimental Animal Laboratory (ATADEM). The animals were housed in groups of six per cage for at least seven days under controlled conditions of constant temperature/humidity and were exposed to a 12-hour light/dark cycle. The rats were randomly allocated into three groups: 1) healthy control group (group 1, n = 6), 2) ovariectomized (OVX) group (group 2, n = 6), and 3) OVX + BORT group (group 3, n = 6).

Experimental models

OVX procedure

A bilateral OVX was performed by making a longitudinal incision (0.5–1 cm) in the midline area of the lower abdomen, removing the ovaries, and closing the skin incision. For the two days following OVX, 25 mg/kg of metamizol sodium was administered to the rats as an analgesic. The OVX rats were separated into two groups (group 2 and 3). The rats in Group 2 were kept alive for 12 weeks. On the eighth week, 0.2 mg/kg of BORT was intraperitoneally applied to group 3, twice a week for a month [12].

Research methods

Histological examination

Each uterus was fixed in 10% formalin solution for 48–55 hours, dehydrated in a graded alcohol series, embedded in paraffin wax, and sectioned using a microtome. For light microscopic histological examination, sections were stained with Hematoxylin & Eosin (H&E). The slides were covered and photographs were taken using a light microscope with a camera attachment.

H&E staining was performed on ten sections with five-µm thicknesses that were obtained systematic randomly from each group's specimens (ten sections were examined in the sampling range of 1/10. After the first section, 11 sections were used. Therefore, tissue samples were obtained in the range of 500 µm). Endometrium and myometrium thicknesses were computed under a ×4 objective microscope.

Table 1. — *Endometrium and myometrium thickness results of experimental groups.*

Groups	Endometrium (µm) mean ± SD	*p* value	Myometrium (µm) mean ± SD	*p* value
Control	526.16±126.64^{a}	0.000	273.13±76.73^{x}	0.000
OVX	137.41±62.29^{b}	0.000	141.21±37.13^{y}	1.000
OVX+BORT	236.67±35.95^{c}	0.000	129.20±36.23^{y}	1.000

The mean of each character (^{a},^{b},^{c}; ^{x} and ^{y}) is statistically differences between groups.

Immunohistochemical staining of NF-κB

Paraffin-embedded lung samples were used for a p65 subunit of NF-κB immunohistochemistry. Tissue sections of five-µm were deparaffinised in xylene and rehydrated in ethanol followed by water and phosphate-buffered saline. Endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide. The tissue sections were then incubated with NF-[kappa] B antibody at a concentration of five µg/ml for one hour at room temperature. Control sections were incubated with phosphate-buffered saline containing normal goat serum without a primary antibody. Immunostaining was then detected with a streptavidin-biotin complex kit and developed with diaminobenzidine tetra-hydrochloride. The sections were counterstained with Mayer hematoxylin followed by light microscopy.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS version 19.0) Software. A comparison of wall thicknesses of the endometrium and myometrium was performed using ANOVA. A $p < 0.05$ was considered significant.

Results

Morphometric results

The results regarding endometrial and myometrial wall thickness are given in Table 1 and Figure 1. When endometrial thicknesses were evaluated, there were significant differences between the OVX and OVX + BORT groups when compared with the control group. There were also significant differences between the OVX and OVX + BORT groups ($p < 0.05$).

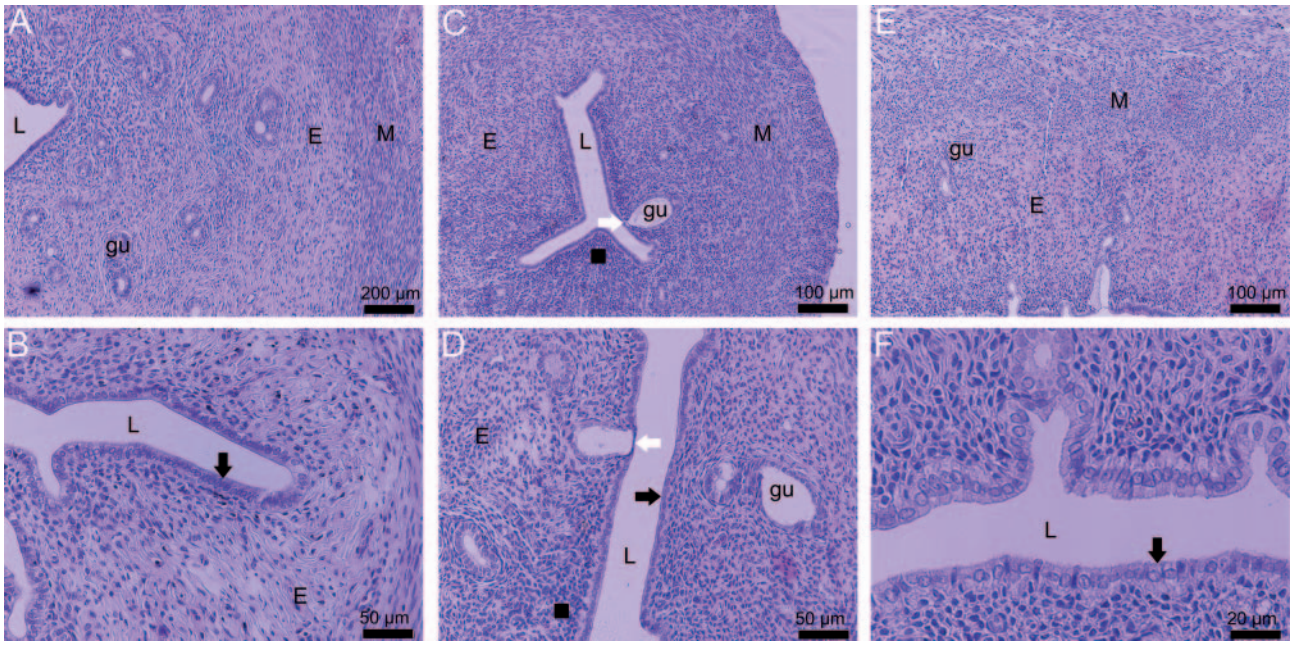

Figure 2. — In OVX groups, uterine histopathological changes are observed including a decrease in the number of uterine glands and their degeneration (white arrow). In OVX and OVX + BORT groups, histopathologic changes are also seen at columnar epithelium cover (black arrow). (Control groups = A-B, OVX groups = C-D, OVX+BORT groups = E-F; L: uterine lumen, E: endometrium, M: myometrium, gu: uterine gland, black arrow: uterine epithelium, white arrow: degeneration of uterine gland, black square: inflammatory cell infiltration). Dye: H&E.

Regarding myometrial thickness, there were significant differences between the OVX and OVX + BORT groups when compared with the control group ($p < 0.05$), but there were no significant differences between the OVX and OVX + BORT groups ($p > 0.05$).

Histopathological results

Control group

All layers of the uterine histological structure had a normal appearance. That is, the endometrium, myometrium, and perimetrium were distinctly observed. The lumen of the uterus was centrally located (Figure 1A). The lamina epithelium of the endometrium was composed of simple columnar epithelium and its euchromatic nucleus had a normal appearance (Figures 2A-B). The vascular structure and glandule uterine, which were located on the endometrium, appeared normal (Figure 2A). The circular and longitudinal smooth muscles of the myometrium layer had a normal appearance (Figure 1A).

OVX group

When histopathological changes were determined in the OVX group, there were some marked differences in the endometrium (Figure 1B) and myometrium. For example, the epithelial layer of the endometrium had changed into simple cuboidal or squamous epithelial cells (Figure 2D). Inflammatory cell infiltration was observed in the endometrium (Figure 2C). There was a decrease in the thickness of the myometrium as compared to the control group. The muscle cells of the myometrium had small and hyperchromatic nuclei.

OVX + BORT group

In this group's sections, general uterine structure was protected when compared with the OVX group. The epithelial layer of the endometrium was composed of simple columnar cells; however, in some areas, there were limited cuboidal and squamous cells (Figure 2F). There was a decrease in the density of the inflammatory cells' infiltration of the endometrium as compared to the OVX group (Figures 2E-F). The smooth muscle cells of the myometrium had small and hyperchromatic nuclei.

Immunohistochemical results

In the control group's section, NF-κB p65 immuno-positivity was only observed in the vascular structure and endothelial cells (Figures 3A-B). In the OVX group, many of the endothelial cells were immune-positive. Endometrial stromal cells had also a slight immuno-positive reaction for NF-κB p65 (Figures 3C-D). When looking at the OVX + BORT group's section, there were a few immuno-positive cells (Figures 3E-F).

Discussion

In this study, the authors examined the effects of administering BORT, a proteasome inhibitor agent, to the uterus in the post-menopausal period using morphometric, histopathological, and immunohistochemical methods. In the first part

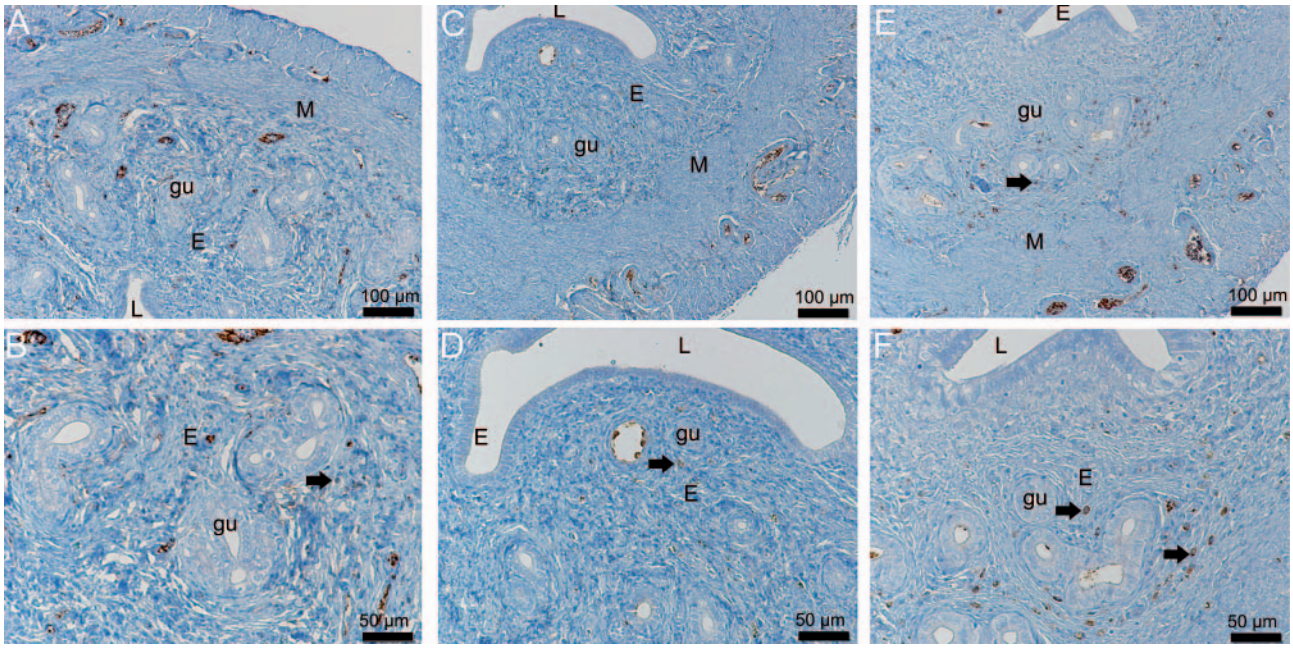

Figure 3. — In OVX + BORT groups, NF-kB immunopositive cells (black arrow) are detected in the basal layer of the endometrium in rat. (Control groups = A-B, OVX groups = C-D, OVX+BORT groups = E-F; L: uterine lumen, E: endometrium, M: myometrium, gu: uterine gland, black arrow: NF-kB (p65) immunopositive cells). Dye: NF-kB (p65) antibody.

of this evaluation, a significant increase was detected in the level of uterine weight against estrogenic activity when the various estrogen treatments' effects on OVX rats were comparatively examined. This case was expressed as an increase in the thicknesses of the endometrium and myometrium. The myometrium had more improved than normal under the estrogen effects. This improvement was proven by both the growth and proliferation of muscle cells [13]. Many smooth muscle cells began to have the ultrastructural features of protein-secreting cells related to their estrogen usage and actively synthesized collagen. Thus, the uterine collagen level significantly increased [14-16].

There was a significant decrease in endometrial thickness in the experimental groups when compared with the control group. The highest level of endometrial thickness was 526.16±126.64 µm, which belonged to the control group. The lowest level of endometrial thickness was 137.41 ± 62.29 µm, which belonged to the OVX group.

In this case, it has been shown that OVX and the disappearance of female sex hormones had a significant impact on the structural elements of a single epithelial layer and the connective tissue of the endometrium. This effect is probably emerging as a quantitative decrease in blood vessels, collagen amounts, and connective tissue cells. It was detected that endometrial thickness of the OVX + BORT treatment group (236.67 ± 35.95 µm) was statistically different from the other two groups, but it was less than 526.16 ± 126.64 µm in the control group and more than 137.41±62.29 µm in the OVX group. In other words, it was observed that BORT treatment inhibited the negative effects of OVX on endometrial thickness.

The second part of the present discussion focused on histological detections. In their histopathological study, Fawcett *et al.* [17] observed 1) atrophy on OVX and non-treated rat uteri, 2) the transformation of prismatic epithelium to cubic epithelium, 3) atrophy in endometrial glands, 4) regulation of stromal connective tissue fibres to tight-intensive form, and 5) shrinkage and hyperchromasia of stromal cells and smooth muscle cells [18, 19].

In a study by Iguchi *et al.*, the authors proved that atrophic changes occurred in uterine smooth muscle [20, 21]. Regarding this issue, the present study is consistent with the current literature data. In addition, BORT treatment may be morphometrically and histopathologically protective. The natural structure of the uterus was protected by itself when the OVX + BORT treatment group was histologically examined.

The third part of the present discussion focused on the immunohistochemical results. Normally, IκB is phosphorylated on the terminal serine residues and thus is targeted to poly-ubiquinone. Then it is degraded by the proteasomal pathway. The degradation of IκB enables NF-κB to enter into the core as a transcriptional factor and bind to the target gene region on the DNA [22, 23]. However, there is a second pathway in which NF-κB can be activated. In this case, there is no need for proteasomal degradation of IκB. Therefore, it is not affected by a proteasome inhibitor such as BORT. This is called an atypical pathway. It is not necessary for NF-κB to enter into the core by nuclear transcription and be activated by proteasome in an atypical pathway. This second NF-κB pathway depends on phospho-

tyrosine. Aging may be responsible for chronic activation of NF-κB. Therefore, NF-κB induction is thought to be one of the cellular effects of aging that may lead to activation of the atypical pathway [24].

In the present study, immunopositivity was not observed on the muscle bundles of muscular layers, uterine glands, and single epithelial layer of the endometrium of the uterus in all experimental groups, when NF-κB p65 immunoreactivity was evaluated on the endometrium, myometrium, and perimetrium layers of the uterus. One of the common findings in all the present study groups was that NF-κB p65 immunoreactivity was clearly positive only in blood vessels and endothelial cells. Strong nuclear and cytoplasmic NF-kB involvement was observed in the stromal cells of the endometrium, especially in some stromal cells in the endometrium basalis in the OVX group. NF-kB immunoreactivity was evident in the stromal cells but cytoplasmic involvement was related to the OVX + BORT treatment group.

In light of the data the present authors obtained, they can say that first there was marked degeneration in the endometrium (connective tissue) and myometrium (smooth muscle tissue) layers of the uterus during the menopausal period, as well as a lack of sex hormones (especially estrogen). In other words, the preventive and curative effects of BORT (proteasome inhibition) may be partially considered. The current literature indicates that proteasome inhibition (BORT treatment) is equal to NF-kB inhibition [11]. As a consequence, this study demonstrated that OVX will have some adverse effects on the uterus (as in the literature) [18-19]. A limited preventive effect of proteasome inhibition was detected by using morphometric and immunohistochemical methods. This study can contribute two important findings to the literature on BORT: first, estrogen deficiency activates the NF-κB pathway in the endometrium, and second, NF-κB exhibits a cytoplasmic reaction in a proteasome-independent way.

References

[1] Edward B.J., Li J.: "Endocrinology of menopause". *Periodontol. 2000*, 2013, *61*, 177.

[2] Porter V.R., Greendale G.A., Schocken M., Zhu X., Effros R.B.: "Immune effects of hormone replacement therapy in post-menopausal women". *Exp. Gerontol.*, 2001, *36*, 311.

[3] Riggs B.L., Khosla S., Melton L.J. 3rd.: "A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men". *J. Bone Miner. Res.*, 1998, *13*, 763.

[4] Do K.A., Green A., Guthrie J.R., Dudley E.C., Burger H.G., Dennerstein L.: "Longitudinal study of risk factors for coronary heart disease across the menopausal transition". *Am. J. Epidemiol.*, 2000, *151*, 584.

[5] Rapp P.R., Morrison J.H., Roberts J.A.: "Cyclic estrogen replacement improves cognitive function in aged ovariectomized rhesus monkeys". *J. Neurosci.*, 2003, *23*, 5708.

[6] Suzuki S., Brown C.M., Dela Cruz C.D., Yang E., Bridwell D.A., Wise P.M.: "Timing of estrogen therapy after ovariectomy dictates the efficacy of its neuroprotective and antiinflammatory actions". *Proc. Natl. Acad. Sci. USA*, 2007, *104*, 6013.

[7] Mani A., Gelmann E.P.: "The ubiquitin-proteasome pathway and its role in cancer". *J. Clin. Oncol.*, 2005, *23*, 4776.

[8] Traenckner E.B., Wilk S., Baeuerle P.A.: "A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B". *EMBO J.*, 1994, *13*, 5433.

[9] Sethi G., Sung B., Aggarwal B.B.: "Nuclear factor- kappaB activation: from bench to bedside". *Exp. Biol. Med. (Maywood)*, 2008, *233*, 21.

[10] Pugh T.J., Chen C., Rabinovitch R., Eckhardt S.G., Rusthoven K.E., Swing R., *et al.*: "Phase I trial of bortezomib and concurrent external beam radiation in patients with advanced solid malignancies". *Int. J. Radiat. Oncol. Biol. Phys.*, 2010, *78*, 521.

[11] Sorolla A., Yeramian A., Valls J., Dolcet X., Bergadà L., Llombart-Cussac A., *et al.*: "Blockade of NF kappaB activity by Sunitinib increases cell death in Bortezomib-treated endometrial carcinoma cells". *Mol. Oncol.*, 2012, *6*, 530.

[12] Gomez A.M., Vrolix K., Martínez-Martínez P., Molenaar P.C., Phernambucq M., van der Esch E., *et al.*: "Proteasome inhibition with bortezomib depletes plasma cells and autoantibodies in experimental autoimmune myasthenia gravis". *J. Immunol.*, 2011, *186*, 2503.

[13] López-Belmonte J., Nieto C., Estevez J., Delgado J.L., del Prado J.M.: "Comparative uterine effects on ovariectomized rats after repeated treatment with different vaginal estrogen formulations". *Maturitas*, 2012, *72*, 353.

[14] Gómez-Zubeldia M.A., Corrales S., Arbués J., Nogales A.G., Millán J.C.: "Influence of estradiol and gestagens on oxidative stress in the rat uterus". *Gynecol. Oncol.*, 2002, *86*, 250.

[15] Ceylan-Isik A.F., Tulmaç Ö., Aktan F., Ari N., Ozansoy G.: "Effects of estrogen replacement therapy on lipid peroxidation and antioxidant enzyme activities of ovariectomized and ovariectomized- diabetic rats". *Dicle Med. J.*, 2007, *34*, 275. [In Turkish, English abstract].

[16] Windahl S.H., Andersson N., Chagin A.S., Mårtensson U.E., Carlsten H., Olde B., *et al.*: "The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice". *Am. J. Physiol. Endocrinol. Metab.*, 2009, *296*, 490.

[17] Fawcett D.W., Deane W.H.: "The effect of cortisone on uterine growth in ovariectomized rats receiving estradiol". *Journal of Cell Science*, 1951, *92*, 385.

[18] Francisco A.M., Carbonel A.F., Simões R.S., Soares J.M. Jr., Baracat E.C., Haidar M.A.: "Do extracts of oral soybean augment the trophic effect of estrogen on the rat uterus?" *Climacteric*, 2013, *16*, 161.

[19] Oner H., Oner J., Kukner A., Ozan E.: "Effects of estrogen and/or progesterone on the changes occuring in the uterine luminal epitheIum of ovariectomızed rats". *Acta Veterinaria*, 2002, *52*, 97.

[20] Iguchi T., Todoroki R., Yamaguchi S., Takasugi N.: "Changes in the uterus and vagina of mice treated neonatally with antiestrogens". *Acta Anat. (Basel)*, 1989, *136*, 146.

[21] Tan J., Li H., Liu H., Yang S.: "Morphometric evaluation on the pathologic changes in ovariectomized endometria influenced by estrogen use". *Hua Xi Yi Ke Da Xue Xue Bao*, 1993, *24*, 179. [In Chinese, English abstract].

[22] Cullen S.J., Ponnappan S., Ponnappan U.: "Proteasome inhibition upregulates inflammatory gene transcription induced by an atypical pathway of NF- kappaB activation". *Biochem. Pharmacol.*, 2010, *79*, 706.

[23] Saccani S., Marazzi I., Beg A.A., Natoli G.: "Degradation of promoter- bound p65/RelA is essential for the prompt termination of the nuclear factor kappaB response". *J. Exp. Med.*, 2004, *200*, 107.

[24] Kriete A., Mayo K.L.: "Atypical pathways of NF-kappaB activation and aging". *Exp. Gerontol.*, 2009, *44*, 250.

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