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Combating endometriosis by blocking proteasome and nuclear factor-κB pathways

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BACKGROUND: The objective of this study is to investigate the effect of pyrrolidine dithiocarbamate [PDTC; a nuclear factor-kappaB (NF-κB) inhibitor] and bortezomib (Velcade; a proteasome inhibitor) on the development of experimental endometriotic implants in rats. METHODS: Endometriosis was surgically induced in 30 rats using the method of Vernon and Wilson. Three weeks later the viability and volume of the implants were recorded and classified. Afterwards, rats were put into three groups with equal numbers. The groups were labelled as the control, the PDTC and the bortezomib groups. Seven days after treatment, a third laparotomy was done and the volume of implants was measured again. The animals were then sacrificed, and the implants were stained with Ki67, proliferating cell nuclear antigen (PCNA), CD34, CD31 and Masson's trichrome histochemical staining. RESULTS: In 80% of the implanted rats, vesicles at the suture region were observed, and the rats graded according to average vesicle diameter (*D*) as: Grade 1 (no vesicle, 20% of rats), Grade 2 (*D* < 2 mm, 33.3% of rats), Grade 3 (2 mm<*D* > 4.5 mm, 26.7% of rats) and Grade 4 (*D* > 4.5 mm, 20% of rats). After treatment with PDTC or bortezomib, these percentages were decreased for Grades 3 and 4, and increased in Grade 1. The post-treatment implant volumes were decreased in the PDTC and bortezomib groups (*P* < 0.002 and *P* < 0.001), and slightly increased in the control group (*P* = 0.279). In the PDTC and bortezomib groups, CD34, CD31, PCNA and Ki67 expression levels were similar but were significantly reduced compared with the control group. CONCLUSIONS: PDTC and bortezomib may represent a novel therapeutic strategy for treatment of endometriosis.

Keywords: NF-κB inhibitor; proteasome inhibitor; inflammation; endometriosis

Introduction

The mammalian inflammatory response is a complex physiological process, and it is critically important for homeostasis and ultimate survival of organism. Nuclear factor-kappaB (NF-κB) is a transcription factor, which induces the expression of many genes that participate in immune and inflammatory responses (McKay and Cidlowski, 1999). It represents a group of structurally related and evolutionarily conserved proteins, with five members in mammals: p50/p105, p65/RelA, c-Rel, RelB and p52/p100 (Ghosh *et al*., 1998). NF-κB is usually found in the cytoplasm of resting cells conjugated to an inhibitory protein termed as IκB (Wang *et al*., 2005). This binding prevents its translocation into the nucleus (Ghosh *et al*., 1998). Phosphorylation of IκB by IκB kinase following inflammatory signal transduction leads to degradation of IκB via proteosome, resulting in the transfer of NF-κB into the nucleus and its activation there (Israël *et al*., 2000). In the nucleus, NF-κB binds to target DNA elements and positively regulates the transcription of genes involved in inflammatory responses, cell growth control and apoptosis (Baldwin, 1996; D'Acquisto *et al*., 2002).

Endometriosis is a chronic inflammatory disease involving multifactorial etiology characterized by implantation and growth of endometrial glands and stroma outside the uterine cavity (Ho *et al*., 1997). NF-κB can be activated in a wide variety of cell types including endometriotic cells (McKay and Cidlowski, 1999; Wang *et al*., 2005). Studies have revealed an important regulatory role of NF-κB in the proinflammatory response of endometrial stromal cells from women with endometriosis (Lebovic *et al*., 2001; Yamauchi *et al*., 2004). The presence of NF-κB response elements in the promoter region

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is a main feature of several inflammatory genes involved in the pathogenesis of endometriosis (McKay and Cidlowski, 1999). Hence, NF-κB is an excellent potential candidate to target the inflammatory response in endometriotic cells (Sakamoto *et al*., 2003). Numerous pro-inflammatory cytokines, such as TNFα and IL-6 that are NF-κB-target genes have been shown to promote proliferation in endometriotic cells (Lin *et al*., 2006). p65, a subunit of NF-κB, has been identified in endometrial stromal cells from women with deep endometriosis (Matsuzaki *et al*., 2005). Most of the investigational medications for treating endometriosis act by inhibiting production of pro-inflammatory cytokines (Wieser *et al*., 2005). The inhibition of inflammation can be realized in two ways: (i) direct inhibition of NF-κB activation through the use of NF-κB inhibitors, and (ii) prevention of NF-κB precursors from processing into mature forms by proteasome inhibitors (Guo, 2007). Proteasomes are key regulators of cells being responsible for the degradation of many intracellular proteins, thus helping to maintain cellular homeostasis during biological processes (Adams *et al*., 1999). Dysregulation of proteasome expression and related substances may contribute preferentially to the development of the ectopic endometrial lesions due to their reduced sensitivity to apoptosis (Ilad *et al*., 2004). Moreover, dysregulation of proteasomal function in implants may induce inflammation due to the proteasome being responsible for the consequent constitutive activation of NF-κB (Adams *et al*., 1999).

Pyrrolidine dithiocarbamate (PDTC) is an antioxidant and potent NF-κB inhibitor (Demirbilek *et al*., 2006), and bortezomib (Velcade; previously known as PS-341) is a dipeptide boronic acid derivative that is a highly selective, potent and reversible proteasome inhibitor (Adams *et al*., 1999). The activation of NF-κB and proteasome pathways participates in the pathophysiology of endometriosis by inducing growth and inflammation of endometriotic lesions (Guo, 2007). Both PDTC and bortezomib have been demonstrated to play a protective role in different types of cells or tissue injuries *in vitro* and *in vivo* (Krunkosky *et al*., 2003; Ferreira *et al*., 2007). Thus, the inhibition of NF-κB and/or proteasome may be considered as a potential treatment option for endometriosis due to aberrant inflammation. Yet, to our knowledge, the roles of proteasome and NF-κB inhibitors in the treatment of endometriosis have not been investigated. The present study in this perspective was undertaken to evaluate the efficacy of intraperitoneally (i.p.) administered bortezomib and PDTC in suppressing implant growth in rats with experimentally induced endometriosis *in vivo*.

Materials and Methods

This study was carried out in the Experimental Research Laboratory of the Inonu University Faculty of Medicine, complying with the approval of the ethic committee and the guidelines for care and use of experimental animals. Thirty adult female Wistar rats each weighing between 250 and 350 g were purchased from Inonu University Animal Laboratory. All rats were examined by a veterinarian and determined to be in good health. The rats were housed in plastic cages and they were kept under standard conditions: 12-h light and 12-h dark periods, 20°C constant temperatures and a humidity range between 40 and 60%. The rats had free access to standard dry pellets *ad libitum* and tap water until the end of the study.

Before transplantation, all animals were hormonally synchronized in their 4-day estrus phase to exclude the differences in the steroid synthesis, cell adhesion and growth, and thus, endometriosis development between the individual animals owing to hormonal variations. Synchronization was performed by administration of the subjects with two subcutaneous injections (55 mg/kg body weight estradiol) with 24 h intermission, followed by one injection (7.5 mg/kg body weight of progesterone) 20 h after the last estradiol injection (Gross, 1977). Daily vaginal smears of the rats were taken to establish the estrous cycle of each animal. Behavioural estrus occurred 4 h after the injection of progesterone. Vaginal smears were taken by cotton swab: the swab was inserted into the vagina and rotated 360° clockwise direction, then the swab was smeared onto a glass slides and fixed with ethanol. Smears were stained with the usual Papanicolau method and then evaluated by light microscopy by an experienced cytopathologist who was unaware of the groups. The estrous cycle was determined as follows: proestrus period (centrally nucleolated many epithelial cells), estrus period (cornified epithelial cells without nucleus), metestrus period (leukocyte, mucus and a few cornified cells), diestrus period (various epithelial cells, mucus and leukocyte). Rats observed for at least two successive 4-day estrous cycles.

Endometriosis was induced surgically by using the method described by Vernon and Wilson (1985) during estrus. A 0.5 × 0.5 × 0.1 cm piece excised by microscissors from the uterine horn was attached onto the peritoneum only on the right side of the ventral abdominal wall close to an artery via the surgical autotransplantation technique. The rats were individually caged after the operation and were left for a recovery period. After 3 weeks their daily vaginal smears were monitored and a second laparatomy was performed in their estrous phase to determine the attachment and viability of endometrial implants. Of the 30 experimental rats, 6 did not develop any signs of vesicles, and therefore these were excluded from the study. The vesicles at the sutures region were observed and the rats were graded according to average vesicle diameter (***D***) as: Grade 1 (for cases in which the implant had disappeared or, if it was visible, never became a cyst, 20% of rats), Grade 2 ($\boldsymbol{D} < 2$ mm, 33.3% of rats), Grade 3 ($2\text{ mm} < \boldsymbol{D} > 4.5$ mm, 26.7% of rats) or Grade 4 ($\boldsymbol{D} > 4.5$ mm, 20.0% of rats). The 24 rats with endometriosis were randomized (using random number tables) into three equal groups: (1) the control group; (2) the PDTC (Sigma-Aldrich Chemie, GmbH, and Steinheim, Germany) group and (3) the bortezomib (Velcade, Ben Venue Laboratories Inc., USA) group. The pretreatment implant volumes in each group were calculated by measuring their dimensions (length, width and height, in milimeters). For volume calculation the ellipsoid volume formula ($\pi/6 \times$ length × width × height) was used. The rats in Groups 2 and 3 were then treated with PDTC (100 mg/kg body weight per rat, i.p.) or bortezomib (0.2 mg/kg body weight per rat, i.p.) respectively for 7 days. These doses were chosen because, in rats, 0.2 mg/kg of bortezomib induces blood proteasome inhibition equivalent to that in human clinical trials (Henninger *et al*., 2006), and 100 mg/kg of PDTC is the physiologically acceptable dose for antioxidant effect and NF-κB inhibition (Demirbilek *et al*., 2006). It has not been stated in any comments by the manufacturers whether PDTC or bortezomib have any effect on the estrous cycle. Therefore, during the treatment period daily vaginal smears were monitored and after the permanence of the estrous cycle was confirmed, a third laparotomy was performed while the rats were fixed in the supine position. The volumes and sizes of the implants were measured again with the same method by the same researchers who were blinded to the groups. Since the

bortezomib and PDTC were diluted in isotonic saline, an identical amount of isotonic saline was used as control in the endometriosis-vehicle group (0.1 ml/day per rat, i.p.) for 1 week.

The endometrial implants were then excised and processed for histological and immunohistochemical studies. Formalin-fixed specimens were embedded in paraffin and cut into 5 μm thick sections and stained with haematoxylin and eosin and Masson's trichrome staining. The sections were also stained for proliferating cell nuclear antigen (PCNA), Ki67, CD34 and CD31 immunohistochemistry. The histological diagnosis of endometriosis was based on the morphological identification of endometrial glandular tissue (GT) and stroma: glands and stroma of the endometrial type, with epithelial lining and luminal formation. All proliferating nuclei in the stromal cells, glandular and luminal epithelial cells were stained with mouse monoclonal antibody against PCNA (Clone PC10; Sigma corp., Missouri, USA) which is a nuclear protein which has peak expression during the S phase of the cell cycle, and has been used previously to identify proliferating cells monoclonal anti-PCNA clone PC10 from mouse ascites fluid were diluted to 1/1000 and applied to 5 μ paraffin sections deparaffinized in xylene using the labeled strepavidin biotin method. The sections, for Ki67 detection, were incubated in 10 mmol/l citrate buffer (pH 6.0) at 98°C for 20 min, and then quenched in Super Block (ScyTek Laboratories, Utah, USA) for 5 min at room temperature. Primary antibodies for Ki67 (rabbit polyclonal, ready to use ScyTek Laboratories) were applied to the samples and incubated at room temperature for 30 min. Immunodetection was performed using Ultra Tek HRP Anti-Polyvalent Lab Pack (ScyTek Laboratories). Ki67 is expressed in the cell during M, G1, S and G2 phases of cell cycle and is absent in resting cells (G0). It shows a good correlation to mitotic indices in human endometrium. Endothelial cells were stained using mouse monoclonal antibody against CD31 and CD34 antigens, a glycoprotein expressed on the luminal surface of endothelial cells (ready to use, clone QBEnd/10; Novocastra, Newcastle, UK). Antigen retrieval was made with enzyme digestion using trypsin. The slides were quenched in Super Block (ScyTek Laboratories) for 5 min at room temperature. Immunodetection was performed using Ultra Tek HRP Anti-Polyvalent Lab Pack (ScyTek Laboratories). In each case the final products were visualized by aminoethylcarbazole chromogen, and counterstaining was performed with haematoxylin. Rat peritoneal vessel served as a positive internal control for CD34 and CD31. For PCNA, human tonsil tissue served as positive control. Negative controls (primary antibody was omitted) were routinely performed on adjacent serial sections.

Histological slides were evaluated for stromal tissue (ST), GT, vascularity, and PCNA, Ki67, CD31 and CD34 immunoreactivity under light microscopy. A semiquantitative grading system was used to score the degree of histological change of endothelial cells, stromal cells, glandular and luminal epithelial cells. The scalings were conducted for: **a:** the degree of PCNA immunoreactivity of implant cells [0, no PCNA positive cells per high power field (×40); 1, between 1 and 10 PCNA positive cells; 2, between 10 and 20 PCNA positive cells and 3, >20 PCNA positive cells]; **b:** the degree of Ki67 immunoreactivity of implant cells [0, no Ki67 positive cells per high power field (×40); 1, between 1 and 10 Ki67 positive cells; 2, between 10 and 20 Ki67 positive cells and 3, >20 Ki67 positive cells]; **c:** the degree of CD34 and CD31 immunoreactivity of endothelial cells (0, no CD34/CD31 positive cells per high power field; 1, between 1 and 10 CD34/CD31 positive cells; 2, between 10 and 20 CD34/CD31 positive cells and 3, >20 CD34/CD31 positive cells]; **d:** the degree of vascularitity (0, no blood vessels; 1, 1–5 blood vessels; 2, 5–10 blood vessels; 3, >10 blood vessels); **e:** the degree of GT [0, no secretory glands per high power field (×40); 1, between 0 and 5 secretory glands; 2, between 6 and 10 secretory glands and 3, >10 secretory glands] and **f:** the degree of ST (0, no stromal tissue; 1, <25% stromal tissue; 2, 25–50% stromal tissue and 3, >50% stromal tissue).

Statistical analysis

The Statistical Package for Social Sciences, version 11.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. The normality of variables obtained from the groups was tested by the Shapiro–Wilk test; the variables did not show a normal distribution. Therefore, the non-parametric Kruskal–Wallis test was conducted on variables to compare all groups together. For detecting inter group differences, the Bonferroni Mann–Whitney *U*-test was used permutatively. The data were presented in mean ± SEM. For all comparisons, statistical significance was defined by $P < 0.05$.

Results

It was observed that 80.0% of the implanted rats developed vesicles in the suture zone, as represented in Fig. 1. The rats that somehow did not develop any vesicle were assigned as Grade 1 (20%). As mentioned earlier, in 33.3% of the subjects the diameter of vesicles was <2 mm (Grade 2), in 26.7%, the vesicles diameter were between 2 and 4.5 mm (Grade 3) and in 20%, the vesicles diameter were >4.5 mm (Grade 4). After the treatment with PDTC or bortezomib, the percentage of Grades 3 and 4 vesicles decreased, whereas the percentage of Grades 1 and 2 vesicles increased. In contrast, the percentage of Grade 4 vesicles increased, whereas the percentage of Grade 2 vesicles decreased in the control group (Fig. 2). As demonstrated in Fig. 3, the pretreatment volumes in the control, PDTC and bortezomib groups were found to be 72.5 ± 28.7, 64.6 ± 24.7 and 67.3 ± 35.1 mm^3, respectively. These pretreatment volumes did not show any significant statistical difference between three groups ($P > 0.05$). The post-treatment volumes in the control, PDTC and bortezomib groups were found to be 80.4 ± 30.1, 31.9 ± 20.0 and 32.8 ± 20.1 mm^3, respectively. When the pre and post-treatment volumes were compared in the same group, it was found that the post-treatment volumes

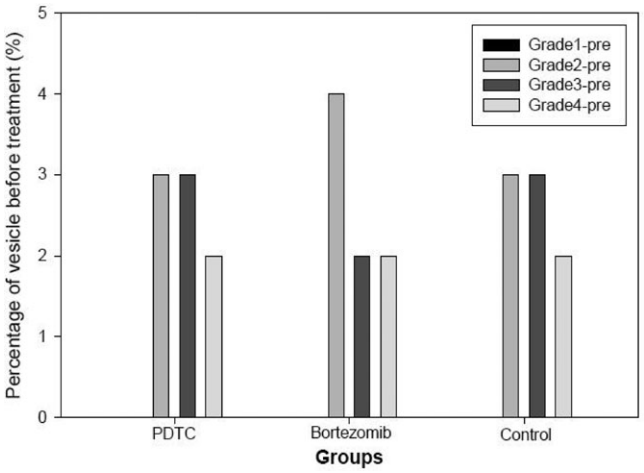

Figure 1: Graphical representation of the implants grading before treatment.

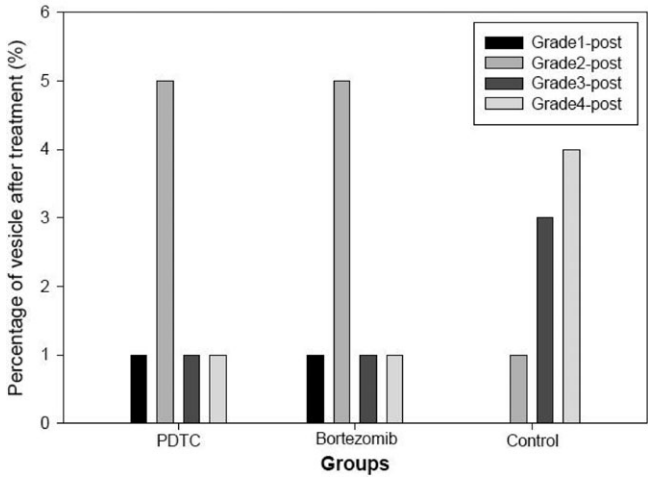

Figure 2: Graphical representation of the implants grading after treatment.

decreased significantly in the PDTC and bortezomib groups ($P < 0.002$ and $P < 0.001$). In the control group, the post-treatment volumes were higher than those of pretreatment volumes, but the differences were not significant statistically ($P = 0.279$). The post-treatment volumes in the PDTC and bortezomib groups were lower than those of in the control group ($P < 0.001$ and $P < 0.001$). In addition, post-treatment volumes in the PDTC and bortezomib groups were comparable ($P = 0.798$).

The immunohistochemical detection of PCNA, Ki67, CD31 and CD34 revealed proliferation of many stromal and glandular cells and endothelial cells in implants of control animals (Figs 4 and 5). In contrast, endometriotic implants of PDTC- and bortezomib-treated rats developed only a few proliferating cells, which were mainly localized within the endometrial stroma (Fig. 4). As shown in Fig. 6, none of the histological parameters studied (endothelial cells, proliferative indices and immunostaining scores for glandular, luminal epithelial

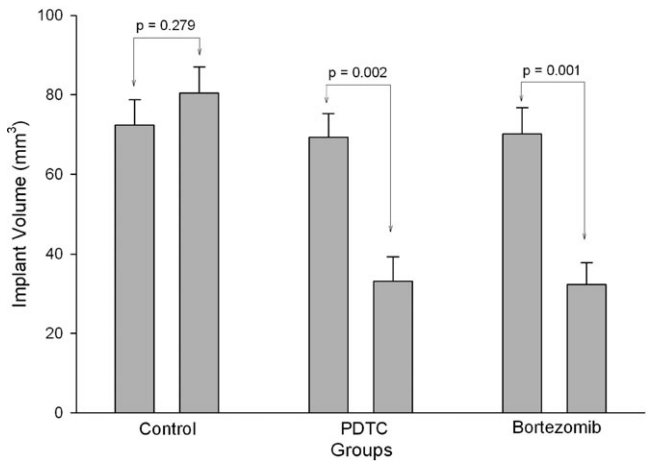

Figure 3: Graphical representation of pre and post-treatment implant volumes of control, PDTC and bortezomib groups.
Each column represents the mean value of implant volumes. $P < 0.05$ indicates the significance of the difference between pre and post-treatment volumes.

and stromal cells) showed any significant differences between animals treated with PDTC or bortezomib. Glandular, luminal, stromal, endothelial cells and fibrous tissue demonstrated higher histochemical staining for CD34, CD31, PCNA, Ki67 or trichrome in the control group than did those in the PDTC and bortezomib groups (Fig. 6). The expression of CD34, CD31, PCNA, Ki67 and trichrome did not show any significant differences between the PDTC and bortezomib groups (Fig. 6). The development of new blood vessels could be observed in each of experimental groups (Fig. 5). Angiogenesis was characterized by formation of capillary buds and sprouts ingrowing into the endometrial implants. Owing to their peculiar architecture, the newly formed microvessels could be clearly distinguished from the host striated muscle capillaries of the rat, which display a typical parallel arrangement. As shown in Fig. 6, the mean scores for vascularity in the PDTC and bortezomib groups were significantly lower than that for the control group ($P < 0.05$). Similarly, the scores for both ST and GT in the PDTC and bortezomib groups were found to be decreased compared with the control group ($P < 0.05$). Negative control reactions, in which the PCNA, CD34, CD31 and Ki67 antibody were omitted, were performed on adjacent serial sections; no specific staining was observed (Fig. 5).

Discussion

Evidence showing that NF-κB may be one of the major culprits in pathogenesis of endometriosis is extensively discussed in the literature (Lebovic *et al*., 2001; Yamauchi *et al*., 2004; Guo, 2007). Activation of NF-κB may be responsible for angiogenesis, invasiveness and inhibition of apoptosis, and for increased production of pro-inflammatory cytokines and estrogens (Lebovic *et al*., 2001; Yamauchi *et al*., 2004; Guo, 2007). In women with endometriosis, NF-κB was found to be highly expressed in both eutopic and ectopic tissues compared with normal endometrium (Wang *et al*., 2005). Increased production of pro-inflammatory cytokines and chemokines has also been observed in ectopic endometrium (Arici, 2002). Compounds that suppress NF-κB activation via different means are already available (Orlowski *et al*., 2002). Some of them have been employed in clinical trials for treating some cancers too (Orlowski *et al*., 2002).

The capacity of NF-κB for being activated by inflammatory cytokines, and then to regulate genes involved in inflammatory function raised the question of whether NF-κB dysregulation could be associated with inflammatory diseases such as endometriosis (Ghosh *et al*., 1998; Baldwin, 1996). The inhibition of NF-κB within the endometrial implant may be achieved either with NF-κB inhibitors or by preventing the release of NF-κB precursors using proteasome inhibitors. As PDTC and bortezomib have been recently shown to diminish NF-κB activity in several cell lines (Adams *et al*., 1999; Demirbilek *et al*., 2006), here we investigated whether these preparations could affect the endometriotic implant growth in rats. Treatment with PDTC or bortezomib effectively reduced the size of implants, and thus, prevented the progress of endometriosis. The effects of PDTC and bortezomib in reducing the implant

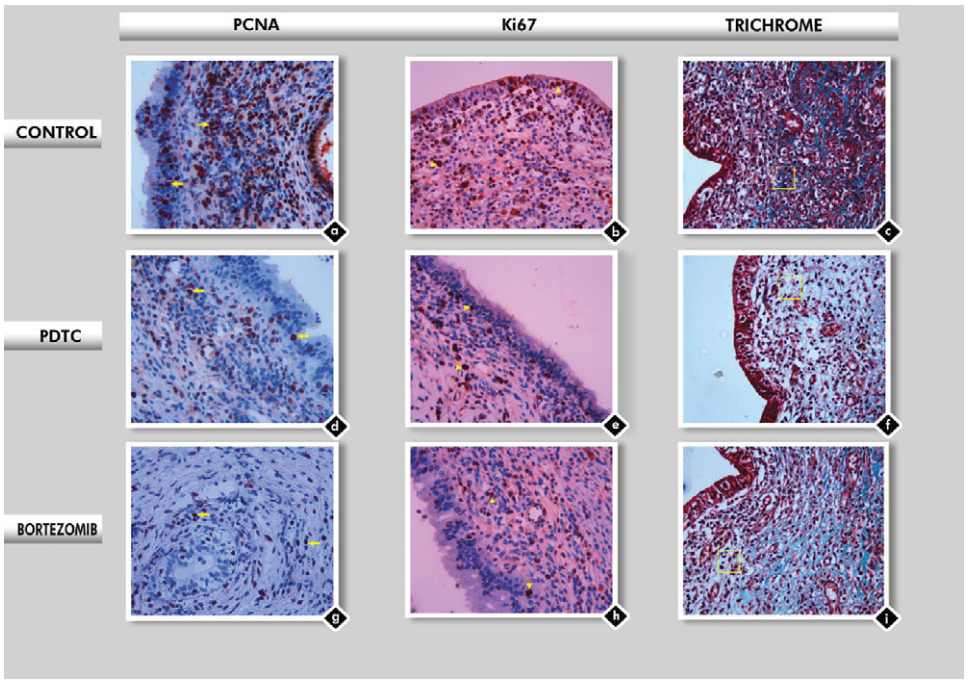

Figure 4: Representative photographs of the implants obtained from control, PDTC and bortezomib groups after treatment.
In PDTC (**d**–**f**) and bortezomib groups (**g**–**i**), PCNA, Ki67 and trichrome expressions were similar but were significantly reduced as compared to control group (**a**–**c**). Arrows indicate PCNA (×400) stained stromal and glandular cells (a, d and g). Arrow heads indicate Ki67 (×400) stained stromal and glandular cells (b, e and h). Squares indicate (×400) trichrome stained fibrous tissue (c, f and i).

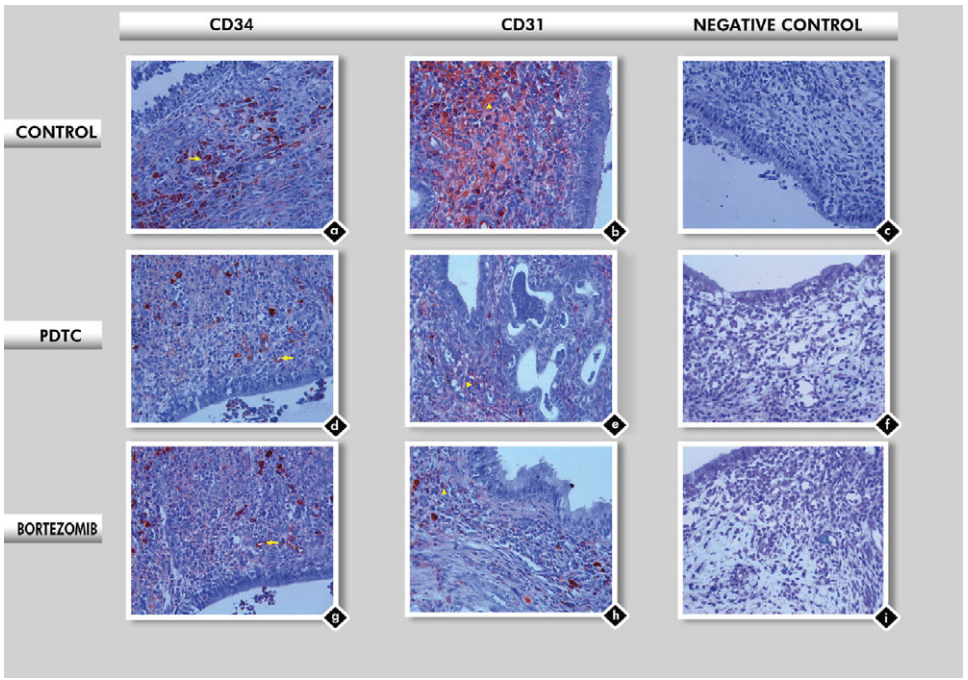

Figure 5: Photograph of the newly formed microvascular networks of endometrial grafts at Day 7 after treatment of a control, a PDTC-treated and bortezomib-treated rat.
Note that all endometrial grafts are characterized by a newly formed microvascular network. However, the PDTC-treated and bortezomib-treated animals revealed a significantly reduced microvessel density and smaller vascularized area when compared to control animals (**d, e, g** and **h**). A rat peritoneal vessel provides a positive internal control for CD34 and CD31. Arrows indicate CD34 (×400) stained endothelial cells (**a**, **d** and **g**). Arrow heads indicate CD31 (×400) stained endothelial cells (**b**, **e** and **h**). Negative control reactions, in which the CD34 and CD31 antibody were omitted; no specific staining was observed (**c, f** and **i**).

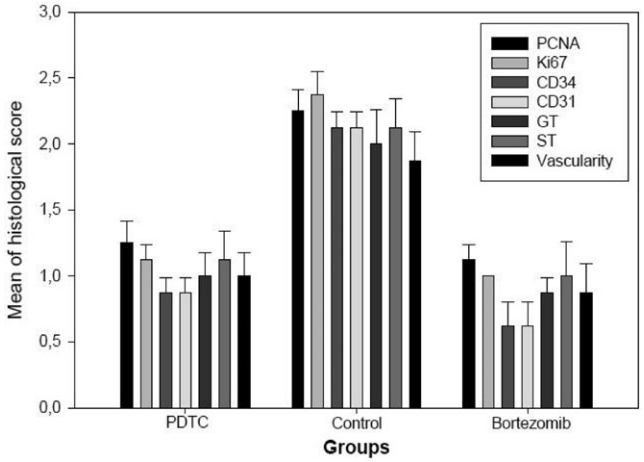

Figure 6: Graphical representation of the mean score of PCNA, Ki67, CD34 and CD31 vascularity, glandular and stromal tissue of endometriotic implants obtained from control and treatment groups.

sizes were comparable. The GT and ST scores in the treated groups were also significantly lower than in the control group. This finding shows, histologically, a significant regression in the endometriotic implants, and also suggests that the local inflammatory state may play an important role in stimulating growth of endometrial implants.

Consistent with its essential role in inflammation, NF-κB is known to be the target of anti-inflammatory compounds (Barnes and Karin, 1997). Various studies have been done in attempt to suppress inflammation mediated by the NF-κB pathway (Bruck *et al*., 2002; Long *et al*., 2003). Progestin and other therapeutic regimens for the treatment of endometriosis have been found to suppress the NF-κB activity (Wieser *et al*., 2005). In fact, progestins, which are physiological NF-κB inhibitors, and danazol attenuate TNFα induced IL-8 production via suppression of NF-κB activation (Horie *et al*., 2005). Nonhormonal therapeutic agents such as the nonsteroidal anti-inflammatory drugs also can target the NF-κB pathway. Wieser *et al*. (2005) reported that sulindac inhibits activation and DNA binding of NF-κB in endometrial stromal cells. Based on our findings, it is asserted that PDTC seems to have a protective effect against implant growth through inhibition of NF-κB activation owing to its abilities to diffuse into the cell and remain stabile in solutions at physiological pH and to hold antioxidant properties (Muià *et al*., 2006). Németh *et al*. (2003) reported that treatment of intestinal epithelial cells with PDTC *in vitro* suppresses the activity of NF-κB. (Cuzzocrea *et al*. 2002) reported the ability of PDTC to suppress the transfer of NF-κB from the cytoplasm into the nucleus due to IκB-α degradation. It has been declared that the increased production of proinflammatory cytokines in endometriotic tissue would activate NF-κB and perhaps other transcription factors (Wu and Ho, 2003). The activation of NF-κB would further increase the production of pro-inflammatory cytokines and chemokines, and promote proliferation and angiogenesis, inhibit apoptosis and increase the invasive capability by upregulating their respective target genes (Kumar *et al*., 2004). Moreover, NF-κB may activate aromatase promoter II which increases the local production of estrogen (Fan *et al*., 2005). It is considered that as NF-κB is inhibited by PDTC these genes are subsequently blocked, and therefore apoptosis is permitted, while proliferation, oxidative stress, angiogenesis, invasion and production of pro-inflammatory cytokines and estrogen are all restricted. These activities altogether would lead to a reduction in the implant volume.

Another possible cause of the reduction in implant growth may be the inhibition of COX-2 expression. Inflammatory reactions with subsequent release of COX-2 have been documented to play a very important role in implant growth (Dogan *et al*., 2004). NF-κB regulates several genes coding for inflammatory enzymes, such as COX-2 (Lähde *et al*., 2000). Inhibition of NF-κB activity has been demonstrated to attenuate the size of different types of tissue or cell injuries via down-regulation of COX-2 expression (Lähde *et al*., 2000). Factors such as TNFα and IL-1β that are inducers of NF-κB activation are known to promote proliferation of endometriotic stromal cells by inducing IL-8 gene and COX-2 expression (Orlowski *et al*., 2002; Wu *et al*., 2005). So, PDTC may suppress the production of COX-2 and lead to a reduction in implant size. In addition to its anti-inflammatory activities, the reduction in the PCNA, Ki67, CD31 and CD34 expression levels found through immunohistochemical examinations demonstrates a direct inhibitory effect of PDTC on cell proliferation in the endometrial implant. Whatever the mechanism is, it can be revealed that inhibition of NF-κB by PDTC offers an effective therapeutic strategy for combating endometriosis.

The proteasome is a multicatalytic threonine protease responsible for intracellular protein turnover in eukaryotic cells, including the processing and degradation of several proteins involved in cell cycle control and the regulation of apoptosis. Preclinical studies have shown that treatment with a proteasome inhibitor results in decreased proliferation, induction of apoptosis and sensitization of tumour cells (Orlowski *et al*., 2002). Endometriosis possesses many features of a benign neoplastic process with the potential for malignant transformation (Varma *et al*., 2004). Another prominent future of endometriotic cells that sets them apart from their eutopic endometrial counterpart is their invasiveness (Zeitvogel *et al*., 2001). Some of the proteases associated with the invasive capacity of endometriotic cells include urinary plasminogen activator and MMPs which have been well documented elsewhere (Fernández-Shaw *et al*., 1995; Mizumoto *et al*. 2002).

The major biological effect of bortezomib is the inhibition of the NF-κB, induction of apoptosis and inhibition of angiogenesis. Inhibition of the proteasomal function results in stabilization and accumulation of its substrates, which include cyclins, transcriptional factors, tumour suppressor proteins and I-κB which inhibits NF-κB (Orlowski *et al*., 2002). As we demonstrated in this *in vivo* study, administration with bortezomib results in the regression of endometriotic lesions in a short period such as 7 days. As discussed previously, this effect is most probably due to the inhibition of NF-κB, an inhibition of cell proliferation and angiogenesis which was observed through the reduced vascularized area of newly formed microvascular networks and reduced expression of CD34, CD31, PCNA and Ki67 within these endometriotic

implants. Others have reported the antiproliferative effect of bortezomib against tumour cells as well (Orlowski *et al*., 2002). These results might offer the opportunity to apply bortezomib as a selective drug not only for the anticancer therapy of different tumour types, but also for the treatment of endometriotic lesions.

It is known that NF-κB is a critical early regulator of the inflammatory response in endometriotic tissues. The inhibition of NF-κB activation may therefore reduce this inflammation and inhibit implant growth. Beside their anti-inflammatory activity, we demonstrated an inhibitory effect of bortezomib and PDTC on cell proliferation within the endometrial implant, as indicated by the down-regulation of Ki67 and PCNA expression. Immunohistochemical examinations of endometriotic lesions were also further demonstrated a reduction in proliferating activity of stromal and endothelial cells.

With the association of all these information, a new therapeutic application of PDTC and/or bortezomib as an antiendometriotic agents in the treatment of endometriosis may be suggested. This study warrants clinical studies to further elucidate the potential role of these agents, which represent a novel therapeutic strategy for the treatment of endometriosis.

Authors' contribution

O.C., S.H. and K.E. performed study design and statistical analysis; O.C., S.H. and N.E.A. collected data; O.C., S.H., K.E., M.E.T., B.G. and N.E.M. done data interpretation and manuscript preparation; O.C., S.H., K.E., M.E.T. and B.G. done literature collection; N.E.M. preformed histological analysis.

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