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ORIGINAL ARTICLE

Anti-tumoral Action of Octreotide and Bromocriptine on the Experimental Rat Prolactinoma: Anti-proliferative and Pro-apoptotic Effects

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Abstract

OBJECTIVES: The purpose of the study was to compare the effects of bromocriptine (BC) – D-2 receptor agonist and octreotide (OCT) – somatostatin analog on the tumor weight, prolactin (PRL) secretion, cell proliferation and apoptosis in the diethylstilboestrol (DES)-induced rat prolactinoma.

MATERIAL AND METHODS: Male four-week Fisher 344 rats were used in the experiment. The animals were implanted subcutaneously (s.c.) with capsules containing DES. Six weeks after the implantation the rats were given OCT (2 x 25 μg/animal/24 h s.c.) or BC (3 mg/kg b.w./24 h s.c.) for 10 days. The incorporation of bromodeoxyuridine (BrDU) into the tumor cell nuclei was used as an index of cell proliferation (labeling index – LI). The labeling of nuclear DNA fragmentation according to the TUNEL method was considered as an index of apoptosis (AI). PRL was measured by radioimmunoassay (RIA).

RESULTS: It has been found that OCT and BC significantly decreased the tumor weight and LI of tumor cells to the same extent. Both OCT and BC suppressed the PRL levels, but the inhibitory effect of BC was stronger than that of OCT. BC and OCT significantly enhanced the number of apoptotic cells in the tumor, but the pro-apoptotic effect of BC was more pronounced. The joint treatment exerted additive effects on tumor mass reduction, PRL secretion and cell proliferation, but OCT attenuated the pro-apoptotic effect of BC.

CONCLUSIONS: Summing up, both OCT and BC inhibit PRL secretion and cell proliferation. The anti-tumoral action of BC, and to some extent the action of OCT, is also connected with induction of apoptosis.

Abbreviations and units

AI	index of apoptosis
b.w.	body weight
BC	bromocriptine (Bromocriptine mesylate)
BrDU	bromodeoxyuridine
C	control
DAB	diaminobenzidine tetrahydrochloride
DES	diethylstilboestrol
GH	growth hormone
i.p.	intraperitoneal
kg	kilogram
LI	labeling index
mg	milligram
min	minute
ml	millilitre
mm	millimetre
µg	microgram
µl	microlitre
ng	nanogram
OCT	octreotide (Sandostatin)
P/A	proliferation/apoptosis
PRL	prolactin
RIA	radioimmunoassay
s.c.	subcutaneously
SEM	standard error of the mean
SST	somatostatin
TdT	terminal deoxynucleotidyl transferase
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling

Introduction

It is well known, that bromocriptine (BC) and other D-2 dopamine receptor agonists inhibit prolactin (PRL) secretion and exert the anti-proliferative action on pituitary PRL cells in humans [1,2] and animals [3–5]. D-2 dopamine agonists inhibit the parameters for lactotroph proliferation such as pituitary weight, DNA synthesis and mitotic activity in normal pituitaries as well as in estrogen–induced [6], transplantable [7] and spontaneously formed pituitary tumors. BC and D2 receptor agonists are also known to induce the regression of human prolactinomas and are commonly used in medical therapy of these tumors (for review see [8, 9]).

Somatostatin (SST) and its analogs have been also demonstrated to exert the anti-proliferative effects in normal and neoplastic tissues both *in vivo* and *in vitro* studies. The anti-proliferative effects of SST and its analogs concern also normal and tumorous pituitary [10–13]. Octreotide (OCT) and other SST analogs are applied in the therapy of GH – secreting pituitary tumors [9]. Although there is a lot of evidence demonstrating the anti-tumoral effects of both BC and OCT, the mechanisms of their action are not sufficiently recognized.

The purpose of our study was to compare the effects of BC, OCT and the combination of both drugs on the experimental rat prolactinoma and to investigate whether the anti-tumoral action of these drugs involves the induction of apoptosis.

Apoptosis is a programmed cell death, which occurs in several physiological and pathological conditions, including neoplasia. Apoptosis in the pituitary gland was investigated only in the limited number of studies. Most of the authors found that the apoptotic index in both non-tumorous and adenomatous pituitary tissues is low [14–16]. The induction of apoptosis after bromocriptine [17, 18] or SST analogs [19] treatment of the pituitary tumors was reported in a few works, however the other studies provide contradictory results [14, 20].

Material and Methods

Experimental protocol: Four week old male Fischer 344 rats weighing 50–70 g, maintained in controlled lighting regime (12L/12D), with free access to standard laboratory food and tap water, were used in the experiment. Capsules containing 8–10 mg of diethylstilboestrol (DES, Sigma) each were implanted subcutaneously (s.c.) in the lumbar region. Such capsules were estimated to release 18–45 µg of DES daily [21].

Six weeks after the implantation of capsules the rats were divided into 5 groups and treated with the following substances for 10 days: **GROUP IA** – control: 0.25 ml of physiological saline s.c., once daily; **GROUP IB** – control: 0.25 ml of 50% ethanol in physiological saline s.c., twice daily; **GROUP II**: octreotide (OCT, Sandostatin, Novartis) at a dose of 25 µg/animal s.c., twice daily; **GROUP III**: bromocriptine (BC, Bromocriptine mesylate, Lek) at a dose of 3 mg/kg b.w. s.c., once daily; **GROUP IV**: OCT + BC at the above doses. BC was dissolved in 50% ethanol in physiological saline.

On the eleventh day the animals were sacrificed. 90 min earlier they had received a single intraperitoneal (i.p.) injection of bromodeoxyuridine (BrDU, Sigma) at a dose of 50 mg/kg b.w.

Blood and pituitary glands were collected. The pituitaries were weighed, fixed in 4% formalin in phosphate buffered saline and then embedded in paraffin wax. In the microscopic preparations three parameters were assessed: the BrDU labeling index (LI), as an index of cell proliferation; the apoptotic index (AI), as an index of programmed cell death; and the proliferation/apoptosis (P/A) ratio. In blood serum PRL concentrations were estimated.

Cell proliferation: The paraffin sections were immunostained using the Cell Proliferation kit (Amersham, UK) to detect the incorporated BrDU. The number of BrDU-immunopositive cell nuclei per 1000 was used as an index of cell proliferation (labeling index – LI). At least 3000 randomly scored nuclei were evaluated in each pituitary gland at 600× magnification.

Apoptosis: Apoptosis was visualized by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method using the In Situ Cell Death Detection Kit, POD (Boehringer Mannheim). The TUNEL method, originally described by Gavrieli et al. [22], was used after necessary modifications. The slides were not treated with proteinase K after

preliminary tests consisting of varying incubation time. Then, 50 µl of TUNEL reaction mixture (450 µl nucleotide mixture in reaction buffer plus 50 µl enzyme TdT from calf thymus) were added to samples. The slides covered with coverslips were incubated in a humidified chamber for 60 min. at 37°C. After the step with TdT the specimens were additionally saturated with 5% normal sheep serum to diminish the background. The exposure to diaminobenzidine tetrahydrochloride (DAB) lasted for 2 min. After that, the tissue sections were counterstained with haematoxylin. Negative control was performed by omitting TdT.

Apoptosis was evaluated by counting of random 3000 cells from each section at 600× magnification. The 0.5 mm boundary around the section was excluded from analysis to circumvent artifactual staining which may occur at the edges of tissue sections. The number of cells containing the apoptotic bodies or nuclei per 1000 cells was used as an apoptotic index (AI).

Prolactin assay: Prolactin was assayed in blood serum using the Rat prolactin 125 I assay system with magnetic separation (Amersham, UK) and expressed in ng/ml. The method sensitivity is ~0.7 ng/tube (7.0 ng/ml).

Statistical analysis: All data are expressed as median, range=max–min (mean±SEM). The data were analyzed statistically using the Mann-Whitney U test. $P<0.05$ was considered as the borderline of statistic significance. As there was no statistically significant difference between both control groups, they have been connected.

Results

Six weeks after the implantation of DES the animals of the control group exhibited the tumorous enlargement of the anterior pituitary gland which contains almost solely the lactotrophs.

Tumor weight: The data concerning the tumor weight are shown on Figure 1. It has been found that both OCT and BC, alone or in combination, significantly reduced the tumor weight: OCT-26.2, 13.7 (24.7±1.6 mg); BC-22.05, 16.5 (22,8±1.7 mg); OCT+BC-19.45, 7.9 (19.7±0.7 mg) as compared to the control group: C-57.65, 35.0 (56.8±2.9 mg). OCT and BC given together were more effective than OCT, but not BC alone.

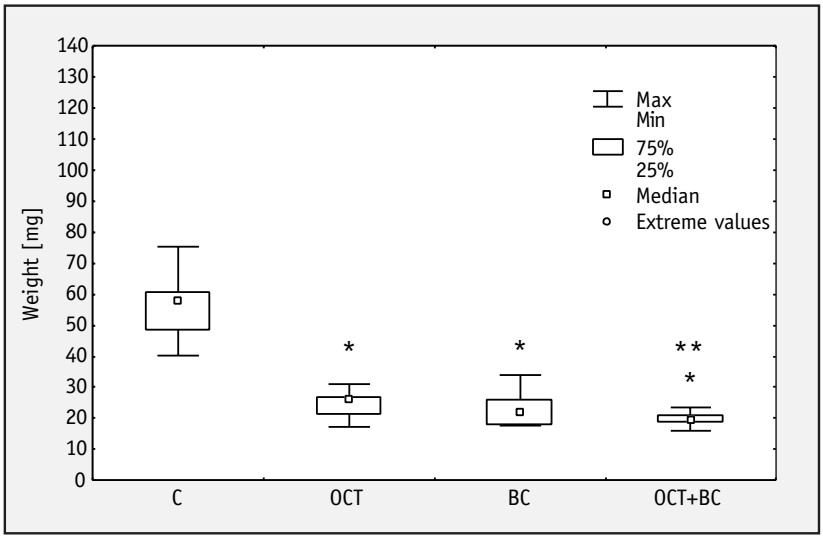

Fig.1. Effects of OCT and BC, alone or in combination, on pituitary tumor weight in the experimental rat prolactinoma. * p<0.0002 vs. control, ** p<0.05 vs. OCT-treated group.

Cell proliferation (LI): Both OCT and BC, administered separately or together, decreased the LI of experimental rat prolactinoma cells (Fig. 2): OCT-2.0, 5.0 (2.4±0.6); BC-2.0, 4.0 (2.6±0.4); OCT+BC-1.5, 3.0 (1.4±0.3) vs. C-14.0, 14.0 (13.9±1.3) (Fig. 3). The combination of OCT and BC proved to be more effective than BC applied alone.

Apoptosis (AI): The treatment with both OCT and BC caused a significant increase in AI (Fig. 4): OCT-3.0, 2.0 (2.75±0.25); BC-28.0, 26.0 (22.1±3.8) vs. C-1.0, 2.0 (1.2±0.3) (Fig. 5). The most pronounced effect was observed in a group receiving BC alone. Unexpectedly, the joint effect of BC and OCT was much lower than that of BC alone: OCT+BC-2.0, 19.0 (5.2±2.2).

Proliferation/apoptosis (P/A) ratio: OCT and BC, alone or in combination, statistically significantly decreased P/A ratio: OCT-1.2, 2.25 (1.1±0.3); BC-0.1, 0.5 (0.2±0.1); OCT+BC-0.5, 2.0 (0.6±0.2) vs. C-8.0, 13.0 (9.9±1.9) (Fig. 6).

Prolactin level: All treatment options resulted in a statistically significant reduction of prolactin serum level: OCT-777.0, 866.7 (729.0±88.2 ng/ml); BC-112.2, 449.5 (190.7±56.0 ng/ml); OCT+BC-58.3, 60.4 (59.5±5.1 ng/ml) vs. C-2380.0, 845.0 (2347.8±115.0 ng/ml) (Fig. 7). The effect of BC was stronger than that of OCT. The joint action of OCT and BC caused a significant decrease of PRL level as compared to OCT alone.

Discussion

The data presented above show that the treatment with either OCT or BC resulted in a significant decrease of the estrogen-induced pituitary tumor weight. Both substances, given alone or jointly, suppressed also effectively the PRL secretion. The inhibitory effect of BC on both PRL secretion and tumor mass of prolactinomas is a very well known phenomenon. Somatostatin

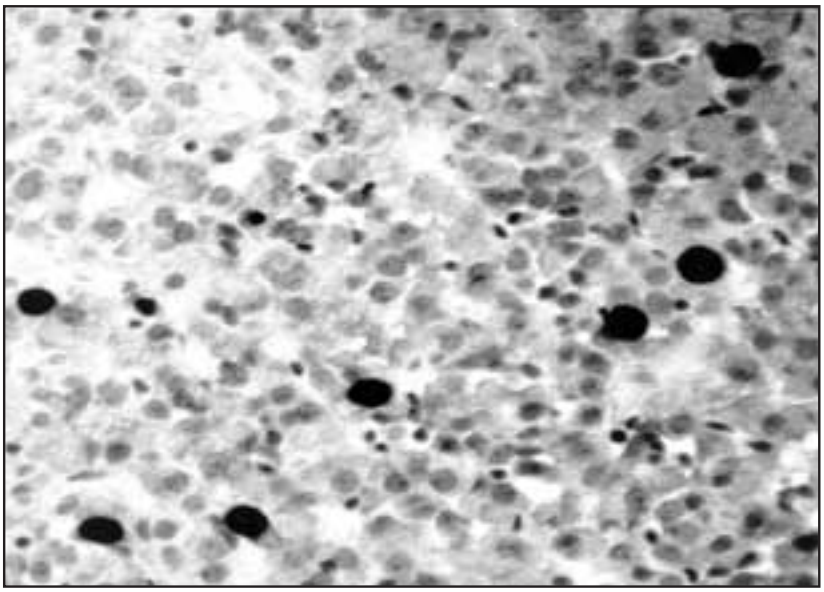

Fig.2. Cell proliferation in the experimental rat prolactinoma (control group) visualized by immunostaining with anti-BrDU monoclonal antibodies.

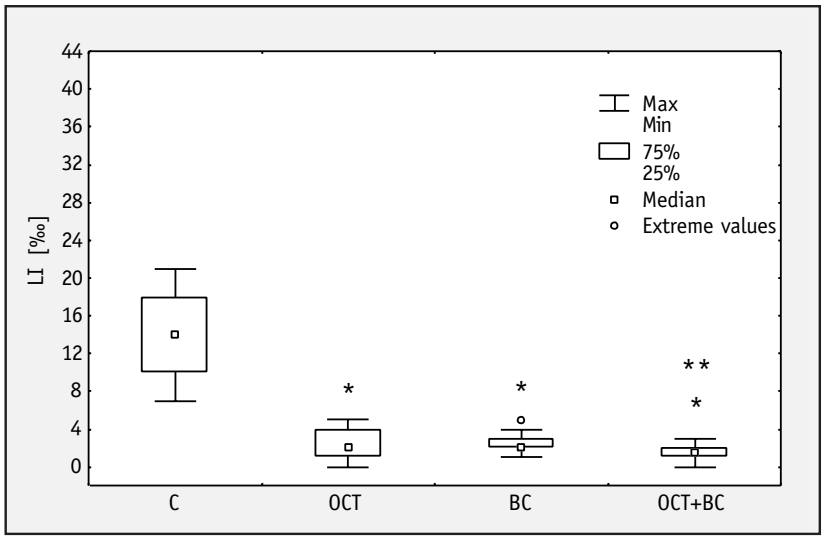

Fig. 3. Effects of OCT and BC, alone or in combination, on BrDU labeling index (LI) in the experimental rat prolactinoma. * p<0.0002 vs. control, ** p<0.05 vs. BC-treated group.

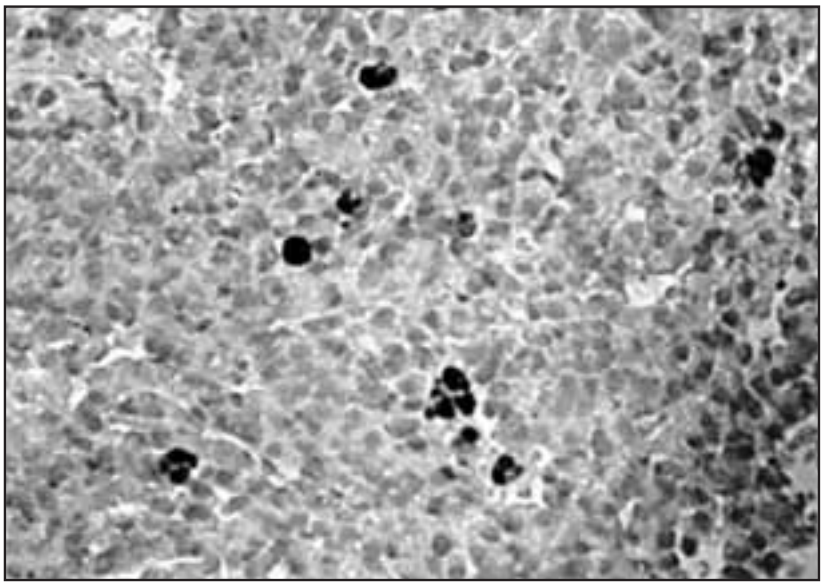

Fig. 4. Apoptosis in the experimental rat prolactinoma treated with bromocriptine visualized by the TUNEL method.

(SST) is not a physiological inhibitor of PRL secretion. However, it was shown that SST exerted the inhibitory effect on PRL secretion in the estrogen-treated pituitary, probably because the up-regulation of sst2 receptors on lactotrophs under the influence of estrogens [23, 24]. In human prolactinomas the suppression of PRL secretion was observed *in vitro* under influence of sst5-receptor selective analog [25]. Both OCT and BC exerted a strong anti-proliferative effect on the investigated tumor and this observation corroborates with the earlier findings from our and other laboratories [10, 11, 13, 26–29]. However, the anti-proliferative action does not explain the rapid regression of tumor mass frequently observed in spontaneous human prolactinomas treated with BC [8]. Another mechanism, which could be taken into consideration, is apoptosis. The investigation of apoptosis using the TUNEL method revealed a sharp increase of the number of apoptotic cell nuclei in BC-treated tumors. This finding corroborates with the data of Drewett and al. [17] and Yonezawa et al. [30] concerning the estrogen-treated rat pituitary, as well as with the observation of Wasko et al. showing the induction of apoptosis in GH3 cell line by BC [18]. In contrast, the treatment with OCT resulted only in a slight, albeit statistically significant, increase of the apoptotic index. It means that both anti-proliferative and pro-apoptotic effects play a significant role in BC-induced tumor regression, whereas in the case of OCT the anti-proliferative effect is prevalent.

The data on SST effects on pituitary apoptosis are scarce and controversial. Saitoh et al. [20] did not observe apoptosis in OCT-treated human somatotropinomas. On the other hand, the apoptotic cells have been revealed by electron microscopy in human somatotropinomas treated before surgery with another SST analog, lanreotide [31]. The relatively low apoptotic index in pituitary tumors treated with SST analogs may result from different reasons. First, the pro-apoptotic effect of SST occurs via sst3 receptors [32]. Octreotide exhibits a lower affinity for sst3 than for sst2 receptors. The higher expression of sst2

receptors in the DES-induced experimental tumor than in normal rat anterior pituitary was found in our laboratory by means of *in situ* hybridization [33]. Although estradiol was shown by other authors [24] to up-regulate not only sst2, but also sst3 receptors in the anterior pituitary, the expression of sst3 receptors in our experimental tumor model remains unknown.

The joint administration of OCT and BC seems to have an additive effect on the regression of tumor weight, inhibition of PRL secretion and inhibition of cell proliferation. These findings support the concept of the combined treatment of pituitary adenomas with both drugs [34]. On the other hand, the joint administration of OCT and BC resulted unexpectedly in the attenuation of the pro-apoptotic effect of the latter. The mechanism by which OCT counteracts the BC-induced apoptosis remains unclear and needs further investigation. Nevertheless, it could be a factor limiting the therapeutic effect of the combined therapy of pituitary adenomas with SST analogs and dopamine agonists.

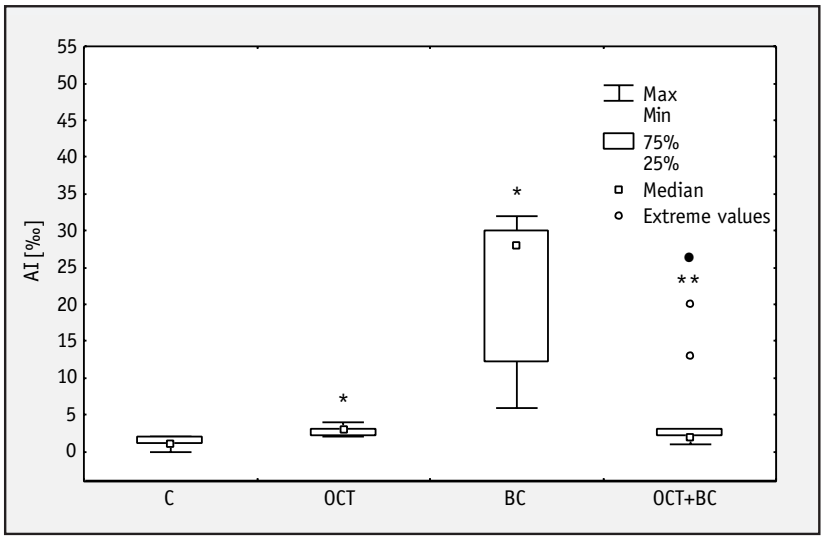

Fig. 5. Effects of OCT and BC, alone or in combination, on apoptotic index (AI) in the experimental rat prolactinoma. * p<0.005, ** p<0.05 vs. control, • p<0.01 vs. BC-treated group.

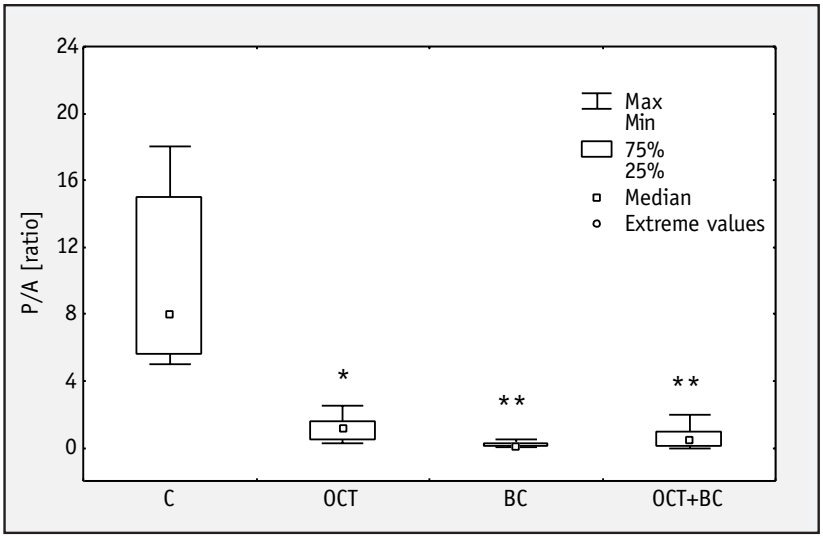

Fig. 6. Effects of OCT and BC, alone or in combination, on proliferation/apoptosis (P/A) ratio in the experimental rat prolactinoma. * p<0.02, ** p<0.002 vs. control.

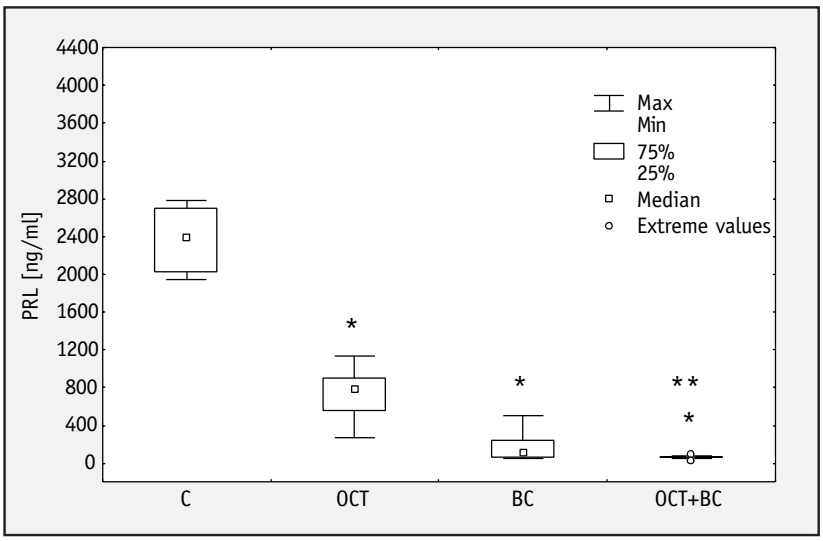

Fig. 7. Effects of OCT and BC, alone or in combination, on prolactin (PRL) serum level. * p<0.0005 vs. control, ** p<0.0005 vs. OCT-treated group.

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