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REPRODUCTIVE MEDICINE

Therapeutic efficiency of Atosiban, an oxytocin receptor blocking agent in the treatment of experimental endometriosis

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Abstract
Purpose The current study investigated the potential therapeutic efficiency of atosiban, an oxytocin receptor antagonist, in an experimental endometriosis model.
Methods Endometriosis was surgically induced in 35 female rats during estrus. Four weeks after this procedure, relaparotomy was performed. The viability and dimensions of the endometriosis foci were recorded. Rats were then randomly divided into three groups. In the first group ($n = 8$), a daily dose of 0.2 ml 0.9 % NaCl was injected intraperitoneally (i.p.) (control cases). In the second and third groups ($n = 8$ and $n = 8$), 0.5 mg/kg/day i.p. atosiban and 1 mg/day i.p. diltiazem were given, respectively. At the end of the treatment, laparotomy was performed, and the dimensions of the endometriosis foci were recorded. The endometrial implants were processed for histological and immunohistochemical studies. The volumes of endometriotic implants were measured, and immunohistochemical analyses were performed, and compared between the groups.
Results After the treatment with atosiban, volumes of endometriotic implants decreased significantly. Proliferating cell nuclear antigen expression levels were significantly reduced in the atosiban and diltiazem groups compared with the control group.
Conclusions In a rat endometriosis model, atosiban, an agent used for the first time for the medical treatment of endometriosis, has shown significant therapeutic efficiency.

Keywords Endometriosis · Receptors · Oxytocin · Atosiban · Therapy

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Introduction

Endometriosis is a chronic disease with an unknown etiology characterized by the presence of the endometrial gland and stroma out of the uterus [1, 2]. It is mainly a disease of women of reproductive age, and its estimated incidence is 10 % [2, 3]. A diverse group of agents (e.g., progestins, gonadotropin-releasing hormone analogs (GnRH-a), combined oral contraceptives, and danazol) is used in the medical treatment of endometriosis; however, none of these agents has a marked effect upon the progress of the disease [4]. Thus, there is a need to explore alternative treatment options, and new treatment modalities (e.g., inhibitors of the arachidonic acid pathway, proteasome-inhibitors (bortezomib), and nuclear factor-kappa B inhibitors (dithiocarbamates)) are being investigated [4–6].

It has been previously shown in animal models that endometrial cells contain oxytocin receptors (OTRs) and that oxytocin (OT) has the capacity to trigger the production of

prostaglandin (PG) E2 and F2α from these cells [7, 8]. Furthermore, Mechsner et al. [9] have reported that OTRs are expressed in smooth muscle cells and in the epithelial cells of the peritoneal endometriotic lesions and ovarian endometriotic cysts of premenopausal women9. OTR activation leads to an increase in intracellular calcium coupled with a stimulating effect on estrogen production via the aromatase pathway [10]. It is well known that PGE2 is one of the most potent stimulators of the expression of cytochrome p 450 aromatase mRNA in endometrial stroma [11]. Thus, a hyperactivated autocrine/paracrine OT/OTR system that already exists within the cells of the endometrial epithelium could be another key factor in the pathogenesis of endometriosis, and the inhibition of OTRs by specific inhibitors might be a useful approach for the treatment of the disease. Based on this background, we aimed to investigate the therapeutic effects of OTR blockage on the endometriotic lesions in an experimental endometriosis model. Since OT leads to a marked increase in intracellular calcium concentrations in the target tissues, diltiazem, a well-known calcium channel blocker, was used as positive control in the present study.

Materials and methods

Animal care

This study was carried out in the Experimental Research Laboratory of the Inonu University, complying with the approval of the ethics committee and the guidelines for the care and use of experimental animals. Thirty-five adult female Wistar rats, each weighing between 300 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 temperature, and a humidity range between 40 and 60 %. The rats had free access to standard dry pellets ad libitum and tap water throughout the study. Before transplantation, all animals were hormonally synchronized in their 4-day estrus phase to exclude differences in steroid synthesis, cell adhesion, and growth, and thus, endometriosis development between the individual animals owing to hormonal variations. Synchronization was performed by administering two subcutaneous injections (55 mg/kg body weight estradiol) with a 24-h intermission, followed by one injection (7.5 mg/kg body weight progesterone) 20 h after the last estradiol injection [12]. Daily vaginal smears of the rats were taken to establish the estrus cycle of each animal. Behavioral 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; then the swab was smeared onto a glass slide and fixed with ethanol. Smears were stained with the usual Papanicolau method and then evaluated by light microscopy by a cytopathologist who was unaware of the groups. The estrus cycle was determined as follows: proestrus period (many centrally nucleolated epithelial cells), estrus period (cornified epithelial cells without nuclei), metestrus period (leukocytes, mucus, and a few cornified cells), and diestrus period (various epithelial cells, mucus, and leukocytes). Rats were observed for at least two successive 4-day estrus cycles.

Surgery and recovery

Endometriosis was induced surgically using the method described by Vernon and Wilson during estrus [13]. Rats were divided randomly into two groups: Group A or the experimental group ($n = 28$) and Group B or the sham group ($n = 7$). All rats were anesthetized intraperitoneally (i.p.) with 20–30 mg/kg ketamine hydrochloride (Rotex, Germany). After perp and drep of the skin, a midline incision was made to enter the abdominal cavity. A $0.5 \times 0.5 \times 0.1$ cm piece excised by micro-scissors from the right uterine horn was attached to the peritoneum only on the right side of the ventral abdominal wall close to an artery via the surgical auto-transplantation technique. For the sham group ($n = 7$), 4-0 nylon sutures, with or without fat tissues, were attached to the peritoneum, except in the auto-transplantation of endometriotic implants.

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 estrus phase to determine the attachment and viability of endometrial implants.

Of the 28 experimental rats, four did not develop any signs of endometriosis, and therefore, these were excluded from the study. In the sham group, the fat tissues showed no growth. The vesicles at the sutures region were observed, and the rats were graded according to average vesicle diameter (D) as follows: Grade 1 (for cases in which the implant disappeared or, if it was visible, never became a cyst, n:7), Grade 2 ($D < 2$ mm, n:8), Grade 3 (2 mm $> D <$ 4.5 mm, n:5), or Grade 4 ($D > 4.5$ mm, n:4). The pretreatment implant volumes in each group were calculated by measuring their dimensions (length, width, and height, in millimeters). For volume calculations, the ellipsoid volume formula (p/6 × length × width × height) was used.

Drug preparation and treatment

The 24 rats with endometriosis were randomized (using random number tables) into three groups: (1) Control group ($n = 8$), (2) Atosiban group ($n = 8$), and (3) Diltiazem group ($n = 8$).

In the first group, a daily dose of 0.2 ml 0.9 % NaCl was injected intraperitoneally (i.p). The rats in Group 2 were then treated i.p. atosiban with a dose of 0.5 mg/kg/day. Diltiazem (5 mg/kg/day) was administered i.p. to rats in Group 3. The drug doses were chosen based on previous biological studies related to the atosiban and diltiazem in experimental rat models [14, 15]. The treatment was continued for 21 consecutive days at approximately the same time each day. A third laparotomy was performed while the rats were fixed in the supine position at the end of the treatment. The volumes and sizes of the implants were measured again with the same method by the same researchers who were blinded to the groups. The distributions of the implant grades after the treatment were as follows: Grade 1, n:9; Grade 2, n:6; Grade 3, n:5; and Grade 4, n:5.

Histological and immunohistochemical analysis of endometriotic implants

The endometrial implants were then excised and processed for histological and immunohistochemical studies. Formalin-fixed specimens were embedded in paraffin, cut into 5-mm-thick sections, and stained with hematoxylin and eosin staining. The sections were also stained for proliferating cell nuclear antigen (PCNA) immunohistochemistry. The histological diagnosis of endometriosis was based on the morphological identification of endometrial glandular tissue (GT) and stromal tissue (ST). All proliferating nuclei in the stromal, glandular, and endometrial epithelial cells were stained with mouse monoclonal antibody against PCNA (Clone PC10; Sigma corp., Missouri, USA). PCNA is a nuclear protein that has its peak expression during the S-phase of the cell cycle, and it has been used previously to identify proliferating cells. Monoclonal anti-PCNA clone PC10 from mouse ascites fluid was diluted to 1/1000 and applied to 5-mm paraffin sections deparaffinized in xylene using the labeled streptavidin–biotin method. For PCNA immunostaining, human tonsil tissue served as the positive control. Negative controls (primary antibody omitted) were routinely performed on adjacent serial sections. Histological slides were evaluated for ST, GT, and PCNA immunoreactivity under light microscopy. A semi-quantitative grading system that was previously described by our research team was used to score the degree of histological change of stromal and glandular epithelial cells [5]. The scalings were conducted to determine 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). The scalings of PCNA immunoreactivity were not separately evaluated for stromal and glandular cells. They were also conducted to determine the degree of GT (0, no secretory glands per high-power field (×40); 1, between 1 and 5 secretory glands; 2, between 6 and 10 secretory glands; and 3, >10 secretory glands). Finally, scalings were conducted to determine the degree of ST (0, no ST; 1, ST/GT ratio less than 25 %; 2, ST/GT ratio 25–50 %; and 3, ST/GT ration >50 %).

Statistical analysis

Statistical analyses were performed using the SPSS for Windows version 15.0 program. Normality for continuous variables in the groups was determined by the Shapiro–Wilk test. The normally distributed variables ($P > 0.05$) were compared by a one-way ANOVA test. The variables with non-normal distributions were compared by the Kruskal–Wallis test. For multiple comparisons of the groups, the Tukey test and the Conover test were used where appropriate. A paired-sample t test was used to compare the pre-treatment and post-treatment implant volumes within each group. A value of $P < 0.05$ was considered statistically significant.

Results

The pre-treatment implant grades were not significantly different between the groups. The distributions of the post-treatment implant grades in each group were as follows: Control group: Grade 1, n:1 (12.5 %); Grade 2, n:3 (37.5 %); Grade 3, n:2 (25 %); Grade 4, n:2 (25 %); Atosiban group: Grade 1, n:6 (75 %); Grade 2, n:1 (12.5 %); Grade 4, n:1 (12.5 %); Diltiazem group: Grade 1, n:2 (25 %); Grade 2, n:2 (25 %); Grade 3, n:2 (25 %); Grade 4, n:2 (25 %). After the treatment with atosiban, we did not find any Grade 3 vesicles, whereas the percentage of Grade 4 vesicles decreased. After the treatment with diltiazem, the percentage of Grade 3 and 4 vesicles increased, whereas the percentage of Grade 2 vesicles decreased. Post-treatment implant grades were similar between the groups (Table 1).

As demonstrated in Table 2, the pre-treatment volumes of endometriotic implants were similar between the groups.

Table 1 Comparison of endometriotic implant grades between the groups

Groups	Pretreatment grade Median (min–max)	P	Posttreatment grade Median (min–max)	P
Control (n:8)	2 (1–4)	0.16	2.5 (1–4)	0.07
Atosiban (n:8)	3 (1–4)		1 (1–4)	
Diltiazem (n:8)	2 (1–4)		2.5 (1–4)	

Kruskal–Wallis H test was used

Table 2 Comparison of endometriotic implant volumes between the groups

Variable	Groups	Mean + SD	*P*
Pretreatment implant volume (mm^3)	Control	64.62 ± 5.60	0.49
	Atosiban	67.12 ± 4.79	
	Diltiazem	68.25 ± 7.61	
Posttreatment implant volume (mm^3)	Control	73.06 ± 5.89	<0.001
	Atosiban	51.31 ± 4.11	
	Diltiazem	62.75 ± 3.65	

One-way ANOVA test was used

Post-treatment implant volumes were significantly lower in the Atosiban group ($P < 0.001$) and Diltiazem group ($P < 0.001$) than in the Control group. In addition, post-treatment volumes of implants in the Atosiban group were significantly lower than those of diltiazem-treated rats ($P < 0.001$). When the pre- and post-treatment volumes of endometriotic implants were compared in the same group, it was found that the post-treatment volumes decreased significantly in the Atosiban ($P < 0.001$) and Diltiazem ($P = 0.02$) groups. Nevertheless, in the Control group, the post-treatment volumes were significantly increased ($P = 0.008$).

The proliferative indices and immunostaining scores of the groups are presented in Table 3. The histological scores for both GT and ST were found to be significantly lower in the Atosiban group than in the Control and Diltiazem groups ($P < 0.001, P = 0.001$, respectively). Glandular and stromal cells of the endometriotic implants demonstrated higher immunohistochemical staining for PCNA in the Control group than those in the Atosiban and Diltiazem groups ($P < 0.001$). The immunohistochemical detection of PCNA revealed the proliferation of many stromal, glandular, and endothelial cells in the implants of control animals. In contrast, endometriotic implants of atosiban- and diltiazem-treated rats developed only a few proliferating cells, which were mainly localized within the endometrial stroma (Fig. 1).

Discussion

The results from the present study have provided a new insight: Atosiban, an OTR antagonist, has therapeutic potential in the treatment of endometriosis, a chronic disease with unclear etiology. Despite being the first peptide hormone to be characterized and synthesized, the effects of OT were considered to be restricted mainly to the periods of labor and puerperium. It was believed that OT is released from hypothalamic nerve terminals of the posterior pituitary into the circulation where it stimulates uterine contractions during parturition and milk ejection during lactation. However, OT is expressed in many other organ systems, including endometrial cells, and it may have the potential to affect the normal cellular functioning of these tissues [9, 16–19].

Medical treatments for endometriosis are usually aimed at reducing endogenous steroid production. Combined oral contraceptives, progestins, danazol, and GnRH-a are all effective in the symptomatic treatment of endometriosis, and they are also effective in causing the short-term regression of endometriotic lesions. However, in addition to their high cost, they carry the risk of osteoporosis when used for long periods of time, they produce several troublesome side effects due to their strong anti-estrogenic effects, and relapse may occur after the cessation of treatment. Thus, alternative medical treatment options are currently needed.

Previously, it was shown that both eutopic endometrium and endometriotic cells are the sites of the synthesis and release of OT, which is stimulated by the rising tide of follicular estradiol [9, 20]. Increases in locally released OT secondary to increased estrogenic activity result in intensified peristaltic stimulation of the circular muscle of the uterus and increased intrauterine pressure in the follicular phases of the cycle [21]. Although the current knowledge on this subject in the relevant literature is limited, hyperperistalsis and increased intrauterine pressure may contribute to the development and progression of endometriosis. Hyperperistalsis constitutes a mechanical trauma resulting in desquamation of fragments of basal endometrium and, in combination with an increased retrograde uterine transport capacity, in the enhanced trans-tubal dissemination of these fragments [22]. Salamanca et al. [23] have investigated the different patterns of sub-endometrial-myometrial contractile waves in the menstrual phase by sonographically assessing patients with and

Table 3 Comparison of the hisopathological scores of groups after the treatment

Groups	Variable					
	Glandular tissue Median (min–max)	*P*	Stromal tissue Median (min–max)	*P*	PCNA expression Median (min–max)	*P*
Control (N:8)	2.0 (1–3)	<0.001	3.0 (2–3)	<0.001	3.0 (3–3)	<0.001
Atosiban (N:8)	1.0 (1–2)		2.0 (1–2)		1.0 (1–2)	
Diltiazem (N:8)	2.0 (1–3)		3.0 (3–3)		2.0 (2–2)	

Kruskal–Wallis H test was used

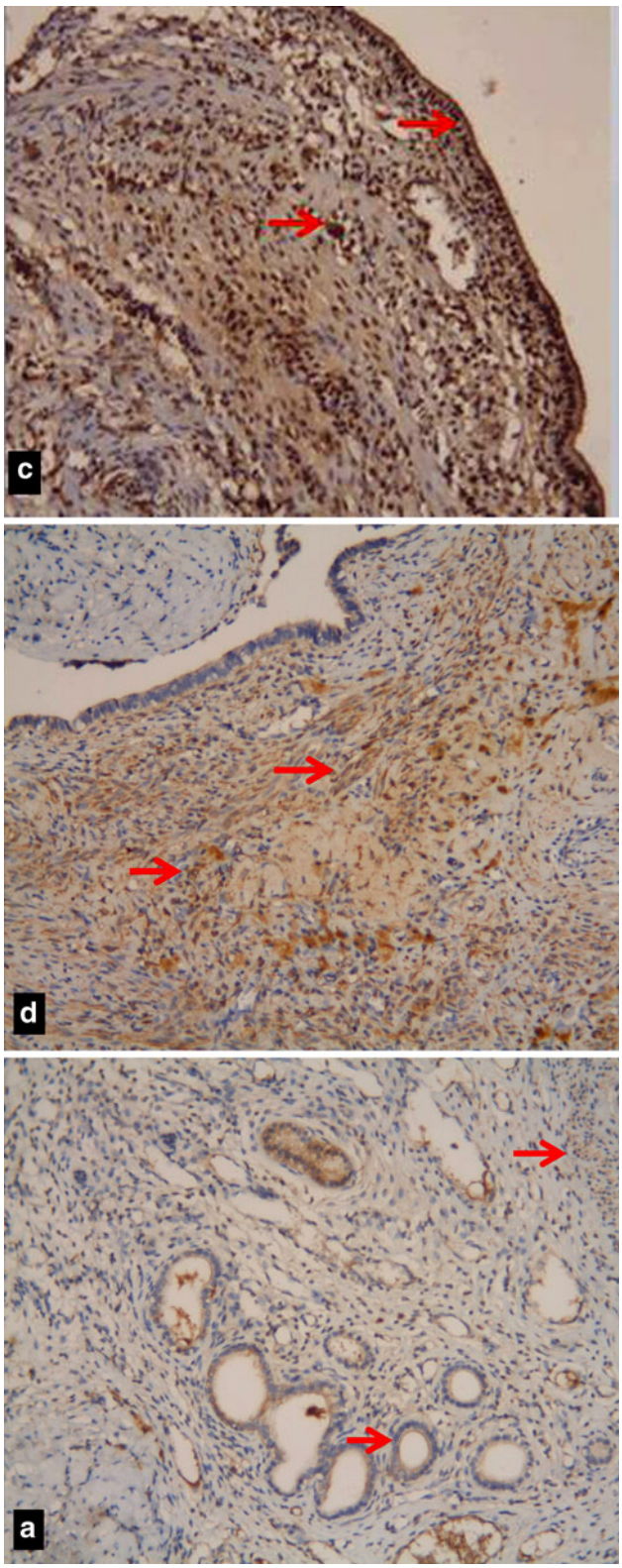

Fig. 1 Representative photographs of the implants obtained from control (*c*), atosiban (*a*), and diltiazem (*d*) groups after treatment. In atosiban-treated rats, PCNA expressions were significantly reduced as compared with control and diltiazem groups. *Arrows* indicate PCNA (×20)-stained stromal and glandular cells

without endometriosis23. They showed that patients with endometriosis have predominant retrograde uterine contraction patterns, whereas healthy subjects have normal antegrade patterns. Therefore, blocking OTRs in order to decrease retrograde menstruation may be an important target point to combat endometriosis. In accordance with this hypothesis, in the present study, post-treatment volumes of endometriotic implants were significantly reduced in the rats treated with atosiban. To the best of our knowledge, this is the first time such an observation has been reported in an experimental endometriosis model.

Another very important point in the pathogenesis of endometriosis is the hyperestrogenic environment in the endometriotic foci secondary to the increased conversion of androgens into estrogen by the aromatase enzyme complex. It is well known that endometriotic implants have markedly high levels of aromatase activity, and both human and animal studies have shown that the inhibition of the aromatase enzyme complex could lead to the complete regression of endometriotic cysts and pain relief in cases with endometriosis [11, 24–26]. Prostaglandin E2, which is produced in very high levels in endometriotic tissues, was found to be the most potent inducer of aromatase activity in endometriosis-derived stromal cells [7, 8]. Asselin et al. [7] found that OT stimulates the production of PGF2α and PGE2 in cultured endometrial epithelial cells7. Similarly, Danet-Desnoyers et al. reported an OT-related increase in the production and release of PGF2α and PGE2 in bovine endometrial cells [8]. Moreover, Mechsner et al. [9] reported high levels of OTR expression in smooth muscle cells and epithelial cells of peritoneal endometriotic lesions and ovarian endometriotic cysts9. All of these reported findings point to another potential beneficial effect of OTR blockage for the treatment of endometriosis: In endometriotic foci, OTR blockage may affect the indirect inhibition of the aromatase enzyme complex and the control of the hyperestrogenic state of the implants. In the present study, we also found significantly decreased GT and ST and significantly lower levels of PCNA expression in the endometriotic implants of rats treated with atosiban compared with control cases.

Another interesting finding in the present study relates to the inhibition of the growth of endometriotic implants using daily injections of diltiazem. Diltiazem, as a member of the benzothiazepine family, is selective blocker for slow calcium channels at the plasma membrane. In the present study, daily intraperitoneal administration of diltiazem at a dosage level of 5 mg/kg caused a significant reduction in endometriotic implant volumes and PCNA expression of glandular and stromal cells.

Calcium ions have a wide variety of biological roles in hormonal secretion and reproduction [27]. It has been

observed that the prompt rise in cytosolic calcium induced by follicle-stimulating hormone (FSH) in rat Sertoli cells indicates the role of calcium in FSH signal transduction [28]. Morad et al. [29] reported that the chronic administration of diltiazem to adult female rats can lead to a significant reduction in the circulating blood levels of sex steroid hormones29. Therefore, the prevention of steroidogenesis by blocking calcium mobilization within the steroid hormone-producing cells may be an important target for the treatment of endometriosis. To the best of our knowledge, the therapeutic potential of calcium channel blockers has not been studied previously in an experimental endometriosis model.

There are several limitations to the present study. This is an experimental study with small numbers of rats per group. This was due to our ethical concern regarding conforming to the 'principle of reduction' in animal experiments; however, much larger numbers would be needed to find smaller histological and immunohistochemical differences between the groups. The second limitation was that we used a subjective scoring system to evaluate the histological changes of the implants. Using an image-analysis program that allows an objective/automated interpretation of histological changes would be more accurate to make a clear conclusion; however, this technique was not available in our setup. Another limitation of the present study was the lack of biochemical evidence regarding the decreased aromatase activity in the Atosiban group due to the blockage of the OT/OTR system. Measurement and comparison of estradiol levels or aromatase activity between the groups would be more valuable when discussing the topic in future.

In conclusion, the discovery of a locally active OT/OTR system in endometriotic implants provides us with another point of view for the treatment of endometriosis. We are introducing a new research trend based on a novel concept. Evidence linking OTR blockage and the successful treatment of endometriosis in our study could provide insights into the new endometriosis treatment modalities, which is an area of ongoing research. The preliminary information obtained in this study suggests that atosiban, as an OTR-blocking agent, can be used as an alternative option in the medical treatment of endometriosis.

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Conflict of interest None.

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