Effectiveness of different immunomodulator agents in the treatment of experimentally induced endometriosis

Deneysel endometriozis tedavisinde farklı immünomodülatör ajanların etkinliği

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Abstract

Objective: : We aimed to investigate the effects of levamisole, interferon alpha-2b and leuprolide on peritoneal implants in experimentally induced peritoneal endometriosis in rats.

Material and Methods: Peritoneal endometriosis was induced in 48 rats by Vernon and Wilson's method. Four weeks after this procedure, peritoneal implants were evaluated histopathologically, and rats were randomized to four groups treated respectively with: a) a single 1 mL intraperitoneal dose of 0.9% saline solution (control group); b) a single 1 mg intramuscular dose of leuprolide acetate; c) interferon alpha-2b, 100.000 U intraperitoneally; d) levamisole 2 mg intraperitoneally for 3 days, then 3 days rest, and continuing in this fashion over 6 weeks. Rats were sacrificed six weeks after the initiation of treatment, and endometriotic implants were evaluated macroscopically and histopathologically. Microscopic evaluations were scored as (+), (++), (+++) corresponding respectively to normal, mild or severe atrophy of the glands and stromas. Results were evaluated with chi-square test by SPSS 10.0 package program. A value of $p < 0.05$ was considered significant.

Results: Comparing treatment groups with the control group, leuprolide ($p < 0.05$) and interferon alpha-2b ($p < 0.05$) were effective, but levamisole was ineffective ($p > 0.05$).

Conclusions: There is substantial evidence that immunologic factors play a role in the pathogenesis of endometriosis, and it could be expected that immunomodulator agents would be useful in its treatment. In this group of agents, interferon alpha-2b was effective, but levamisole was ineffective. Further studies will certainly bring more insight into this disease and will determine the effectiveness of different immunomodulator agents in its treatment.

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Key words: Experimental endometriosis, treatment, levamisole, interferon, leuprolide

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Özet

Amaç: Deneysel peritoneal endometriozis oluşturulan ratlarda, levamizol, interferon alfa-2b ve löprolidin, peritoneal implantlara etkilerini araştırmayı amaçladık.

Gereç ve Yöntemler: Vernon ve Wilson yöntemi ile 48 ratta peritoneal endometriozis oluşturuldu. Bu işlemden dört hafta sonra peritoneal implantlar histopatolojik olarak değerlendirildi ve ratlar dört gruba ayrıldı: a) birinci kontrol grubundakilere tek doz %0.9 salin solüsyonu, 1 ml, intraperitoneal; b) ikinci gruptakilere tek doz löprolid asetat, 1 mg/rat, intramüsküler; c) üçüncü gruptakilere tek doz interferon alfa-2b, 100.000 U, intraperitoneal ve son olarak d) dördüncü gruptakilere üç gün boyunca levamizol, 2 mg/rat, intraperitoneal, üç günlük aralarla altı hafta süreyle verildi. Ratlar altı haftalık ilaç uygulamaları sonunda sakrifiye edildiler. Endometriotik implantlar makroskopik ve histopatolojik olarak değerlendirildi. Mikroskopik değerlendirmeler, implantların bez ve stromalarının atrofilerine göre normal, hafif ve şiddetli atrofiye karşılık gelecek şekilde sırasıyla (+), (+ +) ve (+ + +) olarak skorlandı. Sonuçlar SPSS 10.0 paket programı kullanılarak, χ^2 testi ile değerlendirildi. $p<0.05$ anlamlı olarak kabul edildi.

Bulgular: İlaç uygulanan gruplar kontrol grubu ile karşılaştırıldığında, löprolid ($p<0.05$) ve interferon alfa-2b ($p<0.05$) etkili, levamizol ise etkisizdi ($p>0.05$).

Sonuç: Endometriozisin patogenezinde immünolojik faktörlerin rol oynadığına dair güçlü kanıt vardır. Tedavide immünomodülatör ilaçlardan fayda beklenebilir. Bu grup ilaçlardan interferon alfa-2b etkiliyken, levamizol etkisizdir. İleri çalışmalar bu hastalığı anlamaya yarayacak ve tedavisinde farklı immünomodülatör ajanların etkinliğini belirleyecektir. (J Turkish-German Gynecol Assoc 2009; 10: 84-8)

Anahtar kelimeler: Deneysel endometriozis, tedavi, levamizol, interferon, löprolid

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Introduction

Endometriosis is defined as the presence of a functional endometrial layer, with endometrial glands and stroma, outside the uterine cavity. Although it is common, its etiopathology is not known. Research into its pathogenesis has focused on anatomic, hormonal, immunologic and genetic factors (1-4), while the histogenesis of the disease and the survival mechanism of ectopic endometrial tissue has not been clearly explained.

Endometrial cells that have entered the peritoneal cavity are destroyed by peritoneal cytolysis and cleaning. It has been demonstrated that cytotoxity against endometrial cells is decreased in women with pelvic endometriosis. As a result of se-

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Table 1. Atrophy findings in endometriotic implants according to groups

Treatment Groups	Group 1 (Control) n=12	Group 2 (Leuprolide) n=12	Group 3 (Levamisole) n=12	Group 4 (Interferon α-2b) n=12
Glandular structure	+	+++	+	++
	(10/12)	(9/12)	(9/12)	(8/12)
Stroma	+	+++	+	++
	(10/12)	(9/12)	(9/12)	(8/12)

(+) normal
(++) mild atrophy
(+++) severe atrophy

rial interactions caused by immunogenic substances originating from the ectopic endometrial cells, the non-specific immune response (inflammation) is prolonged and an antigen-stimulated adaptive specific responce is triggered (1-3, 5, 6). Endometrial cells are thus activated to implant and proliferate.

It has been demonstrated that the immune system, especially cell mediated immunity, is activated in endometriosis. Because of the presence of autoantibodies and because it often accompanies other autoimmune diseases, endometriosis is thought to be an autoimmune disease (2, 3, 7, 8).

Estrogen stimulates the development of endometrial implants, and these implants respond to hormonal therapy in the same way as normal endometrial glands. The aim of hormonal therapy is to supress estrogen synthesis. GnRH analogues, such as leuproide, are potent suppressors of this synthesis, and prevent stimulation of implants and suppress the menstrual cycle by means of the hypoestrogenic status they produce (9, 10).

Immunomodulators are agents which modify immune system functions. Cytokines, including interferon, belong to this family. Interferon-alpha 2b (IFN-α2b) not only affects the immune response but also has antitumoral, antiangiogenic and antiproliferative properties (11-13).

Levamisole is a synthetic drug which is a nicotinic acetylcholine receptor agonist. It has been used as an antihelmenthic against nematodes for a long time. Besides this effect, it also has immunostimulant characteristics and is used as an adjuvant in the treatment of colon cancer, chronic hepatitis B, nephrotic syndrome and aphthous stomatitis (14-17).

Because immune factors take part in the etiopathogenesis of endometriosis, immunomodulators may be useful in its treatment. Therefore, we aimed to evaluate the effectiveness of different immunomodulators in the treatment of endometriosis by creating peritoneal endometriosis in rats and comparing the effect of two different immunomodulators (IFN-α2b and levamisole) with GnRH analogue (leuprolide) therapy.

Material and Methods

The experimental procedures were approved by the Committee for Animal Research. All animal studies conformed strictly to the animal experiment guidelines of the Committee for Humane Care. A group of 55 female mature rats, weighing 190-240 grams, was used for the study. Animals were fed ad libitum and housed in pairs in steel cages having a temperature-controlled environment (22±2°C) with a light period between 06.00 to 18.00. The procedures were performed stepwise on the rats as described below.

Initial surgical procedure (institution of surgical endometriosis): Surgical endometriosis was instituted using sterile technique, disregarding the phase of the menstrual cycle. All rats were anesthetized using ketamine hydrochloride (Ketalar® flakon, Eczacıbaşı) administered intraperitoneally at a dose of 100 mg/kg. The rats were positioned supinely on wooden plates designed for the surgical procedure. The abdominal area was prepped with povidone iodine solution (Batticon® solution, Adeka), the peritoneal cavity was accessed through a 4 cm midline incision, and the uterus and ovaries were exposed. A 2 cm segment of the right uterine horn was excised and the redundant portion was sutured using 4/0 polyglactin (Vicryl®, Ethicon). The removed segment was preserved in isotonic saline solution in a petri dish and dissected into 2x2 mm longitudinal pieces. These segments were implanted surgically onto the pelvic peritoneum discontinuously using 4/0 monofilament polypropylene (Prolene®, Ethicon), leaving the endometrium facing into the pelvic cavity. The peritoneum, fascia and muscle layers were closed anatomically using 4/0 Vicryl® and the dermal wound was sutured using 3/0 silk (İpek sütür, Doğsan). Animals were not given any postoperative antimicrobial prophylaxis or estrogen therapy.

Secondary surgical procedure:

At the end of the 4th week following the initial operation, the implants were surgically examined for viability and cystic degeneration through laparotomy incision. Rats (n=48) in which endometriosis had developed on the left-sided endometrial implants were included in the study. These rats were randomly assigned to four groups of 12 animals at the end of the first week of the secondary surgical procedure.

Group 1 (control): At the end of the first week following the secondary surgical intervention, 1 cc of isotonic saline solution was administered intraperitoneally.

Group 2: At the end of the first week following the secondary surgical intervention, 1 mg of leuproide asetate (Lucrin Depot-3M®, Abbott) was administered intramuscularly.

Group 3: At the end of the first week following the secondary surgical intervention, 2 mg of levamisole sodium (Dewo-inject®, Bremer Pharma) was administered intraperitoneally for 3 consecutive days, followed by a 3 day drug-free interval, and continuing in this fashion until the end of 6 weeks.

Group 4: During the secondary surgical procedure, a single dose of 100.000 U IFN-α2b (Intron A®, Schering-Plough) was administered intraperitoneally.

All the rats were sacrificed by cervical dislocation six weeks following the secondary surgical intervention. The implants were examined macroscopically and excised for microscopic evaluation. The excised specimens were fixed in 10% neutral formalin solution, embedded in paraffin, and sections cut with a cryostat at 4 μm thickness. Specimens were then deparaffinized and routinely stained with hematoxylin and eosin. A BH-2 Olympus microscope was used for microscopic evaluation, with both the proportion of stromal component (stromal volume per 10 high power fields <25%, 25-50%, >50%) and glandular activity (number of glands per 10 high power fields 1, 2-3, ≥4) taken into consideration. Non-atrophied glands were characterised by cuboidal or columnar epithelial cells, atrophic glands by flattened epithelial cells. The microscospic evaluation was scored using a semiquantitative scale as (+), (++) and (+++), corresponding to normal cells, mild atrophy and severe atrophy respectively, according to the architecture of the glands and the proportion of stromal component in the implants. Mean weights for the rats before and after treatment did not differ significantly ($p > 0.05$).

Statistical analysis:
Statistical analysis was carried out using SPSS for Windows v.10.0 (SPSS Inc.; Chicago, Ill). All values were expressed as mean ± SD and p values less than 0.05 were considered statistically significant. Group comparisons were made by χ^2 test. This study was approved by the Ethics Committee.

Results

All of the implants were observed as unilocular cystic and vascularised formations at the end of the 4th week after the initial surgical procedure.
Histopathologic examination of the samples taken from these cysts showed endometrial epithelial cells lying on the endometrial stromal layer of the cyst wall. The cystic cavity was lined with a single layer of cuboidal epithelium, and the stroma beneath this epithelium had disappeared in most fields. The cyst was surrounded by a thin layer of smooth muscle and connective tissue.
Group 3 and group 1 rats did not differ significantly in macroscopic and microscopic appearance ($p > 0.05$). Group 4 and group 2 rats both displayed a significantly higher degree of atrophy when compared with group 1 rats ($p < 0.05$). Group 4 rats displayed a significantly higher degree of atrophy when compared with group 3 rats ($p < 0.05$). Group 2 rats displayed a significantly higher degree of atrophy when compared with group 3 and group 4 rats ($p < 0.05$) (Table 1).

Discussion

Because of ethical difficulties in investigating the effectiveness of new drugs to be used in the treatment of endometriosis on humans, there has been interest in developing animal models to enable studies on the etiology and treatment of this disease. Since Vernon and Wilson developed an endometriosis model in rats (18), many investigators have reported that it is possible to create intraabdominal endometriosis in mice, rats, rabbits and monkeys by different techniques (19).
We prefered to use rats as an endometriosis model, since they are more readily available and cheaper, and determined that the method described by Vernon and Wilson can succesfully be put into practice. Other reasons for choosing the rat model were shortening the time for the investigation because of the 70-80 estrus cycles rats undergo annually, and the short duration of the rat menstrual cycle of 4-5 days, consisting predominantly of an estrus cycle with a very short luteal phase. For this reason, we did not need to determine the phase of the menstrual cycle when making the endometrial transplantation to the peritoneum.
It has been noted that, when producing experimental endometriosis models, it is possible to administer additional estrogen, but this application makes no difference in the development of endometriosis when compared with cases that have not been administered estrogen (19-20). In our study, we observed that the transplant can develop without any extra estrogen administration. Although there are many data indicating the deterioration of the peritoneal immune response in endometriosis where a local pelvic inflamatory process is activated, published reports of investigating the use of immunomodulatory agents in the treatment of disease are limited (21-25).
Levamisole is used in the treatment of diseases such as colon cancer, chronic hepatitis B, zona zoster, aphthous stomatitis and chronic brucellosis, because of its immunomodulatory effect (26-30). Another immunomodulator, IFN-α2b, has been found effective in the treatment of endometriosis (31, 32).
In the light of this knowledge, our starting point was that levamisole, being an immunomodulator like IFN-α2b but cheaper and more easily available, might be an alternative choice with similar effectiveness but cheaper. Keenan et al. evaluated the effectiveness of loxoribine and levamisole administered intraperitoneally in the rat endometriosis model and found loxoribine effective but levamisole ineffective. In that study, 2 mg levamisole was administered on three consecutive days with a week interval between courses. We concluded that the ineffectiveness of levamisole could be the result of dosing at infrequent intervals and adopted the standard veterinary practice (34) of using a repetitive three days on three days off schedule. However, the outcome in our study was not different, and this regimen was equally ineffective in producing atrophy of implants. On the other hand, a recent study by Öcal et al (35) found a remarkable effect of levamisole on regression of implants when administered 2 mg weekly. However, they administered levamisole subcutaneously for eight weeks, so this effect of levamisole may only become evident when levamisole reaches supraphysiologic levels. Besides a complex effect of levamisole on the immune system by augmentation of Natural Killer cells and by activating T cells, the suppression effect of levamisole on angiogenesis and tumor development has been demonstrated in vivo and in vitro (35). The variety of findings indicate that establishment of the optimal effective dose necessary to produce the intended immunostimulatory effect for the drug is required. It may also be necessary to use primates to produce results relevant to humans.
Interferon, which has been shown to be beneficial as an immunomodulator in the treatment of urinary bladder tumors, chronic hepatitis B, and chronic HCV and HPV infections, has also been tried in the treatment of endometriosis (38, 39). It was found that IFN-α2b suppressed the development of endometrial cells and DNA synthesis in these cells (30), caused shrinkage of endometrial implants (39), and completely cured some early-stage cases, regressing signs and symptoms (31). It

has also been reported that the use of this agent with retinoic acid caused apoptosis and inhibition of cell growth in a series of human breast cancer cells and the inhibition of cell growth in patients with cervical cancer (32).

In our study, although the moderate atrophic effect of IFN-α2b treatment on implants was greater than that observed in the control group ($p > 0.05$), we found no increase compared with leuprolide. We found leuprolide the most effective drug, with a strong atrophic effect on glands and stroma structures. Leuprolide, as a GnRH agonist, produces its effects both by receptor downregulation in the pituitary and by suppressing the ovaries. The demonstration of GnRH receptors in an endometrial cell series shows that leuprolide can also be directly effective on endometriotic focuses (40).

GnRH analogues are being used effectively in the treatment of endometriosis and there is a consensus in the current literature that these agents are effective (41-43). However, in addition to having high costs, they carry the risk of osteoporosis when used for long periods of time, produce several troublesome side effects because of their strong antiestrogenic effects, and relapse may occur after the cessation of treatment.

Although the evidence is not sufficient to determine the cause of endometriosis, present findings point to the involvement of an immune system abnormality. When everything is taken into consideration, there are grounds for hope that immunomodulator drugs will prove useful in the treatment of peritoneal endometriosis, whether as drugs of choice for primary treatment or as adjuvant treatment with drugs of proven clinical effectiveness. Further investigations are needed to demonstrate the effectiveness of various agents for this purpose.

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