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ARTICLE

Atorvastatin causes regression of endometriotic implants in a rat model

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Abstract Endometriotic implants were induced surgically in female Wistar albino rats, which were randomly divided into three groups. The rats in group I ($n = 10$) and group II ($n = 9$) were given 2.5 mg/kg/day intraperitoneal and oral atorvastatin, respectively, for 28 days. Group III ($n = 9$) was given no medication (control). The mean volume and weight of explants in group I were significantly lower (both $P < 0.05$) compared with group III. Histopathological score of the implants was significantly lower in groups I and II, when compared with group III ($P < 0.01$ and $P < 0.05$, respectively). There were significant reductions in explant concentrations of vascular endothelial growth factor and matrix metalloproteinase 9 in group I ($P < 0.01$ and $P < 0.001$, respectively) and group II (both $P < 0.01$) compared with group III while staining due to tissue inhibitor of metalloproteinase 2 was significantly higher in group I ($P < 0.01$) and group II ($P < 0.01$) compared with group III. Moreover, explant concentration of superoxide dismutase was significantly increased in groups I and II compared with group III (both $P < 0.05$). In conclusion, atorvastatin causes significant regression of endometriotic implants in rats. Moreover, intraperitoneal atorvastatin seems to be more effective than oral atorvastatin.

KEYWORDS: atorvastatin, rat endometriosis model, superoxide dismutase, VEGF, MMP-9, TIMP-2

Introduction

Endometriosis is defined as the presence and proliferation of endometrial glands and stroma outside the uterine cavity. One widely accepted mechanism for the development of peritoneal endometriotic lesions is the adhesion and growth of endometrial fragments deposited into the peritoneal cavity via retrograde menstruation (Sampson, 1927). Retrograde menstruation is likely to carry highly pro-oxidant factors, such as haem and iron, into the peritoneal cavity,

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as well as apoptotic endometrial cells, which are well-known inducers of oxidative stress.

Oxidative stress has been proposed as a potential factor involved in the pathophysiology of the endometriosis (Van Langendonckt et al., 2002). Reactive oxygen species (ROS) are intermediaries produced by normal oxygen metabolism. It has been suggested that ROS or free radicals may increase growth and adhesion of endometrial cells in the peritoneal cavity, promoting endometriosis and infertility (Murphy et al., 1998). ROS exert their cytotoxic effect by causing peroxidation of membrane phospholipids, which results in increased cell membrane permeability, loss of membrane integrity, enzyme activation, structural damage to DNA, and, in effect, cell death (Halliwell, 1994).

To protect themselves from the deleterious effects of ROS, cells have developed a wide range of antioxidant systems to limit production of ROS, inactivate them and repair cell damage (Van Langendonckt et al., 2002). One of them is superoxide dismutase (SOD), an enzyme, which out-competes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. In addition, ROS degrade polyunsaturated lipids, forming malondialdehyde (MDA), which is a reactive aldehyde and one of the many reactive electrophile species that cause toxic stress in cells and form advanced glycation end products (Farmer and Davoine, 2007). This aldehyde is used as a biomarker to measure the level of oxidative stress in an organism (Del Rio et al., 2005).

Angiogenesis and matrix metalloproteinase (MMP) secretion may be involved in the mechanisms proposed for endometriosis, i.e. retrograde menstruation, transplantation, attachment, survival and proliferation of exfoliated endometrium (Healy et al., 1998; Sillem et al., 1998; Taylor et al., 1997). Previous studies have shown that many MMP were more highly expressed in ectopic endometrium than in eutopic endometrium in women with endometriosis (Shan et al., 2006). It has been shown that blocking MMP activity inhibits the formation of ectopic lesions in experimental models (Bruner et al., 1997, 1999). The presence of vascular endothelial growth factor (VEGF) has been demonstrated in human endometrium and it may be important in both physiological and pathological angiogenesis (Charnock-Jones et al., 1993; Smith, 1996). Moreover, antiangiogenic agents inhibited the growth of explants in an in-vivo model of endometriosis by disrupting the vascular supply (Hull et al., 2003).

Atorvastatin, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, has been widely used clinically in patients with atherosclerotic disease and hyperlipidaemia. However, various experimental studies have shown statins to also have antioxidant action (Kishi et al., 2008; Rosenson, 2004; Wassmann et al., 2002) and favourable effects on VEGF (Alber et al., 2002; Kodama et al., 2006; Zhang et al., 2007) and MMP (Chandrasekar et al., 2006; Cheng et al., 2007; Tziakas et al., 2004), all of which may play a role in the regression of endometrial tissue. Nonetheless, there is only one study in the literature which was designed to determine the effects of atorvastatin on experimentally induced endometriosis in a rat model (Oktem et al., 2007). Furthermore, there is no study in the literature investigating the effects of atorvastatin treatment on endometriotic tissue concentrations of SOD, MDA, VEGF, tissue inhibitor of metalloproteinase 2 (TIMP-2) and MMP-9 in rats.

The aim of the present study was to evaluate the effect of atorvastatin on regression of endometriotic explants and on tissue concentrations of SOD, MDA, VEGF, TIMP-2 and MMP-9 in a rat model.

Materials and methods

Animals

Thirty-two mature non-pregnant female Wistar albino rats weighing 190—230 g were used as a model for experimental induction of endometriosis. They were caged in a controlled environment of 22°C with 12-h light/dark cycles. Food and water were provided *ad libitum*. The animals were allowed to acclimatize to these conditions for at least 1 week. The study was approved by the institutional review board of Ankara Educating and Research Hospital and carried out at Animal Research Centre of Ankara Education and Research Hospital, Ankara, Turkey. The guidelines for care and use of animals approved by the institutional review board were followed.

Surgical procedures

First surgery

Endometriosis was surgically induced in 32 rats by transplanting an autologous fragment of uterine tissue onto the inner surface of the abdominal wall as described by Vernon and Wilson (1985) with minor modifications by Lebovic et al. (2004). Briefly, each rat was anaesthetized with an intramuscular injection of ketamine hydrochloride (40 mg/kg i.m. alfamin 10% (100 mg/ml); Woerden, The Netherlands) and xylazine hydrochloride (2 mg/kg i.m. alfazyne 2% (20 mg/ml); Woerden). Before surgery, the abdominal skin was shaved and antisepsis was obtained by 10% povidone iodine solution. Using sterile techniques, a 3-cm vertical midline incision was made and both uterine horns were exposed. A distal segment, 1 cm in length, was resected from the right uterine horn. The segment was placed in phosphate-buffered saline (PBS) at 37°C and split longitudinally, and a 5×5 mm piece was sectioned. This piece of uterine tissue was transplanted without removing the myometrium onto the inner surface of the right abdominal wall with the serosal surface apposed and secured with nonadsorbable 4—0 polypropylene sutures at two edges (**Figure 1A**). Before closure of the abdominal wall, 2 ml of saline was administered into the abdominal cavity to prevent drying and minimize adhesion formation. The midline incision was closed in two layers with the use of a simple interrupted 2—0 polyglactin 910 suture for the peritoneum fascia and for the skin. All of the operations were performed by the first three authors. After the first surgical operation, all rats were observed for 4 weeks in their individual cages without any medication. Body weight of the rats was monitored regularly.

Second surgery

Two rats died during the 4 weeks after the first surgery. Two of the remaining 30 rats underwent a second exploratory laparotomy to detect viability of endometrial implants as in the previous two studies (Lebovic et al., 2004; Uygur

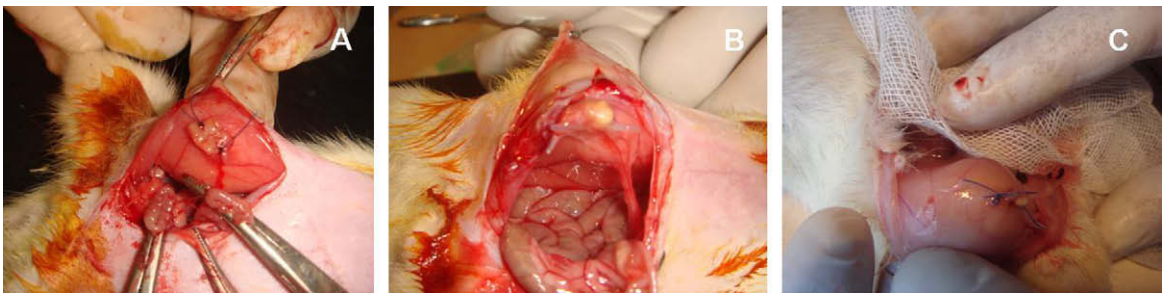

Figure 1 (**A**) Transplanted uterine tissue onto the inner surface of the right abdominal wall with the serosal surface apposed and secured with nonadsorbable polypropylene sutures at two edges. (**B** and **C**) Sample views of an endometriotic implant 4 weeks after the start of the treatment for control (**B**) and atorvastatin-treated rats (**C**).

et al., 2006). Ectopic uterine tissues were identified and measured in three dimensions (length × width × height in millimetres) using a caliper by the fourth author. The spherical volume of each ectopic uterine tissue was calculated using the prolate ellipsoid formula: V (mm^3) = 0.52 × A × B × C, where A, B and C denote width, length and height, respectively. Tissues were photographed using a digital camera and measurements were recorded. Then, the remaining 28 rats were randomly allocated to three groups blinded to the surgeons using a computer-generated randomization (Random Numbers Generator Pro; Segobit Software trial version). The rats in group I (intraperitoneal atorvastatin group, 10 rats) were given 2.5 mg/kg/day intraperitoneal atorvastatin (Lipitor tablet; Pfizer, Istanbul, Turkey) as in the previous study (Aarons et al., 2007) for 4 weeks. The rats in group II (per oral atorvastatin, 9 rats] were given 2.5 mg/kg/day per oral atorvastatin via an orogastric tube. The rats in group III (control, 9 rats) were given no medication. The medications were given by the laboratory personnel. All the rats were observed for 4 weeks. None of the rats was treated with oestrogen.

Third surgery

Four weeks after the beginning of the treatments (24 h after the last dose of atorvastatin), a third laparotomy was performed and all rats were killed by ketamine anaesthesia. The sizes of the implants were measured again with the same caliper method by the fourth investigator who was blinded to the groups. The endometrial explants were quickly excised by the first three authors and weighed (in milligrams) by the laboratory technician who was blinded to the groups. The endometrial explants were divided equally into two longitudinal sections. One half was placed in formaldehyde solution for routine histopathological examination by light microscopy. The other half of the endometrial explant was washed with physiological saline for biochemical analyses of MDA and SOD. The biochemist and pathologist assessing the specimens were also blinded to the treatment groups.

Biochemical analysis

SOD activity determination

Uterus-derived SOD activity (expressed as U/mg protein) was determined using the commercial kit BIOXYTECH SOD-525 (OxisResearch, Portland, OR, USA). The SOD assay is based upon change in absorbance (525 nm) that results from an increased rate of SOD-mediated auto-oxidation of 5,6,6a,11b-trihydroxybenzo[c]flourene under alkaline conditions. Both types of samples were pre-treated (1 min) with 1,4,6-trimethyl-2-vinylpyridinium to remove potentially interfering mercaptans. The ratio of autoxidation of 5,6,6a,11b-trihydroxybenzo[c]flourene in the presence (sample; V_S) and absence (water blank; V_C) of SOD was used to determine SOD activity (U). The total protein content was measured with Bradford's method (1976). Inter-assay variation was avoided by analysing all samples from an individual in the same run and intra-assay coefficient of variation was 7.32%.

Malondialdehyde determination

Samples were subsequently homogenized in buffer and assayed for MDA content using the thiobarbituric acid reaction, as described by Uchiyama and Mihara (1978). Briefly, 0.5 ml of homogenate (10% concentration) was mixed with 3 ml of 1% H_3PO_4. After addition of 1 ml of 0.67% thiobarbituric acid reagent, the tubes were heated in boiling water for 45 min. The colour was formed with 4 ml of *n*-butanol and centrifuged. The colour intensity of the butanol layer was estimated by spectrophotometric absorbance at 532 nm and centrifuged at 3000 × g for 15 minutes. MDA content was then expressed as nanomoles per gram of tissue (nmol/g). Inter-assay variation was avoided by analysing all samples from an individual in the same run and coefficient of intra-assay variation was 3.8%.

Histopathological examination

Evaluation of persisting epithelium in endometrial autografts

The formalin-fixed endometriotic foci were embedded in paraffin blocks, sectioned at about a 4-μm thickness, stained with haematoxylin and eosin, and examined under a light microscope. The pathological evaluation of the uterine autografts was performed according to a previously published method (Keenan et al., 1999) as follows: a well-preserved epithelial layer scored 3, a moderately preserved epithelium with leukocyte infiltrate scored 2, a poorly preserved epithelium (occasional epithelial cells only) scored 1 and no epithelium scored 0.

Immunohistochemical examination

Formalin fixed paraffin embedded tissue sections, 5 micron thick, were incubated for one night at 37°C and for 1 h at 60°C for deparaffinization. Xylol applications were performed twice (15 min). The slides were then laid in 96% absolute alcohol and 80% ethanol for 10 min, followed by distilled water, twice for 5 min. They were boiled in a high temperature microwave oven in 10% citrate buffer for VEGF immunperoxidase. After 20 min at room temperature, the tissue was encircled with a hydrophobic pen (Super PAP PEN IM3580, Immunotech, a Beckman Coulter Company, 13009, Marseille, France). After washing with distilled water and phosphate-buffered saline (PBS), hydrogen peroxide (3%) was added dropwise. After washing with PBS, ultra V block (TA-125-UB; Thermo Fisher Scientific, Fremont, CA, 94539, USA) was applied. After a 1-h application of primary antibody including VEGF (rabbit polyclonal antibody, RB-222-PO; Labvision/NeoMarkers Corporation, Fremont, CA, USA), TIMP-2 (MS-1485-P; Thermo Fisher Scientific) and MMP-9 (RB-9234-P; Thermo Fisher Scientific), the samples were washed with phosphate-buffered saline (PBS) and a post-PBS concentration was applied (biotinylated goat anti-polyvalent and streptavidin peroxidase, respectively). After rewashing with PBS, the specimens were placed in 3-amino,9-ethylcarbazole chromogen for 10 min (TA-125-HA; Thermo Fisher Scientific). Finally the counterstain with Mayer's haematoxylin was performed for 5 min. Dilutions of the primary antibody were 1:100, 1:200 and 1:500 for VEGF, TIMP-2 and MMP-9, respectively. The amount of antibody added to each sample was 100 l and large volume UltrAb diluent was used for dilution (TA-125-UD; Thermo Fisher Scientific). Biotinylated goat anti-polyvalent was used as second antibody (TP-125-BN; Thermo Fisher Scientific). All slides were evaluated with Leica DMI 4000 B light microscope (Leica, Wetzlar, Germany).

To prevent inter-individual bias, all tissues were evaluated by the same histologist (NL), who was blinded to the origin of the samples. The relative intensity of immunoreactivity staining was assessed quantitatively as previously described by McCarty et al. (1985), taking into account both the intensity and the distribution of a specific staining. A value of HSCORE (histological score) was derived from the sum of the percentages of positively stained epithelial cells multiplied by the weighted intensity of staining. HSCORE = Pi(I + 1), where I represents staining intensity (0 = no expression, 1 = mild, 2 = moderate and 3 = intense) and Pi is the percentage of stained cells for each intensity (Budwit-Novotny et al., 1986).

Statistical analysis

All values are given as median, min—max and mean ± SD. Normality was tested by Kolmogorov—Smirnov test. The Kruskal—Wallis test was used for comparison of the variables between the groups. The Mann—Whitney *U*-test with Bonferroni correction was used for multiple comparisons if the difference was significant at $P < 0.0167$ (0.05/3 multiple comparisons = 0.0167). A P value <0.05 was considered as statistical significance in other analyses. Statistica 7.0 statistical software (StatSoft Inc. Tulsa, OK, USA) was used for statistical analysis.

Results

Two rats died after the first surgery, possibly as a result of complications related to surgery. The standardized surgical procedures and the administration of the protocols were well tolerated by the remaining animals. All laparotomy sites were intact and none of the animals had an incisional hernia.

Four weeks from the time of uterine transplantation, two rats were killed to examine the endometriotic explants. In these animals, the explant appeared as well vascularized, unilocular cystic structures containing clear, serous fluid. Average volume and weight of the explants were 171 mm^3 and 204 mg, respectively. Histologically, the epithelia were well preserved, with a score of 3 for both samples.

After the third surgery, it was found that ectopic implants developed in all rats. There were adhesions between the implants and other abdominal viscera, particularly in group III (**Figure 1B**), whereas the majority of the atorvastatin-treated rats had degenerated, pellet appearance (**Figure 1C**). The median (min—max range) volume of implants in group I [12 (3—203)] was significantly smaller ($P < 0.05$) than those in group III [182 (15—1609)]. Similarly, these two groups were significantly different ($P < 0.05$) with respect to weight [48 (22—579) for group I and 212 (56—1446) for group III]. Although, median volume and weight of the implants in group I were smaller than group II [70 (7—450) and 256 (41—457), respectively], the difference was not statistically significant. Moreover, groups II and III did not differ significantly with respect to volume and weight of the explants (**Table 1**).

Neither control nor atorvastatin-treatment groups had any observable adverse effect regarding organ-specific changes such as follicular atresia in the ovaries (**Figure 2A** and **B**) and none of the rat had tissue necrosis in eutopic uterine tissue in any group (**Figure 2C** and **D**) as verified by histological comparison. Nevertheless, cystic implants of group III (**Figure 2E**) were similar to the eutopic endometrium because they both contained endometrial epithelium and stromal cells. The inner lining of the cysts contained simple columnar epithelium. The uterine autograft from atorvastatin-treated rats showed marked epithelial changes and regression of the explant (**Figure 2F**). Moreover, the median score (min—max ranges) of the histopathological examination of the implants at the end of the treatment was significantly lower in groups I and II when compared with group III [1.0 (0—2) versus 3.0 (2—3), $P < 0.01$ and 2.0 (0—2) versus 3.0 (2—3), $P < 0.05$, respectively; see **Table 1**].

As demonstrated in **Table 1**, there was a significant reduction in VEGF and MMP-9 immunoreactivity in group I [1 (0—2) and 0.5 (0—1), respectively; $P < 0.01$ and $P < 0.001$, respectively] and group II [1 (0—2) and 1 (0—2), respectively; both $P < 0.01$] when compared with group III [2 (1—3) and 2 (1—3), respectively]. However, TIMP-2 immunoreactivity was significantly more intense in group I [2 (1—3); $P < 0.01$] and group II [2 (1—3); $P < 0.01$] than in group III

Table 1 Comparison of post-treatment measurements in the atorvastatin-treated and control groups.

	Intraperitoneal atorvastatin (group I) (n = 10)	*Peroral atorvastatin (group II) (n = 9)*	*No treatment (group III) (n = 9)*	*P-value*
Volume (mm^3)	12 (3–203) [46 ± 72][a]	70 (7–450) [128 ± 158]	182 (15–1609) [398 ± 506]	0.013
Weight (mg)	48 (22–579) [117 ± 174][a]	256 (41–457) [211 ± 157]	212 (56–1446) [456 ± 479]	0.026
Histological score	1.0 (0–2) [1.1 ± 0.7][b]	2.0 (0–2) [1.4 ± 0.7][a]	3.0 (2–3) [2.6 ± 0.5]	0.001
VEGF score	1 (0–2) [0.70 ± 0.67][b]	1 (0–2) [0.89 ± 0.78][b]	2 (1–3) [2.11 ± 0.60]	0.002
TIMP-2 score	2 (1–3) [2.30 ± 0.67][b]	2 (1–3) [1.78 ± 0.67][b]	1 (0–2) [1.00 ± 0.50]	0.002
MMP-9 score	0.5 (0–1) [0.50 ± 0.53][c]	1 (0–2) [0.78 ± 0.83][b]	2 (1–3) [2.33 ± 0.71]	<0.001
MDA (nmol/g)	103 (81–181) [112 ± 31]	108 (79–152) [114 ± 27]	121 (65–167) [117 ± 39]	NS
SOD (U/mg protein)	6.6 (5.3–13.8) [7.6 ± 3.0][a]	8.2 (4.6–10.0) [7.9 ± 2.0][a]	4.6 (1.6–7.0) [4.4 ± 2.0]	0.014

Values are presented as median (min–max) [mean ± SD]; MDA = malondialdehyde; MMP = matrix metalloproteinase; NS = not statistically significant; SOD = superoxide dismutase; TIMP = tissue inhibitor of metalloproteinase; VEGF = vascular endothelial growth factor. *P*-values were calculated with Kruskal–Wallis test.
[a] $P < 0.05$ compared with group III.
[b] $P < 0.01$ compared with group III.
[c] $P < 0.001$ compared with group III.

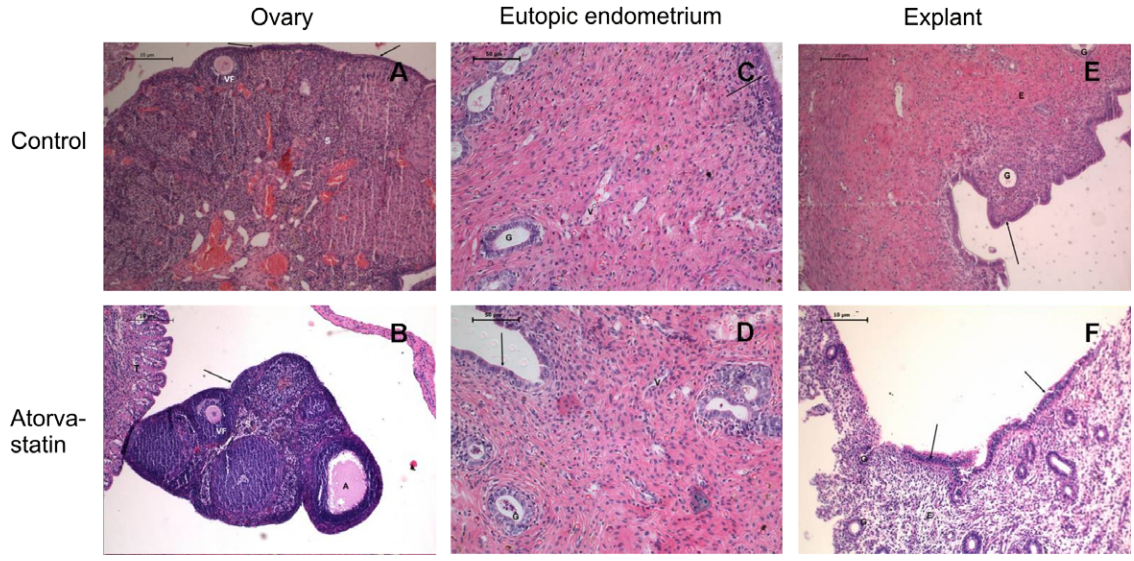

Figure 2 Histology of ovary, eutopic uterus and ectopic uterine tissue with haematoxylin and eosin staining. (**A**,**C**, and **E**) group III, control; (**B**,**D**, and **F**) group I, intraperitoneal atorvastatin: (**A** and **B**) germinal epithelium (arrow), stroma (S) and vesicular follicle (VF); (**C** and **D**) normal-appearing endometrium with associated luminal epithelium (arrow) and glandular structure (G); (**E**) endometriotic implant cyst lumen with epithelial cell lining (arrow) and endometrial gland (G); and (**F**) destruction of columnar epithelium (arrow) and endometrial oedema, glandular structure (G) and disorganization of the explant. Bars = 10 μm.

[1 (0–2)]. Figure 3 shows staining of the specimens for VEGF, TIMP-2 and MMP-9.

The activity of SOD and concentration of MDA were measured in endometrial explants obtained during the third surgery. The median endometrial explants' MDA concentrations did not show statistically significant difference among group I [103 (81–181)], group II [108 (79–152)] and group III [121 (65–167)]. However, the activity of SOD in group I [6.6 (5.3–13.8)] and group II [8.2 (4.6–10.0)] was significantly higher (both $P < 0.05$) than the mean activity found in rats in group III [4.6 (1.6–7.0); see Table 1].

Discussion

The present study was carried out to test the effect of atorvastatin on the growth of ectopic uterine tissue in the rat model of surgically induced endometriosis. In contrast to rats in

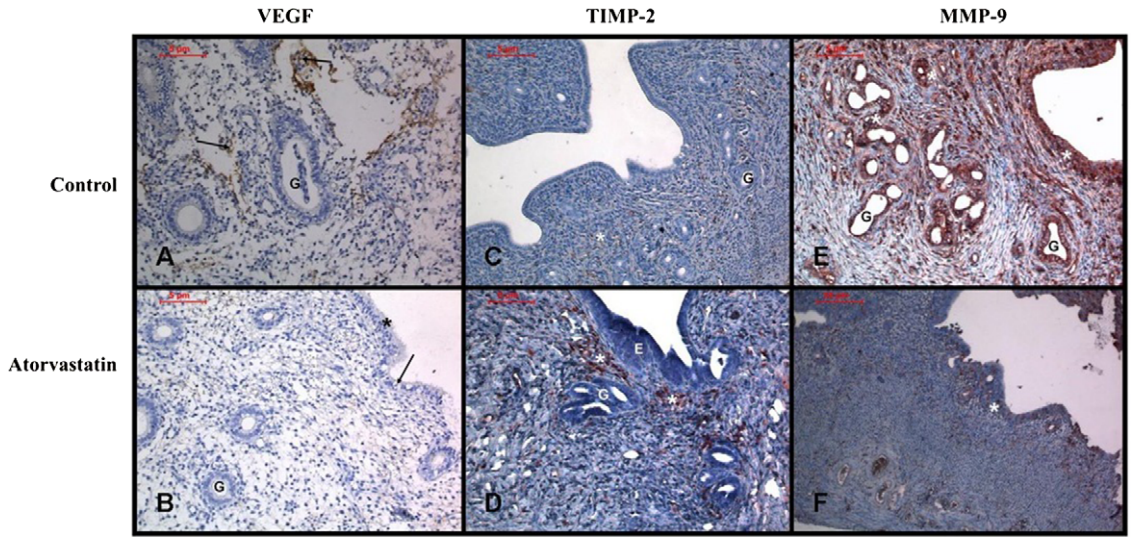

Figure 3 Vascularendothelial growth factor (VEGF), tissue inhibitor of metalloproteinase 2 (TIMP-2) and matrix metalloproteinase 9 (MMP-9) immunoreactivity of ectopic uterine implant. (**A**,**C**, and **E**) group III, control; (**B**,**D**, and **F**) group I, intraperitoneal atorvastatin: (**A**) increased VEGF immunoreactivity (arrow) around uterine gland; (**B**) decreased VEGF immunoreactivity (asterisk) and uterine gland (G); (C) decreased TIMP-2 immunoreactivity (asterisk); (**D**) increased TIMP-2 immunoreactivity (asterisk), epithelial layer (**E**), uterine gland (G); (**E**) increased MMP-9 immunoreactivity (asterisk); and (**F**) decreased MMP-9 immunoreactivity (asterisk) and uterine gland (G). Bars = 5 μm.

group III, atorvastatin treatment for 4 weeks was found to be effective in inducing regression of the endometriotic implants by inhibiting the decrease of antioxidant enzyme (SOD) concentration, decreasing VEGF and MMP-9 concentrations and increasing TIMP-2 concentration in endometriotic implants of the rats. Moreover, intraperitoneal administration of the atorvastatin seems to be more effective than oral administration for regression of endometrial explants.

Endometriosis is an oestrogen-dependent disease. GnRH agonists, progestins, androgenic agents or oral contraceptives, either in a continuous or cyclic fashion, are used in its medical treatment with the principal aim of down-regulating ovarian oestrogen production. Unfortunately, these treatments are often associated with side effects and high recurrence rates (Mihalyi et al., 2006; Valle and Sciarra, 2003). Thus, there is a definite need to develop new drugs to provide specific and more efficient therapeutic alternatives that eliminate endometriotic lesions, prevent recurrences and do not interfere with the fertility potential.

Atorvastatin, a HMG-CoA reductase inhibitor, has been largely used in patients with atherosclerotic disease and hyperlipidaemia (Vaughan et al., 2000). The drug has pleiotropic benefits in addition to cholesterol-lowering effects including antioxidant action (Kishi et al., 2008; Rosenson, 2004; Wassmann et al., 2002), modulation of angiogenesis (Alber et al., 2002; Kodama et al., 2006; Zhang et al., 2007) and MMP (Chandrasekar et al., 2006; Cheng et al., 2007; Tziakas et al., 2004). Moreover, atorvastatin has recently been shown to cause significant reduction in adhesion formation in rats (Aarons et al., 2007; Yilmaz et al., 2009). Despite these facts, as far as is known, just one in-vivo trial in the literature investigated the effect of atorvastatin on the rat models of endometriosis (Oktem et al., 2007), where authors reported that high-dose atorvastatin significantly reduced the size of experimentally induced endometriotic implants and VEGF concentrations in peritoneal fluids of rats.

As the 2.5 mg/kg/day oral atorvastatin has been shown to cause significant regression of explant tissue in the rat endometriosis model (Oktem et al., 2007), the same dose but two different routes of atorvastatin have been compared in the present study. This study noted that intraperitoneal atorvastatin caused a significant regression of endometriotic implants with respect to group III ($P < 0.05$). On the contrary, oral atorvastatin decreased the size of the endometriotic implants when compared with group III but the difference was not statistically significant. Likewise, in a previous study by Aarons et al. (2007), adhesions in rats administered intraperitoneal atorvastatin (30 mg/kg) were decreased significantly, whereas; there was no significant reduction in adhesion formation in rats which were given an oral dose of atorvastatin (30 mg/kg) when compared with vehicle controls. This may be explained by the pharmacokinetic mechanism of the statins which are, at least partially, metabolized by the liver. If given orally, the first pass effect possibly causes the statins to reach the target tissue in a concentration too low to have any effect. However, when histological findings were compared with group III, the regimens of both groups I and II were found to be significantly effective in causing degeneration on explant tissue ($P < 0.01$ and $P < 0.05$, respectively). Therefore, further studies with sufficient sample sizes may show significant effects of the oral route for regression of endometriosis. Power calculations indicate that a minimum of eight rats in each group would detect a maximum 3.5 U/mg protein difference (SD = 2.0 U/mg protein) in SOD concentrations among the groups ($\alpha = 0.05$, power = 0.8).

Effect of statins on endometrial cells has also been evaluated by three recent studies (Bruner-Tran et al., 2009; Esfandiari et al., 2005; Piotrowski et al., 2006). Esfandiari et al. (2005) examined the inhibitory effect of a statin (lovastatin) on angiogenesis in a 3-D culture of human endometrial fragments *in vitro*, which were obtained from the fundus of the uterine cavity. A concentration-dependent effect of lovastatin was shown to be effective in inhibiting the mechanisms of cell proliferation and angiogenesis in an experimental model for the development of endometriosis-like tissue. Similarly, Piotrowski et al. (2006) evaluated the effects of statins on growth of human endometrial stromal cells which were cultured in the absence and in the presence of serum and with or without mevastatin and simvastatin. Mevastatin and simvastatin induced a concentration-dependent inhibition of DNA synthesis and viable cell count in chemically defined media and in the presence of serum. Finally, authors demonstrated for the first time that statins inhibited the growth of endometrial stromal cells. More recently, Bruner-Tran et al. (2009) reported that simvastatin exerted a potent inhibitory effect on development of endometriosis in the nude mouse possibly by inhibition of MMP-3. Therefore, the results of Esfandiari et al. (2005), Piotrowski et al. (2006), Oktem et al. (2007), Bruner-Tran et al. (2009) and the present study suggest a unique therapeutic potential for atorvastatin in medical treatment of endometriosis.

The growth of newly formed blood vessels is of pivotal importance in the development of endometriosis, so inhibition of angiogenesis may offer a new opportunity for treatment. VEGF is the most prominent and most studied pro-angiogenic factor in endometriosis and is the main stimulus for angiogenesis. It has been observed that VEGF gene expression is higher in ectopic than in eutopic endometrium (Bourlev et al., 2006). Moreover, a significant decrease in microvessel density and number of established endometriotic lesions has been reported with the use of anti-angiogenic agents, including TNP470, endostatin, anginex and anti-human VEGF antibody (Nap et al., 2004; Nisolle et al., 2000).

Previous studies showed that atorvastatin decreased the plasma concentrations of VEGF (Alber et al., 2002; Kodama et al., 2006) and VEGF mRNA concentrations in cerebral endothelial cells (Zhang et al., 2007). As far as is known, there is only one study in the literature evaluating the effect of atorvastatin on VEGF in rat endometriosis model, where authors reported that 2.5 mg/kg/day oral atorvastatin for 21 days significantly decreased the VEGF concentrations in peritoneal fluid (Oktem et al., 2007). Likewise, the same dose of atorvastatin given either intraperitoneally or per orally significantly decreased the VEGF concentrations of endometrial implant tissue in rats in this study.

MMP and TIMP are involved in reproductive function and in regulating the dynamic structural changes that occur in the endometrium during the menstrual cycle (Kokorine et al., 1996). It has been shown that endometrium from women with endometriosis has altered expression of MMP and TIMP (Osteen et al., 2003). In patients with endometriosis, endometriotic cyst tissue contains and expresses higher concentrations of MMP-9 than eutopic endometrium (Di Carlo et al., 2008). Moreover, MMP-9 secretion was found to be elevated in the eutopic endometrium of women with endometriosis compared with normal women (Collette et al., 2004).

TIMP-2 is the main inhibitor of MMP-2 (Brew et al., 2000). Studies have shown that eutopic endometrium from patients with endometriosis was shown to express higher concentrations of MMP-2 and lower concentrations of TIMP-2 than the endometrium from normal women (Chung et al., 2002). It was also found that there was a negative correlation between MMP-2 and TIMP-2 expression in endometriotic lesions (Uzan et al., 2004).

Atorvastatin has been shown to inhibit MMP-9 expression in human coronary artery smooth muscle cells (Chandrasekar et al., 2006) and to reduce serum concentrations of MMP-9 in patients with unstable angina (Tziakas et al., 2004). Recently, Cheng et al. (2007) reported that atorvastatin decreased protein expression and enzyme activity of MMP-2 and 9 in the left ventricle tissue of arteriovenous fistula-treated rats. As far as is known, this is the first study of the literature investigating the effects of atorvastatin on the implant concentrations of VEGF, TIMP-2 and MMP-9 in a rat endometriosis model.

Statin treatment inhibits the activation of the oxidant enzyme system NAD(P)H oxidase, likely by preventing a membrane translocation of the small G protein rac-1 (Maack et al., 2003; Wassmann et al., 2002), which might contribute to reduced vascular oxidant stress after statin treatment. Moreover, a recent study suggests that atorvastatin has antioxidant effects in the rostral ventrolateral medulla of stroke-prone spontaneously hypertensive rats (Kishi et al., 2008). However, as far as is known, no study has been conducted to investigate the antioxidant effects of atorvastatin by means of tissue concentrations of SOD and MDA in a rat model of endometriosis.

The exaggerated expression of SOD in the endometrium throughout the menstrual cycle suggests that superoxide plays a key role in infertility in endometriosis and adenomyosis (Ota et al., 1999). In the present study, SOD enzyme concentration in the endometrial implants was significantly increased in the intraperitoneal and peroral atorvastatin groups, which may be due to lesser consumption of the enzyme related to decrease in oxidative stress. Decreased enzyme activity in endometrial explants would indicate a high degree of oxidative stress (Guney et al., 2008), therefore, and the decreased SOD activity may be an ameliorating, adaptive response to the endometrial explant in the control rats. However, MDA concentration in surgically induced endometriotic explants did not differ between the three groups. This might be due to the dose used in this study so that 2.5 mg/kg/day dosage of atorvastatin was not enough to improve tissue concentrations of MDA.

Four weeks after the first surgery, only two rats were examined to evaluate size or viability of endometriotic implants and the remaining rats did not receive a second laparotomy for evaluation and were randomized into three groups. Thus, it might be better to perform a second laparotomy to evaluate the volume of endometriotic explants in each rat before starting medical treatment and giving the data as a reduction rate (%). Although this seems to be a weak point of this study, results of the present study regarding beneficial effects of atorvastatin on implant concentrations of SOD, VEGF, TIMP-2 and MMP-9 support the

finding that atorvastatin treatment reduces the size of endometriotic implants in rats.

By conducting a dose—response experiment, important data would be retrieved regarding the efficacy of different doses of atorvastatin but only two routes (intraperitoneal versus peroral) with same dose (2.5 mg/kg/day) were tested here. This is another limitation of the present study.

Rats are different from women with respect to reproductive anatomy and physiology and they do not undergo menstruation or spontaneous endometriosis. Therefore, the potential therapeutic benefit of atorvastatin for prevention and treatment of endometriosis could be defined by a more relevant preclinical animal model, such as baboon, and a large-scale epidemiological study or randomized clinical trial.

In conclusion, this study has shown that atorvastatin reduced the size of endometrial explants in rats, which was clearly supported in this study by the histological, immunohistochemical and biochemical findings regarding the explant concentrations of SOD, VEGF, TIMP-2 and MMP-9. In addition, intraperitoneal atorvastatin seems to be more effective than oral one. Further experimental and clinical studies should be undertaken to assess the effect of atorvastatin on endometriosis with different doses and routes of administration.

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