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Original Article

Ozone (03)-oxygen mixture therapy inhibits endometrial implant growth

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Abstract: The aim of this study was to investigate potential therapeutic efficiency of ozone therapy in the treatment of experimental endometriosis in rats. Fifteen rats were divided into three groups, which were labeled as the (1) sham control, (2) the ozone (treated with intraperitoneal ozone-oxygen mixture) and (3) the GnRH-agonist (given single dose (1 mg) leuprolide acetate depot formulation) group. Endometrial implant activity of superoxide dismutase (SOD), malondialdehyde (MDA), interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and vasculary endothelial growth factor (VEGF) were measured after ozone-therapy. Furthermore, peritoneal fluid activity of SOD, MDA, and TNF-α were also measured before and after ozone-therapy. Serum AMH levels of the rats those were given ozone-therapy and control groups were measured. The rats given ozone-therapy showed significantly reduced endometriotic implant volumes. After ozone-therapy, a significant increase in activity of SOD in peritoneal fluid was detected. Conversely, implant levels of SOD in rats given ozone therapy was found to be significantly decreased. Both peritoneal fluid and implant levels of MDA were significantly decreased after ozone-therapy. Implant levels of TNF-α, IL-1β, and IL-6 were significantly increased following ozone-therapy. VEGF levels of implant was found to be unchanged after ozone-therapy. Serum AMH levels of animals were given ozone-therapy and control groups were similar. The number of both primordial and preantral follicles were significantly decreased after ozone-therapy. However, the number of atretic follicles were similar in ozone-therapy and control groups. Repeated administration of ozone-oxygen therapy in non-toxic doses inhibits growth of endometrial implants.

Keywords: Endometriosis, ozone, oxidative stress, rat

Introduction

Although, ozone therapy has found different fields of application in medical practise, potential toxicity is a major factor preventing widespread use of it [1]. It has been reported that ozone is a pro-drug and does not need to receptor for its biological action [2].

Ozone therapy prevents oxidative stress-related cell damage by inducing adaptive mechanisms [3]. In agreement with studies reported that low dose ozone therapy supported an oxidative preconditioning by inhibiting cellular damage induced by reactive oxygen species (ROS) [4, 5]. In nontoxic doses, ozon leads to regulation of the biochemical pathways with the production of several critical precursors [3]. Accordingly, some antioxidant enzymes which could be regulated by low dose ozone therapy support the possible impact of ozone in many pathological conditions including endometriosis.

Presence of an ozone-like mediator during inflammation has been reported [6]. By causing oxidative stress, excessive inflammation may lead to the pathologic condition to continue. Given that endometriosis is a disorder associated with inflammation and oxidative stress [7, 8], we have postulated that intraperitoneal ozone treatment may protect antioxidant systems and regulates the physiological concentrations of inflammatuar substances inside the peritoneal implants. In this study, possible impacts of ozone therapy on rat peritneal endometriosis have been investigated by using histomorphological and biochemical markers.

Materials and methods

This study was carried out in the Experimental Research Laboratory of the Firat University Faculty of Medicine, complying with the approval of the ethic committee, the guidelines for care and use of experimental animals. Twenty one adult female Wistar rats each weighing between 200 and 240 g were supplied from Firat University Animal Laboratory. All rats were examined by a veterinarian and determined to be in good health conditions. The rats were housed in plastic cages and kept under standard conditions: 12-h light and 12-h dark periods, 20°C constant temperatures and a humidity ranging from 40 to 60%. The rats had free access to standard dry pellets ad libitum and tap water until the end of the study.

Considering the effect of menstrual cycle and estradiol that is known to be antioxidant, the two subcutaneous injections of estradiol that is known to be antioxidant all rats were hormonally synchronized before surgery in their 4 day estrus phase to exclude the differences in the steroid synthesis, cell adhesion and growth in implanted tissues. Synchronization was realized two subcutaneous injections of estradiol within 24 h intermission, followed by one injection of progesterone 20 h after the last estradiol injection. Daily vaginal smears of the rats were taken to establish the estrous cycle of each animal. Rats observed for at least two successive 4-day estrous cycles. Endometriosis was induced surgically by using the method described by previously [9, 10] during estrus. From the uterine horn, a 5 × 5 × 1 mm piece was excised by microscissors that was attached onto the peritoneum only on the right side of the ventral abdominal wall close to an artery by using the surgical auto transplantation technique. After 3 weeks their daily vaginal smears were monitored and a second laparotomy was performed in their estrous phase to determine the attachment and viability of endometriotic implants. The pretreatment implant volumes in each group were calculated by measuring their dimensions (length, width and height, in millimeters). For volume calculation the ellipsoid volume formula (π/6 × length × width × height) was used. Superficial peritoneal attachment of implants and non-rigid nature of the rat peritoneum allowed the measurment of implant's length, width, and height without resection. Rats with volume calculation is not possible due to heavy invasion of peritoneal implants were excluded from the study.

Of the 21 experimental rats, 2 rats died after operation and 4 rats developed abscess at implantation site and therefore these were excluded from the study. Afterwards, 15 rats were put into 3 groups of 5 rats by using random number tables as the sham control (group 1), the ozone-oxygen mixture (group 2), and the gonadotropin releasing hormone-agonist (GnRH-agonist, group 3). For the sham group, 4-0 nylon sutures, with or without fat tissues, were attached to the peritoneum except the auto transplantation of endometriotic implants. The rats in group 2 were treated with ozone-oxygen mixture (1 mg/kg body weight per rat, intra-peritoneally) every other day for a period of ten days. As a result, animals had received treatment for five days. The rate of ozone at the mixture is 3% percent. The rats in group 3 exposed to single dose leuprolide acetate depot formulation (1 mg/kg body weight per rat, s.c, Lucrin Depot-3M®; Abbott, Cedex, Istanbul, Turkey). This dose was determined based on a previous study in which 1 mg/kg leuprolide acetate was found to be optimal for female rats [11]. It has not been stated in any comments by the manufacturers whether ozone has any effect on the estrous cycle. Therefore, daily vaginal smears were monitored during the treatment period and after the permanence of the estrous cycle was confirmed, a third laparotomy was performed while the rats were fixed in the supine position. The volumes of the implants were measured again with the same method by the same researchers who were blinded to the groups. The cardiac blood samples were obtained to evaluate AMH levels. The endometrial implants and ovaries were then excised and processed for histological and biochemical studies. Peritoneal fluids of animals were collected before and after treatment for evaluating the biochemical markers. The endometriosis was diagnosed according to the histologic identification of endometrial glandular tissue and stroma.

Formalin-fixed endometriotic implants were embedded in paraffin, cut into 5-mm-thick sections, and stained with hematoxylin and eosin. the endometriosis was diagnosed according to the histologic identification of endometrial grandular tissue and stroma. Likewise, formalin-fixed ovarian tissues were exhaustively sec-

tioned at a thickness of 5 mm using a microtome perpendicular to the long axis of the ovary. At least five slices were selected from the each ovary using systematic random sampling rules. Sections representing the largest two dimensional profile of each slice were selected for the follicle counting. The number of the follicles at each section were counted as previously described [12].

The follicles were classified into five types based on the classification of Mazaud [13]. In the light microscopic examination of ovary sections belonging to all groups, follicle classification was carried out according to characteristics noted below:

Primordial follicle: Oocyte was surrounded either partially or completely by granulose progenitor cells.

Primary follicle: Follicle, in which a single layer of cubic granulose cells was observed around oocytes.

Antral (secondary) follicle: Follicle, in which oocyte was covered with more than two layers of granulosa cells and in which antrum formation commenced.

Tertiary (Graafian) follicle: Follicle that possesses a single and big space (antrum), in which a decreasing number of granulosa cells surround an antrum full of follicular fluid, and that oocyte surrounded by granulosa cells.

Atretic antral: Degenerated oocyte along with pycnosis in granulosa cells, low number of pycnotic granulosa cells, and hypertrophic theca interna. For each rat, the total number of atretic follicles was calculated as decribed by Zhang [14]. A method by Pederson and Peders for determining the total follicle number was used to determine the possible impact of ozone therapy on follicular environment of the ovary [15].

Biochemical analysis was performed on blood samples, peritoneal fluid, and endrometrial implants. Samples were stored at -80°C until assay. The biochemist was blinded to the samples. In order to evaluate the possible prooxidant-antioxidant activity of ozone gas, we measured the endometrial implant levels of SOD, MDA, IL-1β, IL-6, TNF-α, and VEGF after ozone treatment. Furthemore, we have also measured the peritoneal fluid activity of SOD, MDA, and TNF-α before and after ozone therapy. At the end of study in order to determine possible harmful effect of ozone therapy on ovarian follicles serum levels of AMH were also measured in all groups.

After centrifugation of both endometrial implant and peritoneal fluid with an equal volume of an ethanol/chloroform mixture (5:3, volume per volume [v/v]) at 5000 × g for 30 min, the clear upper layer (the ethanol phase) was taken and used in the SOD activity assay. All preparation procedures were performed at 4°C. Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun [16].

Malondialdehyde (MDA) levels in endometrial implants and peritoneal fluid were determined by the method of Ohkawa [17] which is based on the reaction with thiobarbituric acid (TBA) at 90-100°C. In the TBA test reaction, MDA or MDA-like substances and TBA react with the production of a pink pigment having an absorption maximum at 532 nm. The results were expressed according to a standard graphic, which was prepared from a standard solution (1, 1, 3, 3-tetramethoxypropane).

Interleukin-1 beta (IL-1β) levels in endometriotic implant were determined with an enzyme-linked immunosorbent assay (ELISA) (Rat IL-1β ELISA-Catalog Number EK0393, USA); which could measure IL-1β in serum with a detection limit of 3 pg/ml. The standart curve range and lower detection limit for this kit is 3.12-2000 pg/ml and 1 pg/ml respectively. Both the intra- and inter-assay coefficients of variation (CV) were <10%.

Interleukin-6 levels in endometrial implant were determined with an enzyme-linked immunosorbent assay (ELISA) (Rat IL-6 ELISA-Catalog Number EK0412, USA); which could measure IL-6 in serum with a detection limit of 62 pg/ml. The standart curve range and lower detection limit for this kit is 62.5-4000 pg/ml and 5 pg/ml respectively.

TNF-α ELISA analysis on the endometriotic tissue and peritoneal fluid was carried out in duplicate in a blinded fashion using commercially available ELISA kit (Rat TNF-α ELISA-Catalog number EK0526, Boster USA). The standart curve range and lower detection limit

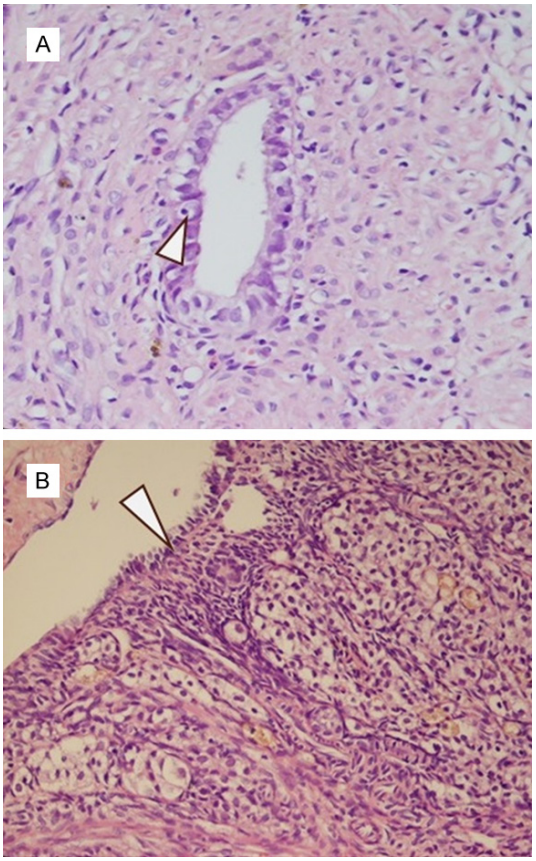

Figure 1. A. Well defined endometriotic implant in GnRH agonist group (arrow head). B. Endometriotic implant with defective epithelium after ozone treatment (arrowhead) (H&E × 200).

for this kit is 7, 8-1000 pg/ml and 1 pg/ml respectively. The intra- and inter-assay CV were <5% and <10% respectively. IL-1β, IL-6 and, TNF-α concentration was measured from absorbance of each well was read at 450 nm using with an auto-analyzer (ELX800). Background absorbency of blank wells was subtracted from the standards and unknowns prior to determination of sample concentrations.

VEGF levels in endometrial implant were determined with an enzyme-linked immunosorbent assay (ELISA) (Rat IL-6 ELISA-Catalog Number EK0540, USA); which could measure VEGF in serum with a detection limit is 15 pg/ml. The standart curve range and lower detection limit for this kit is 15.6-1000 pg/ml and 1 pg/ml respectively. TNF-α concentration was measured from absorbance of each well was read at 450 nm using with an auto-analyzer (ELX800).

Serum levels of AMH were determined with an enzyme-linked immunosorbent assay (ELISA) (Rat IL-6 ELISA-Catalog Number E-EL-R0640, USA); which could measure VEGF in serum. The standart curve range and lower detection limit for this kit is 0.16-10 ng/ml and 0.1 ng/ml respectively. AMH concentration was measured from absorbance of each well was read at 450 nm using with an auto-analyzer (ELX800). Both the intra- and inter-assay CV were <10%.

Statistical analysis

Data analysis was performed by using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, USA). Normality of distributions of continuous variables were determined by Shapiro Wilk test. Levene test was used for the evaluation of homogeneity of variances. In addition to pre- and post-treatment implant volumes SOD, MDA, IL-1β, IL-6, TNF-α, and VEGF levels were compared by Kruskal Wallis test. A *p* value smaller than 0.05 was considered statistically significant. Data are presented as mean ± SD. For all multiple comparisons, the Bonferroni Correction was applied for controlling Type I error.

Results

Rats given ozone therapy showed significantly reduced endometriotic implant volumes (P = 0.034) (**Figure 1A**, **1B**). After ozone therapy, a significant increase in activity of SOD in peritoneal fluid was detected. Conversely, implant levels of SOD in rats given ozone therapy was found to be significantly decreased (P<0.028). Both peritoneal fluid and implant levels of MDA were significantly decreased in rats given ozone (**Tables 1** and **2**). Implant levels of TNF-α, IL-1β, and IL-6 were significantly increased following ozone therapy (P<0.009, P<0.009, and P<0.009 respectively). VEGF levels of implant was found to be unchanged after ozone therapy (P<0.175). Serum AMH levels of animals were given ozone therapy and control groups were similar. The number of both primordial and preantral follicles were significantly decreased after ozone therapy (**Table 3**; **Figure 2A**, **2B**) however, the number of atretic follicles were similar in ozone therapy and control groups. When compared to lucrin group TNF-α, IL-1β, IL-6, and MDA levels of endometrial implants in rats given ozone therapy significantly de-

Table 1. The concentration of TNF-α, IL-1β, IL-6, VEGF, MDA, and SOD in endometriotic implants after ozone therapy

Groups	TNF-α	IL-1β	IL-6	VEGF	MDA	SOD	AMH
1-Control group	0.62±0.32	3.19±0.39	1.86±0.47	0.47±0.37	14.56±3.81	134.55±11.65	1.13±0.3
2-Ozone group	1.76±0.38	18.41±4.03	6.32±1.19	0.76±0.21	7.53±1.35	117.37±4.14	1.17±0.1
3-Lucrin group	0.46±0.22	12.30±1.85	2.32±0.24	1.13±0.36	1.58±0.36	140.96±5.32	1.14±0.2
P value							
1 vs. 2	0.009	0.009	0.009	0.175	0.009	0.028	0.754
1 vs. 3	0.251	0.009	0.117	0.028	0.009	0.754	0.175
2 vs. 3	0.009	0.016	0.009	0.076	0.009	0.009	0.251

The values are presented as mean and standard deviation. *P* value was significant at <0.05.

Table 2. Peritoneal fluid TNF-α, MDA and SOD levels before and after ozone therapy

Groups	Before treatment	After ozone therapy	*P* value
Control group			
TNF-α	10.24±1.79	16.72±1.40	0.024
MDA	2.47±0.13	1.48±0.09	0.030
SOD	164.89±6.03	251.19±15.58	0.034
Ozone group			
TNF-α	17.05±4.58	38.87±2.56	0.016
MDA	2.46±0.25	1.46±0.13	0.028
SOD	257.98±19.46	327.23±15.57	0.009
Lucrin group			
TNF-α	17.64±3.68	36.29±4.30	0.009
MDA	2.59±0.27	1.50±0.91	0.029
SOD	277.60±17.46	255.85±26.59	0.754

The values are presented as mean and standard deviation. *P* value was significant at <0.05.

creased. Conversely, when compared to ozone group VEGF and SOD levels of endometrial implants in rats treated with lucrin significantly increased. Serum levels of AMH in both lucrin and ozone groups were similar (P<0.251).

In the lucrin treated animals MDA levels of endometrial implants were significantly decreased (P<0.009). SOD levels of implants in lucrin group were found to be unchanged (P<0.754). In contrast to ozone group, implant levels of VEGF significantly increased after lucrin treatment (P<0.028). When compared to control group implant levels of TNF-α and IL-6 in lucrin group were similar (P<0.251, P<0.117 respectively). IL-1β levels in the endometrial implants of rats treated with lucrin were decreased significantly (P<0.009). Similar to ozone group, serum AMH levels of rats given lucrin were not changed significantly (P<0.175).

In the lucrin group while peritoneal fluid levels of MDA decreased (P<0.029) SOD levels did not change (P<0.754). Peritoneal fluid levels of TNF-α in both ozone and lucrin groups increased significantly (P<0.016, P<0.009, respectively). The number of both primary and antral follicles were significantly decreased after lucrin treatment (P<0.034, P<0.036 respectively). Similar to ozone group, the number of atretic follicles were similar in rats given lucrin. There were no significant difference between ozone and lucrin groups in terms of follicle numbers.

The mean pretreatment volume of endometriotic implants in the sham control, ozone, and GnRH-agonist groups were found to be 71.2± 4.3, 69.4±9.1, 70.2±8.1 respectively. There were no statistically significant differences among the groups in regard to pretreatment volumes of endometriotic implants. The post-treatment volumes of endometriotic implants in the control, ozone and GnRH-agonist groups were noted to be 77.1±2.2, 40.2±46.1, 46.4± 75.1 respectively. There were significant differences in the percentage of decline between pretreatment and post-treatment volumes of endometriotic implants in the ozone and lucrin groups (**Table 4**).

Discussion

Preliminary results of our study have clearly demonstrated that ozone-02 mixture therapy has a therapeutic potential in the treatment of peritoneal endometriosis. Concordantly, we detected a significant reduction in the volume of endometriotic implants after ozone-02 treatment. Although this mixture leads to significant decline in the number of both primordial and preantral follicles the number of atretic follicles unchanged. Likewise, serum levels of AMH in

Table 3. Comparison of the mean follicle number of control, ozone therapy and lucrin groups (mean ± SD)

Groups	Primordial follicle	Primary follicle	Preantral follicle	Antral follicle	Atretic follicle	Corpus luteum
1-Control	11.8±2.4	6.4±1.8	9.2±1.4	7.8±2.5	3.4±1.1	14.0±3.8
2-Ozone	6.4±1.3	6.4±4.0	5.4±2.1	7.4±3.3	3.8±1.9	9.8±4.9
3-Lucrin	7.0±4.3	3.2±1.9	6.8±2.1	3.8±1.9	2.6±0.8	19.0±4.2
P value						
1 vs. 2	0.008	0.916	0.025	0.916	0.914	0.173
1 vs. 3	0.171	0.034	0.055	0.036	0.228	0.093
2 vs. 3	0.916	0.207	0.454	0.085	0.228	0.024

The values are presented as mean and standard deviation. *P* value was significant at <0.05.

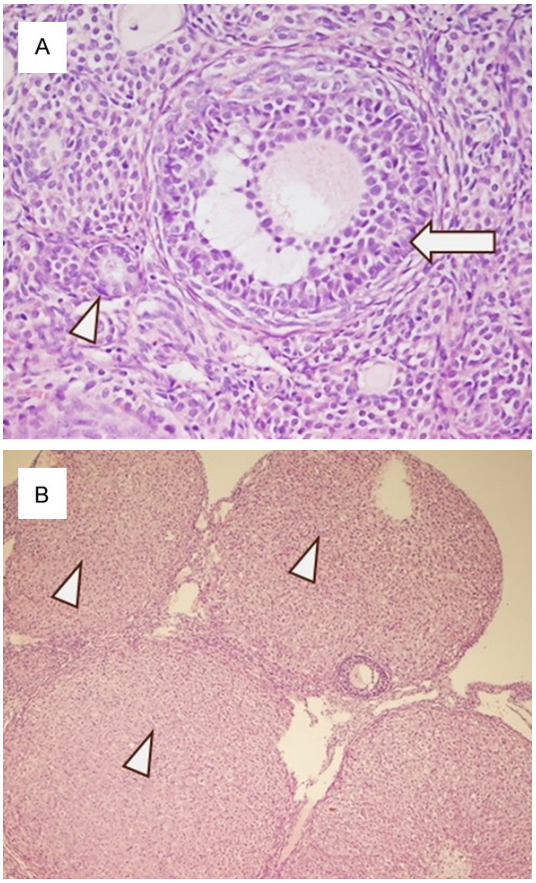

Figure 2. A. Decreased primordial (arrowhead) and preantral follicles (arrow) in ozone given animals. B. Unchanged corpus luteum number after ozone treatment (arrow heads) (H&E × 20).

animals were given ozone therapy and control groups were similar. Collectively, we can strongly propose that inhibitor effect of ozon therapy on endometrial implant growth occured with minimal ovarian side effect.

Possible mechanisms of action of ozone therap on implant growing is unclear. It is most likely that, ozone-O2 mixture therapy prevented oxidative stress, normalized levels of MDA and activated the peritoneal fluid SOD levels. Another mechanism of action of ozone treatment on impant growing might be pharmacodynamic and superoxide scavenger properties of this pro drug. Although ROS can originate from different sources a great number of intracellular impacts are mediated by different ROS [18]. Elevated ROS levels may stimulate the expression of some gene and gene products exert an oxidative activity in endometriotic implant cells. This might lead to increase in implant survival. Ozone mediated oxidative preconditioning might improve a moderate oxidative stress which, in turn, increases antioxidant enzymes protecting against further growth of endometrial implants. As a consequence, ensuring the redox homeostasis [18] inside the implant cells, ozone therapy may lead to increase in the expression of antioxidants enzymes that may prevent ROS-mediated induction of implant growing.

Imbalance in oxidant/antioxidant status in endometriotic implants has been noted [19]. It has been reported a significant suppression of peritoneal fluid antioxidant levels in women with endometriosis [20]. Concordantly, peritoneal fluid of women with endometriosis contain significantly lower levels of SOD [21] and in close aggrement, we also noted a significant increase in the activity of SOD in peritoneal fluid after ozon therapy. Conversely, implant levels of SOD in rats given ozone therapy was found to be significantly decreased. Different concentration of SOD in both peritoneal fluid and inside implant might seem paradox. Decreased SOD levels inside the endometrial implants after ozone therapy may be secondary to ozone-related oxidative preconditioning. Accordingly, a great majority of SOD in endometrial implant might be used for removal or neutralization of oxidant product including MDA. In the present study both peritoneal fluid and implant levels of MDA were significantly decreased after ozone therapy. Concordantly, it

Table 4. Comparison of endometriotic implant volumes between the control and treatment groups

Groups	Endometriotic implant volüme (mm^3)		
	Before treatment	After treatment	*P* value
1-Sham control	71.2±4.3	77.1±2.2	0.56
2-Ozone-O2 mixture	69.4±9.1	40.2±46.1	0.001
3-GnRHa	70.2±8.1	46.4±75.1	0.027
P value			
1 vs. 2	NS	0.001	NS: Not significant
1 vs. 3	NS	0.001	GnRHa: Agonist
2 vs. 3	NS	NS	O2: Oxygen

The values are presented as mean and standard deviation. *P* value was significant at <0.05.

has been reported that low doze ozone therapy improves an oxidative preconditioning by enhancing of antioxidant enzymes [4, 5].

For continued growth and survival of endometriotic implant abnormal inflammatory reaction catalyzed by nuclear transcriptional factor kappa B (NF-kB) is required. Relatedly, the classic NF-κB pathway is induced by TNF-α and IL-1β [22, 23]. In the present study ozone therapy leads to increase in TNF-α, IL-1β, and IL-6 expression in the endometriotic implants. Moreover, peritoneal fluid TNF-α levels increased significantly in ozone given animals. Although the endometrial implants shrink, cytokine levels within the implants increase and that requires detailed explanation. Accordingly, molecular mechanisms responsible for the increased cytokine levels after ozone therapy may be related to the administered dose of ozone. It is well known that high dose ozone-O2 mixture therapy induces severe oxidative stress by activation of NF-κB end up with an inflammatory response [24]. In contrast, administration of low ozone therapy stimulates mild oxidative stress which activates the nuclear factor erythroid 2-related factor 2 as well as SOD [24]. Although we have not measured the endometrial implant levels of NF kB, our results are more consistent with the last hypothesis. Together, our findings suggest that ozone therapy might inhibit the progression of endometriotic implants by regulating the oxidant-antioxidant balance within the implant cells.

Decline in the numbers of both primordial and preantral follicles after ozone therapy may be secondary to potential toxicity of ozone on ovary. Nevertheless, atretic follicle numbers in rats given ozone were not changed significantly. Furthermore, the number of both primary and antral follicles were significantly decreased after lucrin treatment. Taken together, decreased follicle number in ozone and lucrin given animals might be related with follicle counting methods. When reviewing the literature we did not find any information in terms of ozone gas and ovarian follicle number. Unchanged serum AMH levels after ozone therapy supports this idea. In order to recommend to use of ozone gas in the treatment of endometriosis the possible impact of ozone on the ovarian follicles must be clarified.

Although some biological effects of ozone therapy in vivo are likely mediated by blood factors, a direct cellular impact cannot be ignored, especially when ozone is administered by insufflation in a tissue cavity. Relatedly, intraperitoneal insufflation of the ozone may prevent the growth of endometriotic implants. Furthermore, intrauterine ozone insufflation may be tried in order to restorate endometriosis-associated implantation defects.

This is the first study evaluating the effect of ozone therapy on implant growing in experimental endometriosis model. In this study, we have demonstrated that endometrial implants and peritoneal fluid of rats with endometriosis exhibit increased oxidative stress. By inducing the activation of endogenous antioxidant capacity ozone-O2 mixture therapy have provided a significant regression of endometriotic implants.

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Disclosure of conflict of interest

None.

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