Abstract. – OBJECTIVE: Disulfiram (DSF) exerts its therapeutic effect through oxidative, proteasome, and nuclear factor kappa beta (NF-κB) pathways. The study was planned to test the impact of DSF on growing of endometriotic implants in rats with experimentally induced endometriosis.

PATIENTS AND METHODS: Thirty rats were labeled as the control (n = 8), sham (n = 6), GnRH-agonist (n = 8) and the DSF (n = 8) groups. The rats in the group 3 exposed to single dose leuprolide acetate. The rats in group 4 were treated with DSF for 21 days. The serum activity of oxidant and antioxidant markers, total oxidant status (TOS), total antioxidant status (TAS), interleukin-1β, and tumor necrosis factor-α (TNF-α) were determined. Implants were processed for NF-κB, PCNA, and CD34 immunostaining.

RESULTS: The serum concentration of malondialdehyde in the DSF group was significantly higher than those in other groups. The concentration of TAS, TNF-α, and interleukin-1β in the DSF group considerably decreased compared to control group. Following treatment with DSF while the percentage of Grade 1 and 2 implants increased the percentage of Grade 3 and 4 implants decreased. The implants disappeared totally in two cases in the DSF group and one case in the GnRH-agonist group. The mean H-Scores of implant NF-κB and PCNA in DSF treated animals were found to be significantly lower than those of the control group.

CONCLUSIONS: By decreasing NF-κB expression, angiogenesis, and cell proliferation DSF prevents the growth of endometriotic implants.

Key Words: Endometriosis, Disulfiram, NF-κB, Oxidative stress.
The dithiocarbamate (DC) drug, disulfiram (DSF) is a NF-κB inhibitor5,6). It can affect both oxidative and proteasome pathways in healthy and pathological tissues7-9. Conventional effect of DSF on proteasome pathway is inhibition7-9. NF-κB activity has been demonstrated in eutopic and ectopic endometrial tissues10,11. NF-κB assists the transcription of a variety of immune and inflammatory molecules that involved in the development of endometriotic implants10,11. NF-κB also contributes the regulation of cell proliferation, apoptotic events, adhesion, invasion, and angiogenic processes in many cell types12,13. Concordantly, DSF has been shown to reduce angiogenesis14,15 and induce apoptotic cell death in healthy and cancerous tissues16.

The pathophysiological pathways involved in the development of the endometriosis also operate in normal tissues. Hence, there are many concerns about the possible side effects of the current medical agents that targeting these pathways in human endometriosis. Although DSF has not been used for the treatment of endometriosis in animals and humans it has currently been used for the treatment of bacterial and fungal infections and to break off alcohol abuse in humans with relatively mild side effect8,17. Therefore, DSF is a valuable molecule for investigating its effects in the endometriosis treatment. In a previous study18, the positive effect of the first proteasome-inhibitor PS-341 and DC on endometriotic implants were demonstrated by our team. This one encouraged us to use of DSF in the treatment of endometriosis. In the current study, we planned to test the effects of DSF as a candidate NF-κB and proteasome inhibitor on endometriotic implants of rats with experimentally induced endometriosis. Since DSF is a lipophilic and pro-oxidative drug the balance between oxidant and antioxidant systems during implant development was taken into account.

**Materials and Methods**

**Experimental Animals**

This study was carried out in the Experimental Research Laboratory of the Inonu University Faculty of Medicine, complying with the approval of the ethic committee, the guidelines for care and use of experimental animals. Thirty-five adult female Wistar rats each weighing between 250 and 300 g were included. 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.

**Methods**

Endometriosis was induced surgically by using the method described by previously by our team and others18,19. Detailed information could be find elsewhere18. Briefly, A 0.5 × 0.5 × 0.1 cm piece excised by micro scissors from the uterine horn was attached on the right side peritoneal wall close to an artery. This surgical method had been defined as auto transplantation technique. The second laparotomy was performed after 3 weeks in estrous phase to determine the attachment and viability of implants. The vesicle-like appearance at the sutured peritoneum was evaluated and animals were graded according to average vesicle diameter (D) as: Grade 1 (for cases in which the implant had disappeared or, if it was visible, never became a cyst), Grade 2 (D < 2 mm), Grade 3 (2 mm < D > 4.5 mm) or Grade 4 (D > 4.5 mm). Five out of 35 experimental rats were not developed any signs of vesicles and therefore these animals were excluded. The remaining 30 rats were put into four groups. The groups were labeled as control (n = 8), sham control (n = 6), GnRH-agonist (GnRH-a; n = 8), and the DSF (n = 8) groups. For the sham group, the only suture was attached to the peritoneum. The rats in group 4 were treated with DSF (100 mg/kg body weight per rat, intraperitoneally) for 21 days. The rats in group 3 were exposed to subcutaneous single dose leuprolide acetate depot formulation (1 mg/kg body weight per rat, Lucrin; Abbott, Cedex, Istanbul, Turkey). This dose was determined based on a previous study in which 1 mg/kg leuprolide acetate was found to optimal for female rats20). Since the DSF was diluted with an isotonic saline solution containing 1% carboxymethylcellulose, an identical amount of this solution was used in control group (0.1 ml/day per rat, ip) for 21 days. Due to DSF exhibited similar absorption rates when it was given orally or intraperitoneally in the current study peritoneal administration was chosen. Following administration DSF rapidly metabolizes to the diethyldithiocarbamate-glucuronide and inorganic sulfate. Previously, the micromolar concentration of DSF was shown to inhibit the proteasome in the human embryo kidney cell line6. Likewise, NF-κB inhibiting effect of DSF analogue PDTC occurs with the dose of 100 mg/kg in rats. Therefore, in the DSF group we used minimum 100
mg/kg (0.3 µmol/kg) DSF in order to inhibit implant NF-kB pathway. These doses were also chosen because a recent study indicates that micromolar concentrations (0.16 µmol/L) of DSF inhibited proteasome activity in cell-based screening assay9. It has not been stated in any comment by the manufacturer whether DSF has any impact on the estrous cycle we accepted the DSF does not alter cycle characteristic of rats. Following the confirmation of estrous a third laparotomy was performed. The sizes of the implants were measured again with the same method by the same researchers who were blinded to the groups. Due to the small size of the endometriotic implants blood samples were used for measuring oxidative stress markers and cytokines. The endometriotic implants were excised and processed for immunohistochemical studies.

Biochemical Analysis
All biochemical determinations were performed on serum obtained after centrifugation using spectrophotometric methods. Samples were stored at -80°C until assay. The biochemist was blinded to the blood samples.

SOD and GSH-Px Measurement
Total SOD activity was determined according to the method of Sun et al21. GSH-Px activity was measured by the method of Paglia and Valentine22. The activity of SOD and GSH-Px was expressed as U/L.

GSH and PON1 Measurement
Reduced glutathione (GSH) content was determined according to Ellman23. The results were expressed as µmol/L. Paraoxonase-1 (PON1) activity was measured using the commercially available kit (Relassay, Rel Assay Diagnostics, Gaziantep, Turkey) and results expressed as U/L.

ADA and XO Measurement
Adenosine deaminase (ADA) activity was estimated spectrophotometrically by the method of Giusti24. Results were expressed as units per liter (U/L). Xanthine oxidase (XO) activity was measured spectrophotometrically by the formation of uric acid from xanthine. Results were expressed in units per liter plasma (U/L).

MPO and MDA Measurement
Myeloperoxidase (MPO) activity was determined using a 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by H2O2, and changes in absorbance at 510 nm were recorded25. Data were presented as U/L. Malondialdehyde (MDA) level was determined by the method of Esterbauer and Cheeseman26 which is based on the reaction with thiobarbituric acid at 90-100°C. The results were expressed according to a standard graphic, which was prepared from a standard solution.

PC Measurement
The protein carbonyl (PC) content was determined spectrophotometrically by the method based on the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone27. Results were expressed as µmol/dL.

IL-1β and TNF-α Measurement
Interleukin-1 beta (IL-1β) levels were determined with an enzyme-linked immunosorbent assay (Rat IL-1β Platinum ELISA – Catalog Number BMS630, eBioscience, Inc. Vienna, Austria); which could measure IL-1β in serum with a detection limit is 4 pg/ml. The intra- and interassay coefficients of variation (CV) were < 10% and < 10%, respectively. The rat TNF-α ELISA analysis on the serum was carried out in duplicate using commercially available ELISA kit (Rat TNF-α Platinum ELISA, BMS622, eBioscience, Vienna, Austria). The intra and interassay CV were < 5% and < 10% respectively. The TNF-α concentration was measured from the absorbance of each well was read at 450 nm using with an autoanalyser (Alisei system, SEAC Radim Group, Caserta, Italy). The results were presented as ng/ml.

Total Antioxidant Status Assay (TAS)
Total antioxidant assay (TAS) was determined with an enzyme-linked immunosorbent assay (Antioxidant Assay Kit ELISA – Item No. 709001, Cayman Chemical Co. Ann Arbor, MI, USA) which could measure the total antioxidant capacity of cell lysates. The intra- and interassay CV were < 3.4% and < 3% respectively.

Total Oxidant Status Assay (TOS)
The total oxidant status (TOS) was determined using a novel automated measurement method as described previously28. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalents per liter (µmol H2O2 Equiv/L).
Implant Immunohistochemistry

Formalin-fixed endometriotic implants were embedded in paraffin cut into 5 mm thick sections and stained with hematoxylin and eosin. The sections were also stained for NF-κB/p65 (Rel A) Ab-1, proliferating cell nuclear antigen (PCNA), and CD34 immunohistochemistry.

NF-κB/p65 (Rel A) Ab-1

Four micrometer paraffin sections were dewaxed in xylene, rehydrated in ethanol and then incubated for 10 min in 3% hydrogen peroxide to block endogenous peroxidase. After washing in phosphate buffer saline (PBS), the sections incubated 8 min at ultra V block. The immunoreaction was performed for 60 min with ready to use NF-κB/p65 Ab-1 antibody (NeoMarkers, Labvision co., Fremont, CA, USA). After washing in PBS, slides were incubated with horseradish peroxidase kit. The third trimester human placenta served as the positive control.

PCNA

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 (Clone PC10; Sigma-Aldrich, St. Louis, MO, USA). Human tonsil tissue served as the positive control. Negative controls (primary antibody omitted) were routinely performed on adjacent serial sections.

CD-34

Endothelial cells were stained using mouse monoclonal antibody against CD34 antigen, a glycoprotein expressed on the luminal surface of endothelial cells (ready to use, clone QBEEnd/10; Novocastra, Newcastle, UK). Rat peritoneal vessel served as an internal positive control for CD34.

Timm’s Staining for Copper

Timm’s copper stain is a silver technique where copper sulfate is converted to silver sulfide which is then visualized as black deposits in the light microscopic sections. Tissues from liver cirrhosis were used as positive control.

Histological slides were evaluated for NF-κB p65, PCNA, and CD34 immunoreactivity under light microscopy. The H-Score method was used to score the degree of histological change of stromal and glandular epithelial cells. This semi-quantitative method consists of the percentages of positively stained cells multiplied by a weighted intensity of staining: H-Score = ΣPi (i+1), where Pi is the percentage of stained cells in each intensity category (0–100%), and i is the intensity indicating weak (i = 1), moderate (i = 2), or strong staining (i = 3). Due to weak Timm’s staining for copper in treatment and control groups H-score method could not be implemented.

Statistical Analysis

Data distribution was tested using the Kolmogorov-Smirnov test. Comparison among the groups was performed using the Kruskal-Wallis analysis of variance and post-hoc Mann-Whitney U tests for continuous variables. Data was presented as mean and standard deviation (SD) for continuous variables. p < 0.05 was accepted as significant.

Results

Concentration of Oxidative Stress Markers, TAS and TOS

Insignificant difference in serum SOD activity was detected between the group treated with DSF, GnRH-agonist, and the control groups (p = 0.15 and p = 0.67 respectively). SOD activity in the DSF group was significantly different from than the sham group (p = 0.004). Despite high levels of reduced-GSH were detected in the DSF group this did not reach the statistical significance when compared to the GnRH, control, and sham groups (p = 0.16, p = 0.61, and p = 0.20 respectively). Likewise, the mean serum activity of GSH-Px in the DSF group was not different from the GnRH-a, control, and sham groups (p = 0.19, p = 0.61, and p = 0.75 respectively) (Table I). Additionally, the ratio of reduced GSH to GSH-Px was not different in the DSF group compared to other three groups (p = 0.37, p = 1, and p = 0.12 respectively). However, the concentration of serum ADA considerably decreased in the DSF group when compared to the control and sham groups (p = 0.032 and p = 0.008 respectively). Further, significant difference was found regarding serum ADA levels between DSF and the GnRH-a group (p = 0.002). Serum concentrations of XO were the same all groups.

The concentration of serum MDA, end product of lipid peroxidation, was significantly different in the DSF group than the control and sham control groups (p = 0.05 and p = 0.003, respectively). The concentration of serum MPO was not different among the groups. Further, the serum concentra-
The concentration of IL-1β in the DSF group was significantly lower than the control and the sham group (p = 0.007 and p = 0.02 respectively). However, in the DSF group, the concentration of IL-1β was not different from the GnRH-a group (p = 0.66). The serum concentration of PON was the same all groups (Table II).

**Grade of Endometriotic Implants**

After the treatment with DSF and GnRH-a, while the percentage of Grade 1 and 2 vesicles increased the percentage of Grade-3 and -4 vesicles decreased. The endometrial foci disappeared totally in two of the cases in the DSF group and one case in the GnRH-a group.

**H-Score of NF-κB, PCNA and CD34**

Immunohistochemical analysis confirmed the ELISA findings in each group. The increased NF-κB p65 immunoreactivity was predominantly localized to the cytoplasm of luminal and glan-

Table II. Comparison of PON, IL-1β and TNF-α levels among the groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Disulfiram (n = 8)</th>
<th>GnRH agonist (n = 8)</th>
<th>Control (n = 8)</th>
<th>Sham (n = 6)</th>
<th>p₁</th>
<th>p₂</th>
<th>p₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON, U/L</td>
<td>17.88 ± 4.99</td>
<td>16.44 ± 4.08</td>
<td>26.71 ± 24.01</td>
<td>22.92 ± 6.72</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.08 ± 0.02</td>
<td>0.15 ± 0.07</td>
<td>0.29 ± 0.36</td>
<td>0.21 ± 0.28</td>
<td>NS</td>
<td>0.007*</td>
<td>0.02*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.06 ± 0.02</td>
<td>0.09 ± 0.03</td>
<td>0.11 ± 0.07</td>
<td>0.07 ± 0.02</td>
<td>NS</td>
<td>0.006*</td>
<td>NS</td>
</tr>
</tbody>
</table>

The values are presented as mean and standard deviation. p₁-value was presented for comparison between the DSF group with GnRH-a group. p₂-value was presented for comparison between the DSF group with the control group, and p₃-was presented for comparison between the DSF group with sham control group. *p-value was significant at < 0.05.
dular epithelial cells in the DSF group (Figures 1, 2). The mean H-Score of NF-κB p65 expression in the endometriotic implants of disulfiram group was significantly lower than those of the control group (30.0 ± 27.38 vs. 98.13 ± 75.21, p = 0.03). The mean H-Scores of PCNA and CD34 in the DSF group were also lower than those of controls (30.63 ± 25.42 vs. 112.50 ± 64.97 and 27.38 ± 18.41 vs. 128.75 ± 81.84, p = 0.02 and p = 0.007 respectively). In the GnRH-agonist group, the mean H-Score of NF-κB p65 expression was not significantly different from the DSF (68.13 ± 61.29 vs. 30.63 ± 25.42, p = 0.43) and the control group (p = 0.23). The mean H-Score of PCNA in the GnRH-a group was statistically different from those of the control (31.88 ± 23.29 vs. 112.50 ± 64.97, p = 0.02) but not from the DSF group (p = 0.87). In GnRH-a group, while the mean H-Score of CD34 was not different from those of the control group (93.13 ± 69.94 vs. 128.75 ± 81.84, p = 0.34) it was significantly lower than the DSF group (p = 0.02). The comparisons of mean H-Score among the groups were presented in Figure 3.

Discussion

In the present work, we demonstrated a significant reduction in the size of the endometriotic implant after DSF treatment. In line with this, both CD34 and PCNA immunoreactivity of implants also decreased that reflecting the reduced cell proliferation and angiogenesis in the animals were exposed to intraperitoneal DSF. The anti-endometriotic activity of DSF might depend on its impact on NF-κB pathway. Concordantly, studies demonstrated that DSF inhibited the NF-κB pathway both directly and indirectly through proteasome inhibitory action and/or antioxidative mechanisms[8,9,31]. We showed significantly decreased NF-κB-65 expression after DSF treatment. Therefore, reduced cell proliferation and angiogenesis in the DSF treated animals may be through the reduced NF-κB activity within the implants.

Both the growth and survival of implants depend on inflammatory pathways including NF-κB. The classical NF-κB pathway is induced by TNF-α and IL-1β[31-33]. The regulation of NF-κB

**Figure 1.** Representative photographs of the endometriotic implants obtained from control, GnRH-a and Disulfiram groups after treatment. In DSF (G-I, X10, X40, X10) group, H-Score for NF-κB/65 (Rel A), PCNA and CD34 were significantly reduced as compared to control (A-C, X10, X40, X10) and GnRH-a (D-F, X40, X40, X40) groups.
involves positive feedback stimulation through the NF-κB-mediated synthesis of IL-1β and TNF-α. Concordantly, we clearly showed that DSF attenuated the TNF-α and IL-1β expression in the endometriotic implants. The decreased NF-κB expression within the implants can be due to the decreased cytokine levels or vice versa. Whatever the mechanism, we can strongly suggest that DSF inhibits the progression of implant development by reducing the expression of inflammatory and growth-promoting cytokines.

Despite high serum TOS activity detection of low TAS activity let us think that treatment with DSF can lead to the emergence of oxidative stress within the implants. The possible role of oxidative stress on implant development has been reported by several clinical and experimental studies. In cases with advanced stage endometriosis copper, ceruloplasmin and oxidative stress markers were found to be associated with the development of endometriosis. Likewise, increased miR-210 expression in the hypoxic conditions enhances survival rates of endometriotic cells. As supportive, in the present study, both the markers of reactive oxygen species (ROS) mediated protein oxidation and markers oxidative damage of membrane lipids were significantly elevated in the animal on DSF. Our findings disagree with the previous studies indicating the antioxidative effect of DSF as a possible mechanism for indirect inhibition of NF-κB. Nevertheless, oxidative stress-induced NF-κB activation was noted to be cell-specific events and mostly facilitatory rather than causal. Therefore, in the endometriotic implants, DSF does not seem to inhibit the NF-κB through antioxidant mechanisms. As supportive, we found a similar level of PON1, a marker of antioxidant protection against lipid peroxidation, and decreased levels of ADA, a potent inhibitor.
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of oxy-radical injury of neutrophils, in the DSF treated group. Moreover, low serum levels of antioxidant SOD and similar levels of reduced GSH, GSH-Px, and GSH/GSH-Px ratio in all groups support our idea that DSF does not inhibit the NF-κB activation through antioxidant mechanisms.

Dithiocarbamates (DCs) are known to exhibit both pro-oxidant and antioxidant effects in both cell-free and biological systems. DC oxidizes reduced glutathione through a glutathione peroxidase-like activity. They also inhibit glutathione S-transferases. Low levels of DC induce oxidative stress leading to necrosis and cell death. Likewise, some oxidants have been reported to cause a similar dose-dependent cytotoxicity. Therefore, in addition to NF-κB inhibitory action, DSF also leads to oxidative stress and cell death in the endometriotic implants.

This research has some limitations such as relatively small size of groups and lack of the measurement of tissue oxidant status. The evaluation of the oxidative tissue parameters would certainly be more valuable to discuss the effects of DSF on either local or systemic redox status in the endometrial implants. However, in the current experimental study, we could not split up the implants due to their small size.

**Conclusions**

We showed for the first time that DSF exerts a toxic pro-oxidant effect on endometriotic implants. It also inhibits angiogenesis, cell proliferation, and inflammatory events in the implants. This anti-endometriotic activity may depend on the ability of the DSF (1) to inhibit NF-κB pathway and/or (2) stimulate oxidative stress. Overall, the present paper demonstrated that DSF reduced the serum levels of IL-1β, TNF-α, and TAS activity. DSF also increased the serum MDA, PC, and TOS activity, whereas attenuated the NF-κB expression, angiogenesis, and cell proliferation suggesting a possible molecular mechanism of DSF for controlling the implant.
growing. DSF did not induce toxicity in kidney and liver cells and is well tolerated clinically. Considering the relatively mild side effects and the large clinical experience of the clinical drug studies using DSF in treatment of endometriosis and/or endometrioma appears to be warranted.

The Authors declare that there are no conflicts of interest.

**Conflict of Interest**

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**References**


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