Lipid Peroxidation Products and NO Levels After Adnexal Torsion/Detorsion in Rats Pretreated with Aminoguanidine

AMİNOGUANİDİNE İLE TEDAVİ EDİLEN RAT ADNEKSİAL TORSİON/DETORSİON MODELİNDE OVARİAN LİPİT PEROKSİDASYON ÜRÜNLERİ VE NO DÜZEYLERİ

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———————— Abstract ————————

Objective: This experimental study was designed to determine the changes in ovarian tissue nitric oxide (NO), malondialdehyde (MDA), reduced glutathion (GSH) and xanthine oxidase (XO) levels and the effect of aminoguanidine (AMG) on these parameters after ovarian torsion/ detorsion in rats.

Material and Methods: Thirty-two adult female albino rats were divided into four groups (n=8): sham operation, saline/detorsion, AMG/detorsion and torsion. The control rat underwent a sham operation wherein the right ovary was rotated 360° and then immediately relieved. Rats in the torsion group were killed after 360º clockwise adnexal torsion for 3 h. Aminoguanidine was injected intraperitoneally 30 min before detorsion in the AMG/detorsion group and saline was administered in the saline/detorsion group. After 3 h of adnexal detorsion in both of these groups, the rats were killed and adnexa was removed. A part of the adnexa was preserved in formalin with the remainder stored at -30°C until analysis to determine the tissue levels of NO, MDA, GSH and XO.

Results: NO levels in saline/detorsion group were increased significantly when compared to sham operation and ischemia groups (p<0.05). NO levels in the AMG group were lower than saline/detorsion group, and differences between the two groups were statistically significant (p<0.05). Malondialdehyde and XO levels in saline/detorsion group were increased significantly when compared to sham operation group (p<0.05). MDA and XO levels in the aminoguanidine group were lower than saline/detosion group and differences between the two groups were statistically significant (p<0.05). GSH levels in the saline/detorsion group were decreased signifcantly when compared to sham operation group (p<0.05). GSH levels in the aminoguanidine group were higher than saline/detosion group, and differences between the two groups were statistically significant (p<0.05).

Conclusion: This results indicates that the protective action of AMG on torsion-detorsion injury, could be attributable either by its capacity to scavenge free-radicals or its ability to inhibit iNOS.

Key Words: Adnexal torsion/detorsion, Rat, NO, GSH, MDA, XO, Histopathology

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———————— Özet ————————

Amaç: Bu deneysel çalışma rat ovarian torsion/detorsion modelinde amnioguanidin tedavisinin over dokusunda nitrik oksid (NO) ve lipid peroksidasyon ürünleri olan malondialdehid (MDA), redükte glutatyon (GSH) ve ksantin oksidaz (XO) düzeyleri üzerine olan etkisini saptamak için planlandı.

Gereç ve Yöntemler: Otuziki erişkin dişi-albino rat eşit olarak 4 gruba ayrıldı (n=8): sham operasyon grubu, saline/detorsion grubu, aminoguanidine/detorsion grubu ve torsion grubu. Sham grubundaki ratlarda sağ over 360º rotasyon yapılıp hemen düzeltildi. Torsion grubundaki ratlar 360º rotasyonu takiben 3 saat sonra öldürüldü ve overleri toplandı. Aminoguanidine ve salin grubunda 3 saatlik ovarian torsion yapıldı. Bu iki grupta detorsiondan 30 dk önce intraperitoneal salin ve aminoguanidine uygulandı. Daha sonra 3 saatlik detorsionu takiben overler uzaklaştırıldı. Overlerin bir kısmı histopatolojik inceleme için formalide saklanırken bir kısmı da doku NO, MDA, GSH ve XO düzeyleri bakılmak amacıyla -30ºC'de saklandı.

Bulgular: Sham ve iskemi grubu ile karşılaştırıldığı zaman saline/detorsion grubunda NO düzeyleri anlamlı olarak yüksek bulundu (p<0.05). AMG grubunda ise NO düzeyleri saline grubuna göre anlamlı olarak düşük bulundu (p<0.05). İskemi/reperfüzyon grubunda MDA ve XO düzeyleri sham grubuna göre anlamlı olarak yüksek bulundu (p<0.05). Aminoguanidine tedavisi MDA ve XO düzeylerini anlamlı olarak düşürdü (p<0.05). Sham grubu ile kıyaslandığı zaman, iskemi/reperfüzyon grubunda GSH düzeyleri anlamlı olarak düşüktü (p<0.05). Aminoguanidine verilmesi GSH düzeylerinde anlamlı bir artışa yol açtı (p<0.05).

Sonuç: AMG tedavisi rat ovarian iskemi-reperfüzyon modelinde serbest radikal süpürücü etkisi ile veya NO sentezini bloke ederek overleri hasarlanmadan korur.

Anahtar Kelimeler: Adneksial torsion/detorsion, Rat, NO, GSH, MDA, XO, Histopatoloji

Ischemia-reperfusion (I/R) is associated with activation of neutrophils, inflammatory cytokines, and generation of oxygen-derived free radicals. Reactive oxygen species have been implicated in

the pathogenesis of tissue injury after ischaemia-reperfusion. Free radicals are also generated by tissues in response to an ischemic reperfusion injury causing additional damage to the tissue whose initial damage reflects the duration of the ischemia.[1] The major source of $O2^-$ and $H2O2$ with reperfusion is the hypoxanthine-xanthine oxidase system.[1] Toxic products of XO reaction are generated in quantities that overhelm the capacity of endogenous free radical scavengers and inflict significant injury on the previously ischemic tissues. These ROS interact rapidly with almost every cellular macromolecule including lipids, proteins and DNA, and produces structural or functional changes in these biomolecules.[2] Removal of excess ROS or suppression of their generation by antioxidants may be effective in preventing oxidative cell injury. Ovarian torsion is a gynecological emergency referred to as acute abdomen, because early diagnosis and surgical intervention determine the prognosis of future fertility. Misdiagnosis and inappropriate treatment lead to female factor infertility. Conservative management include detorsion of the twisted segment. However, detorsion of the torsioned adnexes would have local and systemic consequences due to reperfusion of ovaries.[3] Many experimental studies on ischaemia reperfusion injury in animals suggest a preventative effect of antioxidants.[4] This improvement may be due to an adequate preservation of the cellular mitochondrial redox status. Nitric oxide (NO), a free radical, synthesized from l-arginine by nitric oxide synthase (NOS) in biological systems.[5] Depending on its redox state (6) and cellular source (7) NO may be toxic or protective. During ischemia and reperfusion, increased NO formation also occurs, and this can interact with XO-derived O^-_2, leading to the formation of peroxynitrite ($ONOO^-$).[8] $ONOO^-$ is a potent oxidant that can attack a wide variety of biological molecules and is produced in diverse inflammatory and pathological processes including postischemic injury.[8] Aminoguanidine (AMG), a nucleophilic hydrazine derivative, is currently under investigation as a selective inhibitor of the inducible nitric oxide synthase (iNOS). More recently, specific antioxidant properties of

aminoguanidine have been demonstrated.[9,10] Since there is no information is available regarding the action of aminoguanidine on the ovary, we studied the effects of AMG on rat adnexa subjected to torsion (ischemia) and detorsion (reoxygenation). To determine whether ischemia followed by subsequent reperfusion can induce ovarian oxidative damage, we created a model of adnexal ischemia/reperfusion in rat. We measured NO and lipid peroxidation products in ovarian homogenates obtained from control rats and from rats treated with AMG. Furthermore, ischemic injury and the effect of AMG administration were evaluated by histologic examinations of the ovaries.

Material and Methods

Thirty two adult female Wistar rats were obtained and divided equally into 4 groups (n=8) with varied treatment. Group I, sham; group II, torsion/detorsion plus saline; group III, torsion/detorsion plus AMG (200 mg/kg); group IV, torsion. Rats were anesthetized with ketamine (50 mg/kg body weight, i.p.) and xylazine (5 mg/kg body weight, i.p). After laparotomy the right ovary was found and rotated 360° clockwise and it was sutured to the abdominal muscles. Rats in the sham operation group underwent a surgical procedure similar to the other groups but the adnexa was not occluded. Rats were killed with an overdose injection of ketamine before the ovaries were harvested.

1. Sham operation group: After laparatomy, the rats were killed and the adnexa were surgically removed.

2. Saline/detorsion group: Saline was injected intraperitoneally (i.p.) 30 min before detorsion. The rats were killed after 3 h of reperfusion and the adnexa were removed.

3. AMG/detorsion group: AMG (200mg/kg) was injected i.p 30 min before detorsion. The rats were killed after 3 h of reperfusion and the adnexa were removed.

4. Torsion group: The adnexa were surgically removed after 3 h of torsion.

Biochemical Analysis

GSH, XO and MDA analysis: The ovary tissues were homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.5) with a homogenizer (IKA ultra turrax T 25 basic) at 16,000 r.p.m for 3 min. The homogenats were used to measure the levels of MDA, GSH, and XO. All procedures were performed at + 4 °C. MDA levels were assayed spectrophotometrically at 535 and 520 nm according to the method of Uchiyama and Mihara.[11] Results were expressed as nmol per gram wet tissue. GSH levels was measured by the method of Ellman.[12] GSH is reacted with 5,5 dithiobis-2-nitrobenzoic acid (DTNB) resulting in the formation of a product which has a maximal absorbance at 410 nm. Results were expressed as nmol per gram wet tissue. XO activity was determined spectrofotometrically by following in absorbancy at 292 nm upon the aerobic oxidation of xanthine to uric acid.[13] Results were expressed as units per mg protein.

NO analysis: Firstly, all ovarian tissue samples were deproteinized. Briefly, for every 200 μl sample, 400 μl of 10% zinc sulfate and 400 μl of 0.5 N sodium hydroxide was added. The samples were then vortexed and santrifuged at 25.000 g for 5 min at 4°C. For this study, 200 μl deproteinized aliquot or water blank was incubated in a final volume of 750 μl containing 75 μl of 0.32 mol/L potassium phosphate buffer (pH 7.5), 25 μl of nitrate reductase (10 U/ml, Sigma) with NADPH (50 μmol/L, Sigma) and FAD (50 μmol/L, Sigma) as coenzyme, and 650 μl of water for 2 hours. After reducing nitrate to nitrite with nitrate reductase, total nitrite was determined with Greiss reagent by mixing equal volumes of the reduced samples with Greiss reagent, 1:1 0.1% α-naphtylamine in water/ 1% p-aminobenzene sulfamide in 5% phosphoric acid. The samples were allowed to stand for 15 minutes and then read in a spectrophotometer at an absorbance of 548 nm. A range of sodium nitrite standards (0-100 μmol/L) was prepared, and a standard curve was used to convert sample measurements to micromoles per liter of nitrite. The reaction was linear from 0.25 to 100 μmol/L. Assays were performed as duplicates.[14] Results were expressed as nmol per gram wet tissue for NO. Protein levels were measured according to Lowry's method.

Histological analysis: At the end of each experiment the ovaries were removed and fixed in 10% neutral buffered formalin solution and then embedded in paraffin as usual. Serial sections were cut using the microtome at a thickness of 4 μm and stained with hematoxylin&eosin. The histologic sections were examined for the presence of intersititial edema, vascular dilatation, hemorrhage and PMNs infiltrations with a microscope and photographed.

Statistical analysis: The Statistical Package for Social Sciences (SPSS), version 10.0, was used for statistical analysis. Individual group parameters were assessed with One sample Kolmogorov Smirnov Z test and found abnormal ($p < 0.05$). The Kruskal-Wallis analysis of variance and post-hoc multiple comparison test (LSD) were performed on the data of the biochemical variables to examine differences between the groups. The results are given in the text as means ± STD. For all comparisons, statistical significance was defined as $p<0.05$.

Results

The ovarian tissues NO, MDA, GSH and XO activity levels are shown in Figure 1. The concentration of NO in the saline + I/R group (1130.2±528.5 nmol/g wet tissue) was significantly higher than that of the sham operation and ischemia groups ($p<.000$). In the AMG + I/R group the NO levels (465.5±132.0 nmol/g wet tissue) were significantly lower than in the saline + I/R ($p<.000$) and ischemia groups ($p<.049$). The levels of MDA and XO in the saline + I/R group (471.4 ± 132.9 nmol/g wet tissue, 108.6 ± 23.2 U/mg prot respectively) were significantly higher than that of the sham operation ($p<.000$) and ischemia groups ($p<.000$). In the AMG + I/R group the MDA level (392.5±50.8 nmol/g wet tissue) was significantly lower than in the saline + I/R group ($p<.043$), higher than the sham operation and ischemia groups ($p<.000$). The level of XO in the AMG group (78.2±16.7 U/mg prot) was significantly lower than the saline + I/R group ($p <.002$), higher
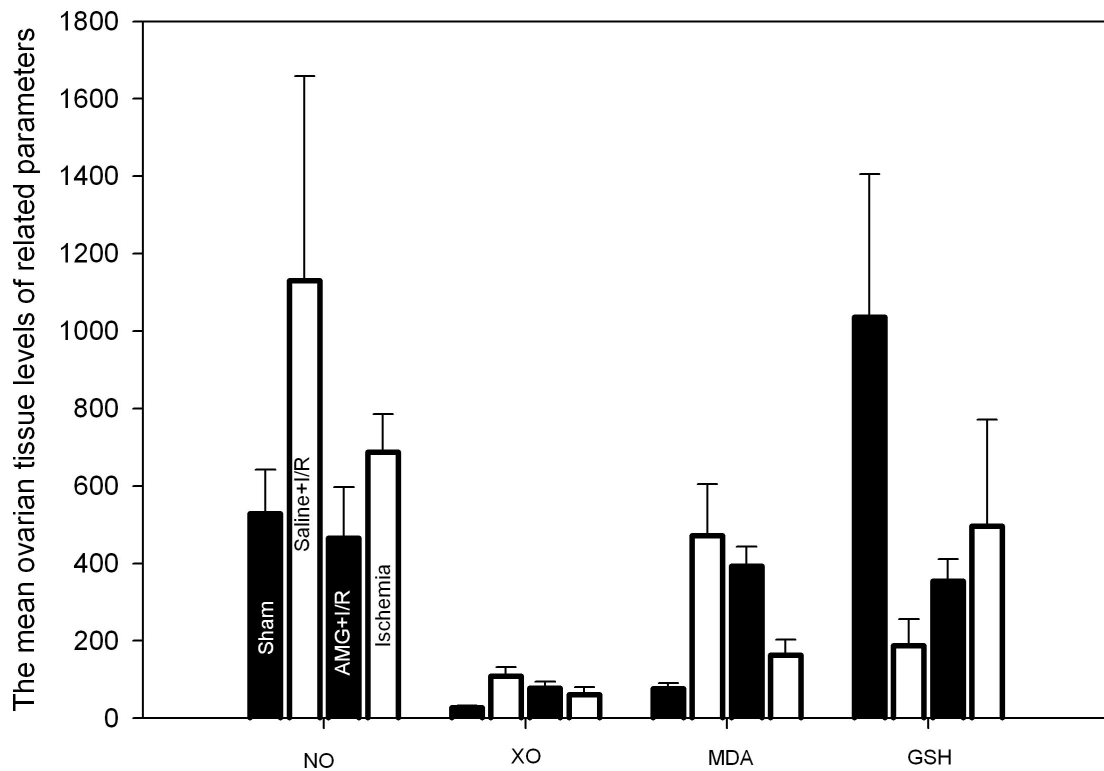
than the sham operation group (p<.000) but similar to the ischemia group. The level of GSH in the AMG + I/R group (354.4±56.7 nmol/g wet tissue) was significantly higher than the saline + I/R group (p<.016), lower than the sham operation group (p<.000) but similar to the ischemia group. Histopathological examination of the ovary in the ischemia group showed diffuse hemorrhage (Figure 2). Microscopic examination of the ovary after I/R revealed an acute infiltration by PMN, vascular dilatation and edema (Figure 3). Histologic analysis revealed a better conservation of ovarian morphology in rats treated with AMG (Figure 4) compared with the I/R and ischemia groups. The ovaries from sham operation group had a normal appearance.

Discussion

Nitric oxide is a very simple molecule produced by the activity of very complex enzymes, nitric oxide synthases (NOS). There are three NOS isoforms that are named after the tissue from which


Figure 2. Photomicrograph of ovary showed intense heamorrhage in ischemia group (H&E, x100).

they were first cloned and numbered in the order in which they were cloned.[15,16] Two of the NOS enzymes are present continuously and are thereby termed constitutive NOS (cNOS). Two types of cNOS exist: one, first detected in the vascular endothelium and called endothelial NOS (eNOS, type III NOS), and the other, initially localized in neu-
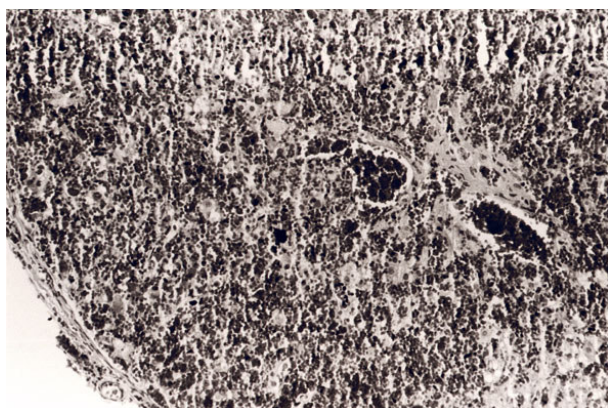
Figure 3. Microscopic examination of ovary after I/R. Notice striking infiltration by PMNs, vascular dilatation and edema (H&E, X 100).
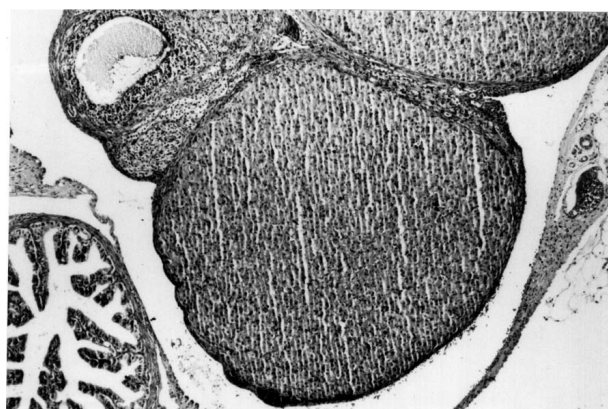
Figure 4. Histopathologic appearance of ovary in the I/R+Aminoguanidine group. Aminoguanidine administration reduced the PMN infiltration, edema, and vascular dilatation (H&E, x 100).

rons and called neuronal NOS (nNOS, type I NOS). Inducible NOS (iNOS, type II NOS) is not typically expressed in resting cells and is induced by various substances and conditions such as pro-inflammatory cytokines, bacterial lipoplysaccha-rides as weel as inflammation, hypoxy. However, overproduced NO may be toxic and damage nor-mal tissues.[17-20] Disruption of any of the physio-logical processes mediated by NO could have dele-terious effects on living tissues. NO may worsen damage by causing energy failure, damaging DNA[21,22] and forming the peroxynitrite anion (ONOO⁻). Because of an unpaired electron, NO is by definition a free radical. NO˙ also inhibits the enzyme activity of a number of enzymes including gluthathione peroxidase,[23] cytochrome c oxidase,[24] and NADPH oxidase.[25,26]

In the present study, it was showed that the levels of tissue NO were significantly increase after reperfusion. Compared to I/R group, NO ac-tivity was significantly lower in the AMG + I/R group. Since scavenging effect of aminoguanidine towards ˙OH and peroxyle (RO2 •) radicals has been demonstrated,[10] decrease in tissue NO levels in the AMG group could be attributable both by its capacity to scavenge free-radicals and its ability to inhibit iNOS gene expression. Yildiz et al.[27] report that AMG has direct scavenging activities against H_2O_2, HOCl, ˙OH and ONOO. Another explanation of reduced NO levels may be the result of the re-dox sensitive eukaryotic nuclear transcription fac-tor NF-kB activation. ROS that are generated dur-ing hypoxemia results in the activation of the tran-scription factor NFkB which translocates into the nucleus and induces the expression of genes regu-lated by NFkB.[28,29] Although the present study did not examine NF-kB, it is likely that it plays an important role in the induction of iNOS gene ex-pression in macrophages following hypoxemia.[30,31]

Lipid peroxidation is a free radical process that leads to the degradation of polyunsaturated fatty acids in cells. Elevated concentrations of MDA reflect the level of lipid peroxidation in tis-sues and increased MDA levels considered as a marker of tissue injury.[32] In the present study, the levels of tissue MDA, an index of lipid peroxida-tion, was significantly increase following ische-mia-reperfusion. On the other hand, MDA levels cleared by intraperitoneally administered AMG in the torsion/detorsion plus AMG group. Decrease in MDA levels in the AMG group is probably due its antioxidant and free radical-scavenging effect.[9,10] A recent experimental study performed in strepto-zotocin-induced diabetes in rats showed that AMG was able to decrease erythrocyte MDA levels.[33] Decreased levels of MDA thus suggest that AMG may have an additional beneficial effect as an anti-oxidant against lipid peroxidation in ovarian reper-fusion.

XO is an important source of oxygen free radicals in biological cells and tissues and has a particularly important role in oxygen radical generation and pathogenesis of injury following postischemic reperfusion.[2] Reduced blood supply is characterized by an overproduction of the superoxide anion. It led to the accumulation of hypoxanthine and stimulated XO activity in tissues. Another consequence of the inadequate perfusion is the conversion of xanthine dehydrogenase to xanthine oxidase. Then with the onset of reperfusion there is a concomitant increase in ROS production by xanthine oxidase. The present study demonstrated that there was a significant increase in the XO levels compared to AMG +I/R group. Since antioxidant properties of AMG are well documented, decrease in XO levels in the AMG group is probably due its antioxidants effect. Philis-Tsimikas et al.[34] reported an antioxidant effect of AMG at high concentrations on the LDL peroxidation initiated by copper ions. A recent experimental study performed in rat retina cells are submitted to an oxidative stress in the presence of AMG, both free radical production and lipid peroxidation product formation are inhibited.[35] Another possible explanation of decreased XO levels may be that AMG may act as an inhibitig factor in the process of XDH to XO conversion during ovarian reperfusion.

All cells contain some enzymatic and non-enzymatic antioxidant defense mechanisms to protect themselves from hazardous effects of the oxidative attack. This enzymatic scavengers which include SOD, CAT, GSH-Px are widely distributed within cells. The balance between ROS and antioxidants is constantly challenged by the external and internal environments and plays an important role in maintaining the redox homeostasis of the living system.[36] GSH, an essential component of the cellular defense mechanisms agaisnt radical-mediated tissue injury, has been used as indicator of oxidative stress induced by ROS in the proccess of ischemia reperfusion.[37] In our study, a reduction in GSH levels occured after a period of ischemia followed by reperfusion. Depletion of tissue GSH

content enhance cellular damage caused by oxidant stress.[38] The drop in GSH levels during I/R was probably due to conversion of GSH to GSSG. Because detoxification of these radical species involves conversion of GSH to GSSG. Increased GSH levels in the AMG group is probably due its antioxidant and free radical-scavenging effect.

It is known that ROS are involved in the pathogenesis of I/R injury.[39] They are associated with both local and remote changes in vascular permeability. In I/R group, the number of PMN and degree of vascular dilatation and edema were elevated over than that of the sham ovaries with the effect being greatly reduced by AMG (Figure 3) Those effects of AMG may occur through its inhibitor effect on iNOS expression and antioxidant properties.

The protective effect of AMG on induced tissue damage due to NO produced by iNOS has been observed previously in a number of experimental models but this is the first study in which it has been shown to be protective againist I/R injury in ovarian tissue. The results of the present study indicate that reperfusion caused increased production of MDA, XO and iNOS-derived NO by macrophages. Because elevated lipid peroxidation products and NO levels have been implicated in producing cellular and organ dysfunction therapeutic interventions that specifically depress iNOS activity following reperfusion may be helpful in decreasing tissue damage after reperfusion. As it occurs with the histological findings, aminoguanidine treatment seems to afford protection against oxidative stress as observed by the increased levels of reduced glutathione and decreased levels of NO, MDA and XO.

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