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The Effect of Agomelatine on Oxidative Stress Induced with Ischemia/Reperfusion in Rat Ovaries

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Ovarian ischemia and reperfusion can lead to serious and irreversible health problems.
Objectives. The aim of this study is to investigate the protective effect of agomelatine against ovarian ischemia/ /reperfusion injury in rats using biochemical methods.
Material and Methods. Thirty female rats were divided into three groups (the number of animals in each group = 10), a control group in which ischemia/reperfusion was established (IRC), an ischemia/reperfusion + agomelatine (IRA) group and a healthy group given a sham operation (SG). Total glutathione (tGSH) and malondialdehyde (MDA) levels and glutathione peroxidase (GPx), superoxide dismutase (SOD) and myeloperoxidase (MPO) enzyme activity were measured in ovarian tissue extracted at the end of the experiment.
Results. Biochemical results revealed MDA levels of 19.1 ± 2.03, 5.8 ± 1.5 and 5.5 ± 1.4 μmol/g protein in ovarian tissue in the IRC, IRA and SG groups, respectively. MPO activity in the IRC, IRA and SG groups was 7.87 ± 2.7, 4.0 ± 2.0 and 3.0 ± 1.0 U/g protein, respectively. tGSH levels were 1.87 ± 1.13, 4.37 ± 1.4 and 5.87 ± 1.64 nmol/g protein, respectively. GPx activity in the IRC, IRA and SG groups was 7.37 ± 1.68, 18.6 ± 3 and 17.75 ± 3.2 U/g protein, and SOD activity 31.1 ± 2.9, 45.3 ± 3.7 and 54 ± 4.2 U/g protein, respectively. The level of 8-OH/ /Gua, a product of DNA damage, was 2.18 ± 0.2 pmol/L in the IRC group, 1.28 ± 0.2 pmol/L in the IRA group and 0.93 ± 0.01 pmol/L in the SG group.
Conclusions. Agomelatine prevented ovarian ischemia/reperfusion injury **(Adv Clin Exp Med 2014, 23, 5, 715–721).**

Key words: agomelatine, rat, ovary, antioxidants, ischemia, reperfusion.

Ischemia emerges as the result of subtotal or total interruption of blood flow to tissue for various reasons. Ovarian ischemia is a pathological condition seen as a result of the ovary twisting around its own vascular structures (torsion) [1]. Prolonged exposure to ischemia may lead to irreversible damage in ovarian tissue. In order to prevent irreversible damage, the ovary exposed to torsion is reperfused through detorsion [2]. However, reperfusion of ischemic tissue may lead to more severe injury than that caused by ischemia in the tissue [3]. High levels of molecular oxygen and overproduction of toxic oxygen radicals with reperfusion of ischemic tissue are implicated as causes of reperfusion injury. Overproduction of toxic oxygen radicals causes severe damage in cells by leading to lipid peroxidation and DNA damage [4]. The use of drugs with antioxidant activity is therefore being tested in the prevention and treatment of ischemia/reperfusion injury [5, 6]. The agomelatine used in our study is a synthetic analogue of melatonin. Like melatonin, agomelatine stimulates MT_1 and MT_2 membrane receptors [7]. Turkoz et al. reported that melatonin prevents ovarian torsion-associated ischemia/ /reperfusion in animals by lowering MDA levels and raising GSH [8]. Melatonin is also known

to exhibit a powerful antioxidant effect through ML1 receptors. Membrane ML1 receptors are also known to be present in ovarian tissue [9]. The fact that agomelatine both stimulates melatonin receptors and antagonizes serotonin receptors indicates that the drug may be useful in the prevention of ischemia/reperfusion injury.

Scans of the literature revealed no data concerning the protective effect of agomelatine against oxidative damage induced with ischemia/reperfusion in rat ovaries. The purpose of this study was therefore to investigate whether agomelatine does have a protective effect against oxidative stress induced with ischemia/reperfusion in rat ovaries.

Material and Methods

Animals

The study was performed with 30 female albino Wistar rats weighing 225–235 g. These were obtained from the Atatürk University Medical Experimental Practice and Research Center. In order to adapt to their environment, animals were kept and fed for a while at normal room temperature (22°C) in the Department of Pharmacology laboratory where the experiment was to be conducted.

Chemical Substances

Agomelatine 25 mg tablets were obtained from Les Laboratoires Servier, France. All other chemical substances used for the experiments were provided by IE Ulagay, Turkey.

General Procedure

The female rats to be used in the experiment were divided into 3 groups, a control group (N = 10) in which ischemia/reperfusion was to be established (IRC), an ischemia/reperfusion + + agomelatine (IRA) group (N = 10) and a healthy group (N = 10) given a sham operation (SG). Surgical procedures were performed with 25 mg/kg intraperitoneal (*i.p.*) thiopental sodium anesthesia.

Surgical Procedures

Before rat ovaries were subjected to the ischemia/reperfusion procedure, the IRA group was given a 25 mg/kg dose of agomelatine by tube. The IRC and SG group rats were given distilled water as solvent by the same route. Once the drugs had been administered, the inferior part of the abdomen was accessed through a 2–2.5 cm vertical incision. An arterial clip was later attached to the lower part of the right ovary (the region where the ovary joins the uterus) of the rats in the IRC and IRA groups, and ischemia was established for 3 h. No ischemia was established in the SG ovaries. At the end of that period the arterial clip was removed and 2-h reperfusion was established. Once reperfusion had been established all animals were sacrificed with high-dose anesthesia, and biochemical analyses were performed. The biochemical findings from the IRA group were analyzed by comparing them with those from the IRC and SG groups.

Biochemical Analysis of Ovarian Tissue

Specimen Preparation

At this stage, 0.2 g were weighed from each ovary extracted. The ovaries were homogenized in 0.5% HDTMAB (0.5% hexadecyltrimethylammonium bromide) containing pH = 6 potassium phosphate buffer for MPO assay, 1.15% potassium chloride solution for MDA assay and pH = 7.5 phosphate buffer for other measurements, all made up to 2 mL in an iced environment. They were subsequently centrifuged at 10,000 rpm for 15 min at +4°C. The supernatant part was used as a specimen for analysis.

Chemical Parameter Screening

Malondialdehyde (MDA) Assay

Based on spectrophotometric measurement at an emission wavelength of 532 nm of the absorbance of the pink complex formed at high temperature (95°C) by thiobarbituric acid (TBA) and MDA [10].

Myeloperoxidase (MPO) Activity Assay

Oxidation reaction performed with MPO-mediated H_2O_2 containing 4-aminoantipyrene/phenol solution as substrate was used to determine MPO enzyme activity [11].

Total Glutathione (tGSH) Assay

DTNB [5,5'-Dithiobis (2-nitrobenzoic acid)] is a disulfide chromogen easily reduced by sulfhydryl group compounds. The resulting yellow color is measured spectrophotometrically at 412 nm [12].

Glutathione Peroxidase (GPx) Assay

The enzyme GPx reduces H_2O_2 to water in the presence of H_2O_2 and catalyzes the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). The GSSG that forms reduces glutathione reductase again to GSH through the glutathione reductase reaction in which it is used as reducing substrate of NADPH. The absorbance decrease resulting from the oxidation of NADPH to NADP is measured spectrophotometrically at 340 nm and GPx enzyme activity calculated from this [13].

Superoxide Dismutase (SOD) Activity Assay

This is based on measurements at a wavelength of 560 nm of the absorbance of formazan, a purple compound forming with the reduction of nitroblue tetrazolium (NBT) in the reaction environment by O_2^- radicals produced by the addition of the enzyme xanthine oxidase to the reaction environment. The intensity of the reduction reaction depends on the activity of the Cu/Zn SOD enzyme activity in the specimen. The more enzyme in the environment, the less O_2^- radical there is to react with NBT. The intensity of the purple color emerging with the presence of the formazan thus decreases in proportion [14].

Isolation of DNA from Ovarian Tissue

Ovarian tissue was drawn and DNA isolated using Shigenaga et al.'s modified method [15]. Samples (50–200 mg) were homogenized at 4°C in 1 mL of homogenization buffer (0.1 mol/L NaCl, 30 mmol/L, Tris, pH 8.0, 10 mmol/L EDTA, 10 mmol/L 2-mercaptoethanol, 0.5% (vol/vol) Triton X-100) with 6 passes of a Teflon-glass homogenizer at 200 rpm. The samples were centrifuged at 4°C for 10 min at 1000 g to pellet nuclei. The supernatant was discarded, and the crude nuclear pellet re-suspended and re-homogenized in 1 mL of extraction buffer (0.1 mol/L Tris, pH 8.0, 0.1 mol/L NaCl, 20 mmol/L EDTA) and re-centrifuged as above for 2 min. The washed pellet was resuspended in 300 µL of extraction buffer with a wide-orifice 200 µL Pipetman tip (Eppendorf, Germany). The resuspended pellet was subsequently incubated at 65°C for 1 h with the presence of 0.1 mL of 10% sodium dodecyl sulfate, 40 µL proteinase K, and 1.9-mL leukocyte lysis buffer. Afterward, ammonium acetate was added to the crude DNA sample to give a final concentration of 2.5 mol/L, and centrifuged in a microcentrifuge for 5 min. The supernatant was removed and mixed with 2 volumes of ethanol to precipitate the DNA fraction. After centrifugation, the pellet was dried under reduced pressure and dissolved in sterile water. The absorbance of this fraction was measured at 260 and 280 nm. Purification of DNA was determined as A260/280 ratio 1.8.

DNA Hydrolysis with Formic Acid

Approximately 50 mg of DNA was hydrolyzed with 0.5 mL of formic acid (60%, v/v) for 45 min at 150°C [16]. The tubes were allowed to cool. The contents were then transferred to Pierce micro-vials, covered with Kleenex tissues cut to size (secured in place using a rubber band), and cooled in liquid nitrogen. Formic acid was then removed by freeze-drying. Before analysis by HPLC, they were re-dissolved in the eluent (final volume 200 µL).

Measurement of 8-Hydroxy-2 Deoxyguanine (8-OH Gua) with a High Performance Liquid Chromatography (HPLC) System

The amount of 8-OH gua and guanine (Gua) was measured by using a HPLC system equipped with an electrochemical detector (HP Agilent 1100 module series, E.C.D. HP 1049 A), as described previously [16].

Statistical Analysis

The results obtained from the experiments are expressed as "mean ± standard deviation" (x ± SD). One-way ANOVA was used to determine degree of significance in differences between groups. Fisher's *post hoc* least significant differences (LSD) test was then performed. All statistical procedures were performed on SPSS 18.0. Significance was set at $p < 0.05$.

Results

As shown in Fig. 1, the level of MDA in the IRC group ovarian tissue in which ischemia/reperfusion was established was 19.1 ± 2.03 µmol/g protein, compared to 5.8 ± 1.5 µmol/g protein in the IRA group receiving agomelatine. The MDA level in ovarian tissue in the SG sham operation group was 5.5 ± 1.4 µmol/g protein. There was a statistically significant difference between the IRA group and the IRC group in terms of MDA

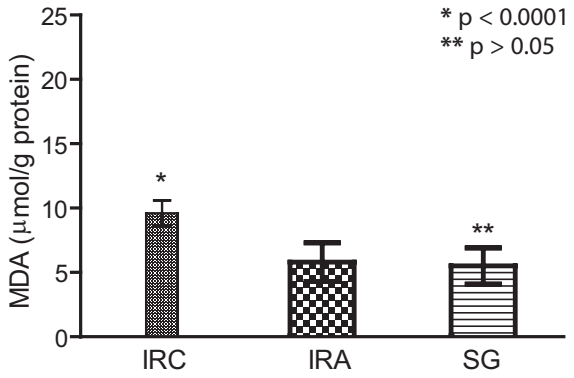

Fig. 1. Comparison of malondialdehyde (MDA) levels of groups. MDA levels defined in µmol/g protein. Bars are means ± standard deviation. IRC, control group in which ischemia/reperfusion would be established; IRA, ischemia/reperfusion + agomelatine treated group; SG, healthy group given a sham operation

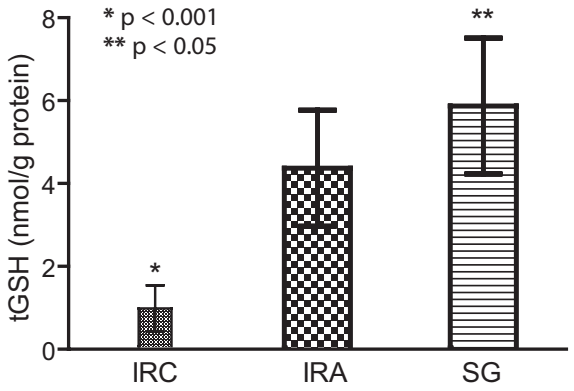

Fig. 3. Comparison of total glutathione (tGSH) levels of groups. tGSH levels defined in nmol/g protein. Bars are means ± standard deviation. IRC, control group in which ischemia/reperfusion would be established; IRA, ischemia/reperfusion + agomelatine treated group; SG, healthy group given a sham operation

levels ($p < 0.0001$) but no significant different with the SG group ($p > 0.05$). MPO activity in the IRC, IRA and SG groups' ovarian tissues was 7.87 ± 2.7, 4.0 ± 2.0 and 3.0 ± 1.0 U/g protein, respectively (Fig. 2). tGSH levels were 1.87 ± 1.13, 4.37 ± 1.4 and 5.87 ± 1.64 nmol/g protein, again respectively (Fig. 3). There was a statistically significant difference between the IRA group and the IRC group in terms of MPO activity ($p < 0.001$) but none with the SG group ($p > 0.05$). There was a statistically significant difference in terms of tGSH levels between the IRA group and the IRC group ($p < 0.001$) and also the SG group ($p < 0.05$). GPx activity in the IRC, IRA and SG groups was 7.37 ± 1.68, 18.6 ± 3 and 17.75 ± 3.2 U/g protein, respectively (Fig. 4), and SOD activity 31.1 ± 2.9, 45.3 ± 3.7 and 54 ± 4.2 U/g protein,

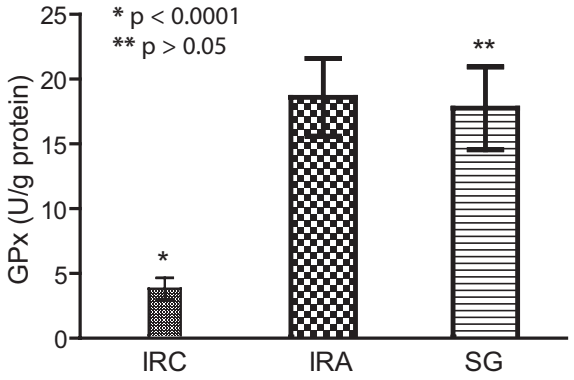

Fig. 4. Comparison of glutathione peroxidase (GPx) enzyme activities of groups. GPx enzyme activities defined in U/g protein. Bars are means ± standard deviation. IRC, control group in which ischemia/reperfusion would be established; IRA, ischemia/reperfusion + agomelatine treated group; SG, healthy group given a sham operation

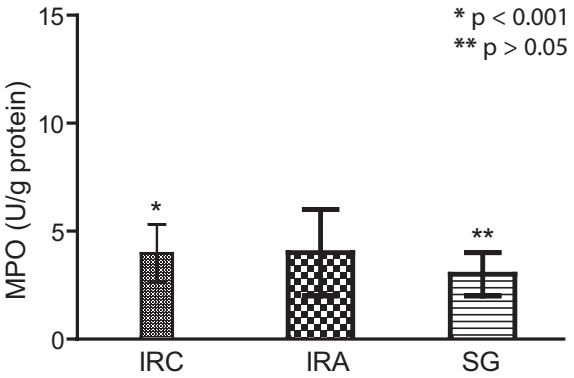

Fig. 2. Comparison of myeloperoxidase (MPO) enzyme activities of groups. MPO enzyme activities defined in U/g protein. Bars are means ± standard deviation. IRC, control group in which ischemia/reperfusion would be established; IRA, ischemia/reperfusion + agomelatine treated group; SG, healthy group given a sham operation

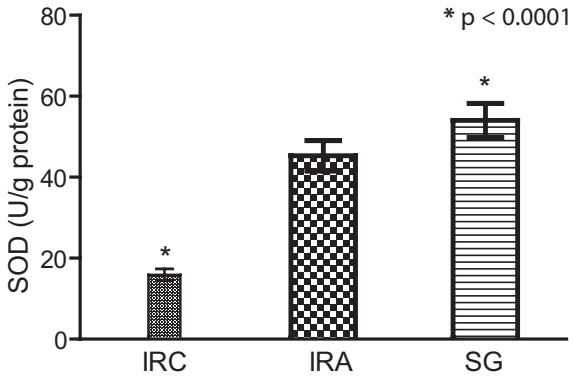

Fig. 5. Comparison of superoxide dismutase (SOD) enzyme activities of groups. SOD enzyme activities defined in U/g protein. Bars are means ± standard deviation. IRC, control group in which ischemia/reperfusion would be established; IRA, ischemia/reperfusion + agomelatine treated group; SG, healthy group given a sham operation

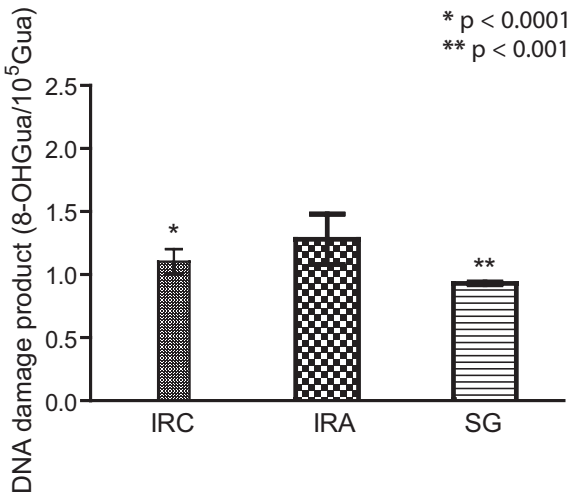

Fig. 6. Comparison of DNA damage product levels of groups. DNA damage product levels defined in 8-OHGua/10^5Gua. Bars are means ± standard deviation. IRC, control group in which ischemia/reperfusion would be established; IRA, ischemia/reperfusion + agomelatine treated group; SG, healthy group given a sham operation

respectively (Fig. 5). The difference in GPx activity was statistically significant between the IRA and IRC groups ($p < 0.0001$), but there was no statistically significant difference with the SG group ($p > 0.05$). There was a statistically significant difference in SOD activity between the IRA group and the IRC ($p < 0.0001$) and SG groups ($p < 0.0001$). Levels of 8-OH/Gua, a product of DNA damage, were 2.18 ± 0.2 pmol/L in the IRC group, 1.28 ± 0.2 pmol/L in the IRA group and 0.93 ± 0.01 pmol/L in the SG group (Fig. 6). There was a significant difference in terms of DNA damage product between the IRA group and the IRC ($p < 0.0001$) and SG ($p < 0.001$) groups.

Discussion

This study investigated the protective effect of agomelatine against oxidative stress induced with ischemia/reperfusion in rat ovaries. Ovarian ischemia emerges as the result of torsion of the ovaries for various reasons. The detorsion procedure performed in order to reperfuse the ischemic ovaries leads to reperfusion injury. Organ-protective surgery is most appropriate in young girls, since ovarian functions need to be protected [17]. Therefore, detorsion with a conservative treatment option is increasingly recommended [18]. The fact that oxidative injury is more severe in ischemic ovaries not receiving antioxidant treatment and in which post-ischemic reperfusion was established shows the importance of conservative therapy before surgery [5, 6]. As our experimental results show, agomelatine therapy significantly prevented oxidative injury arising in the right ovaries of rats in which ischemia/reperfusion was established. We elected to use the right-side ovaries because in literature the pathology is in the right ovary in 2/3 of patients [19]. Agomelatine significantly reduced the ischemia/reperfusion-associated rise in MDA in ovarian tissue compared with the IRC group. MDA is the final product of lipid peroxidation and one of the important parameters in the evaluation of ischemia/reperfusion associated oxidative injury [20]. Lipid peroxidation leads to impaired cell membrane permeability, decreased membrane potential and cell damage. Cell damage further intensifies with MDA formation [21].

Another parameter known as an oxidant in the cell is the enzyme MPO. In the presence of chloride ions, MPO reduces hydrogen peroxide to hypochlorous acid. Hypochlorous acid is a powerful oxidant and leads to tissue damage as it can easily enter into reaction with several biological molecules [22]. The fact that MPO activity in ovarian tissue receiving agomelatine was lower than in the IRC group reveals that it protects ovarian tissue against ischemia/reperfusion injury. Kumbasar et al. showed that MPO activity in ovarian tissues in which ischemia/reperfusion was established was lower compared to that in healthy tissues [23]. This information from the literature agrees with our own experimental results and indicates that agomelatine is effective in the prevention of ovarian ischemia/reperfusion injury.

The level of GSH in the IRA group receiving agomelatine was significantly higher than that in the IRC group. There are known to be various antioxidant defense mechanisms that eliminate the harmful effects of toxic oxidants in tissues. The oxidant/antioxidant balance altering in favor of oxidants leads to oxidative tissue damage. The important effects of antioxidants protect against oxidation in target molecules such as proteins, nucleic acids and carbohydrates, as well as cell membrane lipids [24]. GSH is an important antioxidant and reducing agent. It protects cells against oxidant damage by entering into reactions with glutathione, free radicals and peroxides. It prevents protein oxidation by holding SH groups in an unreduced form [25]. Drugs that protect against ischemia/reperfusion injury in ovarian tissue have been shown to prevent a decrease in GSH in ovarian tissue [26]. The mechanisms of injury established by ischemia/reperfusion in many organs, such as the brain, heart, lungs, liver, stomach, ovaries and intestines, have been investigated and free radicals have been reported to be one of the major components of ischemia/reperfusion injury [27, 28]. This information from the literature is also compatible

with our study findings. GPx and SOD activity were significantly higher in ovarian tissue in which ischemia/reperfusion injury was established and receiving agomelatine compared to the IRC group. GPx is one of the important enzymatic antioxidant parameters establishing reduction of peroxide and organic hyperoxides [29].

Agomelatine significantly prevented DNA oxidation in ovarian tissue in which ischemia/reperfusion injury was established. The product reflecting oxidation of DNA in tissue is 8-hydroxyguanidine (8-OHGua) [30]. Studies have shown that 8-OH -Gua levels in damaged tissue rise in parallel to increases in oxidant parameters [3]. These facts from the literature are also in agreement with our results. In conclusion, ischemia/reperfusion leads to oxidative damage in ovarian tissue. Agomelatine significantly prevented the oxidative damage caused by ischemia/reperfusion in ovarian tissue. This in turn shows that agomelatine may be useful in the prevention of damage associated with ovarian torsion and detorsion.

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