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The Effect of Controlled Reperfusion in the Prevention of Infertility Caused by Ischemia Induced in the Contralateral Ovary in Rats with Unilateral Ovariectomy

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Key Words
Ischemia · Ovary · Reperfusion · Rat · Sterility

Abstract
Background/Aims: To investigate the effectiveness of controlled reperfusion (CR) on ovarian tissue malondialdehyde, total glutathione and 8-hydroxyguanine levels and infertility rates in a rat model of induced ischemia-reperfusion (I/R) injury with unilateral oophorectomy. ***Methods:*** A total of 135 adult female albino Wistar rats were divided into 9 groups (n = 15 for each group): unilateral ovariectomy + ovarian I/R (OIR), unilateral ovariectomy alone (OEG), a sham operation group (SG), and unilateral ovariectomy + CR performed at different intervals (the clips were released 10 times for 10, 8, 6, 4, 2 or 1 s and closed again 10 times for 10, 8, 6, 4, 2 or 1 s; OCR-1–6, respectively). Five rats from each group were sacrificed, and their ovaries were removed. ***Results:*** Higher ovarian tissue malondialdehyde and 8-hydroxyguanine levels and lower ovarian tissue total glutathione levels were found in the OIR group compared with the SG, OEG and OCR-4–6 groups. The number of rats giving birth during the study period was found to be similar among the SG (n = 8), OEG (n = 8) and OCR-6 (n = 7) groups. ***Conclusion:*** These results suggest that sterility and ovarian oxidative stress caused by I/R injury decreases in parallel to the shortening of CR duration.

Introduction

Ovarian ischemia generally occurs as a result of ovarian torsion (twisting). It is a pathological condition leading to the obstruction of the ovarian artery that supplies blood to the ovaries [1, 2]. Ovarian torsion is seen in cysts or neoplasms, in an excessively long adnexal mesovarium, in conditions that lead to adnexal venous congestion and in tubal diseases. Delays in the diagnosis and treatment of ischemic ovaries may result in ovariectomy [3].

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Torsion in the contralateral ovary may lead to sterility in girls who have undergone unilateral ovariectomy. Early diagnosis and treatment of ovarian torsion therefore plays an important role in the preservation of ovarian functions [4]. Detorsion of the twisted adnexa and evaluation of the tissue reperfusion are proposed to prevent future infertility in the case of adnexal torsion. This ovarian torsion-detorsion process is called ischemia-reperfusion (I/R) injury [1]. The primary purpose of the reperfusion procedure applied to ischemic ovaries is to provide urgent protection against life-threatening complications from ischemia [5]. In addition, the reperfusion procedure is a surgical technique intended to restore normal ovarian functions and to prevent potential sterility [6].

Moreover, reperfusion injury oxidizes cell membrane lipids and causes the formation of excessive amounts of reactive oxygen species (ROS) and malondialdehyde (MDA) [7]. Excessive production of ROS and their toxic molecules lead to cell damage by the interaction with lipids, proteins and nucleic acids [8]. Also, increased ROS and MDA levels lead to alkaline changes in nucleic acids and chain breaks in DNA; thus, DNA oxidative damage occurs [9]. One of the oxidative damage products of DNA, 8-hydroxyguanine (8-OH-Gua), is used as an indicator of DNA damage [9]. Various antioxidants such as glutathione (GSH) and superoxide dismutase increase to eliminate ROS and to reduce potential oxidative cell damage [8]. However, it has been reported that reperfusion following ischemia suppresses the ovarian GSH levels [10]. These findings suggest that reperfusion may cause severe compromise of organ and tissue functions, and researchers have reported more severe injury in tissue following reperfusion than that caused by the ischemia [11, 12].

Sterility has been shown to develop in rats undergoing reperfusion of ovarian tissue [13]. Sterility is a significant finding that indicates severe ovarian function impairment. Various techniques have therefore been developed in order to reduce reperfusion injury to a minimum [11, 14]. Controlled reperfusion (CR) has been shown to be beneficial in the prevention of ovarian ischemic injury [11].

We hypothesized that CR applied at different intervals to ischemic ovarian tissue may be useful in preventing tissue damage and sterility due to an ovarian I/R injury. Therefore, we evaluated contralateral ovarian GSH, MDA and 8-OH-Gua levels and fertility rates following a CR procedure applied at different intervals after an I/R injury in a rat model with unilateral oophorectomy.

Materials and Methods

Animals

This study was performed in accordance with the National Institutes of Health's approved guidelines, and the study protocols were approved by the Animal Research Ethics Committee of Ataturk University (protocol No. 35). For this study, 135 adult female albino Wistar rats weighing 200–208 g were purchased from the Ataturk University Medical Experimental Application and Research Centre. The animals were housed between 20 and 22°C under a 12-hour light/12-hour dark cycle. They had free access to water and were given a standard pellet diet for rats.

Experimental Groups

All rats were divided into 9 groups (n = 15 for each group): unilateral ovariectomy + ovarian I/R (OIR), unilateral ovariectomy alone (OEG), a sham operation group (SG), and unilateral ovariectomy + CR performed for different intervals (OCR-1–6; the ovarian artery being opened and closed 10 times at intervals of 10, 8, 6, 4, 2 and 1 s, respectively).

Anesthesia

Surgical procedures were performed under sterile conditions in an appropriate laboratory environment under 25 mg/kg intraperitoneal thiopental sodium (Pentotal®, IE Ulagay, Istanbul, Turkey). Following the administration of anesthesia, sufficient time was allowed to elapse before the surgical procedure was performed. Once the animals remained immobile in the prone position, this was regarded as the appropriate time for surgery [11].

Surgical Procedures

The ovaries of rats in the OEG, SG, OIR and OCR-1–6 groups (n = 15 for each group) were accessed through a vertical incision 2–2.5 cm in length in the lower abdomen. No procedure was performed on the ovaries of the SG group rats, and the lower abdominal incision was sutured and closed. The left ovaries were extracted from the rats in the OEG, OIR and OCR-1–6 groups. Subsequently, the lower abdominal incision in the OEG group was sutured with no procedure performed on the right ovary. Clips were attached to the lower part of the right ovaries in the OIR and OCR-1–6 groups, and ischemia was established for 2 h. At the end of that period, the OIR group ovaries were reperfused for 2 h. Before reperfusion was established in the OCR-1–6 groups, the clips were released 10 times for 10, 8, 6, 4, 2 or 1 s and closed again 10 times for 10, 8, 6, 4, 2 or 1 s in order to apply CR. Following CR, normal reperfusion was established for 2 h. At the end of that period, 5 rats from each group were sacrificed with a high dose of anesthesia (intraperitoneal thiopental with a dose of 50 mg/kg). Then, the ovaries were removed for biochemical analysis. All remaining rats (n = 10 for each group) were kept in a normal laboratory environment for 10 days postoperatively. They were subsequently kept in an appropriate laboratory environment together with male rats for 3 months in order to reproduce. Rats that did not become pregnant and give birth during that period were regarded as sterile [13].

The removed ovaries were washed with 0.9% NaCl and then stored at –80°C for biochemical analysis to determine MDA levels as an indicator of lipid peroxidation, total GSH (tGSH) as a nonenzymatic endogenous antioxidant and 8-OH-Gua levels as an indicator of DNA damage.

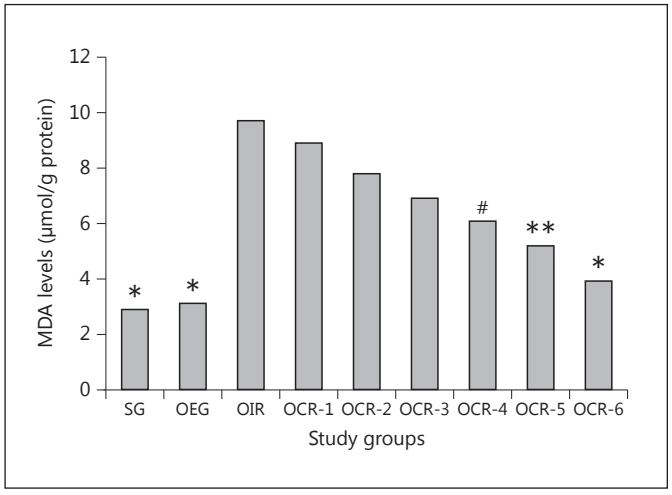

Fig. 1. MDA levels in the study groups. * $p < 0.0001$, ** $p < 0.001$, # $p < 0.05$ compared with the OIR group.

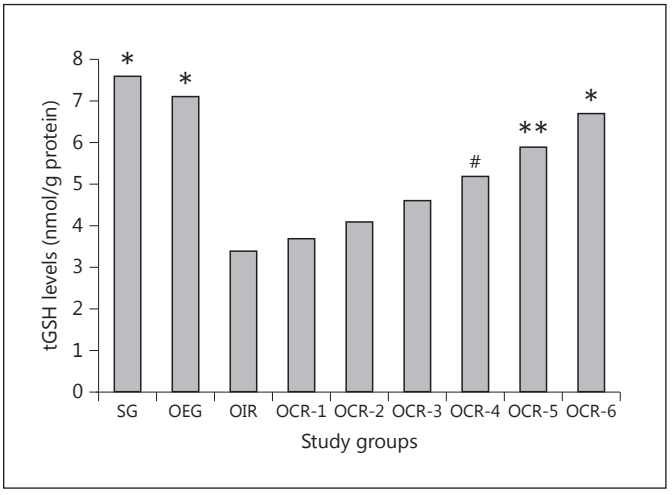

Fig. 2. tGSH levels in the study groups. * $p < 0.0001$, ** $p < 0.001$, # $p < 0.05$ compared with the OIR group.

Biochemical Analysis

Tissues were made up to 9 ml in a 1.15% potassium chloride solution for the MDA assay and in a pH 7.5 phosphate buffer for the tGSH assay and homogenized in 0.9% NaCI solution (10% wt/vol) with an Omni International (Warrenton, Va., USA) tissue homogenizer. Obtained tissue homogenates were centrifuged for 15 min at 18,000 *g*, and then the supernatants were removed for analysis. The protein levels of homogenates were determined according to the method described by Bradford [15]. All spectrophotometric measurements were performed using a Beckman DU 530 spectrophotometer (Beckman Coulter, Fullerton, Calif., USA).

Ovarian Tissue MDA Analysis

Tissue MDA levels were determined spectrophotometrically according to the method described by Ohkawa et al. [16]. This method is based on the spectrophotometric measurement at a wavelength of 532 nm of the absorbance of the pinkish complex formed by thiobarbituric acid with MDA at a high temperature (95°C). Results were expressed as μmol/g protein.

Ovarian Tissue tGSH Analysis

Tissue tGSH levels were measured according to the method described by Sedlak and Lindsay [17]. DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] in the measurement environment is a disulfide chromogen that is easily reduced by sulfhydryl group compounds. The resulting yellow color was measured spectrophotometrically at 412 nm. Results were expressed as nmol/g protein.

Measurement of Ovarian Tissue 8-OH-Gua Levels

Initially, the DNA was isolated from the ovarian tissue according to the method described by Shigenaga et al. [18]. The sample of DNA (50 mg) was hydrolyzed with 0.5 ml of formic acid (60%, v/v) for 45 min at 150°C [19]. Formic acid was removed by freeze-drying. After the DNA samples were redissolved in the eluent (final volume, 200 μl), the amount of ovarian tissue 8-OH-Gua was measured with a high-performance liquid chromatography system equipped with an electrochemical detector (HP Agilent 1100 module series, E.C.D. HP 1049 A), according to the method described previously [19, 20]. Results were expressed as the number of 8-OH-Gua molecules/10^5 guanine molecules (pmol/l) [21].

Statistical Analysis

All data were analyzed using one-way ANOVA on SPSS 18.0 software. Differences among groups were determined using the Tukey multiple comparison test, and significance was set at $p < 0.05$.

Results

Biochemical Results

Ovarian Tissue MDA, tGSH and 8-OH-Gua Levels

Ovarian tissue MDA levels (μmol/g protein) were found to be higher in the OIR group (9.7 ± 0.74) compared with the SG (2.9 ± 0.07), OEG (3.1 ± 0.09), OCR-4 (6.1 ± 0.38), -5 (5.2 ± 0.27) and -6 (3.9 ± 0.09) groups ($p < 0.05$ for the OCR-4 group, $p < 0.001$ for the OCR-5 group and $p < 0.0001$ for the other groups). However, the OCR-1 (8.9 ± 0.66), -2 (7.8 ± 0.43) and -3 (6.9 ± 0.35) groups had similar ovarian tissue MDA levels compared to the OIR group ($p > 0.05$ for all; fig. 1).

Ovarian tissue tGSH levels (nmol/g protein) were found to be lower in the OIR group (3.4 ± 0.72) compared with the SG (7.6 ± 0.67), OEG (7.1 ± 0.71), OCR-4 (5.2 ± 0.62), -5 (5.9 ± 0.67) and -6 (6.7 ± 0.74) groups ($p < 0.05$ for the OCR-4 group, $p < 0.001$ for the OCR-5 group and $p < 0.0001$ for the other groups). However, the OCR-1

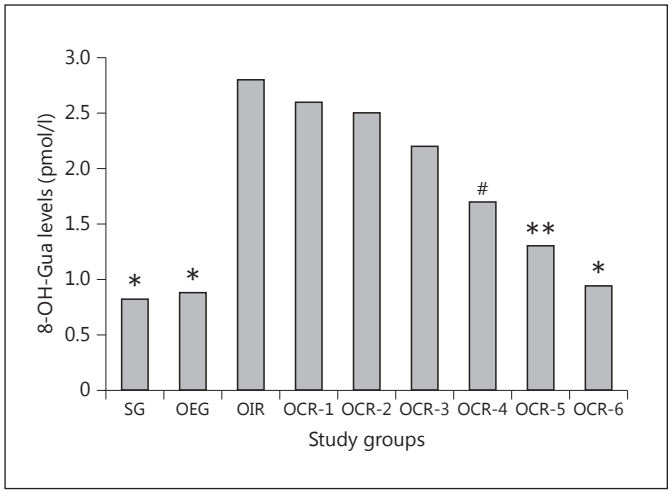

Fig. 3. 8-OH-Gua levels in the study groups. * $p < 0.0001$, ** $p < 0.001$, # $p < 0.05$ compared with the OIR group.

Table 1. Fertility rate and mean time intervals until birth in the study groups

Group (n = 10 for each group)	Rats giving birth	Time until birth, days
SG	8 (80)	50.3±3.6
OEG	8 (80)	58.6±8.5[b]
OIR	0	–
OCR-1	0	–
OCR-2	0	–
OCR-3	0	–
OCR-4	3 (30)[a, c]	80.0 ± 4.5[a, c]
OCR-5	4 (40)[a, c]	74.2 ± 3.7[a, c]
OCR-6	7 (70)	68.0 ± 5.1[a, d, e]

Values are expressed as n (%) or mean ± standard deviation.
[a] $p < 0.001$, [b] $p < 0.05$ compared with the SG group; [c] $p < 0.001$, [d] $p < 0.05$ compared with the OEG group; [e] $p < 0.05$ compared with the OCR-4 group.

(3.7 ± 0.68), -2 (4.1 ± 0.68) and -3 (4.6 ± 0.45) groups had similar ovarian tissue tGSH levels compared to the OIR group ($p > 0.05$ for all; fig. 2).

Similar ovarian tissue 8-OH-Gua levels (pmol/l) were found among the OIR (2.8 ± 0.12), OCR-1 (2.6 ± 0.14), -2 (2.5 ± 0.11) and -3 (2.2 ± 0.13) groups ($p > 0.05$ for all), whereas the OIR group had higher ovarian tissue 8-OH-Gua levels (pmol/l) compared with the SG (0.82 ± 0.04), OEG (0.88 ± 0.06), OCR-4 (1.7 ± 0.09), -5 (1.3 ± 0.07) and -6 (0.94 ± 0.06) groups ($p < 0.05$ for the OCR-4 group, $p < 0.001$ for the OCR-5 group and $p < 0.0001$ for the other groups; fig. 3).

Fertility Levels in the Study Groups

Fertility rate and mean time intervals until birth in the study groups are presented in table 1. No rats from the OIR and OCR-1–3 groups that were removed for reproduction gave birth during the 3-month study period. The number of rats giving birth during the study period was found to be similar among the SG (n = 8) OEG (n = 8) and OCR-6 (n = 7) groups ($p > 0.05$). However, the mean time until birth (in days) was found to be longer in the OCR-6 group (68.0 ± 5.1) compared with the OEG (58.6 ± 8.5) and SG groups (50.3 ± 3.6; $p < 0.05$ and $p < 0.001$, respectively; table 1). Three rats in the OCR-4 group and 4 rats in the OCR-5 group were sterile during the study period ($p < 0.001$ for both groups, compared with the SG group).

While the total number of pups born was significantly greater in the OEG group (17) compared with the OCR-4 (6) and -5 (8) groups ($p < 0.0001$ and $p < 0.001$, respectively), the OEG and OCR-6 groups (16) had a similar total number of pups born ($p > 0.05$; table 2). The percentage of male pups born was significantly greater in the OCR-4 (83%), -5 (75%), -6 (75%) and OEG groups (65%) compared with the SG group (36%; $p < 0.0001$ for all; table 2). Also, the mean total weights (in grams) of the male pups born were significantly greater in the OCR-4 (5.18 ± 0.07), -5 (5.10 ± 0.10), -6 (5.09 ± 0.14) and OEG groups (5.09 ± 0.85) than in the SG group (4.50 ± 0.43; $p < 0.001$ for the OEG group, $p < 0.05$ for the other groups; table 3).

Discussion

This study investigated whether CR applied for varying intervals was effective in preventing I/R-related sterility in rats. Also, ovarian tissue MDA (an indicator of lipid peroxidation), tGSH (a nonenzymatic endogenous antioxidant) and 8-OH-Gua levels (an indicator for DNA damage) were measured biochemically in the study groups. The experimental results of this present study showed that a CR process applied at different intervals creates a protective effect on ovarian tissue and prevents sterility in varying degrees. In addition, we observed decreases in ovarian tissue MDA and 8-OH-Gua levels, and increases in ovarian tissue tGSH levels in parallel to the brevity of the CR procedure.

An I/R injury is described in the literature as a complex pathological phenomenon that begins with ischemia,

Table 2. Number of female and male pups born in the study groups

Group (n = 10 for each group)	Total number of pups born	Number of male pups born	Number of female pups born
SG	50	18 (36)	32 (64)
OEG	17[a]	11 (65)[a]	6 (35)[a]
OIR	0	0	0
OCR-1	0	0	0
OCR-2	0	0	0
OCR-3	0	0	0
OCR-4	6[a, b]	5 (83)[a, d]	1 (17)[a, d]
OCR-5	8[a, c]	6 (75)[a, d]	2 (25)[a, d]
OCR-6	16[a]	12 (75)[a, d]	4 (25)[a, d]

Values in parentheses are percentages.
[a] $p < 0.0001$ compared with the SG group; [b] $p < 0.0001$, [c] $p < 0.001$, [d] $p < 0.05$ compared with the OEG group.

Table 3. Birth weight of male and female pups in the study groups

Group (n = 10 for each group)	Total weight of male pups, g	Total weight of female pups, g
SG	4.48±0.43	4.19±0.36
OEG	5.09±0.85[a]	5.08±0.07[b]
OIR	–	–
OCR-1	–	–
OCR-2	–	–
OCR-3	–	–
OCR-4	5.10±0.10[b]	5.00[b]
OCR-5	5.04±0.16[b]	4.97±0.04[b]
OCR-6	5.11±0.12[a]	5.02±0.12[a]

Values are expressed as mean ± standard deviation.
[a] $p < 0.001$, [b] $p < 0.05$ compared with the SG group.

continues with oxidative stress and broadens with inflammatory reaction [8]. Reperfusion injury causes an increase in oxidant parameters and a decrease in antioxidant levels [7, 8]. MDA, the final product of lipid peroxidation leads to cell damage in the case of oxidative stress. GSH, a nonenzymatic antioxidant, has an important role in protecting cells from oxidative damage [8], and 8-OH-Gua is one of the oxidative damage products of DNA [9]. To our knowledge, this is the first study to investigate the effect of CR at varying intervals on ovarian tissue damage and I/R-related sterility in rats. In this present study, I/R injury led to an increase in ovarian MDA and 8-OH-Gua levels and a decrease in ovarian tGSH levels. Also, these increases and decreases were parallel to the brevity of the CR procedure. Similar to our results, CR protected the rat ovaries against oxidative damage caused by I/R in the study by Ingec et al. [11]. Experimental studies involving animals show that the I/R procedure causes severe oxidative damage in ovarian tissue [13, 22]. Consistent with our study, Celik et al. [23] reported an increase in oxidant parameters including MDA levels and myeloperoxidase activities (an indicator of neutrophil infiltration) and a decrease in antioxidant enzymes including GSH peroxidase and superoxide dismutase activities in ovarian tissue subjected to an I/R injury. The oxidative stress caused by ovarian I/R injury has been reported to lead to sterility in rats [13]. Altuner et al. [24] reported that sterility developed in cisplatin-related oxidative ovarian damage and that antioxidant activity was important in preventing this sterility with mirtazapine. However, although melatonin exhibits more powerful antioxidant activity than thiamine pyrophosphate, it is reportedly ineffective in preventing sterility caused by post-ischemia reperfusion [13]. It therefore appears that the sterility seen in an I/R injury is not dependent on a single cause, and the evaluation of oxidant/antioxidant balance is insufficient for assessing the function of organs and tissues.

In this study, none of the rats in the OCR-1–3 groups (in which CR was applied at intervals of 10, 8 and 6 s) gave birth. The groups whose functions were best preserved after ovarian I/R injury were, in order, OCR-6, -5 and -4. However, the time to pregnancy in these groups was significantly longer than that in the healthy group. Under physiological conditions, since the gestation period in rats varies between 21 and 23 days, we concluded that it was the time to pregnancy rather than the duration of pregnancy that was prolonged. Previous studies have reported that sterility may develop and pregnancy time may be prolonged in rats administered with I/R and simple sham operations [13]. The prolongation of the time to pregnancy may be due to the psychological stress associated with surgical procedures in animals. It has also been reported in the literature that changes may be seen in adult rats' mating and social behavior if social experience is not acquired in the early period of life [25].

Additionally, in this present study, the number of pups born in groups undergoing unilateral ovariectomy was lower than that in the SG group, although body weight (birth weight) was higher. The number of rats at birth is known to affect birth weights [26]. In other words, the

number of pups is inversely correlated with birth weight, which is regarded as a marker of intrauterine growth [26, 27]. Guerra and Nunes [28] reported that the birth weight of pups fell as the number of pups increased. This information from the literature is compatible with our study results.

An interesting and unexpected finding from our study was that the number of male pups in the groups subjected to ovariectomy was greater than that in the healthy group. Various and contradictory opinions on this subject have been published in the literature. Mechanisms affecting sexual identity and orientation are thought to be associated with the effect of testosterone in the developing brain [29]. The production of testosterone and its conversion into dihydrotestosterone are thought to result in the development of the male sex organ and a lack of androgen in the female sex organ [30]. I/R is known to lead to oxidative stress. It is suggested in the literature that stress affects sexual differentiation in the brain. Stress has been observed to increase the release of a mother's adrenal gland hormones and to prevent a constant uptake of sex hormones, particularly testosterone [31]. We suggested that stress experienced by the fetus is a more important factor in the development of sexual identity than stress experienced by the mother. The low number of pups in the uterus of ovariectomized fetuses may have permitted the pups to develop better and to be protected from stress.

In conclusion, an I/R injury induced in the contralateral ovary leads to ovarian oxidative damage and infertility in a rat model with unilateral ovariectomy. Sterility and ovarian oxidative stress caused by an I/R injury declines in parallel to the shortening of the CR duration. CR interval time is important in the prevention of infertility and oxidative damage caused by an I/R injury. This CR technique may be beneficial in preventing sterility in patients with unilateral ovariectomy and torsion in the contralateral ovary.

Disclosure Statement

The authors report no conflicts of interest.

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