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Does sildenafil have protective effects against ovarian ischemia-reperfusion injury in rats?

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Abstract
Purpose The aim of this study was to evaluate the protective activity of sildenafil treatment against ischemia-reperfusion damage created experimentally in rat ovaries.
Methods For this study, 42 female Wistar rats were used, and the rats were separated randomly into six groups consisting of seven rats each: sham, torsion, torsion-detorsion, torsion-detorsion + saline, torsion-detorsion + sildenafil 0.7 mg/kg and torsion-detorsion + sildenafil 1.4 mg/kg. With the exception of the sham group, an ovarian torsion procedure was implemented in all other groups for 2 h. Then, a detorsion procedure was implemented to the groups for 2 h, with the exception of the torsion group. Medications were given intraperitoneally, one-half hour before the detorsion procedure in the saline, 0.7 and 1.4 mg/kg sildenafil groups. Finally, 2 ml of blood samples was drawn for markers of oxidative stress, while the ovaries which were torsioned for the histological examination were extracted from all rats.
Results According to the histopathological damage scores, the least damage was seen in the sham group and the most damage was seen in the torsion-detorsion group. The sildenafil treatment appeared to be effective in decreasing tissue damage; however, there were no differences between the dosages. Additionally, it was determined that the oxidative stress levels were higher in the torsion-detorsion group, while the sildenafil treatment caused a significant decrease in the oxidative stress levels.
Conclusions The results of the current study showed that the sildenafil treatment can be effective in preventing tissue damage and oxidative stress induced by the ischemia-reperfusion created in rat ovaries.

Keywords Adnexal torsion · Ischemia-reperfusion injury · Sildenafil · Rat model

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Introduction

Ovarian torsion (OT) can be defined as the prevention of blood flow to the ovary, and it is the result of the complete or partial rotation of ovarian ligaments around their own axes [1]. It is generally observed in women in the reproductive age group, and it constitutes around 2.7 % of all gynaecological urgent surgical interventions [2, 3]. While the conventional treatment method is the extraction of the torsioned ovary, ovarian detorsion must be implemented for patients in whom the protection of fertility is desired. However, it has been asserted that free oxygen radicals (FOR), which occur after detorsion, cause more damage in ischemic ovarian tissue [1, 4, 5]. Therefore, to decrease the

damage in ovarian tissue exposed to ischemia-reperfusion, the determination of new pharmacological agents is an important clinical target.

Sildenafil is a vasoactive medication that is commonly used for the treatment of erectile dysfunction [6, 7]. In experimental animal models, the protective effect of sildenafil against ischemia-reperfusion damage (IRD) has been shown in the heart, liver and brain [6, 8, 9]. It is asserted that the protective effect of sildenafil is arise from nitric oxide production and antioxidant properties [6, 8, 10, 11].

Although there are many studies which show the protective effect of sildenafil against the IRD in various tissues, there are no studies on ovarian IRD, thus far. Therefore, we hypothesized in this study that sildenafil use in the ovarian torsion-detorsion implemented rat model may have both histopathological and biochemical protective effect.

Materials and methods

Experimental animals

Our work protocol was reviewed and approved by Dicle University and Dollvet Animal Testing Local Ethics Committee (date 07.02.2014; number 2014/05). The care of the rats and all surgical operations to be implemented were planned in accordance with the "Guide for the care and use of laboratory animals".

Forty-two female Wistar rats with weights of 180–240 g were procured from Dicle University Animal Testing Laboratory in Diyarbakır, Turkey. The rats were kept in steel cages until the experiment was carried out; and they were fed standard rodent feed and water under a suitable ambient temperature (24–25 °C), humid environment (55–60 %) and controlled photoperiod (12:12 h light: dark).

Anaesthetic agents

For all surgical interventions; ketamine (Ketalar®; Parke Davis, Eczacibasi, Istanbul, Turkey) and xylazine (Rompun®; Bayer AG, Leverkusen Germany) were used in combination.

Experimental design

In total, forty-two female Wistar rats were separated randomly into six equal groups ($n = 7$). The groups were created as follows: sham (S), torsion (T), torsion-detorsion (T-D), torsion-detorsion + saline (T-DT + S), torsion-detorsion + sildenafil (0.7 mg/kg) (T-DT-Sil0.7) and torsion-detorsion + sildenafil (1.4 mg/kg) (T-DT-Sil1.4). The sildenafil dosage and the injection method used in the experiment were obtained from previous studies carried out on rats [6]. Additionally, a second group (T-DT-Sil1.4) was created to assess whether increasing the dose of this drug is effective in preventing injury.

Surgical method

All surgical interventions were carried out in a proper laboratory environment, under sterile conditions. After weighing the rats, ketamine (50 mg/kg) and xylazine (10 mg/kg) were injected intramuscularly for anaesthesia. Then, the rats were placed in a dorsally recumbent position, and the surgical area was disinfected and dressed with sterile coverings. The abdomen was entered by making a longitudinal midline incision of approximately 2 cm on the sub-abdominal area. After this process, the surgical interventions implemented to all groups were carried out as described below:

Sham group: After localizing the right uterine horn and right adnexa sensitively, the abdominal wall was closed via 4-0 nylon sutures. After 2 h, the right ovary was extracted via re-laparotomy and approximately 3 mL of blood was drawn from the inferior vena cava to measure oxidative stress parameters. All rats were killed after the surgical operation.

Torsion group: Vascular clamps were placed approximately 1 cm under the adnexal structure to simulate ovarian torsion, and the incision line was closed via 4-0 nylon suture. After 2 h, the blood and ovary samples required for the study were collected as described in the sham group via re-laparotomy after 2 h.

Torsion-detorsion group: After 2 h of ovarian torsion, the vascular clamps were opened, and the incision line was closed via 4-0 nylon suture. Reperfusion was allowed for 2 h. After the reperfusion phase, blood and tissue samples required for the study were obtained.

Torsion-detorsion-saline group: After one-half hours of ovarian torsion, saline was administered via intraperitoneal injection. Half hour later, a re-laparotomy was performed and reperfusion was allowed for two hours. After the reperfusion phase, blood and tissue samples required for the study were obtained.

Torsion-detorsion + sildenafil (0.7 mg/kg) group: After one-half hours of ovarian torsion, 0.7 mg/kg of sildenafil was given via intraperitoneal injection. After the reperfusion phase, blood and tissue samples required for the study were obtained.

Torsion-detorsion + sildenafil (1.4 mg/kg) group: After one-half hours of ovarian torsion, 1.4 mg/kg of sildenafil was given via intraperitoneal injection. After the reperfusion phase, blood and tissue samples required for the study were obtained.

Analysis of serum oxidative stress parameters

The blood samples drawn from the inferior vena cava were subjected to centrifugation of 3,000 cycles for 3,000 rev/10 min. The supernatant obtained was placed into plastic tubes and stored at −80 °C until the assays were performed. No preservatives were added to the samples because this study was comparative in nature. The serum oxidative stress parameters, including the total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI), were measured as described previously [12].

Total antioxidant status: The TAS was measured in the samples via a spectrophotometric technique using commercial kits (Rel® Assay). The results were expressed as the mmol Trolox equivalent/L.

Total oxidant status: The TOS was measured in the samples via a spectrophotometric technique using commercial kits (Rel® Assay). The results were expressed as the μmol H_2O_2 equivalent/L.

Oxidative stress index: The OSI was defined as the percentage ratio of the TOS levels to TAS levels. The results are represented as the mmol Trolox/L.

Histopathological analysis

The ovarian tissues taken from the rats for histopathological analysis were fixed in 10 % buffered formalin for 48 h. The tissues were removed from solution and longitudinal tissue dissected towards hilus of the ovary and whole material on the section was placed into the tissue cassette. Then, tissue samples embedded in paraffin blocks. The paraffin blocks were cut by using 4 μm microtomes and it was obtained four sections from each paraffin block. After, all the preparations were stained with hematoxylin and eosin. For the evaluation of the histological sections, a light microscope (Olympus® Inc. Tokyo, Japan) was used to take pictures. During microscopic examination, at least five best fields were examined with microscopy under ×200 magnification. At least five microscopic areas were examined to determine the existence of tissue damage, as well as its severity. The histopathological changes were defined as oedema, congestion, haemorrhage, leukocyte infiltration and follicle degeneration (Fig. 1 a–e). Scoring was determined between 0 and 3 according to the severity of damage: 0 = no pathological findings, and 1, 2 or 3 were defined as <33, 33–66 and >66 % of the area analysed, respectively [13]. The total score was calculated by adding the scores for each parameter.

Statistical analysis

The SPSS 16.0 software (SPSS for Windows, Chicago, IL) program was used for the statistical analysis. While biochemical results were presented as the mean and standard deviation, histopathological results were presented as the mean plus range. The Kolmogorov–Smirnov test was carried out for analyzing the data distribution, while the one-way ANOVA was used for the comparisons between the groups and the Bonferroni correction was used as the post hoc test. The Pearson's test was used for the correlation testing among the data, and those data with "p" values of less than 0.05 were accepted as statistically significant.

Results

Histopathological results

With the exception of the sham group, the ovaries of the other groups appeared macroscopically as "strawberry" red and were purported to be haemorrhagic. The distribution of congestion, haemorrhage, leucocyte infiltration, follicle degeneration and interstitial oedema scores by groups, which are used in the evaluation of histopathological damage, are presented in Table 1. According to the histopathological damage scores, While the least damage was found in the sham group, the most damage was seen in the T-DT group (1.00, 8.29, $p < 0.001$, respectively) (Fig. 1f). When the T-DT group and T-DT-Sil0.7 vs. T-DT-Sil1.4 groups were compared, it could be seen that the sildenafil treatment was effective in decreasing the tissue damage (total damage score average 8.29, 4.57, 4.00, $p < 0.001$, $p < 0.001$, respectively); however, there was no significant difference between the dosages ($p = 0.966$) (Fig. 1g–h).

Biochemical results

The TAS, TOS and OSI levels, which are considered to be serum oxidative stress markers, are presented in Table 2.

TAS levels were observed at the lowest level in the T-DT group. It was determined that sildenafil use increased the TAS level significantly when compared to the T-DT group. The TAS level was highest in the T-DT-Sil1.4; however, a significant difference was not seen between the sildenafil dosages with regard to the TAS ($p = 0.872$).

The highest TOS values were seen in the T-DT group in the analysis of the samples. However, a significant difference was not seen among the other groups with regard to the TOS level.

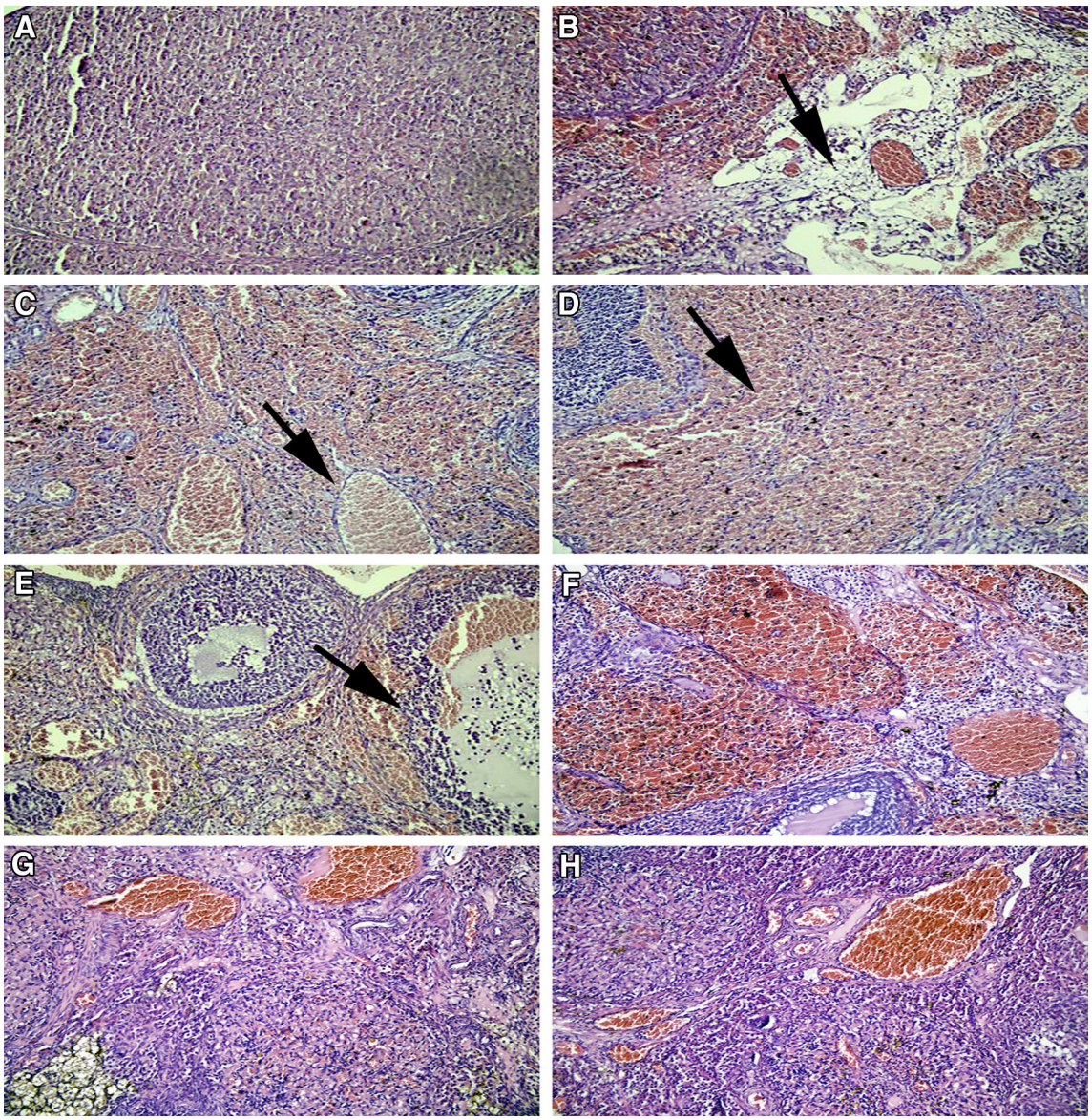

Fig. 1 Light microscopic appearance of rat ovarian section (Hematoxylin-eosin, ×200). **a** Normal ovarian morphology. **b** Remarkable interstitial edema. **c** Congestion in the ovarian parenchyma. **d** Significant hemorrhage in the ovarian parenchyma. **e** Disruption of the follicle cells. **f** Striking diffuse hemorrhage, interstitial edema and congestion in torsion-detorsion group. **g** Ovarian histology after 0.7 mg/kg sildenafil treatments of rats; decreases hemorrhage, interstitial edema and congestion. **h** Decreases hemorrhage, interstitial edema and congestion in torsion-detorsion + sildenafil (1.4 mg/kg) group

The lowest OSI levels were found in the sildenafil groups, but a significant difference was not determined between the T-DT-Sil 0.7 and T-DT-Sil1.4 groups (5.45 ± 1.21, 4.90 ± 0.58, $p = 0.997$, respectively).

According to the Pearson correlation test carried out between the serum OSI levels and ovarian tissue histopathological damage score, a positive significant relationship was determined ($r = 0.409$, $p = 0.007$).

Discussion

The torsion of the ovarian vascular pedicle around its own axis results in a decrease in the blood flow and the obstruction of lymphatic drainage [4]. A decrease in the blood flow causes an increase lactic acid, hypoxanthine and lipid peroxide level in the ovarian tissue [14, 15]. In patients undergoing ovarian detorsion, there is an increase

Table 1 Distribution of histopathologic damage according to groups (mean, range)

	Sham	T	T-DT	T-DT-S	T-DT-Sil 0.7	T-DT-Sil 1.4	$p^{\S}$
Congestion	0.29 (0–1)	1.71 (1–2)	2.29 (2–3)	2.14 (1–3)	1.57 (1–2)	1.57 (1–3)	<0.001[a]
Hemorrhage	0.29 (0–1)	1.71 (1–2)	2.43 (1–3)	2.57 (1–3)	–	1.43 (1–2)	<0.001[b]
Leucocyte inf.	0.29 (0–1)	0.43 (0–1)	1.29 (1–2)	0.86 (0–3)	0.29 (0–1)	0.29 (0–1)	0.022[c]
Follicle deg.	0.00 (0–0)	0.29 (0–1)	0.57 (0–1)	0.29 (0–1)	0.29 (0–1)	0.29 (0–1)	0.373
Int oedema	0.14 (0–1)	1.14 (0–2)	1.71 (1–2)	1.71 (1–2)	0.86 (0–2)	0.43 (0–1)	<0.001[d]
Total score	1.00 (0–2)	5.29 (4–8)	8.29 (7–10)	7.43 (5–10)	4.57 (3–6)	4.00 (2–7)	<0.001[e]

§ One-way analysis of variance (ANOVA)

[a] Comparison of sham group and T, T-DT, T-DT-S, T-DT-Sil0.7, T-DT-Sil1.4 groups ($p = 0.001$, < 0.001, < 0.001, 0.004 and 0.004, respectively)

[b] Comparison of T-DT-Sil1.4 group and sham, T-DT-S groups ($p = 0.029$, $p = 0.029$, respectively)

[c] Comparison of T-DT group and T-DTSil0.7, T-DT-Sil1.4 groups ($p = 0.045$, $p = 0.045$, respectively)

[d] Comparison of T-DT-Sil1.4 group and T-DT, T-DT-S groups ($p = 0.002$, $p = 0.002$, respectively)

[e] Comparison of T-DT-Sil1.4 group and T-DT, T-DT-S groups ($p < 0.001$, $p < 0.001$, respectively); T-DT-Sil0.7 group and T-DT, T-DT-S groups ($p < 0.001$, $p = 0.005$, respectively)

Table 2 Mean total antioxidant status, total oxidant status and oxidative stress index levels determined for rats in all groups (mean ± standard deviation)

	Sham	T	T-DT	T-DT-S	T-DT-Sil 0.7	T-DT-Sil 1.4	$p^{\S}$
TAS	0.92 ± 0.10	0.91 ± 0.13	0.83 ± 0.20	0.88 ± 0.05	1.06 ± 0.11	1.13 ± 0.11	0.001[a,b]
TOS	50.99 ± 8.13	53.43 ± 7.51	72.88 ± 15.24	63.20 ± 15.44	57.67 ± 15.60	55.41 ± 8.08	0.024[c]
OSI	5.57 ± 0.97	5.93 ± 0.88	9.52 ± 4.51	7.28 ± 2.10	5.45 ± 1.21	4.90 ± 0.58	0.003[d]

TAS total antioxidant status, *TOS* total oxidant status, *OSI* oxidative stress index

§ One-way analysis of variance (ANOVA)

[a] Comparison of T-DT-Sil0.7 group and T-TD group ($p = 0.026$)

[b] Comparison of T-DT-Sil1.4 group and sham, T, T-DT and T-DT-SF groups ($p = 0.039$, 0.026, 0.001 and 0.007, respectively)

[c] Comparison of TOS levels between sham and T-DT groups ($p = 0.029$)

[d] Comparison of T-DT-Sil0.7 and T-DT-Sil1.4 groups and T-DT group with regard to OSI levels ($p = 0.014$ and 0.004, respectively)

in the neutrophil infiltration, production of nitric oxide, cytokines and FOR in ovarian tissue [3, 15, 16]. In this case, it is described as IRD, which actually causes more tissue damage when compared to ischemia [3, 4]. Therefore, it is asserted that the use of antioxidant pharmacological agents during or before reperfusion could be useful [17].

Sildenafil is a specific phosphodiesterase type 5 inhibitor traditionally used in the treatment of erectile dysfunction [8]. More recently, it has been used in the diseases induced by hypoxia, such as pulmonary hypertension, chronic heart failure and indomethacin-induced gastropathy, due to its features of vasodilation and the regulation of blood flow [8, 10, 18]. Additionally, there has been research that shows its protective effects due to its anti-oxidant features against IRD in tissues such as the heart, liver, lungs, kidney, colon and testicle [6, 8, 9, 19, 20]. It has been asserted that sildenafil regulates blood flow by increasing nitric oxide production, and decreases FOR by inhibiting leucocyte infiltration and adhesion [9, 10]. In our study, we hypothesized that sildenafil may have protective effects on the blood oxidant-antioxidant balance and ovarian tissue damage in ovarian torsion-detorsion implemented rats.

In ovarian detorsion implemented cases, the oxygen concentration in the tissues increases secondarily to reperfusion. This increasing oxygen reacts with hypoxanthine and xanthine oxidase in ischemic tissue and finally, FOR formation, which causes tissue damage, is triggered [21]. Increasing FOR production causes a greater increase in damage in ischemic tissue by raising the peroxidation of cell membranes and mitochondrial lipids [3]. Due to an important correlation between the oxidative stress levels and TAS, TOS and OSI being shown previously [3, 12], the scaling method mentioned above was used in our study for scaling the blood oxidative stress level. It was determined that the TOS and OSI levels were significantly higher in the T-D group when compared to the other groups; however, sildenafil use caused a significant decrease in the oxidative

stress parameters. A significant difference was not determined between sildenafil dosages and the oxidative stress parameters used in this experiment.

In the detorsion procedure implemented cases, leucocyte deposition occurred in the activated ovarian tissue and, accordingly, an increase in FOR production [3, 14]. FOR that cannot be inactivated through antioxidants constitute direct cellular components, and contribute in the morphological damage formation in ovarian tissue [13, 14]. In our study, the scoring system defined by Kara et al. [13] in 2012 was used for determining the ovarian histopathological damage depending on ischemia-reperfusion. As expected, histopathological damage was not determined in the sham group. The highest tissue damage score was observed in the T-D group and sildenafil treatment was efficient in decreasing the tissue damage score. It was determined that the average leucocyte infiltration rate in the sildenafil groups was the same as in the sham group (leukocyte infiltration total score 0.29). Therefore, we believe that the reducing effect of the sildenafil treatment on tissue damage was connected with the reduction in the FOR production by inhibiting leucocyte infiltration.

Consequently, this study makes one believe that sildenafil treatment can be effective in the reduction or prevention of tissue and biomolecular oxidative damage arising from ovarian reperfusion. The use of antioxidant pharmacological agents like sildenafil can be useful in the abatement of tissue and cell oxidative damage in ovarian detorsion implemented cases.

Conflict of interest The authors have stated explicitly that there are no with this any financial support or relationships that may pose potential conflict of interest in this article.

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