Histological study on the effect of vitamin C on ischemia–reperfusion injury in the adult rat ovary
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Background
Ovarian torsion may cause serious complications such as infertility in young women. Conservative management includes detorsion and reperfusion of the twisted segment. However, it may have local and systemic consequences due to production of large amounts of reactive oxygen species during reperfusion of ovaries.

Aim of work
The present work aimed to study the possible histological and immunohistochemical changes due to ischemia–reperfusion injury in rat ovaries and the possible protective effect of vitamin C as an antioxidant.

Materials and methods
A total of 32 albino rats were divided into four groups. Group I was the control sham-operated group (either sham operated only, or with vitamin C administration). In group II rats, ovarian ischemia was induced by torsion of the right adnexa. In rats of group III, 4 h of ischemia followed by reperfusion was performed. In rats of group IV, 4 h of ischemia was followed by 50 mg/kg vitamin C administration, which was injected intravenously, and then reperfusion was performed. Except for the ischemia group, all other groups were subdivided into two subgroups from which the right ovaries were surgically removed either after 5 h or after 2 weeks of starting the experiment. From the ischemia group ovarian samples were taken after 5 h only. Specimens were processed for paraffin sections and stained with H&E and with an immunohistochemical stain for apoptotic marker p53. Image analysis and statistical analysis of the obtained results were carried out.

Results
Severe vascular congestion, edema, hemorrhage, and increased P53 immunoreaction were detected in the ovaries after ischemia, which became less marked after reperfusion and considerably improved with vitamin C administration, especially after 2 weeks.

Conclusion
Vitamin C treatment can help in protecting the ovaries from ischemia–reperfusion injury after detorsion.

Keywords:
antioxidant, immunohistochemistry, ischemia, ovary, reperfusion, vitamin C

Introduction and aim
Torsion of the ovary is a surgical emergency. Immediate treatment is required for preserving fertility and saving the twisted adnexa [1]. Ovarian ischemia is the result of torsion and leads to cell death because of insufficient perfusion of the tissues [2]. Conservative management includes detorsion of the twisted segment. After detorsion and maintaining the circulation of the ovary, a pathologic process called reperfusion injury occurs [3]. Thus, reperfusion of the ischemic tissue may paradoxically lead to more serious damage to the tissue than the damage caused by ischemia itself in some cases. During the detorsion process, an excess amount of molecular oxygen is supplied to the ischemic tissues, and acute ischemic injury may be worsened by the release of reactive oxygen species (ROS) [4]. Many studies have focused on the protective effects of special medications such as antioxidants, calcium channel blockers, and vasodilators to ameliorate the hazardous effects of the reperfusion process [5]. Vitamin C is important for survival. It is an electron donor, and this property accounts for its most important known functions. As an electron donor, vitamin C is a potent water-soluble...
antioxidant. Human diseases such as atherosclerosis and cancer might occur in part from oxidant damage to tissues, and under septic conditions vitamin C acts as the main regulator of neutrophil attraction and function [6]. Thus, this study aimed to investigate the possible histological and immunohistochemical changes due to ischemia–reperfusion (I/R) injury in adult rat ovaries and the possible protective effect of vitamin C as an antioxidant.

Materials and methods
Animals and experimental procedure
The present study included 32 adult female albino rats of 180–230 g body weight; they were obtained from and housed in the animal house of Kasr-El-Aini Faculty of Medicine, Cairo University. They were selected according to their estrous cycle and were isolated from male rats to avoid pregnancy. This study was approved by the ethics committee for animal research in the animal house of Kasr-El-Aini Faculty of Medicine, Cairo University, Egypt, following international ethics and regulations for animal research in laboratory applications [7].

The animals received a standard diet for rodents and were allowed free access to water. They were divided into four main groups, each containing eight rats. Group I was the control group. Rats in this group were either sham operated only or were sham operated with vitamin C administration, and sacrificed either after 5 h or after 2 weeks. Group II was the ischemia group, in which ischemia was induced. In group III, 4 h ischemia was followed by reperfusion (I/R). In group IV, 4 h ischemia was followed by vitamin C injection and then reperfusion (I/R+vitamin C). Groups III and IV were subdivided into subgroup A, sacrificed after 5 h, and subgroup B, considered the recovery group, sacrificed after 2 weeks of starting the experiment.

Vitamin C (Cevarol ampoule containing 500 mg vitamin C product obtained from Memphis Company, Cairo, Egypt) 50 mg/kg was injected intravenously into group IV and the corresponding control group.

Operative procedure
For all operative procedures, animals were anesthetized using thiopental sodium 50 mg/kg injected subcutaneously. In all animals a midline 2 cm laparotomy was performed. The right adnexa, including the ovaries and tubal and ovarian vessels, was exposed only in the control group, whereas in other groups it was rotated 360° in clockwise direction and fixed to the abdominal muscles with a 5/0 silk suture. The skin of all animals was sutured with 5/0 silk. Four hours later, relaparotomy was performed through the previous incision sites in all groups. Vitamin C was injected into rats of group IV and the corresponding controls, and reperfusion by detorsion was performed in groups III and IV. One hour after relaparotomy half of the animals of each group were sacrificed and the right ovaries were surgically removed. The remaining animals in the I/R group and in the group taking vitamin C with I/R, in addition to the corresponding controls, were sacrificed and their right ovaries were removed after a recovery period of 2 weeks.

Histological and immunohistochemical study
Ovarian samples were fixed in 10% formalin solution, embedded in paraffin, and cut at 6 µm thickness. The sections were stained with H&E to examine the structural changes, and immunohistochemical staining was carried out using primary antisemur to p53 (Catalogue number MS-182-P0; Thermo Scientific Lab Vision Corporation, Westinghouse, California, USA), California, USA). Anti-rat monoclonal antibodies are suitable for immunohistochemical staining of formalin-fixed paraffin-embedded sections using the streptavidin–biotin–peroxidase complex technique. Sections were dewaxed in xylene, rehydrated, and pretreated with 3% hydrogen peroxide for blocking endogenous peroxidase activity. Sections were incubated for 1 h with the primary p53 antibodies at room temperature, washed in PBS, and then incubated with the secondary antibody. The sections were then incubated with streptavidin peroxidase, rinsed, and treated with chromogen. The specimens were then counterstained with hematoxylin, dehydrated, cleared, and mounted. Cells positive for p53 showed nuclear and cytoplasmic brown deposits. Immunoreactivity was absent in negative controls in which the primary antibody was omitted [8].

Morphometric study
Apoptotic marker p53 expression
Area% of positive immunostaining for P53 was measured in 10 nonoverlapping high-power fields for each specimen of all groups at magnification ×400. The data were obtained using the ‘Leica Qwin 500’ image analyzer computer system (Hessen, Wetzlar, Germany).

Statistical analysis
Statistical analysis was performed using SPSS software (Chicago, USA). Data are presented as mean ± SD. Differences among the study groups were detected by one-way analysis of variance as the global test to determine any differences in data before using the t-test to compare two groups. P values less than 0.05 were considered statistically significant [9].

Results
Histological results
In the control group, either sham operated only or administered vitamin C, and sacrificed either after 5 h or after 2 weeks, the rats showed normal ovarian tissue, with follicles at various stages of development embedded in compact stroma. There were no remarkable differences between different control subgroups (Fig. 1).
In the ischemia group, after 5 h of ischemia, rats showed marked edema, congestion, and hemorrhage. Many follicles appeared degenerated, whereas some follicles at different stages of development were still preserved (Fig. 2).

The histological alterations in group III (I/R) were in the form of vascular congestion, interstitial edema, and hemorrhage. Most of the follicles appeared normal in group IIIA (Fig. 3). These changes were less evident after 2 weeks in group IIIB (Fig. 4).

The histological alterations were mild in group IV (I/R+vitamin C group). After 5 h, despite mild edema and congestion, most follicles were well preserved in group IVA (Fig. 5). After 2 weeks, the ovarian tissue showed marked improvement with no remarkable edema or congestion in group IVB (Fig. 6).

Immunohistochemical results

Immunohistochemical expression of P53 in the follicles and stroma of the ovarian tissue was scanty and hardly observed in the control group (Fig. 7). In contrast, extensive immunohistochemical P53 expression was seen in group II (ischemia group) in the follicles and stroma (Fig. 8). There was strong immunoreaction in the I/R group at 5 h in subgroup IIIA (Fig. 9) with decreased intensity to some extent after 2 weeks within the cells in different follicles and in the stromal cells in subgroup IIIB (Fig. 10). P53 expression was found to be moderate in the I/R+vitamin C group at 5 h in subgroups IVA (Fig. 11) but was scanty and hardly observed after 2 weeks in subgroups IVB. (Fig. 12).

Morphometric results

P53 immunostaining as evaluated using analysis of variance showed statistically significant variance between the groups ($P < 0.05$).

Comparison between two groups was performed using the Student $t$-test and the results are summarized in Table 1 and Chart 1:

Table 1 and Chart 1 summarize the results of the mean P53 immunoreaction optical density in different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean P53 Immunoreaction Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.12</td>
</tr>
<tr>
<td>Ischemia</td>
<td>0.67</td>
</tr>
<tr>
<td>5 h I/R Group</td>
<td>0.50</td>
</tr>
<tr>
<td>I/R+vitamin C</td>
<td>0.35</td>
</tr>
</tbody>
</table>

The density of reaction was highest in group II (the ischemia group) followed by group IIIA (5 h I/R group). The control group (group I) and vitamin C group (group IV) showed scanty expression.

there was no statistically significant differences between control group, group IIIB, group IVA and IVB but there was statistically significant differences between them and either group II or group IIIA. However, there were no statistically significant differences between the control group and vitamin C group.
Figure 3. A photomicrograph of a section of a rat’s ovary from group IIa (I/R) showing ovarian follicles at different stages of development (thick arrows) with edema in the stroma (thin arrows) and hemorrhage (curved arrows) after 5 h. H&E, (a) × 100; (b) × 400.

Figure 4. A photomicrograph of a section of a rat’s ovary from group IIIb (I/R) showing persistent marked edema (thin arrows) after 2 weeks among follicles at different stages of development (thick arrows). H&E, (a) × 100; (b) × 400.

Figure 5. A photomicrograph of a section of a rat’s ovary from group IVA (I/R+vitamin C) after 5 h showing ovarian tissue with preserved follicles at different stages of development (thick arrows) with the presence of mild edema (thin arrows), congested blood vessels (crossed arrow), and hemorrhage (curved arrow). H&E, (a) × 100; (b) × 400.

Figure 6. A photomicrograph of a section of a rat’s ovary from group IVB (I/R+vitamin C) showing normally appearing ovarian tissue with preserved healthy follicles at different stages of development (thick arrows) with no remarkable edema or hemorrhage after 2 weeks. H&E, (a) × 100; (b) × 400.
Figure 7. A photomicrograph of a section of a rat’s ovary from the control group showing scanty immunoreactivity for p53 in the cells of the follicles (thick arrow) and stroma (thin arrows). p53 immunostaining, (a) × 100; (b) × 400.

Figure 8. A photomicrograph of a section of a rat’s ovary from group II (ischemia) showing extensive p53 immunoreactivity involving many cells of the ovarian follicles (thick arrows) and mild reactivity in the stroma (thin arrows). p53 immunostaining, (a) × 100; (b) × 400.

Figure 9. A photomicrograph of a section of a rat’s ovary from group IIIA (I/R) showing strong p53 immunoreactivity involving many cells of the ovarian follicles (arrows). p53 immunostaining, (a) × 100; (b) × 400.

Figure 10. A photomicrograph of a section of a rat’s ovary from group IIIB (IR) showing moderate p53 immunoreactivity in some cells of the ovarian follicles (thick arrows) and stroma (thin arrows). p53 immunostaining, (a) × 100; (b) × 400.
In the present study the sections obtained from the control group, either sham operated only or with administration of vitamin C, showed normal ovarian follicles at different stages of development and condensed stroma. Immunostaining for p53 reaction, to determine apoptosis, revealed scanty positive immunoreactions in the ovarian tissue.

Normal individuals, under basal conditions, produce ROS through their aerobic metabolism. Therefore, cells developed antioxidant mechanisms to control overproduction and prevent harmful cell damaging effect, thus maintaining the balance between cellular regeneration and apoptosis [10].

In group II, which was subjected to ischemia only for 4 h, there was significant edema, congestion, and hemorrhage in the ovarian stroma despite the preservation of many ovarian follicles in various stages of development. There was also extensive immunoreaction for P53, which may be an indication of the activation of apoptosis in the follicles and stromal cells of ovarian tissue. It was significantly increased when compared with the control.
group. This may be explained as the reduction in arterial and venous blood flow caused by ovarian torsion may lead to increase in toxic metabolites such as ROS [4]. Cell death after ischemia was previously explained to be due to necrosis only, but further studies indicated that apoptosis may play a key role in cellular damage after ischemia [11]. In addition, if endogenous or exogenous antioxidant molecules do not neutralize free radicals, lipid peroxidation may occur at the cell membrane leading to damage to the cell membrane protein and capillary endothelium. This can enhance adherence of platelets to the vessel walls, increasing its permeability with severe tissue damage [12].

Group III (which was exposed to ischemia and then reperfusion) demonstrated significant structural alterations in the ovary, such as congestion and marked increase in P53 immunostaining in the follicles and stroma, but improvement was seen when compared with the ischemia group. These changes improved further after 2 weeks compared with 1 h after reperfusion. Such damage could be attributed to the release of free radicals following ischemia due to reperfusion. This may not be in agreement with other studies that used longer periods of ischemia in which reperfusion of the tissue results in microvascular dysfunction and there is no reflow phenomenon when the blood circulation is restored. Even if the subsequent perfusion pressure is adequate, most of the capillary vessels are not reperfused and thus early reperfusion can save the tissue [13]. In addition, postischemic reperfusion has experimentally been demonstrated to cause severe damage to the ovary and other tissues [14]. In our study it was not so severe because of the short period of ischemia, with greater improvement seen in the recovery subgroup after 2 weeks’ reperfusion. This can be explained by the observations of Tok et al. [15], who reported that small amounts of oxidant parameters are formed during ischemia and a greater amount of oxidant parameters are produced after reoxygenation of tissues in the reperfusion period. Thus, the mechanism of damage during ischemia is different from that during reperfusion, which may be partly due to the free radicals [16]. In addition to that Kurt et al. [17] found that oxidative damage in the ovarian tissue resulting from the postischemic reperfusion injury was much more severe than that resulting from ischemic damage over a short period which can be decreased by dexamethazone.

There are many studies reporting the protective effects of numerous agents against ovarian I/R injuries. Halici et al. [2] reported that amlodipine preserved the ovary from injury due to I/R. The protective effects of radical scavengers were studied by Kara et al. [3] in the ovarian torsion model of rats. They reported that edaravone was useful in the early treatment of ovarian I/R injuries. There is limited information about the effect of vitamin C on I/R injury. Most of the prior studies performed I/R for 1 and 2 h, respectively [18]. Therefore, in our study, we determined the duration of ischemia as 4 h and reperfusion for either 1 h or for 2 weeks as a recovery model. We therefore studied the effect of vitamin C on ovarian I/R injury in rats by administering it after 4 h of ischemia just before reperfusion.

Vitamin C is an endogenous water-soluble compound that is virtually nontoxic and capable of reducing free radicals. The therapeutic effects of this vitamin may be due to a combination of its antioxidant activity on various free radicals. Vitamin C is known to block lipid peroxidation in the cell membrane and scavenge hydroxyl radicals [19]. Several studies had indicated that vitamin C has protection against reperfusion injury in lung, brain, and skin flaps [20].

For this reason, in the present study we chose vitamin C as a protective antioxidant agent on the ovaries exposed to I/R injury. After 4 h of ischemia, vitamin C was given intravenously and reperfusion was performed for 1 h (group IV), which resulted in decrease in edema and congestion of the ovarian stroma. P53 immunostaining (which is a maker of apoptosis) showed significant decrease in the mean area% of positive reaction compared with group II (ischemia-only group) and group III (I/R group), indicating minimal amount of apoptosis. Thus, it could be assumed that vitamin C can protect the ovary from I/R effect. There was also considerable improvement in the vitamin C group, which appeared to be as normal as the control group after 2 weeks in terms of general morphology and immunoreactivity to P53. These results indicated that most of the ovarian stroma and follicles were preserved from I/R injury after vitamin C administration even in the early period, aiding also in complete recovery.

These results were similar to those of Sagasoz et al. [21], who studied the effects of antioxidant drugs on the ovary and reported that vitamin C reduced I/R injury of the ovary during its early stages, better than verapamil, which is a calcium channel blocker.

Agarwal and Allamaneni [22] reported that vitamin C has an important role in the normal function of the female reproductive system and in the pathogenesis of female infertility, influencing the entire reproductive span of a woman. Free radicals can modulate cellular functions and oxidant stress can impair the intracellular environment resulting in diseased cells and decreased cell survival. Thus, ROS can affect a variety of physiological functions in the reproductive tract.

Recommendations
Vitamin C is a safe and easy medication that can help protect the ovaries from I/R injury due to early detorsion, which is the basic conservative procedure for management of ovarian torsion.

Acknowledgements
Conflicts of interest
There are no conflicts of interest.
References


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المملوكت العربى

دراسة نسيجية عن تأثير فيتامين سي على إصابة نقص الدم و إعادة ضخه في مبايض الجرذان البالغة

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الملخص

قد يسبب التواء المبيض مضاعفات خطيرة مثل العقم في الإناث. وتشمل العلاج التحفظي إصلاح الالتواء وإعادة إعتداله و ضخ الدم للجزء الملتوي. ومع ذلك، قد يكون له عواقب موضعية عامة نتيجة لإنتاج كميات كبيرة من الأكسجين الحر التفاعلي (ROS) خلال ضخه في المبيضين. 

الهدف من الدراسة: العمل الحالي يهدف إلى دراسة التغييرات النسيجية والمناعية الناتجة عن نقص الدم و إعادة ضخه في مبيض الفئران.

الآدوات و الطرق المستخدمة:


٢. تم تجهيز العينات و صباغتها بالهياماتوكسيلين والإيوسين H & E و الصبغة p53 للبروتين P53.

النتائج:

تم الكشف عن الإحتقان الشديد في الأوعية الدموية، و الارتشاد، و وجود نزف، و زيادة التفاعل المناعي للبروتين P53 في المبيض بعد نقص ضخ الدم للمبيض، و التي أصبحت أقل وضوحاً بعد إعادة ضخه و الكثير من التحسن حديثاً بعد حقن فيتامين سي خصوصاً بعد فترة الأسبوعين.

الخلاصة:

العلاج باستخدام فيتامين سي يمكن أن يساعد في حماية المبيض من الإصابة الناتجة عن نقص ضخ الدم للمبيض بعد إعادة ضخه و إصلاح الالتواء.