Effects of folic acid administration on testicular ischemia/reperfusion injury in rats

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Abstract

Purpose: To determine the effect of folic acid (FA) on experimental testicular ischemia/reperfusion (I/R) in rats.

Methods: Sixty male Wistar rats were randomly divided into 6 groups. The control group received physiologic saline orally. The sham-operated group received physiologic saline orally then exposed to midline laparotomy without clamping the IR. The I/R rats received oral gavage of the saline then subjected to 1h ischemia/24h reperfusion, period. In folic acid (2mg/kg+IR) rats received oral gavage of the FA (2mg/kg) then subjected to 1h I/24h R. groups 5-6 received FA (5 and 10 mg/kg), then subjected to 1 h I/24 h, respectively. At the end of the study, semen samples were collected for spermatozoa characteristics. The left testis was removed for histological analysis and superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GPx) measurement.

Results: Spermatozoa mobility, mortality (%) significantly decreased in I/R group (P<0.05). Dose dependent increase observed on spermatozoa mobility, mortality (%) using different levels of the FA (2, 5 and 10 mg/kg) treated rat (P<0.05). Tissue MDA levels significantly increased in I/R rat (P<0.05) while FA (2, 5 and 10mg/kg) in a dose dependent manner decreased I/R-induced MDA (P<0.05). Experimental I/R significantly decreased SOD and GPx activity (P<0.05). Administration of the FA (2, 5 and 10mg/kg) significantly increased tissue SOD and GPx activity in I/R rat (P<0.05). Seminiferous tubules degenerated and loss of spermatogenesis with few spermatocytes was observed in degenerated testis tubules in I/R rat. Orally administration of the FA (5 and 10 mg/kg) improved testis characteristics with few normal seminiferous tubules and spermatocyte in seminiferous tubules in experimental I/R-induced rat.

Conclusion: The treatment of folic acid had a benefit effect against ischemia-reperfusion.

Key words: Folic Acid. Ischemia. Reperfusion. Rats.
Introduction

Testicular torsion is a urologic emergency that occurs frequently in the neonatal and adolescent period. The testicular torsion characterized by a circulatory failure caused via testis revolving around the vascular peduncle. Annual incidence of spermatic cord torsion is 4.5 in 100,000 males 1-25 years of age. The main pathophysiologic event in testicular torsion is ischemia followed by reperfusion; therefore, testicular torsion is an ischemia/reperfusion (I/R) injury to the testis. In the I/R, blood supply of the tissue is interrupted which leads to damage of metabolically active tissues and cellular and tissue damage eventually. The basic pathology in testicular torsion is ischemia which happens because of the torsion followed by tissue damage occurring via the reactive oxygen species (ROS) during reperfusion. Excess production of ROS or decreased antioxidant defenses in the seminal plasma. Activation of several antioxidant defense mechanisms avoids the tissue damage due to ROS. The ROS antioxidants such as SOD, MDA and GPx have an essential effect in human reproduction. Polyunsaturated fatty acids (PUAFAs) are highly concentrated in spermatozoa and vulnerable to be attacked by ROS. To date several anti-inflammatory, antioxidants, and free-radical scavengers were applied for the treatment of testicular I/R-induced male infertility.

Folic acid/ folate (FA), is a water-soluble vitamin. FA is essential for the production of purines and pyrimidines which as precursors of DNA. FA modulates a number of disorders as a result of its antiapoptotic and anti-oxidative properties. It is reported FA (2 mg/kg) has gastroprotective activity against the lipid peroxidation. It is reported FA, the synthetic form of folate has effective antioxidant activity in male infertility. Also, curative effect of the FA observed on sperm parameters and DNA integrity following varicocelectomy. Despite free radical scavenging property of the FA is approved, scarce information exists for its antioxidant activity. Researches were done on antioxidant activity of the FA in liver and digestive system; there is no paper on antioxidant activity of the FA in testis I/R. So, the main purpose of the study was to evaluate the effects of the effect of FA on experimental testicular I/R in rats.

Methods

This study was conducted according to the guidelines of the animal care review board of the Islamic Azad University, Faculty of Veterinary Medicine, adhering to the guide for care and use of laboratory animals; the study was approved by the ethics committee.

Sixty healthy adult male Wistar rats, (weight 250-300 g) were purchased from the Pasteur Institute. Animals kept under constant room temperature of 20±1°C, relative humidity of 42±1%, on a 12-hour light/ dark cycle. All animals had free access to commercial food and filtered tap water.

Experimental groups

Rats randomly divided into 6 experimental groups (n=10). The control group: animals received physiologic saline orally for 7 days via oral gavage. The sham-operated group received physiologic saline orally for 7 days via oral gavage then exposed to midline laparotomy without clamping the IR. The I/R group: rats received oral gavage of the saline for 7 days and then subjected to 1h I /24h R period. Group FA (2mg/kg + IR): rats received oral gavage of the FA (2mg/kg) for 7 days and then subjected to 1 h I/24 h. Group FA (5mg/kg + IR): animals received FA (5mg/kg) for 7 days via oral gavage, then subjected to 1 h I/24 h. Group 10mg/kg FA + IR: animals received
oral gavage of the FA (10mg/kg) for 7 days via oral gavage, then subjected to 1 h I/24 h. The doses for FA selected based on the pilot study (un-published data) and previous report11,12.

**Drugs and detection kits**

Folic acid was obtained from Sigma Co. (Sigma, USA). Assay kits for MDA, SOD and GPx were purchased from the Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom). The dose of FA was obtained from previous studies and our pilot studies11,12.

**Experimental protocol**

All surgical procedures were performed under anesthesia by intraperitoneal injection of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (10 mg/kg) then experimental testicular IR was created13. The upper left abdominal quadrant was approached through a midline laparotomy incision. During the surgical procedures, the body temperature was maintained with a heating pad. The testicular artery and vein of the left testis were occluded with a vascular clamp for 1 h, after this process the clamp was removed and the organ was allowed to reperfusion 24 h13. At the end of the study, rats were euthanized with an overdose injection of pentobarbital (300 mg/kg, i.p.), peritoneum opened and left testis was removed for further investigations. The testicle was divided into two by a sagittal section and one half was fixed in Bouin’s solution. The second half of the testicle tissue was stored at -80°C for the biochemical analysis. Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the current laws of the Iranian government. All experimental procedures were carried in accordance with the Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals.

**Tissue processing**

The tissue was fixed in Bouin’s solution (7.5 mL saturated picric acid, 2.65 mL glacial acetic acid, and 2.5 mL 7% formaldehyde), post-fixed in 70% alcohol, and embedded in paraffin blocks. A tissue section (5µm) were obtained, deparaffinized, and stained with hematoxylin-eosin. The testicular tissue was evaluated in random order with standard light microscopy by an observer who was unaware as to which group the rat had belonged14. Then, testis tissue samples from the experimental rats were fixed at BOuin’s solution for complete fixation and processed for paraffin sectioning. A tissue section about 5µm thickness were taken and stained with hematoxylin and eosin [H & E]. The testis sections were graded numerically to assess the degree of histological changes associated with seminiferous tubule injury as previously described by Johnsen as bellow15:

- 10: complete spermatogenesis and perfect tubules
- 9: many spermatozoa present but disorganized spermatogenesis
- 8: only a few spermatozoa present
- 7: no spermatozoa but many spermatids present;
- 6: only a few spermatids present
- 5: no spermatozoa or spermatids present but many spermatocytes present
- 4: only a few spermatocytes present
- 3: only spermatogonia present
- 2: no germ cells present
- 1: neither germ cells nor Sertoli cells present

**Spermatozoa characteristics**

At the end of the study, semen samples were collected from the Cauda epididymis carefully separated from the testis and placed
in a Petri dish containing Ham’s F10. Epididymal caudal was minced with scissors to release sperm and then was placed in the incubator for 15min\textsuperscript{14}. Approximately, 10μL of the diluted sperm suspension was transferred to each counting chamber of the hemocytometer and allowed to stand for 5 min. The cells which settled during this time were counted by a light microscope at x200 magnification\textsuperscript{17}.

**Antioxidant activity**

The tissue MDA level was determined by a method based on the reaction with thiobarbituric acid (TBA)\textsuperscript{18}. In the TBA test reaction, MDA or MDA-like substances and TBA react with the production of a pink pigment with a maximum absorption at 532 nm\textsuperscript{19}. The SOD activity was expressed as nmol/g tissue. The GPx catalyses the oxidation of glutathione and in the presence of glutathione reductase and NADPH, oxide glutathione converts to the reduced form by changes in oxidation of NADPH to NADP\textsuperscript+\textsuperscript{20}. The GPx level was measured in absorbance of 340 nm\textsuperscript{20}. The GPx activity was expressed as U/mg tissue. Tissue SOD activity was measured according to the method of Paoletti and Mocali\textsuperscript{21}. In brief, the superoxide anions were generated from manganese (II) chloride and mercaptoethanol in the presence of acidethylenediaminetetraacetic acid. The SOD level was determined on the basis of its ability to inhibit nicotinamide adenine dinucleotide oxidation in reaction mixture after the addition of tissue homogenate. Nicotinamide adenine dinucleotide oxidation was measured was measured at 340 nm. The SOD activity was expressed as U/mg tissue.

**Statistical analysis**

Data were prepared in excel, the parametric data analyzed with one-way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean values ± standard error of mean (SEM). Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test. The KruskaleWallis test was used to compare group medians for histopathological scores. P<0.05 was considered to denote significant differences between groups.

**Results**

The effect of different levels FA on Score for assessing in testis and sperm mortality in experimental testicular I/R-induced rat is presented in Table 1. As seen in Figure 1, I/R group had the lowest testis damage grade compared to the other groups (P<0.05). The testis damage grade was higher among the control and sham (P>0.05). A dose dependent difference detected on testis damage grade in FA treated groups compared with I/R group (P<0.05). No difference observed between 2 and 5 mg/kg of the FA groups (P>0.05).

**Table 1** - Effect of different levels FA on Score for assessing in testis and sperm mortality in experimental testicular I/R-induced rat.

<table>
<thead>
<tr>
<th>Group</th>
<th>Score for assessing in testis</th>
<th>Mortality (%)</th>
<th>Mobility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10\textsuperscript{a}</td>
<td>72\textsuperscript{a}</td>
<td>70\textsuperscript{a}</td>
</tr>
<tr>
<td>Sham</td>
<td>3.2\textsuperscript{a}</td>
<td>71\textsuperscript{a}</td>
<td>68.5\textsuperscript{a}</td>
</tr>
<tr>
<td>I/R</td>
<td>4\textsuperscript{b}</td>
<td>40\textsuperscript{b}</td>
<td>39.4\textsuperscript{b}</td>
</tr>
<tr>
<td>FA (2 mg/kg)</td>
<td>7\textsuperscript{c}</td>
<td>43\textsuperscript{c}</td>
<td>42.9\textsuperscript{c}</td>
</tr>
<tr>
<td>FA (5 mg/kg)</td>
<td>8.5\textsuperscript{c}</td>
<td>55\textsuperscript{c}</td>
<td>55.2\textsuperscript{c}</td>
</tr>
<tr>
<td>FA (10 mg/kg)</td>
<td>10\textsuperscript{d}</td>
<td>67\textsuperscript{d}</td>
<td>66.8\textsuperscript{d}</td>
</tr>
</tbody>
</table>

FA: folic acid, I/R: ischemia/reperfusion. Different letters (a-d) indicate significant differences between treatments (P<0.05).
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As seen in Figure 2, spermatozoa mobility (%) significantly decreased in I/R group (P<0.05). Dose dependent increase observed on spermatozoa mobility (%) using different levels of the FA (2, 5 and 10 mg/kg) treated rat (P<0.05). No difference detected among 2 and 5 mg/kg of the FA groups (P>0.05).

According to the results, spermatozoa mortality (%) significantly decreased in I/R group compared to the control and sham groups (P<0.05) (Figure 3). Furthermore, dose dependent increase observed on spermatozoa mortality (%) in FA (2, 5 and 10 mg/kg) treated rat (P<0.05) but no difference observed between groups 2 and 5 mg/kg of the FA (P>0.05).

Effect of different levels FA on tissue values of MDA, SOD and GPx in experimental testicular I/R-induced rat is presented in Table 2. According to the results tissue MDA levels significantly increased in I/R rat (P<0.05) while FA (2, 5 and 10 mg/kg) in a dose dependent manner decreased I/R-induced MDA (P<0.05). Experimental I/R significantly decreased SOD activity compared to control group (P<0.05). Administration of the FA (2, 5 and 10 mg/kg) significantly increased tissue SOD activity in I/R rat (P<0.05). Orally gavage of the different levels of the FA (2, 5 and 10 mg/kg) in a dose dependent manner increased GPx activity in I/R rat (P<0.05).
Table 2 - Effect of different levels FA on tissue values of Malondialdehyde, Superoxide dismutase and Glutathione peroxidase in experimental testicular I/R-induced rat.

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/g tissue)</th>
<th>SOD (U/mg tissue)</th>
<th>GPx (U/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>130.26 ± 12.32 (^d)</td>
<td>3.62 ± 0.14 (^a)</td>
<td>4.11 ± 0.09 (^a)</td>
</tr>
<tr>
<td>Sham</td>
<td>131.37 ± 11.42 (^d)</td>
<td>3.57 ± 0.19 (^a)</td>
<td>4.12 ± 0.10 (^a)</td>
</tr>
<tr>
<td>I/R</td>
<td>172.37 ± 14.20 (^a)</td>
<td>2.11 ± 0.08 (^d)</td>
<td>2.09 ± 0.08 (^d)</td>
</tr>
<tr>
<td>FA (2 mg/kg)</td>
<td>158.14 ± 13.59 (^b)</td>
<td>2.24 ± 0.15 (^c)</td>
<td>2.91 ± 0.09 (^c)</td>
</tr>
<tr>
<td>FA (5 mg/kg)</td>
<td>154.42 ± 12.07 (^b)</td>
<td>2.25 ± 0.11 (^c)</td>
<td>3.10 ± 0.12 (^c)</td>
</tr>
<tr>
<td>FA (10 mg/kg)</td>
<td>141.10 ± 11.23 (^c)</td>
<td>3.22 ± 0.21 (^b)</td>
<td>3.09 ± 0.09 (^b)</td>
</tr>
</tbody>
</table>

FA: folic acid, MDA: malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase, I/R: ischemia/reperfusion. Different letters (a-d) indicate significant differences between treatments (P<0.05).

The effect of FA on testis histopathology is shown in Figures 4 to 9. According to the results, testis section of control (Figure 4) and sham (Figure 5) rats had shown normal seminiferous tubules and spermatogenesis with spermatocytes, Sertoli and spermatozoa.

Figure 4 - Testis section of control rats showing normal seminiferous tubules (arrow) and interstitial cells (arrow head) between tubules (left). Testis section of control rats showing normal seminiferous tubules with spermatogonia (black arrow), spermatocyte (black arrow head) and many spermatozoa (white arrow) (right) (Hematoxylin and Eosin - H&E).

Figure 5 - Testis section of sham rats showing normal seminiferous tubules (arrow) and interstitial cells (arrow head) between tubules (left). Testis section of control rats showing normal seminiferous tubules with spermatogonia (black arrow), spermatocyte (black arrow head) and many spermatozoa (white arrow) (right) (H&E).
Based on the Figure 6, seminiferous tubules degenerated and loss of spermatogenesis with few spermatocytes was observed in degenerated testis tubules in I/R rat.

Based on the results of Figure 7, seminiferous tubules degenerated and loss of spermatogenesis with few spermatocytes was observed in degenerated in orally administration of the FA (2mg/kg) followed by I/R rats.

Figure 6 - Testis section of I/R rats showing degenerated seminiferous tubules (arrow) and loss of spermatogenesis (H&E) (left) and degenerated seminiferous tubules (arrow) with few spermatocyte (arrowhead) in degenerated tubules (right) (H&E).

Figure 7 - Testis section of 7 days orally administration of the FA (2mg/kg) followed by I/R rats showing seminiferous tubules (arrow) with few spermatocyte and interstitial cells (arrow head) between tubules (left) and seminiferous tubules with few spermatocyte (arrow head) and spermatogonia (arrow) (right). H&E.

As seen in Figure 8, orally administration of the FA (5 mg/kg) improved testis characteristics with few normal seminiferous tubules and spermatocyte in seminiferous tubules in experimental I/R-induced rat.

Also, orally administration of the FA (10 mg/kg) improved testis characteristics with few normal seminiferous tubules and spermatocyte in seminiferous tubules in experimental I/R-induced rat (Figure 9).
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Figure 8 - Testis section of 7 days orally administration of the FA (5mg/kg) followed by I/R rats showing seminiferous tubules (arrow) with few spermatocyte and interstitial cells (arrow head) between tubules (left) and seminiferous tubules with f spermatocyte (arrow head) and spermatogonia (arrow) and few spermatid (right). H&E.

Figure 9 - Testis section of 7 days orally administration of the FA (10mg/kg) followed by I/R rats showing many normal seminiferous tubules (arrow) (left) and many spermatocyte (arrow) in seminiferous tubules (right). H&E.

Discussion

To the best of our knowledge, there are limited studies describing the role of FA on spermatozoa characteristics in experimental I/R rat. This study was conducted for the first time to investigate the effect of FA on testis histopathology and semen MDA, SOD and GPx in experimental I/R rat. The most pathogenetic mechanism of I/R injury is the over generation of ROS\textsuperscript{22}. During testicular I/R, the ROS production increased injury on ischemic tissue via oxidation of cell membrane lipids, proteins and DNA\textsuperscript{23}. The ROS have destructive effects on various cellular components in the organism\textsuperscript{24}. This may lead to molecular or genetic changes and pathologic features such as infertility\textsuperscript{7}. As observed in the current study, tissue MDA levels significantly increased in I/R rat while FA (2, 5 and 10 mg/kg) in a dose dependent manner decreased I/R-induced MDA. Experimental I/R significantly decreased SOD and GPx activity. Administration of the FA (2, 5 and 10 mg/kg) significantly increased tissue SOD and GPx.
activity in I/R rat.

MDA is the end product of lipid peroxidation and its elevated level is an indicator for free radical formation in post-ischemic tissue\textsuperscript{25}. Additionally, SOD and GPx are major enzymes that scavenge harmful ROS in male reproductive system\textsuperscript{26}. Based on the reports, I/R increased MDA and decrease SOD as well as GPx which leads to formation of inflammatory mediators\textsuperscript{15}. Spermatozoa membranes contain polyunsaturated fatty acids which are vulnerable to the ROS\textsuperscript{24}. In the testis, MDA levels elevates in case of the lipid peroxidation which leads to infertility\textsuperscript{27}. As observed in this study, spermatozoa mobility and mortality (%) significantly decreased in I/R group (Figures 2-3). Also, seminiferous tubules degenerated and loss of spermatogenesis with few spermatocytes was observed in degenerated testis tubules in I/R rat (Figure 7).

As observed, FA (2, 5 and 10 mg/kg) in a dose dependent manner decreased I/R-induced MDA. The FA (2, 5 and 10 mg/kg) significantly increased tissue SOD and GPx activity in I/R rat (Table 1). Supplementation of the FA decreases the risk of heart and limb defects\textsuperscript{26} and urinary tract anomalies\textsuperscript{29-30}. The I/R injury in testis resulted in decrease in spermatogenesis\textsuperscript{31}. The anti-inflammatory potentials of FA against I/R in rat testes was reported. FA has positive effect in the normal spermatogenesis, maturation and DNA metabolism, synthesis and transcription\textsuperscript{32}. Several researches confirm the repairing and maintenance role of FA during oxidative stress\textsuperscript{32}. However, Raigani reported FA supplementation did not ameliorate sperm quality in infertile men\textsuperscript{28}.

The FA successfully alleviated the SOD, GPx depletion and MDA elevation\textsuperscript{29}. In this study, dose dependent increase observed on spermatozoa mobility, mortality (%) using different levels of the FA (2, 5 and 10 mg/kg) treated rat (Figures 2-3). Orally administration of the FA (5 and 10 mg/kg) improved testis characteristics with few normal seminiferous tubules and spermatocyte in seminiferous tubules in experimental I/R-induced rat (Figures 9-10). In this regard, 74% increase in the sperm count in the men reported after administration of the FA. Also, FA (3 mg/day for 3 months) improved spermatozoa number and motility in infertile men\textsuperscript{29}. FA could protect cells against damage caused by lead\textsuperscript{32}. Therefore, FA supplementation affected positively spermatogenesis\textsuperscript{32}. Orally administration of the FA (2 mg/kg) for 7 days decreased MDA concentration in indomethacin-induced gastropathy in rats\textsuperscript{11}. In a similar study Şener et al\textsuperscript{33} reported administration of the apocynin (20 and 50 mg/kg) in 4 hours torsion and then reperfusion for 4 hours, normalized elevated oxidative enzyme levels in Rat which our results was similar to their findings. Also, in a study on effect of intraperitoneal injection of the Nifedipine (100 mg/kg) on testicular torsion-detorsion injury in rats, Mestrovic et al\textsuperscript{34} reported Nifedipine significantly decreased MDA and increased SOD and GPx levels which our results was in agreement to this report. On protective effect of Urapidil on testicular torsion Meštrović\textsuperscript{35} et al. reported intraperitoneal injection of the 10 ml/kg of the Urapidil 30 min before detorsion significantly significantly decreased MDA and increased SOD and GPx levels on testicular torsion-detorsion injury in rats. Also, Ozbek et al.\textsuperscript{36} revealed intraperitoneal injection of the Apocynin (20 mg/kg) significantly increased SOD, GPx and CAT and decreased MDA levels in 4h torsion followed by 1h detorsion rats.

\section*{Conclusions}

Folic acid prevents the progression of I/R-induced infertility by decrease elevated MDA levels in rat. Also, FA act as antioxidant...
and free radical scavenging activity through increase SOD and GPX levels in I/R-induced rat. Our results indicate FA could be used as an important therapeutic intervention in testicular I/R injury. The new findings of the current study can use as base information and further researches needed to determine effect of FA in human clinical trial.

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