Ovariectomy exacerabates glycerol-induced acute kidney injury in rats

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INTRODUCTION

Clinical and experimental studies have shown that reduced levels of ovarian hormones may play an important role in the pathogenesis of renal diseases. However, it remains unclear whether female hormones are protective or harmful to the kidneys. The influence of ovarian hormones seems to depend on the nature of the insult in acute kidney injury (AKI). In ischemic AKI, the presence of female hormones is associated with improvement of renal function and structure, lower creatinine and urea serum levels, and reduced renal inflammation and apoptosis. On the other hand, ovarian hormones may play an important role in the pathogenesis of renal diseases. However, it remains unclear what the exact role of ovarian hormones is in the development of AKI. Clinical studies have shown that reduced levels of ovarian hormones may play a protective role in the development of AKI.

Methods

Twenty-four female Wistar rats were divided into the following groups (n=6 per group): Sham, sham-operated female rats injected with saline; O VX, ovariectomized female rats injected with saline; Sham+Gly, sham-operated female rats injected with glycerol; OVX+Gly, ovariectomized female rats injected with glycerol. All rats were euthanized 3 days after the injections and the kidneys were removed for histological and immunohistochemical studies. Blood and urine samples were also collected for renal function studies. Results: The OVX+Gly group presented higher creatinine serum levels, as well as greater fractional excretion of sodium and urinary flow than the Sham+Gly group. Histological lesions and tubulointerstitial staining for macrophages, nuclear factor-kappa B, and nitrotyrosine were more pronounced in the renal cortex of the OVX+Gly group compared to the Sham+Gly group. Conclusion: We conclude that ovariectomy aggravates changes in renal function and structure in glycerol-induced acute kidney injury by the intensification of the pro-inflammatory tissue response.

Keywords: Ovariectomy; Glycerol; Rhabdomyolysis; Acute Kidney Injury; Inflammation

Conclusão: Concluímos que a ovariectomia agravou as alterações na função e estrutura renal, na lesão renal induzida por glicerol, pela intensificação da resposta tecidual pró-inflamatória.

Palavras-chave: Ovariectomia; Glicerol; Rabdomiólise; Lesão Renal Aguda; Inflamação.
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anion, hydrogen peroxide, and hydroxyl radical. Under conditions of imbalance in the redox status, nitric oxide (NO) reacts with the superoxide anion to form peroxynitrite, a highly reactive nitrogen species. Peroxynitrite and its breakdown products induce lipid peroxidation and changes in the structure of nucleic acids and proteins, increasing renal injury. Activation of nuclear factor-kappa B (NF-κB) by inflammation and oxidative stress induces the pro-inflammatory mediators' synthesis, which can intensify glycerol-induced renal injury. NF-κB is an important transcription factor that, when stimulated, undergoes translocation from the cytoplasm to the cell nucleus, promoting the transcriptional activation of genes related to inflammation, such as interleukin 1β (IL-1 β) and TNF-α.

Although some studies have shown that female sex hormones exert protective effects on renal tissue, the findings are still conflicting in different experimental models of AKI and hospitalized patients. Furthermore, a study to analyze the influence of female sex hormones in AKI induced by glycerol has not been performed yet. Thus, this study aimed to evaluate the effects of ovariectomy on glycerol-induced renal changes in female rats.

MATERIALS AND METHODS

Animals and Sample Size

Twenty-four female Wistar rats weighing 160–220 g were housed under controlled environmental conditions (12/12 h light/dark cycle and 24±2°C) with food and water ad libitum. All experimental procedures were conducted following the guidelines of the National Council for the Control of Animal Experimentation of Brazil and were approved by the Ethics Committee in Animal Experimentation of the State University of Feira de Santana, Bahia, Brazil (protocol 003/2012). For each of the four experimental conditions, 6 animals were used in each group. The sample size was established according to Sampaio (2007), which demonstrated the number of animals per treatment (n) = (t.SD/IC²), with t = Z value for 95% confidence interval; SD = standard deviation; CI = confidence interval of the variable.

Ovariectomy and Glycerol Injection

Female rats were anaesthetized with ketamine (50mg/kg) and xylazine (2 mg/kg) and submitted to bilateral ovariectomy (OVX) or sham surgery. Under anaesthesia, a small incision was performed on the opposite side. The muscle wall was sutured and the same procedure was repeated. Ovariectomy and Glycerol Injection

These animals were divided into the following groups: Sham, sham-operated rats injected with saline solution (n=6); OVX, ovariectomized rats injected with saline solution (n=6); Sham+Gly, sham-operated rats injected with glycerol (n=6); OVX+Gly, ovariectomized rats injected with glycerol (n=6). The estrous cycle stage was monitored throughout the experiment, according to the methodology previously described. The rats were euthanized 3 days after glycerol injection and the kidneys were removed for histological and immunohistochemical studies.

Blood Pressure

Blood pressure and heart rate were measured, before and post-injection of glycerol, by a programmable tail-cuff sphygmomanometer (LE 5001, Panlab Instruments, Barcelona, Spain). In conscious rats, systemic blood pressure and cardiovascular parameters were monitored with a pulse sensor placed noninvasively on their skin to detect arterial pulsations and non-turbulent blood flow throughout the cycle of tail cuff compression and release. To measure blood pressure accurately, the rats were warmed to 37°C for 5 minutes before readings began. Before measurement, the animals were acclimated to the blood pressure recording. The average of five pressure readings was recorded for each measurement.

Renal function studies

The rats were placed in metabolic cages immediately after glycerol injection. On post-injection day 3, blood and 24-h urine samples were collected to determine osmolality, as well as to quantify sodium, potassium, and creatinine levels. Serum and urine creatinine was measured by the Jaffé method. The levels of sodium and potassium were measured using flame photometry (model 910, Analyser, SP, Brazil) to determine the fractional excretions of these ions. Urinary flow was determined from the total volume of urine within 24 hours. Urine osmolality was determined by an osmometer of vapor pressure (model 5500, Wescor Osmometer, UT, USA). Proteinuria was performed by using a commercial kit (Sensiprot Labtest, MG, Brazil).

Renal Morphology

Kidney samples were fixed in methacarn solution (methanol 60%, chloroform 30%, and 10% acetic acid) and processed for paraffin embedding. Renal sections were stained with hematoxylin and eosin (HE) and examined under light microscopy. The tubulo-interstitial injury was defined as tubular necrosis, tubular lumen dilation, denudated basement membrane, intraluminal casts formation, swelling, and flattening of proximal tubular cells with brush border loss, inflammatory cell infiltrate or intratubular debris. A scale ranging from 0 to 4 was used to grade the renal damage. Thirty-grid fields from the renal cortex measuring 0.245 mm² were evaluated in a section of each kidney. The histological analysis was performed by two independent observers who did not know the treatment groups.
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Immunohistochemical study

Renal sections were deparaffinized and incubated overnight at 4°C with a rabbit polyclonal IgG antibody anti-NF-κB p65 (A) clone sc-109 (Santa Cruz Biotechnology, CA, USA; dil. 1:200), or for 1h at room temperature with a mouse monoclonal antibody anti-CD68 clone ED1 (antibody for rat macrophages/monocytes; AbD Serotec, Oxford, UK; dil. 1:1000), or rabbit polyclonal IgG antibody anti-nitrotyrosine (Millipore Corporation, MA, USA; dil. 1:100). Primary antibodies used were validated by their manufacturers for use in rats. Equivalent concentrations of normal rabbit IgG or mouse IgG were used to replace polyclonal and monoclonal antibodies, respectively. Before incubation with the primary antibody, nonspecific protein binding was blocked by incubation with 20% goat serum in PBS for 20 min. Slides were washed and then biotinylated goat anti-rabbit, or anti-mouse, secondary antibody (EasyPath; Signet Laboratories, USA; dil. 1:200) was added for 30 min at room temperature. Immunohistochemical reactivity was detected by reaction with an avidin-biotin-peroxidase complex (Leica Biosystems Newcastle, UK) and with 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). In sequence, the immunostained sections were counterstained with hematoxylin or methyl green. The number of ED1 positive cells and NF-κB positive nucleus were counted in 30 grid fields (measuring 0.245 mm²) from the tubulointerstitial compartment of the renal cortex, and mean counts per kidney were calculated. The cells labeled positive for ED1 present in capillary lumens were not counted as infiltrating cells. To evaluate immunoperoxidase staining for nitrotyrosine and NF-κB, thirty consecutive grid fields of the renal cortex of each animal (measuring 0.245 mm²) were graded by score (ranging from 0 to IV) and provided an average score for each kidney. The immunohistochemical analysis was performed by two independent observers who did not know the treatment groups.

Statistical Analysis

Data were expressed as mean±SEM and analyzed using GraphPad Prism7 software (San Diego, CA, USA). The level of statistical significance was set at P<0.05. The Kolmogorov-Smirnov test was performed to evaluate the normality of data distribution and the Brown-Forsythe test to analyze the homogeneity of the variances. When there was heterogeneity of variance, logarithmic transformation was applied. Kidney weight and NF-κB immunostaining score data were submitted to Kruskal-Wallis nonparametric test followed by the Dunn post hoc test. All other data were submitted to a two-way analysis of variance followed by the Tukey post hoc test.

RESULTS

Body weight, kidney weight, and cardiovascular parameters: The kidney weight of the glycerol groups was higher than the kidney weight of their respective saline groups (P<0.05), but no difference in this variable was found between control groups (Sham and OVX) or between glycerol groups. There was no difference in body weight, systolic blood pressure, and heart rate between the experimental groups (Table 1).

Table 1. Body weight, kidney weight, cardiovascular parameters, and renal function parameters of sham-operated rats injected with saline solution (Sham), ovariectomized rats injected with saline solution (OVX), sham-operated rats injected with glycerol (Sham+Gly), and ovariectomized rats injected with glycerol (OVX+Gly).

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=6)</th>
<th>Sham+Gly (n=6)</th>
<th>OVX (n=6)</th>
<th>OVX+Gly (n=6)</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>199.3±6.2</td>
<td>204.1±5.8</td>
<td>183.9±14.4</td>
<td>209.2±5.8</td>
<td></td>
</tr>
<tr>
<td>LKW/BW (g (100g)-1)</td>
<td>0.352±0.008</td>
<td>0.336±0.010</td>
<td>0.619±0.029</td>
<td>0.595±0.024</td>
<td>&gt;0.99 0.04 0.01 &gt;0.99</td>
</tr>
<tr>
<td>RKW/BW (g (100g)-1)</td>
<td>0.369±0.004</td>
<td>0.343±0.009</td>
<td>0.637±0.022</td>
<td>0.651±0.025</td>
<td>&gt;0.99 0.04 0.01 &gt;0.99</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>107.5±4.3</td>
<td>105.4±3.5</td>
<td>98.8±4.2</td>
<td>105.1±2.1</td>
<td>&gt;0.98 0.35 0.99 0.62</td>
</tr>
<tr>
<td>Heart rate (beats min-1)</td>
<td>342.5±15.9</td>
<td>361.7±11.5</td>
<td>378.8±11.5</td>
<td>361.1±7.5</td>
<td>&gt;0.99 0.69 0.21 &gt;0.99</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.98±0.04</td>
<td>1.00±0.05</td>
<td>3.04±0.52</td>
<td>5.41±0.60</td>
<td>&gt;0.99 0.08 &lt;0.0001 0.02</td>
</tr>
<tr>
<td>Urinary flow (µl/min)</td>
<td>4.51±0.85</td>
<td>4.56±0.62</td>
<td>14.4±0.85</td>
<td>21.9±3.31</td>
<td>&gt;0.99 0.004 &lt;0.0001 0.04</td>
</tr>
<tr>
<td>FENa+ (%)</td>
<td>0.73±0.03</td>
<td>0.63±0.08</td>
<td>4.48±0.83</td>
<td>12.15±3.49</td>
<td>&gt;0.99 0.47 &lt;0.001 0.03</td>
</tr>
<tr>
<td>FEK+ (%)</td>
<td>66.10±9.25</td>
<td>58.69±5.16</td>
<td>176.60±16.74</td>
<td>261.40±34.11</td>
<td>&gt;0.99 0.004 &lt;0.0001 0.03</td>
</tr>
<tr>
<td>Urinary osmolality (mmol/kg)</td>
<td>1803±82.42</td>
<td>1927±42.94</td>
<td>527.7±33.17</td>
<td>437.2±54.15</td>
<td>0.42 &lt;0.0001 0.66</td>
</tr>
<tr>
<td>Proteinuria (mg/24h)</td>
<td>3.26±0.81</td>
<td>3.15±0.81</td>
<td>26.04±4.03</td>
<td>15.72±2.67</td>
<td>&gt;0.99 &lt;0.0001 0.0005 0.44</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. a Sham versus OVX, b Sham versus Sham+Gly, c OVX versus OVX+Gly, d Sham+Gly versus OVX+Gly. LKW, left kidney weight. RKW, right kidney weight. BW, body weight. SBP, systolic blood pressure. FE, fractional excretion.
Estrous Cycle: The estrous cycle of rats lasts, on average, for four or five days, and is characterized by four stages proestrus, estrus, metestrus, and diestrus. The cycle phases were assessed every day for a week, before glycerol or saline injections, and the results demonstrated stages of proestrus, estrus, metestrus, and diestrus in the Sham female rats. In turn, the OVX female rats showed only histological aspects consistent with persistent diestrus. Persistent diestrus found in the OVX rats reflects a vaginal epithelium under estrogen depletion, thus confirming the effectiveness of ovariectomy\textsuperscript{17}.

Renal Function: Glycerol-injected rats presented higher creatinine serum levels, as well as greater fractional excretion of sodium and potassium, urinary flow, and proteinuria (P<0.05). These animals also demonstrated a decrease in urinary osmolality compared to the Sham and OVX groups (P<0.0001). However, in the OVX+Gly group, serum creatinine, fractional excretion of sodium and potassium, and urinary flow were higher than in the Sham+Gly group (P<0.05) (Table 1).

Renal Morphology: Histological studies showed tubulointerstitial lesions (TIL) areas in the renal cortex of Sham+Gly (2.96±0.19) and OVX+Gly (3.67±0.06) groups compared to the saline groups (Sham: 0.07±0.01 and OVX: 0.03±0.02) (P<0.0001; Fig. 1), but OVX+Gly group had higher TIL score than the Sham+Gly group (P=0.003; Fig. 1). These lesions were characterized by tubular cell necrosis, denuded basement membrane, intraluminal casts, swelling, and flattening of proximal tubular cells with brush border loss and interstitial inflammatory cell infiltrates. Glomerular morphology remained unchanged.

Immunohistochemistry Analysis: The glycerol-injected rats presented a higher number of the macrophages/monocytes with diffuse distribution in the renal cortex, predominantly located in damaged areas (Sham+Gly: 17.08±1.47 and OVX+Gly: 24.79±1.99), compared to saline groups (Sham: 2.78±0.39 and OVX: 2.61±0.32) (P<0.001; Fig. 2). The immunostaining for nitrotyrosine (Sham: 0.09±0.03, OVX: 0.14±0.04, Sham+Gly: 1.25±0.15 and OVX+Gly: 1.81±0.15, Fig. 3) and NF-κB (Sham: 0.10±0.02, OVX: 0.11±0.02, Sham+Gly: 1.37±0.14 and OVX+Gly: 2.03±0.14, Fig. 4) was higher in the tubulointerstitial compartment of the renal cortex of the glycerol-injected animals. Furthermore, the number of NF-κB positive nuclei was also higher in glycerol-injected animals (Sham: 0.16±0.1, OVX: 0.12±0.08, Sham+Gly: 3.73±0.62 and OVX+Gly: 5.87±0.73, Fig. 4). However, OVX+Gly rats showed increased infiltration of macrophages/monocytes, and more intense expression of nitrotyrosine and NF-κB compared to Sham+Gly (P<0.05; Fig. 2, 3, and 4). The number of NF-κB positive nuclei was also higher in the OVX+Gly rats than in the Sham+Gly rats (P<0.05; Fig. 4).
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Figure 2. Effects of ovariectomy in renal tubulointerstitial macrophages/monocytes infiltration in glycerol-induced acute kidney injury. Immunolocalization of ED1+ cells in the renal cortex of Sham (A), OVX (B), Sham+Gly (C), and OVX+Gly (D) rats. Original magnification x200. The scale bar represents 100 µm. Bar graphs showing (E) ED1+ cells quantification. Each field measures 0.245 mm². Data are expressed as mean±SEM. ***P<0.001 versus saline groups; ##P=0.003 versus Sham+Gly.

Figure 3. Effects of ovariectomy on renal nitrosative stress in glycerol-induced acute kidney injury. Nitrotyrosine immunolocalization in renal cortex of Sham (A), OVX (B), Sham+Gly (C), and OVX+Gly (D) rats. Note in D the higher staining intensity. Original magnification x200. The scale bar represents 100 µm. Bar graphs showing (E) nitrotyrosine immunostaining quantification. Data are expressed as mean±SEM. ***P<0.001 versus saline groups; #P=0.02 versus Sham+Gly.
Figure 4. Effects of ovariectomy on renal NF-κB expression in glycerol-induced acute kidney injury. NF-κB immunolocalization in the renal cortex of Sham (A), OVX (B), Sham+Gly (C), and OVX+Gly (D) rats. Note that the tubulointerstitial and nuclear (inset) NF-κB staining is more intense in D than in C. Original magnification x200, scale bar represents 100 µm; inset x 400, and the scale bar represents 50 µm. Bar graphs showing (E) nuclear and (F) tubulointerstitial immunostaining quantification. Data are expressed as mean±SEM. In E, ***P<0.001 versus saline groups; #P=0.03 versus Sham+Gly. In F, **P<0.01 versus saline groups; and #P=0.01 (F) versus Sham+Gly.

DISCUSSION

Our results demonstrate that ovariectomy exacerbated renal changes in the glycerol-induced AKI. Ovariectomized female rats injected with glycerol showed more intense changes in renal function and structure as well as increased macrophages, nitrotyrosine, and NF-κB expression in the renal cortex compared to intact female rats injected with glycerol. There is evidence that the AKI may be modulated by ovarian hormones, especially in ischemic AKI, suggesting the protective effect of estrogens3,4. On the other hand, the data are more conflicting in nephrotoxic AKI. Some experimental studies have shown that females are more susceptible to toxic injury than males2,19. In a clinical study, the female sex was identified as a risk factor for rhabdomyolysis-induced AKI20. Our data showed that the increase in serum creatinine, fractional excretion of sodium, urinary flow, and tubulointerstitial lesions induced by glycerol were more intense in the ovariectomized rats, suggesting the protective action of ovarian hormones in this experimental model. Other studies demonstrated that ovariectomy increased the serum levels of creatinine, blood urea nitrogen, proteinuria, and tubular lesions in ischemia/reperfusion, 5/6 nephrectomy, or Dahl Salt sensitive experimental models21,22.

Interstitial infiltration of macrophages and the abnormal production of proinflammatory cytokines in renal tissue are common finds in renal diseases6,7. AKI induces the production of inflammatory cytokines that contribute to the recruitment of inflammatory cells, including neutrophils and monocytes/macrophages4. Although studies have shown that ovarian hormones modulate the production of cytokines and macrophages23,24, no study reported the involvement of the ovarian hormones in the proinflammatory process of glycerol induced-AKI. As expected, our results showed a large number of macrophages in the renal cortex of all glycerol-injected rats. Notably, this macrophage infiltration was more intense in the renal cortex of the OVX+Gly group. Consistent with our findings, other studies have shown that ovariectomy intensified macrophage infiltration in mice with renal ischemic injury4 and increased the number of infiltrating inflammatory cells in renal tissue of spontaneously hypertensive rats25.

In turn, the involvement of oxidative stress in the pathophysiology of AKI induced by glycerol is widely known6,7. Activated macrophages can produce many cytotoxic compounds, including ROS, reactive nitrogen species (RNS), angiotensin II and cytokines11. Besides, the nitrotyrosine presence in renal tissue is a marker of endogenous production of peroxynitrite (a potent RNS) and renal tubular damage26. Soares et al.6 found that immunostaining for nitrotyrosine in the renal cortex was attenuated by treatment with resveratrol antioxidant on a glycerol-induced renal injury. In the present study, we observed that OVX intensified immunostaining for nitrotyrosine in the renal cortex of glycerol-injected rats. Female sex hormones can reduce the expression of factors related to the production of ROS such as NADPH oxidase and myeloperoxidase, and increase the expression of enzymes related to the synthesis of
glutathione, a natural antioxidant\(^2\). Moreover, Gamal El-Din et al.\(^27\) showed that ovariectomy increased serum creatinine, p38 mitogen-activated protein kinases, and peroxynitrite levels in renal tissue in ischemic AKI.

Our immunohistochemical studies showed that the cortical staining for NF-κB was more intense in the nucleus of tubular cells and the tubulointerstitial compartment of the renal cortex of the glycerol-injected ovariectomized rats. NF-κB activation plays an important role in many types of kidney diseases by inducing the synthesis of cytokines, growth factors, and chemotactic factors for monocytes\(^13\). NF-κB has been associated with proinflammatory and fibrotic processes in the kidney\(^28\). The change in redox state, cytokines, and urinary proteins can activate NF-κB, which induces the synthesis of more proinflammatory mediators\(^29\). Corroborating our results, other work demonstrated that OVX increased the expression of NF-κB in the renal cortex of Wistar rats\(^29\). In this regard, in a cardiovascular disease model, estradiol treatment in OVX mice was found to reduce hepatic levels of active NF-κB\(^30\). These authors suggested a cross-talk between the estrogen receptor and NF-κB that culminates in the inhibiting of this factor. Thus, the renoprotective action of ovarian hormones can also be attributed to the reduction of the proinflammatory response.

CONCLUSIONS

Taken together, our data show that changes in renal function, tubulointerstitial lesions, and expressions of pro-inflammatory and nitrosative stress markers in glycerol-induced AKI are exacerbated by ovariectomy, suggesting a renoprotective action of the ovarian hormones. Future studies with hormone replacement in OVX rats are needed to determine the involvement of estradiol and/or progesterone on renal lesions induced by glycerol.

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