NLRP3 Inflammasome Involvement in the Organ Damage and Impaired Spermatogenesis Induced by Testicular Ischemia and Reperfusion in Mice

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ABSTRACT

We investigated the role of the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome during testis ischemia and reperfusion injury (TI/R) in wild-type (WT) and NLRP3 knock-out (KO) mice. WT and KO mice underwent 1 hour testicular ischemia followed by 4 hours and 1 and 7 days of reperfusion or a sham TI/R. Furthermore, two groups of WT mice were treated at the beginning of reperfusion and up to 7 days with two inflammasome inhibitors, BAY 11-7082 (20 mg/kg i.p.) or Brilliant Blue G (45.5 mg/kg i.p.), or vehicle. Animals were killed with a pentobarbital sodium overdose at 4 hours and 1 and 7 days, and bilateral orchidectomies were performed. Biochemical and morphologic studies were carried out in all groups. TI/R in WT mice significantly increased caspase-1 and interleukin (IL)-1β mRNA after 4 hours and IL-18 mRNA at 1 day of reperfusion (P ≤ 0.05). There was also a significant increase in caspase-3 and terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling–positive cells, marked histologic damage, and altered spermatogenesis in WT mice in both tests after 1 and 7 days of reperfusion. KO TI/R mice, WT TI/R BAY 11-7082, and Brilliant Blue G treated mice showed a significant reduced IL-1β and IL-18 mRNA expression, blunted caspase-1 and -3 expression, minor histologic damages, low terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling activity, and preserved spermatogenesis. These data suggest that the activation of NLRP3 plays a key role in TI/R, and its inhibition might represent a therapeutic target for the management of patients with unilateral testicular torsion.

Introduction

Testis torsion is one of the most common testicular lesions in the pediatric population (Fan et al., 2013), with an estimated incidence of 3.5 per 100,000 person-year (Huang et al., 2013). It causes peculiar structural damages, thus leading to infertility (Huang et al., 2013); therefore, it must be treated promptly to avoid ipsilateral testicular dysfunction (Anderson and Williamson, 1986). Events occurring during spontaneous or experimental testicular torsion and subsequent detorsion basically depend on the extension of ischemia (Filho et al., 2004) and are representative of the ischemia and reperfusion injury observed in other organs (Grace, 1994). Under these circumstances, the testis produces several proinflammatory cytokines, including tumor necrosis factor α and interleukin (IL)-1β (Lysiak et al., 2000). The increased levels of these cytokines can be considered indirect evidence of tissue inflammation.

In addition, testicular ischemia/reperfusion (TI/R) triggers the apoptosis cascade, leading to the degeneration of the germinal epithelium (Tripathi et al., 2009). Apoptosis is required for normal spermatogenesis in mammals at different stages of differentiation to ensure cellular homeostasis (Blanco-Rodríguez, 1998). After TI/R, increased expression of Bax and caspase-1 and -3 were observed (Lysiak, 2004), and interleukin-1β and IL-18 mRNA expression, blunted caspase-1 and -3 expression, minor histologic damages, low terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling activity, and preserved spermatogenesis.
indicating that both initiator and executioner caspases were involved in the apoptotic cascade triggered by the experimental procedure. It was suggested that the regulation of germ cell apoptosis might be central in the development of experimental strategies to prevent their damage (Minutoli et al., 2009).

Inflammasomes are multimolecular complexes assembled in response to various activators and leading to the intracellular activation of the inflammatory cascade, particularly by raising the cleavage of the inactive precursor of IL-1β to its active form (Schroder and Tschopp, 2010). Several inflammasomes have been identified (Tian et al., 2009; Duéñez-Guzmán and

![Fig. 1. Representative Western blot analysis of caspase-1 at 4 hours (A) and caspase-3 at 1 (B) and 7 days (C) in testes of sham WT and KO mice, TI/R + vehicle and CL + vehicle WT and KO mice, and BAY 11-7082– and BBG-treated TI/R and CL WT mice. *P < 0.05 versus sham WT mice; **P < 0.05 versus WT I/R + vehicle mice; †P < 0.05 versus sham KO mice. Bars represent the mean ± S.E. of seven experiments.](image-url)
Haig, 2014). The nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is currently the most fully characterized, and it is formed by the NOD-like receptor NLRP3 by an adapter protein called apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and procaspase-1. Furthermore, NLRP3 is of special interest because it responds to different physical and chemical stimuli, leading to many diseases when out of control (Chen and Sun, 2013), and can be assembled following a wide variety of stimuli, including tissue damage (Gross et al., 2011). The actual activation of the NLRP3 inflammasome is a poorly understood event, which involves the integration of many signals indicative of tissue damage or stress (Gross et al., 2011). The inflammasome NLRP3 is an important regulator of caspase-1 (Martinon et al., 2002; Stutz et al., 2009; Kayagaki et al., 2011), which can trigger the sequential activation of caspase-3 (Stutz et al., 2009) and regulate the maturation of the proinflammatory cytokines IL-1β and IL-18 or the rapid inflammatory form of cell death called pyroptosis.

Specific small molecule inhibitors, such as BAY 11-7082 (Juliana et al., 2010) and Brilliant Blue G (BBG) (Zhao et al., 2013), are able to inhibit the proinflammatory and proapoptotic effects of the NLRP3 inflammasome. More in detail, BAY 11-7082, a kinase-β inhibitor, selectively inhibited NLRP3 inflammasome activity in macrophages independently of its inhibitory effect on the priming step following nuclear factor κB activation (Juliana et al., 2010). BBG blocks the membrane-bound purinergic P2X7 receptor (Diaz-Hernández et al., 2009), which in turn suppresses the expression of ASC and inhibits NLRP3 inflammasome activation (Zhao et al., 2013).

In light of this background and to better understand the role of the NLRP3 inflammasome during testicular torsion, we investigated TI/R injury in wild-type (WT) and NLRP3 knockout (KO) mice.

Materials and Methods

Experimental Protocol. All animals were housed and maintained under specific pathogen-free conditions at the animal facility of the School of Medicine at the University of Messina, Italy. All experimental procedures complied with the Declaration of Helsinki, with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the United States National Institutes of Health and the Animal Research: Reporting of In Vivo Experiments guidelines (Kilkenny et al., 2010) and were authorized by our local institution. WT (C57Bl/6) male mice and KO (Nlrp3tm1bhk) (25–30 g) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals were provided a standard diet ad libitum, with free access to tap water, and were maintained on a 12-hour light/dark cycle. Both WT and KO mice (total number = 126; each group consisted of seven animals) were anesthetized with an intraperitoneal injection of 80 mg/kg of pentobarbital sodium and torsion of the left testis and spermatic cord was performed as previously described (Minutoli et al., 2009). Then, the same testis was detorted. WT and KO animals of all experimental groups underwent testicular ischemia for 1 hour, followed by 4 hours and 1 day and 7 days reperfusion (TI/R). WT and KO sham mice were operated as previously indicated for experimental groups, except for testicular torsion and consequent ischemia. Furthermore, only WT mice were treated either with BAY 11-7082 (20 mg/kg) or Brilliant Blue G (BBG) (45.5 mg/kg) every 2 days (Diaz-Hernández et al., 2009) or vehicle (a 1:3 solution of dimethylsulfoxide and 0.9% NaCl) for up to 7 days. Animals were killed at the above indicated time points after reperfusion with an overdose of pentobarbital sodium, and bilateral orchidectomies were performed. The testes were collected, weighted, and treated for different experimental technical procedures.

Drugs and Chemicals. The following compounds were supplied as indicated: BAY 11-7082 by Adipogene (San Diego, CA), BBG by Sigma-Aldrich (Milan, Italy), and sodium pentobarbital by Intervet (Milan, Italy).

Isolation of Soluble Proteins. Isolation of soluble proteins from testis samples (about 30 mg) of mice was performed in 1 ml of lysis buffer [25 mM Tris/HCl (pH 7.4), 1.0 mM EGTA, 1.0 mM EDTA, 0.5 mM phenyl methylsulfonyl fluoride, 1% NP40, and aprotinin, leupeptin, and pepstatin A (10 mg/ml each)] and homogenized with a Dounce homogenizer (Sigma Aldrich, Milan, Italy; Paterniti et al., 2013). The homogenate was centrifuged at 15,000g for 15 minutes, and the supernatant was collected and used for protein determination with the Bio-Rad protein assay kit (Richmond, CA).

Determination of Active Caspase-1 and -3 by Western Blot Analysis. Total proteins (30 μg) were denatured with reducing buffer [62 mM Tris (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 0.003% bromophenol blue], separated by electrophoresis, and then transferred onto a polyvinylidene difluoride membrane using a transfer buffer [39 mM glycine and 48 mM Tris (pH 8.3)] at 200 mA for 1 hour. To block membrane proteins, 5% nonfat dry milk in Tris-buffered saline (TBS)–0.1% Tween for 1 hour at room temperature was used, followed by three washes with TBS–0.1% Tween for 10 minutes each, and then incubated with a primary antibody for caspase-1 and -3 (Abcam, Cambridge, UK) and diluted 1:200 in TBS–0.1% Tween. The following secondary antibodies were used: anti-mouse (Jackson Laboratory, Bar Harbor, ME) and anti-rabbit (Jackson Laboratory, Bar Harbor, ME) and diluted 1:1000 in TBS–0.1% Tween. The membranes were washed, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, washed again, and then developed with the enhanced chemiluminescence (ECL) system (GE Healthcare, Piscataway, NJ). Blots were scanned, and intensity was measured with the Quantity One software (Bio-Rad). Total proteins (30 μg) of each testis sample were analyzed by Western blot analysis. NLRP3 mRNA levels were normalized to 18S levels by densitometry analysis using Quantity One software.

Fig. 2. mRNA expression for IL-1β at 4 hours (A) and IL-18 at 1 day (B) in the testes of sham WT and KO mice, TI/R + vehicle and CL + vehicle WT and KO mice, and BAY 11-7082- and BBG-treated TI/R and CL WT mice. *P < 0.05 versus sham WT mice; **P < 0.05 versus WT I/R + vehicle mice; ***P < 0.05 versus WT I/R + vehicle KO mice; ****P < 0.05 versus sham KO mice. Bars represent the mean ± S.E. of seven experiments.
At 7 days, in sham WT and KO mice testes, no TUNEL-positive cells are observed. (A) In TI/R WT mice at 1 day, isolated TUNEL-positive cells (arrow) are evident. (B) In TI/R + vehicle WT mice at 1 day, many TUNEL-positive germ cells (arrows) are present (scale bar: 50 μm). (C) In TI/R WT + BAY 11-7082 1 day, few TUNEL-positive cells are present (scale bar: 50 μm). (D) In TI/R WT + BBG 1 day, few groups of TUNEL-positive germ cells (arrow) are present. (E) In TI/R KO mice at 1 day, occasional TUNEL-positive germ cells (arrows) are present in the outer part of the tubules. (F) At 7 days, in sham WT and KO mice testes, no TUNEL-positive cells are observed. (G) In TI/R + vehicle WT mice at 7 days, some peripheral groups of TUNEL-positive germ cells are evident (arrow). (H) In TI/R KO mice at 7 days, occasional TUNEL-positive germ cells (arrows) are present in the outer part of the tubules. (I) In TI/R BAY 11-7082–treated WT mice at 7 days, rare TUNEL-positive germ cells (arrow) are observed. (J) In TI/R BBG-treated WT mice at 7 days, few groups of TUNEL-positive germ cells (arrow) are present (scale bar: 50 μm).

Fig. 3. Assessment of apoptosis with TUNEL technique at 1 and 7 days after the surgical procedure in TI/R WT and KO mice. (A) At 1 day, both sham WT and KO mice testes show no TUNEL-positive cells. (B) In TI/R + vehicle WT mice at 1 day, many TUNEL-positive germ cells (arrows) are present in the seminiferous tubules. (C) In TI/R KO mice at 1 day, peripheral groups of TUNEL-positive germ cells (arrow) are evident. (D) In TI/R WT + BAY 11-7082 1 day, isolated TUNEL-positive cells (arrow) are evident. (E) At 7 days, in sham WT and KO mice testes, no TUNEL-positive cells are observed. (F) In TI/R + vehicle WT mice at 7 days, some peripheral groups of TUNEL-positive germ cells are evident (arrow). (H) In TI/R KO mice at 7 days, occasional TUNEL-positive germ cells (arrows) are present in the outer part of the tubules. (I) In TI/R BAY 11-7082–treated WT mice at 7 days, rare TUNEL-positive germ cells (arrow) are observed. (J) In TI/R BBG-treated WT mice at 7 days, few groups of TUNEL-positive germ cells (arrow) are present (scale bar: 50 μm).
spermaticis present; 9, no germ cells or spermaticis, but many spermatocytes present; 10, only a few spermatocytes present; 11, only spermatogonia present; 12, no germ cells, but only Sertoli cells present; 13, no germ cells and no Sertoli cells present.

As to the extratubular compartment, a previously described method (Minutoli et al., 2005) for the evaluation of edema, hemorrhagic extravasation, vessel dilation, and Leydig cell change evaluation was used, and each parameter was quantified on the basis of the following scale: 0, absent; 1, mild; 2, moderate; and 3, severe.

**Terminal Deoxynucleotidyl Transferase-Mediated Digoxigenin-Deoxyuridine Nick-End Labeling Technique.** For the terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) technique, an apoptosis detection kit (TUNEL Universal Apoptosis Detection Kit; GenScript, Piscataway, NJ) was used on paraffin-embedded 5-μm sections cleared with xylene and dehydrated in graded ethanol. Protein was digested with proteinase K (20 μg/ml PBS), and endogenous peroxidase activity was blocked with 3% H2O2 in methanol. Sections were incubated with terminal deoxynucleotidyl transferase enzyme and biotin-11-deoxyuridine, then with the streptavidin-peroxidase substrate, and finally with the diaminobenzidine solution. Two trained observers without knowledge of the treatment blindly assessed the micrographs, which were obtained and processed as previously indicated for the histologic specimens. From each group, the percentage of tubules with apoptotic cells and the apoptotic index (mean number of TUNEL-positive cells per tubule in 100 seminiferous tubules) (Tsounapi et al., 2012) were calculated.

**Statistical Analyses.** All data are expressed as the mean ± S. E., with the exception of the percentage of tubules with apoptotic cells, where absolute values are provided. Data were analyzed with one-way analysis of variance using Tukey’s test as the post hoc procedure or Student’s t test. A P value of ≤ 0.05 was considered statistically significant.

**Results**

**Active Caspase-1 and Caspase-3 Expression in TI/R.** No caspase-1 activity was demonstrated in the testes of either sham WT or KO mice (Fig. 1A). After 4 hours of reperfusion, TI/R injury determined a significant increase of caspase-1 expression, which was demonstrated in the testes of sham WT or KO mice (Fig. 1A). After 4 hours of reperfusion, TI/R injury determined a significant increase of caspase-1 expression, which was demonstrated in the testes of sham WT or KO mice (Fig. 1A). After 4 hours of reperfusion, TI/R injury determined a significant increase of caspase-1 expression, which was demonstrated in the testes of sham WT or KO mice (Fig. 1A). After 4 hours of reperfusion, TI/R injury determined a significant increase of caspase-1 expression, which was demonstrated in the testes of sham WT or KO mice (Fig. 1A). After 4 hours of reperfusion, TI/R injury determined a significant increase of caspase-1 expression, which was demonstrated in the testes of sham WT or KO mice (Fig. 1A). After 4 hours of reperfusion, TI/R injury determined a significant increase of caspase-1 expression, which was demonstrated in the testes of sham WT or KO mice (Fig. 1A).

**TABLE 2A**

<table>
<thead>
<tr>
<th>Tubular Compartment</th>
<th>MSTD (μm)</th>
<th>Johnson's Score</th>
<th>%TWAC</th>
<th>Apoptotic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham WT</td>
<td>179.4 ± 8.87</td>
<td>9.61 ± 0.33</td>
<td>3</td>
<td>0.23 ± 0.34</td>
</tr>
<tr>
<td>Sham KO</td>
<td>177.3 ± 9.12</td>
<td>9.49 ± 0.47</td>
<td>5</td>
<td>0.29 ± 0.55</td>
</tr>
<tr>
<td>WT TI/R + vehicle 7 days</td>
<td>136.9 ± 8.81a</td>
<td>4.95 ± 0.82a</td>
<td>63a</td>
<td>6.96 ± 1.73a</td>
</tr>
<tr>
<td>WT CL + vehicle 7 days</td>
<td>150.6 ± 8.89</td>
<td>6.98 ± 0.64</td>
<td>42</td>
<td>4.4 ± 1.22</td>
</tr>
<tr>
<td>WT TI/R + BAY 11-7082 7 days</td>
<td>161 ± 7.73b</td>
<td>7.48 ± 0.83b</td>
<td>12b</td>
<td>1.24 ± 0.52b</td>
</tr>
<tr>
<td>WT CL + BAY 11-7082 7 days</td>
<td>168.5 ± 6.38</td>
<td>8.11 ± 0.97</td>
<td>6</td>
<td>0.67 ± 1.12</td>
</tr>
<tr>
<td>WT TI/R + BBG 7 days</td>
<td>160.2 ± 8.12b</td>
<td>7.44 ± 0.52b</td>
<td>11b</td>
<td>1.16 ± 0.44b</td>
</tr>
<tr>
<td>WT CL + BBG 7 days</td>
<td>168.3 ± 9.15</td>
<td>7.81 ± 0.67</td>
<td>9</td>
<td>0.82 ± 0.71</td>
</tr>
<tr>
<td>KO TI/R 7 days</td>
<td>159 ± 9.23c</td>
<td>7.35 ± 0.6c</td>
<td>15c</td>
<td>1.21 ± 0.95c</td>
</tr>
<tr>
<td>KO CL 7 days</td>
<td>168 ± 11.87</td>
<td>8.25 ± 0.72</td>
<td>10</td>
<td>0.92 ± 0.68</td>
</tr>
</tbody>
</table>

MSTD = mean seminiferous tubule diameter; TWAC, tubules with apoptotic cells.

aP < 0.05 versus sham WT mice.
bP < 0.05 versus WT TI/R + vehicle mice.
cP < 0.05 versus sham KO mice.
expression was significantly lower in the ipsilateral and CL testes of WT mice treated with both BAY 11-7082 and BBG and in KO mice (Fig. 1A).

No caspase-3 expression was observed in the testes of both sham WT and KO mice at 1 and 7 days (Fig. 1, B and C). On the contrary, TI/R injury induced an increased expression of caspase-3 in the ipsilateral testis of WT animals after 1 and 7 days of reperfusion (Fig. 1, B and C). At 7 days, the levels of caspase-3 further increased when compared with those at 1 day (Fig. 1, B and C). Caspase-3 expression was significantly lower in the ipsilateral testes of WT mice treated with both BAY 11-7082 and BBG and in KO mice (Fig. 1, B and C).

Fig. 4. Histologic findings at 1 day after the surgical procedure in seminiferous tubules stained with hematoxylin and eosin. (A and B) Sham WT and KO mice show a normal morphology. (C) In TI/R + vehicle WT mice, degenerative changes of the germinal cells (arrowhead) and edema of the extratubular compartment (*) are evident. (D) CL testes of WT mice show disorganized germinal epithelium (arrows) and hemorrhagic extravasation (h). (E, G, and I) BAY 11-7082– and BBG-treated WT TI/R and KO TI/R testes. Only mild changes of the germinal epithelium and a normal extratubular compartment are present. (F, H, and J) BAY 11-7082– and BBG-treated WT CL and KO CL testes. No evident morphologic changes are recognized in both the tubular and extratubular compartments (scale bar: 50 μm).
Similar results were observed in the CL testes of WT and KO mice even if the absolute values were lower.

**IL-1β mRNA and IL-18 mRNA Expression in TI/R.**

Testes from sham animals of both strains had negligible basal levels of IL-1β mRNA expression (Fig. 2A). TI/R injury induced an increased expression of IL-1β mRNA in the ipsilateral testes of WT animals following 4 hours of reperfusion (Fig. 2A). IL-1β mRNA expression was significantly lower in the ipsilateral testes of WT mice treated with both BAY 11-7082 and BBG and in KO mice (Fig. 2A). Similar results were observed in the CL testes of WT and KO mice even if the absolute values were lower.

Testes from sham animals of both strains had very low basal levels of IL-18 mRNA expression (Fig. 2B). After 1 day of reperfusion, the expression of IL-18 mRNA was significantly increased in WT animals (Fig. 2B). IL-18 mRNA expression was significantly lower in the ipsilateral testes of WT mice treated with both BAY 11-7082 and BBG and in KO mice (Fig. 2B). Similar results were observed in the CL testes of WT and KO mice even if the absolute values were lower.

**Morphologic Evaluation of Apoptosis.** No TUNEL-positive germ cells were observed in the seminiferous tubules from the sham groups of both the WT and KO mice at 1 and 7 days of reperfusion (Fig. 3, A and F; Tables 1A and 2A).

After 1 day of reperfusion, in TI/R WT mice, many TUNEL-positive germ cells were observed in the wall of the seminiferous tubules (Fig. 3B; Table 1A). In TI/R KO mice, few TUNEL-positive germ cells were present in the periphery of the seminiferous tubules (Fig. 3C; Table 1A). Similarly, in BAY 11-7082–(Fig. 3D; Table 1A) and BBG-treated TI/R WT mice (Fig. 3D; Table 1A), testes showed a low number of TUNEL-positive cells only in the peripheral layer of the tubules.

After 7 days of reperfusion, in TI/R WT mice, many peripheral TUNEL-positive germ cells were evident (Fig. 3G; Table 2A). On the contrary, in TI/R KO mice (Fig. 3H; Table 2A) and BAY 11-7082–(Fig. 3I; Table 2A) and BBG-treated TI/R WT mice (Fig. 3J; Table 2A), few isolated TUNEL-positive germ cells were observed in the periphery of the seminiferous tubules.

**TABLE 1B**

Effects of ischemia/reperfusion on the extratubular compartment in the testes of WT and KO sham, TI/R and CL of WT plus vehicle, BAY 11-7082– and BBG-treated WT, and KO mice at 1 day after the surgical procedure

<table>
<thead>
<tr>
<th>Extratubular Compartment</th>
<th>Edema</th>
<th>Hemorrhagic Extravasation</th>
<th>Vascular Dilation</th>
<th>Leydig Cell Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham WT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sham KO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>WT TI/R + vehicle 1 day</td>
<td>2.1 ± 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.55 ± 0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.15 ± 0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT CL + vehicle 1 day</td>
<td>0.95 ± 0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.44 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8 ± 0.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT TI/R + BAY 11-7082</td>
<td>0.41 ± 0.34&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.41 ± 0.14&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.38 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.19 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 day</td>
<td>0.3 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT CL + BAY 11-7082</td>
<td>0.48 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.66 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.55 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 day</td>
<td>0.29 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.31 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT TI/R + BBG 1 day</td>
<td>0.45 ± 0.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.55 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4 ± 0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KO TI/R 1 day</td>
<td>0.34 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.31 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.51&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> <sup>P < 0.05 versus sham WT mice.</sup>
<sup>b</sup> <sup>P < 0.05 versus WT TI/R + vehicle mice.</sup>
<sup>c</sup> <sup>P < 0.05 versus sham KO mice.</sup>

No significant variations were observed in the CL testes of both strains of mice (morphologic data not shown).

**Histologic Evaluation.** After 1 day of reperfusion, the sham groups of both the WT and KO mice showed seminiferous tubules and extratubular compartments with normal morphology (Fig. 4, A and B; Tables 1A and 1B).

In the WT mice, the TI/R group showed massive tubular disorganization, with degenerative changes of the germinal cells, interstitial edema, hemorrhagic extravasation from slightly dilated vessels, and Leydig cell changes (Fig. 4C; Tables 1A and 1B). CL testes of the same mice showed small tubules with disorganized germinal epithelium and vascular dilation with hemorrhagic extravasation (Fig. 4D; Tables 1A and 1B).

In BAY 11-7082–(Fig. 4E; Tables 1A and 1B) and BBG-treated WT mice (Fig. 4G; Tables 1A and 1B) and KO mice (Fig. 4I; Tables 1A and 1B), TI/R testes showed only mild changes of the germinal epithelium, but a normal extratubular compartment. In the same groups (Fig. 4, F, H, and J; Tables 1A and 1B), CL testes showed well preserved sperm cells and a normal extratubular compartment.

After 7 days of reperfusion, the sham groups showed normal morphology of both the seminiferous tubules and extratubular compartment (Fig. 5, A and B; Tables 2A and 2B).

In the WT mice, the TI/R group presented small tubules with a highly disorganized germinal epithelium and some spermatagonia along their periphery. The extratubular compartment was damaged, and Leydig cells showed mild-to-moderate structural changes consisting of pale cytoplasm and darker and condensed nuclei (Fig. 5C; Tables 2A and 2B). CL testes of WT mice at the same time had larger, less damaged tubules, while the extratubular compartment showed well evident edema (Fig. 5D; Tables 2A and 2B).

In BAY 11-7082–(Fig. 5E; Tables 2A and 2B) and BBG-treated WT mice (Fig. 5G; Tables 2A and 2B) and KO mice (Fig. 5I; Tables 2A and 2B), TI/R testes showed a germinal epithelium, with many immature spermatids, reduced spermatooza, and some intercellular clefts. The extratubular compartment exhibited mild edema and few dilated vessels. In the same groups (Fig. 5, F, H, and J; Tables 2A and 2B), CL
testes showed better preserved sperm cells and a normal extratubular compartment.

Discussion

Experimental studies have suggested that innate pattern recognition receptors may prime tissue and organ damage during ischemic states (Shigeoka et al., 2007, 2010; Haneklaus et al., 2013). Testicular torsion is characterized by tissue damage and represents a urological emergency (Ringdahl and Teague, 2006), which, when misdiagnosed and inappropriately treated, can lead to male infertility (Antonuccio et al., 2006). Several mechanisms have been implicated in the development
of testicular damage following the torsion and detorsion processes (Lysiak et al., 2000; Nadiri et al., 2006; Srinivasan et al., 2007; Minutoli et al., 2009, 2012), which have been considered similar to ischemia-reperfusion injuries observed in different organs (Eltzschig and Eckle, 2011).

Ischemia and reperfusion is a pathologic event characterized, at first, by a reduction of blood supply followed by the reestablishment of perfusion and related reoxygenation (Eltzschig and Eckle, 2011). It induces early tissue injuries, such as reactive oxygen species (ROS) generation (Lei et al., 2014), interstitial edema (Widgerow, 2014), and a damaged barrier function of endothelial cells, due to reduced adenylate cyclase activity and intracellular cAMP levels. In particular, during ischemia and reperfusion, nucleotides in the form of ATP promote tissue inflammation and activate the NLRP3 inflammasome (McDonald et al., 2010).

Ischemia and reperfusion activates various programs of cell death, among which necrosis and apoptosis play a significant role. Necrosis is characterized by a swelling of cells and organelles, with rupture of their membranes and release of their contents. These cells trigger the inflammatory process and cytokine production. In contrast, apoptosis is characterized by a cellular and nuclear shrinkage and plasma membrane integrity (Hotchkiss et al., 2009).

In particular, TI/R results in exaggerated production of ROS, activates the mitogen-activated protein kinases family, and triggers the inflammatory cascade and apoptosis machinery. This pathologic cascade is responsible for the testicular atrophy and impaired spermatogenesis observed at a later stage (Filho et al., 2004; Antonuccio et al., 2006; Nadiri et al., 2006; Srinivasan et al., 2007; Minutoli et al., 2009, 2012). Even if the importance of early events is well recognized, the late phase of TI/R is crucial from a clinical point of view as the testis morphology and function could be severely impaired (Altavilla et al., 2012; Cvetkovic et al., 2015).

Increased levels of tumor necrosis factor α and IL-1β have been measured during testicular ischemia and reperfusion (Lysiak et al., 2000), thus confirming a role of inflammation in testis twisting. Inflammation works in concert with apoptosis to induce late organ damage and may be responsible for infertility.

Apoptosis, further than a physiologic process that entails the programmed cell death, can also induce testicular injury. In fact, this process is essential during normal spermatogenesis in both humans and animals; however, TI/R injury causes apoptosis of germ cells (Tripathi et al., 2009). All these findings, taken together, clearly suggest that inflammation and apoptosis are important components of the organ damage and impaired spermatogenesis induced by testicular twisting and untwisting.

Muckle-Wells syndrome (MWS) is an inflammatory disease consisting of recurrent symptoms of ocular and joint inflammation, fever, and skin rash, which is due to NLRP3 gene mutations causing an impairment in the activity of the gene product cryopyrin (Agostini et al., 2004). Cryopyrin induces caspase-1 and, in turn, the cleavage of IL-1β and IL-18 that causes the typical inflammatory disease of MWS (Agostini et al., 2004; Hoffman et al., 2001).

Recently, a study suggested that the NLRP3/ASC/caspase-1 axis regulated the proinflammatory cytokines IL-1β and IL-18 (Xie et al., 2014).

Interestingly, patients suffering from MWS have oligozoospermia and/or azoospermia and show subfertility or infertility (Tran et al., 2012). On the basis of this observation and of other experimental data (Lech et al., 2010), as NLRP3 is involved in the molecular cascade of inflammation and apoptosis in response to cellular ischemia, we thought it was worthy of interest to investigate its role in the altered spermatogenesis induced by TI/R injury.

IL-1β and IL-18 were markedly reduced in the TI/R inflammasome–deficient mice compared with the TI/R WT animals. These data suggest that the inflammatory cascade induced by TI/R injury is blunted in the absence of this molecular platform.

Also, contralateral testes showed evident structural damages, even if they were milder than those of the I/R testes. Several mechanisms have been proposed to understand the contralateral testicular damage, such as a decrease in testicular blood flow after an afferent stimulus (Prillaman and Turner, 1997; Andiran et al., 2000; Minutoli et al., 2005), ROS generation after detorsion and overproduction of nitric oxide (NO), or autoimmunization against the spermatagonia

### Table 2B

<table>
<thead>
<tr>
<th>Extratubular Component</th>
<th>Edema</th>
<th>Hemorrhagic Extravasation</th>
<th>Vascular Dilation</th>
<th>Leydig Cell Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham WT</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Sham KO</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>WT TI/R + vehicle 7 days</strong></td>
<td>2.25 ± 0.44</td>
<td>0.92 ± 0.61</td>
<td>1.07 ± 0.59</td>
<td>0.55 ± 0.51</td>
</tr>
<tr>
<td><strong>WT CL + vehicle 7 days</strong></td>
<td>1.85 ± 0.71</td>
<td>0.72 ± 0.3</td>
<td>0.65 ± 0.49</td>
<td>0.7 ± 0.65</td>
</tr>
<tr>
<td><strong>WT TI/R + NLRP3 KO 7 days</strong></td>
<td>0.66 ± 0.25</td>
<td>0.82 ± 0.27</td>
<td>0.53 ± 0.14</td>
<td>0.53 ± 0.4</td>
</tr>
<tr>
<td><strong>WT CL + NLRP3 KO 7 days</strong></td>
<td>0.33 ± 0.19</td>
<td>0.49 ± 0.18</td>
<td>0.39 ± 0.22</td>
<td>0.41 ± 0.27</td>
</tr>
<tr>
<td><strong>WT TI/R + vehicle 7 days</strong></td>
<td>0.55 ± 0.49</td>
<td>0.71 ± 0.35</td>
<td>0.54 ± 0.44</td>
<td>0.44 ± 0.41</td>
</tr>
<tr>
<td><strong>WT CL + vehicle 7 days</strong></td>
<td>0.46 ± 0.33</td>
<td>0.42 ± 0.26</td>
<td>0.33 ± 0.31</td>
<td>0.28 ± 0.5</td>
</tr>
<tr>
<td><strong>KO TI/R 7 days</strong></td>
<td>0.62 ± 0.51</td>
<td>0.65 ± 0.6</td>
<td>0.65 ± 0.51</td>
<td>0.35 ± 0.6</td>
</tr>
<tr>
<td><strong>KO CL 7 days</strong></td>
<td>0.47 ± 0.5</td>
<td>0.4 ± 0.41</td>
<td>0.35 ± 0.48</td>
<td>0.4 ± 0.41</td>
</tr>
</tbody>
</table>

*P < 0.05 versus sham WT mice.

bP < 0.05 versus WT TI/R + vehicle mice.

P < 0.05 versus sham KO mice.
Role of NLRP3 Inflammasome in Testicular Ischaemia

Harrison et al., 1981; Sarica et al., 1997; Shiraishi et al., 2001). In this context, the role of NO is of primary importance as the endothelial nitric oxide synthase and neuronal nitric oxide synthase are: 1) constitutively expressed in the testis, 2) activated transiently in response to the increase in intracellular Ca\(^{2+}\) mobilization, and 3) contribute to the physiologic regulation of vascular tone in both the testes and other target organs (Shiraishi et al., 2001; Esposito and Cuzzocrea, 2009). Moreover, the delicate balance between NO and peroxynitrite catalysts appears as an interesting therapeutic target in ischemia/reperfusion injury (Esposito and Cuzzocrea, 2009).

In the present study, we also studied caspase-1 and -3 expression after reperfusion in both WT and KO animals. Our results demonstrated that KO mice exhibited a reduced expression of both caspas when compared with WT animals during TI/R. Accordingly, a large number of TUNEL-positive germ cells were observed in the seminiferous tubules from TI/R WT mice either at 1 or 7 days, mostly in the outer layer of the seminiferous tubules, suggesting that apoptosis is acutely increased in the tubular tissue of the twisted testes. NLRP3 KO mice showed a reduced number of TUNEL-positive cells, thus suggesting that the absence of this inflammasome reduces the activation of the apoptotic machinery. Therefore, an important finding of our paper is that NLRP3-deficient mice respond to the ischemia and reperfusion insult, with a lower activation of the inflammatory and apoptosis cascade than in WT animals.

The most susceptible function to testicular twining is spermatogenesis. In fact, WT animals subjected to TI/R had a very poor Johnsen’s score, which is a method to evaluate spermatogenesis (Johnsen, 1970; Erdemir et al., 2012). Interestingly, the lack of NLRP3 produced an improvement in spermatogenesis following the surgical insult of TI/R. This finding is of particular interest. In fact, it is possible to speculate that this molecular platform represents one of the most prominent mechanisms for the development of the late complications associated with testicular torsion.


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Authorship Contributions

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Conducted experiments: Puzzolo, Irrera, Rinaldi, Bitto, Santoro, Pisanii, Pizzino, Antonuccio.

References


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