THE EFFECTS OF UNILATERAL TESTICULAR TORSION UPON IMMUNITY MODULATION AND APOPTOSIS OF GERMINAL CELLS IN THE CONTRALATERAL TESTIS
An Experimental Study in Rats

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ABSTRACT
Since the report of Krarup in 1978, deteriorating effects of unilateral testicular torsion to the contralateral testis are generally accepted, but the mechanism of the damage remains controversial. This study aims to explain the mechanism of spermatogenesis disorder of the contralateral testis after unilateral testicular torsion that underlies infertility. This study used 40 Wistar strain Rattus norwegicus white rats divided into 4 groups of 10 rats each with a post test control group design. Two groups of white rats were subjected to left sided testicular torsion three times counter-clockwise for 4 hours and 24 hours respectively, and two other groups, which served as control, were subjected to sham operation. Subsequently, the determination of serum testosterone was carried out using radioimmunoassay and investigations on the proportion of apoptosis of the germinal epithelial cells, the percentage of IL-4 producing lymphocytes and the IgG-producing plasma cells in the contralateral testis was performed by means of an immunohistochemical method. The results of multivariate analysis showed that unilateral torsion in white rats for 4 hours induced significant increase in the percentage of IL-4 producing lymphocytes in the contralateral testis, but no significant change was demonstrated in serum testosterone level, the percentage of IgG-producing plasma cells, or the proportion of apoptotic germinal epithelial cells in the contralateral testis. Unilateral testicular torsion for 24 hours could induce significant decrease in serum testosterone level along with significant proportional increase of apoptosis of the germinal epithelial cells in the contralateral testis. No significant change in the percentage of IL-4 producing lymphocyte and the IgG-producing plasma cells in the contralateral testis could be measured. It can be concluded that the mechanism of spermatogenesis disorder post-unilateral testicular torsion can be explained by the immunopathobiology paradigm. Immunity modulation was found in the rats subjected to testicular torsion for 4 hours, as reflected by an increase in the percentage of IL-4 producing lymphocyte and pathobiological modulation occurred in rats subjected to testicular torsion for 24 hours as reflected by the reduction in serum testosterone level along with an increase in the proportion of apoptosis of the germinal epithelial cells in the contralateral testis.

Keywords: testicular torsion, testosterone, immunity modulation, germ cell, apoptosis, white rats

INTRODUCTION
Testicular torsion results in twisting of the spermatic cord and occlusion of the vascular supply to the testis. If not untwisted immediately complete infarction of the testis may results. If it lasts for 3 hours or less all the twisted testis remain viable after detorsion, if it lasts for 5 hours the testicular viability will decrease to 83 – 90% and if it last for 24 hours or longer the testis is rare viable (Sonda and Wang, 1988). The degree of torsion is also a critical factor in determining testicular viability and most of the cases had three times (1080°) torsion (Haynes et al, 1983). The frequency of tests torsion has been estimated at 1 an 4000 males younger than age 25 years, making it relatively common (Williamson, 1976, Barada 1989).

In Surabaya at Emergency Department of Dr. Soetomo Hospital for 2 years 1989 and 1990 a number of 75 patients of testicular torsion has been treated (Alif, 1992). The majority of cases of testicular torsion occur in boys between ages 12 and 18 years (Scorer and Farrington, 1971; Williamson, 1976; Alif, 1991). Since the report of Krarup in 1978 that has proven the evidence of spermatogenesis disorder after unilateral torsion (Krarup, 1978) the deteriorating effects of unilateral testicular torsion to the contralateral testis are generally accepted (Bartsch et al, 1980; Harrison, 1981; Consentino et al, 1985; Handerson et al, 1985; Anderson dan Williamson, 1986; Awojobi and NKposong, 1986; Anderson and Williamson, 1990; Heindel et al, 1990; Tarmono, 1998). To date, however, the actual mechanism of contralateral damage remains uncertain and still debatable. Some data suggest that the damaged organ may allow immunological injury to the opposite un involved testis (Nugle and de Vere White, 1982; Henderson et al, 1985; Turek, 1997). It is also suggest that there is an increase of apoptosis in the contralateral testis after unilateral torsion.
Twisting of the spermatic cord causes occlusion of the vascular supply to the testis and it makes the testis containing cells become hypoxia and anoxia. The anoxic Sertoli cells damage the blood testis barrier system and allow germinal epithelial cell releasing protein enter blood circulation and recognized by immunocompetent cells as an autoantigen and trigger the immune response.

Furthermore, the differentiation and activation of T cells may secrete various cytokines. The cytokine IL-4 induces activation, proliferation, and maturation of B lymphocytes, resulting in its differentiation to become plasma cells, which is producing antibody that may induce damage in contralateral testicular germinal epithelial cells. The complex of antigen antibody activates the complements, generating lysis in target cells and replaced with connective tissue (Goldsby et al, 2000 and Elyana 2001).

Testosterone level may reduce due to various factors, such as psychological, physical, and metabolic stress (Vasankari, 1983; Mc Grady, 1984; Bergendahl et al, 1989; Veldhuis, 1997). The reduction of testosterone level produced by Leydig cells increases apoptosis (Nandi et al, 1999). The reduction of testosterone level due to the ischemia in Leydig cells results in Fas expression at the surface of germinal epithelial cells as well as the expression of Fas L at the surface of Sertoli cells. The binding of Fas and Fas L brings about apoptosis in germinal epithelial cells (Lee et al, 1997; Nandi et al, 1999) through the activation of Fas Associated Protein with Death Domain (FADD), which subsequently activates caspase, and, thereafter, activates DNAase, resulting in chromosome fragmentation, DNA damage, and, finally, apoptosis occurs (Emari, 1998; Kuby, 2000). The purpose of this study was to explain the mechanism of spermatogenesis disorder of contralateral testis after unilateral testicular torsion that underlies infertility.

MATERIALS AND METHODS

This was an experimental study involving male Wistar strain Rattus norvegicus white rats as samples subjected to treatment of left testicular torsion for 4 and 24 hours, and as control of each treatment that were subjected to sham operation. This study used Posttest Control Group Design, variables measurement was carried out only after treatment. Samples were taken from breeding laboratory at Gadjah Mada University, Yogyakarta, with the following criteria: male, aged 10 - 12 weeks, bodyweight ranged 150 - 200 grams, healthy and without any disabilities.

According to the design, four groups of samples were required. Grouping was carried out in random using random number (Snedecor and Cochran, 1974). Sample size was determined by means of Danniel's formula (1987), which revealing 7.85 rats/group. As there was possibility of drop out, it was decided to use 10 rats in each group, so that there were totally 40 rats involved in this study. These rats were adapted from 2 weeks and divided into 4 groups, treatment groups E1 and E2, and their respective control groups C1 and C2. E1 was subjected to left testicular torsion by twisting spermatic cord counter-clockwise 3 x 360 degrees. After 4 hours, detorsion was undergone to the left testis and followed immediately with dextral orchidectomy for examining apoptosis in germinal epithelial cells, IL-4 producing lymphocytes, and IgG-producing plasma cells, and blood was taken by cardiac puncture for serum testosterone level examination. Similar procedure was also underwent in E2 group, with the difference that detorsion of the left testicle and right orchidectomy was performed after 24 hours. C1 and C2 groups were subjected only to sham operation, in which scrotal skin was incised, without carrying out testicular torsion and the wound closed.

Analysis unit and dependent variables in this study was serum for testosterone level examination and contralateral testicular tissue for examining germinal epithelial cell apoptosis, IL-4 producing lymphocytes, and IgG-producing plasma. The examination of serum testosterone level was done using radioimmunoassay (RIA), while apoptosis in germinal epithelial cell was examined by means of tunnel assay apoptag method, a combination of molecular reaction and immunohistochemistry. Apoptosis in germinal epithelial cells is marked by positive reaction in apoptag staining. The number of apoptotic germinal epithelial cells was counted using light microscope with 400 x magnification in 5 sections of seminiferous tubules.

IL-4 producing lymphocytes examination was done using indirect immunohistochemistry staining with monoclonal antibody (anti IL-4). The cytoplasm of IL-4 producing lymphocytes induces positive light reaction presenting in yellowish brown color. Counting was done using light microscope with 400 x magnification on 5 locations taken randomly from contralateral testicular sections. IgG- producing plasma cells examination was performed immunohistochemically using monoclonal antibody (anti IgG), and IgG-producing plasma cells reacted positively, showing yellowish brown cellular cytoplasm. The number of IgG-producing plasma cells was counted using light microscope with 400 x
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Magnification on 5 locations taken randomly from contralateral testicular sections.

Data obtained in this study were processed using several statistical tests with level of confidence of 95%. Statistical test and analysis were variance analysis (Anova) for homogeneity test of rats bodyweight, Kolmogorov-Smirnov normality test to find the distribution of data obtained, and multivariate analysis (Manova) using Hotelling T test to examine the difference of all variables in treatment and control groups and to test the difference of changes (delta) in the variables in E1 and E2 groups. Experimental animals were handled from treatment to their sacrifice according to International Guiding Principal for Biomedical Research Involving Animals from the Council for International Organizations of Medical Sciences.

RESULTS

To confirm the internal validity, the homogeneity test for sample body weight was done using one way Anova, and the result showed that white rats bodyweight in those four groups were not significantly different (p > 0.05). The variables in this study included serum testosterone level, the apoptotic proportion of germinal epithelial cells, the percentage of IL-4 producing lymphocytes, and the percentage of IgG-producing plasma cells in contralateral testis.

The results of normal distribution test to the four variables in four groups using Kolmogorov Smirnov (K-S) test revealed that the variable of serum testosterone level and the proportion of apoptotic germinal epithelial cells in contralateral testis had normal distribution (p > 0.05). Therefore, both variables were further examined using multivariate analysis of variance (MANOVA) with Hotelling T statistical test. It was found that the percentage of IgG-producing plasma cells and IL-4 producing lymphocytes was not normally distributed (p < 0.05). Therefore, the data was further analyzed using non-parametric Kruskal Wallis or Mann Whitney tests.

Table 1. Combined results of discriminant test in 4 variables between 4 groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean of variables in groups</th>
<th>F</th>
<th>Prob.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum testosterone level</td>
<td>236.30 115.65 212.95 63.60</td>
<td>5.520</td>
<td>0.003</td>
</tr>
<tr>
<td>The proportion of apoptotic contralateral testicular germinal epithelial cells</td>
<td>1.48 1.00 0.86 115.86</td>
<td>8.693</td>
<td>0.000</td>
</tr>
<tr>
<td>The percentage of IL-4 producing lymphocytes in contralateral testis</td>
<td>0.10 0.96 0.14 0.12</td>
<td>-</td>
<td>0.001</td>
</tr>
<tr>
<td>The percentage of IgG-producing plasma cells in contralateral testis</td>
<td>0.18 0.40 0.06 0.10</td>
<td>-</td>
<td>0.063</td>
</tr>
</tbody>
</table>

The results of analysis of the four variables between 2 control groups are shown in the following table:
Table 2. Results of discriminant test between C1 and C2

<table>
<thead>
<tr>
<th>Variables</th>
<th>C1 group</th>
<th>C2 group</th>
<th>F</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Testosterone Level</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>The proportion of apoptotic contralateral testicular germinal epithelial cells</td>
<td>1.48</td>
<td>1.39</td>
<td>0.86</td>
<td>1.03</td>
</tr>
<tr>
<td>The percentage of IL-4 producing lymphocytes in contralateral testis</td>
<td>0.10</td>
<td>0.20</td>
<td>0.14</td>
<td>0.27</td>
</tr>
<tr>
<td>The percentage of IgG-producing plasma cells in contralateral testis</td>
<td>0.18</td>
<td>0.26</td>
<td>0.06</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Hotelling’s Trace = 0.075, p = 0.540

Above table indicates that the four observed variables between groups C1 and C2 are not significantly different. Table 3 below displays the results of analysis of four variables between groups C1 and E1 using MANOVA and Hotelling T statistical test for the level of serum testosterone and the proportion of apoptotic germinal epithelial cells using Mann Whitney test for the percentages of IgG-producing plasma cells and IL-4-producing lymphocytes.

Table 3. Results of discriminant test between C1 and E1

<table>
<thead>
<tr>
<th>Variables</th>
<th>C1 Group</th>
<th>E1 Group</th>
<th>F</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Testosterone Level</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>The proportion of apoptotic germinal epithelial cells</td>
<td>1.48</td>
<td>1.39</td>
<td>1.00</td>
<td>0.59</td>
</tr>
<tr>
<td>The percentage of IL-4 producing lymphocytes in contralateral testis</td>
<td>0.10</td>
<td>0.20</td>
<td>0.96</td>
<td>0.78</td>
</tr>
<tr>
<td>The percentage of IgG-producing plasma cells in contralateral testis</td>
<td>0.18</td>
<td>0.26</td>
<td>0.40</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Hotelling’s Trace = 0.279, p = 0.124

Table 3 shows that there is significant difference between groups C1 and E1 only in the variable of IL-4 producing lymphocytes (p = 0.002), while other variables are not significantly different. Analysis was carried out using MANOVA with Hotelling T statistical test for serum testosterone level and the proportion of apoptotic germinal epithelial cells in contralateral testis as well as Mann Whitney test for the percentage of IgG-producing plasma cells and the percentage of IL-4-producing lymphocytes. The results are shown in the following table:
Table 4. Results of discriminant test between C2 and E2

<table>
<thead>
<tr>
<th>Variables</th>
<th>C2 Group</th>
<th>E2 Group</th>
<th>F</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum testosterone level</td>
<td>212.95</td>
<td>63.60</td>
<td>18.885</td>
<td>0.000</td>
</tr>
<tr>
<td>The proportion of apoptotic germinal epithelial cells</td>
<td>0.86</td>
<td>115.86</td>
<td>8.733</td>
<td>0.008</td>
</tr>
<tr>
<td>The percentage of IL-4 producing lymphocytes in contralateral testes</td>
<td>0.14</td>
<td>0.12</td>
<td>-</td>
<td>0.655</td>
</tr>
<tr>
<td>The percentage of IgG-producing plasma cells in contralateral testes</td>
<td>0.06</td>
<td>0.10</td>
<td>-</td>
<td>0.871</td>
</tr>
</tbody>
</table>

Hotelling's Trace = 1.692, p = 0.000

The results of analysis as seen in Table 4 indicate that there is significant difference in both groups for the variable of serum testosterone level (p < 0.001) and the proportion of apoptotic germinal epithelial cells (p = 0.008). However, significant difference was not found in the percentage of IL-4-producing lymphocytes (p = 0.655) and the percentage of IgG producing plasma cells (p = 0.871).

Table 5. Results of discriminant test between variables E1 and E2

<table>
<thead>
<tr>
<th>Variables</th>
<th>E1 Group</th>
<th>E2 Group</th>
<th>F</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Testosterone Level</td>
<td>115.65</td>
<td>63.60</td>
<td>4.180</td>
<td>0.056</td>
</tr>
<tr>
<td>The proportion of apoptotic germinal epithelial cells</td>
<td>1.00</td>
<td>115.86</td>
<td>8.712</td>
<td>0.009</td>
</tr>
<tr>
<td>The percentage of IL-4 producing lymphocytes in contralateral testes</td>
<td>0.96</td>
<td>0.12</td>
<td>-</td>
<td>0.002</td>
</tr>
<tr>
<td>The percentage of IgG-producing plasma cells in contralateral testes</td>
<td>0.40</td>
<td>0.10</td>
<td>-</td>
<td>0.042</td>
</tr>
</tbody>
</table>

Hotelling's Trace = 0.817, p = 0.006

Table 5 shows that groups E1 and E2 have significant difference in the variable of the apoptotic germinal epithelial cells proportion (p = 0.009) and the percentage of IL-4 producing lymphocytes (p = 0.006). However, significant difference is not found in the variable of serum testosterone level (p = 0.056) and the percentage of IgG-producing plasma (p = 0.063).
Results of Immunohistochemical Staining

Immunohistochemical staining in this study was done to seminiferous tubule in contralateral testis. The results are as follows:

Figure 1. Incision of seminiferous tubule of contralateral testis from Wistar strain in treatment group (E2) using apoptag staining with tunel assay method.
A: magnification 100x  B: magnification 400x  P: apoptotic cells with dark brown nucleus.

Figure 2. Incision of seminiferous tubule of contralateral testis from Wistar strain in treatment group (E2) using immunohistochemical staining with IgG monoclonal antibody.
A: magnification 100x, B: magnification 400x, P: IgG-producing cells (cytoplasm appears brown).

Figure 3. Incision of seminiferous tubule of contralateral testis from Wistar strain in treatment group (E2) using immunohistochemical staining with IL-4 monoclonal antibody.
A: magnification 100x, B: magnification 400x, P: IL-4-producing cells (cytoplasm appears brown).
DISCUSSION

It can be seen in Table 1 that serum testosterone level in four studied groups demonstrate significant difference (p = 0.003), while Table 2, comparing serum testosterone level between 2 control groups, shows no significant difference (Hotelling's Trace = 0.075; p = 0.540). Table 3, comparing serum testosterone level in control group (C1) to that in treatment group of testicular torsion for 4 hours (E1), also shows no significant difference (p = 0.060). Table 4 that compares serum testosterone level between groups C2 and E2 shows significant difference (p < 0.001). Conclusively, the decrease of testosterone level occurred in experimental animals subjected to unilateral testicular torsion, and the decrease was significant in testicular experimental animals subjected to unilateral testicular torsion for 24 hours. It is apparent that unilateral testicular torsion results in the decrease of serum testosterone level, and the decrease is more remarkable in the torsion for 24 hours. Savas also found similar findings in his study using Wistar strain rats subjected to testicular torsion (Savas et al, 2003). Bartsch et al (1980) investigated serum testosterone level in 30 patients who experienced testicular torsion at least for 2.5 years and found that the level was normal. This might be due to the compensatory function from the existing Leydig cells.

Table 1 shows that in four studied groups there are significant difference in the variable of apoptotic germinal epithelial cells proportion (p < 0.001). Table 2, comparing the proportion of apoptotic germinal epithelial cells in contralateral testis between 2 control groups, significant difference is not found using Hotelling's Trace value of 0.075 and p = 0.540. This was because both groups were not subjected to treatment. Table 3, which compares the proportion of apoptotic germinal epithelial cells in contralateral testis between control group (C1) and group receiving testicular torsion for 4 hours (E1), shows no significant difference (p = 0.328). Conversely, significant difference is found with Hotelling's Trace value of 1.692 and p < 0.001 in Table 4, where the proportion of apoptotic germinal epithelial cells in contralateral testis is compared between that in control group (C2) and that in treatment group receiving testicular torsion for 24 hours (E2) is compared. Table 5, comparing the proportion of apoptotic germinal epithelial cells in contralateral testis in treatment group receiving testicular torsion for 4 hours (E1) and that for 24 hours, demonstrated significant difference with p = 0.009.

Those data lead to the conclusion that there was an increase of apoptotic proportion in contralateral testis due to unilateral testicular torsion for 24 hours. Correlation between the reduction of testosterone and the increase of apoptotic germinal epithelial cells had been proved by Nandi et al (1999) who found that the administration of ethane 1,2 dimethanesulfonate (EDS), a toxic substance for Leydig cells, was followed by the increase of apoptosis in germinal epithelial cells. In contrast, apoptotic process can be inhibited by the administration of glucocorticoid (Jazawa, 2001). Additionally, apoptosis can also be prevented by providing Capsaicin, a blocker to the afferent nerve fiber, which works by preventing blood flow reduction in contralateral testis (Sarioglu-Buke, 2001).

Table 1 showed that the percentage of IL-4 producing lymphocyte in contralateral testis in four studied groups had significant difference (p = 0.001). However, significant difference was not found (p = 0.889) in Table 2 where the percentage of IL-4 producing lymphocyte in contralateral testis is compared in two control groups (C1 and C2). Table 3 demonstrated significant difference (p = 0.002) in the percentage of IL-4 producing lymphocyte in contralateral testis between control group (C1) and treatment group receiving testicular torsion for 4 hours (E1). No significant difference (p = 0.655) is shown in Table 4 that compares the percentage of IL-4 producing lymphocyte in contralateral testis between that in control group (C2) and that in group subjected to testicular torsion for 24 hours (E2).

It can be concluded that the increased percentage of IL-4 producing lymphocyte in contralateral testis only occurred in group treated with testicular torsion for 4 hours. Although the role of IL-4 in immune response has long been recognized, its correlation with infertility immunology remains unclear. Several types of cytokines that have been studied in relations with infertility immunology are IL-1, IL-2, Tumor Necrosis Factor (TNF) and interferon gamma (Anderson and Hill, 1988; Haney et al, 1992, Hill et al, 1989).

Table 1 shows that there is no significant difference (p = 0.063) in the percentage of IgG-producing plasma cells in contralateral testis in four studied groups. Similarly, significant difference (p = 0.263) is not shown in Table 2, in which the percentage of IgG-producing plasma cells in contralateral testis in C1 group is compared to that in control C2 group. Table 3, comparing the percentage of IgG-producing plasma cells in contralateral testis between that in C1 and E1 groups, indicates no significant difference (p = 0.203). Insignificant difference is also seen in Table 4 that compares the percentage of IgG-producing plasma cells in contralateral testis between that in C2 and that in E2 groups. It is apparent from these data that the treatment of testicular torsion for 3 and 24 hours not or not yet result in the change of IgG-producing plasma cells
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percentage. From the occurrence of general response until the formation of immunoglobulin took time for 1 - 2 weeks. In relations with the infertility immunology, a subject that has been commonly studied is the antisperm antibody (ASA) (Hendry et al, 1977; Marthur, 1981; Cerasaro et al, 1984; Meinertz, 1992; Naz and Menge, 1994; Peters and Coulan, 1992). The antisperm antibody can be found in serum, semen, and binding with sperm (Turek, 1997). So far, there is no study has been performed to examine the presence of immunoglobulin in testicular tissue was negative.

CONCLUSIONS AND SUGGESTIONS

Unilateral testicular torsion for 4 hours may result in the increase of the percentage of IL-4 producing lymphocyte in contralateral testsis, but not changing of the serum testosterone level, the percentage of IgG producing plasma cells, and the proportion of apoptosis in germinal epithelial cells in the contralateral cells testsis. Unilateral testicular torsion for 24 hours may result in the reduction of serum testosterone level along with the increase of the proportion of apoptosis in germinal epithelial cells in the contralateral testsis, but no changing of the percentage of IL-4 producing lymphocyte and the percentage of IgG producing plasma cells in contralateral testsis. Conclusively, immunity modulation occurs in experimental animals subjected to unilateral testicular torsion for 4 hours, as reflected by the increase of the proportion of apoptotic germinal epithelial cells in contralateral testsis. It can also be suggested that the management of testicular torsion should consider the duration of torsion to provide rational treatment. Further studies are warranted to identify the role of immunosuppressant as an adjuvant therapy for patients with testicular torsion for 4 hours or less, and to disclose the role of apoptotic inhibitor in patients with testicular torsion for 4 hours or more.

REFERENCES

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