



Role of methylprednisolone on mRNA expression of BAX, BCL-2 gene in testicular torsion-detorsion of male albino Wistar rats

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ABSTRACT

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Testicular torsion (TT) is a surgical emergency that most common in adolescent. The primary complication of TT is ischemia due to venous and subsequent occlusion of the arteries accompanied by reperfusion damage. Increasing level of the reactive oxygen species (ROS) is related to the ischemia-reperfusion (IR) injuries and the return of the blood circulation after the ischemia. The mechanism of apoptotic cell death in a classical IR injury on TT has no well known. BCL-2 gene promotes cell survival by inhibiting apoptosis, while BAX, promotes cell apoptosis by blocking BCL-2 genes. Methylprednisolone has an anti-inflammatory effect that can decrease the formation of ROS through inhibition of phospholipase A2 and decreased leukocyte activity. The study aimed to investigate the role of methylprednisolone on mRNA expression of BAX, BCL-2 gene in testicular torsion-detorsion of male albino Wistar rats. This is an experimental study using post-test only control group design involving 24 male rats. The rats were randomly divided into four groups with six rats each group, i.e. testicular torsion (T), testicular torsion-detorsion (TD), testicular torsion with methylprednisolone administration (T-MP) and testicular torsion-detorsion with methylprednisolone administration (TD-MP). mRNA expression of BAX and BCL-2 were measured using quantitative real time-polymerase chain reaction (qRT-PCR). Statistical analyses were performed using SPSS Version 23 and Graph Pad Prism 7 and statistical significance was set at a $p < 0.05$. No significant difference in BAX mRNA expression between groups was observed. However, BCL-2 mRNA expression were statistically significant between T group compared with TD group on right testis (ipsilateral) with $p = 0.006$ and also between T group compared with T-MP group on the left testis (contralateral) with $p = 0.001$. In conclusion, administration of methylprednisolone in TT could be a protective mechanism against germ cell apoptosis in testicular tissue shown by the increase of anti-apoptotic gene expression.

ABSTRAK

Torsio testis (TT) adalah kegawatan daruratan bedah yang umum dijumpai pada orang dewasa. Komplikasi utama TT adalah iskemia akibat penyumbatan vena dan arteri yang diikuti kerusakan reperfusi. Kenaikan kadar *reactive oxygen species* (ROS) dikaitkan dengan cedera iskemia-reperfusi (IR) dan kembalinya sirkulasi darah setelah iskemia. Mekanisme kematian sel akibat apoptosis pada cedera IR belum diketahui dengan baik. Gen BCL-2 menyebabkan sel hidup dengan menghambat apoptosis, sedangkan BAX menyebabkan apoptosis dengan memblokir gen BCL-2. Metilprednisolon mempunyai efek anti inflamasi yang dapat menurunkan pembentukan ROS dengan menghambat fosfolipase A2 dan menurunkan aktivitas leukosit. Penelitian ini bertujuan untuk mengkaji peran metilprednisolon pada ekspresi mRNA gen BAX, BCL-2 pada torsio-detorsi testis tikus Wistars putih jantan. Ini merupakan penelitian eksperimental dengan rancangan *post-test only control group* menggunakan 24 ekor tikus. Tikus dibagi secara acak menjadi empat kelompok dengan masing-

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masing enam ekor tikus yaitu kelompok tersio testis (TT), tersio-detorsio testis (TD), tersio testis dengan pemberian metal prednisolon (T-MP), dan tersio-detorsio testis dengan pemberian metal prednisolon (TD-MP). Ekspresi mRNA gen BAX dan BCL-2 diukur menggunakan *quantitative real time-polymerase chain reaction* (qRT-PCR). Analisis statistik dilakukan menggunakan SPSS versi 23 dan Graph Pad Prism 7. Signifikan secara statistik bilai nilai $p < 0,05$. Tidak ada perbedaan bermakna ekspresi mRNA BAX antar kelompok yang diukur. Namun demikian ekspresi mRNA BCL-2 bermakna antara kelompok T dengan TD pada testis kanan (ipsilateral) dengan nilai $p = 0,006$ dan juga antara kelompok T dengan T-MP pada testis kiri (contralateral) dengan nilai $p = 0.001$. Dapat disimpulkan, pemberian metilprednisolon pada TT dapat sebagai mekanisme protektif melawan apoptosis selgerminal jaringan testicular sebagaimana ditunjukkan dengan kenaikan ekspresi gen anti apoptosis.

INTRODUCTION

Testicular torsion (TT) is a surgical emergency that it was the most common in adolescent.¹ In males aged before 18 years old, the annual incidence is about 3.8%, while the incidence in men aged 18 to 25 years of approximately 1.1 to 4.5 per 100,000 person.^{1,2} Testicular torsion is more common on the left side with a 1.2: 1 ratio, possibly caused by slightly longer spermatic cords on the left.³

The primary complication of TT is ischemia of the testis due to venous and subsequent occlusion of the arteries accompanied by reperfusion damage after recovery. Hemorrhagic necrosis and cell apoptosis contribute to inflammatory response and increased oxidative stress in the testicles.⁴ Several studies have shown unilateral TT affecting spermatogenesis in about 50% of patients and 20% with borderline spermatogenesis damage. In long-term studies, normal semen analysis were found in 5-14% of patients with a history of TT.⁵ In several studies, it has been shown that sparing non-viable testis or a testis with severe damage causes damage to the contralateral testis compared to patients who had been performed orchidectomy.⁶ Contralateral testicular biopsies are found to be abnormal in 57-88% of cases after unilateral torsion.⁷

Ischemia-reperfusion (IR) injury of the testis is the major pathophysiology of the testicular torsion-detorsion. Reactive oxygen species (ROS) was regulated after reperfusion injury to the testes,

where the testis are very sensitive to this type of damage.⁵ Increasing level of the reactive oxygen species (ROS) is related to the IR injuries and the return of the blood circulation after the period of the ischemia. This pathogenic mechanism of IR injury is mainly attributed to the overproduction of ROS in some organs, one of which is the testis. Due to the disruption of the blood flow, it will make hypoxic condition in the ischemic phase of the IR process.^{8,9}

The mechanism of apoptotic cell death in a classical IR injury on TT involves the activation of caspase 9, which heavily regulated by BCL-2 (B-cell lymphoma 2) genes family.¹⁰ Family members of BCL-2 can either facilitate cell survival (BCL-2, BCL-XL, BCL-W, and MCL-1), or support cell death (BAX, BAK, BCL-XS, BID, BIK, HRK, and BCK).¹¹ BCL-2 gene promotes cell survival by inhibiting apoptosis (anti-apoptotic), while BAX (BCL-2 associated X protein), promotes cell apoptosis (pro-apoptotic) by blocking BCL-2 genes.¹² Increased rates of BAX expression in the mitochondrial membranes induce the release of pro-apoptotic molecules that cause effector caspase activation, resulting in the release of numerous inflammatory mediators and free radicals. This process is followed by hypoxia-caused tissue injuries and oxidative stress due to reperfusion.^{13,14} The secretion of BAX by leukocytes could directly stimulate apoptosis in germ cells, the secretion of ROS also may increase BAX levels and stimulate a mitochondrial-driven apoptotic

pathway.¹⁰ Methylprednisolone is a steroid that inhibits ROS by blocking the enzyme phospholipase A2 and inhibiting the activation of leukocyte.^{15,16} Steroid was associated with reduced vascular neutrophil infiltration and decreased germ cell apoptosis in an animal model of testicular torsion¹⁶ This study aimed to investigate the effects of methylprednisolone on expression of pro apoptotic gene (BAX) and anti-apoptotic gene (BCL-2) in rat model of TT-detorsion.

MATERIALS DAN METHODS

Animal

This was an experimental, post-test only control group study. All of the experimental procedures were performed in the Integrated Research Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. Animal laboratory study protocols were approved by the Medical and Health Research Ethic Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. The study involved 24 male rats weighing 250-300g and randomly divided into four groups with six rats in each group. The rats were fed with standard laboratory food and conditions through out the experimental procedures with temperature at 24°C.

Procedure

The rats were divided into the following four groups: testicular torsion (T), testicular torsion-detorsion (TD), testicular torsion with methylprednisolone administration (T-MP) and testicular torsion-detorsion with methylprednisolone administration (TD-MP). Before all of the experimental procedures, ketamine (2mg kg⁻¹) were administered intramuscularly (IM) to all rats as an anesthetic medication.

For T group, testicular access was made by a midline incision through the scrotum, the right testis was rotated 720° clockwise and fixated within scrotum using 5-0 silk suture for 4 h. After 4 h, all of the rats in the group underwent bilateral orchiectomy. For T-MP, all of the rats underwent testicular torsion procedure as in Tgroup, then 30mg kg⁻¹ methylprednisolone were administered (IM) 3 h after initial testicular torsion and followed by bilateral orchiectomy procedures after 1 h. In TD group, all of the rats underwent testicular torsion procedure as in T group. After 4 h of testicular torsion, the right testis underwent detorsion procedure (720° anti-clockwise rotation) for 1 hand followed by bilateral orchiectomy. In the last group, TD-MP, all of the rats underwent testicular torsion procedure as in T-MP group, After 4 h of testicular torsion, the right testis underwent detorsion procedure for 1 hand followed by bilateral orchiectomy.

All of the rats in this experimental study were terminated using cervical dislocation under anesthetic state. Collected testicular samples after bilateral orchiectomy were fixated with 10% buffered formalin. mRNA expression of BAX and BCL-2 were conducted using Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) using primer presented on TABLE 1, in Anatomical Pathology Laboratory, Department of Anatomical Pathology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta. RNA purification from testicular tissue were conducted using Ribospin™ II (GeneAll®) kit and NEXpro™ 1-step qRT-PCR 2x Master Mix (SYBR) were used in this study. All of the procedures followed the manufacturer's recommendations. Normal testicular tissue was used as the internal control and the relative BAX and BCL-2 expression were calculated using the equation .

TABLE 1. BAX and BCL-2 multiplex primer sets (SIGMA®)

	Forward primer	Reverse primer
GAPDH	5'-TGC MTC CTG CAC CAC CAA CT-3'	5'-YGC CTG CTT CAC CAC CTT C-3'
BAX	5'-GTT TCA TCC AGG ATC GAG CAG-3'	5'-CAT CTT CTT CCA GAT GGT GA-3'
BCL-2	5'-CCT GTG GAT GAC TGA GTA CC-3'	5'-GAG ACA GCC AGG AGA AAT CA-3'

GADPH: Glyceraldehyde 3-phosphate dehydrogenase; BAX: BCL-2 associated X protein; BCL-2: B-Cell Lymphoma 2

Statistical analysis

Independent t-test analyses were performed to compare means between experimental groups. Statistical analyses were performed using SPSS Version 23, GraphPad Prism 7 and statistical significance was set at a $p < 0.05$.

RESULTS

Mean mRNA expression of BAX were

higher in the right testis (ipsilateral) compared to the left testis (contralateral) (TABLE 1). The highest mRNA expression of BAX for right and left testis were in TD group, 40.97 ± 17.08 and 26.83 ± 14.58 , respectively (FIGURE 1). The lowest mRNA expression of BAX for right and left testis were in TD-MP group, 26.99 ± 6.64 and 17.82 ± 4.13 , respectively (FIGURE 1).

TABLE 2. mRNA expression (mean \pm SD) of BAX and BCL-2

Group	n	Position	BAX	BCL-2
T	6	Right	28.50 ± 8.85	7.51 ± 1.72
	6	Left	20.08 ± 3.07	12.88 ± 1.54
T-MP	6	Right	32.44 ± 17.36	12.59 ± 6.78
	6	Left	18.17 ± 8.26	21.52 ± 3.59
TD	6	Right	40.97 ± 17.08	3.45 ± 2.33
	6	Left	26.83 ± 14.58	12.52 ± 5.36
TD-MP	6	Right	26.99 ± 6.64	22.15 ± 11.04
	6	Left	17.82 ± 4.13	42.50 ± 12.95

BAX: BCL-2 associated X protein; BCL-2: B-Cell Lymphoma 2; T: torsion; T-MP: torsion+methylprednisolone; TD: torsion-destorsion; TD-MP: torsion-detorsion+methylprednisolone

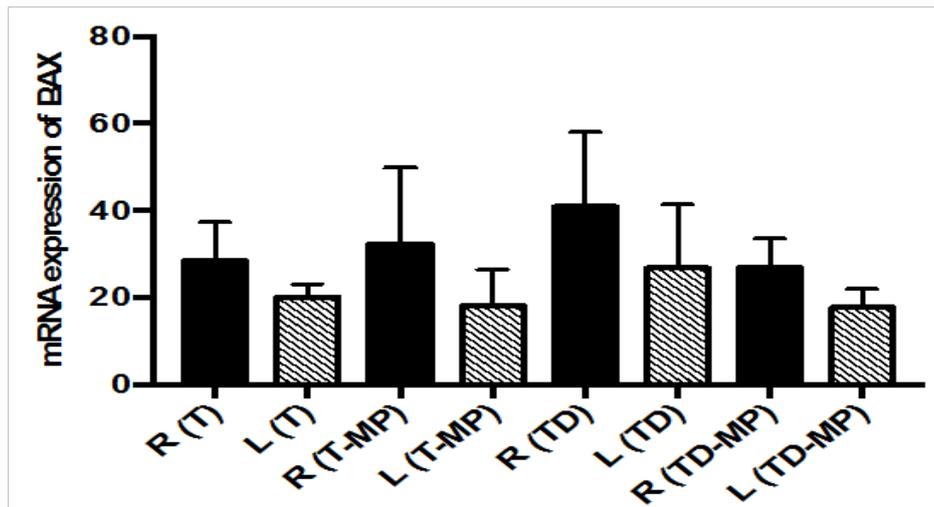


FIGURE 1. mRNA expression of BAX in all of the experimental groups. Values are expressed as mean±standard deviation (SD). R, Right testis. L, Left testis.

Mean mRNA expression of BCL-2 were higher in the left testis compared to the right side. The highest mRNA expression of BCL-2 for right and left testis were in TD-MP group, 22.15 ± 11.04

and 42.50 ± 12.95, respectively (FIGURE 2). The lowest mRNA expression of BCL-2 for right and left testis were in TD group, 3.45 ± 2.33 and 12.52 ± 5.36, respectively (FIGURE 2).

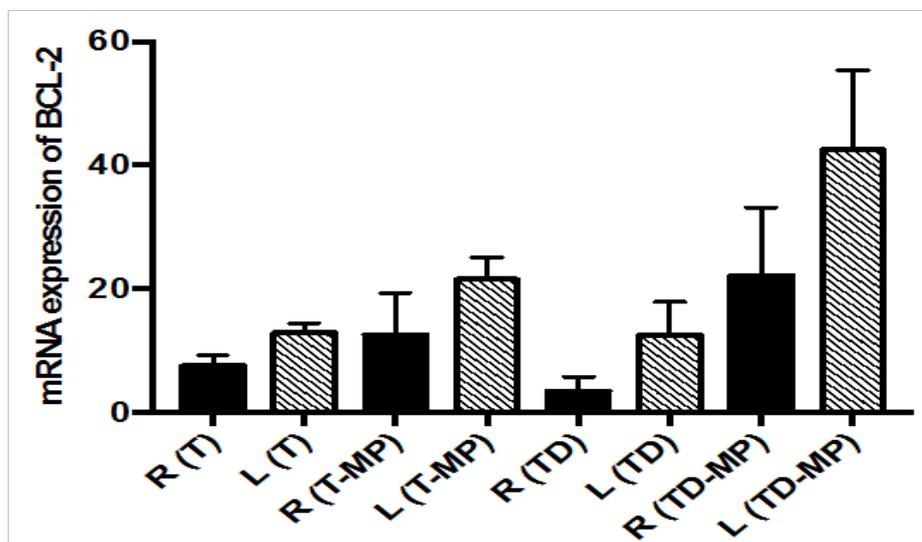


FIGURE 2. mRNA expression of BCL-2 in all of the experimental groups. Values are expressed as mean ± standard deviation (SD). R, Right testis. L, Left testis.

TABLE 3. Independent t-test comparing group means of BAX mRNA expression

Experimental groups		Right testis (p)	Left testis (p)
T	TD	0.144	0.314
T	T-MP	0.631	0.612
TD	TD-MP	0.107	0.197

BAX: BCL-2 associated X protein; T: torsion; T-MP: torsion+methylprednisolone; TD: torsion-destorsion; TD-MP: torsion-detorsion+methylprednisolone

TABLE 4. Independent t-test comparing group means of BCL-2 mRNA expression

Experimental groups		Right testis (p)	Left testis (p)
T	TD	0.006*	0.876
T	T-MP	0.129	0.001*
TD	TD-MP	0.008*	0.001*

*p<0.05; BCL-2: B-cell lymphoma2; T: torsion; T-MP: torsion+methylprednisolone; TD: torsion-destorsion; TD-MP: torsion-detorsion+methylprednisolone

Using independent t-test analyses to compare means between experimental groups, none of the BAX mRNA expression were statistically significant (TABLE 3). Comparison of means in BCL-2 mRNA expression were statistically significant when T group compared with TD group on right testis (ipsilateral) with p value of 0.006 and also, a significant difference of BCL-2 mRNA expression observed when T group compared with T-MP group on the left testis (contralateral) with p value of 0.001 (TABLE 4).

DISCUSSION

There were increase in mRNA expression of BAX and BCL-2 in ipsilateral and contralateral testis from the expression of normal testicular tissue in all groups (TABLE 2). On the previous studies, ischemic tissues undergo apoptosis, resulting in tissue damage.^{13,15} Alternatively, the rates of BAX gene expression in the contralateral testis

are substantially increased, indicating concomitant apoptosis. Although the resulting damage to the contralateral testicles has not been clearly identified, the following mechanisms have been proposed to explain contralateral testicular injury: autoimmunity to spermatogonia, decrease in testicular blood flow caused by a sympathetic reflex, formation of ROS after detorsion and overproduction of nitric oxide.^{15,17}

Comparison of means using independent t-test between T group and TD group showed no significant difference of BAX mRNA expression both in right and left testis. However, BCL-2 mRNA expression showed significant difference on the right testis (p=0.006). In previous testicular torsion studies, testicular cells are ischemic, resulting in mitochondrial disruption and apoptosis activation.^{13,15,17} Previous studies have shown that BAX is the predominant pro-apoptotic molecule in the rat testis and displays increased expression following torsion. Results showed that both the

existence of BAX and the passage of BAX to the mitochondria following IR.¹⁸ TT have a common mechanism of action at the testicular level by causing oxidative stress due to increased accumulation and degradation of reactive oxygen and nitrogen species (ROS/RNS) in the oxidative defense system. Oxidative stress caused the activation of p53, p73, and ASK/p38 MAPK which activates the activation of proapoptotic proteins, including BAX.¹⁹

In previous study, TT followed by torsion repair induces all the hallmarks of classical reperfusion injury.¹⁰ Jin-Zhuo *et al.*²⁰ showed increase expression of BAX and decrease expression of BCL-2 in testicular torsion. During detorsion, the addition of blood flow and oxygen will increase such damage through the release of inflammatory agents from circulation to the testis contributing to apoptosis of germ cells, where BAX and BCL-2 genes play major role.¹⁴

BAX mRNA expression both in right and left testis showed no significant difference when comparing means between T group and T-MP group. However, BCL-2 mRNA expression showed significant difference on the left testis ($p=0.001$). Previous study showed that pretreatment with methylprednisolone has been linked with reduction in lipid peroxidation products and decrease protein oxidation.¹⁵ Mertoglu *et al.*¹⁵ had previously demonstrated the protective role of methylprednisolone after testicular torsion-detorsion in rats. The secretion of ROS may increase BAX levels and stimulate a mitochondrial-driven apoptotic pathway.²¹ Methylprednisolone is a steroid that inhibits ROS by blocking the enzyme phospholipase A2 and inhibiting the activation of leukocyte.^{10,15,16} This study showed no significant difference in BAX mRNA expression after methylprednisolone was administered, these results were varied due to the different route of methylprednisolone administration.

In this study, methylprednisolone was administered by intramuscular injection, slower drug absorption compared to intraperitoneal injection performed in previous studies. Thus, there were variation in efficacy of methylprednisolone. In this study, BCL-2 mRNA expression increased in methylprednisolone groups. These results showed that methylprednisolone prevent cell apoptosis through BCL-2, the increased levels of BCL-2 expression reduced ROS levels.¹⁵ Yazawa *et al.*²² showed that another steroid drug (dexamethasone) was associated with decreased vascular neutrophil infiltration and decreased germ cell apoptosis in animal models with testicular torsion.

Preoperative therapy with methylprednisolone may lead to decrease mRNA expression of BAX in ipsilateral and contralateral testicular tissue. Methylprednisolone could also increase mRNA expression of BCL-2 in ipsilateral and contralateral testicular tissues. Further studies are needed to investigate the efficacy of methylprednisolone as preoperative therapy for testicular torsion.

CONCLUSION

The results of the study show that administration of methylprednisolone in testicular torsion could be a protective mechanism against germ cell apoptosis in testicular tissue shown by the increase of anti-apoptotic gene expression.

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