THE EFFECT OF NIFEDIPINE ON SURVIVE CELL, APOPTOTIC CELL AND NECROTIC CELL OF IPSILATERAL TESTICULAR GERMINAL EPITELIAL CELLS ON MALE WISTAR RATS WITH UNILATERAL TESTICULAR TORSION

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ABSTRACT

Objective:*This study aimed to compare the number of survival, apoptotic and necrotic cells of ipsilateral testicular germinal epithelial cells in male wistar rats with unilateral testicular torsion between nifedipine given and control groups.* ***Material & Methods:*** *Thirty male wistar rats aged 10-12 weeks were randomly divided into 5 groups, each consisted of 6 rats. The negative control group (KN) underwent a sham procedure and left orchidectomy. Positive control group 4 (KP4) and 10 (KP10) performed left torsio testis 3 x 360 degrees medially for 4 hours and 10 hours respectively, then performed orchidectomy 4 hours after detorsion. The 4-hour (N4) and 10 hours (N10) nifedipine treatment group received the same treatment with positive control, but 30 min before detorsion performed, nifedipine were given intraperitoneal 100µg/kg. Within 1 hour after orchidectomy, cell count was calculated using flow cytometry.* ***Results:*** *It was found that the 4 (N4) and 10 hours (N10) nifedipine treatment group had a higher survival cells and also a lower number of apoptotic and necrotic cells compared to the positive control group. It was found that the 10 hours nifedipine treatment group (N10) had a lower number of apoptotic and necrotic cells compared to the 10 hour positive control group (KP10). The difference was statistically significant with p value <0.05. However, in KP4 and N4 group compared with KP10 and N10 group, higher apoptotic cells was obtained. This was a new phenomenon that needs to be investigated more deeply.* ***Conclusion:*** *Intraperitoneal administration of nifedipine prior to testicular detorsion may reduce the number of apoptotic and necrotic cells of testicular germinal epithelial cell, and may increase the number of survival cells in ipsilateral testes with unilateral testicular torsion.*

Keywords: *Survive cell, apoptosis, necrosis, nifedipine, unilateral testicular torsion, flow cytometry.*

ABSTRAK

Tujuan: *Tujuan penelitian ini adalah untuk membandingkan jumlah sel survive, apoptosis dan nekrosis sel epitel germinal testis ipsilateral pada tikus putih jantan strain wistar dengan torsio testis unilateral antara kelompok yang diberi nifedipin dan kelompok kontrol.* ***Bahan & Cara:*** *Tiga puluh tikus putih jantan strain wistar usia 10-12 minggu secara acak dibagi menjadi lima kelompok. Pada kelompok kontrol negatif menjalani operasi sham kemudian diambil testis kirinya. Kelompok kontrol positif 4 (KP4) dan kelompok kontrol positif 10 (KP10) dilakukan torsio testis kiri 3 x 360 derajat ke arah medial selama 4 jam dan 10 jam, kemudian didetorsi dan ditunggu 4 jam, kemudian dilakukan orchidectomy. Kelompok perlakuan nifedipin 4 jam (N4) dan 10 jam (N10) mendapatkan perlakuan yang sama dengan kontrol positif, 30 menit sebelum detorsi diberikan nifedipin 100µg/kg intraperitoneal. Dalam waktu 1 jam setelah orchidectomy, dilakukan pemeriksaan flow cytometry.* ***Hasil:*** *Didapatkan bahwa pada kelompok torsio testis 4 jam (N4) dan 10 jam (N10) yang mendapatkan nifedipin, memiliki jumlah sel survive lebih tinggi dibandingkan dengan kelompok kontrol positif. Pada sel apoptosis, didapatkan bahwa pada kelompok torsio testis 4 jam (N4) dan 10 jam (N10) yang mendapatkan nifedipin dibandingkan dengan kelompok kontrol positif, memiliki jumlah sel apoptosis lebih rendah. Pada sel nekrosis, didapatkan bahwa pada kelompok torsio testis 10 jam yang mendapatkan nifedipin (N10) memiliki jumlah sel nekrosis lebih rendah dibandingkan dengan kelompok torsio testis 10 jam (KP10). Perbedaan tersebut secara statistik bermakna dengan nilai p<0.05. Namun pada kelompok KP 4 dan N4 jika dibandingkan jumlah apoptosisnya dengan kelompok KP10 dan N10, didapatkan jumlah apoptosis yang lebih tinggi. Hal ini merupakan fenomena baru yang perlu diteliti lebih dalam.* ***Simpulan:*** *Pemberian nifedipin secara intraperitoneal sebelum detorsi testis dapat mengurangi jumlah sel apoptosis dan nekrosis sel epitel germinal, serta dapat meningkatkan jumlah sel survive pada testis ipsilateral dengan torsio testis unilateral.*

Kata Kunci: *Sel survive, apoptosis, necrosis, nifedipin, torsio testis unilateral, flow cytometry.*

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INTRODUCTION

Testicular torsion, the rotating or twisting of the testes and spermatic funicle, is a pathological condition that results in severe acute scrotum pain and is an emergency situation in urology.[1] To prevent the occurrence of ischemia and necrosis that may lead to subfertile or infertile, surgical therapy should be performed promptly.[2] The incidence of testicular torsion in men at the age of 1 until 25 years old was 4.5 cases per 100.000 a year, and 3.5 cases per 100.000 men a year at the age below 25 years old. Testicular torsion most commonly suffered by children during puberty (12-20 years).[2,3] In Indonesia, there was no accurate record of exact incidence data of testicular torsion. Latest data from the Emergency Room of Soetomo General Hospital in 2011-2015 reported 20 patients with testicular torsion, the most number was in the 15-20 years age group, that equal to 70% of all cases.[4]

Testicular torsion in adolescence is associated with abnormal testicular supporting system that causes the testis and epididymis move easily in the tunica vaginal sac and hang on the spermatic funicle.[3] Other predisposing factors are testicular tumors, testes with horizontal position, history of cryptorchidism, trauma, and spermatic funicle with long intrascrotal sections.[5] To prevents ischemia and necrosis, surgical therapy should be done immediately. If suspicion of testicular torsion can be detected within 6 hours, more than 90% of patient's testes with torsion can be saved. However, if it was diagnosed more than 24 hours, the success rate decreases to <10%. Data from Soetomo General Hospital Surabaya in 2011-2015 showed testicular torsion incidence was 20 cases, which 16 patients (80%) patients came to Emergency Room more than 6 hours. That makes orchidectomy in testicular torsion case in Soetomo General Hospital was quite high.

Testicular torsion and also detorsion procedures can induce morphological cell changes as well as biochemical changes that are largely due to ischemia/reperfusion injury in testicular tissue.[6,7] Testicular ischemia-reperfusion stimulates intracellular cascade signals in endothelial cells that trigger neutrophil recruitment, enhancement of intracellular reactive oxygen species (ROS), and cell-specific apoptosis that may lead to hypoxia, anoxia, impaired functioning to death (necrosis) of ipsilateral and contralateral testicular cells, including testicular germinal epithelial cells.[8-14] The current management of testicular torsion is to free the twisted funicle with either manual detorsion or performing orchidectomy, while the management of spermatogenesis disorders associated with fertility is not widely practiced.[15]

Nifedipine is a drug that inhibit calcium channel (Calcium Channel Blockers), so it can relax the smooth muscle of blood vessels that cause vasodilatation. This vasodilation effect can provide an advantage of inhibiting the progression of tissue damage due to ischemia. Calcium channel blockers also have antioxidant effects. Some literature suggests that calcium channel blockers may be used to prevent tissue damage due to post-ischemic reperfusion of heart problems (myocardial infarction), and stroke.[16] Nifedipine is relatively inexpensive and easy to obtain and also has vasodilation and antioxidants effect, so it may be possible to be used as a therapy in testicular torsion. This experiments will determine the effect of nifedipine on the number of survival, apoptotic and necrotic testicular germinal epithelial cell in unilateral testicular torsion.

OBJECTIVE

This study aimed to compare the number of survival, apoptotic and necrotic cells of ipsilateral testicular germinal epithelial cells in male wistar rats with unilateral testicular torsion between nifedipine given and control groups.

MATERIAL & METHODS

Experimental animals used in this experimental study were 30 male white rats (Rattus Norvegicus) wistar strains (150-200 grams weight, 10-12 weeks ages) obtained from the Pharmacology Laboratory of Brawijaya University Malang. First, all the rats was performed adaptation process in the cage/research environment (in Pharmacology Department of Brawijaya University Malang) for 2 weeks with 12 hours light, 12 hours dark cycle. Animals were randomly divided into 5 groups, each group consist of 6 rats. Five groups of experimental animals consisted of (1) negative control group KN, (2) positive control group KP4 (performed unilateral testicular torsion for 4 hours, then performed detorsion), (3) positive control group KP10 (performed unilateral testicular torsion for 10 hours, then performed detorsion), (4) group N4 (performed unilateral testicular torsion for 4 hours, then

performed detorsion, 30 min prior to detorsion was given nifedipine 100 µg/kg), and (5) N10 group (performed unilateral testicular torsion for 10 hours, then performed detorsion, 30 min prior to detorsion was given nifedipine 100 µg/kg).

The control group in this study is a negative control group (KN) that only performed sham surgery. In positive control group and treatment group, the testicular torsion was performed by rotating the left spermatic funicle by 3x360º to the medial (anti-clockwise) seen from the caudal. Left testicular detorsion is performed by rewinding the left spermatic funicle in opposite direction with the torsion, 3x360º laterally (clockwise) seen from the caudal. All experimental animals that performed testicular torsion and testicular detorsion were put to sleep using anesthetic, an intramuscular ketamine 75 mg/KgBB. In the treatment group N4 and N10, the animals was given 100 µg/kg nifedipine dissolved in 2 cc normal saline (Wida® Normal Saline 0.9%, Pasuruan, Indonesia) intraperitoneally 30 minutes before performed testicular detorsion. Nifedipine HCl used was Adalat 5 mg Infusions flasche, 0.01% solution; Bayer®, Germany obtained from Soetomo General Hospital Surabaya.

Four hours after detorsion performed, orchidectomy was performed on the left testes. Within 1 hour after the testes was taken, the sample was processed for the flow cytometry examination by making the cell suspension obtained from the testes. The suspension was made by crushed the left testes with fine curved scissors, then added calcium and magnesium free phosphate buffered saline (PBS) and then aspirated to disperse the cells washed it with PBS in 10 minutes. Resuspend the pellet in 1 ml PBS and filtered it through nylon mesh. All samples fixed in 70% chilled ethanol and store it at 4°C. Resuspend cells in annexin V binding buffer at a concentration of 0.25-1.0 x 10 cells/ml. Transfer 100 µl of cell suspension in a 5 ml tube. FITC Annexin V added 5 µl. And then add 10 µl of Propidium Iodida (PI). Add 400 µl Annexin V Binding Buffer for every tube and perfomed cell count by automated cell counter flowcytometer.[17] The results of the calculation of survival, apoptotic, and necrotic cells in germinal epithelial cells (numerical variables). Hypothesis is determined based on the value of significance obtained. This research is significant if the value of $p<0.05$. We use one-way anova, then the next step was to do multiple comparison test or Post Hoc Test Tamhane and LSD.[18] All technical data processing were analyzed using SPSS 20 for Windows, with $p<0.05$ indicating statistically significant difference.

RESULTS

Based on the result of data analysis, we found significant difference of survival cell number in each group with p value <0.05. It can be seen that the effect of testicular torsion for 4 hours (KP4) and 10 hours (KP10) decreased the number of survival cells significantly with p value <0.05. The number of survival cells in the 4 hour testicular torsion nifedipine given group (N4) was higher than the 4 hour testicular torsion group (KP4), but that was not statistically significant with p value >0.05. The 10 hours testicular torsion nifedipine given group (N10) had a higher survival rate compared with the 10 hours testicular torsion group (KP10) and was statistically significant with p value <0.05.

Based on the result of data analysis, there was significant difference of apoptotic cell count in each group with p value <0.05. Based on the analysis of Post Hoc LSD test, testicular torsion for 4 hours (KP4) and 10 hours (KP10) increased the number of apoptotic cells significantly with p value <0.05. The 4 hour testicular torsion group nifedipine given (N4) had a lower number of apoptotic cells compared with the 4 hour testicular torsion group and statistically

Table 1. Comparison of survive cell number in each group.

Group	n	Mean ± SD	Normality Test	Test of Homogeneity	One way Anova
Control	6	90.36 ± 1.23^{a}	0.816	0.014	0.01
Testicular Torsion 4 hours	6	70.45 ± 7.04^{b}	0.653		
Testicular Torsion 10 hours	6	24.17 ± 1.45^{c}	0.316		
Testicular Torsion 4 Hours + Nifedipine	6	74.33 ± 4.33^{b}	0.467		
Testicular Torsion 10 hours + Nifedipine	6	48.91 ± 1.04^{d}	0.569		

* = statistically significant

significant with p<0.05. Similarly, the 10 hour testicular torsion group nifedipine given had a lower number of apoptotic cells compared with the 10 hour testicular torsion group and statistically significant with p<0.05.

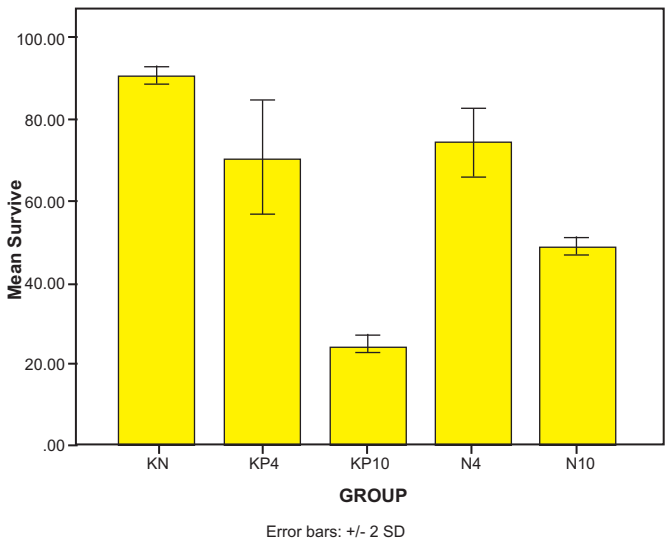

Figure 1. Comparison of Survive cells number in each group.

Table 2. Comparison of apoptotic cells number in each group.

Group	n	Mean ± SD	Normality Test	Test of Homogeneity	One way Anova
Control	6	02.51 ± 0.66[a]	0.343	0.062	0.00*
Testicular Torsion 4 hours	6	28.83 ± 7.30[b]	0.668		
Testicular Torsion 10 hours	6	17.10 ± 2.75[c]	0.576		
Testicular Torsion 4 Hours + Nifedipine	6	24.34 ± 4.66[b]	0.502		
Testicular Torsion 10 hours + Nifedipine	6	10.67 ± 3.35[d]	0.561		

* = statistically significant

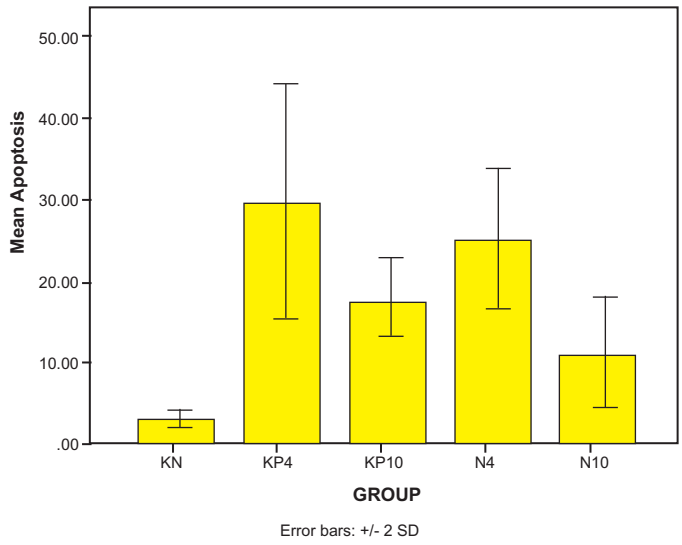

Figure 2. Comparison of apoptotic cells number in each group.

Table 3. Comparison of the necrotic cell number in each group.

Group	N	Mean ± SD	Normality Test	Test of Homogeneity	One way Anova
Control	6	07.14 ± 1.10^{a}	0.248	0.032	0.00*
Testicular Torsion 4 hours	6	00.73 ± 0.29^{b}	0.840		
Testicular Torsion 10 hours	6	58.74 ± 1.85^{c}	0.939		
Testicular Torsion 4 Hours + Nifedipine	6	01.32 ± 0.49^{b}	0.815		
Testicular Torsion 10 hours + Nifedipine	6	40.42 ± 3.24^{d}	0.767		

* = statistically significant

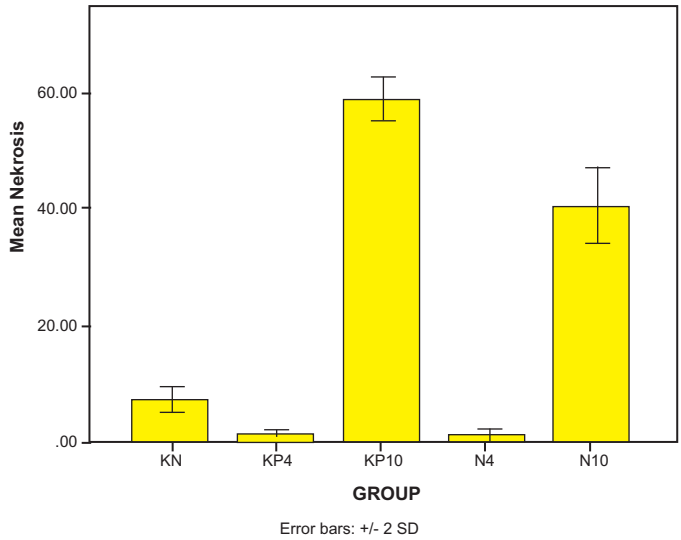

Figure 3. Comparison of necrotic cells in the treatment of testicular torsion in each group.

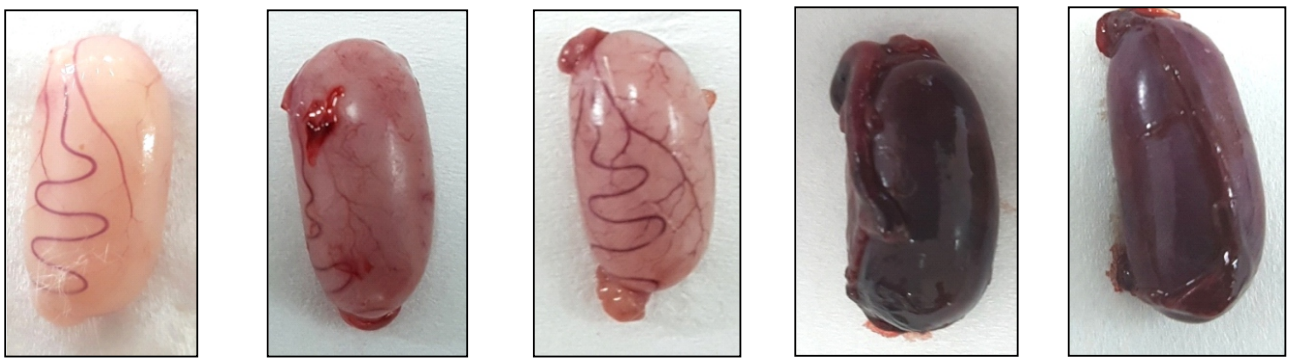

Figure 4. Macroscopic preparation of left unilateral testis after testicular torsion.
A: Negative control (KN)
B: Testicular torsion 4 hours groups (KP4)
C: Testicular torsion 4 hours with nifedipin (N4)
D: Testicular torsion 10 hours groups (KP10)
E: Testicular torsion 10 hours with nifedipin (N10)

Based on result of data analysis, there was significant difference of necrotic cell number in each group with p value <0.05. In this study, it can be seen that necrotic cells were more common in the control group (KN) than in the 4 hours testicular torsion group (KP4) and this was statistically significant with p<0.05. However, different result were found in the 10 hours testicular torsion group(KP10). Higher necrotic cells was found compared to the control group (KN) and was statistically significant with p<0.05. Administration of nifedipine in the 4 hours testicular torsion group (N4) descriptively had higher necrotic cells compared with the 4 hours testicular torsion group (KP4), although it did not differ significantly. In this study, it can also be seen that in the 10 hour testicular torsion group given nifedipine (N10) had a lower necrotic cells than the 10 hours testicular torsion group and this was statistically significant.

After the left testes removed by orchidectomy, the testes appearance was clinically different in each group. The appearance of the testis in the control group appears in bright color with a good testicular anatomical structure accompanied by normal-looking blood vessel and shows a normal and viable testicular picture. Therefore it was called a negative control group.

In the 4 hour testicular torsion group (Kp4), the testicular structure of the anatomy was still good with the color starting to dull and the blood vessels began to darken. Compared to the 4 hours testicular torsion group which given nifedipine before detorsion (N4), it has a better macroscopic testicular structure, with a brighter testicular color with vascular features seen more clearly, almost appeared like negative controls. This is in accordance with the theory that the degree of tissue damage associated with the duration of the testicular torsion.

In the 10 hour testicular torsion group (Kp10), the testicular structure began to deteriorate, with the color became black, and no more visible blood vessels of the testes. The impression of the testes was non-viable. In the 10 hour testicular torsion group given nifedipine prior to detorsion (N10), the macroscopic structure was almost identical to that of a 10 hours positive control group (KP10), with the macroscopic structure of the testes transformed into black, with vascular features was not visible. However, if it observed more closely, the testes with nifedipine treatment (N10) had the color that a little brighter than the 10 hours positive control group (KP10).

Group which performed 4 hours of testicular torsion showed a clinical appearance similar to the negative control, because theoretically treatment of the testicular torsion less than 6 hours, 90% of the testes are still viable and can be saved. In the 4 hours testicular torsion group treated with nifedipine prior to detorsion (N4), the clinical appearance did not differ significantly from the 4 hours positive control groups (KP4).

DISCUSSION

From the description above, can be seen that there is correlation between testicular torsion and duration of the torsion in affecting the cell damage rate. Longer time of the torsion will increasingly affect the number of survive, apoptotic and necrotic testicular cells. It can be seen from the significant increase in the number of cells undergoing apoptosis and necrosis, accompanied by a significant decrease in the number of survive cells between the negative control group (KN), 4 hours testicular torsion positive group (KP4) and 10 hours testicular torsion positive group (KP10).

Testicular torsion that occurs due to twisting the spermatic cord directly result in decreasing of blood flow to the testes that will cause ischemia in the entire testis tissue. Ischemia leads to decreased intracellular ATP levels, in addition to hypoxic conditions, resulting in elevated intracellular Ca^{2+} levels. The transfer process of calcium into the cell is caused by the opening of L-type Ca^{2+} channel. Because of the elevated levels of high intracellular calcium, the activity of Endoplasmic Reticulum (ER) increases by trying to uptake calcium levels. However, this process does not work well because the low levels of ATP produced by mitochondria does not meet the needs of ER. This results in ER reuptake process inhibited and intracellular calcium multiplied. ER failure in reuptake causes high levels of cytoplasmic calcium. High levels of calcium makes the mitochondrial cytoplasm attempts to take over the role of ER by activating the mitochondrial buffer system. This process does not last long because the calcium is kept in due to ischemia, so the calcium level remains high. This resulted in the failure of the mitochondrial buffering capability, resulting in mitochondrial stress. This mitochondrial stress activates Bcl-2 family, causing permeability transition (PT) pore activation on the mitochondrial matrix. Mitochondria then releases C-cytochrome into the cytosol, and then apoptotic protease

activating factor 1 (Apaf-1) and dATP lead to active the procaspase-9 into caspase-9, so apoptosis process occurs.[19]

If the testicular torsion is not treated immediately, the ischemic process becomes longer and causes testes cell death. The cell death triggers an inflammatory reaction that results in the release of pro-inflammatory cytokines TNF-α. TNF-α then binds to TNF-α receptor (TNF-αR) on the cell surface, causing the activation of FADD (protein-associated death-domain) protein-caspase-DNAase, ending with germ cell apoptosis. Inflammatory reactions resulting from prolonged ischemia can also cause neutrophile adhesion to the vascular endothelium. This leads to the increasing of reactive oxygen species (ROS) production. Excessive ROS production leads to the decreasing antioxidants such as superoxide dismutase (SOD), so that apoptotic pathways become active.[16]

Intraperitoneal administration of nifedipine is expected to inhibit apoptosis and necrosis in unilateral testicular torsion, so survive cells remains high. This study showed that intraperitoneal administration of nifedipine in 30 minutes before testicular detorsion decreased the number of apoptotic and necrotic cells and significantly increased the number of survival cells in the 10 hours treatment group (N10) compared with the 10 hours positive control group (Kp10). Intraperitoneal administration of nifedipine 30 minutes prior to detorsion of the testis showed decreased apoptotic and necrotic cells and higher survival rates in the 4 hours treatment group (N4) compared with the 4 hours positive control group (KP4).

However, the statistical test was not significantly different in that group with $p>0.05$. In this study, we also found that the apoptotic cell count in the 4 hourspositive control group (KP4) and the 4 hours treatment group (N4) was higher than the 10 hour positive control group (KP10) and the 10 hours treatment group (N10). This is a new phenomenon that needs to be investigated further. It may occur possibly due to ischemic reperfusion injury effects that occur in 4 hours torsion when detortion done so that free radicals activated apoptosis process significantly. In 10 hour testicular torsion group the reperfusion effect is more dominant in the direction of necrotic cells because of the high ROS formed due to longer ischemic effects. Previous research by Higuchi M, Honda T, Proske RJ, Yeh ET. 1998, which states that ROS may induce apoptosis and necrosis through the role of caspase. At low concentrations Xantine Oxidase (0.025 U/ml) may induce apotosis, whereas at high concentrations (0.1 U/ml) may damage cell membranes in order to induce the occurrence of necrosis.[20] The exact mechanism of this phenomenon needs further investigation.

Nifedipine is a Calcium Channel Blocker (CCB) drug, which can inhibit the incoming ions through the slow channels of active cell membranes. Nifedipine inhibits the L-type Ca2+ channel in the plasma membrane. This barrier will lower intracellular calcium levels because it inhibits calcium influx from extracellular stores. The decreasing in intracellular levels of the ischemia causes vasodilation in the blood vessels resulting in increased blood flow. The vasodilation will decrease the effects of ischemia directly on cell death process. The subsequent effects lead to decreased inflammatory reactions in ischemia process. Pro-inflammatory cytokines TNF-α bounded with TNF-αR on the surface of spermatogonium cells will also decreased. Declining of these bonds resulted in decreased activity of FADD-caspase-DNAase protein so apoptosis process can be prevented. The administration of nifedipine results in decreasing of inflammatory reaction, then causes decreasing of ROS production. It caused the ratio between scavenger and ROS increasing so that neuro-neutralizing ROS will eventually decrease the number of apoptosis in germ cells.[14,16]

The results obtained were in concordance with the study by Metrovi et al., 2014 which suggested that administration of nifedipine prior to testicular detorsion would improve the histologic structure (spermatogenesis) and suppress apoptosis compared with the control group. The study also mentioned that group with nifedipine administration had SOD and glutathione peroxidase (antioxidants) higher than the control group.[16]

LIMITATION

The limitation of this research is not measured the length of the funiculus spermaticus on each subject.

CONCLUSION

The results showed that intraperitoneal administration of nifedipine prior to testicular detorsion may reduce the number of apoptotic and necrotic germinal epithelial cells, and may increase

the number of survive cells in ipsilateral testes with unilateral testicular torsion especially in the 10 hours testicular torsion.

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