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"Protective Effects of Sufentanil and Remifentanil Preconditioning on Testicular Ischemia-Reperfusion Injury"

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ABSTRACT

Testicular torsion is a condition that manifests with acute pain in childhood and adolescence which can lead to infertility despite urgent surgical intervention. The aim of this study is to investigate and compare the protective and preventive effects of sufentanil, remifentanil and fentanyl, a potent analgesic agent on testicular ischemia-reperfusion injury (tIR). 28 adult male Wistar rat were divided into four groups (n=7). Torsion was performed on all left testicles rat 720' clockwise. 1 hour ischemia. 30 minutes before detortion, control group (Nacl 0.9%), fentanyl group (2 mcg/kg), Remifentanil group (1 mcg/kg), Sufentanil group (0.6 mcg/kg) were injected intraperitoneally. During 1 hour of ischemia, the left testes was detorted to restore physiology. 24 hours after reperfusion, rats in euthanasia were taken blood to see Malondialdehyde levels and ipsilateral testes to find germ cell damage. Leydig cells count and percentage tubular necrosis. Administration of Sufentanil before detortion significantly reduced malondialdehyde levels (6.01 ± 0.24SD,$P<0.05$), damage to germ cells (5.93,$P<0.05$), Leydig cells count (20.2 ± 4.5SD,$P<0.05$) and tubular necrosis (3.95 ± 0.62SD,$P<0.05$) which were subjected to torsion compared to the fentanyl and remifentanil groups. However, lipid peroxidation content of malondialdehyde was significantly decline in remifentanil group (3.58 ± 0.23SD,$P<0.05$). Sufentanil in ischemia preconditioning has the most protective effect compare to remifentanil by preventing testicular ischemia reperfusion injury leading to infertility in the future.

1. Introduction

Testicular torsion is twisting of the testis and spermatic cord with acute painful manifestations that often

occurs in children and young adults which requires immediate surgery. Ischemia that occurs during torsion can develop into testicular ischemia reperfusion injury (tIR) when testicular detortion results in infertility [1]. Incidence reaches 4.5 in 100,000 per year. In East Java, Indonesia, 70% of them had to undergo an orchidectomy [2]. Administering fentanyl prior to detortion can reduce lipid peroxidation in malondialdehyde (MDA) levels and reduce testicular tissue damage as seen from germ cell necrosis, Leydig cell count and tubular necrosis [3]. Remifentanil and Sufentanil are analgesics with stronger potency than fentanyl. However, there are no specific in vivo studies using these two opioids. The aim of this study is to investigate and compare protective and preventive effects of Sufentanil, remifentanil and fentanyl in preventing tIR-induced infertility.

2. Material and Methods

This research is a prospective experimental analysis research with a randomized post test only control group design with white male Wistar rats as subjects. This research was conducted at Animal Laboratory The Faculty of Veterinary Medicine, Airlangga University. An ethical clearance (No.2.KEH.112.08.2022) was issued by Ethics Committee of the Faculty of Veterinary Medicine, Airlangga University. There were 28 male wistar rats (weight 150-200 g) which divided into four groups (n=7). Surgical torsion-detorsion procedure with ketamine (75 mg/kg intraperitoneal). Each subject underwent testicular ischemia by torsion of left testis 720' clockwise [3]. Ischemia was carried out for one hour and 30 minutes before detortion, intraperitoneal injection was carried out in each group. Consist of control positive (K+) group (NaCl 0.9%), Fentanyl (KF) group (2 mcg/kg), remifentanil (KR) group (1mcg/kg) and sufentanil (KS) group (0. 6 mcg/kg). After one hour, rat testis is detorted in original direction 720’anticlockwise [3]. 24 hours after rat testicular detortion, euthanasia was performed by taking blood samples to see levels of malondialdehyde and ipsilateral testes to see germ cell necrosis, Leydig cells and tubular necrosis.

2.1 Biochemical Analysis and Histopathological Examination

Blood is taken from the heart and placed in a heparin tube and then centrifuged. Separated plasma will be examined for Malondialdehyde which is an indicator of increased lipid peroxidation in tIR. After euthanasia the ipsilateral testes will be placed in 10% formaldehyde solution and stained with Haematoxylin and eosin (H&E) after deparaffinised. Degree of testicular germinal necrosis will be assessed using the Johnson Tubulus Biopsy Score which consists of 4 levels. In addition, viabel of Leydig cell counts and tubulus seminiferous necrosis was calculated.

2.2 Statistical Analysis

Data were analyzed using SPSS 25.0 for windows. Body weight of each group was measured and homogeneity test was carried out. Dependent variable is presented in box plot graph. Then performed a data normality test with the Saphiro-Wilk test. The data in this study were normally distributed and homogeneous. Then data was continued with ANOVA test followed by a Post-Hoc Test to determine the differences between groups. The limit of significance degree if $p \leq 0.05$ with 95% confidence interval.

3. Result

The experimental animal used Wistar strain male white rat. For rat body weight is also in almost the same range. Descriptive calculation results showed that average body weight of K+ (178.57$\pm$17.17SD), KF (179.86$\pm$13.86SD), KR (178.00+16.07SD) and KS (178.86$\pm$14.26SD). The results of normality test with Shapiro Wilk show that all group data are normally distributed $P>0.05$. The results of ANOVA test obtained a significance level of 0.997. This significance value is greater than 0.05 ($p> 0.05$), which means that there is no significant difference in the body weight of the rats or between groups are homogeneous.

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The first aim of study was to examine differences in MDA levels between groups. The descriptive calculations showed that MDA level in K+ (10.56±2.85SD,P<0.05), KF (8.21+0.54SD,P<0.05), KR (3.58+0.23SD,P<0.05) and KS (6.01±0.24SD,P<0.05). The mean value indicated that the remifentanil group gave lower MDA results than the other groups. ANOVA test result show significance level of 0.000 (P<0.05) which means that there is a significant difference MDA level between 4 groups (figure.1).

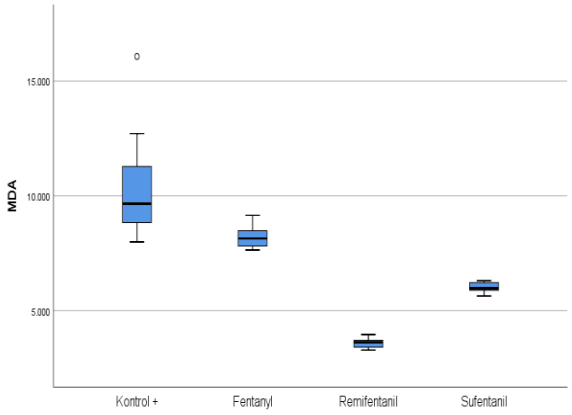

Figure 1. Malondialdehyde Level (µM/ml)

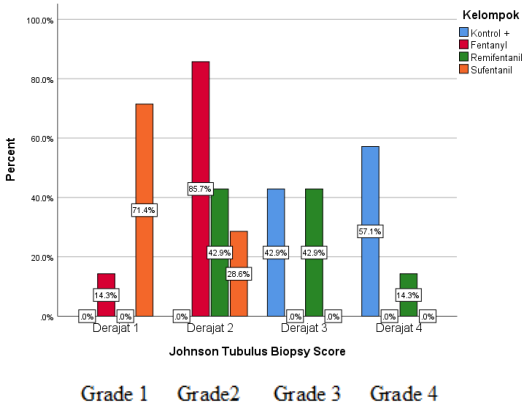

Figure 2. Johnson Tubulus Biopsy Score

Differences in germ cell necrosis based on the Johnson Tubulus Biopsy Score vary from degrees 1 to 4. Grade 1 is normal testicular shape with an orderly arrangement of germinal cells. Grade 2 less orderly, non cohesive germinal cells and closely pack seminiferous tubules. Grade 3 disordered, sloughed germinal cells with shrunken, pyknotic nuclei and less distinct seminiferous tubule borders. Grade 4 seminiferous tubules that were closely packed with coagulative necrosis of germinal cell [4]. The percentage results for each degree in all groups can be seen in figure 2. Grade 1 is more in the sufentanil group which is 71.4%, then grade 2 is more in the fentanyl group 85.7%, grade 3 is the same in both control positive and remifentanil group 42.9%. In grade 4 is more in control positive group 57.1%. Descriptive calculations showed that the mean rank of K+ (23.64,P<0.05), KF (10.79,P<0.05), KR (17.64,P<0.05) and KS (5.93,P<0.05). The mean value results indicated that sufentanil group showed the lowest germinal cell necrosis based on Johnson Tubulus Biopsy Score compared to the others. There is macroscopic view of the left testis (ipsilateral) after torsion-detorsion for 1 hour. From each group where there were differences between four groups macroscopically (figure 3).

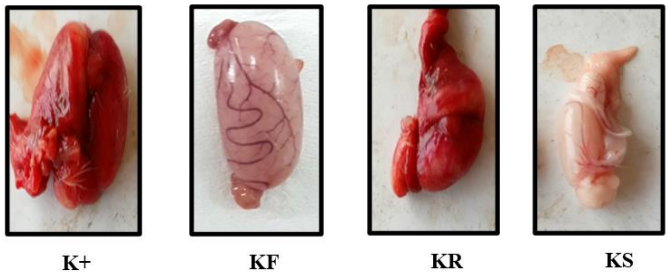

Figure 3. Ipsilateral Testis Post Torsion-Detorsion

Apart from seeing necrosis germinal cells based on Johnson Tubulus Biopsy Score. From microscopic picture we assess the number of Leydig cells that are still viable. There is a microscopic view of Leydig cells between groups in figure 4.

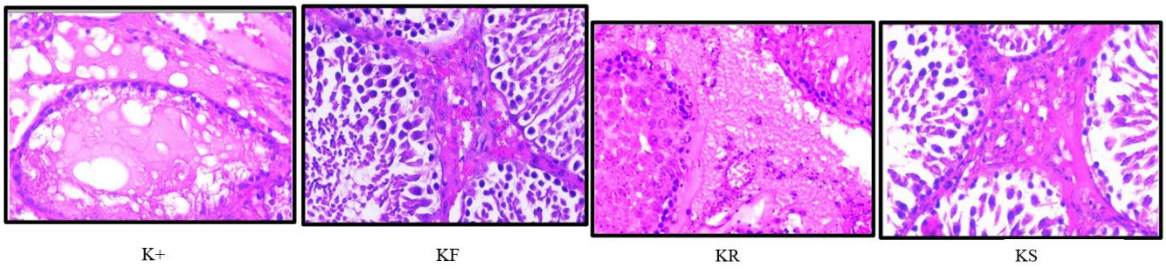

Figure 4. Leydig cells (H&E stain, original magnification x400)

The descriptive calculations showed that mean Leydig cell for K+ (3.60+0.97SD,P<0.05), KF (8.37+2.41SD,P<0.05), KR (14.85+3.38SD,P<0.05) and KS (20.25+4.59SD,P<0.05). Mean value results indicated that sufentanil group had a greater number of viable Leydig cells than other groups (Figure 5).

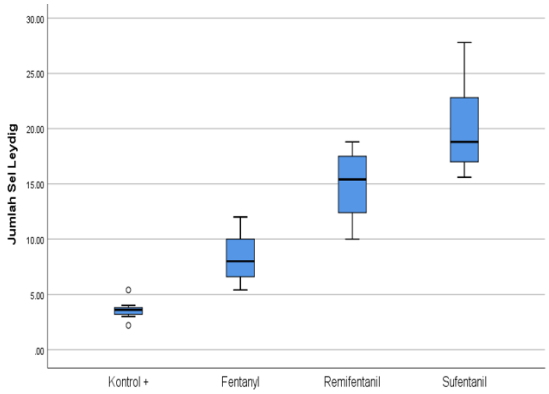

Figure 5. Viabel Leydig cells

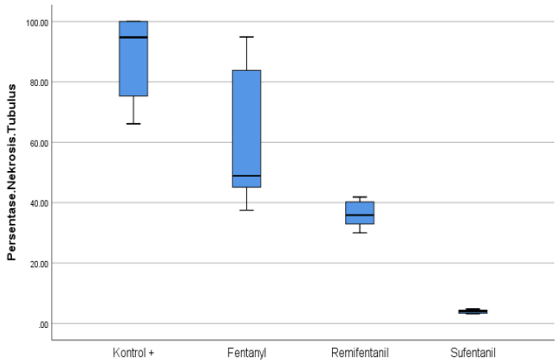

Figure 6. Necrotic of Tubulus Seminiferous

Microscopically, the percentage of tubular necrosis for each group could be calculated. The following is a picture of tubular necrosis with 100x magnification figure 7. Descriptive calculations showed that mean percentage of tubular necrosis in K+ (87.35+14.63SD,P<0.05), KF (62.73+24.51SD,P<0.05), KR (36.30+4.55SD,P<0.05) and KS (3.95+0.62SD,P<005). The results of mean value indicated that sufentanil group gave a smaller percentage of tubular necrosis than other groups (Figure 6).

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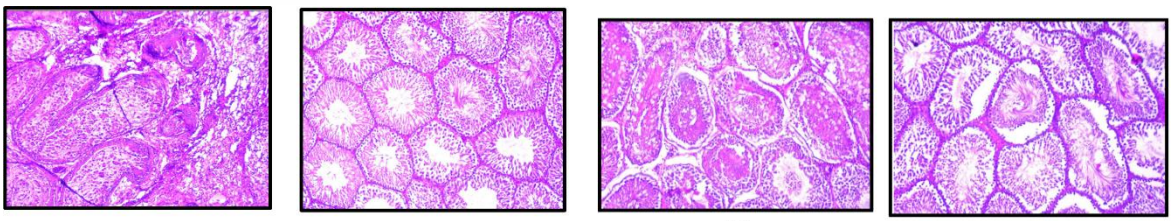

Figure 7. Necrosis of Tubulus Seminiferous(H&E stain, original magnification x100)

4. Discussion

In this study, the first objective was to see comparison of malondialdehyde levels after administration opioids in testicular ischemia reperfusion injury. free radicals will bind to peroxyloxidase in membrane cell to form lipoperoxidase and produce toxic metabolites malondialdehyde [5]. Result From this study, found that administration remifentanil could reduce MDA levels. A study which used remifentanil in rat with dose 1 mcg/kg in ischemic reperfusion injury in small intestine [6]. However, there were differences in preconditioning organ damage intestine, while this study looked at the effects of IRI on testicular organs. This is due to the absence of research references using remifentanil as a specific protective agent in tIR. As a clinical implication this is very important when compared with other opioids such as morphine and fentanyl. High doses of remifentanil intraoperatively will not cause respiratory depression and impaired postoperative intestinal motility [7].

Several other studies have also shown protective effect remifentanil on myocardium, nerve cells and liver by comparing MDA levels [8]. Although we know that remifentanil is basically μ receptor agonist. However, studies in myocardial IRI model showed a protective effect of remifentanil not only through μ receptor agonist but also at κ and δ receptor [9], [28]. It is estimated that in this study remifentanil also stimulated κ and δ receptor agonists. A study which saw tramadol effect on changes MDA levels in testes. We knew that tramadol did not only work on μ receptors but also on κ and δ receptors [10]. In addition, opioid agonists have an inhibitory effect on proinflammatory activity that produces cytokines (Tumor Necrosis Factor α and Interleukin-1) by stimulating toll-like receptor 2 and this is only mediated by opioid receptors [11], [28]. Another thing that needs attention is whether use of high dose remifentanil in acute pain with a short half-life can cause harmful changes at cellular level, especially germinal cells. If we look at several studies, including a dose of 1 mcg/kg remifentanil is protective against liver ischemic reperfusion injury in rats [12]. However, compared to another research, it showed that dose of 10 mcg/kg of remifentanil could increase the degree lipid peroxidation of malondialdehyde levels [13].

Sufentanil has the advantages of stronger lipophilicity, higher plasma protein binding rate, relatively smaller volume distribution, strong affinity opioid receptor and longer duration [14]. But so far, there has been no report on protective effect of sufentanil against IRI that occurs in testicular torsion. In this study, sufentanil group was able to reduce MDA levels although the results were not as low as the remifentanil group. This shows that sufentanil can suppress oxidative stress in target cells according another research who looked at MDA levels in myocardium IRI. Oxidative stress is an important mechanism in tIR. When compared to K+ group, MDA levels were higher than others. So it showed that K+ group can reduce activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) [15]. In contrast sufentanil group where MDA levels were lower when compared to the K+ which showed a more protective effect.

Hypoxia induce inflammatory process in several ways. one of which is Hypoxia inducible Factor-1 alpha (HIF-1α) which plays a major role in nuclear transcription activator and cytokine accumulation. Cytokines also play a role in inflammation of testicular torsion. Including IL-1 and TNF-α, both of which are

synthesized by macrophages. TNF-α activates neutrophils and lymphocytes, also synthesizes adhesion molecules and other inflammatory mediators such as IL-1 and IL-6. The result of neutrophil activation and adhesion will increase tissue and organ damage during IRI. Therefore Sufentanil has a role in suppressing proinflammatory cytokines that occur during testicular torsion. This is reinforced in antoher research, the use of sufentanil can reduce IL-1, IL-6 and TNF-α in acute lung injury [16].

In the Fentanyl group when compared to the remifentanil and sufentanil groups, there was not much difference in reducing MDA levels. However, much better meaningful than K+. This is in line which found fentanyl can decrease Malondialdehyde levels and increase Catalase (CAT) [3]. In addition, it is also supported by tIR studies when use morphine which acts on μ receptor. This proves that use of opioids can increase CAT which functions as an excess ROS scavenger and suppresses Malondialdehyde lipid membrane peroxidation [17]. As a note from this study that fentanyl administration can reduce MDA levels because it can prevent ROS formation which initiated by Polymorphonuclear (PMN) cells. However CAT examination is needed in future studies to further strengthen the research results.

fentanyl that we used in this study can reduce MDA levels by initiating μ opioid receptors. Initiation opioid receptor will activate protein kinase C (PKC). K(ATP) dependent channels whose function is initially impaired during periods of ischemia will open and calcium levels in mitochondria will increase and decrease in cytosol both of them will have a protective effect against lipid peroxidation in membrane cell. This process protects the tissue by increasing extracellular adenosine production, accumulating cellular energy and decreasing PMN adhesion [18].

Testicular torsion is quite common in children and young adults with severe acute pain manifestations. Early administration of opioids before surgical detortion is the starting point in reducing germinal cell damage due to tIR. In this study also assess the extent to which opioids can suppress germinal cell necrosis. This can be seen from various histopathological result by assessing the Johnson Tubular Biopsy Score, viabel of Leydig cells and necrotic seminiferous tubules. In this study, found that sufentanil group suppressed germinal cell necrosis better than the other groups. This can be seen from the Johnson Tubular Biopsy score where the sufentanil group has a grade of 1-2. This is caused primary stress in testicular ischemia due to decreased ability of mitochondria to produce Adenosine triphosphate (ATP). The depletion of ATP results in swelling of the cell and mitochondria, dilation of endoplasmic reticulum and protrusion formation on plasma membrane called blebs. Blebs are the result of ATP depletion and disruption of cells in controlling cell volume. After ischemia period, the formation of blebs rapidly reverses after reoxygenation [19].

However, prolonged ischemia will cause necrosis of cell death. Prior to cell death, which is characterized by increased mitochondrial permeability, lysosomal disruption, coalescing blebs and cell swelling make glycine sensitive to chloride-conducing anions. This makes defense of plasma membrane fragile due to blebs rupture. Plasma membrane destruction causes the contents and various cellular enzymes to come out and when viewed with stains such as trypan blue, a picture of cell necrosis can be seen [20]. There are no studies using sufentanil specifically looking at level cellular effects on testes. However, there is another study using sufentanil in cerebral ischemia reperfusion injury model, where sufentanil is able to inhibit excessive inflammation and cell death in brain tissue [21]. As well as research on sufentanil in cardiac ischemia reperfusion injury model, it also shows a protective effect on preventing necrosis in myocardium [22].

In this study, remifentanil was not as good as sufentanil and fentanyl. We know that in torsion there is an

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inflammatory process which releases various mediators including IL-1 and TNFα. During this process neutrophils will migrate rapidly from intravascular to proinflammatory tissue. During initial migration phase, *rolling* of neutrophil along endothelial cells through the endothelial cell monolayer (ECM). Neutrophil will undergo morphological changes from rounded, relatively smooth cells and become more elongated and irregular pseudopodia. opioids have an effect on neutrophil adhesion, transmigration and expression of intercellular adhesion molecule [23]. So, sufentanil is better at inhibiting neutrophil migration than remifentanil [24].

Testes consist of convoluted loops seminiferous tubules which are arranged in an orderly manner separated by an interstitium which contains components of Leydig cells, blood vessels, macrophages, and various proteins. The seminiferous epithelium is formed by Sertoli cells which are located at base of the cells and function to provide sperm nutrition and blood testis barrier. Meanwhile, Leydig cells function was regulate intratesticular testosterone levels [25]. In this study also assessed sufentanil, remifentanil and fentanyl affect Leydig cell viability and seminiferous tubular necrosis.

Leydig cells, Sertoli cells, and spermatocytes are able to express endothelial Nitric Oxide Synthase (eNOS) and inducible Nitiric Oxide Synthase (iNOS). We know Nitiric Oxide (NO) is a free radical that is important in cellular signaling molecules and vasodilatation. NO can diffuse freely because its molecule is able to dissolve in water and fat with a shorter half-life and is formed from L-Arginine and oxygen molecules (NOS isoform) [26]. Another research showed that reperfusion for 24 hours after testicular torsion with ischemia time on 30 minutes can increase the expression of iNOS which leads to an increase in NO [27]. In this study, Sufentanil found that the ability of Leydig cells to defend themselves or be viable in ischemic reperfusion injury was better than remifentanil and fentanyl. This is because sufentanil is able to suppress iNOS. If NO levels are too high it can reach toxic levels. NO reacts with superoxide to produce highly reactive peroxynitrite which can damages DNA, RNA, lipids and proteins and depletes calcium deposits from endoplasmic reticulum. It showed sufentanil was able to reduce iNOS levels in myocardial ischemia reperfusion injury model [15].

Seminiferous tubule necrosis can occur due to hypoxia or intermittent ischemia due to blood flow disturbances that affect germinal cells and Sertoli cells. In figure 7 will see coagulative necrosis of Sertoli cells, both focal and segmental with tubular disruption and an infiltration process of inflammatory cells around the seminiferous tubules. In general, Sertoli cells are able to survive cell death due to tight junctions between cells that form a blood-tubular barrier. Barrier can decrease if ischemia testis occurs which leads to Sertoli cell death and irreversible tubular necrosis. Sertoli cells death with surrounding tubular necrosis will result in connective tissue. This can affect infertility after testicular torsion [25]. A study which compared several opioids, in this case fentanyl and morphine, it turned out that showed a pro-angiogenesis effect. These effects through opioid receptors on mitogen activated protein kinase (MAPK) pathway [29]. Another study administration sufentanil in hepatic ischemia reperfusion injury model has a protective effect by activating p38MAPK phosphorylation pathway [30]. Meanwhile, remifentanil shows an anti-inflammatory effect by inducing lipopolysaccharide through MAPK pathway [31]. When compared with previous studies, protective effect of sufentanil in celluler level appeared to be better than remifentanil and fentanyl as seen from viability of the number Leydig cells and percentage of necrosis seminiferous tubules. It is hoped that by activating these opioid receptors, death of germinal cells, Leydig cells, Sertoli cells and formation of connective tissue can be inhibited and infertility will not occur.

5. Conclusion

Sufentanil in ischemia preconditioning has the most protective effect compare to remifentanil by preventing

testicular ischemia reperfusion injury leading to infertility in the future

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