THE EFFECT OF ERYTHROPOIETIN SUPPLEMENTATION ON SPERM MOTILITY AND MORPHOLOGY IN WISTAR RAT AFTER LIGATION RELEASE OF THE VAS DEFERENS

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ABSTRACT

Objective:*The patency rates after vasectomy reversal ranges from 71-97%, but there is 26-72% possibility of persistent infertility. Dysfunction or obstruction of the epididymis and oxidative stress are thought to be the important cause of male infertility by disrupting spermatozoa maturation process, causing poor sperm quality. Human erythropoietin or better known as EPO is a glycoprotein hormone that has been purified since three decades ago. Research on the EPO has evolved and become a major research topic the researchers aimed as a therapeutic agent. The cloning and expression of erythropoietin has developed recombinant erythropoietin as a drug that serves as an anti-oxidant, anti-apoptotic and anti-inflammatory. This study aimed to determine the effect of erythropoietin supplementation on sperm motility and morphology in Wistar rat after the release of the vas deferens' ligation.* ***Material & Methods:*** *Twenty four male Wistar rats were randomly divided into four groups (6 each). On the vasectomy group, the vas deferens were serially ligated for 7 weeks using a non-absorbable suture. The vasectomy reversal group get the same surgical treatment and after 7 weeks the ligation were released. While as in the erythropoietin group, recombinant eryhtropoietin (1000 IU/kg) was administered intraperitoneally three times for 1 week after releasing the ligation. Normal control animals received no surgical manipulation and followed by sperm retrieval for analysis. Eosin-stained slides were prepared to assess the motility and morphology of sperm cells and observed under a light microscope.* ***Results:*** *Ligation of vas deferens significantly decreased sperm motility and morphology. Releasing the ligation of the vas deferens did not improve the sperm motility and morphology. Supplementing eryhtropoietin 1000 IU/kg 3 times for a week after releasing the ligation did not improve the sperm motility and morphology.* ***Conclusion:*** *Erythropoietin supplementation did not improve the sperm motility and morphology in Wistar rat after releasing the ligation of vas deferens.*

Keywords: *Vasectomy, reversal, eryhtropoietin, sperm motility, sperm morphology.*

ABSTRAK

Tujuan: *Tingkat patensi setelah prosedur pengembalian vasektomi berkisar 71-97%, tapi ada 26-72% kemungkinan terjadinya infertilitas persisten. Disfungsi atau obstruksi epididimis dan stres oksidatif diperkirakan menjadi penyebab penting infertilitas pria dengan mengganggu proses pematangan spermatozoa, sehingga menghasilkan kualitas sperma yang buruk. Erythropoietin manusia atau lebih dikenal sebagai EPO adalah hormon glikoprotein yang telah dimurnikan sejak tiga dekade lalu. Penelitian tentang EPO sebagai agen terapeutik telah berkembang dan menjadi topik penelitian utama para peneliti. Kloning dan ekspresi erythropoietin telah mengembangkan erythropoietin rekombinan sebagai obat yang berfungsi sebagai anti-oksidan, anti-apoptosis dan anti-inflamasi. Penelitian ini bertujuan untuk mengetahui efek pemberian erythropoietin terhadap motilitas dan morfologi sperma tikus Wistar setelah dilakukan release ligasi vas deferens.* ***Bahan & Cara:*** *Dua puluh empat tikus Wistar jantan secara acak dibagi menjadi empat kelompok (masing-masing 6). Pada kelompok vasektomi, vas deferens diikat selama 7 minggu dengan menggunakan benang non-absorbable. Kelompok vasectomy reversal mendapatkan tindakan bedah yang sama dan setelah 7 minggu ligasi pada vas deferens dilepas. Sedangkan pada kelompok erythropoietin, rekombinan eryhtropoietin (1000 IU/kg) diberikan secara intraperitoneal sebanyak tiga kali selama 1 minggu setelah ligasi dilepas. Hewan pada kelompok kontrol normal tidak mendapat manipulasi pembedahan dan dilanjutkan dengan pengambilan sampel sperma untuk analisis. Preparat dengan pengecatan Eosin disiapkan untuk menilai motilitas dan morfologi sel sperma dan diamati di bawah mikroskop cahaya.* ***Hasil:*** *Ligasi dari vas deferens menurunkan angka motilitas dan morfologi sperma secara signifikan. Melepaskan ligasi dari vas deferens tidak meningkatkan motilitas dan morfologi sperma. Pemberian eryhtropoietin 1000 IU/kg sebanyak 3 kali selama seminggu setelah dilakukan release ligasi tidak meningkatkan motilitas dan morfologi sperma.* ***Simpulan:*** *Pemberian erythropoietin setelah dilakukan release ligasi vas deferens tidak meningkatkan motilitas dan morfologi sperma tikus Wistar.*

Kata Kunci: *Vasektomi, reversal, eritropoietin, motilitas sperma, morfologi sperma.*

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INTRODUCTION

Vasectomy is a procedure that is commonly performed for birth control. Most of the men asked for the reversal procedure to restore their fertility. Advances in surgical techniques have produced excellent patency rates, with success depending on the time that has elapsed since the vasectomy was done. However, although the rate of patency ranged from 71-97%, there is a 26-72% possibility of persistent infertility. It is also mentioned that alterations in the epididymal epithelium's function may occur over time after vasectomy, causing poor sperm quality after reversal procedure.[1]

One of the important cause of male infertility lately leads to oxidative stress. Iwasaki and Gagnon found elevated levels of reactive oxygen species (ROS) in seminal fluid in approximately 40% of infertile men in their study.[1,2]

Erythropoietin (EPO) is a cytokine that is induced by hypoxia stimulates erythropoiesis through the promotion of cell proliferation and differentiation of erythroid precursors. Recent research suggests that EPO mRNA obtained in Sertoli cells and peritubular myoid cells.[2] In addition to its function in the maintenance of normal hematocrit levels, EPO receptor expressed in a number of tissues other than the kidney, including the central nervous system, cardiovascular system and genital organs of women and men. EPO has also been shown to be synthesized in the testes and plays a role in spermatogenesis. EPO is also found in seminal plasma and contributes to the process of spermatogenesis in humans.[3]

First study that describes the effects of erythropoietin or EPO against testicular damage, published by Dobayashi in 2005. Later in 2007, Yazihan on his research on the study of the five groups of mice, explaining that erythropoietin also has anti-apoptotic and anti-inflammatory on testicular torsion. Another research study also gives a positive result and support the importance of the effects of erythropoietin on ischemic damage testicular testis.[4,5] From the research data, explain that erythropoietin can reduce cell damage and apoptosis. Koseoglu concluded that erythropoietin function in maintaining the morphology of seminiferous tubules, lowering the percentage of necrotic tissue in the seminiferous tubules, and lowering the histological damage.[6]

Sperm are very sensitive to oxidative stress response and especially on the lipid peroxidation, due to the high content of polyunsaturated fatty acids in the plasma membrane. Fatty acids are an essential requirement in testicular germ cells to maintain sperm function.[7] ROS can also reduce defense enzymatic spermatozoa, increase the damage of oxidative stress on the membrane of the sperm, proteins, and DNA, in which oxidative stress can lead to damage perioxidative the plasma membrane of sperm which ultimately can lead to loss of sperm function.[8,9]

In a previous study, it was explained that the antioxidant effects of EPO can protect against oxidative damage by inhibiting lipid peroxidation and restore the cystosolic catalase and GPx activity in erythrocytes. Erythropoietin also increase free radical inhibiting activity in astrocytes, as well as having an important role as an antioxidant indirectly by stimulating other antioxidants as a defense mechanism and acting as scavenger itself.[9,10] EPO receptors in the testes found in Leydig cells and EPO also produced by the Sertoli cells. In 2009 research conducted by Tug, stated that the use of rhEPO against human sperm solution at a concentration of 1, 10, and 100 mIU/ml increase the number of sperm motility and sperm progressive motility rate after a 4 hour incubation period compared with the control.[3]

OBJECTIVE

Determined the effect of erythropoietin supplementation on sperm motility and morphology in Wistar rat after the release of the vas deferens' ligation.

MATERIAL & METHODS

Twenty four male Wistar rats (weighing 150-200 g, age 10-12 weeks) used in this experimental study were obtained from animal laboratory of Veterinary Medicine, Airlangga University, Surabaya. Animals were housed in standard laboratory conditions with a temperature of 25 ± 2°C with a normal photoperiod (12 h light/12 h

dark). All treatments in these experimental animals have received an ethical clearance from Faculty of Veterinary Medicine, Airlangga University. Animals were randomly divided into 4 groups, with each group consisting of 6 rats. The normal control group, the vasectomy group, the vas deferens ligation release group and the group with the addition of eryhtropoietin after releasing the ligation.

All of the surgical procedures were performed in sterile conditions. All of the animals were given prophylactic antibiotics using ceftriaxone 20 mg/kg 30 minutes before surgery. After induction of anesthesia with rat cocktail combination (intraperitoneal injection of 60 mg/kg-Xylazine 7.5 mg/kg-Acepromazine 1mg/kg with 0.3 ml/100gr dose), the abdomen was shaved and swabbed with Betadine. A 1-cm midline incision was made in the lower abdomen, and bilateral vasa deferentia were retrieved and exposed. Using 4.0 Silk suture (Ethicon Inc., a Johnson & Johnson Co., Somerville), the vas deferens was serially ligated, and the procedure was repeated on the other side. The incision were closed using the same suture and the ligation left to remained until 7 weeks. After 7 weeks the ligation were released by reopening the wound and the ligation of the vas deferens were cut using scissor. There was no surgical manipulation performed on the normal control group, only orchidectomy was performed to collect the sperm. Group therapy with erythropoietin receive equal treatment as the positive control group, with the addition of recombinant eryhtropoietyin 1000 IU/kg (Epodion 2000IU/0.5 ml injection solution; Daewoong Infion®, Pasuruan, Indonesia) administered intraperitoneally 3 times for a week after releasing the ligation.

Both of cauda epididymis were slashed to let the spermatozoa out. Cauda epididymis then placed in a petri dish that already contains 1 ml physiological saline and cut into small pieces and allowed 1-2 minutes to provide an opportunity for spermatozoa to came out of the epididymis and spread. For motility examination is done by placing a drop of cement on top of the glass object and add one drop of physiological saline solution, then mixed until homogeneous. Examination were performed under a light microscope with 400x magnification. For Individuals motion of the spermatozoa rated as follows: 0 = if no moving spermatozoa, 1 = slow moving of spermatozoa, 2 = moderate movement, 3 = quick movement of sperm, 4 = very rapid movement of spermatozoa.

For morphology/viability inspection was performed by making preparations of negrosin eosin staining with the composition: 1). Solution A: consisting of negrosin sol. (20 g negrosin Aqua ad.100 ml), stirred and heated, 2). Solution B: Stock buffer or mixture of 20 ml of solution a and 80 ml of solution b (solution a: 21 628 g Na2HPO4-2H20 ad.500 ml Aqua solution b: 22 254 g KH2PO4 ad.500 ml Aqua), 3). Solution C: Stock glucose sol 43.3g glucose ad.500 ml Aqua. Then mix the following ingredients with heating: Solution A 150 ml, Eosin Yellow 5 gram, Solution B 30 ml, Solution C 20 ml, Aqua ad. 300 ml. To determine the percentage of live spermatozoa using the formula: The percentage of spermatozoa (%) = Number of live spermatozoa/ number of live and dead spermatozoa x 100%. Live spermatozoa will not be stained by the dye eosin. Spermatozoa that have died will be red-purplish due to destruction of the cell plasma membrane of spermatozoa. Only the live spermatozoa who were in normal shape were counted. A pathologist will calculate the amount of sperm motility and morphology in all preparations with a covert method (single-blind).

The sperm motility and morphology percentage are an interval data, with the presentation of the mean ± SD. One way Anova is used when the data distribution is normal, and Kruskall Wallis is used when the data distribution is not normal. The hypothesis is determined based on the significant value gained. Post Hoc Test LSD was used, if the significance value <0.05. All using the statistical analysis software SPSS 20 for windows with a value of $p<0.05$ indicated a statistically significant differences.

RESULTS

Shapiro-Wilk test was used and showed that there were normal distribution of the morphology and motility of sperm cells in each group with $p>0.05$. Therefore, One Way ANOVA was used for data analysis. There was significant difference of sperm cell motility and morphology in each group based on the data analysis with $p<0.05$. Thus, the analysis was followed by Post Hoc LSD test to determine differences in each group.

Based on the post hoc analysis, there were significant differences in sperm morphology in the control group compared with the vasectomy group ($p<0.05$). This study showed that the morphology of the sperm cells decreased after vasectomy. The

ligation release did not affect sperm morphology significantly. Furthermore, the administration of EPO after releasing the ligation could increase the viability/morphology of sperm cell compared to the release ligation only group, however this was not statistically significant (p>0.05).

Based on the post hoc analysis, there was a significant difference in the motility of sperm cells in the control group compared with the vasectomy group (p<0.05). The percentage individual movement of sperm cells was very low after vasectomy. However, the ligation release did not affect the individual movement of sperm cells significantly. Descriptively, the individual movement percentage of sperm cells in this group were fewer in number than the vasectomy group, although this was not statistically significant (p>0.05). In this study, it could be seen that the administration of EPO after releasing the ligation had a better percentage of the motility than the vasectomy group and the ligation release group, although it was not statistically significant (p>0.05).

Table 1. Comparison of sperm cell morphology in each group.

Group	n	Mean ± SD (%)	p value
Negative control	6	68.33 ± 11.69	0.00*
Vasectomy	6	11.67 ± 10.80	
Vasectomy + ligation release	6	10.00 ± 8.94	
Vasectomy + ligation release + EPO	6	20.00 ± 17.81	

Table 2. Post Hoc LSD analysis of sperm cell morphology comparison on each group.

Group comparison	Mean Difference	IK 95%		p value
		Lower limit	Upper limit	
Control vs Vasectomy	56.67	41.31	72.02	0.00*
Control vs Vasectomy + Release Ligation	58.33	42.98	73.69	0.00*
Control vs Vasectomy + Release Ligation + EPO	50.83	35.47	66.20	0.00*
Vasectomy vs Vasectomy + Release Ligation	1.67	-13.69	17.02	0.82
Vasectomy vs Vasectomy + Release Ligation + EPO	-5.83	-21.20	9.53	0.44
Vasectomy + Release Ligation vs Vasectomy + Release Ligation + EPO	-7.50	-22.86	7.86	0.32

*: statistically significant

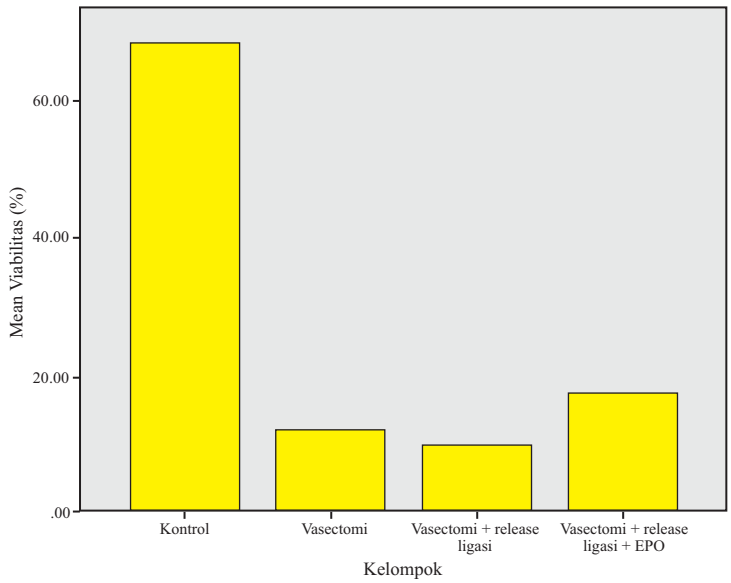

Chart 1. Comparison of sperm morphology in each group.

Table 3. Comparison of sperm cell motility in each group.

Group	n	Mean ± SD (%)	p value
Negative control	6	68.83 ± 11.69	0.00*
Vasectomy	6	11.67 ± 8.16	
Vasectomy + ligation release	6	8.33 ± 7.53	
Vasectomy + ligation release + EPO	6	15.83 ± 10.21	

Table 4. Post Hoc LSD analysis of sperm cell motility comparison on each group.

Group Comparison	Mean Difference	IK 95%		p value
		Lower limit	Upper limit	
Control Vs Vasectomy	56.67	45.18	68.16	0.00*
Control Vs Vasectomy + Release Ligation	60.00	48.51	71.49	0.00*
Control Vs Vasectomy + Release Ligation + EPO	52.50	41.01	63.99	0.00*
Vasectomy Vs Vasectomy + Release Ligation	3.33	-8.16	14.82	0.55
Vasectomy Vs Vasectomy + Release Ligation + EPO	-4.17	-15.66	7.32	0.46
Vasectomy+ Release Ligation Vs Vasectomy + Release Ligation + EPO	-7.50	-18.99	3.99	0.19

*: statistically significant

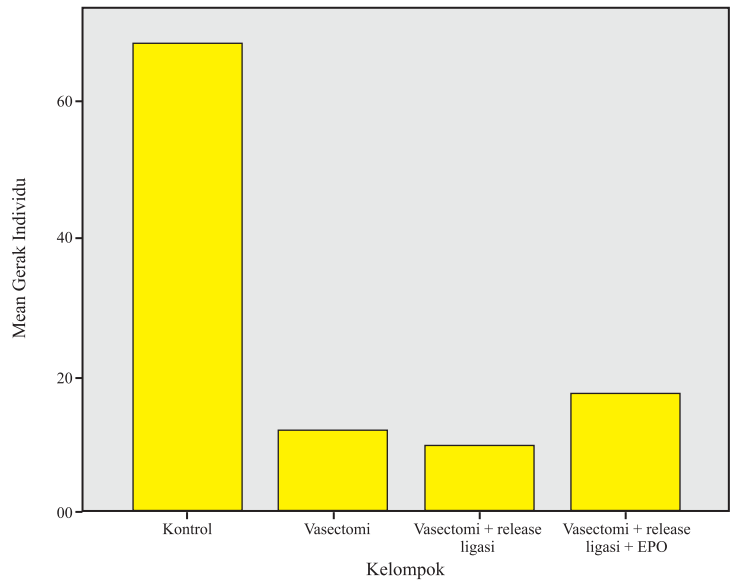

Chart 2. Comparison of sperm cell motility in each group.

DISCUSSION

Ligation of the vas deferens which is analogous to vasectomy will cause obstruction of the vas deferens. This will lead to an increase in hydrostatic pressure within the lumen of seminiferous tubules resulting a buildup of sperm excess and then triggers a local inflammatory response due to the increasing number of leukocytes in the sperm. The increased pressure in the testis, epididymis and vas deferens cause expansion of the epididymis and vas deferens. Vasectomy will cause immunological reactions with the formation of antisperm antibodies due to the exposed sperm were phagocytized by phagocytes and the epididymis epithelium and stimulate the immune system. Vasectomy also trigger an inflammatory reaction characterized by the release of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8 subsequently will increase the activity of ROS (Reactive Oxygen Species). The ligation release will amplify the formation of reactive oxygen species (ROS). This is due to the concentration of leukocytes that remains high as a result of an injury that occurred when the ligation

was done and chronic inflammatory processes, in addition to the concentration of leukocytes and IL-6 that were higher in seminal fluid will continue to increase the production of ROS. This increasing of ROS activity will induce the oxidative stress in the seminiferous tubules.

The administration of EPO is expected to prevent oxidative stress by its function as anti-oxidants that will increase ROS-Scavenger comparison. Besides its function as an anti-apoptosis, EPO binds to EPO receptors in the testes (ie in Leydig and Sertoli cells), thus activates the signaling of Janus Kinase 2 (JAK2) and will activate the signal transducer and activator of transcription (STAT) and Phosphatydilinositol-3 Kinase (PI3K), so that the apoptosis process can be inhibited. EPO also functions as an anti-inflammatory, where in there was an inflammation, then the EPO receptor activates signaling of Janus Kinase 2 (JAK2) and activates p38 MAP kinase, a protein that will inhibit pro-inflammatory mediator such as TNF-α and IL-6 so that the inflammatory process can be prevented. By administering EPO, the inflammation and apoptosis process occur in the case of vasectomy reversal can be inhibited, so that the amount of ROS can be reduced to prevent the higher oxidative stress level that can disrupt spermatogenesis process, and resulting an improvement of sperm quality.

In this study, there were significant differences in sperm cells' morphology and motility in the control group compared with the group that performed vasectomy ($p<0.05$). After ligation of the vas deferens the percentage of individual motion and normal morphology of sperm cells is very low. Supporting the research conducted by Mahabadi et al., 2013, that the sperm motility was significantly decreased in the vasectomy group compared to the control group ($p<0.05$) and the number of immotile sperm vasectomy group increased significantly compared with the sham operation group.[11]

These results indicate that vasectomy on rat causing premature failure of spermatogenesis and reduced sperm motility. In the study conducted by Mahabadi et al., found that there was a significant increase in weight of the epididymis in groups of mice performed a vasectomy as a result of the obstruction caused by vasectomy. Amann et al., reported that the epididymis has a crucial role to sperm maturation by providing a specific environment that is secreted from the epithelium that serves as a place to gain the sperm motility functions. According to Caflisch et Dubose, acid-base balance of epididymal fluid has a significant role to the sperm maturation and motility. Ph levels in situ of the epididymis changes significantly at weeks 4 and 8 after bilateral vasectomy in rats. This is a result of the morphological changes of the epididymis.[11] In this study can also be seen that when ligation release was done did not affect the movement of individual sperm cells and normal sperm morphology significantly. But descriptively the percentage of individual sperm movement and morphology are fewer in number than the vasectomy group, although this was not statistically significant ($p>0.05$). This was probably due to when vasectomy reversal was performed, a process of continuous inflammation will occurs, especially in the area of the anastomosis. This leads to the concentration of leukocytes and IL-6 in the seminal plasma remain high as a result of the chronic inflammation process of the vasectomy, will become higher than before, resulting a higher ROS activity in the emergence of oxidative stress in the tubule seminiferous.[12,13] Nandipati et al., found an increased ROS in the seminal fluid of patients undergoing vasectomy reversal. It is entirely possible that the increased production of ROS inhibit sperm motility by two different mechanisms. First, sperm's flexibility and tail motion will decreased due to ROS-induced peroxidation of the sperm membrane. Sperm membrane contains a large amounts of unsaturated fatty acids thus will be vulnerable to this type of damage. Second, ROS can cause a direct damage the mitochondria of the sperm, thus reducing the availability of energy and inhibit motility. Additionally, Flickinger et al., found that an increase in macrophages in pathological conditions such as epididymitis and post-vasectomy.[14]

In this study, the administration of EPO after vasectomy and ligation release did not have a significant effect ($p>0.05$) on the individual sperm movement and normal morphology of sperm cells, but descriptively it can be seen that the group of mice that received EPO has a better percentage of individual sperm movement and normal morphology than the vasectomy group and the ligation release group. Tug stated that total motility, progressive and non-motile sperm count to be significantly improved. This result depends on the dose of EPO given where at a dose of 1, 10 and 100mIU/ml give a different effect.[3]

Foresta et al., reported that the administration of EPO intravenously at a dose of 60IU/kg improve the production of testosterone, these findings show that epo work directly with the

function of Leydig cells.[15] The function of EPO in spermatogenesis have been described, but it is not known whether the spermatozoa mature also require EPO to maintain normal physiological. Erythropoetin has anti-apoptotic effects that are useful in ischemic disease such as damage secondary to necrosis of tissue hypoxia induced by vasectomy. EPO production is controlled at the transcriptional level, and hypoxia is the only physiological regulator of gene expression of erythropoietin. Erythropoietin produced mainly by the kidneys and a small portion of the heart, but all of the cells basically have the ability to transcribe the EPO gene in hypoxic conditions. It can be said that the EPO could create tissues with bad perfusion more resistant to hypoxia. Unlike most other cells in the body, sperm cells tend to survive hypoxic conditions but these mechanisms can not be explained with certainty. The activities of the EPO in supporting the hypoxic conditions of the epididymis sperm cells, which not only plays a role in biosintesis of steroid hormones and spermatogenesis but also supports the sperm cells to remain alive in hypoxic conditions. Collares et al., conducted a study in 2012 provided supplementation of rhEPO subcutaneously 3 times a week for 5 weeks on rabbits, the results were no significant difference to the rabbit sperm motility and morphology.[15] In a previous study, Tug et al., showed that supplementing the medium used for sperm preparation technique with EPO causes an increased motility in humans. Instead, Temma et al., detect EPO in human seminal plasma and found no correlation between plasma levels of EPO in semen and sperm concentration, morphology, the number of leukocytes or cytoplasmic droplets. Until now there is only a few research that discusses the EPO supplementation to the sperm characteristics and the underlying mechanism. Some research suggests that the effects depend on the dose of the EPO administration. Limitations of this study is because of the time of EPO supplementaton is quite short where as only given 3 times a week while previous studies conducted by Flickinger says that changes in the structure of the epididymis happens after vasectomy remained until four months after vasectomy reversal. Flickinger also noted that the action of vasectomy reversal does not repair damage that occurs in the testes, but can prevent further damage as a result of a vasectomy. In this study the dose given was 1000IU/kg intraperitoneally 3 times for a week. It was possible that this was led to why EPO administration was not statistically significant, therefore it is necessary to conduct further research to assess the effectiveness of the EPO.

CONCLUSION

Giving eryhtropoietin intraperitoneally three times weekly for 1 week after releasing the ligation of vas deferens did not improve the sperm motility and morphology.

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