EFFECT OF ERYTHROPOIETIN ADMINISTRATION ON THE AMOUNT OF SPERMATOGONIUM, SERTOLI CELL, AND LEYDIG CELL ON RATS TESTIS (WISTAR STRAIN)AFTER VAS DEFERENS LIGATION RELEASED

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ABSTRACT

Objective: *To determine the effect of Erythropoietin (EPO) on the number of spermatogonia, Sertoli cells, and Leydig cells in white rats wistar strain testis after the release of ligation vas deferens.* ***Material & Methods:*** *Twenty-four Wistar strain rats were grouped into 4 groups. The control group only performed an orchiectomy for testicular examination, ligation group vas deferens only, group performed release ligation of vas deferens, and group performed release ligation of vas deferens and given EPO injection with dose of 1000 iu/kg BW intraperitoneally for 1 week (3x/week). Observation of spermatogonium, Sertoli cells and Leydig cells by counting the amount on the 5 cross sections of the seminiferous tubules using a 400x light magnification microscope with Haematoxylin Eosin staining.* ***Results:*** *Ligation of vas deferens can significantly decreased the number of spermatogonia and Sertoli cells ($p<0.05$). In Leydig cells there was no significant difference in numbers after ligation of vas deferens ($p>0.05$). Release of vas deferens ligation turned out to be no significant amount difference in spermatogonia, Sertoli cells, and Leydig cells with ligation of vas deferens group. Similarly, the treatment of ligation vas deferens release and an EPO injection for 1 week was also no significant difference in number compared to the ligation release group of vas deferens.* ***Conclusion:*** *The number of Sertoli cells, Leydig cells, and spermatogonia in the ligation release group of vas deferens and given EPO for 1 week hadthe same number with the ligation release group vas deferens.*

Keywords*:Vasectomy reversal, reactive oxygen species, erythropoietin, spermatogonium, Sertoli cell, Leydig cell.*

ABSTRAK

Tujuan: *Mengetahui pengaruh pemberian Eritropoietin (EPO) terhadap jumlah spermatogonium, sel Sertoli, dan sel Leydig pada testis tikus putih strain Wistar setelah dilakukan release ligasi vas deferens.* ***Bahan & Cara:*** *Dua puluh empat tikus strain Wistar dikelompokkan dalam 4 kelompok. Kelompok kontrol hanya dilakukan tindakan orkidektomi untuk pengamatan testis, kelompok ligasi vas deferens saja, kelompok dilakukan release ligase vas deferens, dan kelompok yang dilakukan release ligasi vas deferens dan diberikan injeksi EPO dengan dosis sebesar 1000 iu/kgBB secara intraperitonial selama 1 minggu (3x/minggu). Pengamatan spermatogonium, sel Sertoli dan sel Leydig dengan cara menghitung banyaknya pada 5 penampang sayatan melintang dari tubulus seminiferus dengan menggunakan mikroskop cahaya perbesaran 400x dengan pewarnaan Haematoxylin Eosin.* ***Hasil:*** *Tindakan ligasi vas deferens secara signifikan dapat menurunkan jumlah spermatogonium dan sel Sertoli ($p<0.05$). Pada sel Leydig tidak ada perbedaan jumlah yang signifikan setelah dilakukan ligasi vas deferens ($p>0.05$). Release ligasi vas deferens ternyata tidak ada perbedaan jumlah yang signifikan pada spermatogonium, sel Sertoli, dan sel Leydig dengan kelompok ligasi vas deferens. Sama halnya dengan perlakuan release ligasi vas deferens dan diberikan injeksi EPO selama 1 minggu juga tidak ada perbedaan jumlah yang signifikan dibandingkan dengan kelompok release ligasi vas deferens. Simpulan: Jumlah sel Sertoli, sel Leydig, dan spermatogonium pada kelompok release ligasi vas deferens dan diberikan EPO selama 1 minggu sama besar dengan kelompok release ligasi vas deferens.*

Kata kunci: *Vasectomy reversal,reactive oxygen species, eritropoietin, spermatogonium, sel Sertoli, sel Leydig.*

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INTRODUCTION

Vasectomy is the most popular male contraception used in the world that 40-60 million people do so.[1] After 10 years, about 2% of men who have done vasectomy, choose to do a reversal vasectomy because of the desire to have more children. The majority of men who have done reversal vasectomy have reduced quality of semen fluid and sometimes require additional reproduction techniques in order to achieve a fertilization.[1,2] Men who have done microsurgical reversal vasectomy, patency rate and the success of pregnancy about 97% and 76%. For intervals of 3-8 years after vasectomy about 88% and 53%, 9-14 years about 79% and 44%, and over 15 years patency and success rates in pregnancy were about 71% and 30%.[3,4] However, possibly 26-72% to remain infertile. This may be due to partial obstruction of the vas deferens, epididymal dysfunction or obstruction, antisperm antibodies, or infertility of the woman.[5] Vasectomy will lead to high hydrostatic pressures due to obstruction, excessive sperm accumulation and stimulates local inflammatory reaction processes, characterized by increased leukocytes in sperm and abnormalities of sperm in seminiferous tubules, which play a role in enhancing Reactive Oxygen Species (ROS).[6-8]

When treated with release ligation of the vas deferens, the concentrations of leukocytes and IL-6 in seminiferous tubules that remain stable due to chronic inflammatory processes of vasectomy are multiplied more than ever before, resulting in increased ROS activity as well as higher oxidative stress in the seminiferous tubules.[9] Morphologically and histopathologically, after vasectomy, there is a decrease in the number of Sertoli, spermatogonium, and spermatid cells and an increase in apoptosis of the spermatogenesis process.[10] In Lewis mice reversal vasectomy performed, only 60% improvement of spermatogenesis count was calculated using Testicular Biopsy Score Count (TBSC) at 3-4 months after vasovasostomy. While on the morphological structure there was no significant difference between vasectomy treatment and after reversal vasectomy.[11] According to Gupta et al., the structure of testicular morphology after reversal vasectomy performed improvement after 2nd year in humans, and 3rd month in rat testes.[11,12]

Erythropoietin (EPO) is the most important growth factor that has anti-apoptosis, anti-inflammatory, and antioxidant function by inhibiting Reactive Oxygen Species (ROS).[13] Inside the testes, EPO receptors are found in Sertoli cells and Leydig cells.[14] In the Yazihan et al., study, it was found that in the testes of the mice by the torsio testis then administered EPO at a dose of 1000 IU/kg BW, the morphological improvement of the testicular structure and improvement of the germ cells.[13] Several other studies also explain that EPO can affect the process of steroidogenesis in rat testis by Leydig cells that stimulating testosterone production, and in human intravenous administration of EPO can increase testosterone production in renal failure.[13]

EPO doses typically used in humans range from 150-300 IU/kg BW depending on comorbidity such as renal failure, anemia, chronic infection, or cancerous conditions. How to administer EPO 2-3 times per week provides efficacy and tolerance results, as well as good benefits if given over a long period of time, other than that gradual dosing can prevent side effects from EPO itself.[15] In the study of Koseglu et al., stated that EPO treatment leads to an increase in vascularization among the semineferous tubules and this may indicate the vasoproliferative and neoangiogenic ability of EPO.[16] While Hamed et al., suggests that, the treatment of erythropoietin may be used as an alternative in addition to corrective surgical action and as a new therapeutic approach in the case of testicular torque to correct the harmful effects of ischemic reperfusion.[17] Several studies on the effects of EPO administration on testicular torsion in rats, with doses of 1000 IU or 3000 IU/kg BW, have greatly benefited the improvement of spermatogenesis, spermatogonium, Sertoli, and decreased apoptosis versus those not given EPO.[13,14,16,17] This is the underlying cause, if the administration of EPO can provide benefits by increasing the number of spermatogonium, Sertoli cells, and Leydig cells after a reversal vasectomy.

OBJECTIVE

To determine the effect of erythropoietin on the number of spermatogonia, Sertoli cells, and Leydig cells in white rats wistar strain testis after the release of ligation vas deferens

MATERIAL & METHOD

This research was an experimental research with post-test only control group design. The sample used in this study was 24 white rats (Rattus Norvegicus) Wistar strain (age 10-12 weeks, body

weight 150-200 gram) obtained from breeding laboratory at Faculty of Veterinary Medicine, Universitas Airlangga Surabaya. Experimental animal treated in standardized laboratory with temperature 25 ± 2°C and normal photoperiod (12 h light/12 h dark). All treatments in experimental animals have been certified from the ethical qualifications of the Faculty of Veterinary Medicine of Universitas Airlangga. The samples in the study were grouped into 4 groups randomly, each group consisting of 6 mice. The control group, the group which treated vas deferens ligation was administered for 7 weeks, the group with the treatment of ligation vas deferens release, and the group with the release treatment of vas deferens ligation then given EPO injection for 1 week (3x/week).

All surgical procedures performed with sterile. All animals were given prophylaxis antibiotic ceftriaxone 20 mg/kg 30 minutes before surgery and continued every 12 hours in the first 24 hours. The animals were tried anesthetized using a combination of ketamine 60 mg/kg-xylazine 7.5 mg/kg-acepromazine 1 mg/kg with a dose of 0.3 ml/100gr intraperitoneally. No surgical action in the control group, only an orchidectomy for testicular examination. In the lacation of the vas deferens, midline incision in the lower abdomen to the testis and funikulus spemratikus, then identification of vas deferens and ligation on both sides with silk 3-0 (Ethicon Inc., Johnson & Johnson Co., Somerville) then the wound was covered using the same thread and the ligation was maintained for up to 7 weeks. In the ligation release group, after 7 weeks of ligation release by opening the stitches on both vas deferens. The same was done in the ligation release group given EPO injection with a dose of 1000 iu/kgBB intraperitonally for 1 week (3x/week). Orchidectomy was performed by means of the rats positioned on the back, stitching of the abdominal skin was opened until the right left testis and funiculus were visible, then clamped on 2 adjacent places and cut between them.

The observation of spermatogonia was done by counting the number of spermatogonia on the cross sectional section of the seminiferous tubule using a 400x light magnification microscope with Haematoxylin Eosin staining. Location of spermatogonium is above the basal lamina. If we see spermatogonium under miscroscope, it has an oval-shaped and it has dark or pale colour. Spermatogonium containing solid chromatin that associated with the nuclear membrane.[5]

The observation of Sertoli cells was slim and long cells with irregular borders, extending from the basement membrane to the seminiferous tubular lumen. The core of Sertoli cells was generally more elongated or elongated and contains a small amount of fine chromatin. The striking nucleoli defines it with spermatogenic cells lined up between and around sertoli cells. Observations were made by counting the number of Sertoli cells at 5 cross sections of the seminiferous tubules with 400x enlargement and Haematoxylin Eosin staining.[5]

Leydig cell observations were performed by counting the number of Leydig cells on 5 cross-section of seminiferous tubules with 400x enlargement and Haematoxylin Eosin staining. The oval-shaped Leydig cell was located between the seminiferous tubules, with the eosinophilic cytoplasm, rich in fine endoplasmic reticulum and mitochondria with tubular cristae, which was a character for steroid-producing cells. The examination and counting of the cells studied in each group was carried out by the researcher under the supervision of anatomical pathologist.[5]

From the results of counting on spermatogonia, Sertoli cells, and Leydig cells will be tested for normality and variance test. If the normal data distribution and data variance were the same, then the One Way Anova hypothesis test was used. If the data was not normally distributed or the variant remains uniform, then the alternative was selected Kruskall-Wallis test. Hypothesis was determined based on the value of significance obtained. If the value of significance $p<0.05$, then the next step was to do multiple comparison test or Post Hoc Test by Tamhane. This research was significant if the value of $p<0.05$. All technical data processing was analyzed using computerized statistical product and service solution 20 for windows (SPSS 20).[18]

RESULTS

Based on statistical test by using Shapiro-Wilk test, it can be seen that the distribution of Sertoli cells in each group was normally distributed with $p>0.05$. Therefore, the data were analyzed using One Way ANOVA test. Based on the result of data analysis, there was a significant difference of Sertoli cell count in each group with $p<0.05$. Based on that analysis continued with Post Hoc test to know the difference of each group.

In this study, it was found that the variant of the data was not homogeneous with $p<0.05$ so that

Table 1. Comparison of the number of Sertoli cells in each group.

Group	n	Mean ± SD	p-value
Control	6	14.83 ± 0.85	0.00*
Ligation of vas deferens	6	4.40 ± 1.10	
Ligation release of vas deferens	6	4.07 ± 1.64	
Ligation release + EPO	6	5.4 ± 3.05	

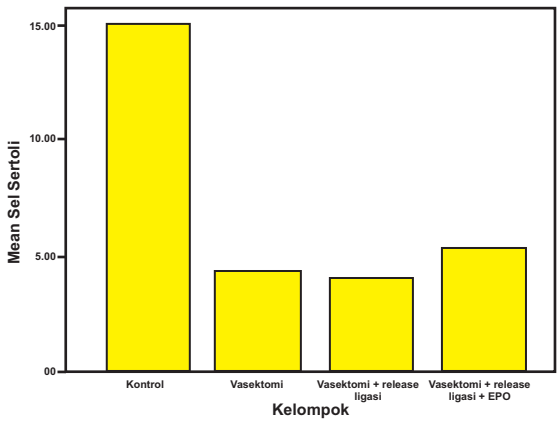
Table 2. Tamhane Post Hoc analysis of Sertoli cell comparison in each group.

Comparison between groups	Mean Difference	IK 95%		p-value
		Lower Limit	Upper Limit	
Control vs ligation of vas deferens	10.43	8.56	12.37	0.00*
Control vs ligation release	10.77	8.10	13.43	0.00*
Control vs ligation release + EPO	9.43	4.38	14.49	0.00*
Ligation vs ligation release	0.33	-2.38	3.05	1.00
Ligation vs ligation release + EPO	-1.00	-5.99	3.99	0.98
Ligation release vs ligation release + EPO	-1.33	-5.99	3.99	0.98

* Statistically significant

Table 3. Comparison of the number of Leydig cells in each group.

Group	n	Mean ± SD	p-value
Control	6	21.43 ± 5.07	0.06
Ligation of vas deferens	6	21.06 ± 5.59	
Ligation release of vas deferens	6	20.80 ± 2.36	
Ligation release + EPO	6	27.23 ± 3.75	



Graph 1. Comparison of Sertoli cells per group.

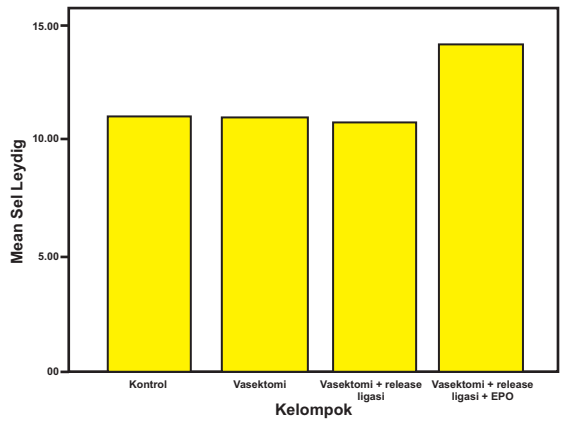
the Post Hoc test used in this study was the Tamhane Post Hoc test.

Based on the post hoc analysis, it can be seen that there was a significant difference in the number of Sertoli cells in the control group compared with the group receiving the vas deferens ligation ($p<0.05$), in which the number of Sertoli cells in the control group had more Sertoli cells than the group performed vas deferens ligation. Release ligation of vas deferens with release ligation and given EPO, there was no significant difference in the number of Sertoli cells. Although not statistically significant but descriptively the number of Sertoli cells on ligation release action and given EPO had higher Sertoli cell count compared with the group performed vas deferens ligation release.

The statistical test using Shapiro-Wilk test obtained Leydig cell distribution in each group with normal distribution with $p>0.05$. Therefore, the data were analyzed using One Way ANOVA test. Based on data analysis result, there was no significant difference of Leydig cell count in each group with $p>0.05$.

In this study, it can also be seen that although not statistically significant, but descriptively it can be seen that EPO administration in the ligation release group has higher Leydig cell count compared

to the group conducted by vas deferens ligation alone or with the group performed ligation release.

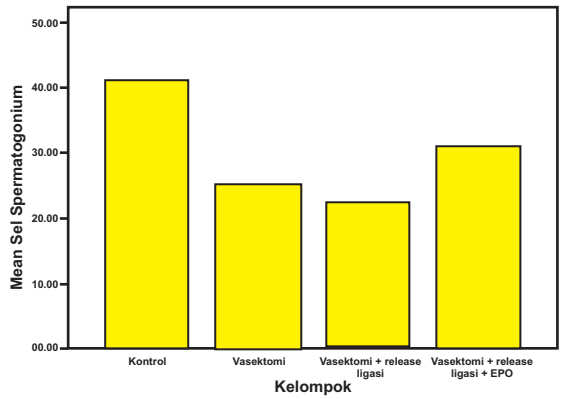


Graph 2. Comparison of the number of Leydig cells per group.

Based on statistical test by using Shapiro-Wilk test it can be seen that spermatogonium distribution in each group was normal distribution with $p>0.05$. Therefore, the data were analyzed using One Way ANOVA test. Based on result of data analysis found there were significant difference from spermatogonium amount in each group with p value <0.05. Based on that, analysis continued with Post Hoc test to know the difference of each group.

In this study, it was found that the variant of the data was not homogeneous with $p<0.05$ so that the Post Hoc test used in this study was the Tamhane Post Hoc test.

Based on the post hoc analysis, it can be seen that there was a significant difference in spermatogonium cell count in the control group compared with the group receiving ligation vas deferens ($p<0.05$). In this study, it was found that the number of spermatogonium cells in the control group was higher than ligated vas deferens group. In this study, statistically it can be seen that ligation release action did not affect spermatogonium cell count as well as on ligation release action and given EPO ($p<0.05$).



Graph 3. Comparison of the number of spermatogonium of each group.

Table 4. Comparison of number of spermatogonium in each group.

Group	n	Mean ± SD	p-Value
Control	6	41.1 ± 4.07	0.00*
Ligation of vas deferens	6	25.13 ± 3.69	
Ligation release of vas deferens	6	24.30 ± 11.88	
Ligation release + EPO	6	31.20 ± 6.63	

Table 5. Tamhane Post Hoc analysis comparison number of spermatogonium in each group.

Comparison between groups	Mean Difference	IK 95%		p-Value
		Lower Limit	Upper Limit	
Control vs ligation of vas deferens	15.97	8.63	23.31	0.00*
Control vs ligation release	16.80	-2.70	36.30	0.09
Control vs ligation release + EPO	9.90	-0.98	20.78	0.08
Ligation vs ligation release	0.83	-18.76	20.43	1.00
Ligation vs ligation release + EPO	-6.06	-16.87	4.74	0.42
Ligation release vs ligation release + EPO	-6.90	-26.26	12.47	0.82

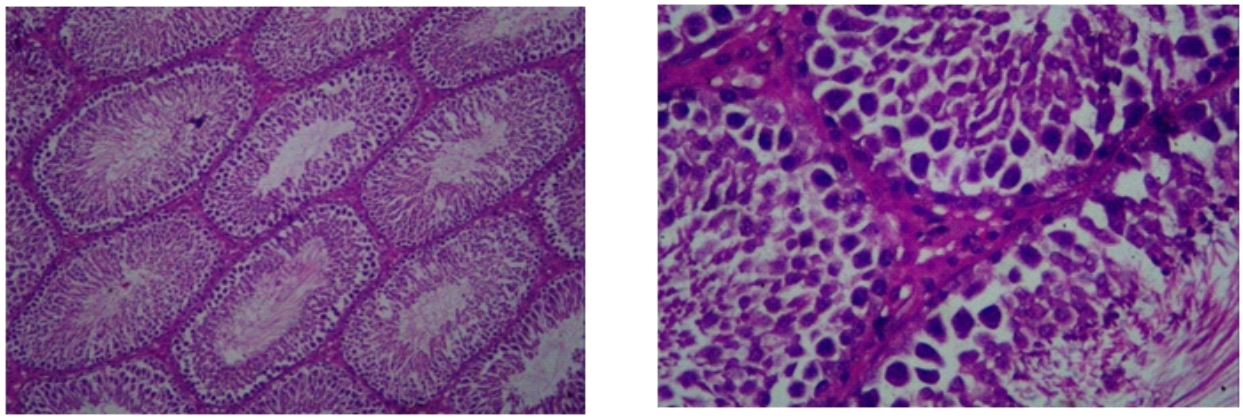


Figure 1. Microscopic cross-sectional view of seminiferous tubules in control group mice (Nikon eclipse e-100 microscope. Optilab Viewer 2.2. 400x).

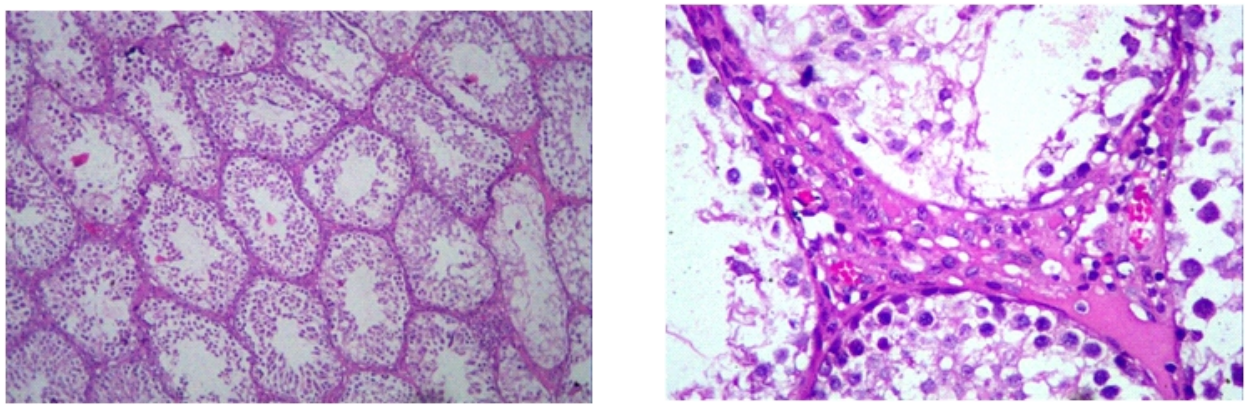


Figure 2. Microscopic cross-sectional view of seminiferous tubules in Group 7 weeks ligation vas deferens mice (Nikon eclipse e-100 microscope. Optilab Viewer 2.2. 400x).

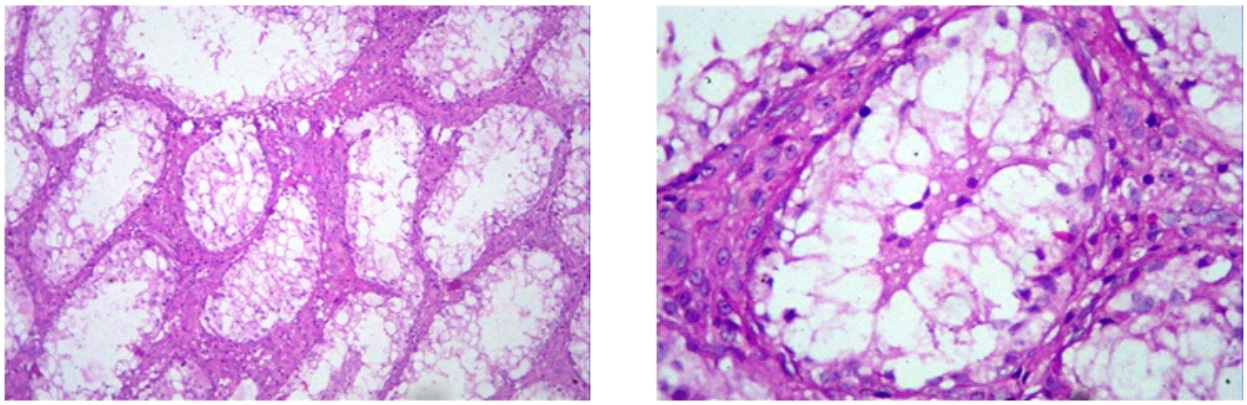


Figure 3. Microscopic cross-sectional view of seminiferous tubules in group mice, release ligation vas deferens (Nikon eclipse e-100 microscope. Optilab Viewer 2.2. 400x).

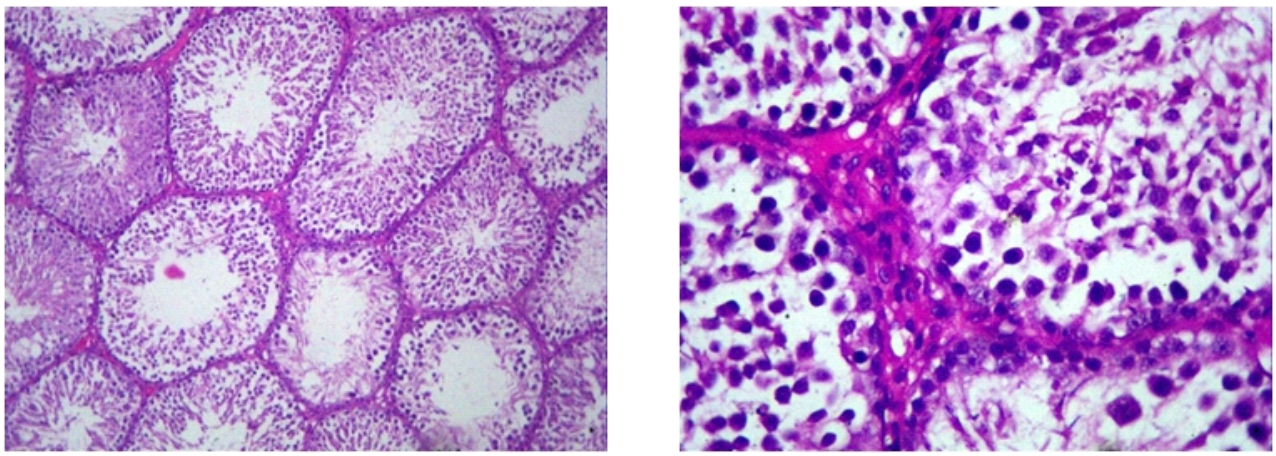


Figure 4. Microscopic cross-sectional view of seminiferous tubules in group mice, release ligation vas deferens + inj. EPO 1 week (3x/week) (Nikon eclipse e-100 microscope. Optilab Viewer 2.2. 400x).

Although not statistically significant, but descriptively it can be seen that ligation release action has lower spermatogonium levels than the ligation group of vas deferens alone, whereas ligation release action and given EPO have higher spermatogonium cell counts than the group performed ligation release of vas deferens.

DISCUSSION

Vasectomy reversal is an act of returning vasectomy by reconnecting the vas deferens either by vasovasostomy or vasoepididymostomy.[1,2] Figures patency and success of pregnancy after vasectomy reversal about 97% and 76%. For intervals of 3-8 years after vasectomy about 88% and 53%, 9-14 years about 79% and 44%, and over 15 years the patency rate and success in pregnancy were about 71% and 30%.[3,4] However, there is still a possibility of 26-72% to remain infertile.[5] The vasectomy action will result in high hydrostatic pressure due to obstruction, excessive sperm accumulation and will stimulate local inflammatory reaction processes, increase leukocytes and secrete mediators of proinflammatory cytokines TNF-α, IL-6, IL-8, NF-κB and TGF- β which will increase the activity of ROS (Reactive Oxygen Species) and iNOS (Inducible Nitric Oxygen Species).[19] In the testes, sensitive cells of hypoxia and expressing TNF-α and IL-6 receptors are spermatogonium.[9,20]

When treated with reversal vasectomy, leukocyte and IL-6 concentrations in the seminiferous tubules multiply more than ever before, increasing ROS activity is also higher and oxidative stress occurs in the seminiferous tubules and may lead to apoptosis in germ cell maturation, which eventually the spermatogenesis process will be disturbed and the number of spermatogonia and Sertoli cells will decrease.[9,20] While in the process of germinal cell maturation apoptosis, it will activate cascade caspase [3, 8, 9], so that will damage cell DNA in seminiferous tubule and also decrease spermatogonia number, Sertoli cell, and Leydig cell.[21,22]

In this study, ligation of vas deferens significantly decreased spermatogonia and Sertoli compared with the control group ($p<0.05$). While in Leydig cells, in this study there was no significant difference in numbers after ligation of vas deferens with a control group ($p>0.05$). This is consistent with a study by Baker et al., That Leydig cells were the interstitial compartment of the testes together with macrophages and lymphocytes, where Leydig cells were located between seminiferous tubules. So when there was a condition that results in a destruction of seminiferous tubules or degeneration of the seminiferous tubules, Leydig cells were the last cells to respond to damage because they are not included in the seminiferous tubules, and were the most resistant to the oxidative stress processes that take place in the testes.[23]

In the mice group performed release ligation of vas deferens and left for 1 week, there was no statistically significant difference of spermatogonium, Sertoli, and Leydig cells with mice group only ligation of vas deferens for 7 weeks ($p>0.05$) but descriptively the number of spermatogonia, Sertoli cells, and Leydig cells after ligation release of the vas deferens has a lower number than the ligation

group of the vas deferens alone. According to Flickinger et al., study, there was no significant difference between mice testicular morphological structure of vasectomy and vasovasostomy for 3 months, but spermatogenesis began to improve about 60% at 3-4 months after vasovasostomy was characterized by the number of young sperm that had not matured within each seminiferous tubule calculated using Testicular Biopsy Score Count (TBSC).[11]

In the treatment of this study, we analogized the vasectomy reversal with the release of vasectomy ligation, because the vasectomy we performed was also an artificial vasectomy by ligating the two vas deferens testis mice for 7 weeks with non absorbable sutures and not cutting either proximal from the vas deferens ligation nor distal from ligation vas deferens. In the lack of this research, we did not perform general vasectomy reversal surgery techniques such as vasovasostomy and vasoepididymostomy due to limited experience in performing such surgical techniques.

In groups of Erythropoietin (EPO) injected mice given 1 week after release of vas deferens ligation, there was no statistically significant difference in spermatogonium, Sertoli, and Leydig cells compared to the rats group by ligation of the vas deferens and the group performed release ligation ($p>0.05$). Descriptively, however, the administration of EPO groups for 1 week had higher spermatogonium, Sertoli, and Leydig cells than the ligation group of vas deferens and the ligation release group of vas deferens.

EPO has a function as an anti-inflammatory, anti-apoptosis, and antioxidants, and it is known that there were EPO receptors located in the testes, namely Sertoli cells and Leydig cells.[13,14] The EPO receptor will provide a transducer signal resulting in a bond between EPO and EPO receptor (r-EPO) enabling Janus Kinase 2 signalling (JAK2) that will activate various transducer and activator of transcription proteins (STAT), p38 MAP kinase, and Phosphatidylinositol-3 Kinase (PI3K). Signal transducer and activator of transcription (STAT) and Phosphatidylinositol-3 Kinase (PI3K) function as apoptotic inhibitors, while p38 MAP kinase acts as an inhibitor of pro-inflammatory proinflammatory mediators TNF-α, IL-6, and NF-κB.[24,25] The increase in ROS can be inhibited and damage to the spermatogenesis process can be prevented, which will directly increase the number of spermatogonia, Sertoli cells, and Leydig cells after reversal vasectomy.

The effectiveness of EPO after release ligation of vas deferens in this study was felt not to give the expected results, because the time constraint of giving EPO only given within 1 week only, whereas in previous research mentioned that start of improvement of spermatogenesis process after done reversal vasectomy in mouse in the 3rd month, and no previous research data does this kind of treatment so that the differences cannot be assessed.

CONCLUSION

The number of Sertoli cells, Leydig cells, and spermatogonia in the group performed ligation release vas deferens and administered EPO for 1 week as large as the group performed ligation vas deferens release. This means that the administration of erythropoietin for 1 week on the treatment of ligation release vas deferens did not increase the number of spermatogonia, Sertoli cells, and Leydig cells.

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