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Erythropoietin Effect on Testicular Germinal Epithelium Cells in Undescended Testis Mice Model

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ABSTRACT

Background: Undescended testis is an absence of testis in the scrotum, the incidence was 15 cases per 1000 from 1974 to 1996 in Europe. At Saiful Anwar Regional Hospital East Java, from January 2015 to July 2019 there were 60 boys diagnosed with undescended testis. A temperature rise of testis located in the abdominal triggers production of reactive oxygen species, causing impairment of the testicular epithelial germ cells and spermatogenesis, leading to many complications. Erythropoietin is a glycoprotein hormone that circulates in the body and has a positive effect on ischemic injury/gonadal reperfusion. **Objective:** To find out ROS involvement in undescended testis and efficacy of EPO as an additional therapy for undescended testis. **Methods:** This study is an experimental study with a post-test only control group design, using 18 male Wistar mice conditioned to be undescended testis for 7 days and underwent orchidopexy and some are given additional erythropoietin 1000iu/Kg 3 times a week. **Results:** Before and after the intervention, the mean body weight of mice did not experience a significant difference, meanwhile testicular volume showed a significant difference between the orchidopexy and EPO groups (p = 0.005 and 0.001). Johnsen 's score were found significant in the EPO group. Malone dialdehyde level in EPO and orchidopexy group showed significant difference *p* = 0.01 and 0.009 when compared to undescended testis group. **Conclusion:** There was the involvement of ROS in undescended testis and additional EPO improve impairment of germinal epithelial cells and spermatogenesis process due to undescended testis.

Keywords: undescended testis, erythropoietin, germinal epithelial, spermatogenesis, Malone dialdehyde.

1. BACKGROUND

The testis is part of the male reproductive system which function as both exocrine and endocrine. The main exocrine function of the testis is to assist in the formation of spermatozoa, so they are considered cytogenic glands. The main endocrine secretion from the testis is testosterone, which is produced by interstitial cells. Undescended testis is a congenital malformation that often occurs in male neonates present by the absence of testis in the scrotum, either unilateral or bilateral. It can cause temperature differences between the scrotum and the abdomen. Complications due to undescended testis include infertility, malignancy, testicular torsion and hernias.

The temperature in the abdominal cavity is ± 1°C higher than the temperature in the scrotum, the high temperature of the testis located in the abdomen can cause damage to the testicular epithelial germ cells. This thermal injury is mediated by reactive oxygen species and heat-shock proteins, one of which is malondialdehyde can damage testicular germ cells. Operative management to the testis is the recommended therapy for treating undescended testis. The main causes of testicular damage are the production of reactive oxygen species, increased intra-mitochondrial calcium concentrations and increased cellular apoptosis rates, and some drugs may be potentially effective in inhibiting reperfusion injury (1-3).

Erythropoietin is a glycoprotein hormone that circulates at about one-hundredth of the concentration of most other hormones in the body. Erythropoietin is produced in the kidneys, it circulates in the plasma and induces the production of red blood cells in the bone marrow, where it binds to erythroid progenitor cells, which are known to have many biological effects. Apart from the kidneys and liver, Erythropoietin messenger RNA (mRNA) is de-

tected in many organs including the testis, but the identity of the erythropoietin-producing cells in the testis is not defined (4, 5).

Erythropoietin has antiapoptotic and anti-inflammatory effects, positive effects against ischemic injury/gonadal reperfusion in previous research on testicular torsion. Based on previous researches and the lack of research on the use of Erythropoietin in undescended testis, this research investigated the effect of giving Erythropoietin on the undescended testis. This study is a laboratory experimental study with a post-test only control group design, which aims to compare several treatment groups.

2. OBJECTIVE

This study aimed to find out reactive oxygen species involvement in undescended testis and the efficacy of EPO as an additional therapy for undescended testis for better improvement of impaired germinal epithelial cell and spermatogenesis process.

3. MATERIAL AND METHODS

Subject

This study is a laboratory experimental study with a post-test only control group design, using 18 male Wistar mice. The mice were then divided into four different groups (control, undescended testis, orchidopexy and EPO). Study inclusion criteria include healthy male mice, age 6 weeks, weighing 130-200 grams with normal testis on both sides.

Before intervention: The mice were acclimatized in the laboratory for 1 week before the onset of the experiment, kept in a cage containing 4 mice, and fed well. Before the surgery: The mice received an intramuscular injection of cefazolin 100mg/kg for prophylactic and for anesthesia, ketamine 75mg/kg with xylazine 5mg/kg was injected intramuscularly.

The mice were then made into undescended testis condition by incising the lower part of the abdomen and locating the testis from the scrotum into the abdomen and fixed it with 4.0 silk. The abdomen was then closed using 2 layered sutures using catgut 4.0 catgut and 4.0 silk.

Score	Characteristics
1	Tubular sclerosis, no seminiferous epithel cell
2	Only Sertoli cell, no germ cell
3	Only spermatogonia
4	No spermatids, arrest of spermatogenesis at the primary spermatocyte stage
5	Many spermatocytes, but no spermatids
6	No late spermatids, arrest of spermatogenesis at the spermatid stage
7	Many early spermatids, but no late spermatids
8	Few late spermatids.
9	Disorganized tubular epithelium with many late spermatids
10	Full Spermatogenesis

Table 1. Johnsen score, a scoring system used to observed spermatogenesis in the testicular tissue of rats (6) Spandidos Publications. All rights reserved.

The testis was kept in the abdomen for 7 days, after 7 days some mice were taken their blood and terminated, while the other mice underwent orchidopexy and left for 7 days, during observation some mice that underwent orchidopexy received 1000iu/kg dose of erythropoietin for 3 times a week subcutaneously. After the observation finished all their blood was taken and the mice were terminated. The testis was taken to the histopathology laboratory and made into a specimen then hematoxylin and eosin staining was conducted. The evaluation of germinal epithelial cell and spermatogenesis was made using the Johnsen score using 400x magnification under the microscope.

Procedure and ethical consideration

The ethics for this study was approved by the Ethical Committee of Animal Care and Use Universitas Brawijaya.

Measures

Study variables include measurement of body weight using scale and testis weight. Malone dialdehyde was examined using *Thiobarbituric acid reactive substance* (TBARs) using serum obtained from the orbital vessels. While histopathology analysis was made using Johnsen Score, using microscope with 400x magnification.

Statistical analysis

A descriptive analysis was used to identify samples' characteristics. T-tests were calculated to determine significant differences in means. All statistical analyses were performed using SPSS Version 23.

4. RESULTS

Characteristic distribution of mice wight in this experiment ranges from 120 grams to 150 grams age 6 weeks old. While the normal testicular weight of mice ranges from 0.92 gram to 0.78 gram. The decline of testis weight due to intervention ranges from 0.41 to 0.38 in the 3 intervention groups. Comparison between body weight and testicular weight of mice in Figure 1. showed declining in testicular volume in all four groups. Whereas in mice body weight, the change of body weight was not as significant compared to testis weight.

Mean body weight before and after the intervention, in four different groups before intervention was 120.5; 134;150; and 145.3 grams for control, undescended testis, orchidopexy and EPO group respectively, while in post intervention within the same group mean body weight were 130.5; 137; 152.4; and 146.8 grams respectively. There was no significant difference in body weight before and after intervention among groups ($p > 0.05$). The mean testicular weight of mice before and after the intervention was measured before and after mice underwent intervention.

Pre-intervention group testicular weights were 0.918; 0.522; 0.764; and 0.782 grams respectively within control, undescended testis, orchidopexy and EPO group. In the post-intervention groups within the control, undescended testis, orchidopexy and EPO group, the testicular weight were 0.895; 0.405; 0.448; and 0.469 grams respectively. In Orchidopexy and EPO group there were statistically significant $p = 0.005$ and 0.001 respectively.

Mean Johnsen score in control, undescended testis, orchidopexy and EPO group were 9.6; 3; 5.86 and 7.2 respectively. When Johnsen score compared within groups, all of them were significant statistically $p < 0.05$, except when Johnsen score was compared among undescended testis and orchidopexy group there were no significant with $p = 0.065$ for each group respectively.

Malone dialdehyde, MDA was measured using TBARs and the mean MDA level within control, undescended testis, orchidopexy and EPO group were 444.11; 520; 526 and 423 ng/mL respectively and it was significant when compared among EPO and Orchidopexy and Undescended group. MDA was found higher in the undescended testis group and orchidopexy group compared to the EPO group shown in Table 4. Statistically when undescended testis, orchidopexy and EPO group were compared against each other EPO group statistically showed significance against the undescended testis and orchidopexy group, $p = 0.01$ and 0.009 respectively.

Figure 1. (A) is described as the Johnsen score 10, where the entire spermatogenesis process is obtained fully. The yellow arrow shows the spermatogonia phase; The black arrow shows the primary spermatocyte phase; The green arrow shows the intermediate spermatocyte phase; The blue arrows show the spermatid phase; and the red arrows indicate the phases of the spermatozoa. In Figure B. the yellow arrow shows the spermatogonia phase, where in Johnsen score 3, only the spermatogonia phase is found. Figure C shows a Johnsen score of 5 presenting, spermatocytes without any spermatids or spermatozoa shown by the black arrows. Meanwhile, Figure D shows a score of 7, where at a score of 7 there are no spermatozoa but spermatids are obtained, which is indicated by a blue arrow (6)

Pre and Post Operative Group	Body weight (gram) Mean + SD	p	Testis weight (gram) Mean + SD	*p*
Control	120.5 ± 9.19	0.063	0.918 ± 0.066	0.080
	130.5 ± 7.77		0.895 ± 0.703	
Undescended Testis	134.0 ± 1.41	0.205	0.522 ± 0.226	0.272
	137.0 ± 2.82		0.405 ± 0.151	
Orchidopexy	150.0 ± 16.96	0.590	0.764 ± 0.156	0.005*
	152.4 ± 12.96		0.448 ± 0.263	
EPO	145.3 ± 19.02	0.747	0.782 ± 0.068	0.001*
	146.8 ± 20.78		0.469 ± 0.142	

Table 2. Paired T-test for body weight and testicular weight before and after mice underwent intervention. * = p < 0.05 (Considered as statistically significant)

Groups	Johnsen Score Mean + SD	p Control	Undescended Testis	Orchidopexy	EPO
Control	9.6 ± 0.35	-	0.000*	0.000*	0.002*
Undescended Testis	3 ± 0.14	0.000*	-	0.065	0.000*
Orchidopexy	5.68 ± 0.97	0.000*	0.065	-	0.000*
EPO	7. 2 ± 0.37	0.002*	0.000*	0.000*	-

Table 3. Histopathology Analysis made using Johnsen Score from 4 different group. * = p < 0.05 (statistically significant)

Groups	MDA Level (ng/mL) Mean + SD	p Control	Undescended Testis	Orchidopexy	EPO
Control	444.11 ± 7.07	-	0.13	0.089	0.44
Undescended Testis	520 ± 55.26	0.13	-	0.085	0.01*
Orchidopexy	526.89 ± 49.25	0.089	0.085	-	0.009*
EPO	423.56 ± 30.93	0.44	0.01*	0.009*	-

Table 4. Malone dialdehyde analysis made using TBARs from 4 different group

5. DISCUSSION

The change in body weight was not statistically significant, due to proper pre and post-operative care and nutrition to minimize complications such as infection and sepsis which can cause rats to experience decreased appetite and systemic impairment causing a change of body weight. The Wistar rat itself is a strong experimental mouse that is widely used as an experimental animal in various studies (7).

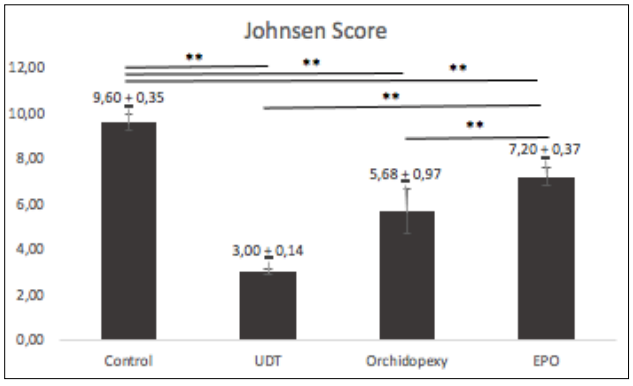

Figure 1. Johnsen score between four groups. ** = p < 0.01

Comparison between Orchidopexy and EPO group there were statistically significant $p = 0.005$ and 0.001 respectively. An increase in the scrotal temperature gradient by only 1-2 ° C is sufficient to suppress spermatogenesis in an experiment. In humans, varicocele and undescended testis can cause male fertility disorders associated with abnormal spermatogenesis. Undescended testis develops in an increase in temperature around the stomach or inguinal tract. This thermal injury is mediated by reactive oxygen species and certain heat-shock proteins, which damage germ cells as well as Sertoli cells, orchiopexy even if performed before 1 year of age does not prevent postnatal morphological changes in the testicles including the size of the testes. The severity of undescended testis contributing to fertility depends on whether one or both testicles fail to descend completely, their position along the inguinal canal, the cause of the incomplete descent and the length of time before

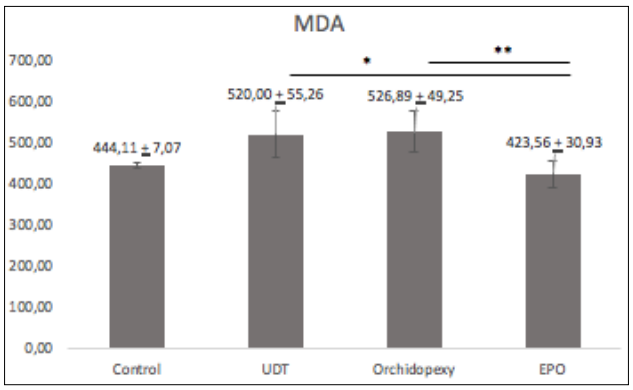

Figure 2. Malone dialdehyde level between four groups. * = p < 0.05, ** = p< 0.01

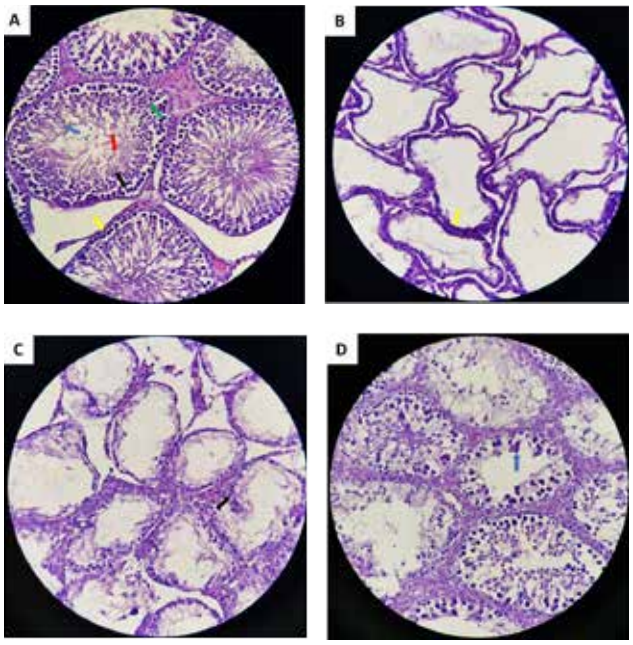

Figure 3. Histopathology images of haematoxylin and eosin staining of rat testicular tissue, 400x magnification in four different group (A) Control, (B) Undescended Testis, (C) Orchidopexy and (D) Orchidopexy with additional EPO.

surgical intervention to reposition the affected testis. into the scrotum (8-10).

In a study conducted by Tseng et.al in 2017, it was stated that orchidopexy in UDT patients could accelerate testicular growth. Forty-five boys out of 134 children (33.4%) of whom underwent orchiopexy before the age of one produced a significantly higher Growth Percentage Ratio (2.02 ± 0.40) than GPR in the second group (1 <age ≤ 2 years, 1.25 ± 0.13, p = 0.004) and third (age > 2 years, 1.24 ± 0.14 p = 0.008) age group.

However, it is said that orchidopexy itself caused a decrease in testicular volume. The most worrying complication of inguinal orchidopexy is testicular atrophy, which occurs when the testicular vessels are damaged. According to a recent systematic review of this topic, the pooled atrophy rates were 1.83% for primary orchidopexy (range 0–4%), 28.1% for single-stage Fowler Stephen (range 22–67%), and 8.2% for two stages Fowler Stephen (range 0-12%)(2, 11).

Histopathology examination using a microscope with 400x magnification was used to assess impairment of epithelial germ cell and spermatogenesis and it was scored by Johnsen score. In This study mean Johnsen score in control, undescended testis, orchidopexy and EPO group were 9.6; 3; 5.86 and 7.2 respectively. When Johnsen score compared within groups, all of them were significant statistically $p < 0.05$, except when Johnsen score was compared among undescended testis and orchidopexy group there were no significant with $p = 0.065$ for each group respectively. The difference in the Johnsen score of each group indicated a morphological change that occurred due to undescended testis when compared to the control group. In the undescended testis group, there was a Johnsen score of 3 which showed only spermatogonia, which should have been found in the normal process of spermatogenesis in all phases of sperm development starting from spermatogonia, primary spermatocytes, secondary spermatocytes, initial spermatids, intermediate spermatocytes, advanced spermatids and spermatozoon as in the control group with Johnsen score 9 to 10. In Johnsen's score of 7, no spermatozoa but many spermatids were found (12, 13). Meanwhile in score 5 and 6 shows many spermatocytes, but no spermatids and no late spermatids, arrest of spermatogenesis at the spermatid stage respectively.

According to Shahat et al. 2020 the heat stressor occurs when the temperature exceeds the physiological range and passes the compensatory ability. Most mammalian testicles are at a temperature of 4-5 ° C cooler than body temperature. Heat stressor either systemically or locally in the testis affects all types of testicular cells, although germ cells are more sensitive than Sertoli or Leydig cells. The increased testicular temperature has a detrimental effect on sperm motility, morphology, and fertility, with effects related to the duration of the increase in temperature. The main consequence of heat stressors on the testis is damage to germ cells due to apoptosis, with pachytene spermatocytes, spermatids, and epididymal sperm being most susceptible. In addition to the involvement of various transcription factors, heat stressors trigger the production of reactive oxygen species (ROS), leading to germ cell apoptosis and DNA damage. The effects of heat stressors on the testes can be divided into three categories: testicular cells, sperm quality, and the ability of sperm to fertilize the oocyte and support its development (14) overwhelming compensatory mechanisms. Most mammalian testes are 4–5 °C cooler than core body temperature. Systemic HS or localized warming of the testes affects all types of testicular cells, although germ cells are more sensitive than either Sertoli or Leydig cells. The increased testicular temperature has deleterious effects on sperm motility, morphology and fertility, with effects related to extent and duration of the increase. The major consequence of HS on testis is the destruction of germ cells by apoptosis, with pachytene spermatocytes, spermatids and epididymal sperm being the most susceptible. In addition to the involvement of various transcription factors, HS triggers the production of reactive oxygen species (ROS.

Meanwhile, research by Rashed et al. in 2013 regarding ischemic injury/reperfusion in testicular torsion said that erythropoietin showed efficacy in reducing changes after ischemia/reperfusion when compared with a similar control group that did not receive erythropoietin (15) group II (sham operation. Malone dialdehyde, MDA was measured using TBARs and the mean MDA level within control, undescended testis, orchidopexy and EPO group were 444.11; 520; 526 and 423 ng/mL respectively and it was significant when compared among EPO, orchidopexy and Undescended group. MDA was found higher in the undescended testis group and orchidopexy group compared to the EPO group shown in Table 4. Statistically when undescended testis, orchidopexy and EPO group were compared against each other EPO group statistically showed significance against the undescended testis and orchidopexy group, p = 0.01 and 0.009 respectively.

It is widely accepted that any type of stress can cause a response in the cells of a living organism, called free radical, quantitative changes in intracellular calcium, reduced energy metabolism, which ultimately lead to the formation of pathology in the cardiovascular, digestive and immunological system, neurodegenerative processes, and mental disorders. The key parameter in changing cellular metabolism is the activation of lipid peroxidation (LPO). Under normal conditions, this process is at normal levels and is necessary for cells to function normally. The intensity of LPO depends on the appearance of the active form of oxygen and its relationship with the degree of functionality of the antioxidant system in cells (16). Malondialdehyde can interact with protein and nucleic acid molecules, causing the formation of intermolecular bonds, where imalondialdehyde can cause structural changes in various receptors, ion channels, cytoskeleton, proteins, enzymes, and nucleic acids. In addition, malondialdehyde can also change the activity of the antioxidant system in cells and the enzymes involved in it. The cell's antioxidant system develops an effective response to maintain cell homeostasis (16).

Similar to the study by Ünsal et al., It was said that the MDA levels in the undescended testis group were significantly higher than the healthy control group (p = 0.014). The study investigated the AMI level of the two groups, there was a statistically significant difference between the two groups (p = 0.008). Because ROS is highly reactive, ROS can attack DNA, lipids, and proteins and change the structure of their biomolecules, oxidative stress occurs as a result of degeneration of biomolecular structures. for the determination of oxidative stress, the level of the modified molecule or the product. occurs as a result of measured oxidative damage. MDA concentrations are widely used as biomarkers for the determination of lipid peroxidation (17) which is known for its antioxidant activity, on a testicular torsion/detorsion model in animals and to help understand how to prevent both ischemic and reperfusion injuries after testicular torsion and detorsion (Material and methods: Six groups of rats (n=7 in each group).

According to Li et al. , MDA reached its highest level on day 6, coinciding with the time of testicular weight loss from day 5 and a large wave of down-regulation of genes on day 7 during which massive apoptosis occurred as previously reported (18). There were also limitations in this study due to the number of samples cannot meet the number of samples required, due to the difficulty of obtaining samples that match the inclusion criteria and with the minimum number of samples obtained there are also dead samples and damaged testes during the study so that lost samples cannot be replaced due to limited time and materials.

6. CONCLUSION

Based on the research that has been done, it can be concluded that undescended testis can cause an increase in ROS where the increase in ROS in undescended testis is marked by an increase in MDA levels. Damage to germinal epithelial cells and disruption of spermatogenesis process caused by undescended can be repaired by orchidopexy and the addition of EPO as an additional therapy for undescended testis did not provide significant differences in germinal epithelial repair and spermatogenesis process. Due to limited data and research available, further research is needed to support this research.

- **Patient Consent Form**: None.
- **Author's Contribution**: A.A and B.B.P gave substantial contributions to the conception or design of the work in acquisition, analysis, or interpretation of data for the work. K.W.A contribute in histological analysis and V.W contribute in statistical analysis. K.P.S had a part in article preparing for drafting or revising it critically for important intellectual content. A.A, B.B.P and K.P.S gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
- **Conflicts of interest**: There are no conflicts of interest.
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