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EFFECT OF 2-METHOXYETHANOL ON SPERM COUNT, LEUCOCYTE AND EPIDIDYMAL EPITHELIAL CELL STRUCTURE IN RATS (*Rattus norvegicus*)

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ABSTRAK

2-Methoxyethanol (2-ME) adalah salah satu agen beracun dari gugus ester phatalate yang berpotensi mempengaruhi struktur spermatozoa. Racun ini cair, mudah menguap, hampir tidak berbau, dan masuk dalam tubuh melalui kulit, terhirup atau makanan. Pada mamalia 2-ME dapat mempengaruhi struktur dan fungsi testis, dan terlibat dalam pengaturan apoptosis spermatosit primer dan lesi testis. Percobaan menggunakan 30 tikus untuk menganalisa kualitas sperma dan epididymis sel epithel tes apoptosis. Dosis 2-ME 200 mg/kg, frekuensi bervariasi dan eksposur durasi 2-ME adalah (1, 3 dan 6 kali/minggu). Hewan-hewan kelompok yang diobati (n = 30) dibagi menjadi dua kelompok, terdiri dari kelompok perlakuan dan kelompok kontrol. Setiap kelompok dibagi menjadi 3 sub kelompok perlakuan (n = 5). Tikus-tikus subkelompok perlakuan diberi injeksi subkutan 0,2 ml 200 mg/kg/2-ME dengan pemaparan frekuensi bervariasi, tetapi subkelompok kontrol disuntik dengan larutan garam fisiologis frekuensi yang sama. Hasil menunjukkan jumlah spermatozoa pada kelompok kontrol dan kelompok perlakuan tidak berbeda nyata (p > 0,05). Jumlah leukosit pada semua kelompok perlakuan meningkat (p < 0,05). Beberapa sel epitel epididymis dari kelompok perlakuan diwarnai dengan HE menunjukkan ungu (gelap) dan memiliki inti lebih kecil dari normal. Apoptosis terdeteksi pada semua kelompok perlakuan dengan DAB noda pada sel epitel epididymis dalam kelompok perlakuan menunjukkan coklat (gelap) dan inti menyusut berwarna hijau sitoplasma. Sitoplasma hijau terlihat pada sel epitel normal. Kesimpulan, paparan 2-ME sebanyak 200 mg/kg/hari meningkatkan jumlah leukosit dan sel-sel apoptosis pada epididymis.

ABSTRACT

2-Methoxyethanol (2-ME) is one of the toxic agent from phatalate ester groups that potentially affect the structure of spermatozoa. This poison is a liquid, volatile, almost odorless, and enters the body through the skin, inhaled or food. In mammals, 2-ME can affect the structure and function of the testes, and is involved in the regulation of apoptosis of primary spermatocytes and testicular lesions. This experiment using 30 mice was to analyze the quality of sperm and epididymis epithelial cell apoptosis test. 2-ME dose 200 mg/kg, frequency and exposure duration of 2-ME was varied (1, 3 and 6 times/week). The animals in treated group (n = 30) were divided into two groups, consisting of treatment and control groups. Each group was divided into three sub-groups of treatment (n = 5). Subgroup treated rats were given subcutaneous injections of 0.2 ml 200 mg/kg/2-ME with exposure frequency varies, while control subgroups were injected with physiological saline solution in the same frequency. Results showed the number of spermatozoa in control and treatment groups were not significantly different (p > 0.05). The number of leukocytes increased in all treatment groups (p <0.05). Some of the epididymis epithelial cells of the treated group stained with HE showed purple (dark) color and had a nucleus smaller than normal. Apoptosis was detected in all treatment groups with the DAB staining of the epididymis epithelial cells in the treatment group, which appeared brown (dark) and showed shrinking core green cytoplasm. Green cytoplasm was seen in normal epithelial cells. In conclusion, exposure to 2-ME as much as 200 mg/kg/day increases the number of leukocytes and apoptotic cells in the epididymis.

Keywords : *2-methoxyethanol, spermatozoa, leukocyte, epididymal epithel, apoptosis*

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INTRODUCTION

Almost every purchase of food, the food must be wrapped in plastic or in a particular container. It is more practical, because we do not need to carry around a container. Buy meatballs, fried, fruit chunks, etc. wrapped in plastic. Vegetable soup in a fast food restaurant, do not need fragile ceramic bowl, enough to throw away Styrofoam bowl after use. Likewise if you want to enjoy hot noodles when camping or on the go, we need not take it bowl.

Packaging materials and containers are so familiar with our lives. However, it turns materials that store dangerous if its use is not appropriate. Plastic is used as one type of packing materials. There are relatively safer to use for food is Poly Ethylene which look clear, and Poly Propylene which is softer and slightly thicker, but

those that are dangerous if prolonged contact with food such as poly vinyl chloride (PVC) is usually used for wrapping candy, rice paper coatings and cover material because it is very thin and transparent. The types of plastics are high hazard varies from plastic material, the type of food that is wrapped, the duration of contact between the food in plastic, as well as food or room temperature storage. One of the chemical compound as the base material of the manufacture of plastics is a dangerous class of phatalate ester is 2-methoxy ethyl phthalate (DMEP). DMEP compounds that enter the body will be hydrolyzed to 2-methoxyethanol (2-ME), which subsequently became metabolized methoxyacetic acid (MAA). Both of these compounds (2-ME and MAA) can cause toxic damage to cells.

Damage and cell death can occur in both normal and abnormal circumstances. Cell damage in the normal state aims to regulation and control of cell activity. During the maturation process of spermatogenesis and spermatogenic cell death also occurs, to maintain the balance of the number of spermatogenic cells in the seminiferous tubules. Some researchers claim that, 2-ME causes damage to spermatogenic cells. MAA exposure (metabolite 2-ME) dose of 650 mg/kg.bw for 24 hours caused apoptosis of pachytene spermatocytes cells and Sertoli cells (Tirado et al., 2003). Dose of 200 mg/kg.bw for 3 weeks cause damage to spermatogenic cells, decrease in diameter and thick epithelium of the seminiferous tubules and decreased number of spermatocytes and spermatids may not find the existence of cells compared to controls (Hayati et al., 2004). 2-ME exposure in the short term (1 week) while causing damage to the testes, but in less than four weeks to recover (recovery) as the normal state (Hayati et al., 2005). The mechanism of cell death due to a pathological toxic materials is caused by the interaction between material toxic with the cell membrane. This situation increases calcium ions influx or intracellular Ca2 + increase, thus causing a decrease and loss of cell membrane integrity. This situation can cause damage and eventually cause cell death.

Exposure of phthalate ester plasticizer (a group of compounds with 2-ME) also cause toxic effects on male reproductive tract and the damage caused is irreversible (Corton and Lapinskas, 2004). Reproductive tract are damaged due to oxidative stress, which stimulates an increase in free radicals (ROS). High levels of ROS lead to damage to sperm membrane proteins (Hayati, 2007). In addition ROS also increase the concentration of lipid peroxidation, because of the hydrogen ions from MAA compound (metabolisis results of 2-ME) reacts with molecular oxygen present in the cell. This reaction will produce hydrogen peroxide. At the cell membrane, these hydrogen ions react with unsaturated fatty acids would then produce hydrogen peroxide and a new lipid radical. Decomposition of these unsaturated fatty acids to form fatty acid fragments with low molecular weight of which is malondialdehyde (MDA). MDA compound used as an indicator of lipid peroxidation in cell membranes which leads to damage to the structure of cell membranes (Kim et al., 2001). Thus, elevated levels of ROS and lipid peroxidation as toxic material is thought to cause inflammation or changes in the structure of epididymal epithelial cells, thus leading to a decrease in sperm quality. This study aims to determine the effect of toxic materials (2-ME) against 1) the number of spermatozoa epididymal, 2) the number of leukocytes epididymal, and 3) epithelial cell damage epididymal.

MATERIALS AND METHODS

This research uses experimental animals rats (*Rattus norvegicus*) male Wistar strain (age 12-14 weeks, weight 125-130 g). 2-ME dose used was a single dose 200mg/kg.bw. Variation 2-ME exposure frequencies used are 1, 3 and 6 times/week. Thirty rats divided into 2 groups: the treatment group and control group. Each group was subdivided into 3 subgroups (n = 5) with variation of frequency of exposure (1, 3 and 6 times/week). The control group were injected once daily with physiological solution with exposure frequency variations such as the treatment group. One day after treatment ended, the rats euthanasized with chloroform, the cauda epididymis were separated from the testis. Rat spermatozoa collected from epididymal cauda and sperm suspensions prepared with 4 ml of physiological solution. Spermatozoa suspension was examined under a light microscope 400x.

The number of spermatozoa was calculated using the method of Goyal et al. (2001). The suspension of spermatozoa in increments of 1 ml is placed into a glass hemocytometer, then covered with a glass lid. Rat spermatozoa amount calculated under light microscope at 400x magnification. How to calculate the spermatozoa are as follows. Sperm suspension was diluted with 4 ml (4x103 ml) physiological saline solution taken 10? L then placed into the room count (hemocytometer). The formation of air bubbles should be avoided at the close count room with glass cover. Spermatozoa counted is located in the center and edge of the chamber (upper right and left chambers) is located on the edge of the spermatozoa to the right and bottom chambers are not counted. The average number of spermatozoa (n) is obtained from the total sum of spermatozoa available in each chamber divided by 4. The length of each chamber of 1 mm and height 0.1 mm, so that the chamber volume equal to 0.1 mm 3 or

1.0 mm2 x 0.1 mm or 1.0 x 10-4 ml. Calculations using the equation the number of spermatozoa sperm cells/ml, ie the number of cells/ml = number of spermatozoa (n) x 104 x dilution factor.

The number of leukocytes in the suspension of spermatozoa was calculated referring to the Eggert-Kruse et al. (2001) that uses a hemocytometer. Spermatozoa suspension dropwise (2-3 drops) on a hemocytometer and diluted with Turk solution (3 ml glacial acetic acid, 1 ml of 1% gentian violet, and 100 ml aquades). Then covered with cover glass and observed under a light microscope and counted 400 x. How to leukocyte count is as follows. Leukocyte count is located in the center and the edge chamber (left top and left chambers) are leukocytes that are located in the booth to the right and bottom are not counted. The average number of leukocytes (n) is obtained from the total number of leukocytes that exist in each chamber divided by 4. Chamber volume equal to 0.1 mm 3 or 1.0 mm2 x 0.1 mm or 1.0 x 10-4 ml. Leukocyte counting using the equation the number of cells/ml = number of leukocytes (n) x 104 x dilution factor.

To observe apoptosis epididymis use APOP tag peroxidase in situ, apoptosis detection kit S7100-S7101. Epididymal tissue that has been separated from the testes were fixed with a solution paraformaldehyde for 1 day, then blocked in paraffin. Endogenous peroxidase deactivated by H2O2 at room temperature, then labeled using the In Situ Apoptosis Detection Kit (S7100-S7101). Furthermore, specimens were incubated with terminal deoxy-NTP and deoxynucleotidyl tansferase at room temperature for 60 minutes. Then it was washed 3 times in PBS, stained with diaminobenzidine (DAB) for 5 minutes at room temperature. The specimen was washed 3 times in distilled water, then counterstained with fast green for 10 minutes, then washed 3 times with distilled water. Preparations then dried and covered with glass cover and observed under a light microscope. Data on the number of spermatozoa and leukocytes that have been collected were analyzed using statistical tests (analysis of variance) and continued with the smallest real difference test with significance level of 5% ($p < 0.05$).

RESULTS

The number of spermatozoa contained in the control group is similar to 2-ME treatment group. The number of spermatozoa in the control group ranged x107 0.98 - 1.02 x107/ml suspension of spermatozoa whereas the treatment group after a 2-ME for 1, 3 and 6 times/week in a row x107 0.94, 0.99, x107; and 0.94 x107/ml suspension of spermatozoa (Table 1). Figures followed by letters are not the same in each column indicate significantly different ($p < 0.05$, $n = 5$). The number of spermatozoa in the control group and treatment about the same and were apparent after the data were analyzed using statistical tests. The test results showed that rats exposed to 2-ME dose of 200 mg/kg.bw every day for 1, 3 and 6 times/week, showed no significant difference in the number of spermatozoa ($p > 0.05$) compared to controls. Collection of spermatozoa contained in the lumen of the epididymis of control and treatment groups mentioned above can be seen in Figure 1.

Table 1. The number of spermatozoa and leukocytes epididymal mice with exposure frequency variation 2-ME

		2-ME Dose (mg/kg.bw/day)	Sperm count (10^7/mL)	Leucocyte count (10^6/mL)
Control	K_1	0	0.98 ± 0.05	0.48 ± 0.08
	K_2	0	1.06 ± 0.16	0.50 ± 0.10
	K_3	0	0.98 ± 0.16	0.48 ± 0.10
Treatment	P_1	1 x200	0.94 ± 0.04	1.26 ± 0.14^a
	P_2	3 x200	0.99 ± 0.09	3.37 ± 0.11^b
	P_3	6 x200	0.94 ± 0.16	4.27 ± 0.07^c

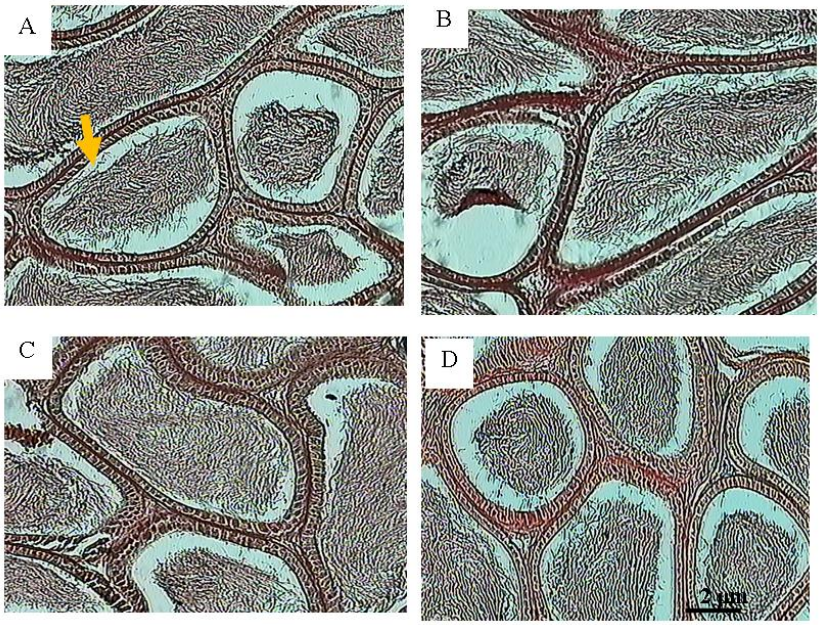

Figure 1. Cross section of the epididymis, was a collection of spermatozoa in the lumen of rat epididymis. A = control group, B, C and D = 2-ME treatment group a dose of 200 mg/kg/day for 1, 3, and 6 times/week. Arrow = a collection of spermatozoa. The density of spermatozoa in the lumen of the epididymis appear almost the same between the control and treatment groups

From Figure 1 can be seen that the collection of spermatozoa that have been separated from the epididymal lumen of the seminiferous tubules packed. Spermatozoa were exposed to 2-ME dose of 200 mg/kg.bw with a frequency of 1, 3 and 6 times/week in Figure 1.b, C and D did not differ by the number of spermatozoa in the control group (Fig. 1 A).

The number of leukocytes in the suspension of spermatozoa epididymal

Counting the number of leukocytes in the suspension of spermatozoa can be seen in Table 1. In the control group, the lumen of epididymal spermatozoa in addition to adults also found leukocytes. The presence of leukocytes is useful to balance the normal functioning of sperm cells. Tues leukocytes play a role in the immune system, presence of foreign cells are not recognized will phagocytosized or neutralized. However, in addition to leukocyte function in the immune system can also damage sperm cells, since leukocytes produce ROS. The number of leukocytes in the suspension of epididymal spermatozoa from the control group x106 between 0.48 - 0.50 x106/ml suspension of spermatozoa, whereas 2-ME treatment group increased significantly ($p < 0.05$) than controls ($p > 0.05$). Increasing the number of leukocytes was inalong with increasing exposure time 2-ME. Leukocytes can be found in the male reproductive tract, but is physiologically much leukocytes derived from the epididymis. According to WHO (1999), which led to the accumulation of leukocytes leukositospermia when its concentration is greater than 1x106 per ml of semen. The number of leukocytes in the control group is smaller than the standard set by the WHO, so that its presence does not affect function of spermatozoa.

Epididymal epithelial cell apoptosis

Rat epididymal cross section with HE staining can be seen in Figure 2. In cross section looks epididymal epithelial cells in the control group composed of basement membranes, cell nuclei purple and pale eosinophilic cytoplasm colored. Epididymal epithelial cell columnar-shaped, cilia-covered artificial and have a role in the transport of spermatozoa. Epididymis which had been exposed 2-ME 200 mg/kg, in addition there is the structure of epithelial cells of normal epithelial cells is also apparent with the shrinking core or smaller than normal epithelial cell nuclei. Cell nuclei had shrunk because of the chromatin core condensation after 2-ME exposure. Core chromatin is easily absorb the alkaline color (color haematoxylin) so that the cell nuclei appear dark purple in color whereas more eosinophilic cytoplasm. Similar picture is also seen in the other treatment groups, in addition to the normal epithelial

cells are also found some epithelial cells with nuclei of cells that picnotic. Structure of epithelial cell nuclei indicates that there has been damage and cell death (Figure 2. BD). By staining diaminobenzidine (DAB) and fast green as a counterstain, epididymal epithelial cells appear normal green as counterstain. These cells are found in the epididymis control group (Figure 3.a). Epithelial cells with damaged or apoptosis seen in all treatment groups (Figure 3. BD).

Epididymal epithelial cells were damaged or apoptotic nuclei have a smaller than normal cell nuclei, more brownish colored and green colored cytoplasm. Brownish color on the damaged epithelial cells derived from degraded DNA or DNA fragmentation. The end of the DNA fragments are bound dye DAB, causing brownish color, whereas intact DNA material that will remain green (counterstain with fast green) because the dye DAB can not bind to DNA intact. Some of the cells were undergoing apoptosis in the treated group very aesthetically.

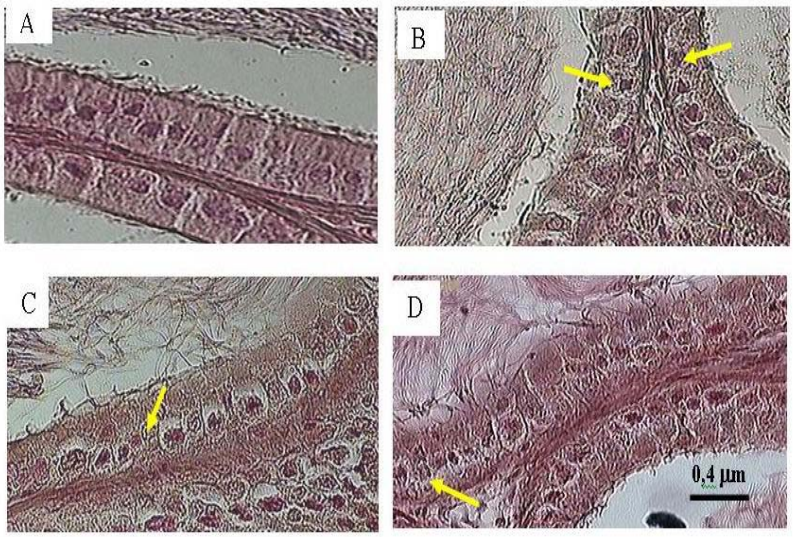

Figure 2. Rat epididymal cross section with HE staining. A = control group; B, C and D = 2-ME treatment group a dose of 200 mg/kg/day after exposure to 1, 3 and 6 times/week. Arrow = nucleus of epithelial cells damaged

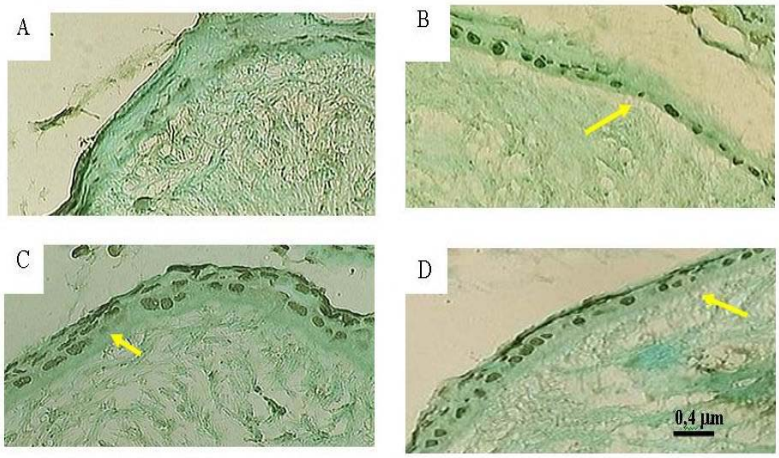

Figure 3. Cross sections stained with DAB rat epididymis. A = control group; B, C and D = 2-ME treatment group a dose of 200 mg/kg/day for 1, 3 and 6 times/week. The arrows = epithelial cell apoptosis, decreases cell nuclei due to chromatin condensation

DISCUSSION

Target cell of the compound 2-ME in rat testis seminiferous tubules were spermatogenic cells and somatic cells. Cell damage caused by 2-ME can inhibit the process of spermatogenesis, leading to the occurrence of olygospermia or azoospermia. Besides compound 2-ME also can damage other cells in the male reproductive tract. Toxic compounds that enter through passive transport across the cell membrane to the other reproductive tract.

Spermatogenic cell damage that occurs in the testes will appear in the epididymis after a time the process of spermatogenesis (54-56 days) plus the transport time spermatozoa leave the testis to the epididymis (12-21 days) (Schrader and Lemasters, 2002). But in this study, giving 2-ME dose of 200 mg/kg/day for over 1, 3, and 6 days did not affect the number of spermatozoa epididymal. This is because the dose and duration of toxic compounds in this study damaged spermatozoa at the molecular level, i.e. some of the proteins making up the membrane of spermatozoa is expressed after administration of 2-ME (Hayati, et al, 2007). So that the cellular level the influence of toxic compounds was not significant compared to the control group ($p > 0.05$).

Increasing the number of leukocytes in 2-ME treatment group compared to the control group caused by 2-ME as a toxic material which causes damage to the reproductive tract, especially the epididymis. The damage to the epididymal epithelial cells thought to facilitate transport of leukocytes from blood vessels into the lumen of the epididymis. In addition, leukocyte migration into the male reproductive tract can be through a thin layer of smooth muscle cells are damaged, inflammation or the presence of fibroblast cell activity (Hess, 2000). The presence of infection, inflammation and damage to the male reproductive tract will cause a condition called leukositospermia of the cement contained many leukocytes (> 1x106/mL) (WHO, 1999). Increasing the number of leukocytes, but not followed by an increase in the number of spermatozoa showed that leukocytes are the source of ROS in addition to abnormal spermatozoa in semen.

MAA compounds as a result of metabolism of 2-ME induces apoptosis in epithelial cells in the epididymis. According Tirado et al. (2003) and Barone et al. (2005), these compounds also cause apoptosis in somatic cells and spermatogenic cells. Apoptosis occurred because MAA activate pro-apoptotic protein caspase in the cytoplasm. Caspase proteins work by damaging the cytoskeleton proteins and core proteins such as DNA repair enzymes. Also activate caspase enzymes destructive enzymes such as DNase-repair during DNA synthesis in the nucleus. Activation of this enzyme leads to cell damage. In addition, caspase activation also results in impaired mitochondrial function. Caspase activation caused a blocking of Bcl-2 gene activity and stimulate the activation of Fas and Bax genes. Fas and Bax gene is a gene that causes cell death through increased activity of the p53 protein. Protein p53 is a protein that plays a role in the regulation of cell death (pro-death proteins). Increased expression of this protein leads to the destruction of DNA. DNA damage occurs because the structure of DNA is changed, damaged or lost chains to form DNA fragments (Moustafa et al., 2004). Damage to the changing structure and function of cells results in biochemical and morphological changes in cells and cause apoptosis (Chandrasekaran and Richburg, 2005).

Epididymal maturation of spermatozoa as a place, providing a variety of proteins for spermatozoa in the seminal needs. Protein is derived from the secretion of seminiferous tubules and epididymal epithelial cells. The existence of epididymal epithelial cell damage will affect the balance of micro-environment that can affect the function of epididymal spermatozoa cells (Hess, 2000). The presence of apoptosis in the epididymal epithelium easy transport of leukocytes into the lumen of the epididymis and cause inhibition of metabolism and secretion of steroid hormones, thus decreasing the function of spermatozoa (Jeyaraj et al., 2003; Tirado et al., 2004).

CONCLUSION

From the research results it can be concluded that administration of 2-ME 200 mg/kg per day for 1, 3 and 6 times/week do not affect the number of spermatozoa epididymal, but increasing the number of leukocytes epididymal and increases the incidence of epithelial cell damage epididymal.

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