SPERMATOZOA MOTILITY AND MORPHOLOGICAL RECOVERY PROCESS IN MICE (*Mus musculus*) AFTER THE INDUCTION OF 2-METHOXYETHANOL

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ABSTRACT

This study used 30 male BALB/C mice aged 8 - 9 weeks with bodyweight of 28 - 30 grams. These experimental animals were divided into 6 groups, each comprising 5 mice. Group K1 was given with 2-ME of 200 mg/kg intraperitoneally every day for three weeks. The groups P1, P2, P3 and P4 were given with 2-ME, also for three weeks, and the administration was subsequently ceased for 1, 2, 3, and 4 weeks, respectively. Group K0 (control) was injected with saline solution. Spermatozoa were taken from epididymal cauda. Observation was carried out for the motility of 100 spermatozoa with types A, B, C or D (WHO, 1992), as well as to normal and abnormal morphology in head and tail, and combination of head and tail. Data were tested using one-way variant analysis. Results showed that the administration of 2-ME every day for 3 weeks resulted in azoospermia, so that sperm motility and morphology was not observable. However, by providing recovery period from 1 to 4 weeks, sperm motility and morphology was found to recover into normal. There was a significant difference in sperm motility and morphology after treatment and recovery. As conclusions, 2-Methoxyethanol given to male mice for 3 weeks may result in azoospermia, and the effect of the length of cessation period of 2-ME administration increases the recovery of sperm morphology and motility.

***Keywords**: 2-methoxyethanol, sperm motility and morphology*

INTRODUCTION

2-Methoxyethanol (2-ME) or ethylene glycol monomethyl ether (EGME) is a colorless and volatile glycol ether compound. 2-ME is commonly used as plasticizer in producing plastics (Singh et al., 1972 ; Miller et al., 1982). Plastics are also commonly used in various human activities, for example, as material for home utensils, for packaging, toys, water pipes, and numerous health and medical equipments. Additionally, 2-ME is also used for organic solvent in producing industrial products, such as paint solvent, acetic cellulose, resin, accelerating the drying of varnish, nail polish, and wood polish, also for perfume fixative and photographic film production (Sax and Lewis, 1989). 2-ME and its metabolite in the body may damage cells and tissue. The exposure of the body to 2-ME may occur in workplace, in air, water, soil, and ground water (EPA, 1994; Kezic S, et al., 1997). The 2-ME enters the body through respiration, contaminated foods and drinks, and direct dermal contact (Anonymous, 1994; Dugard, et al., 1984). In the liver, 2-ME is oxidized into methoxyaldehyde (MALD), which is catalyzed with alcohol dehydrogenase. MALD is oxidized further to become methoxyacetate acid (MAA), which is catalyzed with aldehyde dehydrogenase. The metabolite compound is toxic (Moslen et. al., 1995) and teratogenic (Butterworth, et al., 1995; Davis, et al., 1997). This indicates that, in addition to its usefulness, it also produces negative effects on living beings.

The effects of 2-ME on human health and environment are dependent upon its amount, duration and frequency of exposure, as well as on physical condition. 2-ME may increase olygospermia and azoospermia in ship painters, and the substance is also toxicant for reproductive organs, particularly the testis. Emerged abnormalities are the reduction of testicular weight and accessory tissue, the atrophy of seminiferous tubule, the degeneration of pachytene spermatocyte, and the reduction of sperm cells, as well as the disturbance in Sertoli cells (Rumanta et al., 2001; Feuston et al., 1990). However, environmental 2-ME in normal amount is not hazardous for the environment itself (EPA, 1994).

Spermatogenetic cells damaged by MAA inhibit DNA synthesis in pachytene spermatocyte. These cells are the most active ones in synthesizing RNA, so that they are also sensitive towards MAA and subjected much to degeneration. 2-ME also disrupts lactate production in Sertoli cells. If this production is disrupted, the development of spermatocyte that requires lactate as energy substrate will also be disturbed (Gray et al., 1985). However, the regenerative capability of epithelial cells in seminiferous tubule is not damaged entirely by MAA (Valentine et al., 1998). There are spermatogenic cells in seminiferous tubule that are more resistant against MAA. These cells are spermatogonium A,

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which are due to their location in basal compartment, compared to two other types of spermatogenic cells residing the adluminal compartment, they are more protected by the barriers produced by Sertoli cells (Rumanta et al., 2001). The duration of 2-ME cessation leads to the gradual reduction of 2-ME concentration in the body, and finally it ends with total depletion of the substance. At the same time, the damaged or apoptotic spermatogenic cells are quickly absorbed by Sertoli cells. The existing spermatogonium remains actively proliferating, producing new spermatocyte and results in recovery process to produce new and healthy spermatozoa.

METHODS

A number of 30 male mice (*Mus musculus*) aged 8 - 9 weeks, weighed 28 - 30 grams, were grouped to receive six treatments. Each group consisted of 5 mice. Treatment was carried out with intraperitoneal 2-ME solution in each group. K0 was injected with saline every day for 3 weeks. K1 was injected with 0.1 ml 2-ME 200mg/Kg BW daily for 3 weeks. P1, P2, P3, and P4 were injected with 0.1 ml 2-ME 200mg/Kg BW daily for 3 weeks. Treatment was stopped 1, 2, 3, and 4 weeks for respective treatment groups, and those experimental animals were finally operated.

Spermatozoa Removal

Spermatozoa were taken from epididymal cauda (the last 1/3 of the epididymis), cleaned from the attaching lipids, and placed into a petri dish containing 1 ml saline. The spermatozoa were cut and suspended.

Spermatozoa Motility Observation

One drop of suspension was put onto concave object glass and observed under the microscope with magnification of 400 x. The observation was carried out onto 100 spermatozoa with five times replication in each mouse. Sperm motility was grouped into four categories (WHO, 1992), i.e., type A, spermatozoa move rapid progressively; B. spermatozoa move slow progressively; C. Spermatozoa move in place, and D, spermatozoa is immotile. Motility is regarded as normal if A $\geq$ 25% or A+B > 50%.

Observation of Spermatozoa Morphology

A drop of suspension was put onto an object glass, stained with 1% eosin and 10% nigrosin, and smeared. Morphological observation was conducted in 100 spermatozoa with 5 replication in each mouse with magnification of 400 x. The observation data were differentiated based on normal and abnormal morphology. The morphology was regarded as normal when the acrosome is curved like a hook, and the neck is straight with free-end single tail. Abnormal morphology was found when the head was smaller than normal, the neck was broken, the tail was branched or cut, etc.

Data Analysis

The data on type A spermatozoa motility and normal sperm morphology was tested using One Way Variance analysis.

RESULTS

The Effect of 2-ME on spermatozoa morphology

The results of observation using microscope with magnification of 400 x on the morphology of spermatozoa in control group (K0) are displayed in Table 2, while the motility of spermatozoa can be seen in Table 2, while treatment group K1 could not be observed due to azoospermia.

Table 1. The percentage of spermatozoa morphology in treatment group after the administration of 2-ME (K1) and in control group (K0)

Treatment	Abnormal morphology (%)			Normal morphology	Total
	H	T	H-E		
K0	8.7 ± 4.6	0.2 ± 0.2	0.4 ± 0.6	90.7 ± 5.2	100 ± 0
K1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

H: head, T: tail, H-E: head and tail

Table 2. The percentage of spermatozoa motility in various types in treatment group (K1) and control group (K0).

Treatment	Types of Spermatozoa Motility (%)				Total
	A	B	C	D	
K0	84.6 ± 6.4	9.9 ± 4.6	4.0 ± 1.5	1.8 ± 1.2	100 ± 0
K1	0 ± 0	0 0	0 ± 0	0 ± 0	0 ± 0

The effect of the duration of 2-ME cessation on the morphological and motility recovery process in spermatozoa. The results of observation using microscope in magnification of 400 x showed difference in morphological condition and the motility of spermatozoa in control group (K0) and in groups P1, P2, P3, and P4. Along with the period of cessation of 2-ME for 1, 2, 3, and 4 weeks, the presence of spermatozoa started to appear and the observation of normal morphology percentage was getting better. Moreover, morphological condition of spermatozoa in the last treatment group (P4) approximated that of control group. The percentage of spermatozoa count with normal morphology can be seen in Table 3.

Table 3. The percentage of spermatozoa morphology in control group (K0) and treatment groups (P)

Treatment	Abnormal morphology (%)			Normal morphology	Total
	Head	Tail	Head-Tail		
K0	8.72 ± 4.58	0.2 ± 0.24	0.4 ± 1.44	90.72 ± 5.21	100 ± 0
P1	46.44 ± 4.73	8.68 ± 5.28	4.12 ± 1.82	40.28 ± 2.28	100 ± 0
P2	44.16 ± 4.75	5 ± 3.22	1.68 ± 0.79	48.76 ± 1.11	100 ± 0
P3	34.88 ± 3.76	8.08 ± 2.98	3.2 ± 1.44	54.12 ± 4.74	100 ± 0
P4	29.72 ± 6.52	4.96 ± 2.72	1.88 ± 1.88	65.16 ± 1.89	100 ± 0

Table 3 shows that the percentage of spermatozoa with normal morphology is increasing, as shown from P1 (40.28%) to P4 (65.16%) that indicates an increase after 2-ME cessation for 4 weeks. Although the percentage of spermatozoa morphology is increasing (P4), the increase has not reached the percentage of normal morphology (K0 = 90.72%). This indicates that the duration of 2-ME cessation for 4 weeks was not sufficient to recover totally to normal condition. Based on data analysis, there was significant difference in the percentage of spermatozoa with normal morphology between treatment groups, except between P1 and P2, and P2 and P3, which showed no significant difference. In abnormal morphology, presenting as abnormal head, tail, and combination of head and tail, there was significant difference between treatment groups.

The results of spermatozoa motility in groups K0 and P1, P2, P3, P4 showed that after prolonged 2-ME cessation period for 1, 2, 3, and 4 weeks, the presence of spermatozoa and its motility started to become observable. The percentage of spermatozoa in various types of motility can be seen in Table 4 below.

Table 4. The percentage of various types spermatozoa motility in control (K0) and treatment groups (P)

Treatment	Types of Spermatozoa Motility (%)				Total
	A	B	C	D	
K0	84.6 ± 6.4	9.9 ± 4.6	4.0 ± 1.5	1.8 ± 1.2	100 ± 0
P1	0.5 ± 0.2	10.2 ± 5.7	8.4 ± 2.8	79.8 ± 8.1	100 ± 0
P2	4.9 ±2.5	17.6 ± 9.1	10.4 ± 4.9	67.5 ± 11.6	100 ± 0
P3	43.8 ± 2,0	31.8 ± 2.2	17.1 ± 1.4	7.1 ± 1.8	100 ± 0
P4	73.0 ± 1.0	10.8 ± 3.0	7.8 ± 2.7	9.0 ± 1.9	100 ± 0

Prolonged period after the cessation of 2-ME administration results in the increased percentage of motile spermatozoa, particularly in type A. Although group P4 (73%) showed the highest percentage of type A motility, as compared to other treatment groups, it was apparent that 2-ME cessation period for 4 weeks (P4) was not enough to recover the percentage of spermatozoa with type A motility to become similar to that of K0 (84.6%). This indicates that time needed to recover sperm motility to reach the percentage in group K0 was more than 4 weeks. Based on data analysis, it was found that there was significant difference in the percentage of type A spermatozoa motility in treatment groups and control.

DISCUSSION

2-ME induces damage in spermatogenetic process, as proved by the occurrence of azoospermia in treatment group K1, so that the morphology and motility of spermatozoa was not observable. This condition was different from that in control group (K0), which was not given with 2-ME. Azoospermia in K1 occurred due to damaged spermatogenic cells because 2-ME was absorbed by Sertoli cells in seminiferous tubule, resulting in the absence of spermatozoa in epididymis. After the administration of 2-ME was discontinued for 1, 2, 3, and 4 weeks, spermatozoa began to appear and gradually showed better motility and morphology, since during this time the recovery process started.

Spermatozoa were not found in K1. This indicated that those cells were degenerated due to the exposure to 2-ME. In the body, MAA metabolite has toxic and teratogenic feature, so that it may inhibit the formation of DNA and RNA in primary spermatocyte, particularly in pachytene spermatocyte. Pachytene spermatocyte is the mostly active cell in synthesizing RNA. Therefore, these cells are the most sensitive ones against MAA, with the result that they become the mostly degenerated cells (Gray et al., 1985; Rumanta et al., 2001). MAA treatment in mouse may result in DNA fragmentation, characterized with the occurrence of apoptosis, and apoptotic cells are finally absorbed by Sertoli cells. This condition resulted in the occurrence of azoospermia in group K1. MAA can also increase membrane permeability, resulting in excessive influx of Ca2+ (Ca2+ overload). Ca2+ overload may inhibit oxidative phosphorilation, so that energy supply becomes diminished as it is used to pump out Ca2+ ion, while energy is important for spermatozoa motility (Rumanta et al., 2001). This is one factor that leads to the damage of spermatozoa motility. Additionally, the damage is also caused by the presence of abnormal cells. Ca2+ overload may also activate protease and phospholipase enzymes that are able to degrade cytoskeletal proteins required in building cellular structure (Li et al., 1997). The degradation of these cytoskeletal proteins becomes one cause of the damage in the process of cellular structure formation, resulting in the abnormality of spermatozoa morphology. 2-ME also disturbs lactate production in Sertoli cells. In effect, the development of spermatocyte, that requires lactate as energy substrate, is disturbed as well, leading to abnormal morphology of spermatozoa (Gray et al., 1985). 2-ME causes degeneration of spermatogenic cells. Due to this factor, the size of seminiferous tubule reduces, as indicated by the reduction of its diameter and decreasing thickness of germinal epithelial cells, which even results in the formation of vacuola. Vacuolization in the cytoplasma of seminiferous tubule is one indication of reduced activity of Sertoli cells (Rumanta et al., 2001). Sertoli cells have a role in secreting androgen binding protein (ABP) that will bind testosterone and carry it into testicular circulation. Testosterone is a male sex hormone that has influence on the maintenance of optimal condition in spermatogenesis. It is the reduction of Sertoli cells activity that results in damaged

spermatogenesis and produces abnormality in spermatozoa morphology.

Spermatozoa motility or movement has a highly important function in fertilization. After the treatment of 2-ME administration for 3 weeks, spermatozoa was not found in caudal epididymis, although in the subsequent treatment, in which 2-ME was discontinued for different length of time, spermatozoa was found to recover. However, until two weeks cessation, spermatozoa count with type A motility remained less than 25%. After 2-ME cessation for 3 and 4 weeks, type A spermatozoa motility started to increase more than 25%. It is the type A spermatozoa motility (moving straightforward) that can survive until fertilization, while type C (motile in place) and D (immotile) spermatozoa are the factors that prevent spermatozoa to fertilize ovum. One factor causing the reduction of spermatozoa motility is the abnormal morphology of spermatozoa. Reduced spermatozoa motility occurred because mitochondria, as the primary target of MAA, are damaged due to Ca2+ overload. Ca2+ overload may inhibit oxidative phosphorilation, so that energy supply becomes diminished as it is used to pump out Ca2+ ion, while energy is important for spermatozoa motility (Rumanta et al., 2001).

Along with increasing 2-ME cessation period, the motility and morphology of spermatozoa was getting better. This was because the regenerative capacity of epithelial cells in seminiferous tubule was not totally destroyed by MAA (Valentine et al., 1998). There is a spermatogenic cell, spermatogonium A, in seminiferous tubule that is not the target of MAA for it is not at the state of dividing actively (Berndtson, WE and Foote RH, 1997; Ku et al, 1995). The length of 2-ME cessation made the 2-ME influence in the body become reduced and finally disappeared. At the same time, damaged or apoptotic spermatogenic cells due to 2-ME were readily absorbed by Sertoli cells (Albert, et al., 1989). The remaining spermatogonium actively proliferated continuously, produced new spermatocyte, which finally became new spermatozoa with healthy morphology.

CONCLUSIONS

In conclusion, a dose of 200 mg/kg 2-Methoxyethanol given to male mice for 3 weeks results in azoospermia, rendering its morphology and motility unobservable. The length of 2-ME cessation affects the recovery of spermatozoa morphology and motility in mice.

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