

Mucuna pruriens restores spermatogenesis in mice after exposure to 2-methoxyethanol

Putu Oky Ari Tania* and Sri Winarni**

ABSTRACT

BACKGROUND

Increasing industrial activity is causing many problems in reproductive health, such as infertility. The factors causing infertility are hormones, infection, radiation, drugs and chemicals. One of these is 2-methoxyethanol (2-ME). The objective of this study was to evaluate the effect of *Mucuna pruriens* (MP) seed fraction on spermatogenesis, including number of spermatogenic cells and spermatozoal membrane protein profiles after exposure to 2-ME in mice.

METHODS

This study was of experimental design. Thirty mice were randomized into 5 groups, i.e. 2 control groups and 3 treatment groups. All mice were injected subcutaneously with 2-ME doses of 100mg/kg body weight (BW)/day, for a period of 12 days. Positive (PC) and negative controls (NC) were treated with carboxy methyl cellulose (CMC). The three treatment groups were given MP seed fraction in doses of 14 (T1), 28 (T2) and 56 mg/kg BW (T3), respectively, from day 13 until day 64. Data on spermatogenic cells were collected from histological cross-sections and analyzed with one-way ANOVA. The spermatozoal membrane protein profile was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining and were analyzed descriptively.

RESULTS

Administration of MP seed fractions resulted in significantly different numbers of spermatocytes I and round spermatids between PC and T1, T2, and T3. A protein with the molecular weight of 41.6 kDa was expressed in all groups except the positive controls, while a 24.5 kDa protein was expressed in the positive controls only.

CONCLUSION

MP seed fraction effectively recovers spermatogenesis loss in mice due to 2-ME exposure.

Key words : *Mucuna pruriens*, 2-methoxyethanol, spermatogenic cells, mice

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Mucuna pruriens memulihkan spermatogenesis pada mencit setelah paparan 2-methoxyethanol

ABSTRAK

LATAR BELAKANG

Peningkatan aktivitas industri mengakibatkan masalah kesehatan, khususnya mengenai kesehatan reproduksi, yaitu infertilitas (kemandulan). Faktor yang menyebabkan infertilitas antara lain hormon, infeksi, radiasi, obat dan bahan kimia baik alami maupun sintetik, yang dapat berinteraksi dengan sistem endokrin. Salah satu bahan kimia tersebut adalah 2-methoxyethanol (2-ME). Penelitian ini bertujuan untuk menilai pengaruh fraksi biji *Mucuna pruriens* (MP) terhadap spermatogenesis meliputi jumlah sel spermatogenik dan profil protein membran spermatozoa setelah pemaparan 2-ME pada mencit.

METODE

Sebuah rancangan eksperimental digunakan pada studi ini. Tiga puluh ekor mencit jantan dibagi dalam 5 kelompok, 2 kelompok kontrol, yaitu kontrol positif (PC) diberikan 2-ME dan kontrol negatif (NC) diberikan carboxy methyl cellulose (CMC), serta 3 kelompok perlakuan diberikan fraksi biji MP dosis 14 (T1), 28 (T2) dan 56 mg/kg berat badan (T3). Semua mencit diinjeksi secara subkutan dengan 2 MP sebanyak 100 mg/kg berat badan 12 hari sebelum perlakuan. Pengambilan data sel spermatogenik dilakukan dengan pembuatan sediaan histologi, dan dianalisis dengan oneway ANOVA. Profil protein membran spermatozoa diidentifikasi dengan sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), silver staining dan dianalisis jumlah pita secara deskriptif.

HASIL

Pemberian fraksi biji MP meningkatkan jumlah sel spermatosit I dan spermatid bundar secara bermakna pada kelompok T1, T2, dan T3 dibandingkan kelompok PC. Terdapat 13 pita yang muncul pada gel elektroforesis untuk semua kelompok, pita dengan BM 41,6 kDa muncul di semua kelompok, kecuali kontrol positif sedangkan pita dengan BM 24,5 kDa hanya muncul pada kontrol positif.

KESIMPULAN

Fraksi biji MP mampu memulihkan spermatogenesis mencit akibat pemaparan 2-M.

Kata Kunci : *Mucuna pruriens*, 2-methoxyethanol, sel spermatogenik, mencit

INTRODUCTION

In recent years, there are serious problems in reproductive health, including infertility and sterility. The factors that cause infertility are hormones, infection, radiation, and natural and synthetic chemicals, which can interact with the endocrine system.⁽¹⁾

Several studies are currently investigating the causes for infertility. One of these are chemical agents that are toxic to the human body and impair fertility. 2-methoxyethanol (2-ME)

is a chemical agent that is widely distributed in the environment. It is a glycol ether derived from phthalate esters. The latter compounds are used globally as water-based organic solvents in industry and household appliances.⁽²⁾

In the mammalian body, 2-ME is metabolized into methoxyacetic acid (MAA), which has more toxic effects than others.⁽³⁾ Several studies suggested that there are abnormalities in the reproductive system caused by 2-ME. Administration of 2-ME at a dose of 100 mg / kg body weight (BW) can decrease

testosterone levels in male mice,⁽⁴⁾ and also inhibit the secretion of androgen-binding protein (ABP).⁽⁵⁾

Normally, the process of spermatogenesis is regulated by endocrine and paracrine signals. Gonadotropine releasing hormone (GnRH) plays an important role. Pulsatile secretion of GnRH produces follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Production of testosterone is stimulated by LH, and FSH stimulates Sertoli cells to secrete ABP.⁽⁶⁾ The decrease in testosterone and ABP levels due to 2-ME is associated with decreased functioning of the hypothalamus and pituitary that regulates the secretion of LH and FSH.

Testosterone and ABP levels decreased by 2-ME would cause an interruption of spermatogenesis. Reduced testosterone autoregulates expression of some testicular proteins, including those that play a role in spermatogenesis.⁽⁷⁾

Spermatogenesis disruption caused by 2-ME can affect protein synthesis in the spermatozoal membrane. Exposure to 2-ME at a dose of 200 mg / kg BW 3 times weekly significantly reduces the width of the 19 kDa and 24 kDa protein bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).⁽⁸⁾ In order to promote a life style of using natural products and to manage the negative impact of 2-ME, we need natural or herbal substances, such as *Mucuna pruriens* (MP).

Seeds of MP contain highly beneficial natural substances, and MP seeds have been reported to possess anti-diabetic, anti-neoplastic, anti-microbial, aphrodisiac, and learning and memory enhancing properties.⁽⁹⁾ *Mucuna pruriens* seed extract acts as a herbal medicine that reduces stress and improves sperm quality.⁽¹⁰⁾ According to Singh et al., MP efficiently repairs spermatogenic cell damage caused by ethinyl estradiol (EE).⁽¹¹⁾ There have been many previous studies on the effect of MP seed fraction on spermatogenic cells, but not on its effect on exposure of spermatogenic cells to 2-ME in mice. The most important component of MP seed

fraction that improves spermatogenesis is L-3,4 dihydroxyphenyl alanine (L-DOPA).⁽¹²⁾

L-DOPA undergoes biotransformation into dopamine, which is mostly converted to norepinephrine (NE) by dopamine- β -hydroxylase and undergoes methylation to epinephrine (E) in brain ventricles. Norepinephrine affects hypothalamic pulsatile release of GnRH, and frequency and amplitude of GnRH stimulation. As a result, there is increased GnRH stimulation of FSH and LH,⁽¹³⁾ resulting in normal spermatogenesis. In this study, an MP seed extract containing L-DOPA was expected to induce normal spermatogenesis after exposure to 2-ME. The purpose of this study was to evaluate the effect of MP seed fraction on spermatogenesis in mice after exposure to 2-ME, particularly the number of spermatogenic cells and the spermatozoal membrane protein profile.

METHODS

Research design

This study was of an experimental completely randomized design. This study was conducted at Airlangga University (Surabaya), Brawijaya University (Malang), and Gadjah Mada University (Yogyakarta), from January to July 2011.

Experimental animals

Thirty 6-8 week old adult male BALB/C mice (*Mus musculus*) weighing 25-35 g and purchased from Gadjah Mada University, Yogyakarta, were used in this study. The animals were divided into 5 groups, comprising positive controls (PC) and negative controls (NC), and 3 treatment groups (T1, T2, T3).

Plant material

The plant material used in this study was MP seeds from Banyakprada village, Central Java, obtained from and verified by the Institute for Research on Pulses and Root Crops (*Balai Penelitian Tanaman Kacang-kacangan/Umbi-*

umbian), Malang. Acetone, ascorbic acid, ethanol, 2-methoxyethanol, and distilled water were used for MP seed fractionation in the Phytopharmaca-Pharmacognosy Laboratory, Airlangga University.

Histologic examination

Histological sections of testicular tissue were prepared by the paraffin method with Harris's Hematoxylin-Eosin staining at the Airlangga University. Preparation of histological sections of testicular tissues by the paraffin method comprises the following steps: fixation, washing, dehydration, clearing, infiltration, embedding, sectioning, staining and mounting.⁽¹⁴⁾

Intervention

During the first twelve days all groups except the negative control group were injected subcutaneously with 2-ME. From day 13 until day 64, all mice in the treatment groups were treated with MP seed fraction at oral doses of 14, 28 and 56 mg /kg BW/day, respectively, whereas the control groups were given 0.5% carboxy-methyl cellulose (CMC) orally (Figure 1). After completion of the treatment, one testicle of each animal was used for preparation of hematoxylin-eosin paraffin sections and the other for sperm membrane protein isolation.⁽¹⁴⁾

Spermatogenic cell count

Determination of spermatogenic cell numbers in the histological preparations was done by hand counter under a light microscope at 400 x magnification, at the Faculty of Science and Technology, Airlangga University. The number of spermatogenic cells includes spermatogonia (cell number per seminiferous tubule), spermatocytes I (cell number per seminiferous tubule) and round spermatids (cell number per seminiferous tubule). The seminiferous tubules were observed in accordance with the criteria of spermatogenic cell stage 7 of epithelium.⁽¹⁵⁾

Electrophoresis

Isolation and purification of sperm membrane protein using NaCl, potassium phosphate buffer (PPB), MgSO₄, Octyl-beta-D-thioglucopyranoside (OSGP) (1%), glycerol (10%), Tris, and complete mini protease inhibitor, was performed at Brawijaya University, Malang, according to a centrifugal method. Isolation of sperm membrane proteins was performed in several stages. First the spermatozoa were collected from the testicles by enumeration techniques, followed by sperm membrane protein isolation. Finally, the membrane protein profile was determined using

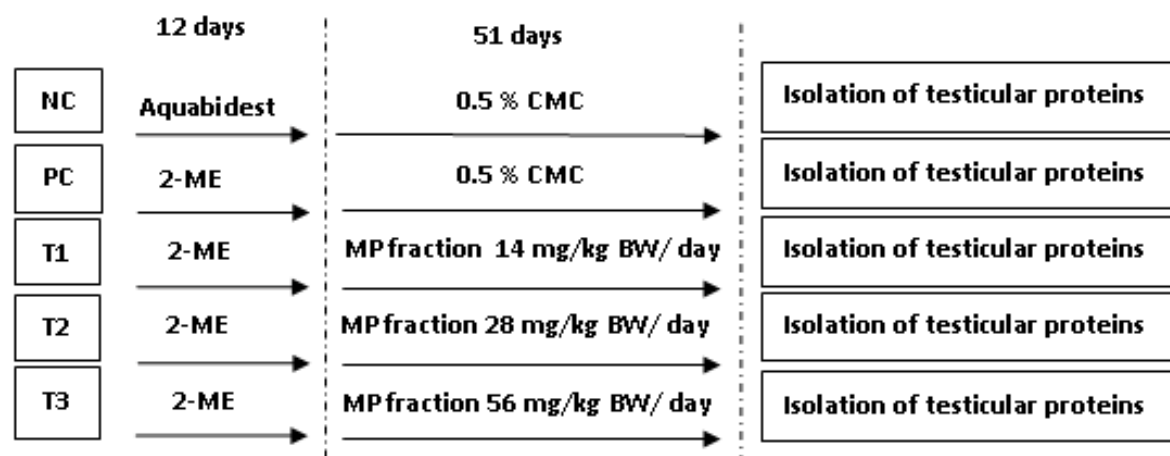


Figure 1. Experimental procedures for treatment (T1-3) and control groups (NC, PC) for 63 days

electrophoresis SDS-PAGE, silver staining, and visualization of the protein bands. Electrophoresis of sperm membrane protein bands by SDS-PAGE with silver staining was completed at Airlangga University.

Histological preparations

Preparation of histological sections of testicular tissues by the paraffin method comprises the following steps: fixation, washing, dehydration, clearing, infiltration, embedding, sectioning, staining and mounting.⁽¹⁴⁾

Data analysis

Data were analyzed by ANOVA and Least significant differences (LSD), with $p < 0.05$. Proteins from the spermatozoal membrane profile were analyzed descriptively.

Ethical clearance

Ethical clearance was issued by the Faculty of Medicine, Airlangga University.

RESULTS

The mean number of spermatogenic cells and LSD test results can be seen in Table 1.

The differing effects of 2-ME on the number of spermatogenic cells can be seen in the PC and NC. The mean of numbers of spermatogonia, spermatocytes I and round spermatids were lower in PC as compared with NC, but there was no difference between PC and NC in the number of spermatogonia.

The effect of MP seed fraction on the recovery of spermatogenesis after administration of 2-ME was seen in PC and T1, T2 and T3. The number of spermatocytes I and round spermatids differed significantly between PC and T1, T2, T3, but the number of spermatogonia showed a gradual but non-significant increase in numbers in T1, T2, and T3. The number of round spermatids also did not differ significantly between T1, T2 and T3.

The effect of administration of MP seed fraction on the seminiferous tubules of mice exposed to 2-ME can be seen in the histological cross sections in Figure 2.

The effect of MP toward spermatozoal membrane protein profile can be seen in Table 2. The protein profile was described by the molecular weight (kDa) of the proteins in testicular tissues, and was visualized by bands in gel electrophoresis.

The sperm membrane protein profile in the control groups and the treatment groups showed 13 protein bands on gel electrophoresis and was analyzed descriptively. In PC, the proteins were visualized by 13 bands of molecular weight 120; 114; 70.6; 66.2; 56.4; 53.4; 49.5; 38.5; 32.2; 28.4; 24.5; 22.7 and 19.4 kDa. While in NC and T1, T2, T3, there were 13 protein bands with molecular weight 120; 114; 70.6; 66.2; 56.4; 53.4; 49.5; 41.6; 38.5; 32.2; 28.4; 22.7, and 19.4 kDa. The protein with molecular weight of 24.5 kDa was expressed in PC, but not in the other groups. The 41.6 kDa protein was present in all groups except PC.

Table 1. Mean numbers of spermatogenic cells in the control and treatment groups

	PC (n=6)	NC (n=6)	T1 (n=6)	T2 (n=6)	T3 (n=6)
Spermatogonium	40.66 ± 2.31 ^a	41.71 ± 3.14 ^a	43.26 ± 3.90 ^a	43.44 ± 3.91 ^a	45.30 ± 3.21 ^a
Spermatocyte I	29.50 ± 1.58 ^a	34.51 ± 4.15 ^b	39.54 ± 3.26 ^{cd}	36.69 ± 2.82 ^{bc}	36.72 ± 2.00 ^{bd}
Round spermatid	80.02 ± 7.44 ^a	81.50 ± 11.34 ^{ab}	107.97 ± 1.98 ^{cd}	98.77 ± 7.43 ^{de}	101.02 ± 8.46 ^{de}

Values represent mean ± SD

PC = positive controls receiving 2-ME; NC = negative controls; T1 = treatment group receiving MP seed fraction at a dose of 14 mg/kg BW/day; T2 = treatment group receiving MP seed fraction at a dose of 28 mg/kg BW/day; T3 = treatment group receiving MP seed fraction at a dose of 56 mg/kg BW/day. Superscripts ^(a,b,c,d,e) indicate a significant difference by LSD test

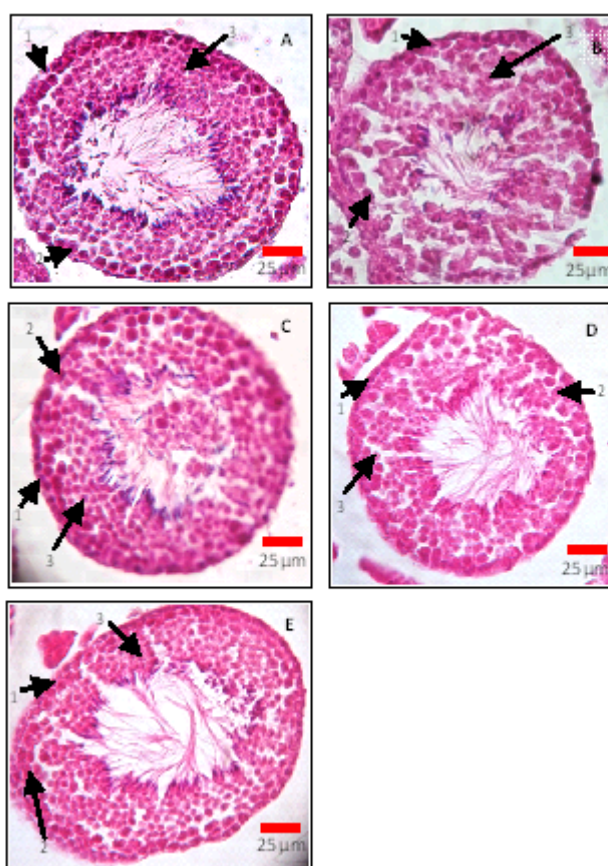


Figure 2. Cross sections of seminiferous tubules of *Mus musculus*. A = NC; B = PC ; C, D, and E = treatment groups T1, T2, and T3, receiving MP seed fraction at doses of 14, 28 and 56 mg/kg, respectively

DISCUSSION

In this study MP seed fraction was shown to induce the recovery of spermatogenic cells after exposure to 2-ME. This finding has previously not been made in other studies. In the treatment groups (T1, T2, T3) the number of spermatogenic cells were shown to be greater than in the negative controls (NC). 2-ME decreases the number of spermatogenic cells, especially spermatocytes I, which are the main targets of 2-ME.⁽⁵⁾

Methoxyacetic acid (MAA), a metabolite of 2-ME, can damage spermatogenic cells, particularly pachytene spermatocytes.⁽¹⁶⁾ Spermatocytes are also the main target of apoptosis induced by MAA.⁽¹⁷⁾

The effect of 2-ME on spermatogenesis can be seen in the NC and PC. The number of

spermatogenic cells in PC was always lower than in NC, and this decrease in spermatogenic cell count indicates disruption of spermatogenesis by 2-ME.

The results of one-way ANOVA on number of spermatogonia in T1, T2 and T3 showed significance differences, but no effect of MP treatment at different doses on the number of spermatogonia. Spermatogonia are resistant to 2-ME and its metabolites,⁽¹⁶⁾ implying that administration of 2-ME does not effect a decrease in the number of spermatogonia. Spermatocytes are the first cells to be affected by exposure to 2-ME. Oxidative stress in spermatocytes due to MAA triggers release of cytochrome C, activation of caspase and apoptotic cell death.⁽¹⁸⁾ Spermatocytes I are the cells that are the most sensitive to 2-ME.⁽¹⁶⁾ Twelve hours after exposure to 2-ME, almost

Table 2. Molecular weight of spermatozoal membrane proteins in PC, NC, and treatment groups T1, T2 and T3

Protein	Molecular weight of spermatozoal membrane protein (kDa)				
	Control Groups		MP seed fraction treatment groups (dose)		
	PC	NC	T1	T2	T3
a.	120	120	120	120	120
b.	114	114	114	114	114
c.	70.6	70.6	70.6	70.6	70.6
d.	66.2	66.2	66.2	66.2	66.2
e.	56.4	56.4	56.4	56.4	56.4
f.	53.4	53.4	53.4	53.4	53.4
g.	49.5	49.5	49.5	49.5	49.5
h.	-	41.6	41.6	41.6	41.6
i.	38.5	38.5	38.5	38.5	38.5
j.	32.2	32.2	32.2	32.2	32.2
k.	28.4	28.4	28.4	28.4	28.4
l.	24.5	-	-	-	-
m.	22.7	22.7	22.7	22.7	22.7
n.	19.4	19.4	19.4	19.4	19.4

PC = positive controls receiving 2-ME; NC = negative controls; T1 = treatment group receiving *mucuna pruriens* (MP) seed fraction at a dose of 14 mg/kg BW/day; T2 = treatment group receiving MP seed fraction at a dose of 28 mg/kg BW/day; T3 = treatment group receiving MP seed fraction at a dose of 56 mg/kg BW/day.

50% of spermatocytes I undergo apoptosis, indicating that spermatocytes are the main targets of 2-ME.

The effect of 2-ME on the histology of the seminiferous tubules can be seen in Figure 1B, where the density of spermatocytes I is greatly diminished. Exposure to 2-ME in laboratory animals causes decrease in testicular weight, histopathological changes in the testes, decreased sperm count and motility, and increased proportion of epididymal sperms with abnormal morphology.^(18,19)

The effect of MP seed fraction at different doses on the number of spermatocytes I are shown by the results of the LSD test. In T1, T2 and T3, the number of the cells increased significantly as compared with PC. Thus the decrease in the number of spermatocytes I caused by 2-ME can be dramatically restored by giving MP seed fraction.

The effect of MP seed fraction on recovery of the number of round spermatids was shown

on LSD testing. The treatment groups given MP seed fraction at various doses showed a significant increase in number of spermatogenic cells compared with PC. This indicates that MP seed fraction has a significant enhancing effect on the number of round spermatids that have been exposed to 2-ME.

MP seed fraction contains 14.7% L-3,4 dihydroxyphenyl alanine (L-DOPA) and it is estimated that L-DOPA can recover the number of spermatogenic cells that had previously been exposed to 2-ME. L-DOPA undergoes biotransformation into dopamine and is converted into norepinephrine (NE). NE affects the increase in hypothalamic pulsatile GnRH, and the frequency and amplitude of GnRH stimulation, which ultimately increases FSH and LH.⁽¹³⁾ Protein bands appearing in PC showed an effect of 2-ME on the spermatozoal membrane protein profile. In PC a 24.5 kDa protein band was visualized that did not appear in the other groups.

In a study by Honda et al.⁽²⁰⁾ testicular proteins with molecular weights of 14-18 kDa and 41-42 kDa were found on the surface of spermatozoal membranes. The proteins act as serine proteases in the penetration of the zona pellucida (ZP). Non-expression of serine proteases in PC can lead to infertility via blocking of acrosine, an enzyme in the acrosome of the spermatozoal head, activated at the time of ZP penetration

In this study, the 41.6 kDa protein band was not expressed in the PC and was also correlated with decreased numbers of spermatocytes I and round spermatids after administration of 2-ME. Because this protein is supposedly a serine protease and estimated to play an important role in fertilization, this suggests that exposure to 2-ME could lead to infertility due to non-expression of protein serine proteases.

Administration of MP seed fraction at different doses was capable of inducing recovery of spermatogenesis, as indicated by the increase in number of spermatocytes I and round spermatids. However, MP seed fraction has no effect on spermatogonia, as indicated by the non-significant increase in their number. *Mucuna pruriens* seed fraction is also capable of inducing the expression of the 41.6 kDa protein.


One limitation of this study was its sole focus on the effect of MP seed fraction on spermatogenesis after exposure to 2-ME, especially on spermatogonia, spermatocytes I and round spermatids. Another limitation was that the molecular weights of the sperm membrane proteins were not exact, but only rough estimates. In the near future we expect that the MP seed fraction will become an alternative medicine for infertile males, especially those at risk of exposure to 2-ME.

CONCLUSIONS

This study showed that administration of MP seed fraction caused increases in the number

of spermatocytes I and round spermatids, but not that of spermatogonia. There were 13 protein bands which appeared in all groups. A 41.6 kDa protein was expressed in all groups except PC, while a 24.5 kDa protein was expressed in PC only.

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