

Finding the Minimal Effective Dose of a Combination of Depot Medroxyprogesterone Acetate and Javanese Long Pepper for Male Contraception on Testicular Fertility Parameters in Male *Sprague Dawley* Rats

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ABSTRACT

To reduce population growth, the Indonesian government promotes a Family Planning Program for fertile couples. Depot-medroxyprogesterone acetate (DMPA) combined with testosterone or herbal androgens as testosterone substitute is intended for hormonal male contraception because it is expected that these combinations can suppress gonadotropin and inhibit spermatogenesis. Various medicinal herbs contain androgens, e.g., Javanese long pepper (JLP) (*Piper retrofractum* Vahl). Traditionally, JLP chili extract (JCE) is used to cure impotency and was proven to increase blood testosterone levels in hypogonad men. The aim of this study is to combine increasing amounts of JCE (0.94 – 3.76 mg) with DMPA (1.25 mg) and to study the effects on testicular parameters using male Sprague-Dawley rats: testis weight, seminiferous tubule diameter, the subpopulations of spermatogenic cells, and Leydig cells. Our randomized study design used six equally sized groups with 6 animals, each. Testis weight only decreased vs. controls in two groups, DMPA + placebo and DMPA + 3.76 mg JCE. Significant decrease occurred in the diameter of seminiferous tubules and at 3.76 mg JCE, in Leydig cell population. Within spermatogenic cell population spermatogonia A, spermatocyte-I preleptotene, spermatocyte-I pachiten, and spermatid population decreased. Conclusion: The combination of 1.25 mg DMPA and 3.76 mg JCE appears to be the minimal dose and a potent candidate for male contraception in our rat model.

Keywords: DMPA, *Piper retrofractum* Vahl, seminiferous tubules, Leydig cells, spermatogenic cells.

INTRODUCTION

In 2025, the Indonesian population is expected to reach 273 million inhabitants even with population growth below 1.5%. To effectively reduce these numbers, the Indonesian government launched a Family Planning Program for couples in fertile age¹. By far less male contraceptives have been available until today as compared to female

contraception methods. Until mid-20th century, male contraceptive methods were used by 30% of couples in the world, such as condoms, vasectomy, coitus interruptus, and sexual abstinence².

In our efforts searching for male contraceptives which are safe, effective, easy to use, reversible, and acceptable by the society³. We used depot medroxyprogesterone acetate (DMPA), a

synthetic progesterone derivative with a long acting and safe effect, when administered via IM injection. DMPA is a potent inhibitor of pituitary gonadotropin secretion and widely used for female contraception⁴⁻⁶. Combined with testosterone it is good prospect for development of male contraceptives providing sufficient effective time which is required for inhibiting the secretion of gonadotropin hormones FSH and LH, thus suppressing spermatogenesis and plasma testosterone levels. This decrease in testosterone levels can be overcome by the administration of exogenous testosterone⁷⁻⁸.

Applying a combination of progestin and testosterone was shown to cause a decrease in testicular volume or weight and shrinkage of the diameter of the tubulus seminiferous⁹⁻¹⁰. Moreover, this combination decreased the population of spermatogenic cells¹¹ by disrupting their development due to constraints on gonadotropin hormones FSH and LH⁷. Progesterone acetate derivatives injected into rams did not only lead to the cessation of spermatogenesis but also to atrophy of Leydig cells¹².

Java chili fruits are traditionally used to cure male impotence, stomach weakness, strong transpiration etc. They contain piperine, piperatin, beta-sitosterol, free amino acids, essential oils, resins, saponins, polyphenols, and other compounds¹³⁻¹⁴. Infusion of Javanese chili fruit extract (JCE) at a dose of 2.1 mg / 10 g body weight of rats showed androgenic and anabolic effects¹⁴. In the Ames test JCE did not show any mutagenic effects and was considered safe for consumption¹⁵. On male chicks, the androgenic effect of 3.75 mg / 100 g body weight of ethanolic JCE extract was not significantly different from the standard dose of 500 mg TU / 100 g BW¹⁶. In 2006, a clinical trial was carried out to determine the androgenic effects of JCE on 9 hypogonadal men¹⁷. The results showed that JCE could increase blood testosterone levels in 7 out of 9 hypogonadal men (78%), did not reduce the levels of FSH and LH, could increase the frequency of coitus and did not cause serious side effects¹⁷.

In previous studies, the minimal effective dose of 1.25mg DMPA had been determined by the concentration and viability of spermatozoa in Vas deferens of rats¹⁸⁻¹⁹. However, testosterone levels decreased by more than 50% and JCE was

administered to maintain the testosterone level. The combination of 1.25 mg DMPA with various amounts of JCE had shown to keep the number and viability of spermatozoa low, but only the combination with JCE 3.76 mg kept the testosterone level at 100% of controls²⁰⁻²¹. According to these studies DMPA can suppress spermatogenesis, while JCE can maintain testosterone levels decreased by DMPA. It is necessary to further investigate the combined effects of DMPA and JCE on spermatogenesis using rats as experimental animals. The purpose of this study is to confirm the results of preliminary studies by investigating testicular fertility parameters.

MATERIALS AND METHODS

Healthy and fertile male rats (*Rattus norvegicus* L) Sprague-Dawley, aged 40-60 days with 250 grams of body weight and Java chili extract (JCE) in the form of gelatin capsules were obtained from BPOM RI.

Most chemicals (benzene, benzyl benzoate, entellan, eosin Y, ethanol, ether, glycerol, paraffin, and xylol) were purchased from Merck AG, Darmstadt, Germany (subsidiary Jakarta), at highest grade available, all other chemicals and instruments from local distributors in Jakarta or Singapore of the companies given in brackets: solution of 1% Na-CMC (Sino-CMC), DMPA (Depogeston-Biowet), picric acid (JT-Baker), aquabidest (IKA), physiological saline (Baxter), hematoxiline (MCB), measure cups (Iwaki), syringes (Terumo), surgical tools (Yamaco-Inox), digital scales (Citizen), rotary microtome (Spencer), beaker glasses and bottles (Schott-Duran), electronic binocular microscope (Nikon), micrometer objective and micrometer ocular (B&L), laboratory counter (Clay-Adams), glass slides and cover glasses (Assistent), razor blades and staining jar (Termo-Sandon). The stomach tubes were handmade in our animal house.

Experimental design

This study used a completely randomized design (CRD) with equal sample size (n=6), 6 groups consisting of the control group (C), a sham control group (CP = rats injected with DMPA and given placebo), treatment group I (TI = rats injected with DMPA and JCE 0.94 mg), treatment group II (TII = rats injected with DMPA and JCE 1.88 mg), treatment

group III (TIII = rats injected with DMPA and JCE 2.82 mg), treatment group IV (TIV = rats injected with DMPA and JCE 3.76 mg).

Treatment of the experimental animals

The rats were acclimatized in their cages for 15 days, fed standard diet with free access to drinking water ad libitum. Rats were injected with DMPA in accordance with the predetermined dose of 1.25 mg, alternately into the right or left thigh. Injections were done twice, the first one at week 0 and the second one at week 12, because the effective time of DMPA in suppressing the secretion of gonadotropin hormones FSH and LH is 12 weeks.

In addition, JCE was administered to the treatment groups from week 7 to week 18. The reason for the start of JCE administration at week 7 was the effective time of DMPA to suppress spermatogenesis. KP group was administered with placebo, group T1 with 0.94 mg of JCE, group TII with 1.88 mg of JCE, group TIII with 2.82 mg of JCE, and group TIV 3.76 mg of JCE. Administration of placebo and JCE was accomplished by means of a stomach tube equipped with a modified syringe tip, every morning at 8 o'clock Western Indonesian Time (WIB).

By the end of experiments at week 18, the rats were anesthetized with ether and dissected: right and left testicles were taken, weighed and then prepared for further data retrieval.

Data collection

1. Weight of right and left testicles was determined by Digital Citizen Scales.
2. The diameter of the seminiferous tubules was measured with an electronic microscope equipped with micrometer objective and ocular at magnification 10x10.
3. Leydig cells were observed and calculated per one optical field under the electronic microscope at magnification 40x10.
4. Spermatogenic cells comprising spermatogonia-A, spermatocytes-I preleptoten, spermatocytes-I pachytene and spermatids were observed using the electric microscope at magnification 40x10.
5. Calculations of spermatogenic cells were performed in the seminiferous tubules

in phase VII and VIII of the seminiferous epithelium cycle, cut one round slice per optical field. Stage VII / VIII was selected because it is long lasting, relatively common in testicular cross section, and the type of spermatogenic cells are found more complete than in other stages. To calculate the actual number of spermatogenic cells, the obtained data were then corrected by the Abercrombie formula²²:

$$P = A \frac{M}{L + M}$$

Legend: P = average number of nuclei per slice (true count), A = number of rough calculation of nuclei per slice (crude count), M = thickness of incision (microns), L = average diameter of nuclei (microns).

Data analysis

Quantitative data were statistically evaluated using computer software Statistical Product and Service (SPSS) release 15 with the following test sequence²³⁻²⁴:

1. Shapiro and Wilk Normality Test and Bartlett Homogeneity of Variance Test.
2. If the data were normally distributed and variance homogeneous, one-way Analysis of Variances (ANOVA) was applied. If the obtained value of p was <0.05, *post hoc* Bonferroni test followed for the average differences between the two groups. For normally distributed data and variance not homogeneous (or the other way around), data had to be transformed. After data transformation, it turned out that the fixed data were still not normally distributed or inhomogeneous, non-parametrical Kruskal-Wallis test was applied. If the obtained value of p was <0.05, *post hoc* Mann-Whitney analysis followed.

RESULTS

Testicle Weight

The average weight of the testicles decreased from 1.59±0.08g in controls (group C) to 1.51±0.24g with 1.25 mg DMPA (group CP). In group TIII, testicle weight slightly decreased to 1.53±0.13g and only in group TIV the weight was a low as in

group CP (1.51 ± 0.23 g). In groups TI and TII, testicle weight did not decrease but increased in both groups to 1.67g. In other words, only the combination of 1.25 mg DMPA and 3.76 mg JCE is as effective as 1.25 mg DMPA alone.

The diameter of the seminiferous tubules

Figure 1 shows the results of the average diameter determination of the seminiferous tubules: group C, $235.5 \pm 17.41 \mu\text{m}$; CP, $205.9 \pm 6.62 \mu\text{m}$; TI, $201.9 \pm 11.45 \mu\text{m}$; TII, $195.2 \pm 7.51 \mu\text{m}$; TIII, $193.0 \pm 11.47 \mu\text{m}$; TIV, $179.1 \pm 9.95 \mu\text{m}$. Data management and calculation with one way ANOVA and Bonferroni post-hoc test demonstrated significant differences in diameter of seminiferous tubules between control and all other groups. Sham group (CP) differed significantly from treatment group TIV. The latter differed also significantly from TI group. Treatment groups TII, TIII, and TIV did not differ significantly. The most effective dose combination with significant decrease in the diameter of seminiferous tubules was DMPA 1.25 mg and JCE 3.76 mg (group TIV).

Spermatogonia-A cells

Figure 2 shows the results of the calculation of average spermatogonia-A cell population for group C, 37.25 ± 3.63 ; CP, 30.75 ± 1.81 ; TI, 27.00 ± 2.26 ; TII, 25.30 ± 2.76 ; TIII, 22.00 ± 2.98 ; TIV, 21.25 ± 1.97 . Data management and calculation with Kruskal-Wallis ($p = 0.000$) and Mann-Whitney post-hoc tests demonstrated that the control group differed significantly from all treatment groups (TI, TII, TIII, and TIV). Sham group CP did not differ significantly from control, but significantly differed from groups TII, TIII, and TIV. TI group differed significantly from group TIV. The most effective dose combination with significant reduction of the spermatogonia-A cell population was DMPA 1.25 mg and JCE 3.76 mg (group TIV).

Spermatocytes-I preleptoten

Figure 3 shows the calculation results of the average population of spermatocytes-I preleptotene for group C, 27.53 ± 4.86 ; CP, 21.10 ± 2.33 ; TI, 20.48 ± 1.51 ; TII, 20.38 ± 2.22 ; TIII, 17.35 ± 1.94 ; TIV, 16.65 ± 2.27 . Data management and calculation with one way ANOVA ($p = 0.000$) and Bonferroni post-hoc test demonstrated significant differences in the population of spermatocytes-I preleptoten between

control and all treatment groups (CP, TI, TII, TIII, and TIV). CP and TI also differed significantly from TIV. The most effective dose combination with significant reduction of spermatocytes-I preleptoten population was DMPA 1.25 mg and JCE 3.76 mg (group TIV).

Spermatocytes-I pachytene

Figure 4 shows the calculation results of the average population spermatocytes-I pachytene for group C, 24.30 ± 1.83 ; CP, 20.70 ± 2.57 ; TI, 20.73 ± 0.47 ; TII, 19.17 ± 1.59 ; TIII, 16.80 ± 1.13 ; TIV, 14.08 ± 1.44 . Data management and calculation with Kruskal-Wallis ($p = 0.000$) and Mann-Whitney post-hoc tests demonstrated that the control group differed significantly from treatment groups TII, TIII, and TIV. Sham group CP and treatment group TI did not differ significantly from control. Treatment group TIV differed significantly from sham group CP and treatment groups TI and TII; TI also differed significantly from group TIII. The most effective dose combination with significant reduction of spermatocytes-I pachytene population was DMPA 1.25 mg and JCE 3.76 mg (group TIV).

Spermatid cells

Figure 5 shows the calculation results of the average spermatid cell population for group C, 44.58 ± 9.40 ; CP, 33.25 ± 5.20 ; TI, 27.83 ± 2.70 ; TII, 24.75 ± 1.13 ; TIII, 19.50 ± 0.63 ; TIV, 18.67 ± 3.33 . Data management and calculation with Kruskal-Wallis ($p = 0.000$) and Mann-Whitney post-hoc tests demonstrated that the control group differed significantly from treatment groups TII, TIII, and TIV. Sham group CP and treatment group TI did not differ significantly from control. Treatment group TIV differed significantly from sham group CP and treatment groups TI and TII; TI also differed significantly from group TIII. Sham group CP differed significantly from treatment groups TII, TIII, and TIV. Treatment group TI differed significantly from groups TIII and TIV and group TII differed significantly from group TIII. The most effective dose combination with significant reduction of spermatid cell population was DMPA 1.25 mg and JCE 3.76 mg (group TIV).

Leydig cells

Figure 6 shows the results of the calculation of the average population of Leydig cells for group C, 508.67 ± 23.32 ; CP, 482.67 ± 16.94 ; TI, 473.50 ± 42.67 ; TII, 473.50 ± 42.67 ; TIII, 475.50 ± 45.85 ; TIV,

364.67±48.80. Data management and calculation with one way ANOVA and Bonferroni post-hoc test demonstrated significant differences in diameter of seminiferous tubules between treatment group PIV and all other groups. The sham group CP and treatment groups TI, TII and TIII did not significantly differ from the control group. The most effective dose combination with the only significant decrease in the number of Leydig cells was DMPA 1.25 mg and JCE 3.76 mg (group TIV).

DISCUSSION

DMPA is a progestin derivative already known for long in hormonal contraception⁸. It

lowers the concentration of androgens in the testes by reducing androgen biosynthesis, altering the metabolism of androgens and competing directly at the androgen receptor (AR) with androgen binding⁴.

By binding to plasma albumin DMPA concentration in serum is generally maintained for about 3 months, only decreasing gradually²⁵. DMPA enters the cell and binds to receptors located between the nucleus and cytoplasm. This ligand-receptor complex forms a dimer before bound to DNA²⁶. The mechanisms of DMPA to inhibit spermatogenesis are multifactorial⁶; it can decrease serum concentrations of sex hormone-binding globuline (SHBG), gonadotropins and

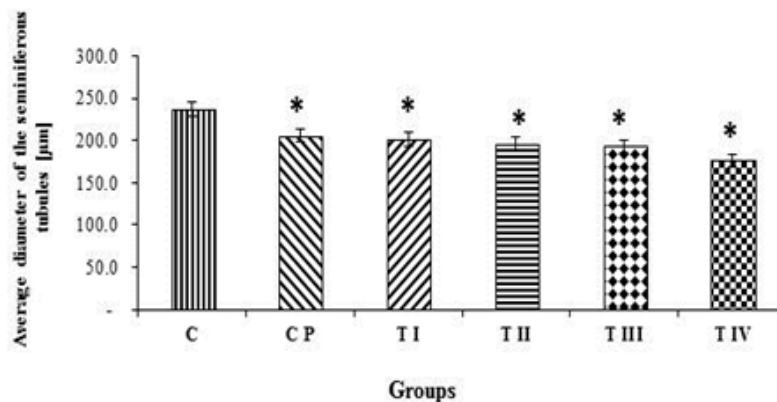


Fig. 1: Average diameter of seminiferous tubules after injection of DMPA and administration of various doses of Java chili extract (JCE). C = Control; CP = Sham control (DMPA + placebo); TI = DMPA + JCE 0.94 mg; TII = DMPA + JCE 1.88 mg; TIII = DMPA + JCE 2.82 mg; TIV = DMPA + JCE 3.76 mg

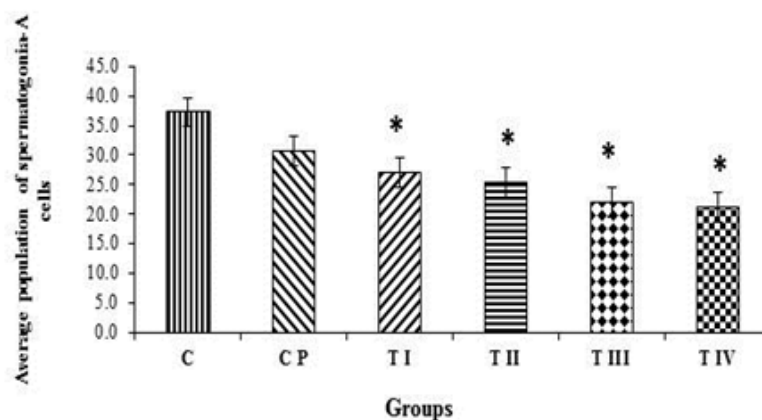


Fig. 2: Average spermatogonia-A population after injection of DMPA and administration of various doses of Java chili extract (JCE). C = Control; CP = Sham control (DMPA + placebo); TI = DMPA + JCE 0.94 mg; TII = DMPA + JCE 1.88 mg; TIII = DMPA + JCE 2.82 mg; TIV = DMPA + JCE 3.76 mg

testosterone. Combined with testosterone DMPA can decrease spermatogenesis, thereby lowering sperma production²⁷⁻²⁸.

As reported in previous studies¹³⁻¹⁴ JCE has androgenic effects. Javanese chili contains β -sitosterol which is converted into testosterone to replenish the loss due to the provision of DMPA¹³.

In previous studies, Yurnadi *et al.*¹⁸⁻²¹ investigated the concentration and the viability of spermatozoa in Vas deferens and the testosterone levels with DMPA 1.25 mg alone and in combination

with JCE. The essentials are summarized in Table 1. Furthermore, the authors investigated safety parameters of the combination of DMPA and its combination with JCE^{19,21}.

From the preliminary results, only the combination of 1.25 mg DMPA + JCE 3.76 mg fulfilled the desired effects (Table 1). Thus, we now investigated whether these results can be confirmed with testicular parameters.

The body weight (BW) of the rats did not differ significantly between all groups²¹. The

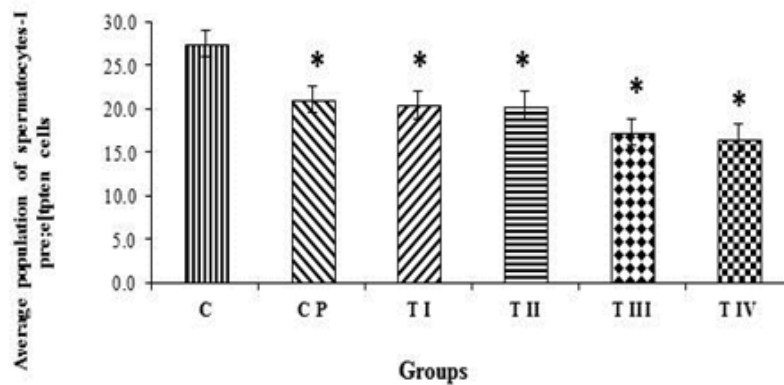


Fig. 3: Average population of spermatocytes-I preleptoten after injection of DMPA and administration of various doses of Java chili extract (JCE). C = Control; CP = Sham control (DMPA + placebo); T I = DMPA + JCE 0.94 mg; T II = DMPA + JCE 1.88 mg; T III = DMPA + JCE 2.82 mg; T IV = DMPA + JCE 3.76 mg

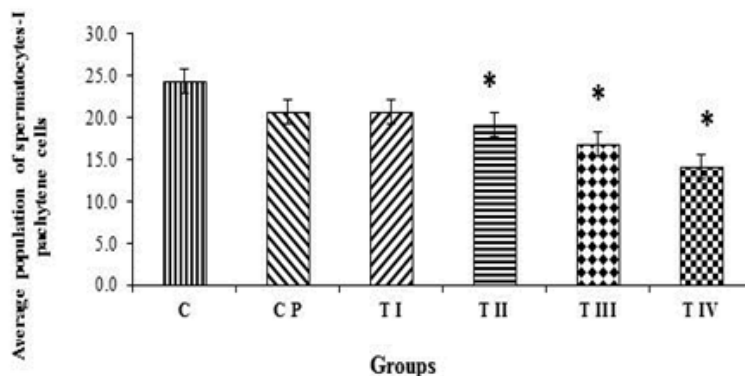


Fig. 4: Average population of spermatocyte-I pachytene after injection of DMPA and administration of various doses of Java chili extract (JCE). C = Control; CP = Sham control (DMPA + placebo); T I = DMPA + JCE 0.94 mg; T II = DMPA + JCE 1.88 mg; T III = DMPA + JCE 2.82 mg; T IV = DMPA + JCE 3.76 mg

estimation of the dose administered in group TIV (1.25 mg DMPA + 3.76 mg JCE per 250 g BW) is equivalent to 5 mg DMPA + 15 mg JCE per Kg BW, in general.

Testicular weight

Berndston and Thompson²⁹ reported that there are many factors that affect the weight of the testes. Increasing age is directly proportional to the weight of Sprague Dawley rat testes. Total body weight gain was also positively correlated with an increase in testicular weight; the older rats and the greater their weight, the greater also the weight of their testes.

According to Amatayakul *et al.*³⁰ a decrease in testis weight is closely connected with decreasing diameter of the seminiferous tubules and production of spermatogenic cells. Marson *et al.*³¹ claimed that gonadal function is positively correlated with the volume of the gonads. Bercovitch and Rodriguez³² reported that testis weight of male rhesus monkeys was positively correlated with sexual activity. This means that the increase in gonadal function would lead to an increase in testicular weight. Research by Kusmana⁹ showed that a combination of DMPA and TE injections caused a decrease in testicular volume of short-tailed male macaques (*Macaca nemestrina* L) and confirmed the same pattern of

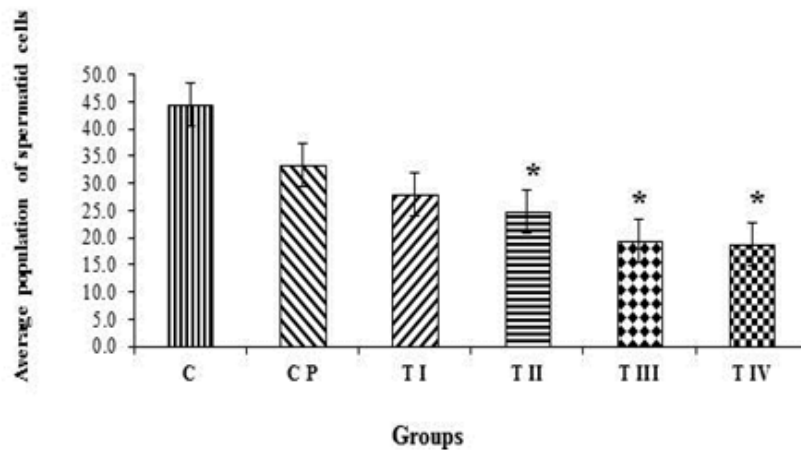


Fig. 5: Average spermatid population after injection of DMPA and administration of various doses of Java chili extract (JCE). C = Control; CP = Sham control (DMPA + placebo); TI = DMPA + JCE 0.94 mg; TII = DMPA + JCE 1.88 mg; TIII = DMPA + JCE 2.82 mg; TIV = DMPA + JCE 3.76 mg

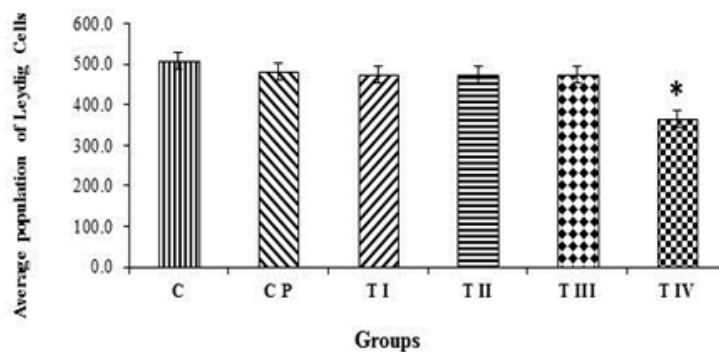


Fig. 6: Average population of Leydig cells after injection of DMPA and administration of various doses of Java chili extract (JCE). C = Control; CP = Sham control (DMPA + placebo); TI = DMPA + JCE 0.94 mg; TII = DMPA + JCE 1.88 mg; TIII = DMPA + JCE 2.82 mg; TIV = DMPA + JCE 3.76 mg

decline in testicular volume that had been reported by Sutyarso¹⁰ with long-tailed macaques (*Macaca fascicularis* L).

In our study the combination of 1.25 mg DMPA + JCE 3.76 mg reduced the testicular weight to the same value as 1.25 mg DMPA + placebo in the sham control.

Seminiferous tubules

Soeharsono³³ reported that medroxyprogesterone acetate (MPA) injections at a dose of 8 mg per rat reduced the number of sperm cells and caused shrinkage of the seminiferous tubules. Kusmana⁹ showed that administration of TE and DMPA caused a decrease in the diameter of the seminiferous tubules and the score of spermatogenesis. This occurred because of the damage of the histological structure of the testes. Such damage could be classified from mild to severe hypoplasia of the germ cells.

The decrease in seminiferous tubule diameter treated by DMPA and JCE may be

caused by hyalinisation as discussed by Nistal and Paniagua³⁴. According to the authors it is usually caused by hormonal mechanisms of interference on the axis hypothalamus-pituitary-testes.

Spermatogenic cells and sub-populations

Spermatogenic cell population and all subgroups investigated (Figures 4,5,6,7) gradually decreased in a dose-dependent manner with the most significant effect in group TIV. In general, it appears that the combination of DMPA and 3.76 mg of JCE is most effective in our entire study.

McLahlan *et al.*¹¹ reported decline in the number of spermatogenic cells subjected to DMPA + testosterone. After 12 weeks of treatment, there was a significant reduction in all sub-types of spermatogenic cells from spermatogonia A to spermatids vs controls. Kusmana⁹ observed with the scoring method of Johnson that DMPA + TE treatment of male macaque monkeys caused bottleneck spermatogenesis if compared with controls. Population decline of spermatogenic cells in experimental animals given DMPA combination and

Table 1: Summary of preliminary studies

	Control	1.25 mg DMPA	1.25 mg DMPA + JCE 3.76 mg	% of control
Spermatozoa				DMPA/ + JCE
Concentration in Vas deferens	94.7x10 ⁶ / mL	22.3x10 ⁶ / mL	22.1x10 ⁶ / mL	23 / 23
Viability	53.8%	32.8%	29.4%	61 / 55
Testosterone	2.26 ng / mL	1.08 ng / mL	2.28 ng / mL	48 / 100

Note: The data in Table 1 are taken from Yurnadi *et al.*¹⁸⁻²¹

Table 2: Compilation of results of the combination of DMPA 1.25 mg + JCE 3.76 mg

Testicular parameter	Control	1.25 mg DMPA	1.25 mg DMPA + JCE 3.76 mg	% of control DMPA/+JCE
Testis weight	1.59±0.08g	1.51±0.24g	1.51±0.23g	95 / 95
Diameter of seminiferous tubules [µm]	235.5±17.41	205.9±6.62	179.1±9.95	87 / 76
spermatogonia-A cells	37.25±3.63	30.75±1.81	21.25±1.97	83 / 57
Spermatocytes-I preleptoten	27.53±4.86	21.10±2.33	16.65±2.27	77 / 60
Spermatocytes-I pachytene	24.30±1.83	20.70±2.57	14.08±1.44	85 / 58
Spermatid cells	44.58±9.40	33.25±5.20	18.67±3.33	75 / 42
Leydig cells	508.67±23.32	482.67±16.94	364.67±48.80	95 / 72

JCE may occur due to constraints on the process of spermatogenesis.

DMPA and JCE cooperate in reducing the concentration of intra-testicular testosterone through a negative feedback mechanism in the hypothalamic-pituitary-testicular axis. It is known that testosterone is necessary in controlling spermatogenesis and a decrease in testosterone level leads to constraints of spermatogenesis by disrupting the process of the cells to develop spermatogenic³⁵⁻³⁶. A decrease in FSH may disturb the Sertoli cells to produce androgen-binding protein (ABP) which is very important for the binding of testosterone. According to Lui *et al.*³⁷ FSH receptor containing Sertoli cells generate support and nutrition for spermatogenic cells during spermatogenesis.

During spermatogenesis, spermatogenic activity of cells is very high, which occurs through both, morphological and biochemical changes. To support these activities, spermatogenic cells are highly dependent on energy sources derived from Sertoli cells. Lue *et al.*³⁸ proved that a decline in FSH leads to significantly reduced numbers and activity of Sertoli cells. Glucose is an important substrate for the survival of spermatogenic cells. The decline in the population of Sertoli cells can block the transport of glucose into the testicles, causing a decrease in the population of spermatogenic cells and vacuolization of seminiferous tubules. Limitation to glucose transport into spermatogenic cells can cause limitation of protein biosynthesis by spermatocytes and spermatids. Instead of glucose pachytene spermatocytes and spermatids also utilize energy sources in the form of lactate and pyruvate, which are supplied by Sertoli cells. In other words, apart from controlling survival, proliferation and differentiation by hormones spermatogenic cells are also controlled by Sertoli cells. Lactate and pyruvate production by Sertoli cells is affected by FSH, namely through increased intracellular level and activity of cAMP¹⁰.

Leydig cells

Low content of LH can also suppress the production of testosterone by Leydig cells and thus, spermatogenic cell proliferation. Leydig cells express LH receptors and in response to LH produced by pituitary gland they produce testosterone³⁹.

Meistrich and Shetty⁴⁰ reported that suppression of gonadotropins and testosterone cause disturbances in differentiation and development of spermatogenic cells. Under normal conditions spermatogenesis is directly related to the differentiation and survival of spermatocytes and spermatids. The decline of FSH level can inhibit the differentiation of spermatogonia. Testosterone also affects normal mitotic spermatogonia and the successful completion of meiosis⁴¹. FSH plays a role in the stimulation of mitosis in type B spermatogonia and in spermatocytes preleptoten activity in preventing apoptosis of pachytene spermatocytes and spermatids in the vicinity. Low levels of testosterone and FSH together affect the adhesion of spermatogenic cells to Sertoli cells and eventually cause increased spermatogenic cell apoptosis⁴².

DMPA also inhibits Leydig cells in their process of steroidogenesis⁴. Leydig cells play a role in the process of steroidogenesis by producing testosterone which is required for spermatogenesis⁴³. Biosynthesis of testosterone by the Leydig cells involve the action of a carrier protein cascade and enzymes of steroidogenesis. Mutations of genes regulating steroidogenesis in Leydig cells can result in testosterone deficiency. The decline in Leydig cell population can cause limited spermatogenesis⁴⁴. Ericson and Dutt¹² injected rams with progesterone acetate derivatives and reported not only the cessation of spermatogenesis but also atrophy of Leydig cells.

The decline of Leydig cell population is likely due to apoptotic mechanisms. Morris *et al.*⁴⁵ reported that apoptosis occurs because of interference of Leydig cells with their extracellular environment. A decrease in the concentration of gonadotropin (LH) seems to be positively correlated with a decrease in Leydig cell population, but the exact relationship between hormonal effects and the decline of Leydig cell population has not yet been explained.

Our results showed differences in Leydig cell population among treatment groups and controls. As seen in most other results, also population decline of Leydig cells in seminiferous tubules was most effective and significant vs control in group TIV at a dose of 3.76 mg JCE. That means, the combination of DMPA and JCE impedes the development of

Leydig cells gradually in a dose-dependent manner, because there is no significant difference of groups CP, TI, TII, and TIII vs control.

Safety and final remarks

During recent years, novel interventions for male contraception have been investigated with an extract from *Justicia gendarussa* leaves in Indonesia⁴⁶ and with VasalgeI™ in India⁴⁷. A clinical trial with intramuscular (IM) injection of a combination of 200 mg norethisterone enanthate (NE) and 1000 mg testosterone undecanoate (TU) turned out effective to almost complete and reversible suppression of spermatogenesis, however, the study was terminated early because of adverse events, such as acne, injection site pain, increased libido, and mood disorders⁴⁸.

The safety and tolerability of DMPA and its combination with JCE in the range of the dosage used in our studies was reported previously from the examination of hematological and biochemical parameters in rats¹⁹⁻²¹.

CONCLUSION

The minimal effective dose for the tested combination is DMPA 1.25 mg and JCE 3.76 mg (Table 2). Further histological and long-term observational fertility studies on effectivity and safety with experimental animals should follow before this combination will be tested in humans.

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