

Induction of Rat Germ Cell Apoptosis by Testosterone Undecanoate and Depot Medroxyprogesterone Acetate and Correlation of Apoptotic Cells with Sperm Concentration

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ABSTRAK

Tujuan: untuk mengetahui keamanan dan efektivitas dosis hormon testosteron undecanoate (TU) dan depot medroxyprogesterone acetate (DMPA) guna mengembangkan potensi kontrasepsi pria. **Metode:** dilakukan penekanan kadar sperma tikus melalui peningkatan apoptosis sel germinal secara in vivo melalui pemberian androgen kerja panjang yang merupakan kombinasi TU dan DMPA. Tikus jenis Sprague-Dawley dewasa diberi TU dengan dosis 2,5 mg setiap 6 minggu dan DMPA 1,25 mg setiap 12 minggu selama 60 minggu, yakni suatu regimen pengobatan yang diketahui dapat mempercepat penurunan produksi progesteron oleh testis dan menyebabkan azoospermia dalam waktu 12 minggu. Data tentang kadar sperma ditransformasi dalam bentuk log terlebih dahulu sebelum dianalisis. Hasil dinyatakan dalam bentuk nilai rerata $\pm$ SEM. ANOVA yang diikuti oleh uji post hoc digunakan dalam penelitian ini untuk menentukan perbedaan lintas waktu dan fase. Uji T dilakukan untuk menentukan perbedaan antar dua kelompok. **Hasil:** ditemukan apoptosis dalam bentuk peningkatan sel-sel germinal (80%) yang mengalami apoptosis secara bermakna ketika tikus diberikan TU+DMPA. Sel-sel germinal yang mengalami apoptosis dapat ditemukan pada beberapa spermatogonia (20%), spermatosit (30%), dan spermatid (50%). **Kesimpulan:** pemberian hormon TU+DMPA dalam dosis tertentu mungkin merupakan cara yang aman dan efektif dalam mengembangkan kontrasepsi pria yang cukup potensial.

Kata kunci: TU, DMPA, kadar sperma, apoptosis.

ABSTRACT

Aim: to investigate the safety and effectiveness of dose TU+DMPA hormones in developing potential male contraceptives. **Methods:** suppression of rat sperm concentration through increased apoptotic germ cells by in vivo administration of a long-acting androgen composed of a combination of testosterone undecanoate (TU) and depot medroxyprogesterone acetate (DMPA) was performed. Adult Sprague-Dawley rats received 2.5 mg TU every 6 week and 1.25 mg DMPA every 12 week for 60 week, a regimen known to rapidly reduce testosterone production by the testes and produce azoospermia within 12 week. Sperm concentration data were log transformed before analysis. Results are expressed as the mean $\pm$ SEM. ANOVA, followed by post hoc test was used to determine differences across time and phase. T test was employed to determine differences between two groups. **Results:** apoptosis revealed significant increase in apoptotic germ cells (80% when rats were administered with TU+DMPA. Apoptotic germ cells can be found in several spermatogonia (20%), spermatocytes (30%), and spermatids (50%). **Conclusion:** dose TU+DMPA hormones may be a safe and effective way to develop potential male contraceptives.

Key words: TU, DMPA, sperm concentration, apoptosis.

INTRODUCTION

Since World Health Organization studies using a prototype androgen-alone regimen, second-generation hormonal regimens featuring a combination of an androgen with a second gonadotropin-suppressing agent have been proven to be more effective than androgen-alone regimens.¹ Although GnRH antagonists are expensive and locally irritating² and estradiol is not sufficiently effective at tolerable doses³, numerous synthetic progestins have become available, with several progestin/androgen combination regimens showing promise for suppression of germ cells.^{4,5} A few studies have used a depot hormonal approach^{6,7} but no efficacy studies of a hormonal depot or an androgen/progestin combination have yet been reported. A desirable feature in a hormonal male contraceptive is freedom from high demands on medication compliance.

In a more recent dose-finding study in Indonesia, spermatogenesis was rapidly and completely suppressed after the administration of 500 mg TU in tea seed oil every 6 week in combination with 250 mg DMPA every 12 week.⁸ One explanation for this result is that the combination of TU and DMPA induces a more profound inhibition of gonadotropin and endogenous T levels, as evidenced by the suppression of serum LH and FSH, and spermatogenesis. One of the most susceptible pathways by which to disrupt spermatogenesis is interference with the function of the hypothalamic-pituitary-gonadal axis. This axis regulates spermatogenesis by controlling circulating levels of luteinizing hormone and follicle-stimulating hormone (FSH) through feedback regulation of the hormones.⁹

A number of approaches for male hormonal contraception using testosterone (T) esters have been widely investigated.¹⁰ In several studies, an androgen ester has been administered to both consistently suppress gonadotropin and serve as an androgen replacement. Azospermia could be achieved in 80% of Chinese men¹¹ and 94.7% of Indonesian fertile men¹² by administration of the androgen ester at 8 week and 6 week intervals, respectively. Intratesticular testosterone is decreased by suppression of gonadotropin secretion. In fact, caspase-3 activation has been shown to occur in rat germ cells within 1 week during in vivo reduction of

intratesticular testosterone, and internucleosomal DNA cleavage in germ cells continues to increase through the subsequent 3 week.¹³ Kim et al. (2001)¹⁴ examined the roles of caspase-3 and CAD activation in relation to the apoptotic death of germ cells. They showed that testosterone withdrawal results in spermatocyte apoptosis, and that this apoptosis is correlated with activation of caspase-3, as well as increased CAD protein expression. These results suggest that spermatocyte apoptosis, resulting from reduced intratesticular testosterone, is mediated by caspase-3 activation and CAD.

The present study is designed to estimate the contraceptive efficacy of a combination of DMPA with testosterone undecanoate at regular intervals.

METHODS

Animals

Adult male Sprague-Dawley rats (150 g to 250 g), purchased from BPOM Depkes RI Jakarta, were housed four per cage in a light-controlled room (12 h light:12 h darkness; lights on at 0700 H). The animals were handled daily for at least 1 week prior to the beginning of experimentation; food and water were available *ad libitum*. Rats were injected *i.m.* with either TU (1.25 mg/kg body weight at 6 week intervals) or DMPA (2.5 mg/kg body weight at 12 week intervals). The animals were killed by administration of an overdose of anesthetic at specific time points, and their testes were removed and weighed. The left and right testes were processed for histology after fixing in Bouin's solution.

Androgen/Progestin Preparation

Injectable testosterone undecanoate (Nebido) was manufactured by Schering AG, Germany and was provided in ampoules containing 1000 mg of the ester in 4 mL of castor oil. The same batch of TU was used throughout the study. DMPA was manufactured and distributed by a pharmacy in Jakarta. The registered trade name for this product is Depo-Geston. The compound was formulated as an aqueous suspension and packaged in 3 mL vials containing 150 mg DMPA.

Study Procedure

The study consisted of a pretreatment phase lasting at least 4 week, a suppression phase lasting 24 week, a maintenance phase lasting

36 week, and a recovery phase lasting 12 week.

During pretreatment phase period, three fluid samples were taken from the left and right cauda epididymis. Upon completion of the pretreatment period, 40 rats were randomly assigned to receive one of the following regimens: a) TU 2.5 mg (every 6 week) plus DMPA 1.25 mg every 12 week (treatment group) for 24 week. b) Control to treatment group (control group) for 24 week.

During the maintenance period, about 36 week, the rats received injections according to the schedule of the group to which they were assigned. During the recovery phase, rats underwent physical and sperm concentration examinations every 6 week for 12 week.

In-situ and Labeling of DNA in Apoptotic Cells

One testis from each rat was fixed in Bouin's fluid and paraffin embedded. Apoptotic cells were identified in tissue sections (5 mm) by ISEL using an in-situ death detection kit, POD (Cat No. 11 684 817 001, Roche). Briefly, sections were deparaffinized in xylene and rehydrated in a graded ethanol series (100%, 95%, 80%, and 70%), followed by phosphate-buffered saline (PBS; 10 mM sodium phosphate dibasic, 1.8 mM potassium phosphate monobasic, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The sections were digested with buffer citrate for 30 min at 95°C, washed with PBS, and then treated with PBS 4°C for 2 min. Sections were incubated with terminal deoxynucleotidyl transferase reaction mixture for 1.5 h at 37°C in a humidified atmosphere and the reaction stopped by immersion in buffer. Sections were then washed in PBS and incubated with peroxidase-conjugated anti-digoxigenin for 30 min at room temperature in a humid atmosphere. Following washing in PBS, sections were incubated with diaminobenzidine (DAB) substrate for 10 min at room temperature, washed in distilled water, dehydrated stepwise in ethanol (70%, 80%, 95%, and 100%) and xylene, and mounted with entelan. For quantification, slides were blinded and all tubules in two different sections of testis were counted for the absence or presence of 1 to 3 or >3 TUNEL-positive germ cells. For every animal, at least 400 tubules were scored for TUNEL positivity (modified method from Rasoulpour and Boekelheide, 2005).¹⁵

Statistical Analysis

Sperm concentration data were log transformed before analysis. Results are expressed

as the mean±SEM. $P<0.05$ was considered to be significant.

ANOVA, followed by post hoc test (software package, SPSS 13) was used to determine differences across time and phase. T test was employed to determine differences between two groups.

RESULTS

Germ Cell Apoptosis During in-vivo TU+DMPA Injection of Rats

The timing of germ cell apoptosis in relation to TU+DMPA injection in-vivo was studied by TUNEL assay to examine the possible involvement of germ cell apoptosis in TU+DMPA-induced azoospermia. Means of germ cell apoptosis in treated groups gradually increased during injections of the first and end regimens, and a significant increase was found at the 6th week of the treatment period when compared with pretreatment values. Means of germ cell apoptosis were maintained at very high levels throughout the 60 week of the treatment period (**Figure 1**). A trend towards a more sustained increase in germ cell apoptosis was observed in TU+DMPA combination groups compared with the control group, and their difference was significant ($P<0.05$). Germ cells began to recover during the recovery period (after cessation of TU+DMPA administration). By the 12th week of the recovery period, mean caspase-3 positive germ cells in the treated group had returned to the normal reference range (39.37 ± 5.32 cells/400 of tubules), and no significant ($P>0.05$) difference in this parameter was observed when values at this

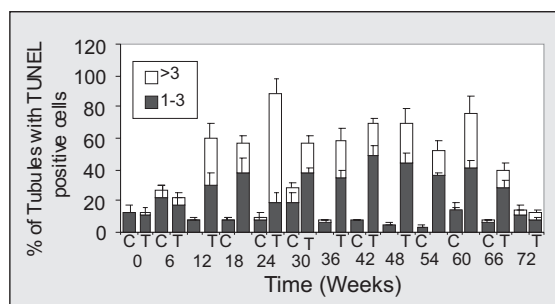


Figure 1. Quantification of the percentage of TUNEL-positive seminiferous tubules with 1 to 3 or >3 TUNEL-positive cells at 0, 3, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 wk (n; 5-5) during TU+DMPA or control (n;5-5) treated rat testes. Significant differences ($P<0.05$) are indicated by different letters. K0, pretreatment; K1, suppression; K2, maintenance; K3, recovery, C = control and T = treatment group

period were compared with the pretreatment of germ cell apoptosis in the untreated and treated groups.

All data were divided into four phases: pretreatment, suppression, maintenance, and recovery; these phases are shown in **Figure 2**.

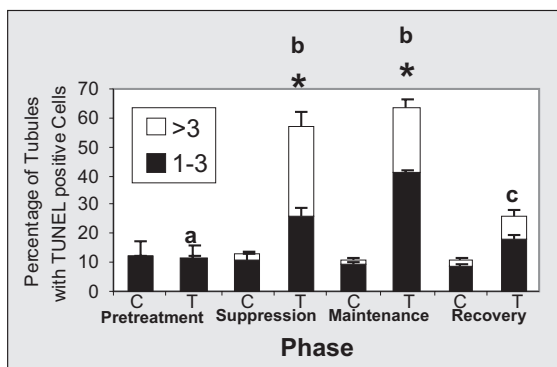


Figure 2. Percentage of tubules with TUNEL positive Cells in each phase during TU combined with DMPA for 72 wk. Values are expressed as the mean $\pm$ SEM.

*_{a,b} $P < 0.05$; C = control group, T = treatment group.

Localization of Germ Cell Apoptosis During TU+DMPA Injection in Vivo

TUNEL assay was used to further examine the possibility of relationship between TU+DMPA-induced apoptosis germ cells (**Figure 3**). Germ cells in the testes of rats were germ cell apoptosis negative (yellow arrow; **Figure 3A**), and cells showed evidence of germ cell apoptosis in the nucleus (blue and red arrows; **Figure 3A-H**).

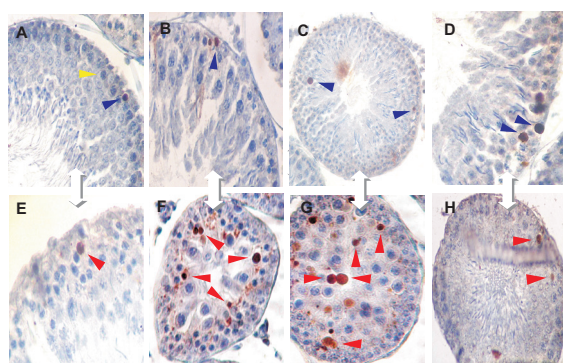


Figure 3. Localization apoptosis germ cells in germ cells pretreatment (A, control; E, treated) suppression (B, control; F, treated), maintenance (C, control; G, treated) and recovery (D, control; H, treated) phase post-TU+DMPA injection.

Sperm Concentration

Distribution of the rats and times for achieving azoospermia or severe oligozoospermia (≤ 3 million/mL during the treatment period) are

shown in **Figure 4**. Azoospermia or severe oligozoospermia was achieved and maintained in all rats during the suppression and maintenance phases, but sperm rebound occurred in one rat [sperm concentrations were 16.33 ± 1.53 million/mL] in the TU+DMPA group. A trend towards a more sustained suppression of spermatogenesis was observed in TU+DMPA combination groups than in the control group, and their difference was significant ($P < 0.05$). Spermatogenesis began to recover during the recovery period. However, recovery of spermatogenesis in the two combination groups was delayed and azoospermia was maintained for an additional 6 week after cessation of the treatment period. By the 12th week of the recovery period (at 72 week), the mean sperm concentration in the treated group had returned to the normal reference range [(109.47 ± 27.52) million/mL], and no significant difference ($P > 0.05$) in this parameter was observed when values at this period were compared with sperm concentrations in the control group (**Figure 5**).

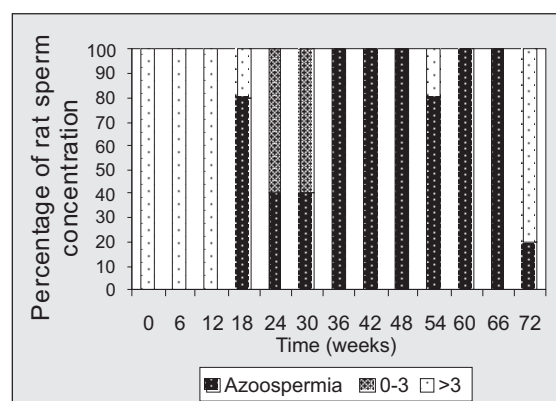


Figure 4. Percentage of azoospermia among rats receiving TU+DMPA per time point of the study

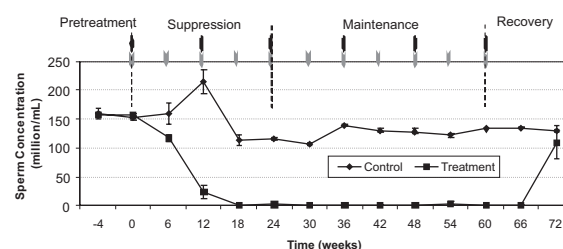


Figure 5. Mean sperm concentrations in each group during TU combined with DMPA at 6 wk injection intervals. Values are expressed as mean $\pm$ SEM. Arrows indicate hormone injections. The y-axis is represented as a log scale. ♦, Control group; ■, Treatment group.

Correlation Between Sperm Concentration and Germ Cell Apoptosis After Administration of TU+DMPA

When data were treated at the cellular level using comparisons with the relative controls, sperm concentration appeared significantly associated ($P < 0.000$) with the presence of germ cell apoptosis (**Figure 6**).

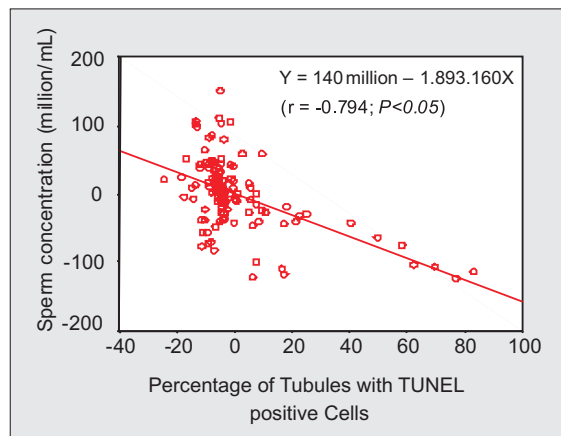


Figure 6. Regression diagram of apoptosis germ cells vs. mean of sperm concentration

DISCUSSION

The present study demonstrated that TU treatment every 6 week and TU+DMPA treatment every 12 week at doses of TU 1.25 mg/kg and DMPA 2.5 mg/kg, significantly influence apoptotic germ cells in rats. TU+DMPA slightly suppressed spermatogenesis during the 60 week treatment, whereas severe suppression resulted after continued treatment for 24 week. Recovery occurred 72 week after cessation of TU+DMPA treatment. However, such a degree of suppression may still lead to azoospermia, a possible explanation for which is that germ cells suppression may be associated with the impaired release of late spermatids, as observed in men and monkeys.⁶ After TU+DMPA treatment for 18 week, 80% of the rats became azoospermic. Few studies have investigated whether or not spermatogenesis, in terms of germ cell numbers, could fully recover after drug treatment that induces gonadotropin withdrawal. Hikim and Swerdloff (1999)¹⁶ reported a complete reversal of late spermatid number, testicular weight, and tubule diameter 6 week after cessation of Nal-Glu-GnRH treatment in rats. The present study also showed the complete restoration of spermatogenesis after TU+DMPA suppression.

During normal adult spermatogenesis,

spontaneous apoptosis occurs at various phases of germ cell development and mainly in pre-meiotic cells. In addition to spontaneous apoptosis, apoptotic cell death of rat germ cells can be induced during administration of TU+DMPA combination (**Figure 1**). The influence of TU+DMPA injection decreases intratesticular testosterone. Adult mammalian spermatogenesis is a testosterone-dependent process, and many studies have shown that testosterone withdrawal from the rat testis results in increased germ cell apoptosis. However, the mechanism by which testosterone regulates spermatogenesis remains uncertain.^{17,18} In Nandi's study,¹⁹ ethane 1,2-dimethanesulfonate (EDS), a Leydig cell toxicant, was used to kill Leydig cells, thus reducing intratesticular testosterone levels in Sprague-Dawley rats. After the treatment, germ cell apoptosis and testicular fas protein expression increased.¹⁹ In another similar study conducted by Woolveridge's group, they found that 8 day after treatment with EDS, germ cell apoptosis index increased 24-fold compared with the control group. After withdrawal of androgen, no significant changes in Bcl-xl, Bak, and Bad levels were observed. However, the expressions of Bcl-2 and Bax were upregulated.²⁰ Cell adhesion has been shown to play an essential role in the regulation of programmed cell death in epithelial cells, known as FSH, and testosterone withdrawal results in the triggering of apoptosis, as well as germ cell detachment from the seminiferous epithelium. After hormone suppression by short-term estradiol benzoate treatment of rats, germ cell apoptosis was observed, and the triggering of apoptosis preceded germ cell detachment.²¹ Addition of TU+DMPA resulted in improved suppression of germ cells to azoospermia. A long-acting TU+DMPA treatment given twice weekly has shown promise in a small number of subjects, producing an azoospermia rate of 80% at 18 week and, as such, is worthy of further consideration. However, problems associated with local injection site reactions, including erythema and induration, continue to arise.

Good correlation was observed between the number of apoptotic germ cell and the number of sperm concentration in all testicular specimens ($P < 0.01$, Spearman's rank = -0.700). Although the causes and molecular mechanisms involved in germ cell apoptosis are far from defined, programmed cell death appears to be important to

adjust germ cell numbers to that of the supporting Sertoli cells and ensure quality control of the gametes produced.

Apoptotic germ cell return decreased at the maintenance phase ($P>0.05$) versus the control or pretreatment phase. Long-acting TU+DMPA injection increased the number of apoptotic germ cells significantly, which was reversible when injection was discontinued. Taken together, the data essentially illustrate the important role of apoptosis in the proposed combination-drug contraception, and the extent of apoptosis was TU+DMPA dose-dependent. Both intrinsic and extrinsic pathways participate in the process.

CONCLUSION

The use of TU+DMPA hormones may be a safe and effective way to develop potential male contraceptives, and further studies on this topic should be carried out.

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