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Extended intervention time and evaluation of sperm suppression by dienogest plus testosterone undecanoate in male rat☆

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Abstract

Background: The potential of using dienogest [DNG, 40 mg/kg body weight (bw)] plus testosterone undecanoate (TU, 25 mg/kg bw) in rats for development of a once-a-month male hormonal contraceptive has been reported earlier in our laboratories.
Study Design: In the present study, we report a separate efficacy evaluation of the same combination, DNG (40 mg/kg bw) and TU (25 mg/kg bw) in which interval of drug administration has been extended further to 45 and 60 days instead of every 30 days.
Results: Complete sperm suppression was observed in rats sacrificed either 60 or 90 days after DNG+TU administration, for two injections at 45-day interval. The neutral α-glucosidase activity in these treated rats remained in the normal range. Germ cell loss due to apoptosis was frequently observed both after 60 or 90 days of combination treatment. Significant decline in serum gonadotropin and testosterone, both serum and intratesticular levels, were observed in the treated rats. Following stoppage of treatment (given at 45-day interval) after two (0 and 45 days) or three injections (0, 45 and 90 days), complete restoration of spermatogenesis was observed by 120 and 165 days, respectively. The sperm suppression, however, could not be sustained when the period of combined drug administration was extended from every 45 to 60 days.
Conclusions: Dienogest plus testosterone undecanoate in the above doses retained contraceptive effectiveness when administered every 45 days but not 60 days. The spermatogenic arrest was completely reversible once drug treatment is stopped. The dose and the frequency of intervention can be extrapolated in future clinical trials.

Keywords: Dienogest; Testosterone undecanoate; Spermatogenic arrest; Reversibility; Germ cell loss; Apoptosis

1. Introduction

Hormonal contraception is based on the regulation of the classical gonadal–pituitary feedback loop, which has been successfully exploited in females with the development of hormone-based pills and injectables. Similar approaches are not available for men as male-directed contraceptive options are extremely limited. Emerging data now indicate that an identical approach could also provide a reversible option for suppression of fertility in men with efficacy similar to that of the female hormonal contraception [1]. Of the various approaches tried for the purpose, testosterone alone was found to be effective in achieving azoospermia [2–5], but due to the lack of high efficacy combined with significant side effects [6,7], alternate protocols using combinations of testosterone and progestins are preferred. These include testosterone plus progestins such as medroxyprogesterone acetate [8], cyproterone acetate [9], desogestrel [10], levonorgestrel [11] and norethisterone enanthate [12]. These combinations have been shown to be highly effective in terms of sperm suppression and do not induce any major changes in clinical and laboratory parameters, thus promising to be safe for long-term use [13].

Another progestin, dienogest (DNG), has also been used to suppress sperm production both in animals [14,15] and men [16] either alone or given in combination with testosterone. It not only provides the benefits of a norprogestin with strong progestational activity, high

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bioavailability, low liver impact but also demonstrates moderate antiandrogenic action and excellent tolerability [16]. We were earlier encouraged to evaluate the contraceptive potential of DNG [40 mg/kg body weight (bw) administered every 4 weeks, three injections] in male rats in combination with testosterone undecanoate (TU, 25 mg/kg bw given every 6 weeks, two injections), which was found to be highly efficacious in arresting spermatogenesis after 60 days. Sperm production was arrested at the round spermatid stage, and the effect was found reversible after withdrawal of treatment [17]. As extended and one-time interventions lead to better acceptability of any contraceptive method, the potential of sperm suppression by the DNG plus TU combination was again reexamined in the present study by extending the interval of drug administration from 30 to 45 and 60 days and administering both the drugs at the same time but at two different locations.

2. Materials and methods

2.1. Drugs and injection

Dienogest (17α-cyanomethyl-17β-hydroxy-estra-4,9-dien-3-one; code STS557) in powder form and TU in oil form were generous gifts from Schering AG, Germany. Dienogest was first dissolved in isopropanol and then made into a homogenous solution in olive oil by adequate vortexing. The mixture was kept open at room temperature overnight for evaporation of the alcoholic portion part before being administered. Testosterone undecanoate was further diluted in olive oil. The concentrations of drugs were made in such a way that 100 μL of each contain the desired dose to be injected intramuscularly. Dienogest 40 mg/kg bw and TU 25 mg/kg bw were administered to rats intramuscularly once every 45 or 60 days until the end of treatment after 60 or 90 days of the first injection; after which the animals were sacrificed at either 60 or 90 days. Irrespective of the duration of treatment, each rat from these groups received at the maximum two injections of both the drugs. To analyze reversibility in sperm production, the treatment was stopped after two (at 60 days) or three (at 90 days) injections and the animals were sacrificed at 75 days after the last injection.

2.2. Animals, tissue preparation and histology

Adult male rats (Wister strain), weighing 200–220 g, were maintained under controlled temperature (25°C±2°C) and constant photoperiodic conditions (12-h light/dark) with food and water ad libitum. The animals received 100 μL of the drug (DNG+TU) or olive oil every 45 days [until sacrificed at 60 (two injections) or 90 days (two injections)] or 60 days [until sacrificed at 60 days (one injection) or 90 days (two injections)] under strict compliance with Standard Institutional Guidelines for Animal Care. To test the effect on sperm suppression and reversal following stoppage of treatment, experiments were carried out in animals subdivided into the following groups with six animals in each group. The animals in the last group (e) were sacrificed 75 days after the last injection to assess the reversibility of sperm suppression.

Set 1		
Treatment (groups)	**Injection at (days)**	**Sacrifice at (day)**
(a) Olive oil (vehicle control)	(0 and 45, two injections)	(60)
(b) TU (25 mg/kg bw) only	"	"
(c) DNG (40 mg/kg bw) only	"	"
(d) DNG (40 mg/kg bw)+TU (25 mg/kg bw)	"	"
(e) DNG (40 mg/kg bw)+TU (25 mg/kg bw)	"	(120 days)
Set 2:		
Treatment (Groups)	**Injection at (days)**	**Sacrifice at (day)**
(a) Olive oil (vehicle control)	(0 and 45, two injections)	(90)
(b) TU (25 mg/kg bw) only	"	"
(c) DNG (40 mg/kg bw) only	"	"
(d) DNG (40 mg/kg bw)+TU (25 mg/kg bw)	"	"
(e) DNG (40 mg/kg bw)+TU (25 mg/kg bw)	(0, 45 and 90, three injections)	(165)
Set 3:		
Treatment (Groups)	**Injection at (days)**	**Sacrifice at (day)**
(a) Olive oil (vehicle control)	(0, one injection)	60
(b) TU (25 mg/kg bw) only	"	"
(c) DNG (40 mg/kg bw) only	"	"
(d) DNG (40 mg/kg bw)+TU (25 mg/kg bw)	"	"
(e) DNG (40 mg/kg bw)+TU (25 mg/kg bw)	(0 and 60, two injections)	(135)
Set 4:		
Treatment (Groups)	**Injection at (days)**	**Sacrifice at (day)**
(a) Olive oil (vehicle control)	(0 and 60, two injections)	90
(b) TU (25 mg/kg bw) only	"	"
(c) DNG (40 mg/kg bw) only	"	"
(d) DNG (40 mg/kg bw)+TU (25 mg/kg bw)	"	"
(e) DNG (40 mg/kg bw)+TU (25 mg/kg bw)	(0, 60 and 120, three injections)	(195)

The animals were sacrificed at the end of 60 and 90 days of treatment, and organ weights of testes and accessory sex organs were recorded. Testes were immediately fixed in buffered formalin and stepwise dehydrated in graded ethanol. Tissues were blocked in paraffin and the sections (4 μm) were stained with hematoxylin and eosin. Cauda epididymis was dissected out, snap-frozen in liquid nitrogen and stored at −20°C until used for estimation of α-glucosidase activity.

2.3. Assay for α-glucosidase

Cauda epididymis was taken out from liquid nitrogen, grinded in phosphate-buffered saline (PBS, pH 7.4) and

centrifuged at 4000 rpm for 10 min. The supernatant was used for the estimation of neutral α-glucosidase activity [18].

2.4. Quantitation of spermatogenesis

Quantitation of spermatogenesis in the testes of control and treated rats (n=6) was carried out as described by Russell and Clermont [19]. Briefly, 20 tubules in each testicular section were randomly picked and included in the analysis. Numbers of different types of germ cells present in a tubule were recorded through microscopic examination. All the nuclear count (crude counts) of the germ cells were corrected for differences in nuclear diameter by the formula of Abercrombie [20], i.e., true count=(crude count×section thickness)/(section thickness+nuclear diameter of germ cells) and tubular shrinkage by the Sertoli cell correction factor [21].

2.5. Evaluation of sperm from epididymis

The contents of the epididymis were collected after finely mincing the tissue in 1 mL of PBS. Percentage of intact sperm in the suspension was examined under the microscope at ×400 magnification [18].

2.6. Evaluation of germ cell apoptosis

In Situ Apoptosis Detection Kit (TA4625; R&D Systems, Minneapolis, MN) was used, which detects DNA fragmentation to identify apoptotic cells in the seminiferous epithelium. Deparaffinized testis sections were utilized. To make the DNA accessible to the labeling enzyme, the cell membranes are permeabilized with proteinase K. Endogenous peroxidase activity was quenched using hydrogen peroxide. Next, biotinylated nucleotides were incorporated into the 3′-OH ends of the DNA fragments by terminal deoxynucleotidyl transferase. The biotinylated nucleotides were detected using streptavidin–horseradish peroxidase conjugate followed by the substrate, diaminobenzidine. The enzyme reaction generates an insoluble colored precipitate where DNA fragmentation has occurred. Diaminobenzidine-stained samples were examined using a Nikon microscope image analyzer and photographed. Randomly, 20 tubules were picked up to count the average number of in situ end-labeling (ISEL)-positive germ cells in each tubule.

2.7. Studies on reversibility of sperm production

A group of animals from experimental sets 1 and 2 stopped treatment of the drug (DNG+TU) after two or three injections, respectively. In set 3, the treatment was stopped after one injection, and in set 4, after two injections, since the duration of drug administration was extended to 60 days. Rats were sacrificed as specified. Spermatogenesis revival was examined in histological sections.

2.8. Hormone estimations

Serum luteinizing hormone (LH; Cusabio Biotech, Hubei, China), follicle-stimulating hormone (FSH; Immunodiagnostic-systems, Biocode-Hycel, Liege, Belgium), testosterone and intratesticular testosterone (DRG Diagnostics, Marburg, Germany) levels were determined using ELISA kits. Samples collected were directly used, and measurements were made according to manufacturer's instructions. For intratesticular testosterone measurement, the testis was homogenized in 8 mL of 10 mM PBS, and the homogenate was extracted with diethyl ether, air-dried and resuspended in PBS. The minimum detection limits of the assay for rat LH was 0.5 mU/mL, FSH 0.2 ng/mL and testosterone 0.083 ng/mL.

2.9. Statistical analysis

Quantitative data were expressed as mean±SEM. Statistically significant differences between normally distributed groups were determined by two-tailed t test and ANOVA. $p<.05$ was considered statistically significant.

3. Results

3.1. Extending intervention time vs. the effect on sperm suppression

While spermatogenesis was normal (Figs. 1A and 2A) in vehicle-treated animals, those receiving combination regimen of DNG+TU every 45 days and sacrificed either at 60 (Fig. 1D) or 90 days (Fig. 2D) demonstrated complete arrest of spermatogenesis. Spermatogenic arrest was accompanied by complete absence of elongated spermatids and sperm from the affected seminiferous tubules. Significant reduction in the number of round spermatids was observed in both treatment groups, which was associated with reduction in preleptotene, leptotene and pachytene spermatocytes (Fig. 3A, B). Spermatogenesis was partially affected in animals treated with either TU (Figs. 1B and 2B) or DNG (Figs. 1C and 2C) given alone but with the same durations as DNG+TU. In conformity with histological observations, there was significant ($p<.001$) decrease in the testis weight of the treated animals. Epididymal preparations were completely devoid of sperm in the DNG+TU-treated group (Tables 1 and 2). The weights of accessory organs, epididymis, seminal vesicle and prostate were identically reduced, more in those sacrificed at 90 days than at 60 days of receiving the first injection. However, no significant change in body weight over time was observed. Neutral α-glucosidase activity was also unaffected in all the treated animals for both durations (Tables 1 and 2). Sperm suppression failed when the duration of the DNG+TU treatment (sets 3 and 4) was extended to every 60 days (data not shown).

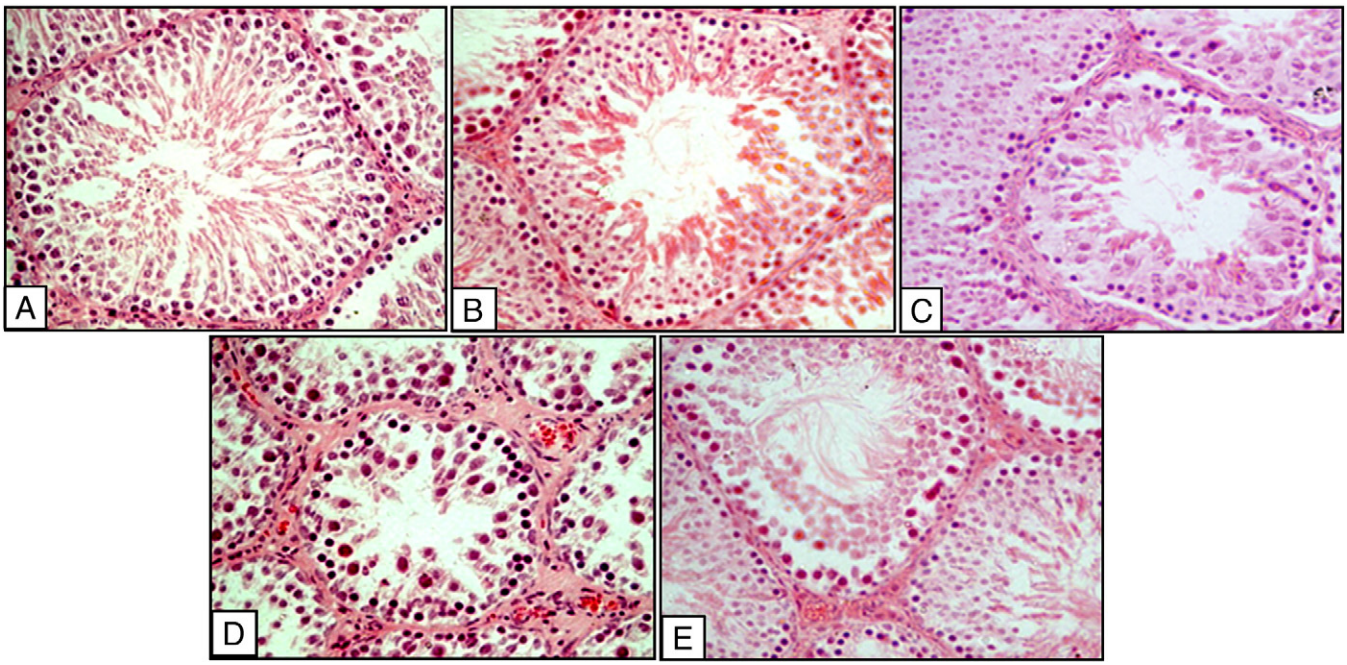

Fig. 1. Histological sections of rat testes stained with hematoxylin and eosin. Normal spermatogenesis is seen in the control testis (A). Spermatogenesis was partially affected with TU 25 mg/kg bw (B) or DNG 40 mg/kg bw (C) given alone every 45 days (0 and 45 days) and animals sacrificed at day 60. Spermatogenesis was, however, completely arrested in rats receiving combination treatment of DNG+TU (D). Full restoration of spermatogenesis was seen when the treatment was stopped after two" injections (at 45 days) and animals sacrificed after 75 days of last injection (E) (n=6/group, original magnification ×400).

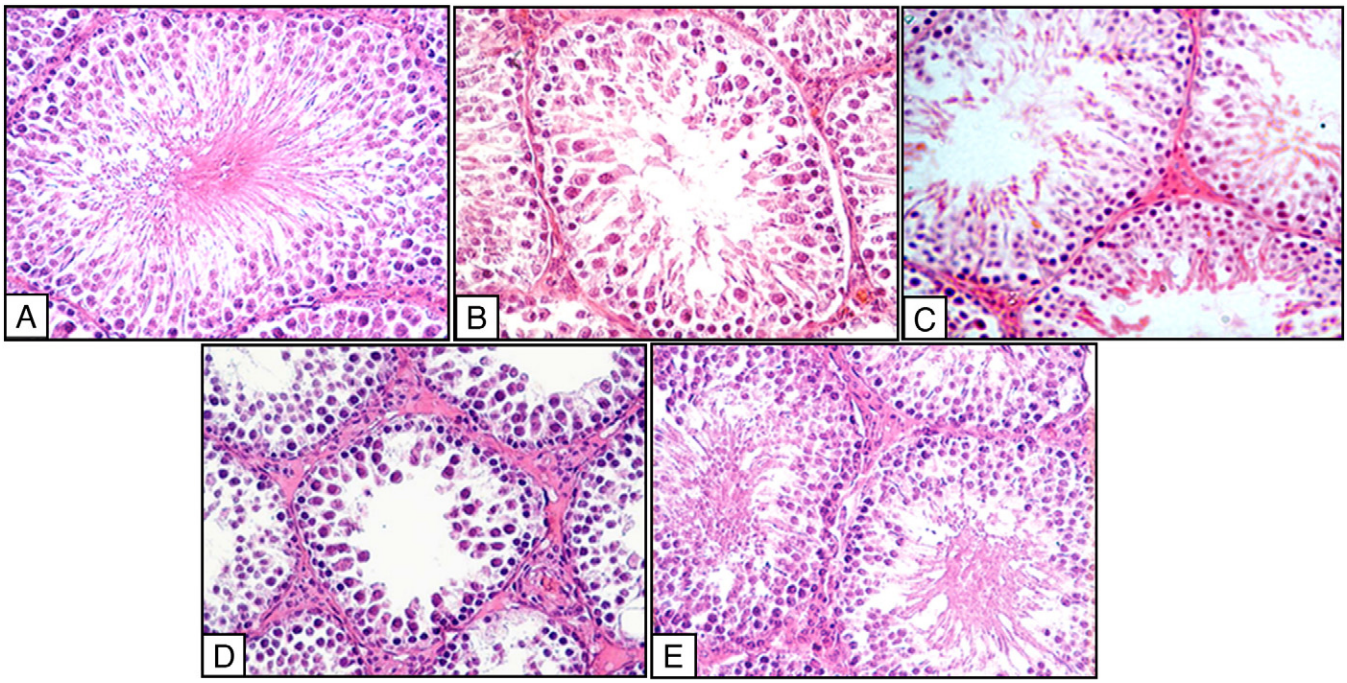

Fig. 2. Histological sections of rat testes stained with hematoxylin and eosin. Normal spermatogenesis is seen in the control testis (A). When the animals were sacrificed at day 90 after the first injection after receiving drugs (0 and 45 days), complete suppression of spermatogenesis is maintained in rats administered with DNG+TU (D). The effect was marginal in rats receiving either TU 25 mg/kg bw (B) or DNG 40 mg/kg bw (C) alone. Full restoration of spermatogenesis was seen when the treatment was stopped after three injections (0, 45 and 90 days) and animals sacrificed after 75 days after last injection (E) (n=6/group, original magnification ×400).

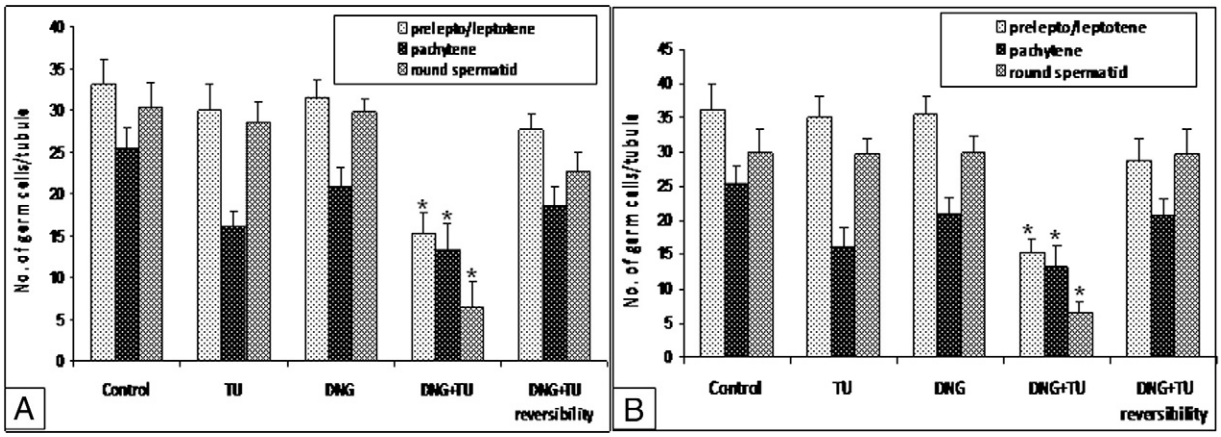

Fig. 3. Quantitation of germ cell numbers per tubule (mean±SE) from 20 randomly selected tubules in each testis section from control vs. treated rats. A significant (∗p<.001) decrease in number of germ cells is seen in DNG+TU treatment group (∗p<.001) analyzed after receiving two injections (0 and 45 days) and sacrificed at 60 days (A) or 90 days (B) (n=6/group, original magnification ×400).

3.2. Apoptosis in testicular cells

A significantly (p<.001) large number of cells were found ISEL-positive in the testicular sections of rats administered with DNG+TU every 45 days and sacrificed either at 60 days (Fig. 4E, f) or 90 days (data not shown) of the initial treatment. Germ cell apoptosis was also more prevalent in the seminiferous epithelium of TU- (Fig. 4C) than DNG-treated (Fig. 4D) rats compared with vehicle-treated controls (Fig. 4D). Fig. 4A represents the negative control. The prevalence of apoptotic cells per tubule in different treatment groups is shown in Fig. 4F.

3.3. Reversibility of spermatogenesis following stoppage of treatment

When DNG+TU treatment was stopped either after 45 (Fig. 1E) or 90 days (Fig. 2E), there was significant repopulation of germ cells in the seminiferous epithelium (Fig. 3A, B). The elongated spermatids and sperm reappeared in the seminiferous epithelium (Figs. 1E and 2E). In conformity with the spermatogenesis revival, sperm density in the epididymal suspensions was significantly restored and found comparable with vehicle-treated controls (Tables 1 and 2).

3.4. Hormones

Significant (p<.01) reduction in the serum levels of LH and FSH was observed in rats sacrificed at 60 (Fig. 5A, C) or 90 days (Fig. 5B, D) of DNG+TU given every 45 days. Following stoppage of the treatment at 45 or 90 days, a complete restoration in the levels of serum gonadotropins was observed when analyzed, respectively, at 120 or 165 days after receiving the first injection (Fig. 5). Serum testosterone levels, on the other hand, declined significantly (TU p<.001, DNG p<.01) in all the treated animals when examined at 60 or 90 days of treatment (Fig. 6A, B). However, the decrease was more pronounced in the animals treated with DNG+TU than with individual treatments. Intratesticular testosterone levels were observed to be significantly (p<.001) low at 60 days (Fig. 6C) in the combination treatment group, which remained unchanged until 90 days (Fig. 6D) of the first treatment.

4. Discussion

The above findings indicate that DNG and TU given every 45 days did not lead to a decline in contraceptive efficacy. However, if the duration of drug administration is

Table 1
Weights of reproductive organs, sperm count and mean±SE neutral α-glucosidase activity in epididymis of rats treated with DNG 40 mg/kg bw+TU 25 mg/kg bw either given alone or in combination every 45 days and sacrificed after 60 days of first treatment

Treatment	Testis (g)	Epididymis (g)	Seminal vesicle (g)	Prostate (g)	Epididymal sperm count (10^6/mL)	Neutral α-glucosidase (U/L)
Control	1.62±0.10	0.951±0.1	1.15±0.102	0.618±.096	375±27.38	6.63±.27
TU	0.97±0.341	0.662±0.242	1.148±0.094	0.405±.053	294.16±22.36	5.01±0.46
DNG	1.05±0.254	0.790±0.077	0.928±0.028	0.442±.137	234.5±14.3	5.36±0.57
DNG+TU	0.388±0.103*	0.445±0.076*	0.489±0.212*	0.388±.028*	Nil	4.30±0.132
DNG+TU reversibility	1.07±0.126	0.941±0.067	1.02±0.211	0.568±.069	295.16±9.61	6.07±0.091

* p≤.001.

Table 2
Weights of reproductive organs, sperm count and neutral α-glucosidase activity (mean±SE) in epididymis of rats treated with DNG 40 mg/kg bw+TU 25 mg/kg bw either given alone or in combination every 45 days and sacrificed after 90 days of first treatment

Treatment	Testis (g)	Epididymis (g)	Seminal vesicle (g)	Prostate (g)	Epididymal sperm count (10^6/mL)	Neutral α-glucosidase activity (U/L)
Control	1.430±0.46	1.11±0.007	0.725±0.037	0.585±0.04	455.23±23.11	4.665±0.327
TU	1.05±0.528	0.980±0.10	1.09±0.37	0.462±0.049	395.09±13.31	5.23±0.96
DNG	1.131±0.073	1.075±0.024	0.834±0.059	0.468±0.096	385.41±24.09	4.09±1.57
DNG+TU	0.391±.022*	0.215±0.067*	0.265±0.134*	0.304±0.155*	Nil	3.44±0.23
DNG+TU reversibility	1.095±0.82	0.983±0.07	0.821±0.93	0.495±1.03	390.19±19.37	4.60±0.91

* p≤.001.

extended to 60 days, it fails to suppress sperm production in the testis. Spermatogenic arrest is associated with an increase in germ cell apoptosis and complete absence of sperm and elongated spermatids from the affected seminiferous tubules. Stoppage of treatment either after two or three injections restores complete spermatogenesis after 75 days of the last injection. The goal of hormonal male contraception is the suppression of spermatogenesis to azoospermia. Arrest of spermatogenesis in tissue sections and complete absence of sperm in the epididymal preparations was the end-point of the study analysis, as azoospermia cannot be examined in the model presently used. Using the same analysis, we earlier reported the dose standardization and contraceptive efficacy of DNG and TU in rats to develop a once-a-month injectable male contraceptive [17]. In the present study, the same efficacious dose of DNG+TU was used, which demonstrated no loss of potency in suppressing spermatogenesis when given every 6 weeks instead of 4 weeks. Dienogest plus testosterone buciclate, a long-acting testosterone ester, was earlier successfully tried as a male contraceptive in bonnet monkeys, but DNG was administered daily at a dose of 12 mg/monkey for 15 weeks [22]. Considering that the weight of the monkeys are around 10 kg, DNG dose per monkey works out to a meager 1.2 mg/kg bw every day. Although the dose appears very low, cumulatively given in oil preparations everyday for 15 weeks, it was very much higher than the dose level presently utilized. A very similar study has been recently reported in which human subjects were given a daily administration of 10 mg of DNG for 21 days. It resulted in profound suppression of gonadotropins and testosterone,

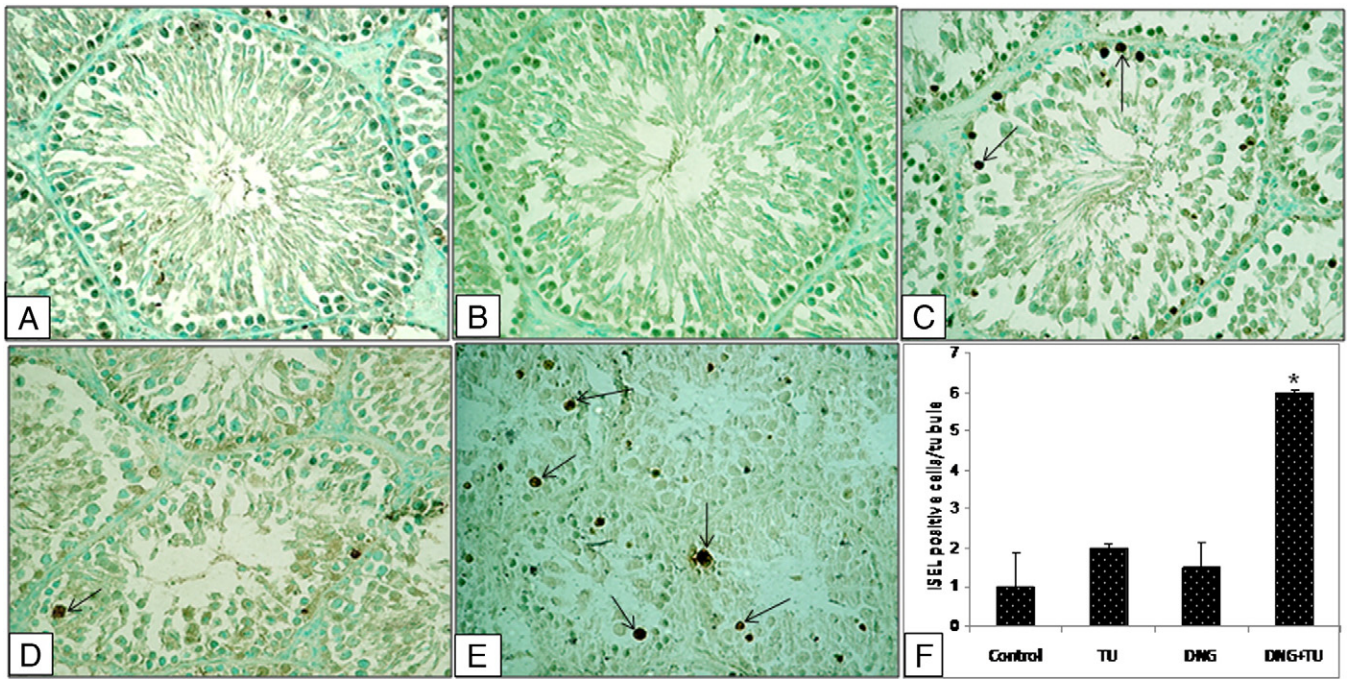

Fig. 4. In situ end-labeling of DNA in testicular sections. A representative section showing ISEL-positive cells/tubule from 20 randomly selected tubules of rats administered with DNG+TU (E) compared with sections from negative control (A), age-matched vehicle control (B), TU alone (C) and DNG alone (D). Compared with other treatments, a significant increase (mean±SE) in ISEL-positive germ cells (→) were observed in tubules of rats receiving combination treatment (F) (n=6/group, original magnification ×400). ∗p<.001.

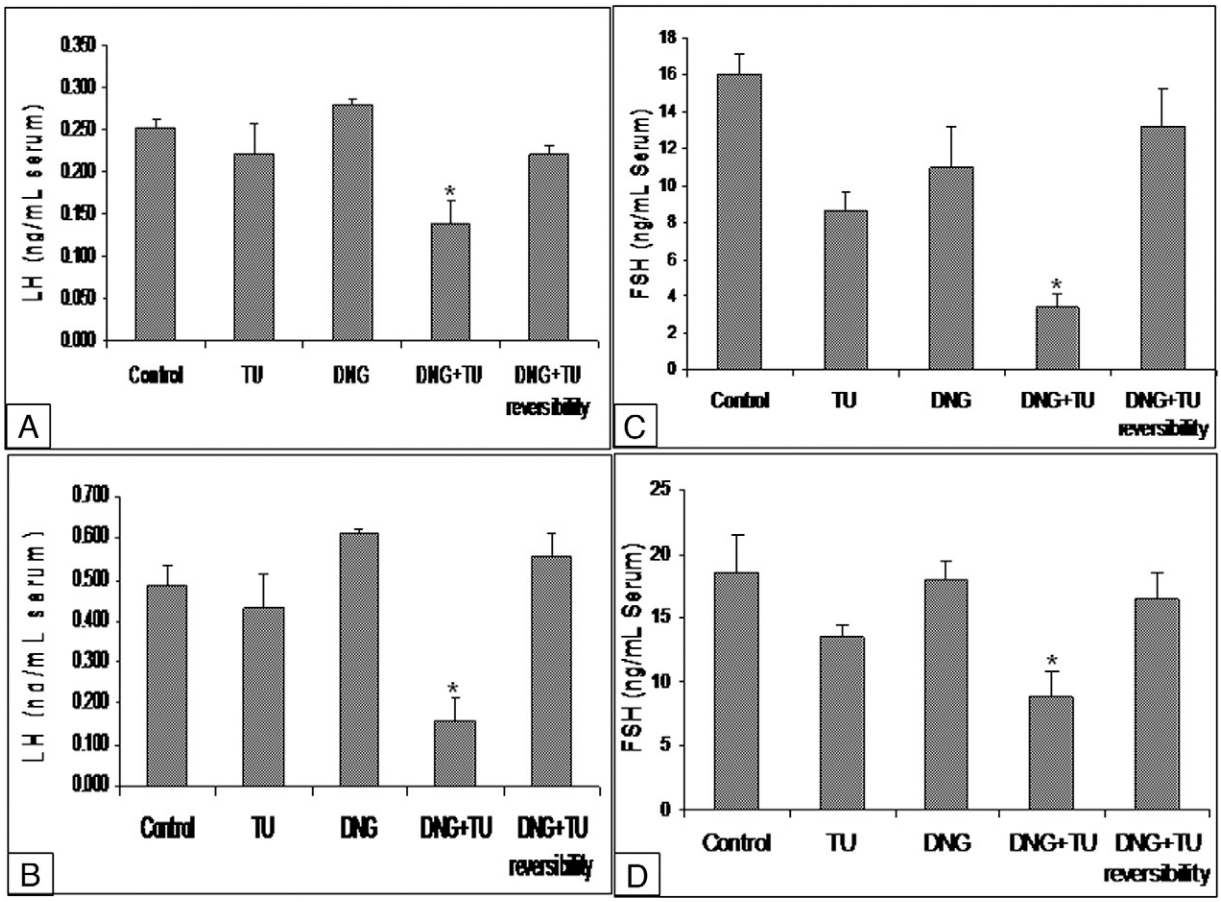

Fig. 5. Serum levels of gonadotropins (mean±SE) in rats treated with DNG+TU combination compared with other treatment groups analyzed following two injections (0 and 45 days). When analyzed at 60 or 90 days of first treatment, both LH (A, B) and FSH (C, D), respectively, demonstrated a significant (∗$p<.01$) decline in the combination treatment group. Although not fully restored, gonadotropin levels showed considerable increase following stoppage of treatment after two injections (n=6/group).

which was associated with a significant decline in sperm concentration compared with baseline groups [16]. Unless detailed pharmacokinetics data are in place, it is difficult to justify such high doses of DNG to suppress sperm production. On the other hand, given in combination with TU, DNG at much lower doses and with less frequent interventions achieves identical gonadotropin (Figs. 5 and 6) and spermatogenesis (Figs. 1 and 2) suppression as observed in the present study. This effect should be considered as synergistic as individual drugs given alone failed to bring down either of the parameters to the levels attained by DNG+TU combination treatment.

A long interval between injections such as monthly to a 3-monthly interval is more acceptable to approximately 40% of the men [23]. Testosterone undecanoate (1000 mg) combined with norethisterone enantate (200 mg) given every 6 weeks over a period of 24 weeks resulted in azoospermia in 13 of 14 volunteers. During this entire treatment period, mean serum testosterone concentrations remained within normal limits while gonadotropins demonstrated marked suppression [24]. Although periodic measurements were not carried out in the present study, identical suppression of gonadotropins was observed (Fig. 5). However, serum testosterone also decreased significantly compared with vehicle-treated controls (Fig. 6). Although serum testosterone declined in all other treatment groups, it was estimated comparatively low in the combination treatment group. Accordingly, the testosterone-dependent accessory organs registered a steady fall in organ weights at the end of 60 (Table 1) or 90 days (Table 2) of treatment.

Many recent studies in which prototype androgen–progestin regimens have been tested indicate that these regimens are promising candidates for male contraception [25]. The purpose of this hormonal combination is to induce a more profound inhibition of gonadotropins and, consequently, of spermatogenesis while avoiding side effects due to any increase in the peripheral androgen concentrations. Instead of increasing, the serum levels of testosterone indeed declined in the present study, which has raised the issue as to what extent it needs to be suppressed on one hand, inhibiting spermatogenesis and maintained on the other hand, fulfilling the required important physiological functions. Optimal

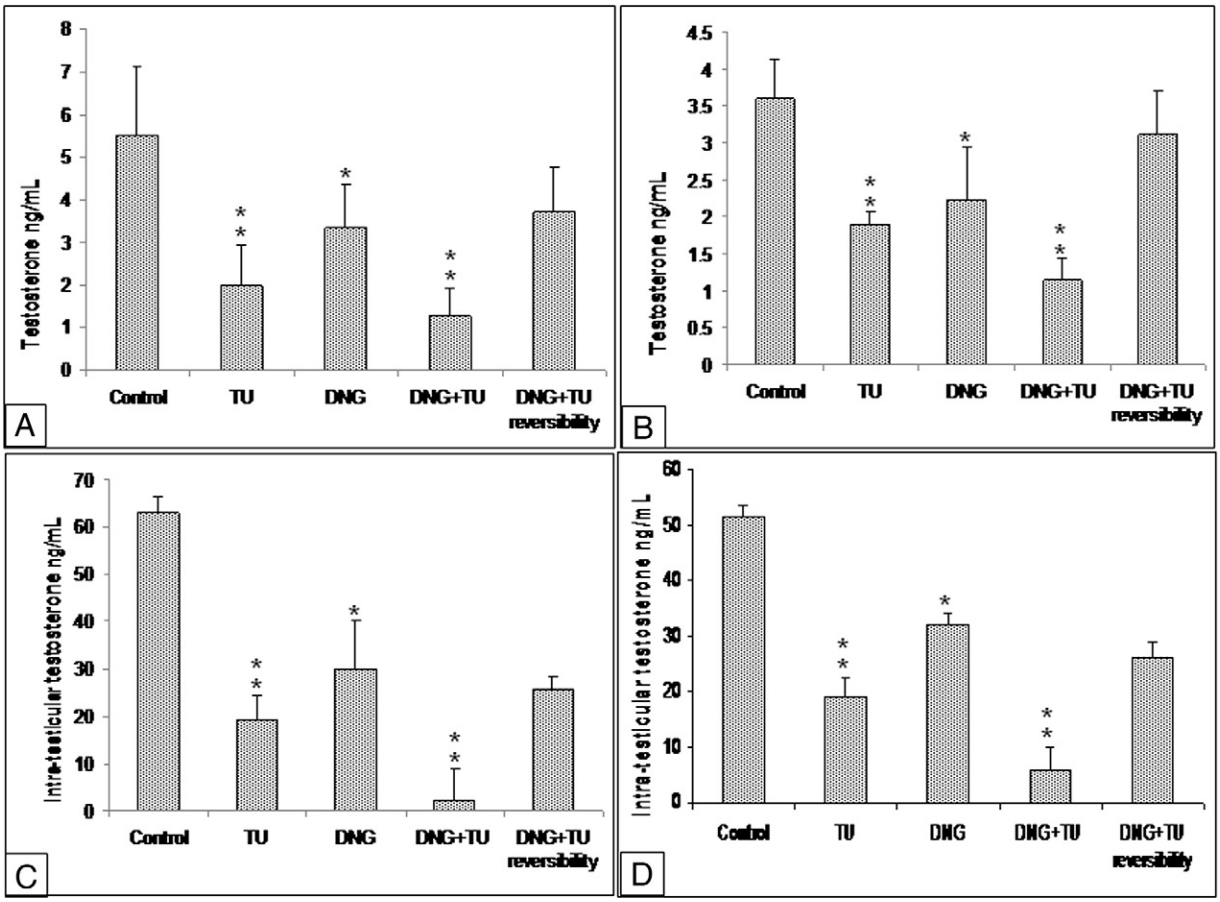

Fig. 6. Serum and intratesticular levels of testosterone (mean±SE) in rats treated with DNG+TU combination compared with other treatment groups analyzed following two injections (0 and 45 days). When analyzed at 60 or 90 days after first treatment, both serum (A, B) and intratesticular (C, D) testosterone, respectively, declined significantly (∗p<.001) in the combination treatment group. Although not fully restored, a complete upward revision of the trend was noted with both the parameters following stoppage of treatment after two injections (n=6/group).

testosterone support is critical for spermatogenesis [13], but high testosterone availability is counterproductive in suppressing spermatogenesis [26,27]. Similarly, intratesticular androgen concentrations might be a key factor for maintenance of sperm production. Higher dihydrotestosterone levels were reported in men who remained oligospermic following administration of testosterone enanthate 200 mg/week [28]. As seen with either DNG or TU alone treatment, a significantly low peripheral and intratesticular testosterone compared with controls has very little effect on sperm suppression in these animals (Figs. 1 and 2). However, if the levels go down further as seen with DNG+TU treatment, spermatogenesis is completely arrested but simultaneously affects the weights of accessory organs. The low basal level of testosterone is, however, able to support the normal epididymal function since neutral α-glucosidase activity remained unaltered in the treated animals (Tables 1 and 2).

Inhibition of spermatogenesis is associated with an increase in germ cell apoptosis in the treated animals (Fig. 4). Although less prevalent under normal conditions, frequency of germ cell apoptosis rises under conditions of hormonal depletion, ischemia or cryptorchidism [29–31]. The insufficiency in the levels of reproductive hormones following DNG+TU treatment results in the severe reduction of number of germ cells per tubule (Fig. 3), which may be subsequent to apoptotic induction and removal of germ cells from the seminiferous epithelium. Stoppage of treatment either after 2 (45 days) or 3 (90 days) injections reverses the adverse effect on seminiferous epithelium with qualitatively full restoration of spermatogenesis (Figs. 1E and 2E). Although the gonadotropin levels are not completely restored, the significant increase in LH concentration following withdrawal modulated an identical increase in serum testosterone (Fig. 6), supporting sperm production. The present findings confirm the potential of the DNG+TU combination as a potential candidate for male contraception but needs to be tested further in clinical trials. Since the metabolism of DNG will be quite different in men, the doses used in the present study may not be directly extrapolated. However, these results will be useful for similar dose standardization studies involving human volunteers.

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