Complete Sperm Suppression in Rats With Dienogest Plus Testosterone Undecanoate Is Facilitated Through Apoptosis in Testicular Cells

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Abstract

Complete suppression of the production of sperm in rats with dienogest (DNG, 40 mg/kg body weight [bw]) plus testosterone undecanoate (TU, 25 mg/kg bw), every 45 days, was found to be associated with a significant increase in germ cell apoptosis. Caspase 3 activity and expression in testis were simultaneously upregulated. Rise in the activities of caspase 8 and 9 was associated with overexpression of upstream marker proteins from extrinsic (Fas [Fatty acid synthase], FasL [Fatty acid synthase ligand], and caspase 8) and intrinsic (Bax [Bcl2-associated-x protein], Bcl2 [B-cell lymphoma 2], and caspase 9) pathways of apoptosis. Apart from the germ cells, interstitial cell apoptosis was also observed along with a decline in the number of functional Leydig cells. It is therefore concluded that complete suppression of the production of sperm with DNG + TU is facilitated mainly through the removal of precursor germ cells through apoptosis. The process is largely modulated by upregulation of upstream and downstream marker proteins from intrinsic as well as extrinsic pathway of metazoan apoptosis.

Keywords

dienogest, testosterone undecanoate, sperm suppression, germ cells, apoptosis

Introduction

Complete suppression of the production of sperm has been one of the best indicators of evaluating contraceptive efficacy for all the testis-targeted approaches investigated till date. Hormonal male contraception, either experimental or clinical, is more or less similar but an indirect approach that aims to inhibit sperm production in the testis completely. However, it has been observed that the extent of spermatogenic inhibition following hormonal interventions is not uniform and also varies with respect to race and ethnic diversity among participants.¹⁻³ Clinically, no regimen so far has been reported to induce consistent azoospermia in all participants. Even with studies reported in rats, a single hormone or hormone combinations have been reported to induce different degrees of spermatogenic inhibition leading to either partial or complete suppression of the production of sperm.⁴ Again, in clinical trials, although a small steroid load is able to induce azoospermia, in others, it fails to achieve the same even with a comparatively high hormonal dose.^{5,6} The apparent reason/reasons for such differential sperm suppression has not been investigated in detail in the past.

The potential of dienogest (DNG) plus testosterone undecanoate (TU) as a male contraceptive has earlier been investigated and reported from our laboratories.⁷ The aspect of differential sperm suppression was also partly addressed as complete suppression of the production of sperm in the

DNG + TU group was reported to be directly related to significant and uniform downregulation of expression of upstream steroidogenic enzyme genes. This was consistent with the fact that no such inhibition of gene expression occurred in the TU or DNG alone treated rats, which demonstrated only incomplete sperm suppression.⁸ Considering that complete arrest of spermatogenesis is the outcome of the successful regulation of various interdependable physiological/biochemical mechanisms, in the present work we explored the aspect of apoptotic induction of germ cells that successfully facilitated the removal of large number of precursor cells out of the meiotic and mitotic loop leading to a practical halt in sperm production. The involvement of other testicular cells in the process and the expression of molecular markers associated with pathways of apoptosis were also investigated.

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Materials and Methods

Animals and Treatments

Adult Wistar rats, weighing 200 to 250 g, were maintained under controlled temperature ($25 \pm 2^\circ\text{C}$) and constant photoperiodic (12-hour light:12-hour dark) conditions with food and water ad libitum. The animals were divided into 5 groups with 8 animals in each group. Both DNG and TU (oil preparation) were a generous gift from Schering AG, Germany. Dienogest was dissolved in isopropanol and suspended in olive oil to make a homogenous solution. The alcoholic portion was allowed to evaporate after keeping the suspension overnight at 37°C . The concentration of drugs in the preparation were made in such a way that 100 μL of the suspension contained the desired dose. Animals ($n = 8$) were administered with the preparation intramuscularly once every 45 days until killed at 90 days. In the last group (e), drug treatment to animals was stopped after 90 days of the first dose and were killed during the recovery, 75 days after (on 165th day) the last treatment. Standard institutional guidelines for animal care as prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals were strictly followed. The dose and the duration of treatment are described as follows:

Treatment (Group)	Drug administered (day)	Sacrificed on (day)
a. Olive oil (vehicle control)	(0, 45)	(90)
b. TU (25 mg/kg bw) only	(0, 45)	(90)
c. DNG (40 mg/kg bw) only	(0, 45)	(90)
d. DNG (40 mg/kg bw) + TU (25 mg/kg bw)	(0, 45)	(90)
e. DNG (40 mg/kg bw) + TU (25 mg/kg bw)	(0, 45, 90)	(165)

Tissue Processing and Histology

All the animals were killed at the end of 90 days from the first treatment; the testes were removed, weighed, and divided into 2 equal groups. The first group of testes was immediately fixed in buffered formalin and processed as described previously.⁷ Testes from the other group were snap frozen and stored in liquid nitrogen till assayed for caspase 3, 8, and 9 enzyme activity and apoptotic gene expressions.

In Situ End Labeling

In situ Apoptosis Detection Kit (TA4625, R & D Systems, Minneapolis, Minnesota), which detects DNA fragmentation was used to identify apoptotic germ cells in the seminiferous epithelium. Testis sections were deparaffinized and cell membranes were permeabilized with proteinase K in order to make the DNA accessible to the labeling enzyme. Hydrogen peroxide was used to quench endogenous peroxidase activity and the labeling was carried out as previously reported.⁴ The 3,3'-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.

Immunofluorescent Staining

Characterization of apoptotic cells in the testicular sections was carried out through immunofluorescence using antibodies against caspase 3, 8, 9, and Bax as described previously.⁹ Briefly, the sections were deparaffinized, rehydrated with phosphate-buffered saline (PBS), and incubated with a blocking solution containing 0.05% Triton-X-100 and bovine serum albumin (0.01%) in PBS. Following washing in PBS, the sections were incubated for 2 hours with primary antibodies (1:100 dilution) to caspase 9, Bax (rabbit polyclonal), caspase 8 (mouse monoclonal) from Santa Cruz Biotechnology, Santa Cruz, California. Caspase 3 (rabbit polyclonal) was from Cell Signaling Technology Inc, Danvers, Massachusetts. Similarly, a rabbit polyclonal 3β -hydroxysteroid dehydrogenase (3β -HSD) antibody (Santa Cruz Biotechnology) was used for characterizing Leydig cells in the testicular sections.

The sections were then incubated with secondary antibody (Santa Cruz Biotechnology) tagged with tetramethyl rhodamine isothiocyanate (for caspase 3, 8, 9, and Bax) or fluorescein isothiocyanate (for 3β -HSD), washed in PBS, mounted with vecta-shield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei. Testis sections processed without primary antibodies were considered as negative controls (data not included). Slides were examined under fluorescent microscope (Nikon Eclipse 80i, Tokyo, Japan), photographed using suitable filters for rhodamine (red), fluorescein (green), and DAPI (blue) wherever required and superimposed using NIS element imaging software (Nikon Instech Co, Ltd, Tokyo, Japan).

Assay for Caspase 3, 8, and 9 Activity

Activity of caspase 3, 8, and 9 was assayed with the help of colorimetric assay kit (BioVision, San Diego, California) as per the manufacturer's instructions. Briefly, testis was isolated, decapsulated, and homogenized in cold lysis buffer (100 mg/100 μL). Tissue lysate were centrifuged for 2 minutes at 10 000g at 4°C . An aliquot of supernatant (100 μg protein/50 μL) was added to 50 μL of reaction buffer containing 200 $\mu\text{mol/L}$ of chromogen (Ac-DEVD-pNA/Ac-IETD-pNA/Ac-LEHD-pNA for caspase 3, 8, 9, respectively), kept at 37°C for 2 hours and terminated by adding stop buffer. The change in the absorbance due to the release of *p*-nitroanilide (*p*-NA) was measured at 405 nm using microtiter plate reader (BioTek Inc, Winooski, Vermont).

Western Blot Analysis

Primary antibodies (rabbit polyclonal), anti-Fas, anti-FasL, anti-Bax, anti-caspase 9 along with (mouse monoclonal) anti-caspase 8, anti-Bcl-2, anti- β -actin, and secondary antibodies (polyclonal) goat anti-rabbit/mouse-HRP, and rabbit anti-goat-HRP were all procured from Santa Cruz Biotechnology. The whole tissue lysates were prepared and Western blotting was carried out as

Table 1. Densitometric Analysis Showing Ratio of Protein to β -Actin Expression $\pm$ SD.

Serial No.	Protein Name	Protein/ β -Actin			
		Control	TU	DNG	DNG + TU
1	Fas	1.126 $\pm$ 0.03	1.156 $\pm$ 0.209	1.266 $\pm$ 0.237	1.812 $\pm$ 0.0261 ^a
2	FasL	1.265 $\pm$ 0.10	1.017 $\pm$ 0.018	1.213 $\pm$ 0.319	1.397 $\pm$ 0.110 ^a
3	Caspase 8	1.031 $\pm$ 0.112	1.009 $\pm$ 0.256	1.01 $\pm$ 0.083	1.474 $\pm$ 0.121 ^a
4	Bax	1.084 $\pm$ 0.43	0.986 $\pm$ 0.26	0.883 $\pm$ 0.067	1.2304 $\pm$ 0.313 ^a
5	Bcl2	1.067 $\pm$ 0.126	0.754 $\pm$ 0.309	0.9419 $\pm$ 0.092	1.173 $\pm$ 0.291 ^a
6	Caspase 9	1.228 $\pm$ 0.039	1.216 $\pm$ 0.109	1.388 $\pm$ 0.265	1.8414 $\pm$ 0.027 ^a
7	Caspase 3	0.933 $\pm$ 0.031	1.125 $\pm$ 0.115	1.005 $\pm$ 0.059	1.426 $\pm$ 0.521
8	Cytochrome c	0.809 $\pm$ 0.109	1.069 $\pm$ 0.134	0.884 $\pm$ 0.128	1.248 $\pm$ 0.181 ^a

Abbreviations: Bcl2, B-cell lymphoma 2; DNG, dienogest; SD, standard deviation; TU, testosterone undecanoate.

^a $p < .001$.

Table 2. Primer-Specific Conditions Used for PCR Amplification of Candidate Genes.

Serial No.	Gene Name	Primer Sequence	Amplicon Size	Tm	Accession No.
1	Fas	F-GGCCCCATTTTGCTGTCAA R-TCCACTTGTGTGCAGTCCT	66	60	NM_139194.2
2	Fas L	F-CCGGTGATATTTTTCATGGTG R-AGGCTGTGGTTGGTGAAGTC	116	60	U03470
3	Caspase 8	F-AGAGCCTGAGGGAAAGATGTC R-TCACATCATAGTTCACGCCAGT	72	60	AF279308
5	Bax	F-GTGAGCGGCTGCTTGTCT R-GTGGGGGTCCCGAAGTAG	73	60	U49729
6	Bcl2	F-GTACCTGAACCGGCATCTG R-GGGGCCATATAGTTCACAA	76	60	LI4680
7	Caspase 9	F-CGCGACATGATCGAGGATA R-TCGAGTCTCAAGATCTATGACCAG	87	60	AF286006
8	β -Actin	F-CTGTGCCCATCTATGAGGGTTAC R-AATCCACACAGAGTACTTGCCT	359	60	V01217

Abbreviations: Bcl2, B-cell lymphoma 2; PCR, polymerase chain reaction.

described.⁸ β -Actin was used to monitor equal loading of protein. Densitometric analysis of the protein bands (Table 1) was performed with image analysis software (Labworks 4.0, Image Acquisition and Analysis software, UVP, Inc, Upland, California).

RNA Isolation and Real-Time PCR Analysis

Total RNA was extracted from testes using TRI reagent (Ambion, Austin, Texas) according to manufacturer's instructions. Complementary DNA was synthesized using 2 μ g of total RNA by omniscrypt RT kit (Qiagen, Hilden, Germany) and real-time polymerase chain reaction (PCR) was performed in the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) using the SYBR Green I Master mix (Roche Diagnostics) as described earlier.⁸ Primer specificity was assessed through melting curve analysis. The sequence, amplicon size of primers, and annealing conditions are detailed in Table 2.

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation. Significant difference between control and experimental groups was analyzed by Student *t* test and one-way analysis of variance

followed by Dunnett test using Graphpad Prism software. $P < .05$ was considered statistically significant.

Result

Apoptosis in Seminiferous Epithelium

Compared to negative (Figure 1A) and vehicle-treated controls (Figure 1B), a significant ($P < .001$) rise in the number of ISEL-positive cells was observed in the seminiferous epithelium of DNG + TU-treated rats (Figure 1E, G). Few ISEL-positive germ cells were also resolved in testis sections of TU- (Figure 1C) and DNG (Figure 1D)-treated rats. Following stoppage of treatment at 90 days (3 injections, 0th day, 45th day, and 90th day) and examined 75 days thereafter, decline in germ cell apoptosis was very much evident with restoration of spermatogenesis (Figure 1F, G). However, few occasional ISEL-positive cells still continued to be resolved (Figure 1F) comparable with that of vehicle-treated controls.

Caspase 3 Immunoexpression in Testis

Caspase 3 positive immunoexpression either in the seminiferous epithelium or in the interstitium was not encountered

Figure 1. In situ end labeling (ISEL) of DNA in testicular sections. A representative section each from (A) negative control, (B) age-matched vehicle control, (C) treatment testosterone undecanoate (TU) alone, (D) dienogest (DNG) alone, (E) DNG + TU, and (F) withdrawal group shows the distribution of ISEL-positive ($\rightarrow$) cells/tubule. Compared to other treatments, a significant rise (E, G) in ISEL-positive ($\rightarrow$) germ cells were observed in seminiferous epithelium of rats receiving DNG + TU treatment ($n = 8/\text{group}$). $\times 400$, $*P < .001$.

in the testis sections of vehicle control (Figure 2A), TU- (Figure 2B), DNG- (Figure 2C) treated rats and even from those in which the treatment was stopped and spermatogenesis was allowed to recover (Figure 2E). However, a marked rise in the number of such cells was observed in the seminiferous epithelium of DNG + TU-treated rats (Figure 2D, $\rightarrow$). Characterization of cell apoptosis through caspase 3 immunoexpression was not only limited to the seminiferous epithelium (Figure 2D, $\rightarrow$) but also extended to include cells in the interstitium (Figure 2D, $\rightarrow$).

was found significantly ($*P < .001$) upregulated in DNG + TU-treated rats (Figure 3A, B, C). Overexpression of these marker proteins were similarly observed in Western blots (Figure 3D, Table 1) too. Expressions of other marker proteins such as Fas, FasL, Bax, B-cell lymphoma 2 (Bcl2), and cytochrome c in Western blots followed an identical trend (Figure 3D, Table 1). Real-time PCR analysis further confirmed a parallel increase in transcript levels of apoptotic markers in the testis extrinsic, Fas, FasL, and caspase 8 or intrinsic, Bax, Bcl2, and caspase 9 (Figure 4).

Activity and Expression of Apoptotic Marker Proteins

The mechanism of cell apoptosis in the testis was further probed through assay of caspase 8, 9, and 3 activity that

Apoptosis in Testicular Cells

In order to confirm that apoptosis in the testis is not limited to germ cells only, immunofluorescence studies with antibodies to

Figure 2. Characterization of cell apoptosis through immunofluorescence using antibody against caspase 3. A representative merged picture in pink (arrow and arrowhead) after 4',6-diamidino-2-phenylindole ([DAPI] blue) and tetramethyl rhodamine isothiocyanate ([TRITC] red) staining of testis sections is shown. Occasional staining may be seen in testis sections from (A) vehicle control, (B) testosterone undecanoate (TU) alone, (C) dienogest (DNG) alone, or (E) treatment withdrawal group. Positive staining of tubular ($\rightarrow$) and interstitial ($\rightarrow$) cells in the testis section of DNG + TU (D) rats can be easily resolved ($n = 8/\text{group}$). $\times 400$.

caspase 8, -9, and Bax (Figure 5) were carried out using testis sections from different treated groups of rats. Irrespective of the antibody used, in each case, positive staining of the cells both in the seminiferous epithelium and in the interstitium was resolved in the DNG + TU-treated group only (Figure 5A, B, C). The testis sections from control, TU, DNG, and the recovery group (Figure 5A, B, C) did not reveal any fluorescence positivity in cells located either in the tubular or in the intertubular compartment.

Functional Characterization of Leydig Cells

Leydig cell function/dysfunction was analyzed by 3β -HSD staining of cells in the interstitium again through immunofluorescence. A significant decline in the number of fluoresced cells

was seen in the testis sections of DNG + TU compared to control, TU, or DNG alone treated rats (Figure 5D). Based on the same criterion, subtle improvement in Leydig cell function (restored 3β -HSD positivity in cells) was resolved in the recovery group (Figure 5D).

Discussion

The present findings establish that complete suppression of the production of sperm in rats following DNG + TU treatment is primarily aided by elimination of precursor cells by apoptosis mediated through extrinsic as well as intrinsic pathways. Again, apoptosis is not limited to the seminiferous epithelium only, but extends to the cells of the interstitial compartment

Figure 3. Activity and expression of apoptosis marker proteins. Activities of (A) caspase 8, (B) caspase 9, and (C) caspase 3 are found upregulated in the dienogest (DNG) + testosterone undecanoate (TU) treatment compared to all other groups. Identical overexpression of markers for (D) Fas, FasL, caspase 8, Bax, Bcl-2, caspase 9, caspase 3, and cytochrome c may be seen in Western blots. ** $P < .001$. * $P < .01$.

probably as a sequel to prolonged Leydig cell dysfunction due to gonadotropin withdrawal.

The hormonal approach to male contraception is primarily based on downregulating gonadotropin secretion resulting in the loss of both endocrine and spermatogenic activity in the testis.^{10,11} This is achieved by surpassing the physiological negative feedback control mechanisms both at the hypothalamic and pituitary level by administration of high doses of androgen or progestogens or by a combination of such agents.¹² Androgen only regimens are effective only to a limited extent as it failed to induce uniform suppression of spermatogenesis leading to azoospermia among diverse ethnic populations. The efficacy of sperm suppression was reported more in Chinese and Asian men than those of Caucasians.¹³ However, if a progestin is added to the androgen, profound suppression of spermatogenesis is confirmed.¹⁴ Therefore, in subsequent clinical studies, androgen

regimens combined with different progestins such as levonorgestrel, desogestrel, medroxyprogesterone acetate, cyproterone acetate, or norethisterone enanthate were tried which successfully demonstrated a uniform and optimal sperm suppression.^{15–22} Even in animal studies recently reported, DNG, a progestin, in combination with TU was found very much effective in achieving complete suppression of the production of sperm in rats. On the other hand, individual regimens of DNG or TU, administered alone, without any alteration in dose and duration, induced only partial suppression of spermatogenesis.^{4,7,8}

The factor/factors responsible for such differential sperm suppression with respect to different contraceptive regimens is not very clear. Data from animal studies suggest that very low levels of residual intratesticular (iT) testosterone may support spermatogenesis even in the absence of gonadotropins.

Figure 4. Real-time polymerase chain reaction (PCR) analysis of apoptotic marker proteins. Transcript levels of extrinsic (FasL [A], Fas [B], and caspase 8 [C]) and intrinsic (Bax [D], Bcl2 [E], and caspase 9 [F]) marker proteins of apoptosis are seen upregulated in dienogest (DNG) + testosterone undecanoate (TU)-treated rats. ** $P < .001$, * $P < .01$.

Significant decline in iT testosterone below a threshold of 17 nmol/L is reported to result complete abolition of sperm production.^{23,24} Progestins being antiandrogenic, probably help in minimizing the effects of residual iT leading to a fast and effective sperm suppression in humans²⁵ and in DNG + TU-treated rats.⁷ As the attenuation in iT testosterone is very much linked to decline in androgen production via Leydig cell steroidogenesis, a significant downregulation of upstream steroidogenic enzyme genes was further observed in animals showing a complete suppression of the production of sperm.⁸ Considering the fact that cell death by apoptosis might also play a significant role in the removal of precursor cells leading to complete suppression of the production of sperm, the same was investigated in detail for the first time in the present study.

Combined treatment of DNG and TU showed a marked rise in ISEL-positive cells over other treatments (Figure 1). Rise in the activities of caspase 3 and its protein expression in the testis (Figure 3C, D) also supported the above observation.

Gonadotropin suppression for longer durations disrupts Leydig cell steroidogenesis. Rats treated with DNG + TU demonstrate significant depletion in serum testosterone levels compared to vehicle-treated controls.⁷ Recently, we reported that persistent stimulation of Leydig cells with human chorionic gonadotropin raises oxidative stress leading to Leydig cell apoptosis in both in vitro and in vivo conditions.^{26,27} However, it is not known whether or not prolonged dysfunction in Leydig cells could also lead to their own demise through apoptosis. This was investigated for the first time in the present study.

Figure 5. A graphical representation of testis sections following immunostaining of cells with 4',6-diamidino-2-phenylindole (DAPI) and rhodamine tagged to caspase 8 (A), caspase 9 (B) and Bax (C), or DAPI and fluorescein isothiocyanate (FITC) tagged to 3 β -hydroxysteroid dehydrogenase (3 β -HSD). Compared to vehicle control, testosterone undecanoate (TU) alone, dienogest (DNG) alone and the withdrawal group, a significant increase in rhodamine positive staining is resolved for DNG + TU (A, B, C). In contrast, 3 β -HSD positive cells declined significantly in the same group (D) compared to vehicle-treated controls. A marginal decline is also observed in TU-treated rats. However, DNG alone or withdrawal group presented a comparable 3 β -HSD positive cell population identical to controls (D). ** $P < .001$, * $P < .01$.

using immunofluorescence as a tool and with the help of antibodies against apoptotic markers caspase 3 (Figure 2), caspase 8 (Figure 5A), caspase 9 (Figure 5B), and Bax (Figure 5C). In all such cases, testis sections only from DNG + TU-treated rats demonstrated a rise in fluorescent positive cells both in the interstitium and in the tubular epithelium. To further confirm that functional Leydig cells have indeed declined following contraceptive treatment for 90 days, testis sections were examined over 3 β -HSD immunostaining (Figure 5D). The findings are consistent with the suppressed spermatogenic status in the DNG + TU-treated rats as functional Leydig cells are encountered only occasionally. Similar decline in the number of functional Leydig cells are also seen in TU only treated rats. On the other hand, the revival of spermatogenesis following stoppage of treatment was supported by an increase in the number of functional Leydig cells in the interstitium (Figure 5D).

A critical balance exists between germ cell proliferation, differentiation, and apoptosis in the seminiferous epithelium. During establishment of spermatogenesis at the pubertal age, an early apoptotic wave occurs aimed to remove abnormal germ cells and subsequently maintaining a proper ratio of

maturing germ cells to Sertoli cells.^{28,29} As a result, approximately 75% of the spermatogenic cells are eliminated during the maturation process.^{30,31} Since the percentage of germ cell apoptosis was very high following DNG + TU treatment (Figure 1E), the process is considered as extremely relevant for achieving complete suppression of the production of sperm under any contraceptive intervention. In fact, it is reported that progestin supplementation with TU enhances significant transcriptional changes in many testicular genes associated with biological processes in receptor binding, signal transduction, cytokine activity, growth factor activity, and apoptosis.³² In the present study, protein (Figure 3D) and transcriptional upregulation (Figure 4) of apoptotic markers for extrinsic (Fas, FasL, and caspase 8) and intrinsic (Bax, Bcl2, and caspase 9) pathways was observed identical to findings reported earlier.^{33,34}

B-cell lymphoma 2 (Bcl2) has been localized exclusively in spermatids³⁵ in humans and the same may also be true for rats. However, the spermatid number declines significantly after DNG + TU treatment.⁷ The Bcl2, being antiapoptotic, a significant rise in its expression (Figure 4) following complete suppression of the production of sperm may be due to compensatory upregulation in

the other prevailing tubular cells like Sertoli cells or spermatogonia. Physiologically, this is recognized as part of a survival mechanism in these cells to ensure a rapid recovery to normal spermatogenesis once the contraceptive intervention leading to apoptotic induction is withdrawn. Rightly so, following stoppage of treatment at 90 days, occasional ISEL-positive cells are resolved in the seminiferous epithelium (Figure 1F).

In conclusion, the above findings support the contention that apoptosis-related mechanisms in the seminiferous epithelium play a major role in the elimination of precursor germ cells leading to complete suppression of the production of sperm as seen in case of DNG + TU treatment. Leydig cell demise through apoptosis in the same group may be attributed to prolonged dysfunction leading to inhibition of steroidogenesis which further supported germ cell removal.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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