Transient Scrotal Hyperthermia and Levonorgestrel Enhance Testosterone-Induced Spermatogenesis Suppression in Men through Increased Germ Cell Apoptosis

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Context: In rodents and monkeys, a combination of hormonal and physical agents accelerates germ cell death.

Objective: A “proof of concept” study was performed to investigate whether addition of heat exposure or a progestin to an androgen induces germ cell death and more complete and rapid spermatogenesis suppression.

Design and Settings: A randomized clinical trial was performed at academic medical centers.

Participants: We treated four groups of healthy male volunteers (18 per group) for 18 wk: 1) testosterone undecanoate (TU) 1000 mg im (first dose), followed by 500 mg im every 6 wk; 2) submersion of scrotum for 18 wk; 1) TU plus heat; and 4) TU plus oral levonorgestrel (LNG) 250 μg/d.

Main Outcome Measures: Semen parameters, testicular histology, and germ cell apoptosis were the main outcome measures.

Results: Heat alone and TU plus heat suppressed sperm counts more than TU alone by wk 6. By wk 9, recovery began in the heat only group, whereas spermatogenesis remained suppressed in the TU plus heat group. Oral LNG plus TU suppressed spermatogenesis earlier and more severely than TU alone at wk 2, significantly greater germ cell apoptosis occurred in heat and heat plus TU subjects, but not in subjects without heat treatment, compared with pretreatment subjects. By wk 9, markedly smaller seminiferous tubule diameters and fewer spermatocytes and spermatids were noted in all 12 biopsies from men receiving TU, TU plus LNG, with most dramatic differences for the TU plus heat group, whereas no differences from pretreatment biopsies were observed in men who received heat treatment only.

Conclusions: Heat causes a rapid and transient suppression of spermatogenesis. TU plus heat resulted in low-sperm output that was maintained by continuous treatment with TU. Addition of an oral progestin accelerated spermatogenesis suppression by TU alone. Increased germ cell apoptosis contributed to suppression of spermatogenesis. (J Clin Endocrinol Metab 92: 3292–3304, 2007)
In this proof of concept study, we address the question whether addition of a second “hit” such as a physical agent (heat) or another gonadotropin suppressing agent [a progestin, e.g., levonorgestrel (LNG)] to exogenous T treatment (first “hit”) will result in accelerated apoptosis, resulting in rapid and increased spermatogenesis suppression. As a secondary objective, results not discussed in this report, we studied the alterations in gene expression of regulators of spermatogenesis at various time points after intervention to identify signaling pathways that may provide novel targets responsive to the “two hits” that can be used in future male contraceptive development.

Subjects and Methods

Subjects

A total of 72 healthy Chinese male volunteers between the ages of 27 and 48 yr \[35.6 \pm 5.4 \text{ (mean \pm sd)}\] were recruited from a glass/lens factory at the Nanjing Center. The subjects had no significant medical history, and all had a normal physical examination during recruitment. None of the subjects was undernourished. They had normal baseline hematology, blood biochemistry, urinalysis, fasting lipid profile, and three consecutive normal semen analyses at 2-wk intervals (sperm concentration > 20 million/ml; motility > 50%; and oval forms > 10%) according to the WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction (23).

Study design

After a pretreatment observation phase of 4 wk, the eligible subjects were randomized in a 1:1:1:1 allocation to four groups for a treatment phase of 18 wk: group 1, testosterone undecanoate (TU); group 2, heat alone; group 3, TU plus heat; and group 4, TU plus LNG group. After randomization, treatment assignments were known to subjects and clinical staff, but not to laboratory and histological evaluators. On the first day of the treatment phase, 1000 mg TU was administered as a deep im injection that was followed by TU 500 mg im injections at wk 6 and 12 in groups 1, 3, and 4. Subjects in groups 2 and 3 returned daily from d 8–13 for testicular warming at 43 C in a water bath for 30 min each day. Subjects in group 4 took a 250 \( \mu g \) LNG capsule orally every morning, starting on d 1 for 18 wk. The treatment phase was followed by a recovery phase of at least 12 wk (wk 30, end of study).

Physical examinations and interviews were performed by a physician at screening, and wk 6, 12, 18, and 30. Digital examination of the prostate was performed at screening and the end of the study. Testicular volume was measured on and off the agent observed using the Prader orchidometer with ellipsoids up to 35 ml. Semen analysis was performed every 3 wk on samples during the 18-wk treatment period and then every 4 wk during the 12-wk recovery period, which was extended if necessary until sperm concentrations returned to pretreatment levels or over \( 20 \times 10^6 \text{ /ml} \). Serum hormones (LH, FSH, T, free T, and SHBG) were collected between 7:00 and 10:00 h at baseline (d 0), and d 5 and 14, then every 3 wk during the treatment period, and then every 4 wk during the recovery period. Blood samples for the measurement of safety laboratory tests were obtained at screening, and wk 6, 12, 18, and 30. A validated questionnaire covered four domains. Sexual desire, sexual enjoyment, sexual activity, and mood were recorded by the subjects daily for 7 consecutive days before clinical visits at baseline, and wk 6, 12, 18, and 30 (24).

Open testicular biopsy was performed on four volunteers at baseline, and four participants in each group at wk 2 and 9 of treatment. Time points were carefully chosen based on our data from rodent and monkey models, and published studies on scrotal hyperthermia and hormonal treatment in men. Week two (day after completion of testicular heat treatment) was selected to reflect the changes in testicular morphology, apoptosis rate, and gene expression studies in response to heat treatment. Week nine was anticipated to represent the effects of gonadotropin suppression and T withdrawal within the testis. Testicular biopsy was performed only once in a given volunteer on one testis. All subjects who had completed their family were asked to participate in the testicular biopsy part of the study on enrollment until the required number of subjects in each group was reached. Refusal to participate in the testicular biopsy portion of the study did not exclude the volunteer from participating in the main study.

This study was approved by the Ethical Committees of the Jiangsu Family Planning Research Institute and Nanjing Medical University, and the institutional review board of Los Angeles Biomedical Research Institute at Harbor-University of California Los Angeles Medical Center. All subjects gave informed written consent. Subject compensation for time loss from work and transportation costs was based on the established guidelines and standards of the Nanjing institution as appropriate for the research participants and approved by the respective institutional review board/ethical committee.

Medication, heat treatment, testicular biopsy

TU (125 mg/ml in tea seed oil) was obtained from Xian-Ju Pharmaceutical Company (Zhejiang, People’s Republic of China), and was used in prior studies in China in hypogonadal men and male contraceptive trials (25–28). TU was administered as 8 ml (4 ml in each buttock) im injection on d 1, and then 500 mg (2 ml) at wk 6 and 12 by the study physician. Tests were obtained at screening, and wk 6, 12, 18, and 30. A validated questionnaire covered four domains. Sexual desire, sexual enjoyment, sexual activity, and mood were recorded by the subjects daily for 7 consecutive days before clinical visits at baseline, and wk 6, 12, 18, and 30 (24).

Open biopsy of the one testis was performed under local anesthesia under the supervision of an experienced surgeon (L-X.Q.), who routinely performed this procedure for the investigation of male infertility in the Nanjing Institute. Open biopsy was performed instead of fine needle biopsy to ensure that good quality testicular tissues were obtained for the studies and to control risk of bleeding (34, 35). After local anesthesia, a 5-mm incision was made through the skin, tunica vaginalis, and tunica albuginea, and a small piece of tissue up to 100 mg was obtained immediately after heating that showed no scrotal skin changes.

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Hormone assays and semen analyses

All serum samples of the subjects were measured in validated hormone assays (36–39) in the Los Angeles center. Serum T levels were assayed by a specific RIA (coat-a-tube; Diagnostic Products Corp., Los Angeles, CA) for the measurement of safety laboratory tests. All subjects who had completed their family were asked to participate in the testicular biopsy part of the study on enrollment until the required number of subjects in each group was reached. Refusal to participate in the testicular biopsy portion of the study did not exclude the volunteer from participating in the main study.

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Angeles, CA) with within and between run coefficients of variation of 4.0 and 5.8, respectively, in the adult male range. Serum-free T was calculated using the equations based on the law of mass action with the serum T, SHBG, and albumin levels (40). Serum FSH, LH, and SHBG were measured by the highly sensitive and specific fluorimmunometric assays with reagents provided by Delfia (Wallace, Gaithersburg, MD), with intraassay and interassay coefficients of variation of less than 6% and 10%, respectively, for all three hormones. All samples from a subject were run within the same assay to minimize the effect of between-assay variability. When results were below the lower limit of quantification, the lower limit of quantification value was reported and used in data analysis.

Semen samples were collected after at least 48 h of abstinence by masturbation into sterile plastic containers and were analyzed in the study clinic in Nanjing according to the recommended methods described in the WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction (23).

**Fixation for testicular histology**

Immediately after obtaining the biopsy, each specimen was fixed in Bouin’s solution overnight, embedded in paraffin, sectioned at 5 mm, and stained with hematoxylin and eosin for routine morphological observations. In situ detection of cells with DNA strand breaks was performed in paraffin-embedded testicular sections by the terminal deoxynucleotidyl transferase-mediated deoxyuridine 5’-triphosphate nick end labeling technique (3, 41) using an ApopTag-peroxidase kit (Chemicon Int., Inc., San Francisco, CA).

**Testicular histology and enumeration of apoptotic germ cells**

The testicular histology and apoptotic germ count were examined with an American Optical Microscope (Scientific Instruments, Buffalo, NY) with 100× objective and a pair of ×10 eyepieces fitted with a square grid with methods previously described (41). The number of apoptotic germ cells and the nonapoptotic Sertoli cell nuclei with distinct nucleoli within the frame of the grid were counted. The rate of germ cell apoptosis or apoptotic index (AI) was expressed as the number of apoptotic germ cells per 100 Sertoli cells (2, 3, 41).

**Tentis morphometry**

The diameters of 15 randomly selected transverse sections of seminiferous tubules were measured across the minor axes of their profiles with an ocular micrometer calibrated by a stage micrometer (41). The volume densities (Vv) of seminiferous tubules and their lumina were determined by point counting, as described previously (41, 42). Testicular sections in each group were examined by an American Optical Microscope, with a 20× objective and a 10× eyepiece fitted with a square lattice containing 121 intersections. The results were expressed as percentage of the testis volume.

**Assessment of germ cells by immunohistochemistry**

Testicular sections were briefly deparaffinized, hydrated by successive series of ethanol, rinsed in distilled water, and then incubated in 2% H2O2 to quench endogenous peroxidases (2, 42). Sections were blocked with 5% normal horse serum for 20 min to prevent nonspecific binding of IgG and subsequently incubated with a 1:100 dilution of a polyclonal anti-Vasa (DDX4) antibody (Abcam, Cambridge, MA). Immunoreactivity was detected using biotinylated antimouse IgG secondary antibody, followed by avidin-biotinylated horse radish peroxidase complex visualized with diaminobenzidine tetrahydrochloride, as per the manufacturer’s instructions (UniTect 228 ABC Immunohistochemistry Detection System; Calbiochem, La Jolla, CA). Slides were counter-stained with hematoxylin and reviewed with a Zeiss Axioskop 40 microscope (Zeiss, Thornwood, NY).

**Statistical analyses**

The four randomized treatment groups were compared for age, body weight, body mass index (BMI), sperm concentration, T, LH, and FSH at baseline before treatment using the nonparametric Kruskal-Wallis tests, due to skewness in the distributions of some of these measures. Changes over time in sperm concentration did not display skewness, and, thus, mean changes within treatment groups and differences in these changes among treatment groups were analyzed with repeated measures ANOVA, implemented with maximum likelihood estimation using unstructured covariance. Sperm motility and morphology and serum hormones were separately analyzed using the same ANOVA models.

Sperm concentration was also analyzed according to percentages of subjects within each treatment group who were suppressed to oligozoospermia (<3 million/ml) or azoospermia (zero measured sperm) at each of the six sperm collection periods between wk 6 and 22. These percentages were compared among treatment groups using Fisher’s exact tests with Bonferroni adjustment for an overall 0.05 level of significance for multiple statistical comparisons for six collection periods. Changes from baseline in complete blood count and clinical chemistry measures were averaged for each subject over all posttreatment measurement periods, and the significance of mean changes within treatment groups were calculated in one-way ANOVAs.

Mean AI at wk 2 for each treatment group was compared with that from biopsies at baseline, before treatment, using ANOVA with Dunn’s correction for multiple comparisons with a control. The associations between AIs and sperm concentrations were measured with Pearson correlations.

**Results**

**Subjects**

There were no significant differences in age, weight, height, BMI, sperm concentration, serum levels of T, LH, and FSH among the subjects in the different treatment groups at baseline before treatment, as shown in Table 1.

**Sperm concentration**

Sperm concentrations at baseline (pretreatment), treatment, and recovery (posttreatment) phases are shown in Fig. 1 (upper panel). At 3 wk, sperm concentration decreased significantly in all groups except the TU alone group. Mean sperm concentration after heat treatment decreased maximally at 6 wk and then gradually returned to baseline levels.

| TABLE 1. Characteristics of participants at baseline |
|---------------------------------|-----------------|----------------|----------------|----------------|----------------|
| Measurement                     | All             | TU             | Heat           | TU + heat       | TU + LNG       |
| n                               | 72              | 18             | 18             | 18             | 18             |
| Age (yr)                        | 36 (31–39)      | 36 (31–38)     | 35 (31–37)     | 36 (30–41)     | 38 (35–41)     |
| BMI (kg/m2)                     | 23.2 (22.1–24.6) | 22.7 (21.5–23.3) | 24.2 (23.6–25.1) | 23.1 (21.7–24.8) | 23.1 (22.6–24.3) |
| Body weight (kg)                | 66 (63–71)      | 64 (61–66)     | 70 (64–72)     | 66 (63–71)     | 67 (65–71)     |
| Sperm concentration (10^6/ml)   | 77.0 (65.0–86.5) | 78.8 (58.0–88.0) | 79.3 (62.5–90.5) | 74.3 (62.5–82.0) | 77.8 (70.0–86.5) |
| T (nmol/liter)                  | 17.4 (14.5–19.9) | 18.1 (16.2–19.9) | 15.8 (12.2–17.8) | 16.9 (14.9–19.2) | 18.2 (15.0–22.6) |
| LH (IU/liter)                   | 4.5 (3.3–5.5)   | 4.1 (2.2–4.8)  | 4.6 (3.7–5.2)  | 5.2 (4.2–6.0)  | 4.5 (2.8–5.9)  |
| FSH (IU/liter)                  | 5.0 (3.8–7.7)   | 4.6 (3.4–5.8)  | 5.4 (4.8–9.5)  | 4.3 (3.2–6.9)  | 4.9 (4.6–9.2)  |

Values are expressed as median (25th–75th percentile).
FIG. 1. Mean sperm concentration in the volunteers in logarithmic scale (upper panel), sperm motility (middle panel), and sperm morphology (lower panel). Left panel shows data from participants who were treated with TU injections alone 1000 mg at d 1, and then 500 mg at wk 6 and 12 (closed circles, ○), heat alone applied from d 8–3 (open square, □), and combined TU plus heat (closed triangles, ▲). Right panel shows those treated with TU alone (closed circles, ○) and TU plus daily oral 250 µg/d LNG (open diamonds, △). Note that in this figure and in Figs. 3 and 4, the TU alone group consists of the same subjects in both the left and right panels.
by wk 12 (left panel). TU alone suppressed sperm output much more slowly and reached maximum suppression at wk 18, the end of the treatment period. The combination of heat plus TU injections suppressed mean sperm concentration markedly by 6 wk and then remained suppressed at low levels until the end of the treatment period. At both 3 and 6 wk, heat only and TU plus heat groups had suppressed mean sperm concentration significantly more than TU alone group \((P < 0.0001)\), but these two groups receiving heat did not differ significantly. At 9 wk, TU plus heat and TU alone groups had further suppression, whereas the heat only group was already recovering. At 9 wk, the suppression of sperm concentration was significantly \((P < 0.0005)\) less for each treatment alone than TU plus heat. At wk 3 and 6, TU plus LNG suppressed sperm concentration significantly greater than TU (same as the group in Fig. 1, left panel) alone \((P < 0.0001)\), but less than TU plus heat \((P < 0.0001)\). The combination of TU plus LNG continued to suppress spermatogenesis to a very low level until the end of treatment. During the recovery period, mean sperm concentration in all groups returned to levels above 20 million/ml by wk 30.

Of subjects that had sperm in the ejaculate, group differences in the mean percentages that were motile followed the same pattern as for sperm concentration (Fig. 1, middle panel). Abnormal morphology rates in ejaculated sperm were similar, with no significant group differences \((P = 0.24)\) in changes in mean percent normal sperm from baseline to 6 wk, and similar \((P = 0.25)\) near return to baseline levels at 30 wk (Fig. 1, lower panel).

**Proportion of subjects with severe oligozoospermia and azoospermia**

No subject in the heat alone group had sperm concentration less than 3 million/ml at any time (Fig. 2). All subjects with sperm concentration less than 1 million/ml at any particular time reached azoospermia at that time, except one subject in the TU plus heat group at wk 18. Azoospermia was achieved at wk 18 by seven of 18, six of 18, and 17 of 18 men in the TU, TU plus heat, and TU plus LNG groups, respectively. Significantly \((P < 0.05)\) more men achieved azoospermia: 1) in the TU plus LNG group compared with each of the
other groups at wk 12–22, 2) in the TU and TU plus heat group compared with the heat group at wk 18, and 3) in the TU plus heat group compared with the heat group at wk 15. In addition, the TU plus heat group had significantly more men achieving oligozoospermia compared with each of the other groups at wk 9.

### Serum hormone concentrations

As shown in Fig. 3, upper panel, mean serum total T appeared to increase, but not significantly \((P = 0.41)\) after heat treatment alone but increased 3 wk after each injection in the TU alone, TU plus heat, and TU plus LNG groups, which did not differ from each other \((P = 0.20)\). Identical results were obtained for serum-free T levels (Fig. 3, middle panel). Mean serum SHBG level was unchanged in the heat only group but showed a small but significant decrease in the TU alone and TU plus heat groups, and a significantly greater reduction (to 45% of baseline levels) in the TU plus LNG group compared with the other TU groups \((P < 0.002)\) (Fig. 3, lower panel).

In synchrony with increases in serum total and free T after each injection, mean serum LH (Fig. 4, upper panel), and FSH (Fig. 4, middle panel) concentrations showed sig-

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**Fig. 3.** Mean serum total T (upper panel), free T (middle panel), and SHBG (lower panel) in the four treatment groups. Left panel shows data from participants who were treated with TU alone (closed circles, ●), heat alone (open square, □), and combined TU plus heat (closed triangles, ▼). Right panel shows those treated with TU alone (closed circles, ●) and TU plus LNG (open diamonds, ◦).
significant decreases 3 wk after each TU injection in the TU and TU plus heat groups (P < 0.05). The TU plus LNG group also showed significant mean LH and FSH decreases 3 wk after the first injection (P < 0.0001), which were maintained less than 0.5 IU/liter from wk 3–18, resulting in significantly (P < 0.003) lower mean concentrations than the other groups over that period. Mean serum inhibin (Fig. 4, lower panel) was not significantly changed in the heat or the TU group but decreased significantly in the TU plus heat group (by 15%; P = 0.05) and in the TU plus LNG group (by 34%; P < 0.0001).

Safety monitoring of the subjects

No adverse events were observed with transient testicular hyperthermia. Three subjects reported pain at the incision site, and a small hematoma was observed in another subject after testicular biopsy, which subsided in a few days without
Treatment. Mild acne was present at baseline in four subjects, and four additional subjects reported acne at the end of treatment. There were no reports of gynecomastia, injection site pain, or other potential treatment-related adverse events. There were no significant posttreatment mean changes in serum liver enzymes, high-density lipoprotein-cholesterol, and prostate-specific antigen levels in any of the four groups of subjects, but there were small but significant mean increases in hemoglobin in the heat (0.34 g/dl), TU plus LNG (0.28 g/dl), and TU plus heat (0.44 g/dl) groups, and in hematocrit in the TU (1.6), heat (1.7), and TU plus heat (2.6) groups. There were statistically significant but small and presumably clinically unimportant increases in low-density lipoprotein-cholesterol in the heat and TU plus LNG groups, and in triglycerides in the TU plus heat group; and total cholesterol decreased significantly in the TU group. Small decreases in mean testis volume by wk 18 were noted in the TU group. Small and in triglycerides in the TU plus heat group; and total cholesterol decreased significantly in the TU group. Small decreases in mean testis volume by wk 18 were noted in the TU (4%; \( P = 0.01 \)), TU plus heat (3%; \( P = 0.08 \)), and TU plus LNG groups (2.5%; \( P = 0.11 \)), but not in the heat alone group (0%; \( P > 0.99 \)).

There were no significant changes in positive and negative mood scores. However, mean scores for sexual desire, enjoyment with partner, sexual activity, erection frequency, percent full erection, and erection satisfaction all significantly (\( P < 0.05 \)) increased through the study, but the magnitude of these increases did not differ among treatment groups, with one exception. The T plus heat group did not show changes in enjoyment with partner and significantly (\( P = 0.0004 \)) differed from the heat only group, which showed the greatest mean increases. These changes were small and may not be clinically important.

**Testicular morphology**

There were no discernible changes in testicular morphology after 2 wk of treatment in any treatment group. No changes in tubule diameter and Vv% of seminiferous tubules and tubular lumens were apparent between baseline and various treatment groups after 2 wk of treatment, except that the seminiferous tubule diameter showed a modest (17.6%) but significant (\( P < 0.05 \)) decrease in the TU plus H group (Table 2). However, striking differences in testicular morphology were noted after 9 wk of treatment with TU plus heat and TU plus LNG (Fig. 5 and Table 2). These differences were consistent within each treatment group for each of the four men providing biopsies. In the TU-treated group, changes included reduction (26.2%) in tubular diameter, and a marked decrease in the number of spermatocytes and spermatids (Fig. 5B). The overall testicular morphology of the TU plus LNG (Fig. 5E) group was similar to that of the TU alone group (Fig. 5B) except for an additional decrease in germ cell numbers. The maximum impairments of spermatogenesis occurred in the TU plus H group. There was a further reduction in tubule diameter, and the morphological alterations were more extensive with complete obliteration of tubular lumen, thickened basal lamina, and complete absence of mature spermatids (Fig. 5D). Quantitative analysis further confirmed histological findings (Table 2) and revealed a marked decrease in tubule diameter (38.5%) and Vv% of the seminiferous tubules (31.7%) in comparison with controls. Vv% of tubular lumens declined markedly to only 3.3% of control values. The morphological appearance of the testis after 9 wk of heat alone was similar to that of the control group.

**Activation of germ cell apoptosis**

Quantitative assessment of germ cell apoptosis (Fig. 6, A–E) was performed in testicular biopsies after 2 wk of treatment, and the incidence of germ cell apoptosis (expressed as numbers per 100 Sertoli cells) in various treatment groups is summarized in Fig. 6F. At 2 wk, no significant differences in the incidence of apoptosis were noted between baseline and TU or TU plus LNG groups. In contrast, heat alone or in combination with TU resulted in a significant increase of germ cell apoptosis when compared with that at pretreatment, with no significant differences between these two groups. No attempts were made in the present study to quantitate the incidence of germ cell apoptosis after 9 wk of treatment because the results may not be meaningful due to: 1) rapid clearance of apoptotic germ cells through phagocytosis by Sertoli cells in severely regressed tubules in subjects treated with TU alone, or in combination with heat or LNG; and 2) the recovery of spermatogenesis in heat alone group after 9 wk. At 2 wk significantly correlated with the decrease in sperm concentration from baseline to 3 wk in the men providing these biopsies (\( P < 0.05 \)), with this association weakening at 6 wk (\( P = 0.15 \)) and disappearing at 9 wk (\( P > 0.99 \)).

**Vasa expression and localization in human testes**

The expression of Vasa, a germ cell specific marker (43, 44), was consistent in all the biopsies from the same treatment group.
group. At 2 wk, Vasa staining was present in spermatocytes and spermatids, which appeared not different among the treatment groups (data not shown). Representative sections of testicular biopsy from each group after 9 wk of treatment showed Vasa localization mainly in spermatocytes and round spermatids, with moderate expression in spermatogonia at baseline and after heat treatment alone (Fig. 7). Marked loss of Vasa positive round spermatids was noted in the T alone group. Vasa positive spermatocytes and spermatids were lost in shrunken seminiferous tubules both in T plus heat and T plus LNG groups, however, spermatogonia remained in these combined treatment groups.

Discussion

In this study, we increased the interval between TU injections that was known to be suboptimal in the suppression of spermatogenesis in Chinese men (25) to allow us to examine potential additive effects of a physical agent such as heat or a progestin like LNG to enhance spermatogenesis by an androgen alone. The results of suppression of spermatogenesis in our study were inferior to those using a loading dose of 1000 mg, followed by 500 mg every 4 wk by design. The levels of T, and free T attained, and the suppression of LH, FSH, and SHBG mirrored those previously reported in Chinese men who were administered 500 or 1000 mg TU in tea seed oil (the same preparation used in this study) every 4 wk (27, 28) and our recent study of TU in castor oil given every 8 wk to non-Asian men (39). The suppression of spermatogenesis by TU alone was slow, and sperm output decreased to azoospermia and severe oligozoospermia only in 50% of subjects at the end of 18 wk of treatment. Our experimental paradigm of testicular warming was based on the previously published wet heating method (33). We found significant decreases in sperm concentration beginning at 3 wk and maximally at 6 wk, which then recovered to baseline by wk 12. These results are similar to the earlier testicular heating by testicular insulation (31, 32) or experimental transient artificial cryptorchidism (45, 46) in healthy volunteers. We also showed that mild transient elevation of testicular temperature in combination with TU significantly enhanced suppression of spermatogenesis by TU alone during the early treatment period between 3 and 9 wk, and heat alone from wk 9–18. Thus, we have confirmed that our observations previously demonstrated in rodents and monkeys (1, 2) were present in men using a similar experimental paradigm. Similar to other reports, we also showed in this study that addition of oral LNG to this suboptimal dosing interval of TU for suppression of spermatogenesis enhanced the effect of the androgen alone on decreasing sperm output throughout the treatment period (29, 36, 47, 48). This enhanced suppression was not accompanied by higher levels of serum total or free T but by more effective and complete suppression of serum gonadotropin levels in the LNG plus T group. Serum inhibin showed significant decreases in the TU combined with heat and LNG groups compared with the TU and heat alone groups, suggesting more severe Sertoli cell dysfunction, supporting our results of more suppression of spermatogenesis. Thus, in this proof of concept study in men, we showed that the combination of the “two hits,” exogenous TU (“hit 1”) and transient testicular hyperthermia (“hit 2”), resulted in accelerated suppression of spermatogenesis in the first few weeks compared with either treatment alone. In addition, we showed the enhancement of exogenous T (“hit 1”) suppression of spermatogenesis by LNG (“hit 2”), which had been demonstrated in many other studies (10, 11, 13, 38).

Histological sections of the testis at 2 wk showed no gross
FIG. 6. A–E, In situ detection of apoptotic germ cells (black arrow) in testicular biopsies from control (A), TU alone (B), heat alone (C), or TU in combination with heat (D) or LNG (E) after 2 wk of treatment. Apoptosis was detected by a terminal deoxynucleotidyl transferase-mediated deoxy-uridine 5c-triphosphate nick end labeling assay. Methyl green was used as a counterstain. Scale bar, 0.05 mm. F, Comparison of mean AI of germ cells among various treatment groups at 2 wk. Heat alone or in combination with TU resulted in significantly greater germ cell apoptosis when compared with that of controls. However, no significant differences in the incidence of apoptosis were noted between controls and TU or TU plus LNG groups. Values are the mean ± SEM. *, P < 0.05 from ANOVA with Dunnett’s adjustment.
differences among all treatment groups compared with the pretreatment biopsies, but morphometric analyses detected a modest (17.6%) decrease in tubule diameter in TU plus heat group compared with baseline values. This implies an additive effect of heat on TU during the early phase of suppression of spermatogenesis. At 9 wk, the combination groups (TU plus heat and TU plus LNG) showed a marked decrease in spermatogenesis compared with the T alone group, whereas in the heat alone group, recovery of spermatogenesis began. This decrease was mainly in spermatocytes and spermatid, as confirmed by the expression of Vasa, a marker for premeiotic and postmeiotic sperm cells (43, 44). A novel finding was that a rapid and marked decrease in sperm concentrations, after mild transient testicular hyperthermia in combination with TU during the early treatment period between 3 and 9 wk, was associated with significant \( (P < 0.05) \) activation of germ cell apoptosis within 2 wk of treatment. Our data constitute the first demonstration of the involvement of apoptosis in suppression of spermatogenesis in healthy men after TU plus heat treatment that is consistent with our earlier studies in rats (1) and monkeys (2), indicating that apoptosis is an important determinant of hormone and heat-induced suppression of spermatogenesis. No attempts were made in the present study to measure the rate of spermatogonia proliferation in these men under various treatment regimens. However, there are studies indicating that the B spermatogonia were the first germ cells to decrease after 2 wk of treatment with T enanthate alone or in combination with depot medroxyprogesterone acetate (17, 49). This implies that inhibition of spermatogonial proliferation is also an important determinant of sperm output after T and/or progestin treatment. Earlier studies by us in rodents (50, 51) as well as by others in primates, including men (17, 20), provided evidence indicating that the failure of spermatogenesis, characterized by the presence of elongated spermatids deep within the seminiferous epithelium, also plays an important role in the suppression of spermatogenesis after hormonal contraception. Unlike those reports, we did not observe failure of spermiogenesis in the present study after any of the treatment regimens. The reason for this difference is not known but could relate to testicular biopsies obtained at different time points after treatment with TU plus LNG compared with earlier studies. Moreover, the failure of spermiogenesis is a “dynamic” event, whereas testicular biopsy was taken at a “static” time point. The marked decrease in sperm concentration at 3 wk in the heat, TU plus heat, and TU plus LNG groups suggests that defects in spermiogenesis is likely. Ethnic differences in gonadal axis regulation, susceptibility of germ cells to apoptosis, or testicular morphology and spermatogenic potential between the Chinese and Caucasian men may also play a role (41, 52, 53). Thus, the mechanisms to explain the suppression of spermatogenesis under various treatment regimens include inhibition of spermatogonial proliferation, activation of germ cell apoptosis, and failure of sperm release.

Although scrotal warming adds to the efficacy of TU to suppress spermatogenesis earlier, our goal in this study was not to use the combined heat plus TU treatment as a practical, reversible method of male contraception but to investigate the mechanisms underlying the heightened spermatogenesis suppression with the combined intervention using an androgen with either heat or a progestin. We plan to define the key signal transduction pathways leading to accelerated apoptosis and decreased spermatogenesis by gene expression studies once our proof of concept study supported our hypothesis. A recent study (54) assessed selected testicular gene expression using quantitative PCR after treatment by a GnRH antagonist with T with or without the addition of desogestrel, a potent oral progestin. They demonstrated that the steroidogenic enzyme genes were markedly reduced,
and Sertoli cell genes such as inhibin α and the spermatocyte marker acrosin-binding protein were suppressed more in the group that received desogestrel in addition to the GnRH antagonist and T. In that study, testicular biopsy specimens were obtained at the end of the treatment period of 4 wk, gene profiling was not performed, and histological examination of the samples was not possible because the testicular biopsies were performed by needle biopsy (54). Thus, the study could have missed important known and unknown genes that may be the important regulators of germ cell proliferation, apoptosis, or spermiation early after initiation of treatment. Our experimental model will allow us to identify novel, testis-specific targets (key signaling pathways, specific kinases and phosphatases, and other druggable proteins) for the development of male contraceptive agents of the future. These new targets will not use hormones or heat but will act on master switches specific to the testis to turn spermatogenesis “off” or “on” for enhancement of male fertility or male fertility regulation.

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