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Effects of sleep deprivation on serum testosterone concentrations in the rat

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ABSTRACT

Sleep deprivation (SD) leads to decreases in circulating levels of testosterone with unknown mechanisms. We tested the hypothesis that decreased testosterone levels associated with SD may be caused by serotonin-mediated inhibition of its production. Male rats were subjected to SD for 24 or 48 h using the dish-over-water-method with a Rechtschaffen apparatus. Serum testosterone, corticosterone and serotonin (5-HT) concentrations were assessed thereafter, as were testicular StAR and 5-HT 2 receptor levels. SD, regardless of duration led to significant decreases in serum testosterone levels and testicular steroid acute regulatory protein (StAR) protein expression, while 5-HT levels were significantly elevated (all $P < 0.05$). Corticosterone concentrations were significantly increased in 48 h SD rats ($P < 0.05$). In primary Leydig cell cultures, 5-HT decreased chorionic gonadotropin-induced testosterone secretion and StAR expression, which appeared to be dependent on 5-HT 2 receptor activation but independent of cyclic AMP signaling. These findings suggest that decreased serum testosterone levels in SD rats may be the result of 5-HT-related inhibition of testosterone production and decreased testicular expression of StAR protein.

Sleep deprivation (SD) is a significant problem among adult men [1,2]. Mounting evidence suggests that SD can have various deleterious effects on human health and functioning [3], including that of the endocrine system [4,5]. Circulating testosterone levels are known to increase during sleep [6], which have been shown to start to rise upon sleep onset and peak during the first episode of rapid eye movement sleep [14]. Findings from human and animal studies have shown that SD is associated with reductions in circulating levels of androgens, including testosterone [7]. This is of clinical importance as decreased testosterone levels can impair gonadal and sexual functioning, and potentially result in decreased fertility [8].

It is unclear how precisely SD influences testosterone production. Testicular function is regulated by a number of neurotransmitters and neuropeptides, including serotonin (5-HT) [9–11]. In addition, both serotonin and serotonin receptors have been localized in Leydig cells isolated from the testes of golden hamsters [10]. Serotonin has also been demonstrated to inhibit testosterone production [10]. Findings from animal studies indicate that serotonin concentrations are elevated during SD [15,17]; hence, the decreased testosterone levels associated with SD may be in part due to serotonin-related inhibition of testosterone production. We tested this possibility by examining serum testosterone, serotonin, and corticosterone concentrations in SD rats, as well as serotonin receptor expression and intracellular signaling in Leydig cells isolated from these rats. We also examined testicular protein levels of steroid acute regulatory protein (StAR) and the 5-HT 2 receptor. We examined StAR expression because this protein transports cholesterol from the outer to the inner mitochondrial membrane, and is the rate limiting mediator of steroidogenesis [21].

A total of 24 adult male Sprague Dawley rats (42 days-old) were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). The rats were acclimated to a temperature (25 °C) controlled animal facility (12 h light:dark cycle from 9:00 am to 9:00 pm) for two weeks prior to the experiments. During acclimatization, two male rats were held in a single stainless steel cage with free access to chow and water. All animal procedures were approved by the Research Animal Resource Center Committee of China Medical University. Rats

Abbreviations: 5-HT, serotonin; SD, sleep deprivation; StAR, steroid acute regulatory protein.

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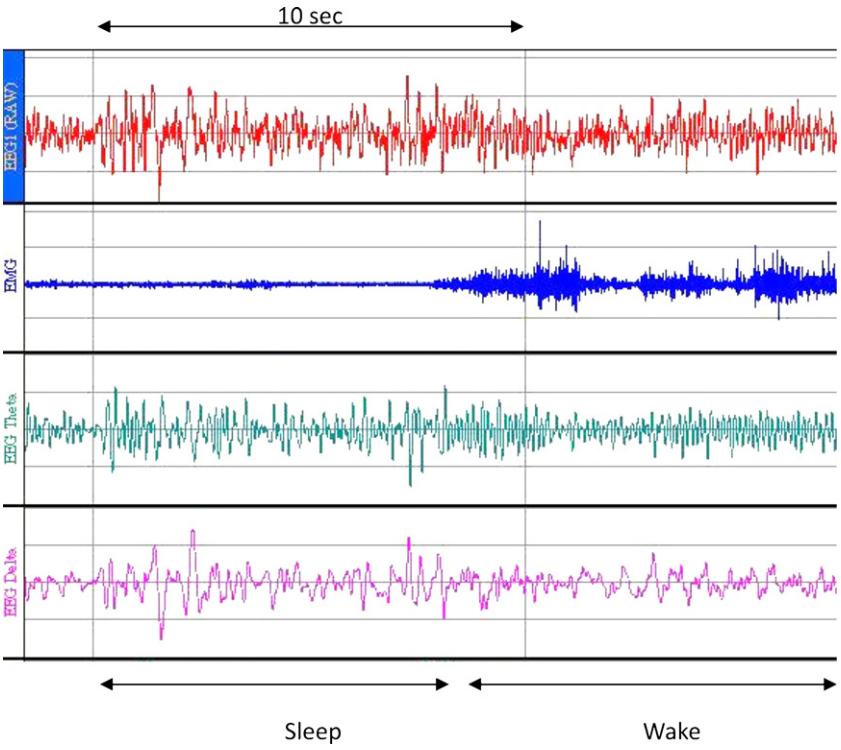

Fig. 1. Electroencephalography of a rat's sleep state. EEG theta and delta waves are indicated on the lower panels. The electromyography (EMG) is indicated in the upper middle panel. EEG1 (RAW) is indicated in upper panel and is the combination of these waves.

were randomly divided into four groups of six as follows: control, sham, SD 1 (SD1: 24 h of SD), and SD 2 (SD2: 48 h of SD).

Rats from both groups were deeply anesthetized with 20 mg/kg tiletamine-zolazepam (Zoletil 50®) and 10 mg/kg xylazine (Rompun®) before surgery. Four stainless steel screw electrodes were implanted (fronto-parietal region) in all rats for electroencephalogram (EEG) recording with commercially constructed electrodes (#E363/20, Plastics One Inc., USA). After surgery, amoxicillin (40 mg/kg/day p.o.) and ketoprofen (5 mg/kg s.c.) were administered and the animals were allowed to recover for 15 days.

The SD deprivation procedure was conducted using the dish-over-water-method with a Rechtschaffen apparatus. The apparatus consists of two rectangular clear plastic chambers (60 cm × 20 cm × 60 cm) placed side by side for the simultaneous study of two rats. Beneath each side of the dish and extending to the walls of each chamber was a tray containing water to a depth of 2–3 cm.

During experimentation, only SD rats with the implanted electrodes were connected to the EEG computer monitoring system (BIOPAC systems, Inc., Goleta, CA) with Acknowledge data acquisition software (ACK100W 3.7.3, BIOPAC). Sleep state was controlled by automatic monitoring of the root mean square of the EEG theta wave. Upon detection of a sleep state, the computer triggered the motor beneath the disc to rotate in a counterclockwise direction at a speed of 3.5 rpm. With rotation of the disc, rats had to move in a clockwise direction in order to avoid being propelled into the water. Disc rotation was ceased when a sleep state was no longer detected. Sham rats underwent the implantation surgery but were not connected to the EEG computer monitoring system and were not placed in the water-disc cage. After surgery, all rats were housed individually to avoid the infection of the surgical sites.

At the end of experimentation, rats were decapitated and blood was collected. Serum was isolated following refrigerated centrifugation (4 °C) at 3000 rpm for 30 min. Samples were stored at −20 °C until the assay. Testosterone, corticosterone and serotonin concentrations were determined using commercial enzyme assays kits (Diagnostic products corp., Los Angles, CA). Samples from all groups were analyzed on the same day for each hormone assay. The following intra- and interassay coefficients of variation (CV = standard deviation/mean × 100) were provided by the manufacturer: testosterone, both <5.3%; corticosterone, both <6.1%; and serotonin, both <6.0%. The sensitivities of the testosterone, corticosterone and serotonin assays were 1.6 ng/ml, 0.04 ng/ml and 5 ng/ml, respectively.

Testes were dissected and stored at −20 °C. Subsequent thawing and homogenization steps were performed in a RIPA lysis buffer with protease inhibitors. The mitochondrial fraction was purified from total testicular protein and was quantified using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Protein aliquots (25 μg) were subjected to standard SDS–PAGE procedures and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) using a semidry electrotransfer apparatus (E-C Apparatus Co., Holbrook, NY). Membrane blocking was for 60 min (PBS buffer containing 0.5% Tween 20 and 4% nonfat dry milk) followed by overnight incubation with anti-StAR (1:400; Serotec, Oxford, England), anti-5-HT R_2A antibody (1: 500; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-β actin (1:5,000; Sigma–Aldrich, Inc., St. Louis, MO) antibodies at 4 °C. Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (1:5,000; Chemicon, Temecula, CA) that were developed using the enhanced chemiluminescence detection system (Biological Industries Co. Kibbutz Beit Haemek, Israel). The intensities of the bands were measured using the MultiImageTM computer-assisted image system (Alpha Innotech Co., San Leandro, CA). Beta-actin was used for the normalization of 5-HT 2 receptor signal; however, because StAR is a mitochondrial protein, no actin normalization was performed.

The Leydig cell isolation was performed according to a previously established protocol [16] with slight modification. Briefly, decapsulated testes were incubated in a stirred water-bath (120 cycles/min) at 37 °C with minimum essential medium α (MEM-

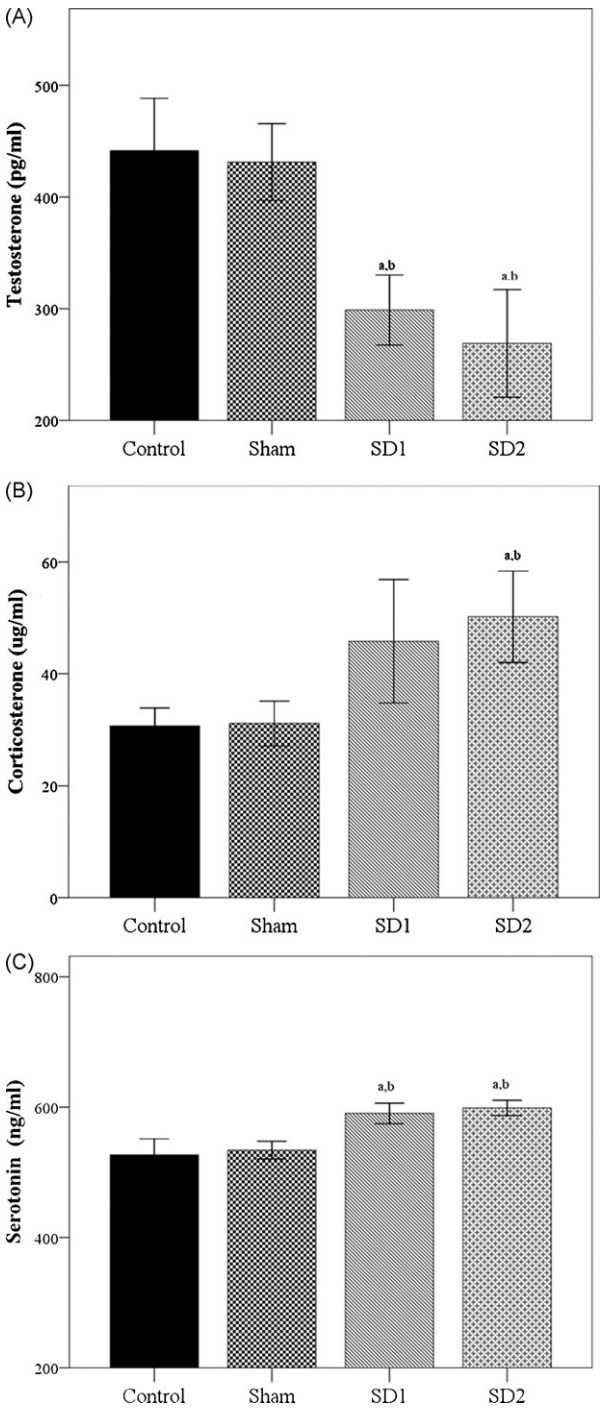

Fig. 2. Summary of serum (A) testosterone, (B) corticosterone, and (C) serotonin concentrations in rats following sleep deprivation (SD). Control and Sham group rats were allowed to sleep; SD1 and SD2 group rats were sleep deprived for 24 and 48 h, respectively. Data are displayed as means ± SD. [a] $P < 0.05$ vs. the control group. [b] $P < 0.05$ vs. the sham group.

α; Hyclone, Logan, UT) containing 4 mM $NaHCO_3$, 25 mM HEPES, 0.06 g penicillin, 0.05 g streptomycin, 1% bovine serum albumin (BSA) and 100 IU/ml collagenase (type II; Worthington Biochemical, NJ) for 30 min. After incubation, cold MEM-α was added to stop the collagenase activity. Seminiferous tubules were separated from interstitial cells by filtering with sterilized gaze. The filtered cell suspension was collected and centrifuged at 300 × *g* for 10 min at room temperature and resuspended in 4 ml of MEM-α containing 0.1% BSA and 0.01% DNase. The cell suspension was loaded on a discontinuous Percoll gradient (20–86% Percoll) and centrifuged at 800 × *g* for 20 min at 18 °C. After centrifugation, fractions at 1.056 and 1.068 g/mL were collected, washed with buffer, and counted. Isolated Leydig cells were resuspended in MEM-α culture medium containing 15 mM HEPES, nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured on 6-well culture plates at a concentration of 0.5 to 0.7 × 10^6 cells/mL of medium. Between 90 and 95% of the isolated Leydig cells were viable as assessed with the trypan blue dye exclusion method. The total number of cells and the percentage of 3β-HSD-positive cells were also determined. The purity of the Leydig cells was between 85 and 90%. For all in vitro experiments, primary Leydig cells were treated with various reagents for 1 h.

Normally distributed variables were compared by one-way analysis of variance (ANOVA). When a significant between groups difference was apparent, multiple comparisons of means were performed using the Bonferroni procedure with type-I error adjustment. Two-way ANOVA tests were used to determine the effects of 5-HT or ketanserin treatment and the interaction of these treatments on testosterone secretion and StAR protein expression. Data are presented as means ± standard deviation. All statistical assessments were two-sided and evaluated at the 0.05 level of statistical significance. Statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL).

An exemplary EEG recording of a sleep state in the rat is depicted in Fig. 1. Detection of EEG theta and delta waves indicated that the rat did sleep during the annotated 10 s period. Meanwhile, the electromyography (EMG) indicated that the rat was subsequently awake. EEG1 (RAW) indicated the combination of waves during sleep. SD rats were monitored during the same time every day (9–11 am) and immediately entered slow-wave sleep upon being sleep deprived.

A significant difference in serum testosterone levels among the 4 groups was observed ($F = 28.52$, df = 3, 20, $P < 0.001$). There was no difference in serum testosterone levels between the control and sham groups, while concentrations in the SD1 and SD2 groups were significantly lower than in both the control and sham groups (all $P < 0.05$; Fig. 2A). A significant difference in corticosterone concentrations among the 4 groups was observed ($F = 11.26$, df = 3, 20, $P < 0.001$). There was no significant difference in testosterone concentrations between the SD1 and SD2 groups. Serum corticosterone levels were significantly higher in the SD2 group compared to both the control and sham groups (both $P < 0.05$; Fig. 2B). A significant difference in serum serotonin levels among the 4 groups was observed ($F = 28.67$, df = 3, 20, $P < 0.001$). There was no difference in serum serotonin levels between the control and sham groups, while concentrations in the SD1 and SD2 groups were significantly higher than in both the control and sham groups (all $P < 0.05$, Fig. 2C). There was no significant difference in serotonin concentrations between the SD1 and SD2 groups.

A significant difference in StAR protein levels among the 4 groups was observed ($F = 23.50$, df = 3, 8, $P < 0.001$). There was no difference in testicular StAR protein levels between the control and sham groups, while levels in the SD1 and SD2 groups were significantly lower than in both the control and sham groups (all $P < 0.05$; Fig. 3A). There was no significant difference in StAR protein levels between the SD1 and SD2 groups. In addition, there were no differences among groups in 5-HT 2 receptor expression (Fig. 3B).

Leydig cell cultures were established to examine the intracellular signaling pathways after 5HT 2 receptor activation. In Fig. 4A, cultures were treated with 0.01 mM 8-Br-AMP, which activates PKA; 0.001 mM forskolin, which activates adenylcyclase; and 5 mIU/ml human chorionic gonadotropin (hCG) for 1 h. Afterwards, the testosterone concentration in the culture medium was determined. 5-HT suppressed hCG-induced testosterone release in Leydig cell cultures in a dose-dependent manner, while 5-HT

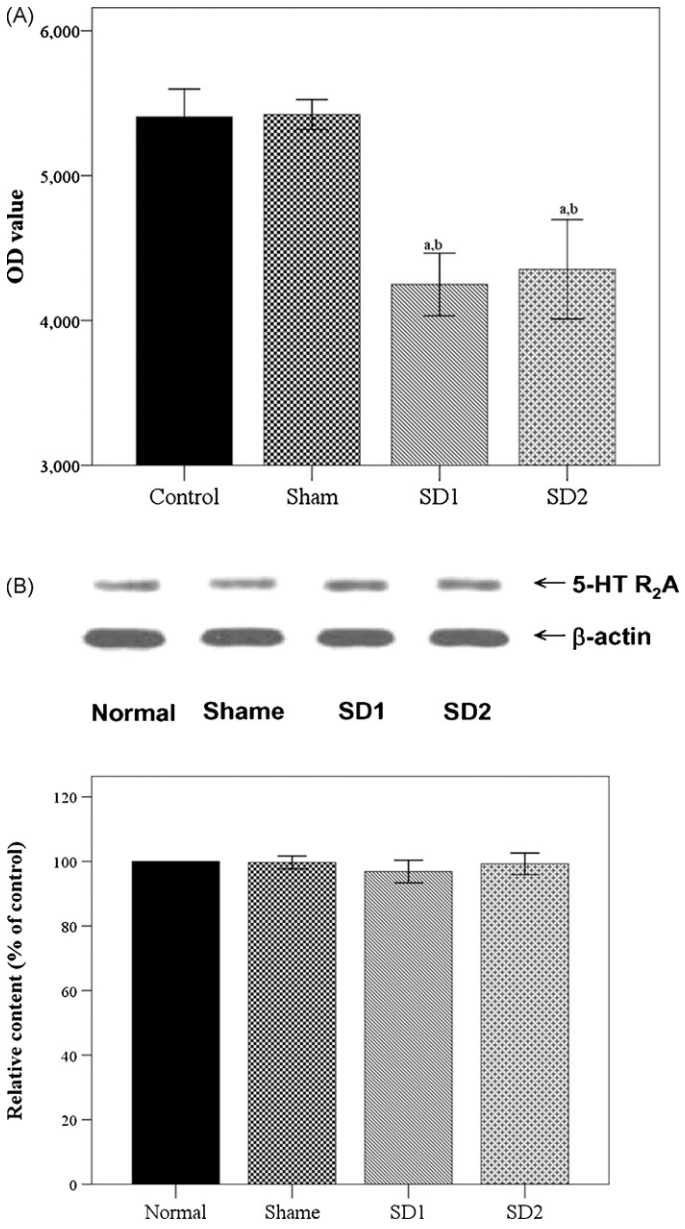

Fig. 3. Summary of (A) testicular steroid acute regulatory protein and (B) 5-HT 2 receptor protein expression levels as determined by Western blotting in rats following sleep deprivation (SD). Control and Sham group rats were allowed to sleep; SD1 and SD2 group rats were sleep deprived for 24 h and 48 h, respectively. Data are displayed as means ± SD. $^{a}P < 0.05$ vs. the control group. $^{b}P < 0.05$ vs. the sham group.

alone had no effect on constitutive testosterone release in Leydig cell cultures. Both 8-Br-AMP and forskolin triggered activation of the PKA pathway, resulting in elevated testosterone secretion to some extent. hCG and LH have similar structures and it has been suggested that LH is increased during sleep deprivation; however, only 15% of adult males exhibit sleep-related LH increases [19]. Furthermore, the responsiveness of cultured Leydig cells to hCG is well documented in the literature [12]. Thus, hCG was used as the stimulus for these cultures that were treated with 5 mIU/ml hCG, 100 ng/ml 5-HT, and 1 mg/ml ketanserin (an antagonist of the 5-HT2A receptor). The results of a two-way ANOVA showed that there was a 5-HT effect ($F = 63.36$, df = 1, 12, $P < 0.001$), ketanserin effect ($F = 70.13$, df = 1, 12, $P < 0.001$) and an interaction of the effects of 5-HT and ketanserin on testosterone secretion ($F = 67.24$, df = 1, 12, $P < 0.001$). Furthermore, we determined the difference among the

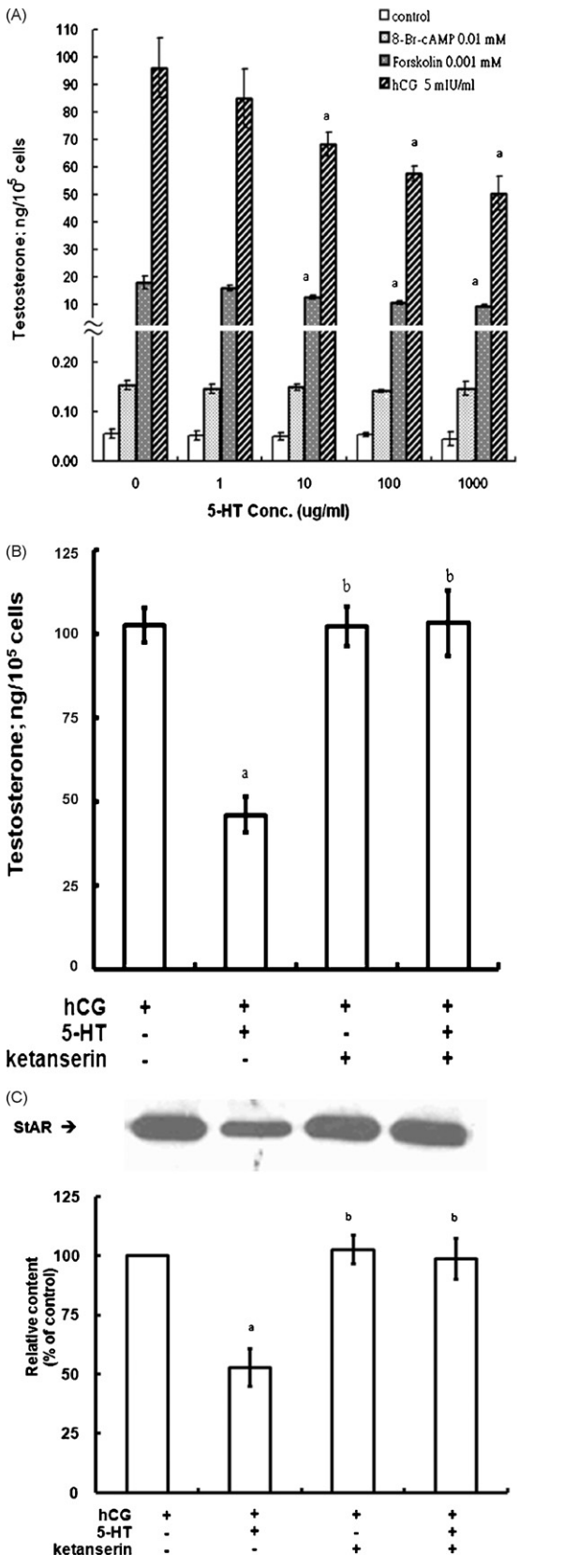

Fig. 4. Effects of 5-HT on testosterone secretion and StAR protein expression in primary Leydig cell cultures. (A) Regulation of testosterone secretion by 8-Br-cAMP (10^{-2} mM), Forskolin (10^{-3} mM) and hCG (5 mIU/ml) in the presence of various doses of 5-HT. Data are displayed as means ± SD. $^{a}P < 0.05$ vs. the control group (0 μg/ml of 5-HT). (B) Serotonin suppressed hCG-induced testosterone secretion. Cells were treated with hCG (5 MIU/ml), 5-HT (100 ng/ml), and/or ketanserin

4 groups [hCG (5 MIU/ml) only, hCG (5 MIU/ml) + 5-HT (100 ng/ml), hCG (5 MIU/ml) + ketanserin (1 mg/ml) and hCG (5 MIU/ml) + 5-HT (100 ng/ml) + ketanserin (1 mg/ml) groups]. In Fig. 4B, 5-HT decreased hCG-induced testosterone secretion, which appeared to be mediated through the 5-HT2A receptor. The results of a two-way ANOVA showed that there was a 5-HT effect ($F = 65.55$, df = 1, 12, $P < 0.001$), ketanserin effect ($F = 78.55$, df = 1, 12, $P < 0.001$) and an interaction of the effects of 5-HT and ketanserin on StAR protein expression ($F = 53.56$, df = 1, 12, $P < 0.001$). In Fig. 4C, the Western blot of StAR demonstrated that 5-HT decreased hCG-induced StAR protein expression. Taken together, these in vitro data demonstrate the role of the 5-HT2 receptor in StAR expression and testosterone secretion.

In this study, we demonstrated that SD for 24 or 48 h caused significant decreases in serum testosterone concentrations. Parallel changes in testicular StAR protein expression levels were also observed; while serum serotonin levels were significantly increased following SD.

The most remarkable finding of our study was the clear-cut reduction in serum testosterone levels after SD. This finding is consistent with that reported by Andersen et al., who found that plasma testosterone concentrations were significantly decreased following one to four days of SD [2]. Several human studies have also reported that SD is associated with decreased testosterone levels [7].

The synthesis of testosterone is dependent on endocrine and neuronal signals which in turn are influenced by physiological conditions such as stress [18]. SD is a known physiological stressor; hence, it is unsurprising that serum testosterone concentrations were altered in rats following SD. Although we did not assess fertility or sexual functionality in this study, decreased testosterone levels are known to deleteriously affect sexual adequacy and fertility humans [8,20]. Further study is required to determine whether SD using the model described herein results in decreased fertility/sperm count.

One neurotransmitter known to regulate testicular function is serotonin, which is produced both intrinsically and externally to the testes [9–11]. In accordance with previous reports [15,17], we found that serotonin concentrations were significantly increased following SD. Serotonin is thought to inhibit testosterone production via binding to type 2 serotonin receptors in Leydig cells [10]. Consistent with these findings in that 5HT 2 receptors are expressed in Leydig cells and appear to be the dominant serotonin receptor subtype in these cells [10,21]. Findings from previous studies have shown that serotonin, upon receptor binding, simulates the secretion of corticotropin-releasing hormone in Leydig cells, which in turn inhibits cyclic AMP (cAMP) generation and subsequent testosterone production [22]. This signaling pathway was examined in cultured Leydig cells and it was demonstrated that 5-HT suppressed hCG-induced testosterone release in Leydig cell cultures in a dose-dependent manner (Fig. 4). This effect was also observed during activation of the PKA pathway, which resulted in elevated testosterone secretion to some extent. These findings suggest that 5-HT suppressed testosterone production by a PKA independent mechanism and support the existence of non-cAMP signaling pathways involved in the regulation of testosterone [13]. Nevertheless, we cannot rule out the possibility that the observed decreased testosterone levels following SD may be mediated by factors other than serotonin. Further in vivo studies of SD rats treated with 5-HT receptor antagonists are also needed.

The rate limiting step in steroidogenesis is the delivery of cholesterol from the outer to the inner mitochondrial membrane, a process that is facilitated by StAR [21]. In the present study, we found that StAR protein levels were significantly decreased in testes from SD rats. To our knowledge, this is the first study to report such a finding. This suggests that decreased testicular StAR expression contributed to the decreased serum testosterone concentrations in SD rats.

In conclusion, we have utilized a unique rat model of SD to investigate potential mediators responsible for the decrease in serum testosterone concentrations following SD. Our findings suggest that testosterone production may be decreased due to a combination of serotonin-related inhibition and decreased StAR expression. Additional research is needed to confirm these findings in vivo and to develop these therapeutic targets for the treatment of testicular dysfunction.

Conflict of interest statement

None declared.

Contributors

JLW, KHF, RSCW, JGY, CCH, and KBC performed experiments. HDT designed the experiments and wrote the manuscript.

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