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Original Article

The role of glucocorticoids in ovarian development of sleep deprived rats

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ABSTRACT

Objective: Sleep deprivation (SD) adversely affects female reproductive function. In this study, we investigated the role of glucocorticoids in ovarian development in sleep deprived rats.
Materials and methods: Female rats were subjected to SD for 1–4 days. Concentrations of serum estradiol and corticosterone were assessed. Betamethasone (BET) and/or recombinant human follicle-stimulating hormone (FSH) were administered to 21-day-old female rats for 2 days to evaluate ovarian status for follicular development. Intact preantral follicles were mechanically dissected from the rat's ovaries and cultured for 72 h with or without FSH in the presence or absence of BET to evaluate follicular development.
Results: SD led to a significant difference in serum estradiol concentrations between the sham and SD groups, and corticosterone concentrations were significantly elevated in groups with more than 2 days of SD ($P < 0.05$). FSH stimulated ovarian growth in immature rats, whereas BET inhibited ovarian development caused by the FSH treatment. Treatment of the preantral follicles with FSH induced an increase in both follicle size and follicular cell number, while follicular cell differentiation was accompanied by enhanced inhibin-α and connexin 43 expression. Inhibition of FSH-stimulated follicular growth through BET treatment exhibited a dose-dependent reciprocal trend; as the BET dose increased (0.001–1 μg/mL), preantral follicular growth decreased. This decrease was associated with a decrease in follicular cell numbers and suppression of a proliferating cell nuclear antigen, inhibin-α, and connexin 43 expression.
Conclusion: The findings suggest that the adverse effects of SD may inhibit follicular development during ovarian hyperstimulation by corticosterone elevation in rat.
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Introduction

Sleep deprivation (SD) is prevalent in highly industrialized countries and is associated with deleterious effects on human health and physiological functions [1–3]. Numerous human and animal studies have shown that SD can cause an irregular estrous cycle and anovulation, which can potentially cause reproductive dysfunction [4–6]. These findings highlight the clinical importance of understanding how SD affects fertility [6]. How SD precisely influences ovarian function is unknown.

The development and maturation of ovarian follicles are fundamental to fertility in the female reproductive system. In addition to gonadotropins, factors such as steroids, cytokines, estrogen, progestin, and neurotransmitters are known to regulate the development and maturation of follicles [7–9]. Steroids such as glucocorticoids (GCs) also regulate the development of follicles. Several studies have shown that GCs regulate reproductive function through direct or indirect suppression of follicular functions. The GC receptor (GR) has been identified in granulosa cells [10–12], which indicates that GCs are directly involved in follicular development. Studies have also shown that GCs can act on ovaries to regulate steroidogenesis [13,14], survival of granulosa cells [15–17], or oocyte maturation [18,19].

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SD increases the secretion of GCs by the adrenal cortex and disturbs reproductive function [20]. Therefore, the disturbed ovarian function associated with SD may be a result of GC-related actions. Although ovarian follicular development is the key element in female fertility [21], few studies have reported that GCs directly affect follicles. To examine whether SD affects preantral follicular development through GCs, we investigated serum estradiol and corticosterone concentrations in totally sleep deprived rats. We also examined the growth of preantral follicles to explore the role of a synthetic GC, betamethasone (BET), in the regulation of early follicular development in rats.

Materials and methods

Experimental procedure and recording of SD in rats

Female Wistar rats (8 wk old or 21 days old) were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan, ROC) and were housed in a light–dark controlled facility (12 h light:12 h dark) at a temperature of 25 °C with food and water provided ad libitum. All animal handling and experimental procedures were approved by the Research Animal Resource Center Committee at Changhua Christian Hospital.

The 8-wk-old rats were divided into eight groups (with or without SD for 1–4 days, respectively, six rats in each group). The surgical procedure for electrode implantation and the SD procedure were performed using the dish-over-water method and a Rechtschaffen apparatus, in accordance with a previous study [22]. Briefly, rats in the sham and SD groups were anesthetized before the electrodes were surgically implanted into their skulls for electroencephalography (EEG) and were left to recover for 14 days. The phase of the estrous cycle was determined through vaginal smearing. The SD experiment was performed for 1–4 days during the estrous phase. Briefly, the rats were placed on a disk in the Rechtschaffen apparatus for 5 days before the SD experiment. An EEG monitoring system (Mp150) (BIOPAC systems, Inc., Goleta, CA, USA) with Acknowledge data acquisition software was connected to the electrodes of the SD rats to monitor their sleep state. When the rats fell asleep (when EEG-theta waves were detected), the disk slowly rotated at a speed of 3.5 rpm to keep the rats awake and walking. The disc stopped rotating when the rats were no longer in a sleep state. The rats in the sham group were not connected to the EEG monitoring system, and they were not in the water-disc cage. Food and water were provided ad libitum during the SD period.

Blood sampling and serum hormone measurement

At the end of the experiment, the rats were decapitated and their blood was collected in glass tubes, which were stored on ice to allow coagulation. Serum was isolated following refrigerated centrifugation (4 °C) at 3000 rpm for 30 min and stored at −80 °C until assays were performed. Estradiol and corticosterone concentrations were determined using commercial enzymatic assay kits, which were purchased from ALPCO Diagnostic Inc. (Salem, NH, USA). The samples from all the groups were analyzed on the same day.

Ovary weight and morphology

To study the effect of GCs on ovarian growth, immature female Wistar rats (21 days old) were treated with an intraperitoneal injection of 10 IU of pregnant mare serum gonadotropin (PMSG) (China Chemical & Pharmaceutical Co. Ltd., Taipei, Taiwan) and/or betamethasone disodium phosphate (4 mg/kg) (China Chemical & Pharmaceutical Co. Ltd.) for 48 h. At the end of the treatment, the rats were sacrificed through cervical dislocation, and the ovaries were removed and dissected from adherent tissues under microscopic visualization. The ovaries were weighed and fixed in 4% paraformaldehyde for histological analysis. The ovaries were longitudinally and serially sectioned at 4 μm; every 10th section (n = 6 per ovary) was mounted on a glass slide, stained with hematoxylin and eosin, and analyzed under a microscope. The area of the largest follicle was measured, and the follicles were counted.

Preantral follicle isolation and culture

Preantral follicles (140–160 μm in diameter) were mechanically and aseptically isolated from the rats (21 days old) in α-minimal essential medium (α-MEM) (Life Technologies, Rockville, MD, USA) with 0.5% bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO, USA) at day 0 using 27-gauge needles. Preantral follicles were cultured individually in 100 μL of medium in 96-well dishes at 37 °C in a moist atmosphere of 5% CO_2 and 95% air. The medium comprised α-MEM with ITS+ (insulin, 10 μg/mL; transferrin, 5.5 μg/mL; linoleic acid, 4.7 μg/mL; and selenium, 5 μg/mL) (Sigma–Aldrich) and 0.5% BSA and Pen/Strep (penicillin, 100 IU/mL; and streptomycin, 100 μg/mL) (Sigma–Aldrich). The follicles that were cultured were selected in three washing steps by using the following criteria: (i) an intact round follicular structure with two or three layers of granulosa cells and at least some adhering theca cells; and (ii) visible, round, and centrally located oocytes within the follicles, as previously described [21]. The selected follicles were pooled and randomly divided into various treatment groups. Two-dimensional maximum lengths of each follicle were measured daily by using an inverted microscope (Olympus, Tokyo, Japan) equipped with an image analysis system (Inspector software version 2.1, Matrox, Quebec, Canada). Interstitial cells and theca cells around the basement membrane were not included in the follicular measurements. The mean diameter of the follicles was calculated by averaging of two measurements. At the end of the 72-h incubation period, the follicles were collected to further analyze cell numbers and developmental markers. Each experiment was repeated three times. Betamethasone disodium phosphate and recombinant human FSH (rhFSH) (Ares-Serono; Geneva, Switzerland) were added on day 0 at the indicated concentrations.

Quantification of viable follicular cells

Follicular cell viability was verified through a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. At the end of the culture, replicates of eight follicles from each treatment group were moved to single wells containing 100 μL of α-MEM with 0.5% BSA and 20 μL of tetrazolium salt (MTS) reagent, which were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h. After incubation, 100 μL of medium from each well was transferred to a new well to determine the absorbance of optical density at 490 nm by using ELISA reader (Dynex Technologies, Chantilly, VA, USA). The follicular cell number was then calculated. Absorbance levels were standardized to known quantities of granulosa cells.

Protein electrophoresis and Western blot analysis

Twenty-five follicles of each treatment group were collected in microcentrifuge tubes at the end of culture and kept frozen at −80 °C until further analysis. The follicles were thawed in a cell lysis buffer (Chemicon Inc., Temecula, CA, USA) and homogenized with a plastic rod. The samples were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sampling buffer (Chemicon), were boiled for 5 min, and were loaded into a

12.5% mini gel through standard SDS-PAGE procedures, along with prestained molecular weight markers (Fermentas Inc., Hanover, MD, USA). Gels were electrophoresed in a running buffer (25 mM Tris-base; pH 8.3, 192 mM glycine, and 0.1% SDS) at 50 mA for 60 min at room temperature. The proteins were electrophoretically transblotted to a polyvinylidene difluoride membrane by using an electrotransfer apparatus (Bio-Rad, Hercules, CA, USA). Protein transfer was conducted for 40 min at 0.8 mA/cm^2 in a transfer buffer (20 mM Tris-base (pH 8.3), 150 mM glycine, 10% methanol, and 0.01% SDS). The membrane was blocked through 60-min incubation in a blocking buffer (phosphate-buffered saline (PBS) containing 0.5% Tween 20 and 1% BSA), followed by overnight incubation with anti–inhibin alpha (1:400) (Serotec, Oxford, England), anti–proliferating cell nuclear antigen (PCNA), or anti-Cx43 (1:1000) (Sigma–Aldrich) in PBS Tween-20 (PBST) with 0.1% BSA at 4 °C. After three washes for 5 min each with the PBST buffer, the membranes were incubated for 60 min with horseradish-peroxidase-conjugated goat antimouse (for mouse antibodies) immunoglobulin G (1:20,000) (Chemicon) in PBST with 0.1% BSA. After three washes with PBST, the membranes were developed by using an enhanced chemiluminescence detection system according to the manufacturer's instructions (Biological Industries Co., Kibbutz Beit Haemek, Israel). The intensity of the bands on Western blots was measured using a MultiImage computer-assisted image system (Alpha Innotech Co., San Leandro, CA, USA).

Statistical analysis

Values were expressed as the mean ± standard deviation of pooled data from three to four experiments. In experiments with more than two groups, significant differences between the groups were determined through ANOVA (SPSS 19.0, IBM Inc., Chicago, IL, USA). The means were then analyzed through multiple comparison using Fisher's probable least-squares difference test.

Results

SD increased serum corticosterone levels in adult female rats

We measured serum concentrations of corticosterone in the rats after 4 days of SD to elucidate whether SD increases serum corticosterone concentrations and to determine whether SD is a stressor. After 1 day of SD, corticosterone concentrations did not change significantly. After 2–4 days of SD, the rats in the SD group had significantly higher corticosterone levels than the sham group ($P < 0.05$, Fig. 1A). We measured serum concentrations of estradiol during the estrus cycle to determine whether the rhythm of the estradiol level that defines the estrous cycle was affected by SD. Cyclical changes of estradiol were absent in the SD group, whereas estradiol levels in the sham group kept the rhythm of the estrus cycle (Fig. 1B).

BET attenuates PMSG-stimulated ovarian growth in 21-day-old rats

To determine whether gonadotropin-stimulated ovarian growth was affected by GCs, immature rats were injected with BET. We assessed the status of the ovaries. Compared with the controls, ovary weight was lower in the rats in the BET with PMSG group and higher in the rats in the PMSG group (Table 1). The ovarian area and follicular size were greater in the rats in the PMSG group than in the controls; follicular growth arrest was observed in the BET with PMSG group (Table 1). These results indicate that PMSG stimulated ovarian growth, which was attenuated by BET.

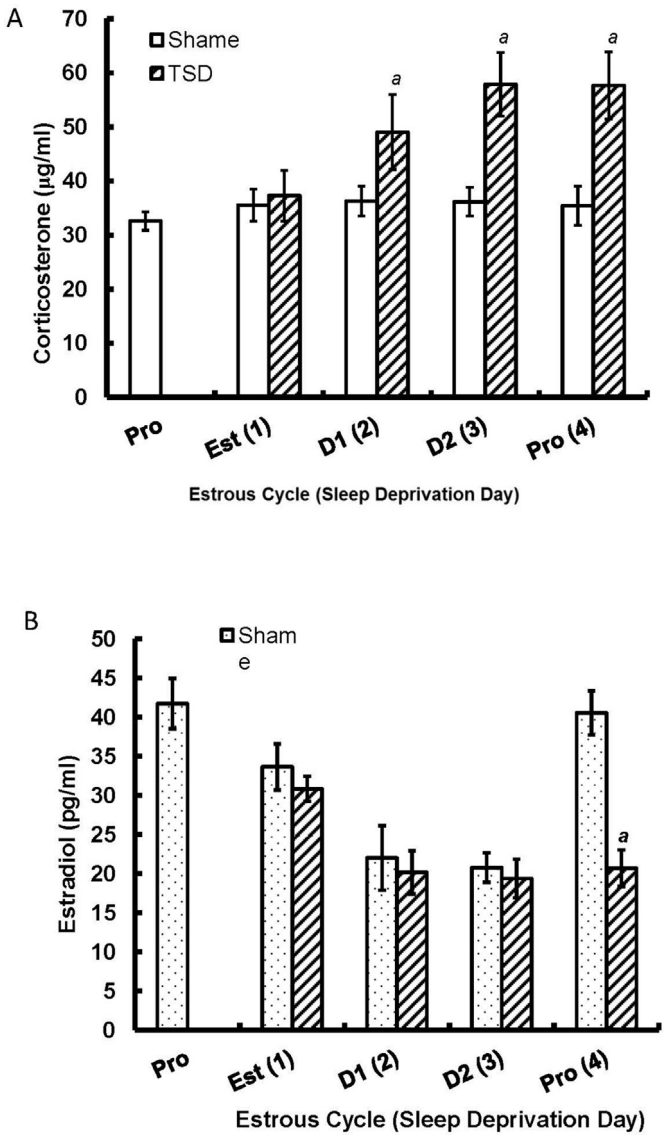

Fig. 1. Stages of the estrus cycle and changes in serum (A) estradiol and (B) corticosterone concentrations in rats following sleep deprivation (SD). Pro., proestrus; Est., estrus; D., diestrus. Rats in the sham group were allowed to sleep; rats in the SD group were sleep deprived for 1–4 days. The number between parentheses indicates the days of SD. Values are each the mean ± standard deviation of six animals. $^aP < 0.05$ vs. the sham group.

BET suppresses FSH-stimulated preantral follicular growth in vitro

We cultured preantral follicles in vitro to assess whether previous ovarian growth arrest resulted from BET regulation of follicular cell growth or development. Preantral follicles cultured over 72 h in a control medium without FSH had minimal growth (72 h vs. 0 h; follicular diameter 150.7 ± 0.9 μm vs. 170 ± 1.44 μm, n = 30) (Fig. 2A), whereas when FSH (1–5000 mIU/mL) was added, follicular size markedly increased at 10 mIU/mL FSH and reached a plateau at 100 mIU/mL (185 ± 2.34 μm, n = 30, $P < 0.01$ vs. control, Fig. 2A). To assess the effect of GCs on the growth of preantral follicles, follicles were cultured with an increasing dose of BET (0.001–1 μg/mL) in the presence of 100 mIU/mL FSH. As shown in Fig. 2B, BET markedly attenuated increases in follicular growth induced by FSH in a dose-dependent manner. At 0.1 μg/mL, BET significantly suppressed FSH-stimulated increases in follicular size (185 ± 2.35 μm to 173 ± 1.77 μm, $P < 0.01$, Fig. 2B) when the

Table 1
Effects of BET on ovarian weight, ovarian area, and follicular development.

	Control (n = 6)	PMSG (n = 6)	PMSG + BET (n = 6)
Ovaries (g)	0.14 ± 0.02	0.25 ± 0.04[a]	0.19 ± 0.02[a,b]
Ovary area (mm^2)	18.4 ± 1.3	29.8 ± 1.5[a]	21.7 ± 1.6[a,b]
Area of largest follicle (mm^2)	0.27 ± 0.06	1.08 ± 0.16[a]	0.63 ± 0.19[a,b]

Values are the mean ± standard deviation.
[a] $P < 0.05$ vs. controls; [b] $P < 0.05$ vs. PMSG group.

incubation time was 72 h. These data reveal that FSH stimulated follicular growth in vitro, and this growth was attenuated by BET. Pregnenolone 16 a-carbonitrile (PCN), a GR antagonist, was used to examine whether a decrease in follicular growth due to BET is mediated by the GR. Preantral follicles received a combined treatment of BET (0.1 μg/mL) and FSH (100 mIU/mL) in the presence or absence of PCN (1 μg/mL). To clarify that changes in follicle diameter represent decreases in follicular cell number, we used an MTS assay to analyze the viable cell number in follicles in the different treatment groups at the end of culture (Fig. 3A). FSH treatment increased the follicular cell number by 15% compared with that of the control group ($P < 0.01$). The addition of BET (0.1 μg/mL) lessened the FSH-stimulated increase in the cell number per follicle (from 1159 ± 29 cells/follicle to 950 ± 21 cells/follicle, $P < 0.01$). Changes in the number of follicular cells were consistent with changes in the cell proliferation marker PCNA (Fig. 3B).

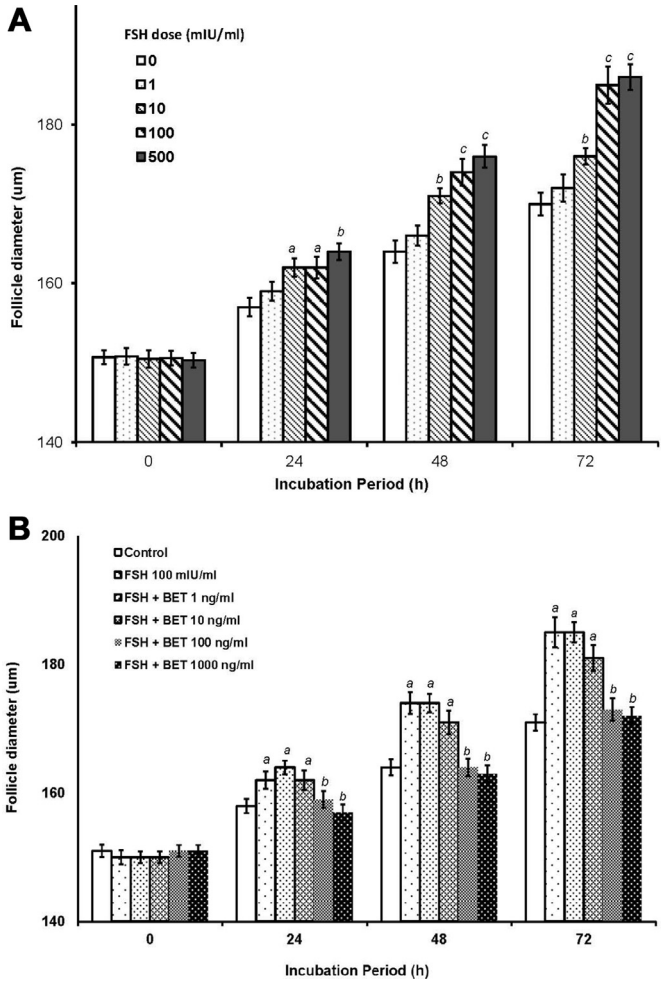

Fig. 2. Preantral follicular diameter change during in vitro culture. (A) Effect of FSH on changes in preantral follicular diameter. Follicles were cultured with an increasing dose of rhFSH (0–500 mIU/mL) for 72 h in serum-free media. [a] $P < 0.05$, [b] $P < 0.01$, and [c] $P < 0.001$ vs. the FSH 0 mIU/mL group for different incubation periods. (B) Effect of BET on changes in preantral follicular diameter. Follicles were cultured with or without rhFSH (100 mIU/mL) in the presence of an increasing dose of BET (0–1000 ng/mL) for 72 h in serum-free media. The diameter of individual follicles was measured every 24 h. The results are expressed as the mean ± standard deviation from 30 preantral follicles in three independent experiments. [a] $P < 0.05$ vs. the control group for different incubation periods. [b] $P < 0.05$ vs. the FSH 100 mIU/mL group for different incubation periods.

Preantral follicular developmental markers downregulated by BET

Expression of connexin 43 (Cx43) and inhibin-α, which have been used as markers for differentiation of granulosa cells and

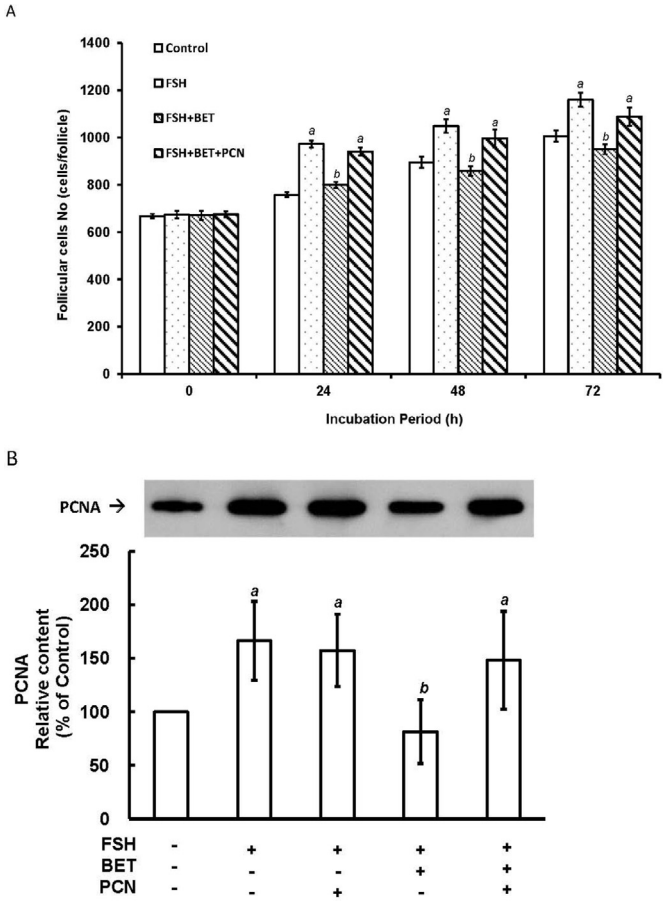

Fig. 3. (A) Effects of BET on number of viable cells after preantral follicles were cultured for 72 h. (B) Effects of BET on PCNA protein expression after follicles were cultured for 72 h. Preantral follicles were cultured and then subjected to a MTS assay as described in the Materials and Methods section. Data are reported as cells per follicle; 24 follicles per group were analyzed. The results are expressed as the mean ± standard deviation from three independent experiments. Preantral follicles were treated with or without FSH (100 mIU/mL), BET (100 ng/mL), and/or PCN (1 mg/mL) for 72 h. [a] $P < 0.05$ vs. the control group. [b] $P < 0.05$ vs. the FSH (100 mIU/mL) group. Protein was extracted from 25 follicles per treatment group after culture for 72 h with different treatments.

follicles, increases with the maturity of follicles [21,23]. We used Cx43 and inhibin-α as developmental markers to determine the effects of BET on the development of cultured follicles. Both Cx43 and inhibin-α antigen were present at low levels in control preantral follicles after 72 h of culture (Fig. 4A, B). By contrast, treatment with FSH increased Cx43 and inhibin-α expression by approximately 1.5 folds. BET treatment attenuated FSH-induced Cx43 and inhibin-α expression ($P < 0.01$) (Fig. 4A, B). PCN may reverse the downregulating effects of BET on Cx43 and inhibin-α expression.

Discussion

Hypothalamus, pituitary gland, and adrenal glands (HPA axis), regulates the body's adaptive response to stress and glucocorticoids are hormonal mediators of stress. This study showed that SD increases corticosterone secretion, which is related to stress. We also found that SD administered animals exhibited complete absence of proestrous and estrous with prolonged period of diestrous. The mean serum level of estradiol showed a steady decline, while the ovaries failed to share the characteristic hormonal activity after 4 days SD. These results indicate that the effect of sleep deprivation on the estrus cycle is associated with an increase in corticosterone. These findings are consistent with previous studies, which have shown that total or paradoxical SD not only elevates serum GC levels but also leads to disturbances in female reproductive function in humans and other animals, such as an irregular menstrual cycle and an extended duration of the reproductive cycle [4,5,22].

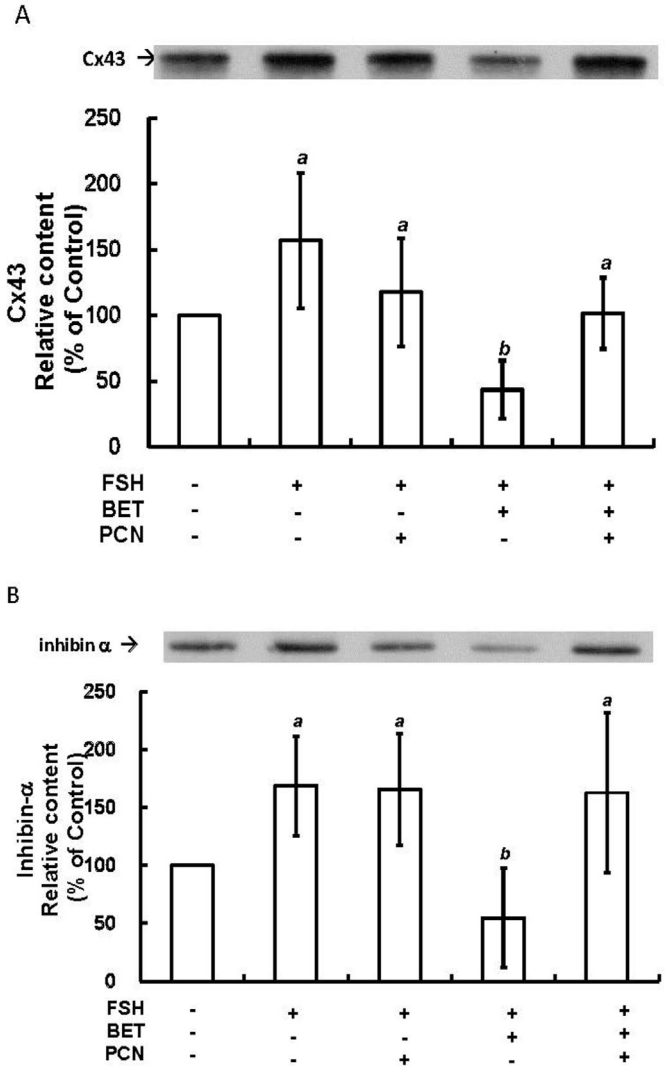

Fig. 4. Effects of BET on inhibin-α and Cx43 protein expression in preantral follicle cultures. (A) BET suppressed FSH-induced inhibin-α. (B) BET suppressed FSH-induced Cx43 protein expression. Preantral follicles were treated with or without FSH (100 mIU/mL), BET (100 ng/mL), and/or PCN (1 mg/mL) for 72 h. The results are expressed as the mean ± standard deviation from four independent experiments. Data are displayed as the mean ± standard deviation. $^{a}P < 0.05$ vs. the control group. $^{b}P < 0.05$ vs. the FSH (100 mIU/mL) group. Protein was extracted from 25 follicles per treatment group after culture for 72 h with different treatments.

The effects of glucocorticoids on estradiol variation during the estrous cycle may occur at the hypothalamus (to decrease the synthesis and release of GnRH), the anterior pituitary gland (to inhibit the synthesis and release of gonadotropins), or the ovary (to modulate steroidogenesis and/or oogenesis) [15]. However, the role of GCs in the development of reproductive disorders caused by SD remains unclear. Immature females are often used to assess whether various substances directly affect ovarian function because immature females are unable to secrete gonadotrophins [24]. Our results showed that PMSG could increase the ovary weight and antral follicular numbers, while BET injection in immature female rats significantly reduced antral follicular numbers and ovarian size. This result suggests that GCs might directly impair gonadotropin stimulated ovarian development. Previous studies have found GR in ovarian cells, and several in vitro studies have suggested that GCs may directly act on oocytes to arrest maturation or to induce granulosa cell apoptosis [15,19,22]. These reports are consistent with our findings that BET directly affects ovarian function.

Using a serum-free culture system of preantral follicles isolated from ovaries of immature rats, we have demonstrated that BET is able to decrease significantly FSH-induced follicular size, cell numbers, PCNA and associated differentiated proteins, inhibin-α and Cx43 levels. The BET effect on FSH-induced follicular size is dose and time dependent (Fig. 2). These observations were supported by a reduction in the FSH-stimulated increases in follicular cell numbers and protein expression of PCNA (Fig. 3). We also demonstrated that PCN can displace BET from the GR reversed the effect of BET on follicular cell numbers and PCNA protein levels. The PCN effect is specific, as this compound by itself does not influence the PCNA, inhibin-α and Cx43 proteins expression induced by FSH. Because GR is expressed constitutively throughout follicular development and luteinization [25], this inhibitory effect is prevented by the GR antagonist PCN, which thus suggests that follicular growth essentially results from GC-receptor-mediated events.

During follicular development, the proliferation of follicular cells and the size increase in oocytes are necessary for follicular maturation [21]; hence, the slow proliferation of follicular cells blocks follicular development. GCs are potent antiproliferative agents in many cell types and are also known to induce G_1 arrest and programmed death of several leukemia cell lines [26,27]. Therefore, the decrease in the FSH-stimulated increase in follicular cell numbers and protein levels of PCNA in preantral follicles after BET treatment was a direct reflection of these processes. PCNA is an auxiliary factor of DNA polymerases δ and ε in eukaryotic cells, which are required for DNA synthesis and repair, and is widely used as a marker of cell proliferation [28]. Because PCNA begins being synthesized in the late G1 period and is required for DNA synthesis, the decrease of PCNA in the preantral follicles due to BET probably led to follicular cells being arrested in the next G1 phase or being blocked from the entry into the next cell cycle. More research is required to confirm these results. Follicular development involves cell proliferation and differentiation. BET attenuates the differentiation of preantral follicles induced by FSH, as demonstrated by the

decrease in inhibin-α subunit and Cx43 expression in this study. Inhibin-α is produced by ovarian granulosa cells, is correlated with the number of developing follicles, and is considered an indicator of follicular development [21]. In developing follicles, gap junctions formed by connexins couple the growing oocyte and its surrounding follicular cells into a functional complex and thereby transport small molecules bidirectionally. Among connexins, Cx43 is particularly vital for follicular development because the absence of Cx43 blocks the preantral to antral follicle transition in mice [29]. To our knowledge, few studies have reported the effects of GCs on intact preantral follicles. Exposure to high GC concentrations attenuates FSH-stimulated estradiol but increases progesterone synthesis by GCs in culture [29].

This study highlights a crucial aspect concerning SD: to enhance the secretion of corticosterone in rats, it might directly act on ovarian follicles and thus lead to disorders of the estrous cycle. We cannot rule out the possibility that SD may also affect the secretion of reproduction-related hormones in the hypothalamus and pituitary gland. However, the results of BET injection in the immature rats showed that GCs might directly attenuate FSH-stimulated ovarian growth, which may directly affect both follicular growth and differentiation in rat preantral follicles in vitro. We cannot yet define the role that GCs play in follicular cell events that lead to cell proliferation and differentiation. However, we show that BET decreases levels of FSH-stimulated PCNA, inhibin-α, and Cx43 expression. Changes in these proteins may reduce follicular cell proliferation and preantral follicular development.

Conflicts of interest statement

The authors have no potential conflicts of interest to disclose.

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