Original Article
Simulated weightlessness by tail-suspension affects follicle development and reproductive capacity in rats

Chunyu Wang[1], Jihua Wu[1], Yan Cui[2], Shujun Song[1], Jun Zhang[1], JJiinlian Zhou[1], Jianzhong Zhang[1]

Departments of [1]Pathology, [2]General Surgery, 306 Hospital of PLA, Beijing, People's Republic of China

Abstract: Weightlessness is known to induce many pathophysiological changes in various organs and systems in simulated weightlessness animal models. However, there is limited data on the effects of weightlessness on the female reproductive system. In the present study, we used the Morey-Holton hind limb suspension model to simulate weightlessness in female rats. We have shown that this weightlessness induces alterations in follicle development, ovarian hormone secretion, proliferation and apoptosis of granulosa cells, and reproductive capacity. These results demonstrate that simulated weightlessness decreases follicle number, enhances follicular atresia, inhibits granulosa cell proliferation, promotes granulosa cell apoptosis, affects levels of hormone secretion, and significantly decreases reproductive capacity. In addition, our results show that the negative effects of microgravity on the reproductive functions in female rats are reversible when gravity is restored.

Keywords: Simulated weightlessness, microgravity, ovary, tail-suspension, granulosa cells

Introduction

Gravity has been a constant physical factor shaping the evolution of life on Earth. Reproduction in all animals is well adapted to Earth's gravitational field, and any gravitational change may have significant effects on reproduction and development [1-4]. Space flight induces many physiological changes in various organs and systems, including fluid shifts, muscle atrophy, bone demineralization, and depressed cellular immunity due to gravity changes. Space flight also causes other conditions such as increased radiation, changes in noise levels, isolation, and disrupted circadian rhythms [3-6]. Partly due to the demands and expense of space experiments, there is limited information on the effects of weightlessness affecting the structure and function of the female reproductive system. Although the absence of gravity cannot be accurately simulated on the ground, several models mimic the body's responses observed after exposure to microgravity in actual spaceflight. Antiorthostatic bed rest, dry immersion in humans, and tail-suspension or hind limb unloading in rodents are the most commonly used models that can mimic many of the physiological alterations in various organ systems [7-9].

Ambitious space exploration plans raise challenges for medical research in space to ensure the safety of male and female astronauts during prolonged spaceflights. Some studies have reported that simulated weightlessness reduces testicular weight, decreases testosterone levels, and reduces the expression of androgen receptor in the testes [10, 11]. Germinal epithelium irregularity, malformed spermatozoa, degeneration, and necrosis were found in the seminiferous tubules along with overexpression of Hsp70 and activation of the apoptotic pathway. These changes contribute to the testicular injury that is detected in experimental animal model studies [12]. However, limited information is available as to whether simulated weightlessness induces pathophysiological alterations to the female reproductive system [13, 14]. Therefore, the present study was designed to investigate the follicular development and reproductive capacity of rats under simulated weightlessness and to determine whether microgravity imposes any associated stress
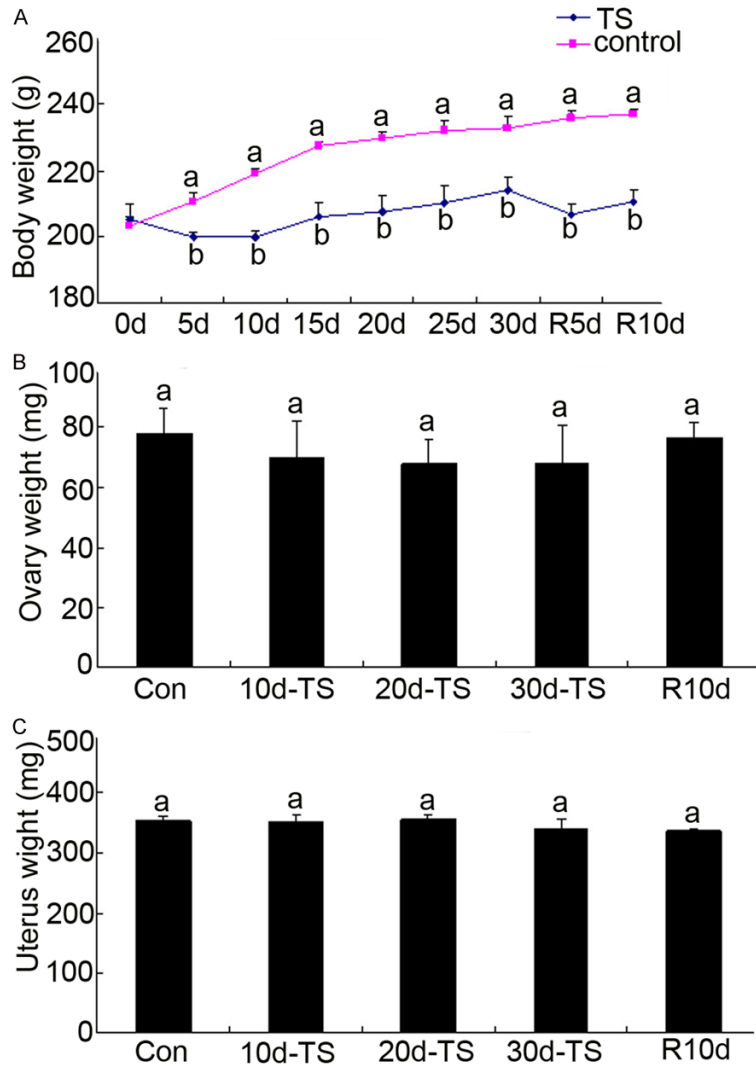
Figure 1. Effects of simulated microgravity on the body weight (A) ovarian weight (B) and uterine weight (C) of rats. The data are represented as mean ± SEM. Different letters indicate a significant difference versus the corresponding control group at the same time or following the same treatment (n = 10, $P < 0.05$).

week after arrival, the animals were randomly assigned to five groups: 10 d-TS group (n = 5), 20 d-TS group (n = 5), 30 d-TS group (n = 10), 10 d-gravity-restored after 30 d-TS group (R10 d group, n = 10), and a control group (n = 10). The rats' tails were cleaned and dried. A thin layer of adhesive tape was applied to the middle of the tail along the medial to lateral sides and a harness made of standard tape was pressed firmly to the glue. A paper clip was used to attach the rat's tail harness to a swivel apparatus on a wire spanning the top of the cage. The height of the animal's hind limbs was adjusted to prevent any contact with the cage bottom, which made the head tilt of 30° downwards. The forelimbs of the animal maintained contact with the cage bottom allowing the rat full access to the entire cage. The control animals were individually maintained in identical cages and allowed free movement across the cage. All rats were weighed at intervals of five days and at the end of the experiment.
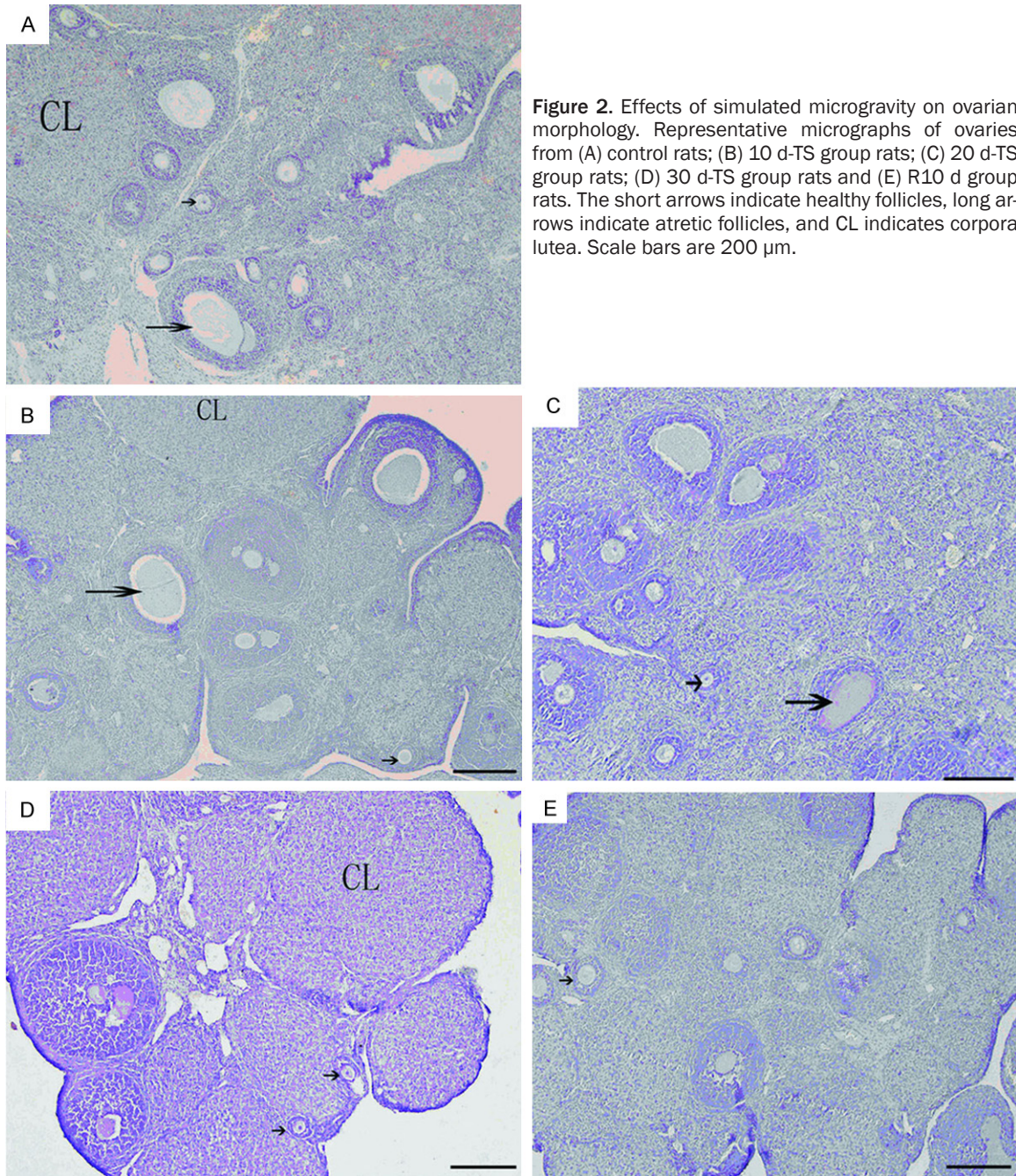
Following the period of suspension, the rats were anesthetized with chloralhydrate. Laparotomies via the middle line were made, and the ovaries and uterus were harvested from all animals and weighed.

Morphometric analysis of ovarian follicles

One side of each rat ovary was immersed and fixed in 4% paraformaldehyde (PFA) at 4°C for 24 h, embedded in paraffin, serially sectioned (8 μm), and mounted on glass slides. Routine hematoxylin and eosin staining were performed for histological examination, and every tenth section was selected for analysis. The follicles were classified as previously described [15]. Briefly, primary follicles were classified as an oocyte surrounded by a single layer of cuboidal

responses and pathophysiological alterations on the ovary.

Materials and methods

Animals

All animal studies were approved by the Ethics Committee for Laboratory Animal Sciences of 306 Hospital of PLA. Wistar female rats weighing 200 ± 20 g were housed under a 12 h light/dark cycle (06:00 to 18:00 h daily) and constant temperature (21 ± 2°C) conditions, and allowed free access to water and food.

The tail-suspension (TS) method was used to establish simulated weightlessness [7]. One

Figure 2. Effects of simulated microgravity on ovarian morphology. Representative micrographs of ovaries from (A) control rats; (B) 10 d-TS group rats; (C) 20 d-TS group rats; (D) 30 d-TS group rats and (E) R10 d group rats. The short arrows indicate healthy follicles, long arrows indicate atretic follicles, and CL indicates corpora lutea. Scale bars are 200 μm.

cells. Secondary follicles comprised an oocyte with a visible nucleolus, two-to-five layers of granulosa cells, but no antrum. Antral follicles contained an oocyte with a visible nucleolus, more than five layers of granulosa cells, and/or a distinguishable antrum. Atretic follicles were defined as having an oocyte and/or granulosa cells that exhibit pyknotic nuclei, cytoplasmic shrinking, and granulosa cells pulling away

from the basement membrane in the follicular fluid.

In order to measure the diameter, only the follicles with the nucleus and nucleolus of the oocytes were selected and the diameter was measured using a calibrated ocular micrometer. Mean diameter was obtained by adding the maximum diameter plus the diameter taken at
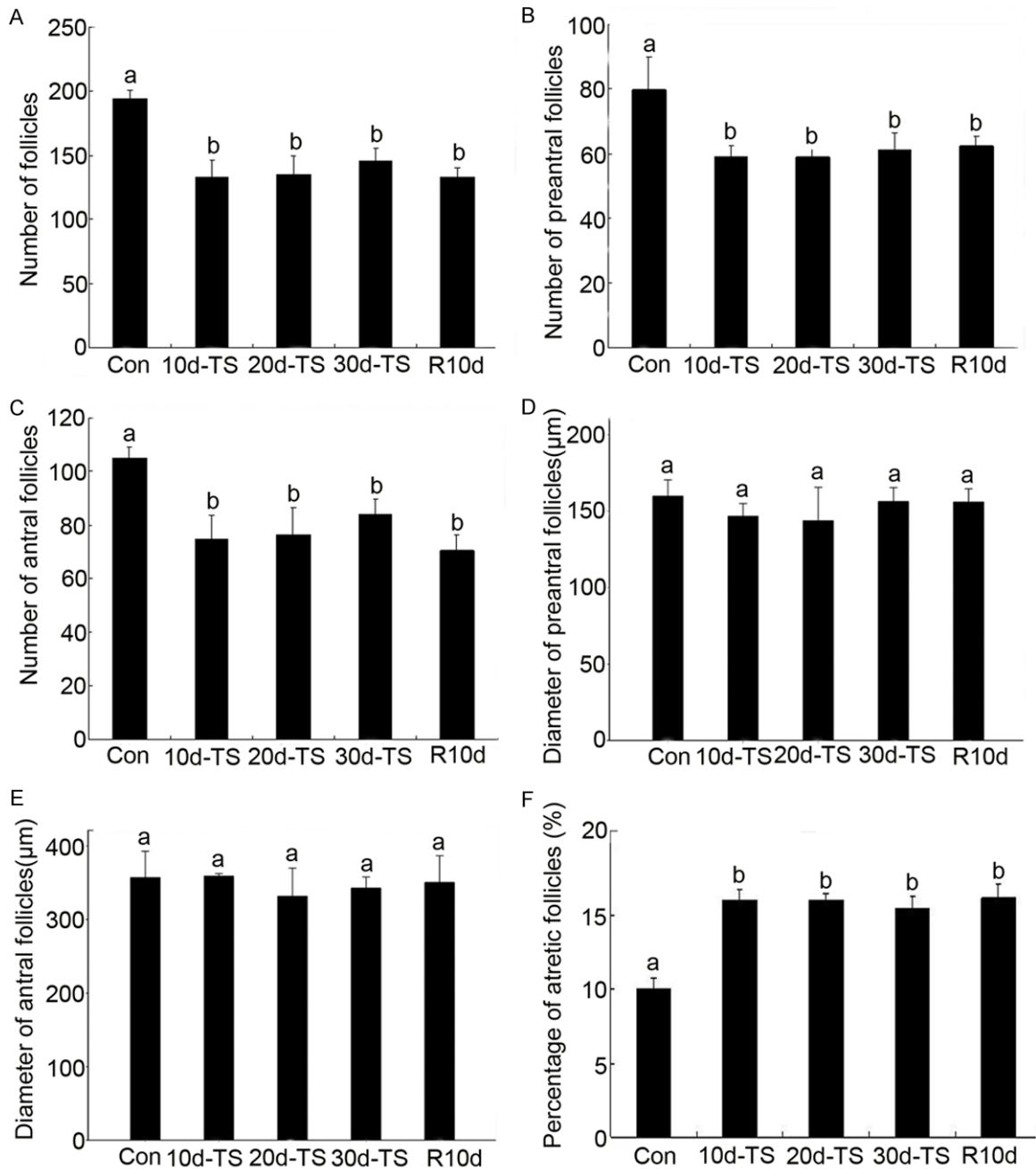
Figure 3. Simulated microgravity decreases the number of follicles and enhances follicular atresia. (A) The total number of follicles; (B) The number of preantral follicles; (C) The number of antral follicles; (D) The diameter of preantral follicles and (E) the diameter of antral follicles. The values represent mean ± SEM (n ≥ 5, $P < 0.05$). (F) The percentage of atretic follicles in TS and control ovaries. The values represent mean ± SEM. Groups with different letters are statistically different (n ≥ 5, $P < 0.05$).

right angles [16]. Data are presented as mean ± SEM from at least 10 ovaries for each time point.

BrdU immunohistochemistry

To study the cellular proliferation of follicles, BrdU (75 mg/kg; Roche Molecular Biochemi-

cals, Sussex, UK) dissolved in PBS was intraperitoneally administered 2 h before the ovaries were collected [17]. The collected ovaries were fixed, dehydrated, embedded in paraffin wax, and 8-μm-thick sections were cut. The immunohistochemical procedures for BrdU detection were performed as previously described [18]. Briefly, the sections were
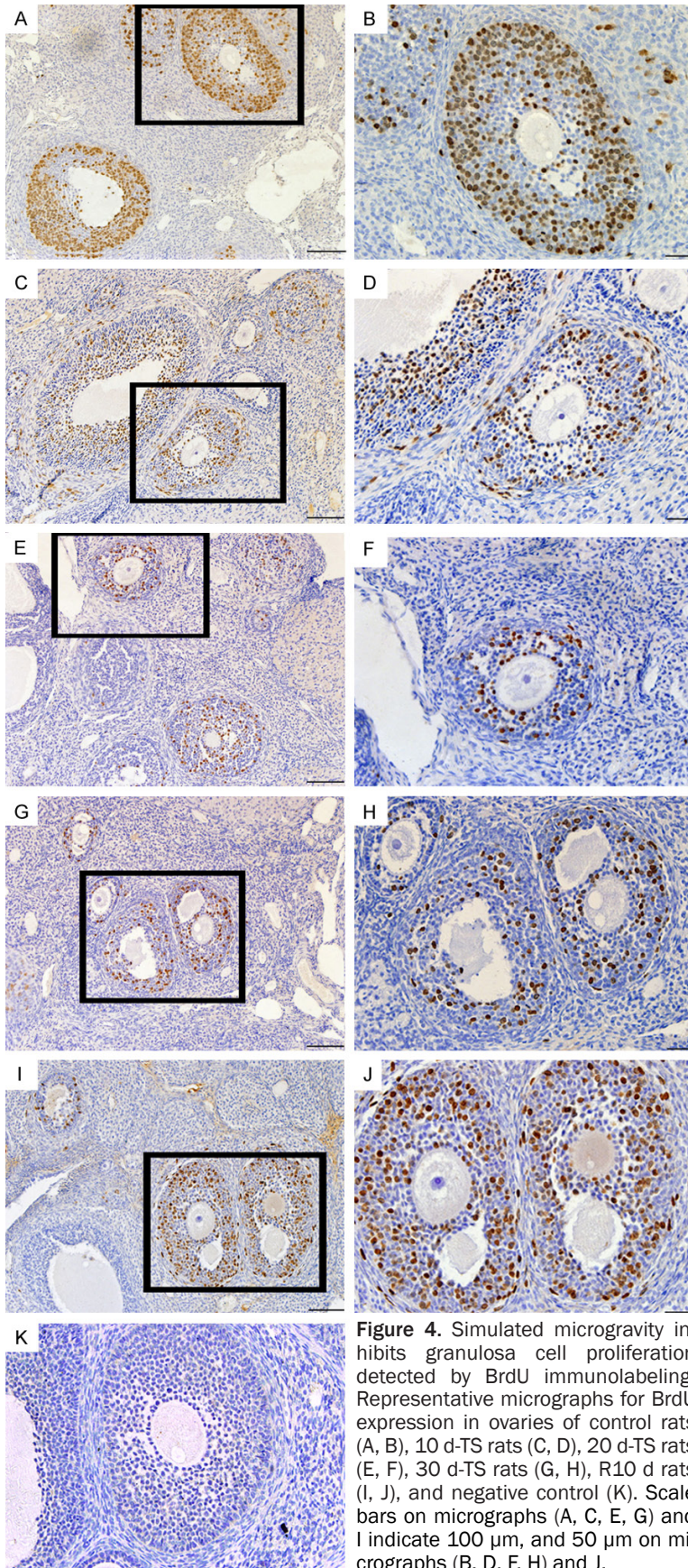
Figure 4. Simulated microgravity inhibits granulosa cell proliferation detected by BrdU immunolabeling. Representative micrographs for BrdU expression in ovaries of control rats (A, B), 10 d-TS rats (C, D), 20 d-TS rats (E, F), 30 d-TS rats (G, H), R10 d rats (I, J), and negative control (K). Scale bars on micrographs (A, C, E, G) and I indicate 100 μm, and 50 μm on micrographs (B, D, F, H) and J.

dewaxed and rehydrated, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H_2O_2) in PBS (0.01 M, pH 7.4) for 30 min. DNA was denatured in 2 M HCL for 30 min at 37°C, and BrdU-incorporated sites were exposed by treatment with 0.01% trypsin (Sigma, St. Louis, MO, USA) for 12 min at 37°C. The sections were rinsed in PBS, treated with 10% normal goat serum in PBS, and incubated with an anti-BrdU primary antibody G3G4 (1:50; Developmental Studies Hybridoma Band, University of Iowa, IA, USA) overnight at 4°C. The sections were then washed in PBS and incubated with biotinylated goat anti-mouse IgG (1:150; Zymed, San Francisco, CA, USA) for 3 h at room temperature. Sections were washed in PBS and incubated with a streptavidin-peroxidase complex (1:150; Zymed) for 3 h at room temperature. After a PBS wash, the sections were subsequently visualized with diaminobenzidine (DAB), and counterstained with hematoxylin.

Apoptosis assays

To detect apoptotic granulosa cells, cleaved caspase-3-positive cells were detected by immunochemistry. The sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in PBS (0.01 M, pH 7.4) for 30 min. Antigen retrieval was performed by microwaving the sections (4 × 4 min at medium power) in 0.01 M sodium citrate buffer (pH 6.0). The sections were then rinsed in PBS, treated with 10% normal goat serum, and
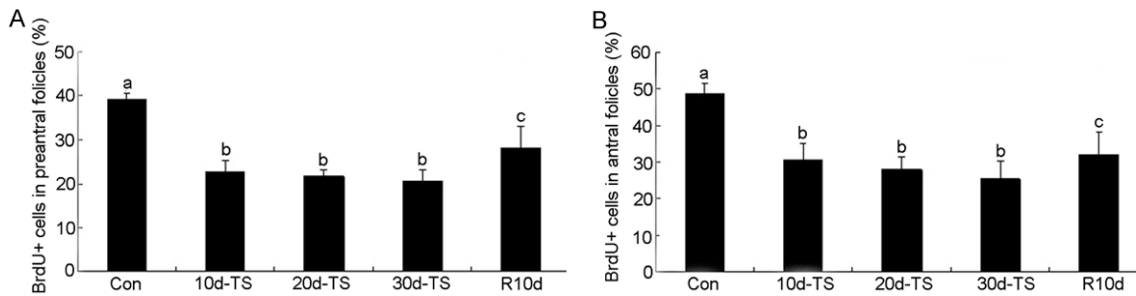
Figure 5. The percentage of BrdU-positive granulosa cells in preantral (A) and antral follicles (B). The values represent mean ± SEM, Groups with different letters are statistically different (n = 5, $P < 0.05$).

incubated with rabbit anti-cleaved caspase-3 monoclonal antibody (1:100; Cell Signaling Technology, Boston, MA, USA) overnight at 4°C, followed by PBS washes, and incubation with biotinylated goat anti-rabbit IgG (1:150; Zymed, USA) for 3 h at room temperature. After an additional wash in PBS, sections were incubated with a streptavidin-peroxidase complex (1:150; Zymed, USA) for 3 h at room temperature. After washing three times in PBS, positive staining was subsequently visualized with DAB and sections were counterstained with hematoxylin.

Hormone assays

Venous blood samples were collected from each rat during diestrus (detected by wet mount smears of vaginal secretion) to minimize natural fluctuations in hormone levels. The samples were centrifuged at 3500 × *g* for 10 min at 4°C. The sera were stored at -20°C until assayed. Concentrations of estradiol, progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) concentrations were determined by radioimmunoassay (RIA) using commercially available kits (Northern Biotechnology, Beijing, China). Estradiol, progesterone, FSH, and LH were assayed using 50 μL, 100 μL, 200 μL, and 200 μL sera, respectively. The sensitivity of the RIA was 2 pg mL^{-1} for estradiol, 2 ng mL^{-1} for progesterone, and 1.0 mIU mL^{-1} and 0.15 mIU mL^{-1} for FSH and LH, respectively. The average intra-assay and inter-assay coefficients of variation were less than 10%.

Reproductive capacity assays

The female Wistar rats in estrus were selected by wet mount smears of vaginal secretions. Male rats were introduced into the cages containing the female Wistar rats from the 30 d-TS (n = 5) and R10 d (n = 5) groups to determine the litter size of newborn rats as compared with the corresponding control animals (n = 5).

Cell counting and statistical analysis

To estimate granulosa cell proliferation and apoptosis, 3-5 follicles from one section of each rat were randomly chosen to be viewed under a microscope. Firstly, we photographed sections of BrdU and cleaved caspase-3 immunohistochemistry with a Leica DMLB microscope equipped with a Leica DFC320 camera (Leica) under a 20 × and 40 × magnification objective, respectively. The BrdU and cleaved caspase-3 immunohistochemical-positive cells and total cells from the micrographs were counted using the manual counter function of the AlphaImager 2200 software (Proteinsimple, Santa Clara, California). All cell counting was performed by a single investigator. The results are expressed as the percentages of BrdU or cleaved caspase-3 immune-positive cells per follicle.

All experiments were repeated at least three times and the results are expressed as the mean ± SEM. Body weight and serum concentration of reproductive hormones, the litter number of rats, and the body weight of pups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS software (SPSS, Chicago, IL, USA). In addition, the number of follicles and the percentage of BrdU or cleaved caspase-3-positive granulosa cells were analyzed using the Kruskal-Wallis test. Differences between groups were considered significant if $P < 0.05$.

Results

Effects of simulated microgravity on body and reproductive organ weights

We examined the bodyweight of each rate after simulated microgravity. As shown in **Figure 1**, there was an obvious decrease in body weight at all days of suspension and all days of gravity recovery compared with the control ($P < 0.05$).
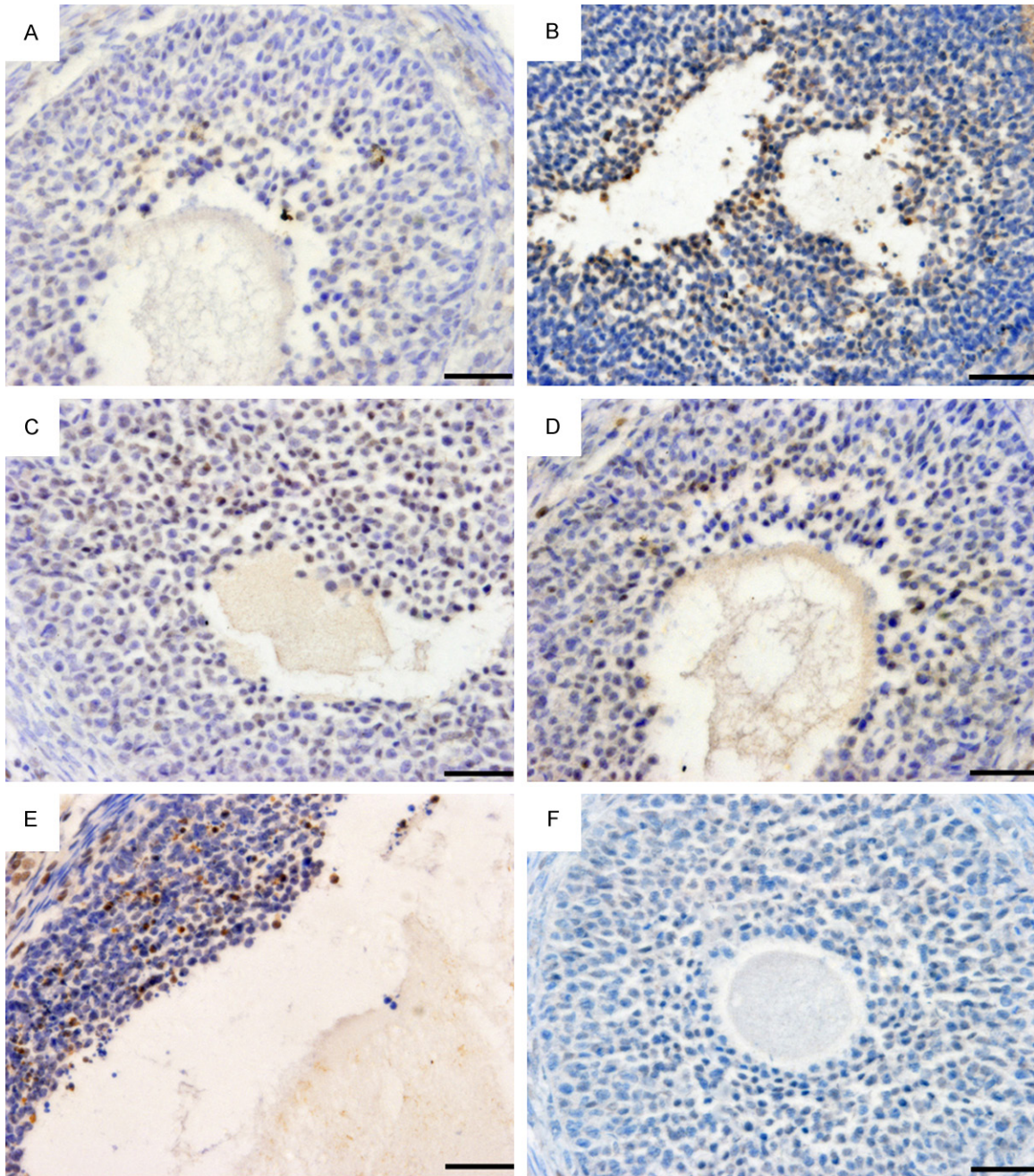
Figure 6. Representative micrographs showing cleaved caspase-3 expression in granulosa cells from antral follicles in control rats (A), 10 d-TS rats (B), 20 d-TS rats (C), 30 d-TS rats (D), R10 d group rats (E), and negative control (F). Scale bars on the micrographs are 30 μm.

There were no significant difference in the weights of the rat ovaries and uteruses subjected to the simulated microgravity compared with those from control animals (**Figure 1**).

Effects of simulated microgravity on follicular development and follicular atresia

To determine the effect of simulated microgravity on follicular development, micrographs were taken (**Figure 2**). The total numbers of follicles, preantral follicles, and antral follicles were counted and the follicular size was analyzed by measuring the follicular diameter. The numbers of total follicles, preantral follicles, and antral follicles were all significantly reduced ($P < 0.05$, **Figure 3A-C**); however, there was no significant change in the preantral follicular diameter and the antral follicular diameter in the TS rats compared with the control rats (**Figure 3D** and **3E**).
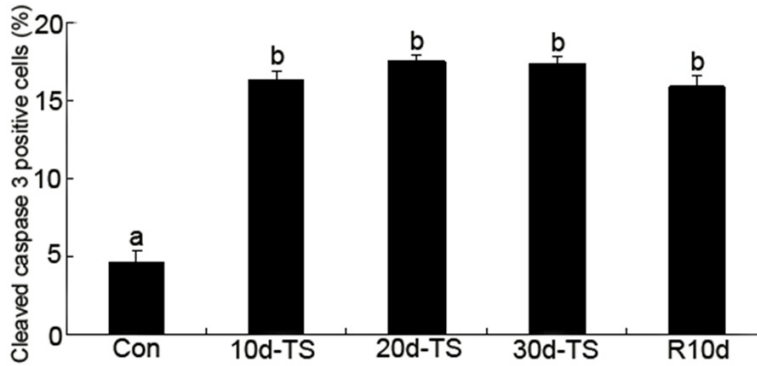
Figure 7. The percentage of cleaved caspase-3-positive granulosa cells in antral follicles. The values represent mean ± SEM (n ≥ 5). Groups with different letters are statistically different ($P < 0.05$).

We counted and compared the numbers of atretic follicles (**Figure 3F**). The results showed that the percentage of atretic follicles was 16 ± 0.8% in 10 d-TS group rats, 16 ± 0.9% in 20 d-TS group rats, and 15 ± 0.9% in 30 d-TS group rats, which was significantly higher than that observed in the control group (10 ± 0.8%). The number of atretic follicles in the R10 d group ovaries (15 ± 1.0%) was not significantly different from that in the TS groups.

Effect of simulated microgravity on BrdU and caspase-3 activities in the ovary

Figures 4 and **5** show BrdU immunolabeling in granulosa cells, theca cells, and luteal cells. However, no staining was observed in the interstitial cells from any of the animals used for this study. As expected, we found a lower number of BrdU-positive cells in TS rats compared with the control rats. The proportion of BrdU positive cells in the control rats was 39 ± 1.1% in the preantral follicles and 50.4 ± 1.9% in the antral follicles. The proportion of BrdU-positive cells in 10 d-TS group rats was 28.7 ± 2.3% in the pre-antral follicles and 44.6 ± 3.2% in the antral follicles, in the 20 d-TS group, the rats had 22 ± 1.1% and 35.1 ± 2.9% positive cells, and 30 d-TS group rats had 21.3 ± 2.9% and 28 ± 6.6% ($P < 0.05$) in the preantral and antral follicles, respectively. We found that the number of BrdU-positive cells in rats in the R10 d group was higher than that in the 30 d-TS rats (26.8 ± 3.4% in the preantral follicles and 45 ± 4.6% in the antral follicles) ($P < 0.05$).

We assessed the apoptotic response of granulosa cells by examining cleaved caspase-3 lev-

els by immunohistochemistry. The results show that cleaved caspase-3-positive cells were more condensed in the antral follicles (**Figures 6** and **7**) than in preantral follicles in all ovaries examined. As expected, the percentage of cleaved caspase-3-immunopositive cell numbers in the TS rats (10 d-TS group rats 16.4 ± 0.5%, 20 d-TS group rats 17 5 ± 0.4%, and 30 d-TS group rats 17.3 ± 0.5%) was also higher than in the control rats (4.7 ± 0.8%). These data suggest that simulated weightlessness enhances follicular atresia. There was no significant difference in the number of cleaved caspase-3-positive cells in R10 d group rats (15.9 ± 0.7%) compared with 30 d-TS group rats (17.4 ± 0.5%) (**Figure 7**).

Effect of simulated microgravity on serum hormone concentration

To examine the effect of simulated microgravity on the hormone secreting functions of the ovary, we analyzed the serum levels of estradiol, progesterone, FSH, and LH. The present results showed that simulated weightlessness had no effect on the level of serum progesterone, but led to a significant reduction in serum estradiol levels (**Figure 8**).

Effect of simulated microgravity on reproductive capacity

In the investigation of simulated microgravity on reproductive capacity, we found that the litter number of rats was significantly less in the 30 d-TS group as compared with the corresponding control group ($P < 0.01$). The litter size increased in the R10 d group, but remained lower than that in the corresponding control group ($P < 0.05$) (**Table 1**).

Discussion

With the rapid development of space technology, astronauts of both genders are able to stay in space for long periods. It is therefore essential to determine the effects of long-term weightlessness on the female reproductive system [5]. Using the tail-suspension rat model, the present study is the first to provide evidence
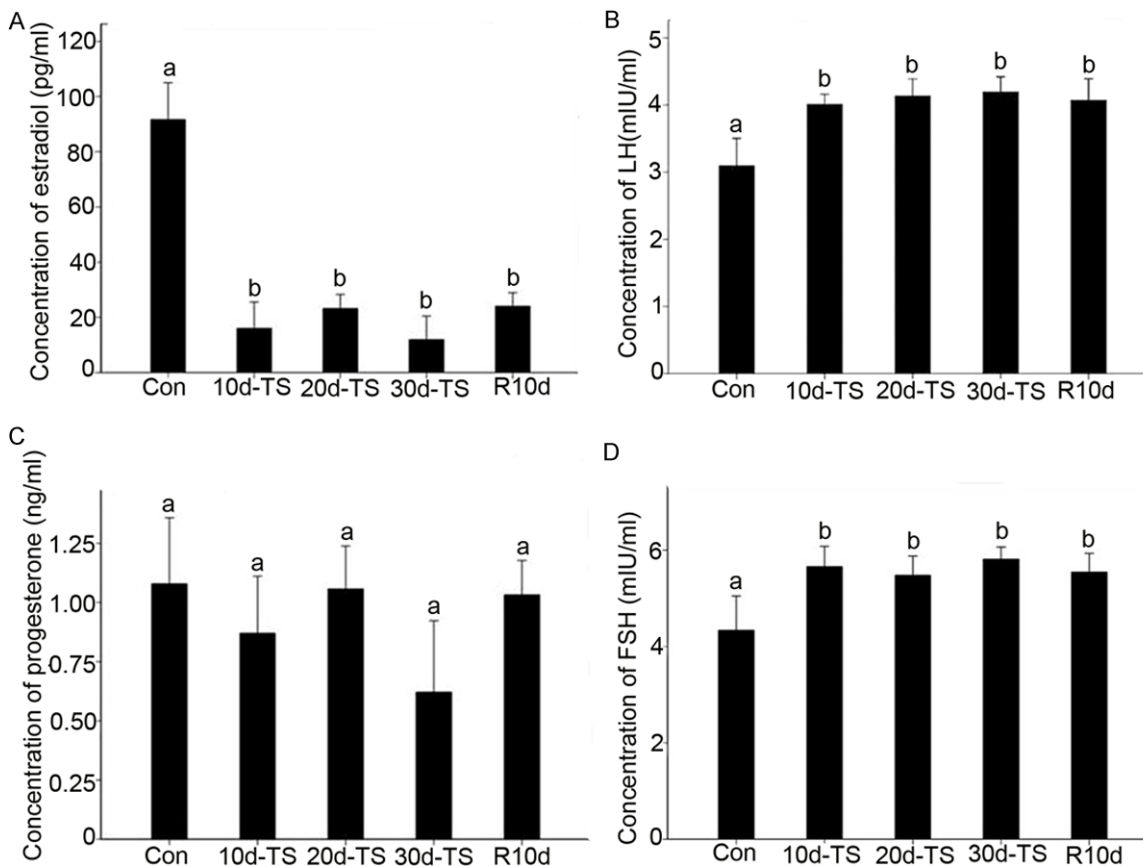
Figure 8. Effect of simulated microgravity on serum estradiol, progesterone, LH, and FSH concentrations. A: The concentration of serum estradiol; B: The concentration of serum LH; C: The concentration of serum progesterone; D: The concentration of serum FSH. The values represent mean ± SEM. Asterisks indicate a statistically significant difference compared with the control (n = 5, P < 0.05).

that simulated weightlessness can reduce follicular number, enhance follicular atresia, and markedly hinder follicular development [7]. However, a 10-day gravity restoration period partially restored reproductive function.

In addition, our data demonstrate that there was a decrease in the bodyweight of each rat on the fifth day of tail-suspension, and that this recovered by the fifth day of gravity restoration, although the bodyweight was not measured each day in the duration of the experiment. A possible explanation is that both microgravity and subsequent gravity recovery stress the rat, which results in a variety of abnormal changes [3, 4, 6]. However, the animals adapt after exposure to long-term weightlessness or gravity recovery, although the related mechanisms need to be determined in future studies.

The results also demonstrate that simulated weightlessness significantly decreases total

follicle number (preantral and antral follicles). A possible reason is that the simulated weightlessness inhibited the activation of primordial follicles, and the follicular transition from primary follicles to preantral follicles did not have any influence on the follicular size. This hypothesis is supported by our data, which show that simulated weightlessness decreases the proportion of antral follicles that make up the total follicles. In addition, the decline in the total follicle number in the simulated weightlessness rat model may account for the enhanced follicular atresia.

We still do not know the mechanism by which simulated weightlessness inhibits follicular development, but successful follicular development and follicle atresia are dependent on the proliferation and survival of their constituent granulosa cells. In addition, increased resistance of granulosa cells to apoptosis is associated with their ability to promote progression

Table 1. Comparison of rat reproduction in different groups (mean ± SEM)

Group (n = 5)	Average litter size	Average Body weight of pups (g)	Range of litter size
Control	11.5 ± 0.57[a]	5.99 ± 0.39[a]	11-12
30 d-TS	3.7 ± 0.57[b]	5.98 ± 0.40[a]	3-4
R10 d	7.0 ± 3.56[c]	5.67 ± 0.37[a]	4–11

Different letters indicate statistically significant differences ($P <$ 0.05).

through the cell cycle [19, 20]. Decreased granulosa cell proliferation and enhanced apoptosis during simulated weightlessness are accompanied by a decline in follicular number. Therefore, the inhibiting effect of the simulated weightlessness on follicular development is closely related to the inhibiting effect on cell proliferation and the enhancement of granulosa cell apoptosis. In addition, simulated weightlessness may inhibit follicular development by influencing the proliferation and apoptosis of granulosa cells, as presented in this study, which are required to maintain the physiological functions of the ovary.

The ovary has two principal functions-the production of sex steroids and ovulation. The present results show that simulated weightlessness had no effect on serum progesterone levels, but that it significantly decreased serum estradiol levels, which is used as a direct index for the function of granulosa cells in mammalian ovaries [21]. However, we did not anticipate that the levels of pituitary gonadotropins, including FSH and LH, would increase in rats under simulated weightlessness. This may be due to the attenuation of the negative feedback that regulates the effect of ovarian estrogen under simulated weightlessness. However, further studies need to be carried out, such as those to test the effect of simulated weightlessness on hypothalamus GnRH, a key factor influencing pituitary gonadotropins [22].

Finally, the results presented here demonstrate that tail suspension in rats inhibits follicle development by suppressing granulosa cell proliferation, enhancing granulosa cell apoptosis, and promoting follicular atresia, which temporally suppresses rat fertility. Furthermore, our study demonstrates that the negative effects on female reproductive function in rats induced by microgravity seem to be reversible. Whether these changes-the number of follicles, follicular atresia, granulosa cell proliferation, granulosa cell apoptosis, hormone secretion levels, and reproductive capacity-are totally reversible should be further studied since female astronauts will in the future have to endure long periods of weightlessness, and understanding the effects on female fertility will be important for the future of space exploration.

Disclosure of conflict of interest

None.

Address correspondence to: Jinlian Zhou, Department of Pathology, 306 Hospital of PLA, Beijing, People's Republic of China. E-mail: drzhoujl@aliyun.com

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