

Full Length Research Paper

Apoptosis of rat's ovarian follicle cells induced by triptolide *in vivo*

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This present study aimed to investigate ovarian follicular apoptosis and development in rats administrated with different dosages of triptolide (TR). Thirty healthy female Sprague Dawley rats were randomly divided into 3 groups (n = 10), including placebo group (water and 0.06% Dimethyl Sulfoxide); high dose (HD) group (120 µg/kg.d TR) and low dose (LD) group (60 µg/kg.d TR). Vaginal smear was daily performed to estimate each rat's estrous cycle, while body weight was measured every 4 days to evaluate rat's growth pattern and adjust the dosage. Left ovary of each rat was resected and then serial section was performed for follicular classification and counts. Apoptosis was detected with TUNEL assay and then positively-stained follicles were classified and calculated. Compared with the placebo and LD groups, the average estrous cycle was significantly longer in the HD group ($p < 0.01$). The secondary follicle in both HD and LD groups was significantly more than the control groups ($p < 0.05$), while there were no significant changes of primordial follicle and antral follicle among three groups. Furthermore, higher proportion of apoptotic follicle in the stage of secondary follicle was found in both HD and LD groups. TR presented dose-dependent inhibition on estrous cycle and apoptosis induction in secondary follicle, which might account for female gonad depression activities.

Key words: Triptolide; ovarian follicle; apoptosis; rat

INTRODUCTION

Triptolide (TR) is one of the main active ingredients of tripterygium wilfordii which is a traditional anti-inflammatory and anti-rheumatic drug. Recent studies have revealed that TR possesses multiple effects including anti-inflammatory, anti-tumor, immune suppression, anti-fertility effects and so on (Corson and Crews, 2007). However, in the past many years, sexual gland suppression such as female amenorrhea and infertility appearing in the application of the mixed preparation "Tripterygium wilfordii multi-glycoside" is attributed to a side effect of TR. Recently, the inhibitory function of TR preparation on ovarian function was investigated, and it was speculated that TR played important roles in the treatment of gynecological diseases such as endometriosis, hysteromyoma and so on (Xu and Zhao,

2008). However, the impact of TR on female reproductive system especially on development of ovarian follicle as well as its mechanism has not yet been clarified. Furthermore, it should be urgent to further investigate the inhibition degree of TR on female gonadal activities and whether TR results in permanent damage of ovarian reproductive potential or not.

MATERIALS AND METHODS

Drugs and reagents

Two bottles of TR standard preparation (20 mg, powder, purity > 99.0%, Batch No: 200502) were purchased from Guangdong Institute for Drug Control. In Situ Cell Death Detection Kit (POD) for TUNEL assay was purchased from Roche (Germany). DAB chromogenic kit was purchased from Guangzhou Whiga Technology Co., Ltd. Referring to the previous method (Pan et al., 2006), 0.6 ml dimethyl sulfoxide (DMSO) was dissolved in 20 mg TR standard preparation, and then the mixture was dissolved in 1000 ml sterile water to be 20 µg/ml of final concentration. Subsequently, the mixture was filtered with a Millipore 0.22 µm bacterial filter and

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Abbreviation: TR, triptolide

then preserved in a 4°C refrigerator. Meanwhile, the equal 0.6 ml DMSO was added into 1000 ml sterile distilled water as placebo.

Pretreatment and grouping of experimental animals

Thirty healthy female SD rats were used in this study, with the following parameters: mean weight of 220 – 250 g, mean age of 10 - 11 weeks, sexual maturity, regular estrous cycle for 4 - 5 days, quality level of specific pathogen-free animal (SPFA), Animal Quality License: SCXK (Guangdong) 2003 - 0001, Guangdong Monitoring Certificate 2007A024. All experimental rats were purchased from SPF breeding room of Laboratory Animal Center, Guangzhou University of Chinese Medicine. The rats were bred in a barrier shield environment, with ventilation with laminar flow, room temperature of 22 - 26°C, day and night alternating time of 10/14 h and free access to drink and food. Two weeks after breeding in the above-mentioned environment, the rats were divided into the following groups: low dose (LD) group: intragastric administration with 60 µg/kg.d TR for 35 days; high dose (HD) group: intragastric administration with 120 µg/kg.d TR for 35 days; placebo group: intragastric administration with equal volume of placebo for 35 days (dose setting based on the previous literature (Wang et al., 1993). Normal estrous cycle of SD rat was 3 - 4 days, and its life span was only 14 - 24 months, while human menstruation cycle was one month.

We observed that most women appear menstruation disorder after 6-10 cycles of TR compounds treatment, thus we chose 35 days (≈10 cycles of rats) as the treatment duration. Within the duration of the experiment, daily smears were performed under microscopes to record the estrous cycle of rats (proestrus, estrus, postestrus and interval) and estrous cycle was calculated based on the interval of two estruses. The body weight of rats in each group was recorded with electronic scales every four days to observe the growth of rats and then accordingly adjust intragastric administration dose of TR.

Specimen collection and preparation

Within 24 h after termination of the study, all rats in each group were anesthetized by intraperitoneal injection with 10% chloral hydrate solution (0.3ml/100 g), and then fixed on special plates, followed by abdominal skin disinfection. A medial longitudinal incision was performed in lower abdomen above the pubic symphysis and the bilateral rufous orbicular-ovate ovary as connection of fallopian tube and uterus free end was identified. Subsequently, the complete left ovary of rats was quickly removed and then surrounding fat tissue and ovarian bursa were removed and immediately immersed and fixed with 10% neutral formalin solution (40% formaldehyde solution: PBS buffer solution = 1:9, PH = 7.2 - 7.4). After the above-mentioned treatments, all rats were sacrificed by cervical dislocation. Within 24 h after specimen collection, conventional dehydration, lucidification, paraffin imbedding and 3 µm serial sectioning were performed in each specimen. The 16th and 18th section through ovarian central axis were selected and then covered on conventional glass slides for HE staining and mounted for preservation. The 20th section was covered on silicified anti-exfoliation glass slides for TUNEL assay (details as later).

Histological examination of rat ovaries

HE stained sections were selected, and then observed under optical microscope with 20 - 40 magnification whether or not there were pathological changes in ovarian tissues? Twenty high-power visual fields were randomly selected in each section to observe all

follicles in oocyte nuclei and count three following kinds of follicles referring to the separate criteria of Gaytán (Gaytán et al., 1996) and Myers M (Myers et al., 2004): Primordial follicle (primordial follicle and primary follicle): primordial follicle comprised a central oocyte and surrounding monolayer flat squamous granulosa cells; primary follicles comprised a central oocyte and surrounding monolayer cubic granulosa cells or at least 3 cubic epithelial cells in monolayer granulosa cells; Secondary follicle (also known as pre-antral follicle): follicles contained two or more layers granulosa cells, without formed antrum folliculi; Antral follicle: follicles contained two or more layers granulosa cells, with formed antrum folliculi.

Follicle cell apoptosis detection

Apoptotic cells in ovarian tissue sections were labeled *in situ* by terminal deoxynucleotidyl transferase [TdT] -mediated deoxy-uridinetriphosphate [dUTP] Nick End Labeling (TUNEL). Referring to the manufactory instruction of *in situ* Cell Death Detection Kit (POD) (Roche Company, Germany) for TUNEL, the detailed procedures were as follows: (A) Deparaffinage of tissue paraffin section for 10 min × 2 times. (B) Hydration with 100 - 70% gradient concentration of alcohol, followed by immersion in double-distilled water for 5 min. (C) Pretreatment for antigen reparation: incubation in proteinase K working solution (20 µg / ml in 10 mM Tris/HCl, pH = 7.6) at 37°C for 20 min, and wash with PBS buffer (0.01 M, pH = 7.2) for 5 min × 2 times. (D) Immersion in 3% H₂O₂-formaldehyde solution for 10 min to block endogenous peroxidase reaction, and then rinsed with PBS buffer for 5 min × 2 times. (E) Permeabilizing: the freshly pre-cooling prepared 0.1% TritonX-100 permeabilization solution with 0.1% citrate buffer was added, and then all slices were placed in a 4°C refrigerator for 8 min, followed by rinsing with PBS buffer for 5 min × 2 times. (F) Positive control: positive control slices were added with DNase (recombination solution (pH = 7.2, 50 mM Tris-HCl +1 mg/ml BSA +1 mg/ml DNase) and then incubated at 25°C for 10 min, followed by rinsing with PBS buffer for 2 times. (G) positive control and other samples slices were added with TUNEL reaction solution (mixture preparation with solution A: solution B = 1: 9), while negative control slices were added just with solution B, and then all slices were incubated in wet boxes at 37°C for 60 min, followed by rinsing with PBS buffer for 3 times. (H) After POD transformation solution C was added, all slices were placed in wet boxes and then incubated in dark at 37°C for 30 min, followed by rinsing with PBS buffer for 4 times. (I) In sequence, DAB coloration and hematoxylin re-staining were performed, followed by rinsing back to blue for 3 min. (J) Dehydration with gradient concentration of alcohol and mounting. Apoptotic labeled color was brown-red while background was hematoxylin-stained light blue.

Twenty consecutive high-power visual fields were observed in each slice to count each level of follicles and positively-stained apoptotic follicles in oocyte nuclei. The criteria of positively-stained apoptotic follicles were as follows: the apoptosis of primordial follicle and primary follicle was marked as apoptotic staining in oocyte nuclei; while secondary follicle and antral follicle as apoptotic staining in oocyte nuclei and / or > 10% of granulosa cells were apoptotic staining in each follicle. The number of each level of positive apoptotic stained follicles were counted and then the apoptotic rate of each level was calculated.

Statistical analyses

Statistical analyses of all observation parameters including body weight, estrous cycle, follicle counting and apoptotic rate were performed by SPSS 13.0 statistical analysis software. Body weight and follicle counting were compared with single factor analysis of variance (Oneway-ANOVA), while estrous cycle and apoptotic rate among groups were compared with non-parametric Kruskal-Wallis

Table 1. Rats' body weight before and after administration (mean \pm standard deviation).

	n	Wt. before administration(g)	Wt. after administration(g)
LD Group	10	216.20 \pm 10.34	238.00 \pm 11.08
HD Group	10	214.50 \pm 11.81	241.00 \pm 12.27
Placebo group	10	211.20 \pm 6.07	237.90 \pm 8.10

Table 2. Rats' estrous cycle change in each group (mean \pm standard deviation).

Group	N	Estrous cycle under the action of TR
HD group	10	5.67 \pm 1.45 [▲]
LD group	10	4.25 \pm 0.70
Placebo group	10	4.31 \pm 0.41 [▲]

Note: [▲] indicates significant difference between the HD and placebo group ($Z = 3.272$, $p = 0.001$).

test.

RESULTS

Growth condition of rats under the action of TR

All the young rats keep on growing in the same way. Before and after administration, there were no significant differences of body weight among groups. The P values before and after administration were 0.513 and 0.762 (Table 1), respectively. No disease, growth inhibiting or death was found due to TR.

Impact of TR on estrous cycle changes of rats

Compared with the placebo groups, significantly prolonged or even missed estrous cycle of rats were found in the HD group. Moreover, no obvious inhibition on estrous cycle was found in the LD group (Table 2). The cyclic estrual activity modification for each representative group of rats is shown in Figures 1, 2, 3). As time goes by, HD group rats missed estrus cycle, stay at the situation of interval for a long time and LD rats still have the cycle, but their cyclicity seems not so regular as that of the placebo group.

Histological manifestation of TR inhibition on rat follicle development

It was observed under microscope that there were clear structure and distinguished tissue structure and corpus luteum in all levels of ovarian follicles, without the manifestations of uncultivated follicle or ovarian degeneration including necrosis, edema, etc (Figure 4 and 5). The observation and counting analyses of rat ovarian follicles revealed that there was significant difference on

distribution of secondary follicles of rat ovarian among treatment groups. The total number of secondary follicles in TR groups significantly increased as compared with the placebo group (Figure 6). Meanwhile, the researches on follicle apoptotic rates also revealed that there were significantly increased secondary follicle apoptosis in the TR groups. However, there were no significant differences on distribution and apoptotic rate of primordial follicle and antral follicle among groups (Table 3).

There were no significant differences of total population of follicles among treatment groups, but there was significant difference on distribution of secondary follicles among treatment groups ($P < 0.01$). Percentage of the secondary follicle increased under TR treatment (HD 19%; LD20%; placebo group only 15%).

DISCUSSION

Since Kupehan et al. (1972) first isolated and purified the monomer compound of triptolide (TR) in the 70s of 20th century, researchers have found that TR may play inhibitory roles in rapidly proliferative cells such as testicular germ cells, ovarian follicles cells, hematopoietic system and so on (Kupehan, 1974). In the long-term use of TR as anti-rheumatic, anti-inflammatory agents and immunosuppressive agents in China, doctors and patients were troubled by the phenomenon of amenorrhea in many females after administration with TR.

Thus, the inhibition of TR on sexual gland limited the clinical application of this kind of drugs. Recently, with the improvement of technology, the active ingredient of TR was extracted and possible mechanisms were further explored. All studies seemed to point at apoptosis inducing activity of TR. The inhibition phenomenon of TR on sexual gland had attracted the attention of gynecologists and endocrinologist. Some domestic

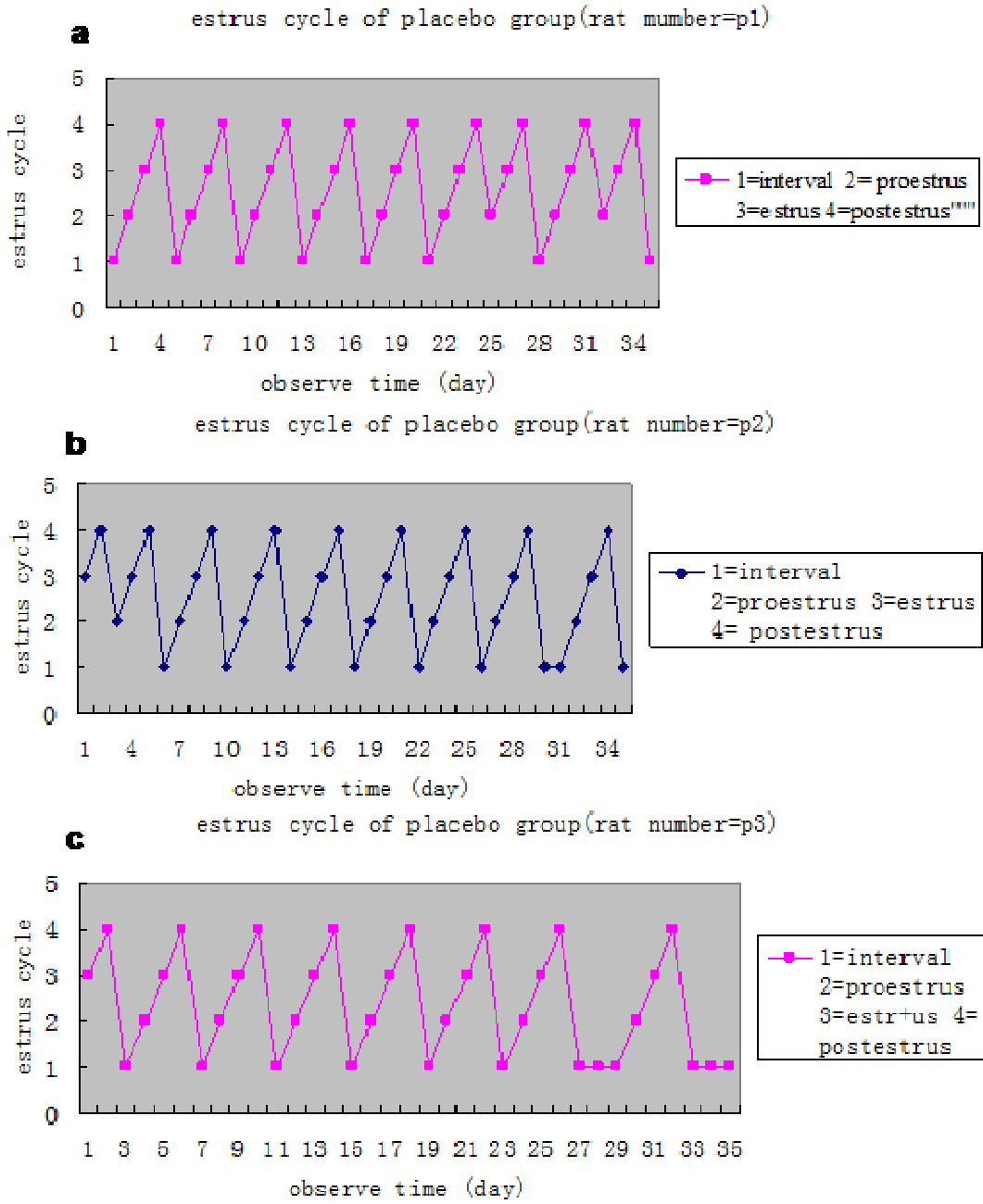


Figure 1. Estrus cycle of the placebo group (rat P1-P3) (a) rat P1 (b) 1.2: rat P2 (c) 1.3: rat-P3.

scholar conducted a retrospective analysis on the phenomenon of amenorrhea after administration with TR and then proposed that TR preparations might lead to women's high gonadotropin amenorrhea and then eventually result in gonadal suppression (Fu et al., 1999; Yang et al., 2006), and ovary might be the action site of TR. Accordingly, this present study aimed to investigate the role of TR on follicles (main functional unit of ovaries). After the treatment of different doses of TR, estrous cycle, the external indicator of rat gonad activities was

carefully observed and prolonged or missed estrous cycle was found in the HD group, with significant difference as compared with the placebo group. It was confirmed that 120 ug/kg.d of TR indeed resulted in estrous cycle disorder of rats in the same premise such as lavage, vaginal smears and so on, which was consistent with the ovulation suppression of rats. On the other hand, ovarian histological study and differential count of different levels of ovarian follicles in the drug groups revealed that high and low doses of TR significantly impacted the

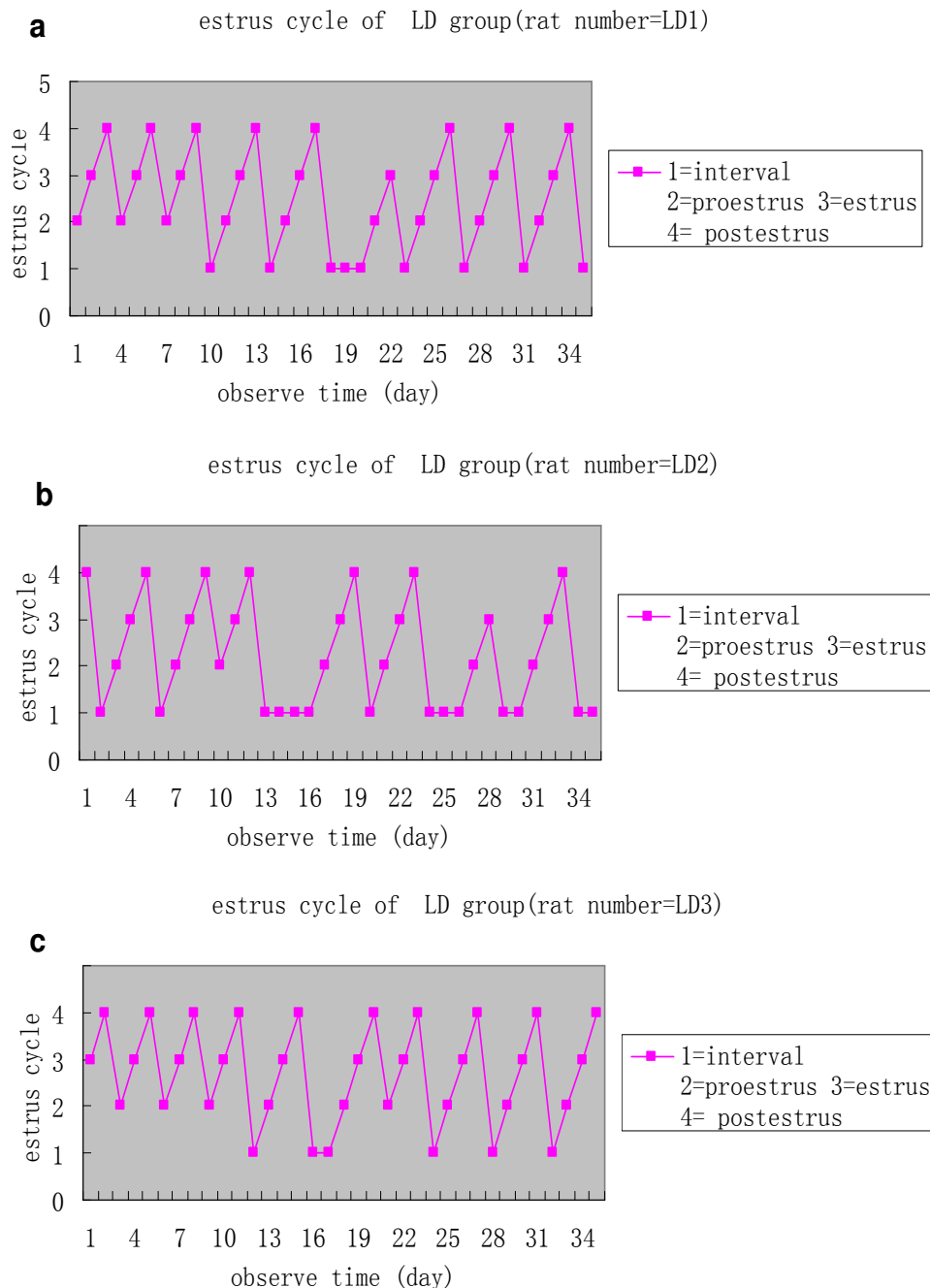


Figure 2. Estrus cycle of TR (low dose) Treatment group (rat LD1-LD3) (a) rat LD1 (b) rat LD2 (c) rat LD3.

distribution of ovarian follicles compared with the placebo groups, indicating that TR might interrupt ovarian follicular development. Further comparison, found that the phenomenon mainly appeared in the stage of secondary follicles.

Study results revealed that prolonged or even missed estrous cycle appeared on day 35 after administration, when no obvious ovarian follicle loss was found.

Contrarily, there were a large number of assembled secondary follicles. This phenomenon was not contradictory with the external manifestations estrous cycle suppression. We still believed that TR played inhibitory roles in ovaries. First of all, because the preliminary aim of this study was to explore the possibility of depressant effect on sexual gland, relatively low dose of TR was selected in this study to avoid its cytotoxicity and

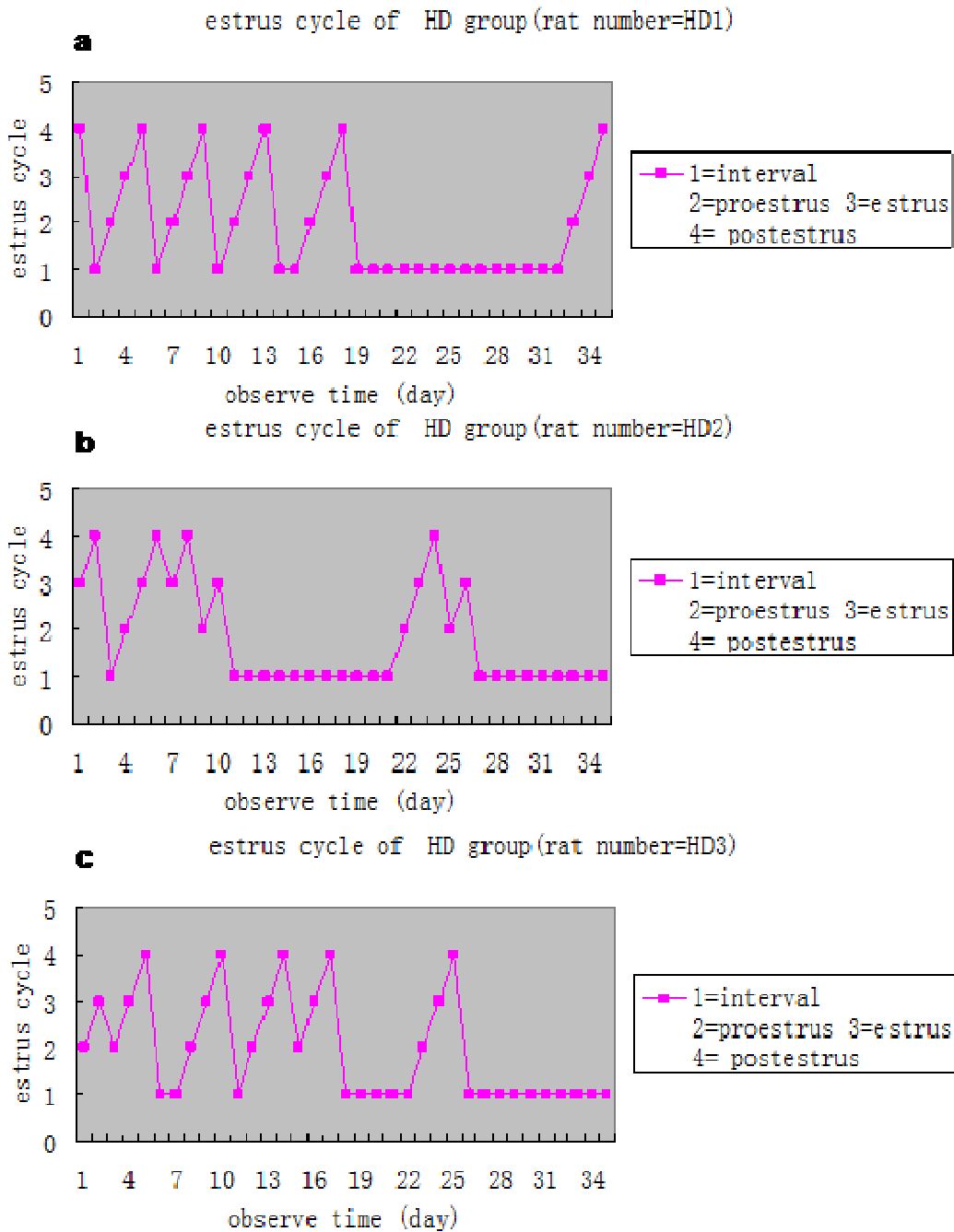


Figure 3. Estrus cycle of TR (high dose) Treatment group (rat HD1-HD3)(a) rat HD1 (b) rat HD2 (c) rat HD3.

immunosuppressive toxicity. Thus, the inhibition of TR on ovaries in this study was not up to ovarian atrophy or the absolute number reduction of ovarian follicles. In other words, TR might have led to the inhibition of ovarian function, but could not be up to organic damage. Second, this phenomenon was similar to high gonadotropin amenorrhea in humans. Due to the inhibition of TR on ovarian function, the feedback mobilization of preserved follicles

could induce follicular development to compensate follicle loss resulted from TR. Thus, a large number of secondary follicles aggregated in ovaries were the manifestation of follicle normal development kinetics suppression. Due to the inhibition of TR, a large number of secondary follicles stopped developing and could not develop into the stage of antral follicles. However, primordial follicle pool was not inhibited, and thus it was

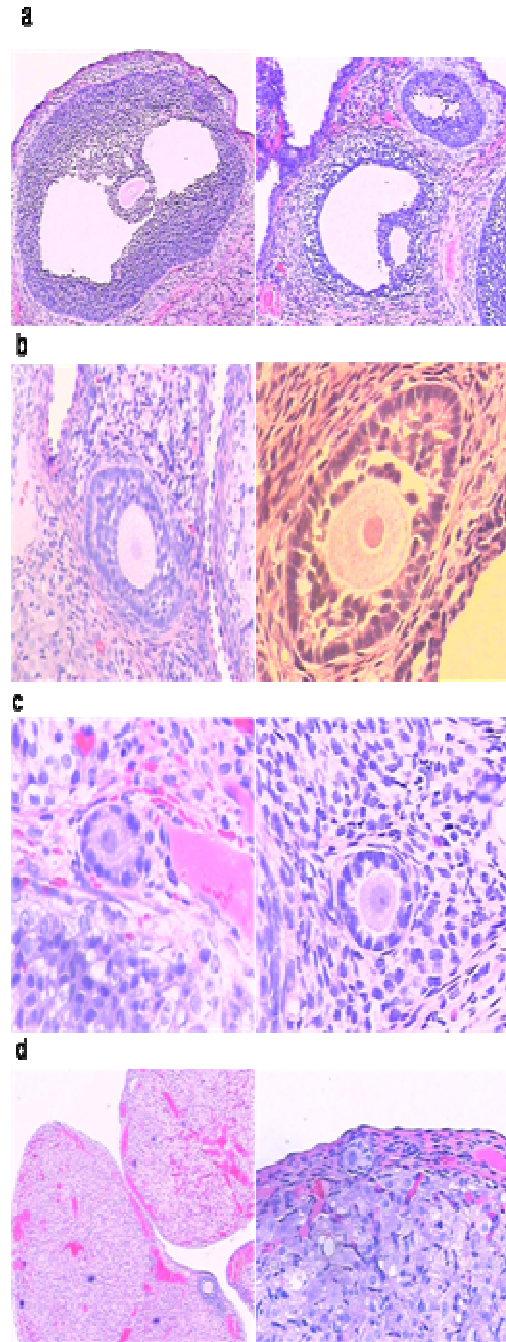


Figure 4. Follicular classification. (a) Rat antral follicles (HE staining). (b) Secondary follicles (preantral follicles) (HE staining). (c) Primary follicles (primordial follicle) (HE staining). (d) Corpus luteum and Primary follicles under high power lens (HE staining).

still effective to mobilize small follicular development, leading to the accumulation of secondary follicles in the intermediate stage. It was confirmed by follicular cell apoptotic rate in this study that there was a significantly increased positively-stained apoptotic rate of secondary

follicles in rat ovaries with the action of TR while no significant difference on positively-stained apoptotic rate of primordial follicle and antral follicle among groups. Thus, secondary follicle apoptosis resulted from TR interrupted the internal link of ovarian follicle development,

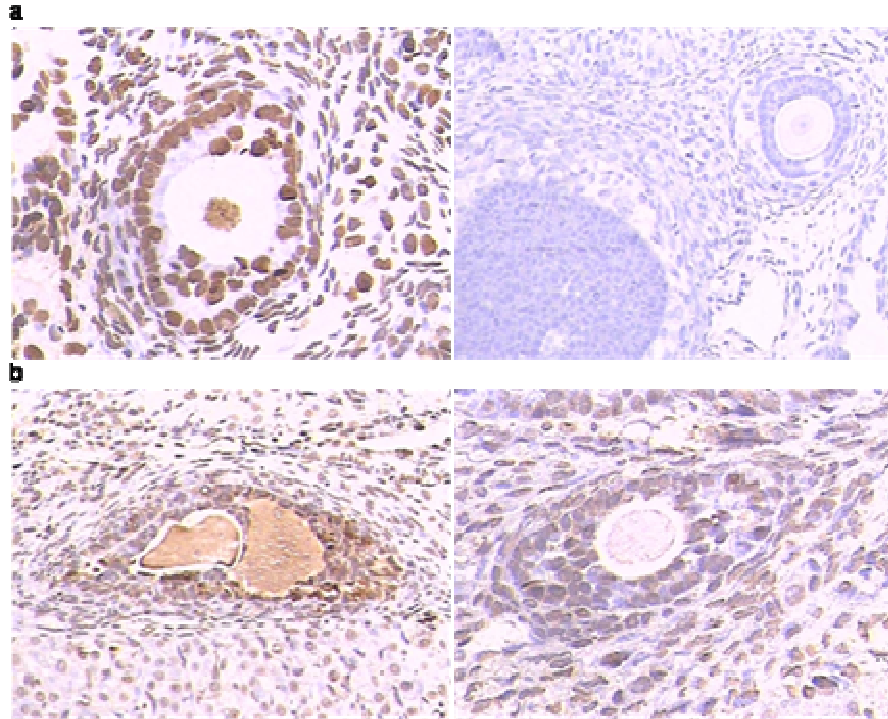


Figure 5. TUNEL apoptotic labeled slices. (a) Positive control (left) and negative control (Right) of Tunnel apoptotic labeled staining. (b) Apoptotic corpora atretica in Tunnel apoptotic labeled staining

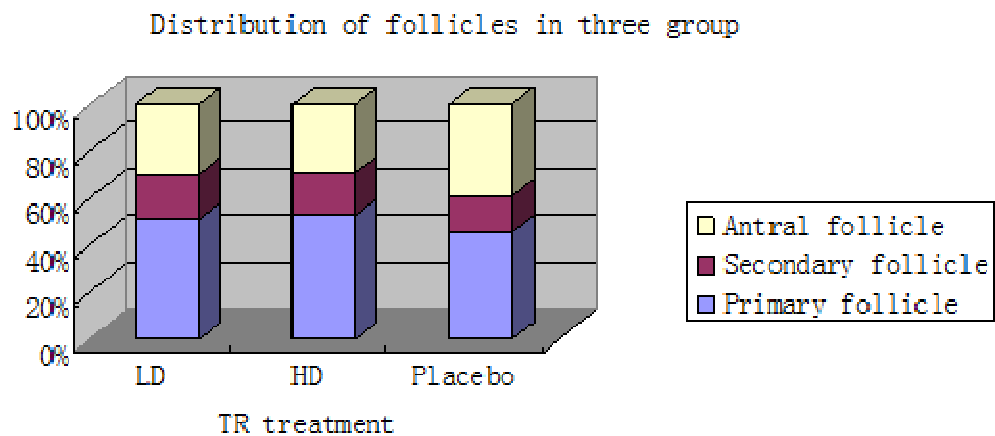


Figure 6. Distribution of follicles counted in different group.

Table 3. Statistical table of follicle differential counting.

Group	Primordial follicle		Secondary follicle		Antral follicle	
	Apoptosis rate	X ± S	Apoptosis rate	X ± S	Apoptosis rate (%)	X ± S
HD group	9 %	36.1 ± 5.62	≥32%	° 13.9 ± 2.33	23	20.7 ± 3.35
LD group	7 %	35.2 ± 5.58	31% [▲]	12.0 ± 1.55*	24	19.4 ± 2.26
Placebo group	7 %	23.2 ± 2.36	≥12% [▲]	° 7.7 ± 0.96*	17	19.8 ± 1.49

while reserved primordial follicles were not damaged, which could be explained that body could still mobilize primordial follicle development into secondary follicles to compensate inhibition. When the compensation still could not make up for the inhibition of TR, it mainly represented the inhibition of ovarian function, including prolonged or even missed estrous cycle, but it was not necessary to accompany with an absolute reduction of follicle number, or even increase. It was found that high dose of TR could induce rat estrous cycle disorder while this effect was not obvious in low dose, also indicating that the inhibitory role of TR on sexual gland was dose-dependent. Good growth condition of all rats was found in all groups, but no significant difference on body weight was found among these groups. It was the most important that the most precious primordial follicles in ovaries were not impacted by TR. The findings in this study may provide theoretical bases to explore safe use of TR's inhibitory action on sexual gland.

In short, it is presumed at present that the apoptosis induction of TR on ovarian cells leads to the inhibition of follicular development. However, duration of this study was set at 10 times of estrus cycle of rats, and it seemed that short term treatment dose not significantly affected the whole ovary follicle number, while increased its apoptosis and interrupted the follicle development procedure. It needs to be further studied whether or not TR participates in the regulatory process through other mechanisms.

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