Low-Intensity Pulsed Ultrasound Ameliorates Cyclophosphamide-Induced Ovarian Damage in Rats (Postprint)

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Abstract

Objective: To investigate the reparative effect of low-intensity pulsed ultrasound on ovarian damage induced by intraperitoneal injection of cyclophosphamide in Sprague-Dawley (SD) rats.

Methods: Sixty-three specific-pathogen-free female Sprague-Dawley (SD) rats were randomly divided, with 13 assigned to the normal group. The remaining 50 rats received intraperitoneal injections of cyclophosphamide at 30 mg/kg for 5 consecutive days to establish a premature ovarian failure animal model. The 43 rats that successfully developed the model were randomly assigned to an experimental control group (n=21) or a treatment group (n=22). The normal group and experimental control group received no intervention, while the treatment group received low-intensity pulsed ultrasound irradiation. The estrous cycles of rats in each group were monitored. Seven days after irradiation, the rats were sacrificed, and serum levels of estradiol (E2), follicle-stimulating hormone (FSH), and anti-Müllerian hormone (AMH) were measured. Morphological changes and follicle counts in the ovaries were observed.

Results: After cyclophosphamide injection, compared with the normal group, the experimental control group exhibited disordered estrous cycles, significantly reduced follicle numbers at all stages, significantly decreased serum E2 and AMH levels, and significantly increased FSH ($P=0.01$). After ultrasound irradiation, compared with the experimental control group, the treatment group showed restored normal estrous cycles, significantly increased follicle numbers, significantly elevated serum E2 ($P=0.01$), significantly reduced FSH ($P=0.01$), and no significant change in AMH ($P=0.50$).

Conclusion: Low-intensity pulsed ultrasound exerts a reparative effect on ovarian damage induced by intraperitoneal injection of cyclophosphamide in SD rats.

Full Text

Preamble

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Abstract

Objective To investigate the reparative effects of low-intensity pulsed ultrasound on cyclophosphamide-induced ovarian injury in SD rats. **Methods** Sixty-three specific-pathogen-free female SD rats were randomly assigned to a normal control group (n=13) or received intraperitoneal injections of cyclophosphamide (30 mg/kg) for 5 consecutive days to establish a premature ovarian failure model. Forty-three rats with successful model induction were randomly divided into an experimental control group (n=21) and a treatment group (n=22). The normal and experimental control groups received no intervention, while the treatment group received low-intensity pulsed ultrasound irradiation. Estrous cycles were monitored throughout the experiment. Seven days after irradiation, rats were sacrificed to measure serum estradiol (E2), follicle-stimulating hormone (FSH), and anti-Müllerian hormone (AMH) levels, and to observe ovarian morphology and follicle counts. **Results** Following cyclophosphamide injection, the experimental control group exhibited disrupted estrous cycles, significantly reduced follicles at all developmental stages, markedly decreased serum E2 and AMH, and elevated FSH compared with the normal group (P=0.01). After ultrasound irradiation, the treatment group showed restored estrous cycles, increased follicle numbers, significantly higher serum E2 (P=0.01), and lower FSH (P=0.01) compared with the experimental control group, though AMH showed no significant change (P=0.50). **Conclusion** Low-intensity pulsed ultrasound demonstrates reparative effects on cyclophosphamide-induced ovarian injury in SD rats.

Keywords: low-intensity pulsed ultrasound; cyclophosphamide; ovarian injury; premature ovarian failure

Introduction

Premature ovarian failure (POF) refers to ovarian insufficiency occurring before age 40 due to follicular depletion or iatrogenic damage, characterized by menstrual disturbances (amenorrhea or oligomenorrhea), hypoestrogenic symptoms such as hot flashes and night sweats, and infertility [1]. POF accounts for 4–18% of secondary amenorrhea cases [2]. Currently, no definitive cure exists for POF; treatment primarily involves hormone replacement therapy, ovulation induction, and donor oocyte-assisted reproduction to alleviate symptoms

[3]. Although widely used, hormone replacement therapy only improves perimenopausal symptoms caused by estrogen deficiency but cannot restore ovarian function, promote follicular development, or increase ovulation rates [4]. Therefore, novel and more effective therapeutic approaches are needed. Low-intensity pulsed ultrasound (LIPUS) delivers energy in pulsed mode at low levels, effectively avoiding thermal damage to cells while offering good tolerability, controllable operation, and absence of radiation. As early as 1978, animal studies demonstrated that low-dose ultrasound could promote follicular formation in juvenile mouse ovaries and advance endometrial decidualization cycles [5]. Recent research has also shown that LIPUS can enhance estrogen receptor protein expression to promote follicular development in perimenopausal rats [6]. Cyclophosphamide induces follicular loss and POF by promoting quiescent follicle proliferation and accelerating apoptosis in growing follicles [7]. Animal models of ovarian injury induced by cyclophosphamide are well-established [8-10]. This study employed cyclophosphamide to establish an ovarian injury model in female SD rats and treated them with LIPUS to observe its effects on ovarian function and explore its reparative mechanisms, potentially providing new therapeutic targets and strategies for clinical POF management.

Methods

Experimental Animals

Specific-pathogen-free female SD rats aged 12 weeks and weighing 200±20 g were purchased from the Experimental Animal Center of Chongqing Medical University. The rats were housed at 23–25°C with a 12-hour light cycle and ad libitum access to food and water. After 7 days of acclimatization, vaginal smears were performed daily between 9:00–10:00 AM for 14 consecutive days. Sixty-three rats that exhibited two regular estrous cycles were selected for the study.

Reagents and Instruments

Cyclophosphamide (CTX) was obtained as a powder (200 mg/vial) and dissolved in 12 mL normal saline to prepare a 10 mg/mL solution for injection. Alkaline methylene blue staining solution was prepared in-house (Solution A: 0.6 g methylene blue dissolved in 30 mL 95% ethanol; Solution B: 0.01 g potassium hydroxide dissolved in 100 mL distilled water; Solutions A and B were mixed thoroughly after complete dissolution). A low-power focused ultrasound experimental device developed by Chongqing Haifu Medical Technology Co., Ltd. was used, with ultrasound frequency adjustable from 0.5–1.2 MHz, a treatment head diameter of 2 cm, and pulsed wave output.

Vaginal Smear Method

Female SD rats were placed on the wire cage lid and restrained with the middle, ring, and little fingers of the left hand while the thumb and index finger held the tail. Any feces or urine were removed, and a sterile cotton swab moistened with sterile saline was gently inserted into the vagina, rotated, and withdrawn. The sample was evenly smeared onto a glass slide, air-dried, fixed in anhydrous ethanol for 3-5 minutes, rinsed under running water for 2 minutes, air-dried again, stained with alkaline methylene blue solution for 5 minutes, rinsed, air-dried, and sealed with neutral resin for microscopic examination.

Establishment of the Premature Ovarian Failure Model

Fifty SD rats received intraperitoneal injections of cyclophosphamide (30 mg/kg) once daily for 5 consecutive days to establish the premature ovarian failure model, following previously described methods [10]. Starting from the first day of injection, vaginal cytology was performed daily between 9:00-10:00 AM for 19 consecutive days to monitor estrous cycles. Continuous diestrus indicated successful model establishment, which was achieved in 43 rats.

Grouping and Intervention

The 43 rats with successful model induction were randomly divided into two groups: experimental control group (n=21) and treatment group (n=22). An additional 13 rats served as the normal control group. The normal and experimental control groups received no intervention. The treatment group received low-intensity pulsed ultrasound irradiation starting when vaginal smears indicated diestrus. Ultrasound parameters were: frequency 900 kHz, intensity 0.8 W/cm^2, pulse interval 2 seconds, and irradiation time 30 minutes daily for 10 consecutive days. Vaginal smears were performed daily between 9:00-10:00 AM for 17 consecutive days starting from the first irradiation to monitor estrous cycle changes.

Sample Collection and Processing

All rats were sacrificed 7 days after treatment completion. Serum, ovarian, and uterine specimens were collected. Prior to sacrifice, rats were fasted for 4 hours and weighed. Anesthesia was induced with 1% pentobarbital (0.3-0.5 mL/kg intraperitoneally). After laparotomy along the linea alba, the right ovary was carefully dissected from surrounding adipose tissue and immediately fixed in 4% paraformaldehyde for 24 hours before paraffin embedding for sectioning. Blood was collected from the heart apex using a 2.5 mL syringe, allowed to clot at room temperature for 1-2 hours, and centrifuged twice at 2000 rpm for 20 minutes at 4°C. The clear supernatant was collected for analysis.

Estrous Cycle Monitoring

Vaginal smears were performed daily between 9:00-10:00 AM from the beginning to the end of the experiment to monitor estrous cycle changes in each rat.

Ovarian Histomorphology and Follicle Counting

Ovarian tissues were embedded in paraffin and serially sectioned at 4 m thickness. Three consecutive sections from the largest ovarian cross-section were stained with hematoxylin-eosin (HE) for histomorphological examination. Follicles at all developmental stages with normal structure were counted under light microscopy (×40) according to established rat follicle classification criteria [11], with counts from three sections summed for each rat.

Serum Sex Hormone Detection

Serum estradiol (E2), follicle-stimulating hormone (FSH), and anti-Müllerian hormone (AMH) levels were measured by enzyme-linked immunosorbent assay (ELISA) following kit instructions. Samples and corresponding antibodies were incubated in coated plates for 30 minutes, followed by addition of enzyme-labeled reagent and color development. Absorbance was measured using a microplate reader, and standard curves were generated using standard solutions.

Statistical Analysis

SPSS 22.0 software was used for statistical analysis. Parametric data meeting normality and homogeneity of variance assumptions were analyzed by one-way ANOVA, while non-parametric tests were used for data not meeting these assumptions. Repeated measures data were analyzed by repeated measures ANOVA. $P<0.05$ was considered statistically significant.

Results

Estrous Cycle Changes

Among the 50 rats receiving cyclophosphamide, 43 exhibited continuous diestrus within 2 weeks after drug administration, yielding a modeling success rate of 86%. This demonstrates that short-term, high-dose intraperitoneal cyclophosphamide injection can successfully establish a premature ovarian failure model. Seven days after ultrasound irradiation, vaginal cytology revealed normalized estrous cycles in 22 rats (22/43, 51.2%), including all 22 rats in the treatment group (22/22, 100%) and none in the experimental control group (0/21, 0%).

Vaginal smear results with alkaline methylene blue staining during different estrous phases were as follows: (1) Proestrus: predominantly oval nucleated epithelial cells with dark blue nuclei and light blue cytoplasm (Figure 1 [Figure 1: see original paper]A); (2) Estrus: predominantly sheet-like, anucleated keratinized epithelial cells stained dark blue (Figure 1B); (3) Metestrus: approximately equal proportions of keratinized epithelial cells, nucleated epithelial cells, and leukocytes (Figure 1C); (4) Diestrus: predominantly leukocytes, which are small and stain dark blue (Figure 1D).

Ovarian Histomorphological Changes After Ultrasound Irradiation

Histopathological analysis revealed that the normal group had large ovaries with active follicular growth, visible follicles at all developmental stages, and abundant follicular fluid (Figure 2 [Figure 2: see original paper]A). In contrast, the experimental control group showed ovarian atrophy with few follicles, most of which were atretic (Figure 2B). Compared with the experimental control group, the treatment group exhibited numerous growing follicles with abundant follicular fluid (Figure 2C).

Follicle Count Comparison After Ultrasound Irradiation

Compared with the normal group, the experimental control group showed significantly increased atretic follicles (P=0.00) and decreased follicles at all other developmental stages (all P=0.00). The treatment group demonstrated significantly reduced atretic follicles (P=0.00) and increased follicles at all other stages compared with the experimental control group (all P=0.00, Table 1).

Serum Sex Hormone Level Changes After Ultrasound Irradiation

As shown in Table 2 , compared with the normal group, the experimental control group exhibited significantly decreased serum E2 and AMH levels and increased FSH levels (all P=0.00). Compared with the experimental control group, the treatment group showed significantly elevated serum E2 (P=0.00) and decreased FSH (P=0.00), but no significant difference in AMH levels (P=0.50).

Discussion

Cyclophosphamide is a cell cycle-nonspecific alkylating agent that exerts toxic effects not only on proliferating cells but also on undeveloped oocytes and pregranulosa cells in primordial follicles, ultimately impairing follicular development [12]. Additionally, cyclophosphamide can damage ovarian function by increasing SDF-1/CXCR4 expression and promoting granulosa cell apoptosis [13-14]. Our results demonstrate that cyclophosphamide disrupts estrous cycles, reduces estrogen levels, and significantly increases atretic follicles while decreasing follicles at all developmental stages in sexually mature female SD rats, confirming successful establishment of a premature ovarian failure model.

The SD rat estrous cycle comprises four stages: proestrus, estrus, metestrus, and diestrus, which indirectly reflect ovarian function. Our study found that after cyclophosphamide administration, 43 of 50 rats (86%) exhibited continuous diestrus without estrus phases, compared with the normal group. This indicates that the chemotherapeutic agent disrupted the estrous cycle and impaired ovarian function.

Estradiol, a steroid hormone primarily secreted by ovarian follicular cells, promotes follicular growth and development, endometrial thickening, and glandular proliferation. Decreased estradiol levels indicate diminished ovarian estrogen secretion [15-16]. Follicle-stimulating hormone (FSH), a glycoprotein hormone secreted by basophilic cells of the anterior pituitary, stimulates follicular growth and participates in follicular recruitment; its elevation signifies ovarian dysfunction [17]. Anti-Müllerian hormone (AMH) is a dimeric glycoprotein primarily secreted by granulosa cells of primary and early antral follicles [18-19]. AMH levels do not fluctuate with the menstrual cycle, and lower values indicate poorer ovarian reserve [20-22]. Our study revealed that after modeling, rats exhibited significantly decreased serum E2 and AMH levels with elevated FSH, mirroring the serological profile of clinical POF patients.

Ovarian histomorphology and follicle counting results showed that cyclophosphamide injection significantly increased atretic follicles and decreased follicles at all other stages compared with the normal group, consistent with follicular loss and increased atresia observed in POF patients.

LIPUS has been applied in multiple research fields, including fracture healing and soft tissue injury repair, with confirmed efficacy [23]. Furthermore, LIPUS can accelerate resolution of chronic inflammation, improve blood circulation in ischemic muscle, promote recovery of nerve conduction blockade, and enhance angiogenesis and healing of ligaments and tendons [24-25]. This study employed pulsed ultrasound at 0.8 W/cm^2 to irradiate ovaries in POF rats. Post-irradiation results showed increased body weight, no damage to local skin or surrounding tissues, significantly increased follicles at all developmental stages, elevated serum E2, and decreased FSH, demonstrating that LIPUS can safely and effectively promote ovarian function recovery in POF rats.

In summary, low-intensity pulsed ultrasound irradiation exhibits significant reparative effects on cyclophosphamide-induced ovarian injury in SD rats, effectively improving ovarian function. However, the underlying mechanisms require further investigation.

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