ISSN 2618-1584 Vol. 6, Issue 5: 15-19, DOI: 10.25236/FMSR.2024.060503

Bone microstructure analysis of ovarian premature aging rat model under dual perspectives

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Abstract: *This study aims to establish an ovarian premature aging rat model induced by cyclophosphamide and explore the effects of CTX on ovarian function and bone microstructure in rats. This study used SD rats as the modeling subjects, divided into a modeling group, a control group, and an aging control group. The ovarian premature aging model was established by intraperitoneal injection of CTX, and vaginal smears were collected for cytological analysis after 14 days. Serum was collected after 14 days for sex hormone determination to verify the success of the model. After successful modeling, femoral head specimens were taken from the rats for histological sectioning, stained with HE, and analyzed for bone microstructure based on the staining results. Mesenchymal stem cells were extracted from the bone marrow of the three groups of rats and labeled with specific markers, then cultured in osteogenic induction medium. Alkaline phosphatase staining was performed after 7 and 21 days to observe the osteogenic differentiation ability of the rats in different groups. Bone microstructure analysis was conducted from the dual perspectives of histological sectioning with HE staining and osteogenic induction differentiation. The results showed that the modeling of ovarian premature aging in rats induced by CTX was successful, with changes in the microstructure of the femoral bone tissue and a decrease in osteogenic differentiation ability.*

Keywords: *Premature ovarian insufficiency, rat modeling, osteoporosis*

1. Introduction

Osteoporosis, as a chronic metabolic bone disease, is continuously increasing globally due to the rise in life expectancy, and this trend is expected to continue over the next few decades[1]. By 2050, it is estimated that half of the osteoporotic fractures worldwide will occur in Asia, particularly in China[2]. Therefore, osteoporosis should receive more attention from the Chinese population. The development characteristics of osteoporosis include changes in bone microstructure and increased bone fragility[3]. Due to the decline in ovarian function in perimenopausal women and the decrease in estrogen secretion (which plays a crucial protective role in bones), osteoporosis is particularly common among middle-aged and elderly women. Premature ovarian insufficiency refers to a decline in ovarian function in women under 40 years old, clinically manifested as amenorrhea, infertility, and other symptoms. A study on women with premature ovarian insufficiency showed that they experience greater and earlier bone loss compared to women undergoing natural menopause[4]. Therefore, for patients with premature ovarian failure, adequate awareness and early prevention of osteoporosis can slow down disease progression. Cyclophosphamide (CTX), as a chemotherapy drug, can be used to establish an animal model of premature ovarian insufficiency as a basis for research.

In this study, experimental rats were divided into a model group, a control group, and elderly rats. The rat model of premature ovarian insufficiency was established using CTX. The model establishment was confirmed by observing the estrous cycle and ovarian morphology of the rats. Through a dual perspective of bone sectioning with HE staining and mesenchymal stem cell osteogenic induction with Alizarin Red and alkaline phosphatase staining, the study aimed to explore the impact of premature ovarian insufficiency on the bone microstructure of rats.

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2. Experimental animals, materials

2.1. Experimental animals

Female SD rats, consisting of 8 rats aged 8 weeks with a body weight of approximately 150-200g each, and 4 elderly rats with a body weight of approximately 200-300g, were purchased from Henan Sbecks Biotechnology Co., Ltd. (License No. SCXK Yu 2020-0005). The handling of experimental animals complied with the ethical regulations for laboratory animals at Henan Polytechnic University.

2.2. Instruments and equipment

Thermo Fisher 371 Carbon Dioxide Cell Culture Incubator; Shanghai Dingke SW-CJ-2D Clean Bench; Anhui Jiawen Instrument Equipment Co., Ltd. JW-3021HR High-speed Refrigerated Centrifuge; Beijing 61 Biological Technology Co., Ltd. WD-2102B Non-medical Fully Automatic Microplate Reader; Olympus BX53 Fluorescence Microscope; Shanghai Tianeng Technology Co., Ltd. Chemiluminescence Imaging and Analysis System; Zhiwei (Xiamen) Instrument Co., Ltd. G180TW Vertical Automatic Pressure Steam Sterilizer; Hubei Bona Medical Technology Co., Ltd. BQ-318D Paraffin Slicer; Jinhua Huasu Technology Co., Ltd. HS-1145 Flat Baking and Paraffin Embedding Machine; Shanghai Yiheng THZ-103B Constant Temperature Cultivation Shaker; Beijing Junyi Dongfang Electrophoresis Equipment Co., Ltd. JY600E Universal Electrophoresis Power Supply, JY-SCZ2+ Vertical Electrophoresis Tank, JY-ZY5 Transfer Electrophoresis Tank; Changzhou Langyue Instrument Manufacturing Co., Ltd. HH-W-600 Digital Constant Temperature Water Bath; UK NEW BRUNSWICK Premium U410 Vertical Ultra-low Temperature Freezer.

3. Experimental methods and content

3.1. Rat modeling

A total of 8 female SD rats aged 6-8 weeks, with a weight range of 150-200g, purchased from Henan Sbecks Biotechnology Co., Ltd. (License No. SCXK Yu 2020-0005); and 4 elderly female SD rats, 20 weeks old, with a weight range of 200-300g, were used in the experiment. The experiment was divided into three groups: the model group consisted of 4 rats labeled as the CTX group; the blank group consisted of 4 rats labeled as the NaCl group; and the elderly control group consisted of 4 rats labeled as the elderly group (Lao group). The initial dose for the model group was 50mg/kg, followed by intraperitoneal injections of 8mg/kg over the next two weeks. The blank group and the elderly group received intraperitoneal injections of an equivalent amount of saline solution during the same period.

3.2. Model validation

Observation of the estrous cycle in rats: Vaginal smears were made the day after each CTX injection to observe the estrous cycle. The process involved: a. holding the rat's tail and suspending it. b. gently inserting a fine cotton swab moistened with physiological saline into the rat's vagina, about 1 cm deep, and rotating clockwise for a few turns before removing it. c. rotating the swab counterclockwise on a glass slide with a few drops of physiological saline to evenly distribute the sample on the slide. d. staining with Gram's stain solution following the four steps of primary staining, mordanting, decolorizing, and counterstaining, and observing the sample under a microscope. This process lasted for two weeks, and after two weeks, the modeling situation was assessed based on changes in the estrous cycle.

3.3. Mesenchymal stem cell induction with alkaline phosphatase staining

a. Immerse the anesthetized rats in 75% ethanol for 10 minutes; b. Transfer the rats to a sterile clean bench, dissect the femur, and cut the femoral head; c. Prepare complete culture medium by mixing basal medium, fetal bovine serum, and antibiotics in a ratio of 100:10:1, rinse the bone marrow cavity with 10% complete culture medium, and filter the medium through a 100μm cell strainer; d. Transfer the medium to culture bottles for cultivation, perform half medium changes at 24 and 48 hours post-transfer, and complete medium change at 72 hours post-transfer; e. When the cells cover 80%-90% of the bottom of the culture bottle, passage the cells. Prepare osteogenic induction culture medium, seed the second-generation cells in a six-well plate, add osteogenic induction culture medium, and culture for 7 days. Perform alkaline phosphatase staining, followed by Alizarin Red staining after 21 days of culture. The

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three groups undergo the same operations. The experimental results are observed and photographed under a microscope, and the area of positive staining is calculated using Fiji image processing software.

3.4. Bone section Hematoxylin and Eosin (HE) staining

a. Retain the femoral head from the previous step; b. After removing the surface muscles, rinse with physiological saline and place the specimen in 10% neutral buffered formalin solution for fixation for at least 24 hours; c. After draining the fixative, rinse the tissue with distilled water three times, each time for 20 minutes; d. Place the tissue in a decalcifying solution, changing the solution daily until a needle can penetrate the tissue without significant resistance, indicating complete decalcification; e. Rinse the tissue with distilled water three times, each time for 20 minutes; f. Treat the tissue with an alkali solution by immersing it twice, each time for 20 minutes. Change the distilled water every 30 minutes to thoroughly remove the alkali solution, and immerse for 6 hours; g. Dehydrate in a gradient of alcohol, following the same steps as for ovarian section staining and observation; h. Clear with xylene; i. Infiltrate with paraffin; j. Embed; k. Cut continuous sections at a thickness of 6μm; l. Perform HE staining and mount the slides in neutral resin for observation under a microscope.

4. Experimental results

4.1. Rat modeling results

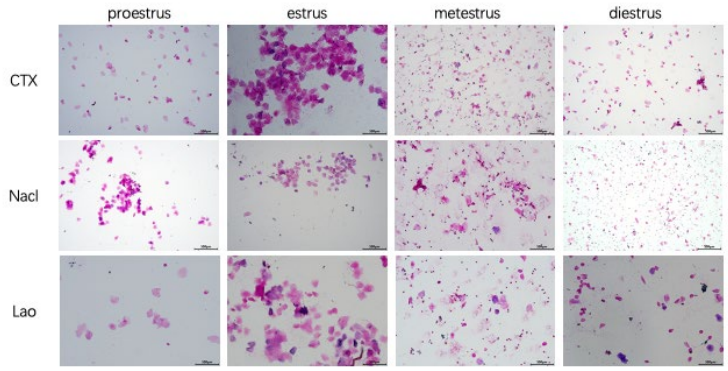

Figure 1: Rat modeling results

The results of the vaginal smear are shown in Figure 1. In Gram staining, the four stages of the estrous cycle in rats are easily distinguishable. In the proestrus stage, mainly nucleated epithelial cells are visible in the images, with purple nuclei, pink cytoplasm, and intact cell membranes. As the transition from proestrus to estrus occurs, the number of non-nucleated keratinized epithelial cells increases until entering estrus. In estrus, predominantly pink non-nucleated keratinized cells are observed, forming irregularly shaped aggregates that can cover the entire field of view. As the transition to metestrus approaches, a large number of deeply stained round or oval-shaped leukocytes and non-nucleated keratinized epithelial cells appear in the field of view. The density of leukocytes is highest in metestrus. In the early diestrus stage, the number of leukocytes does not decrease, and nucleated epithelial cells and keratinized cells coexist. As the transition to proestrus begins, the number of leukocytes and epithelial cells gradually decreases, signaling the start of the next estrous cycle.

4.2. Alkaline phosphatase staining results

Alkaline phosphatase (ALP) is an enzyme associated with the activity of bone cells, and its activity can be used to evaluate the formation and maturation of bone tissue. In the staining results, under normal circumstances, ALP-positive cells will appear purple or blue in color, while negative cells will not stain, and the cell nuclei will appear green. Therefore, a darker staining intensity indicates a higher level of ALP expression. The staining results are shown in Figure 2, where it can be observed that the staining effect of the modeling group is not significantly different from the NaCl group. In the NaCl group, cell nuclei are seen to cluster together, while in the CTX group, they are arranged loosely.

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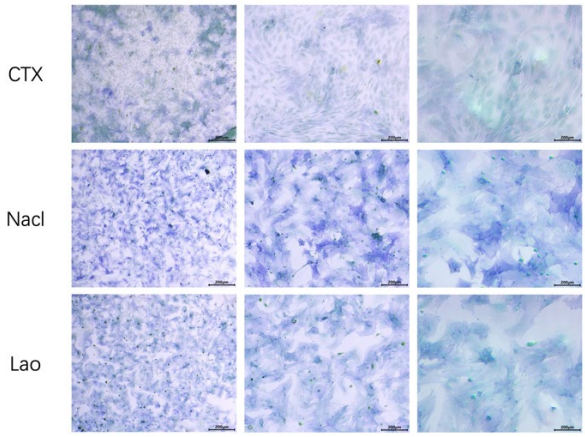

Figure 2: Alkaline phosphatase staining result

4.3. Femoral bone section Hematoxylin and Eosin (HE) staining results

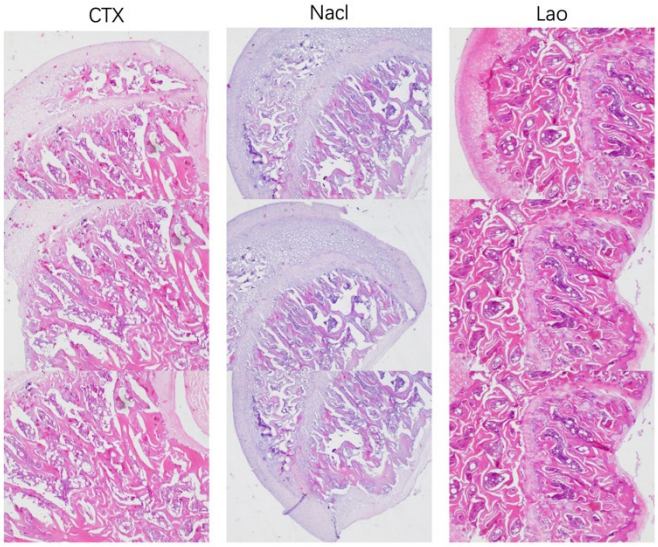

Figure 3: HE staining results

HE staining results are shown in Figure 3.HE staining[5] is a commonly used histological staining method, which is also suitable for staining bone sections. The cell nuclei stained with hematoxylin appear purple or blue, while the cytoplasm stained with eosin appears pink. The staining results show that in the CTX group, there are fewer chondrocytes in the secondary ossification center compared to the NaCl group, indicating a significant lack of cartilage reserves. The chondrocytes are loosely arranged, with sparse texture, and a relatively insufficient level of compactness compared to the NaCl group. Additionally, there is a disordered arrangement of trabeculae with visible fractures. In the NaCl group, the cartilage reserves are sufficient, the cells are closely arranged, and the trabeculae are intact. Furthermore, due to the insensitivity of eosin staining to the cytoplasm of newly formed cells, the cytoplasm of aged cells appears heavily stained, resulting in a noticeable deepening of color.

5. Discussion

Cyclophosphamide (CTX) [6]is a commonly used anticancer drug that works by interfering with the DNA replication and repair processes of cells, thereby inhibiting the proliferation and growth of tumor cells. CTX is primarily activated in the body through chemical conversion into phosphoramide mustard, which is its main therapeutic component. Phosphoramide mustard can bind to nucleophilic sites in DNA molecules, causing DNA strand breaks and inhibiting the connection of DNA double strands, ultimately

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leading to the death of tumor cells. Injection of CTX has been a mainstream method for establishing premature ovarian failure (POF) animal models, and this study further elucidates the ovarian toxicity of CTX.

Premature ovarian failure (POF) is a multifactorial disease characterized by secondary amenorrhea, decreased estrogen, and increased gonadotropins in women under 40 years of age. The incidence of POF is increasing annually, and existing cases can be classified as primary or secondary cases. The long-term progression of POF will have adverse effects on the physiological and psychological well-being of women. Genetic, immune, metabolic, viral, drug-related factors, and abnormalities in certain signaling pathways may all play a role in the etiology of POF[7].

In conclusion, estrogen plays a crucial role in bone formation. This study analyzes the microstructural changes in bone under estrogen deficiency from two perspectives: femoral bone section HE staining and alkaline phosphatase staining results of bone marrow mesenchymal stem cells (BMSCs) during osteogenic induction. HE staining reveals significant defects in chondrocyte reserves and poor development or lack of dynamics in trabecular bone leading to fractures under estrogen deficiency. Alkaline phosphatase staining for osteogenic induction indicates the difficulty in generating calcium nodules under estrogen deficiency conditions. This study validates the rat model of premature ovarian failure and further explores the development of bone health after premature ovarian failure. However, osteoporosis can result from various factors, and while this study focuses on experimental model establishment, further research is needed to delve deeper into the underlying causes of secondary osteoporosis following premature ovarian failure, aiming to lay the foundation for early prevention and treatment of osteoporosis.

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