Effects of Zuogui Pill on Peripheral Blood Cells and Spleen Index of Rats Injured by ^{60}Coγ Ray

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Abstract [**Objectives**] To investigate the effects of Zuogui Pill on peripheral blood cells, sex hormone levels, interleukin 1β (IL-1β) levels, ovarian follicle number and spleen index in SD rats damaged by ^{60}Coγ rays. [**Methods**] Fifty 8-week-old female SPF SD rats were selected, 10 of which were in the normal group and were fed routinely without irradiation; the other 40 rats were irradiated with ^{60}Coγ rays (6.0 Gy, LD_{40}) for 24 h, and then divided into radiation model group, Progynova group, high and low dose of Zuogui Pill groups. Progynova group was treated with 0.09 mg Progynova; high dose Zuogui Pill group was treated with 4.725 g Zuogui Pill crude drug and low dose Zuogui Pill group was treated with 0.945 g Zuogui Pill crude drug; radiation group was treated with 2 mL normal saline by gavage once a day for 21 d. The changes of peripheral blood cells in different time periods were detected; the follicle stimulating hormone (FSH) and estradiol (E_2) in peripheral blood serum were detected by enzyme linked immunosorbent assay (ELISA). The expression levels of E_2, luteinizing hormone (LH) and interleukin 1β (IL-1β) were detected, and the ovarian follicle number and spleen index were measured. [**Results**] After irradiation, the number of peripheral blood cells decreased, especially the number of white blood cells ($P < 0.05$), the content of hemoglobin increased ($P < 0.05$), the level of E_2 decreased, the number of mature follicles decreased, the spleen index decreased, and the expression level of IL-1β increased. After the intervention of Zuogui Pill, the serum E_2 level, number of mature follicles and spleen index increased, while the serum IL-1β, FSH and LH levels decreased, especially in the high dose Zuogui Pill group ($P < 0.05$). [**Conclusions**] Zuogui Pill can promote the repair of ovarian function in rats with radiation injury, which may be related to the promotion of bone marrow hematopoiesis and the improvement of immune function, reflecting the theory of "kidney governing blood vessels".

Key words Radiation injury, ^{60}Coγ ray, Zuogui Pill, Spleen index, Animal experiment

1 Introduction

Premature ovarian failure (POF) has become a major disease that seriously affects women's reproductive health and endocrine function. POF can affect the reproductive function of young women in the short term, and lead to endocrine dysfunction, osteoporosis, cardiovascular diseases and so on in the long term[1]. Physical and chemical factors (radiotherapy, chemotherapy and toxic drugs) have a particularly serious impact on ovarian functions, and the mechanism of its damage is not yet clear. Traditional Chinese medicine believes that physical and chemical factors belong to "toxic evil", and radiation belongs to "heat evil", which directly hits multiple viscera, impairs yin, consumes qi and moves blood, burns blood, accumulates blood heat, stagnates blood and coagulates phlegm, damages viscera, prolongs for a long time, and is stubborn and difficult to cure[2]. Endogenous toxic evil often adheres to pathological products such as phlegm-dampness and blood stasis. "Internal warm toxin burns its blood, its blood will congeal". Pathogenic heat spreads to other viscera for a long time, will damage the heart, spleen and kidney. From the clinical signs and symptoms, when the human body is exposed to excessive electromagnetic radiation, serious people will have insomnia, tinnitus, memory loss, sexual deterioration and reproduction dysfunction and other clinical signs, which is the syndrome of kidney essence damage[3]. Immune dysfunction caused by acute radiation injury is the most common complication. Zuogui Pill, a famous prescription for tonifying the kidney, comes from *Complete Works of Jingyue*[4], and its mechanism of action in treating POF has been studied[5-7]. The protective effect of Zuogui Pill on ovarian functions is mainly reflected in inhibiting the expression of apoptotic proteins and promoting the development of primordial follicles, but there are few reports on Zuogui Pill on immune function and bone marrow suppression caused by radiation injury. Based on the theory of "tonifying the kidney and activating pulses", we explored the effect of Zuogui Pill on the body's immune function and ovarian functions based on the changes of peripheral blood cells and ovarian tissue apoptosis in radiation-damaged rats.

2 Materials and methods

2.1 Experimental animals Fifty SPF SD female rats, aged 8 weeks, with a body mass of (220 ± 20) g. Laboratory Animal License No.: SYXK (Gan) 2015-0005. Feeding conditions: feeding under natural light conditions (day/night 12 h/12 h), feeding and drinking freely, keeping room temperature at 20 - 25 ℃, aseptic feeding (the room was sprayed with potassium permanganate with a concentration of 1∶5 000, the air was irradiated with ultraviolet lamp for 30 min, twice a day, the feed, water and bedding were sterilized by high pressure. After 3 d of feeding, they

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were used for experimental studies. This study followed the ethical requirements of animal experiments and was approved by the Medical Ethics Committee of Gansu University of Chinese Medicine (2020-255).

2.2 Experimental drugs Composition of Zuogui Pill formula granules: Rehmanniae Radix Praeparata (batch No.: 1031263) 24 g, Dioscoreae Rhizoma (batch No.: 1032993) 12 g, Corni Fructus (batch No.: 1030033) 12 g, Lycii Fructus (batch No.: 1010423) 12 g, Cuscutae Semen (batch No.: 1012053) 12 g, Cyathulae Radix (batch No.: 1035963) 9 g, provided by Guangdong Yifang Pharmaceutical Co., Ltd. Testudinis Carapacis Et Plastri Colla (Huixin Pharmaceutical Co., Ltd., batch No.: 1908003) 12 g, Cervi Cornus Colla (Herun Pharmaceutical Co., Ltd., batch No.: 02180515) 12 g. All medicinal materials were provided by the pharmacy of Gansu University of Chinese Medicine Affiliated Hospital, and were identified as authentic products by Yang Xicang, director of the Department of Pharmacy. Estradiol valerate (trade name: Progynova, Bayer Healthcare Co., Ltd., batch No.: J20171038, specification: 1 mg/tablet).

2.3 Reagents and instruments Enzymelinked immunosorbent assay (ELISA) kit, luteinizing hormone [(LH); batch No.: MM-0624R1], follicle stimulating hormone [(FSH, batch No.: MM-0566R1], estradiol [(E_2, batch No.: MM-0575R1] and Interleukin 1β [batch No.: MM-0047R2] were purchased from Jiangsu Feiya Biotechnology Co., Ltd. ONCOR Impression Plut High Energy Linear Accelerator (Siemens, Germany), CX21FS1 Inverted Microscope (Olympus, Japan), Jingzhi 00000246 Electronic Analytical Scale (Sartorius Scientific Instruments (Beijing) Co., Ltd.), KX-21 automatic blood cell counter (Sysmex).

2.4 Molding and grouping From 50 rats, 10 rats were randomly selected as the normal group, which were not irradiated and fed routinely, and the other 40 rats were used to prepare the model. The irradiation conditions were as follows: ^{60}Coγ ray, total body irradiation of 6.0 Gy, dose rate of 3 Gy/min, 6 mV, and irradiation duration of 2.2 min (irradiated in the Department of Radiotherapy, Gansu Provincial Hospital). After irradiation, the rats were randomly divided into radiation group, Progynova group, high and low dose Zuogui Pill groups, with 10 rats in each group.

2.5 Intervention methods Zuogui Pill formula granules were dissolved in heated sterilized purified water to prepare Zuogui Pill decoction with a concentration of 1.89 g crude drug/mL, and the dose of rats was calculated according to the adult dose of Zuogui Pill; the Zuogui Pill high dose group was given 4.725 g of Zuogui Pill, and the low dose group was given 0.945 g of Zuogui Pill. Progynova was calculated according to 70 kg of adults taking 1 mg of Progynova daily, and the dose of rats was 0.09 kg (body mass)/d, which was prepared into 0.018 mg/mL, and the Progynova group was given 0.09 g of Progynova daily; rats in the normal group and radiation group were given 2 mL of normal saline by gavage daily for 21 consecutive days. During the experiment, 4 rats died in the radiation group, 2 in the Progynova group, 1 in the high dose Zuogui Pill group, and 3 in the low dose Zuogui Pill group.

2.6 Observation indicators

2.6.1 Body mass of rats. The rats were weighed at 9:00 every morning, and the general condition of the rats was observed.

2.6.2 Peripheral blood routine. First, 20 μL blood was collected from rats by cutting off their tails, and the content of hemoglobin (Hb), white blood cell (WBC) and platelet (PLT) were detected by automatic blood cell counter.

2.6.3 The serum sex hormone level. Blood was taken from the precordial region of the rat, and the blood sample was put into a 1.5 mL centrifuge tube, centrifuged at 4 ℃ for 10 min with a centrifugal radius of 8 cm and 3 000 r/min, and the centrifuge tube was taken out, and the upper serum was aspirated with a pipette gun and transferred into a new centrifuge tube. Serum levels of FSH, E_2 and LH were measured by 48-well ELISA kit.

2.6.4 Histopathology of rat ovaries. The rats in each group were sacrificed at the end of the experiment. The ovary was taken and fixed, and the changes in the structure of the primitive follicle and the follicle at different stages of development were observed. Pretreated with 2.5% glutaraldehyde/0.1 M phosphate buffer (pH 7.3), fixed with 1% osmium tetraoxide (Sigma) at 4 ℃ for 2 h. After xylene dewaxing, 95% and 80% ethanol dehydration and hematoxylin/eosin staining, the slides were fixed and sealed. Using an ultramicrotome, tissue sections were 4 μm and stained with HE. The follicles of ovarian tissue were observed under the microscope, and 5 slices of each follicle were observed, and the number of follicles in all slices was calculated.

2.6.5 Determination of spleen index and serum IL-1 β. The rats were killed, the spleen taken out, removed the surrounding fat tissue, weighed, and calculated according to the formula: Spleen index = Spleen mass (mg)/Rat body mass (g). The serum IL-1β expression level was detected by ELISA method according to the operation steps of the kit, and the absorbance was detected by microplate reader at 450 nm wavelength.

2.7 Statistical methods SPSS 20.0 statistical software was used to analyze the data, and the measurement data were expressed as $\bar{x} \pm s$. For measurement data following normal distribution, one-way analysis of variance was used to compare multiple groups, *LSD* method and Dunnett method were used to compare groups when the variances were uniform, Tamhane's T2 test was used when the variances were uneven, and pairwise comparisons were made between means; Kruskal-Wallis H test of non-parametric test was used for the data that did not follow the normal distribution; $P < 0.05$ was statistically significant.

3 Results and analysis

3.1 Body mass of rats Compared with the normal group, the body mass of radiation rats decreased significantly, especially on the 7th, 14th and 21st day ($P < 0.05$). After drug treatment, the rats' appetite and diarrhea gradually improved, and the body mass

gradually increased; compared with the radiation group, the body mass of the Zuogui Pill dose groups increased significantly, and the difference was statistically significant ($P<0.05$), as indicated in Table 1.

Table 1 Comparison of body mass of rats in each group at different times ($\bar{x}\pm s$, g)

Group	Number of rats	1st day	7th day	14th day	21st day
A	10	238.82±10.16	244.89±9.36	247.39±8.11	246.84±10.48
B	6	236.76±10.47	214.67±5.54*	202.16±4.42*	207.15±8.34
C	8	238.14±9.79	215.38±12.86	205.92±9.59	207.87±6.67
D	9	239.29±10.07	221.49±16.27△	227.18±16.23△	244.47±7.86△
E	7	241.17±13.37	217.56±16.36	225.55±13.29△	231.45±13.11△

NOTE A. normal group, B. radiation group, C. Progynova group, D. Zuogui pill high dose group, E. Zuogui pill low dose group; * indicates comparison with normal group in the same period, $P<0.05$; △ indicates comparison with radiation group in the same period, $P<0.05$, the same below.

3.2 Content of WBC, Hb and PLT in peripheral blood of rats Compared with the normal group, the contents of WBC, Hb and PLT in the peripheral blood of the radiation model rats decreased significantly, especially on the 7th day after irradiation, and the difference was statistically significant ($P<0.05$). Compared with the model group, the contents of WBC, Hb and PLT in the peripheral blood of the drug group gradually recovered after treatment, especially in the Zuogui Pill high dose group, and the difference was statistically significant ($P<0.05$), as shown in Table 2-4.

Table 2 Comparison of peripheral blood WBC of rats in each group ($\bar{x}\pm s$, $\times10^9$/L)

Group	Number of rats	1st day	7th day	14th day	21st day
A	10	7.77±0.86	10.10±0.42	10.69±0.81	9.25±0.51
B	6	3.42±2.27*	1.16±1.21*	2.45±1.21*	3.87±2.29*
C	8	3.35±1.70*	4.95±2.47△	4.53±2.67	6.71±1.90
D	9	2.43±1.73*	4.85±2.76△	6.25±3.75	9.15±1.34△
E	7	2.13±0.89*△	3.31±1.41*	4.46±3.95	5.31±1.95

Table 3 Comparison of peripheral blood Hb of rats in each group ($\bar{x}\pm s$, g/L)

Group	Number of rats	1st day	7th day	14th day	21st day
A	10	140.60±41.31	173.82±55.34	158.34±48.49	148.23±58.91
B	6	145.27±34.53*	144.20±28.43*	134.30±43.27*	141.30±21.34*
C	8	168.30±23.34*	92.49±56.78△	104.66±32.35	118.12±38.19
D	9	170.20±45.31*	126.8±21.11△	129.20±24.56	138.40±20.07△
E	7	163.20±45.85*△	116.9±20.09*	127.30±16.81	135.80±25.55

Table 4 Comparison of peripheral blood PLT of rats in each group ($\bar{x}\pm s$, $\times10^9$/L)

Group	Number of rats	1st day	7th day	14th day	21st day
A	10	506.00±65.18	601.50±219.91	563.10±49.49	435.10±187.13
B	6	781.00±121.62	84.00±69.99*	171.30±21.15	274.40±20.02*
C	8	668.42±231.34	92.49±52.78*	324.66±172.35*△	380.12±182.19
D	9	649.39±100.01	66.80±23.35*	249.30±122.21*△	412.20±234.09△
E	7	754.30±183.86	112.50±32.21*	213.60±48.89*△	316.40±251.12*

3.3 Levels of FSH, LH and E_2 in serum of rats Compared with the normal group, the serum levels of FSH increased and the serum levels of LH and E_2 decreased in the radiation group ($P<0.05$). After treatment with Progynova and Zuogui Pill, the serum levels of FSH decreased and the serum levels of LH and E_2 increased; compared with the model group, the difference was statistically significant ($P<0.05$), especially the Zuogui Pill high dose, Table 5.

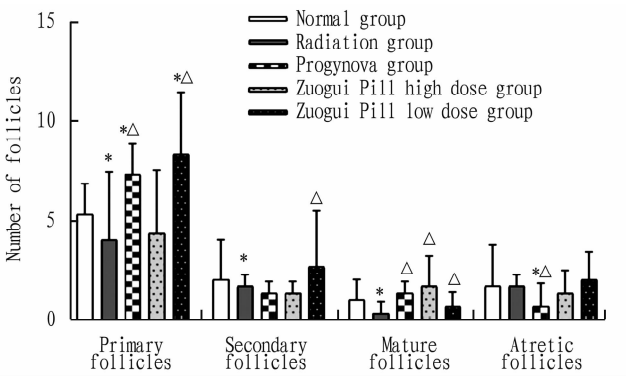
Table 5 Comparison of FSH, LH and E_2 contents in serum of rats in each group ($\bar{x}\pm s$, pg/mL)

Group	Number of rats	FSH	LH	E_2
A	10	1.33±1.09	1.88±1.19	104.58±38.82
B	6	1.97±0.78	1.14±1.03*	49.40±12.54*
C	8	0.75±0.93*△	1.47±1.29△	61.43±28.34*
D	9	1.28±1.12△	1.78±1.39△	75.62±23.67*△
E	7	1.17±1.15*△	1.23±0.26*	62.87±16.68*

3.4 Analysis of changes in the number of follicles of rats in each group In the normal group, the ovaries of rats showed active follicle growth at all levels, large follicle volume, and enlargement of the intermediate lacuna of the follicle. The apoptosis of ovarian oocytes, the reduction of follicular granulosa cell layer, the degeneration and necrosis of granulosa cells, and the infiltration of inflammatory cells in the ovarian interstitial gland and atrophic follicles can be clearly observed in the radiation model rats. The number of atretic follicles is markedly increased, and the number of ovarian granulosa cells is reduced. Compared with the normal group, the primary follicles and mature follicles in the radiation group were significantly reduced ($P<0.05$). After treatment, the

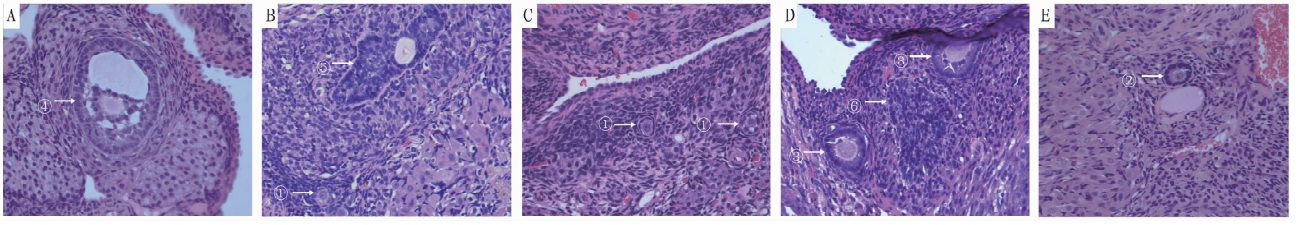
primary follicles and mature follicles were increased in the Progynova and Zuoguiwan groups, and the difference was statistically significant compared with the radiation model group ($P<0.05$), as shown in Fig. 1.

3.5 Ovarian histopathological sections Follicles were normal in normal group; primary follicles and atretic follicles were found in radiation group, and there were some pyknotic granulosa cells in the basement membrane; some antral follicles were found in Progynova group, and the cells were arranged in order. In the Zuogui Pill high dose group, antral follicular nucleus apoptosis was observed, granulosa cells were arranged in order, and a small number of primary oocytes were observed; in the Zuogui Pill low dose group, primary oocytes were observed, no antral oocytes and mature oocytes were observed, and granulosa cells were arranged in order, as shown in Fig. 2.



NOTE * means comparison with normal group, $P<0.05$; △ means comparison with radiation group, $P<0.05$.

Fig. 1 Ovarian follicle number of each group of rats



NOTE ① primary oocyte; ② secondary oocyte; ③ antral oocyte; ④ mature oocyte; ⑤ atretic oocyte; ⑥ corpus luteum; A is the normal group; B is the radiation group; C is the Progynova group; D is the Zuogui Pill high dose group; E is the Zuogui Pill low dose group; Scale = 20 μm.

Fig. 2 Ovarian histopathology of rats in each group (HE, ×400)

3.5 Spleen index and serum IL-1β level in rats Compared with the normal group, the spleen index of rats in the radiation group decreased and the serum IL-1β level increased ($P<0.05$); compared with the radiation group, the spleen index of rats in the radiation group increased and the serum IL-1β level decreased after the treatment of Progynova and Zuogui Pill ($P<0.05$), as shown in Table 6.

Table 6 Comparison of spleen index and IL-1β content in peripheral blood of rats in each group ($\bar{x}\pm s$)

Group	Number of rats	Spleen index // mg/g	IL-1β // ng/L
A	10	3.98 ± 0.24	37.69 ± 1.80
B	6	3.01 ± 0.13 *	48.47 ± 4.04 *
C	8	3.07 ± 0.20 *	42.42 ± 0.44
D	9	3.61 ± 0.11 *△	41.52 ± 8.79 *△
E	7	3.10 ± 0.12 *	32.66 ± 4.45 △

4 Discussion

Studies have found that electromagnetic radiation damage can have an impact on multiple systems such as immunity, nerves, or reproduction[8], but the current basic and clinical research of traditional Chinese medicine is mostly about the reproductive system, especially in patients of childbearing age, who are sensitive to radiation-induced DNA damage[9-10], and radiation-induced apoptosis, which is particularly prone to occur in growing and developing follicles[11]. The immune system is one of the most sensitive target organs of ionizing radiation injury, which is mainly manifested in that radiation can induce the body to produce a series of specific and non-specific immune responses, so that tissues and organs are in a strong state of stress injury, releasing a large number of endogenous harmful factors, and the change of peripheral hemogram is the early manifestation of ionizing radiation injury[12-13]. IL-1 plays an important role in the regulation of lymphocytes, especially in the process of lymphocyte immune activation. Spleen index can reflect the strength of immune function to a certain extent, so it is used as one of the objective indicators to evaluate immune function[14].

Traditional Chinese medicine believes that high-energy rays have the characteristics of "heat evil", and heat-toxin accumulates in the body and fills the triple energizer[15]. It will burn body fluid, harm yin and consume qi, initially injure the lungs, leading to long-term illness and affecting the kidneys, as well as causing blood stasis. Abnormal coagulation function and microvascular occlusion lead to microcirculation disorders, affecting the blood supply of viscera, especially insufficient ovary blood supply and functional decline. That is to say, radiation injury is caused by heat toxin, which is initially manifested as "excessive heat toxin in the interior and deficiency of both qi and blood", which is consistent with the symptoms and pathogenesis of hemorrhage after radiation[16]. Traditional Chinese medicines can prevent and treat electromagnetic radiation damage, and most of them have the effects of benefiting qi, nourishing yin, replenishing essence and tonifying kidney. Zuogui Pill is from *Complete Works of Jingyue*. It is suitable to strengthen the main body of water to cultivate the primordial yin of the left kidney for the syndromes of exhaustion of the essence and dryness of body fluid.

This study showed that the body mass of irradiated rats decreased, and the number of peripheral blood cells decreased sharply, especially white blood cells. The number of platelets increases in the early stage, which may be related to the decrease of body fluid and blood concentration caused by radiation heat. The apoptosis of ovarian tissue in rats was obvious, the follicle cells were

loosely arranged after radiation injury, the oocytes showed nuclear condensation and nuclear lysis, and the necrosis of granulosa cells was seen in the follicle cavity. Serological detection of FSH increased, and the levels of LH and E_2 decreased significantly, which was related to the apoptosis of granulosa cells caused by radiation and the impact on the energy supply of oocytes.

After treatment with Zuogui Pill, the body weight and hematopoietic function of rats gradually recovered, and the number of peripheral blood cells increased, and the Zuogui Pill high dose group was significantly better than Progynova group and the Zuogui Pill low dose group. The number of secondary oocytes in the ovaries increases, immune function is restored, the spleen index increases, and serum inflammatory pro-inflammatory factors decrease. This study shows that the mechanism of POF caused by radiotherapy may be related to "blood stasis", so promoting blood circulation and removing blood stasis is a means of treating POF.

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count; the results of platelet adhesion experiments suggested that extract A can enhance platelet adhesion ability; the results of platelet aggregation experiments showed that extract A can induce platelet adhesion in a rat model. Therefore, extract A can increase the number of platelets, produce platelet adhesion, cause platelet aggregation, and lead to platelet thrombus formation, playing a hemostatic role.

In summary, extract A had hemostatic effects and a significant hemostatic effect on uterine bleeding model of early pregnancy rat. Its function may be induced by affecting blood vessel contraction-relaxation; coagulation factors of the internal and external coagulation systems in the blood; the adhesion and aggregation of platelets, as well as the increase in platelet count, the amount of uterine bleeding, thereby displaying the characteristics of multiple pathways and multi-target effects.

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