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Original Article

Effect of Modified Wuzi Yanzong Pill (五子衍宗丸) on Tip60-Mediated Apoptosis in Testis of Male Rats after Microwave Radiation*

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ABSTRACT **Objective:** To investigate the effect of a modified Wuzi Yanzong Pill (五子衍宗丸, WZYZP) on the male rats' testis after microwave radiation, as well as its potential mechanism. **Methods:** Forty-five male rats were randomly assigned to three groups: the control group, the radiation group, and the WZYZP group. The rats in the radiation group and WZYZP group were exposed to microwave radiation for 15 min once, while the rats in the control group were not exposed to any radiation. The rats in the WZYZP group were given a modified of WZYZP by gavage daily for 7 days. Apoptosis in the testis was evaluated using terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) assay. Histopathological alterations of the testis were observed by haematoxylin-eosin (HE) staining. Tat-interactive protein, 60kD (Tip60) and p53 expressions were determined by Western blotting. **Results:** The apoptosis index (AI) in the radiation group was higher than that of the WZYZP group and control group on day 1 (D1), day 7 (D7) day 14 (D14) after radiation (P<0.05). The seminiferous tubules were of normal morphology in the control group. In the radiation group, the partial seminiferous tubules were collapsed, basement membranes of the seminiferous epithelia became detached. WZYZP could restore the morphological changes. There was no expression of Tip60 among the three groups on D7 and D14. The expression of p53 was higher in the radiation group than in the control group (P<0.05). WZYZP could down-regulate the rising p53 induced by radiation on D7 and D14 (P<0.05). **Conclusion:** A modified WZYZP may affect germ cells, and its protective effects may partly result from its ability to intervene in Tip60 mediated apoptosis.

KEYWORDS microwave, electromagnetic radiation, spermatogenesis, apoptosis, histone acetyltransferases

Microwaves have been used in the military, industry, agriculture, and scientific research, and even in everyday family life. People are exposed to microwave radiation more often than before. In 1995, microwave radiation had been classified as one of the major causes leading to environmental pollution by the United Nations Conference on Human Environment. The effects of microwave radiation on humans are various. The male reproductive system in particular is sensitive to microwave radiation. Epidemiological and experimental studies have found microwave radiation has adverse effects on male reproductive functions.[1-7] Our previous studies[8,9] also found that, compared with non-exposed workers, the testosterone and sperm motility of exposed workers decreased, while the total apoptosis rate of sperm increased significantly in those who had been exposed to microwave radiation occupationally, indicating that microwave radiation could be harmful to the reproductive system and could increase the sperm apoptosis index (AI). However, the mechanism of increasing apoptosis of germ cells induced by microwave radiation is not clear. Apoptosis, also known as programmed cell death, is a kind of mode of cell death distinguished from necrosis.[10] Various internal and external environmental factors are likely to cause

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pathological apoptosis in germ cells.[11] Excessive germ cell pathological apoptosis affects male reproductive functions. Tat-interactive protein, 60kD (Tip60) is an important histone acetyltransferase (HAT) that plays an important role in deoxyribonucleic acid (DNA) damage repair, cell apoptosis, and genome integrity.[12-16] P53 plays a major role in the cellular response to DNA damage and other genomic aberrations. Activation of p53 can lead either to cell cycle arrest and DNA repair or apoptosis.[17] Previous studies have found Tip60 is involved in DNA damage repair by regulating the activity of p53. When DNA damage occurs, Tip60-dependent acetylation of p53 at Lys120 (K120) makes p53 preferentially bind to promoters of pro-apoptotic genes (Bax and Puma), leading to p53-mediated apoptosis.[18,19]

A modified Wuzi Yanzong Pill (五子衍宗丸, WZYZP) is effective in treating male reproductive dysfunction caused by microwave radiation in clinical practice. WZYZP has been used in Chinese medicine for over 400 years; it is widely used as a classical prescription for male reproductive dysfunction with insufficiency of Shen (Kidney) essence, and has been observed to improve spermatogenesis. Our early study in male rats shows that a modified WZYZP could improve the levels of serum sex hormones and promote sperm quality in male rats after microwave radiation exposure.[20] However, the mechanism of the protective effect of modified WZYZP is not clear.

Thus, we hypothesized that modified WZYZP could exert a protective effect on germ cells by regulation of Tip60-p53 mediated apoptosis in male rats after microwave radiation. In the present study, we investigated the impact of a modified WZYZP on sperm apoptosis, testicular morphology, and the expression of Tip60 and p53 in male rats after microwave radiation.

METHODS

Animals and Groups

A total of 45 three-month-old male, specific pathogen free (SPF) class Wistar rats, weighing 230 to 250 g, were provided and reared by the Experimental Animal Center of Military Medical Sciences [Qualification No. SCXK (army 2007-004)]. The rats were randomly assigned to three groups by a random number table: the control group, the radiation group, and the WZYZP group. All the rats were housed in a routine manner in terms of temperature (21–23 ℃), light (12 h of light and 12 h of darkness), relative humidity (60%), and ventilation. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals by the National Institute of Health.

Radiation group: 15 rats were placed in a microwave anechoic chamber, an electromagnetic measuring environment that can mask off external interference and restrain internal multipath echoes, and exposed to systemic microwaves irradiation once for 15 min. The height of the radiation source was 30 cm, and the radius from the radiation source to the rats was 10 cm. The radiation density was 100 mW/cm^2. The ambient temperature was 18±2 ℃, and the humidity was 50% to 70%. The rats were kept in a routine way.

WZYZP group: 15 rats were placed in a microwave anechoic chamber and exposed to systemic microwaves irradiation once for 15 min. Radiation conditions in the WZYZP group were the same as those in the radiation group. The rats were removed from the radiation chamber after radiation, and given a decoction of a modified WZYZP by gavage at 08:00–09:00 daily for 7 days during the radiation.

Control group: 15 rats were placed in the microwave anechoic chamber, but without exposure to microwave radiation. Other conditions were the same as those in the radiation group.

Preparation for Modified WZYZP Extract

The modified WZYZP consisted of *Herba Cistanches* 10 g, *Radix Moromdae Officinalis* 10 g, *Herba Epimedii* 10 g, *Radix Angelicae sinensis* 10 g, *Radix Salviae Miltiorrhizae* 20 g, *Radix Polygoni Multiflori* Preparata 10 g, *Rhizoma Polygonati* 10 g, *Semen Cuscutae* 10 g, *Fructus Ligustri Lucidi* 10 g, *Fructus Lycii* 10 g, *Radix Pseudostellariae* 10 g, *Radix Astragali* 10 g, *Fructus Cnidii* 10 g, and *Radix Rehmanniae* Preparata 10 g, etc. All the herbs were purchased from the Beijing Tong Ren Tang Pharmacy.

The herbs, including the crude drug at a concentration of 1 g/mL, were immersed in water for 1 h and then decocted traditionally to boil for 1 h, filtered, and then concentrated. The decoction was stored in sterile flasks sealed at 4 ℃ before use.

Morphological Analysis

Five rats in each group were killed by cervical spine dislocation on 1, 7, and 14 days after radiation, respectively. Then, the testicles and epididymis were quickly removed.

Testicles fixation: after rinsing with saline, the left testicles were cut into two pieces and fixed in 10% neutral formalin for 24 h in order to prepare for the apoptosis assays and morphological analysis. The right testicles were cut into small pieces and quickly frozen in liquid nitrogen for Western blotting assay. After dewaxing, transparentizing, embedding, slicing, hematoxylin-eosin (HE) staining, and conventional dehydration, the sections of testis were observed under a light microscope for morphological analysis.

Terminal-Deoxynucleoitidyl Transferase Mediated Nick End Labeling Assay

Paraffin-embedded specimens were serially cut into 4-μm sections and mounted on microscope slides. The slides were deparaffinized using xylene and decreasing concentrations of ethyl alcohol and washed in 0.01 mol/L phosphate buffer saline (PBS, pH 7.4). The sections were then incubated with 0.5% Triton X-100 for 1 h at 37 ℃. They were then washed with PBS [with added 0.3% bovine serum albumin (BSA)] for 30 min at room temperature. A mixed solution of terminal DNA transferase (TDT)–marking reaction solution and biotin-labeled nucleotides solution was added to cover each section, and the slides were incubated in a humidified chamber at 37 ℃ for 1 h. The slides were incubated with anti-fluorescein antibody-peroxidase (POD) at 37 ℃ for 20 min. The sections were washed with PBS. Then, the slides were incubated with 3, 3'-diaminobenzidine (DAB) solution and observed under a microscope. Finally, the slides were counterstained with hematoxylin for 3 min.

Terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL)-positive cells were visualized as brown cells. Negative controls were processed as PBS instead of a TDT reaction solution. The AI was determined to record the presence or absence of apoptosis in each specimen. The AI was calculated as the percent of positively stained cells in 5 randomly selected fields at 400× magnification.

Western Blotting Assay

The protein concentrations of the testicles were determined by using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Proteins were separated on 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a nitrocellulose membrane for 80 min at 120 V. Then, the membranes were blocked with a blocking buffer [5% non-fat milk diluted in Tris buffered saline with 0.1% tween (TBST)] for 1 h at room temperature. The membranes were incubated overnight at 4 ℃ with the following antibodies diluted in TBST: mouse anti-p53 antibody (abcam, Cambridge, MA, USA), mouse anti-Tip60 antibody (abcam), or mouse anti-Tubulin antibody (abcam, Cambridge, MA, USA), respectively. After washing with TBST (4 times, 10 min each), the blots were incubated for 45 min at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse antibodies at 1:5000 dilution (Boster, Wuhan, China). Signals were detected using an electrogenerated chemiluminescence method.

Statistical Analysis

The statistical analysis of data was carried out using SPSS 20.0 statistical software (IBM, Armonk, NY, USA). The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and analyzed with a one-way ANOVA. Multiple comparisons among groups were performed with a least significant difference (LSD) *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

TUNEL Assay Results

On D1, D7, and D14, the AI in the radiation group was significantly higher than that in the control group ($P<0.05$). Similarly, the AI in the WZYZP group was significantly lower than that in the radiation group ($P<0.05$), but there was no significant difference between the WZYZP group and the control group ($P>0.05$, Figure 1).

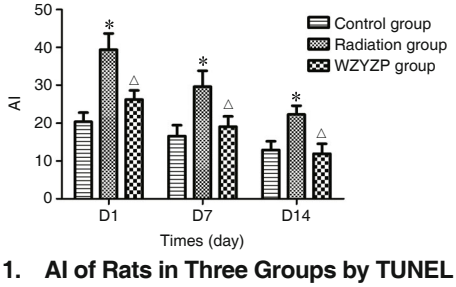

Figure 1. AI of Rats in Three Groups by TUNEL Assay

Notes: *$P<0.05$, compared with the control group; △$P<0.05$, compared with the radiation group

Morphological Analysis

In the control group, seminiferous tubules were integrated, the basement membranes were intact and clear, the thickness of the seminiferous epithelium was moderate, the Leydig cells were arranged in an orderly manner, the capillaries were rich in loose connective tissue, and different stages of germ cells and mature sperm could be found.

On D1 and D7 after exposure, the partial seminiferous tubules were collapsed, basement membranes and thickness of seminiferous epitheliums became thin and detached, and the spermatogenic cells and Leydig cells were arranged in a disorderly manner in the radiation group. On D14 after exposure, the structure of the seminiferous tubule was partially restored.

WZYZP could restore the changes of morphology in testicular tissue after microwave exposure. Compared with the radiation group, seminiferous tubules and basement membranes were integrated, the thickness of the seminiferous epithelium was moderate, and most of the Leydig cells were arranged in an orderly fashion (Figure 2).

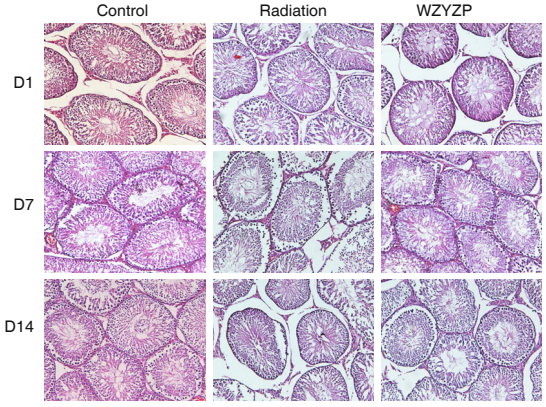

Figure 2. Testicular Morphology of Rats by HE Staining (Images under light microscope, 200×)

Western Blot of p53 and Tip60

Microwave radiation induced a significant increase of p53 and Tip60 ($P<0.05$). WZYZP was able to restore the protein levels of p53 and Tip60 on D1 ($P<0.05$). P53 was expressed on D1, D7, D14, and its expression in the radiation group was higher than in the control and the WZYZP groups ($P<0.05$, Figure 3). There was no expression of Tip60 in the 3 groups on D7 and D14 exposure (Figure 3).

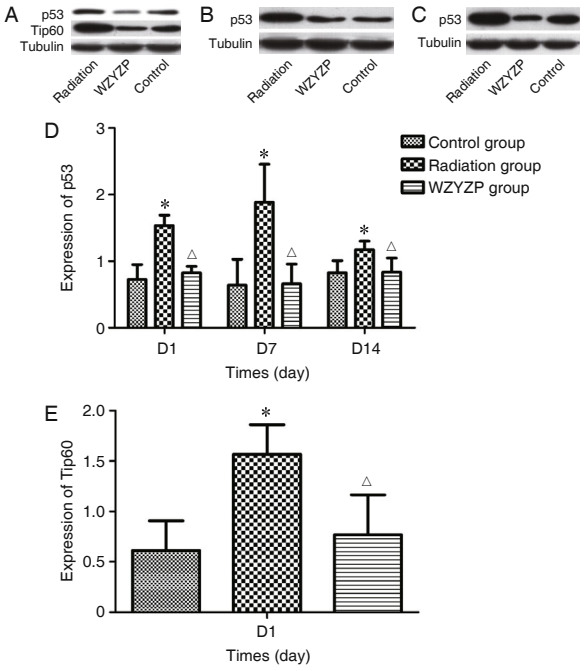

Figure 3. Protein Expressions of p53 and Tip60 in Rat Testis by Western blotting

Notes: A: Expressions of p53 and Tip60 on D1; B: Expressions of p53 on D7; C: Expressions of p53 on D14; D: Expressions of p53/tubulin on D1, D7, and D14; E: Expressions of Tip60/tubulin on D1. $^{*}P<0.05$, compared with the control group; $^{\triangle}P<0.05$, compared with radiation group.

DISCUSSION

The current studies confirmed the male reproductive system is highly sensitive to electromagnetic radiation, and microwave radiation can lead to decreased semen quality, reproductive dysfunction, testicular structure and function injuries, and genetic material injury.[21-23] The results of this study show high-intensity microwave radiation can increase the AI of the testis, affect the morphology of testis, and induce a significant increase of p53 and Tip60 expressions. WZYZP may restore these morphological changes, decrease the AI, and restore the levels of p53 and Tip60.

On the basis of Chinese medicine, microwave radiation can be considered "fire".[24] But if the microwave power is too strong, the exposure time is too long, or there is a lack of appropriate protective measures, it will become "pathogenic fire" and exert adverse effects on the whole body. Pathogenic fire can easily hurt body fluids and qi, resulting in qi and yin deficiency of Shen. The efficacy of Chinese medicine in improving reproductive system damage caused by microwave radiation has received much attention, and most herbs can supplement the Shen and enhance immunity.

The modified WZYZP targets the particular

pathogenesis after microwave radiation. Based on WZYZP, *Fructus Ligustri Lucidi*, *Fructus Lycii*, and *Rhizoma Polygonati tonify* the Shen yin and nourish essence and blood. *Semen Cuscutae*, *Herba Cistanches*, *Radix Moromdae Officinalis*, and *Herba Epimedii* tonify the Shen yang and nourish essence and blood. All the monarch drugs above can tonify the Shen and nourish essence together. *Radix Rehmanniae* Preparata and *Radix Angelicae sinensis* enrich the blood and nourish the yin. *Radix Pseudostellariae* and *Radix Astragali* supplement the qi and promote body fluid. All the minister drugs above could reinforce the efficacy of monarch drugs. *Radix Salviae Miltiorrhizae* promotes blood circulation and removes channel obstructions to assist the monarch drugs and minister drugs. In earlier studies, we found that the modified WZYZP could improve the levels of serum sex hormones and promote the quality of sperm in male rats after microwave radiation.[20] However, the mechanism of the protective efficacy is not clear.

In the present study, the TUNEL assay suggested microwave radiation could lead to increased AI in germ cells, which were consistent with the results of other studies.[5,6] Chinese medicine could significantly reduce increase in AI and play an anti-apoptosis role in male rats.

We further investigated the molecular mechanism of the modified WZYZP. First, we found the increase in p53 expression was consistent with the increased AI in the radiation group, and the decreasing p53 expression was also consistent with descending AI in the WZYZP group, which indicated that p53 expression was closely related to apoptosis in germ cells. This is consistent with previous findings from Levine's study.[17]

Furthermore, Tip60 and p53 in Western blot showed the trend of Tip60 apoptosis was similar to that of p53 on D1. However, on D7 and D14, there was no expression of Tip60 in the radiation group, WZYZP group, or the control group, while the expression of p53 could be found in all three groups. These results indicated p53 was related to Tip60 on D1. There was no significant correlation between p53 and Tip60 on D7 and D14. The discrepancy among D1, D7, and D14 may result from the short half-life of Tip60. Normally, the Tip60 expression level is low.[25] The microwave radiation may stimulate expression of Tip60 temporarily on D1, and subsequently activate downstream molecules, such as p53. When radiation exposure ends, Tip60 expression may decline and lose activity. However, the active downstream molecule p53 could be found and play a continual role.

Current studies show Tip60 can participate in apoptosis by modulating the activity of p53. Therefore, we concluded that a modified WZYZP may exert a protective effect on germ cells by regulating Tip60-p53 mediated apoptosis in male rats after microwave radiation. However, whether other molecules and target are involved in the apoptosis of germ cells remains to be determined by future studies.

In conclusion, this study provides evidence that the protective effects of a modified WZYZP on sperm may partly result from its ability to intervene in Tip60-p53 mediated apoptosis in male rats after microwave radiation. However, the complicated mechanisms of this Chinese herbal medicine require further study.

Conflict of Interest

All the authors do not have any possible conflicts of interest.

Authors Contributions

Hu HX and Sun J contributed to the laboratory work. Gao YJ, Fang H and Xu SQ contributed to the laboratory data analyses. Wei LZ, Luo SB and Dong J contributed to the study design. Shen CY, Zhang QL, and Xie YL contributed to the statistical analyses. Hu HX contributed to the study design, laboratory work, and grant application. All authors read and approved the final manuscript.

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