Volume 24, No 2 | International Journal of Radiation Research, April 2026

Improving oxidative stress and radiation-induced injury in diminished ovarian reserve rats by warming needle moxibustion via Keap1-Nrf2-ARE pathway

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► ***Original article***

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Received: July 2025
Final revised: September 2025
Accepted: September 2025

Int. J. Radiat. Res., April 2026; 24(2): 435-439

DOI: 10.61186/ijrr.24.2.20

Keywords: *Warming needle moxibustion, diminished ovarian reserve, oxidative stress, radiation injury, Keap1-Nrf2-ARE pathway, fertility preservation.*

ABSTRACT

Background: The Diminished ovarian reserve (DOR) is often linked with oxidative stress (OS) and aggravated by chemotherapy and radiation. The Keap1-Nrf2-ARE pathway regulates antioxidant defense. This study evaluated whether warming needle moxibustion alleviates OS and radiation-induced ovarian injury in DOR rats. ***Materials and Methods:*** Thirty-six female Sprague-Dawley rats were assigned to control, model, treatment, and sham groups. DOR was induced with cyclophosphamide, busulfan, and localized pelvic radiation. The treatment group received warming needle moxibustion at Guanyuan (CV4) and Sanyinjiao (SP6) for 4 weeks. Estrous cycle, oxidative stress biomarkers (SOD, GSH-Px, MDA), sex hormones (FSH, LH, E2, AMH), and Keap1-Nrf2-ARE proteins were assessed. ***Results:*** Compared with model rats, treatment significantly improved estrous cycle regularity, increased SOD and GSH-Px, reduced MDA, normalized FSH and LH, and elevated E2 and AMH (all $P < 0.05$). Western blot showed decreased Keap1 and increased Nrf2, HO-1, and GCLC, confirming pathway activation. ***Conclusion:*** Warming needle moxibustion attenuated oxidative and radiation-induced ovarian damage in DOR rats through Keap1-Nrf2-ARE modulation. These results support its potential as a fertility-preserving, non-pharmacological therapy for patients undergoing chemo-radiotherapy.

INTRODUCTION

Diminished ovarian reserve (DOR) is a prevalent gynecological disorder characterized by a decline in the number and quality of ovarian follicles, leading to menstrual irregularities, reduced fertility, and early onset of perimenopausal symptoms [1, 2]. In recent years, the incidence of DOR has increased significantly due to delayed childbearing and heightened exposure to environmental and iatrogenic risk factors. Alarmingly, the age of onset is trending downward, making DOR a growing global concern in reproductive health.

The pathogenesis of DOR is multifactorial, involving genetic predisposition, autoimmune dysfunction, environmental toxin exposure, and notably, iatrogenic ovarian injury from chemotherapy and pelvic radiation therapy-common treatments for malignant diseases [3]. These cancer therapies are known to generate oxidative stress (OS) in ovarian tissue, further aggravating follicular damage.

Under physiological conditions, the ovarian microenvironment maintains redox balance through intrinsic antioxidant mechanisms. However, excessive reactive oxygen species (ROS), as seen in response to radiation and cytotoxic agents, can disrupt mitochondrial function, induce lipid peroxidation, damage DNA and proteins, and activate apoptotic pathways. This cascade accelerates follicular depletion and compromises ovarian function irreversibly [4].

Currently, hormone replacement therapy (HRT) remains the standard Western treatment for DOR. However, long-term HRT carries risks such as increased susceptibility to breast and endometrial cancers, prompting the search for safer, more holistic alternatives [2].

Traditional Chinese medicine (TCM), particularly acupuncture, offers a promising complementary approach. With over 2,000 years of clinical application, acupuncture is known to restore the balance of qi, blood, yin, and yang, and has been effectively used in treating a variety of gynecological conditions [5-7].

Modern research has progressively clarified the biological basis of acupuncture's efficacy. Its multi-target, systems-level regulation has been shown to influence the neuro-endocrine-immune network, particularly the hypothalamic-pituitary-ovarian (HPO) axis. By improving ovarian microcirculation and modulating local and systemic signaling pathways, acupuncture supports follicular development and hormonal balance [8].

Warming needle moxibustion, a technique that combines acupuncture with the thermal stimulation of burning moxa, enhances the biological effects of both modalities. The infrared radiation emitted during moxibustion penetrates deep into tissue,

[DOI: 10.61186/ijrr.24.2.20]

promoting local blood flow and stimulating cellular metabolism. Additionally, warming needle moxibustion activates temperature-sensitive ion channels, such as members of the transient receptor potential vanilloid (TRPV) family, which are implicated in regulating neurotransmitter release, immune activity, and oxidative stress responses [9,10]. In clinical practice, it has shown superior results compared to acupuncture alone, especially in conditions involving ovarian dysfunction and blood stasis.

This study aims to systematically investigate the molecular mechanism by which warming needle moxibustion improves ovarian function in a rat model of DOR induced by chemotherapy and radiation. The intervention's effects were assessed across multiple dimensions, including estrous cycle regulation, serum sex hormone levels, follicle count, and oxidative stress parameters. Particular focus was given to the Kelch-like ECH-associated protein 1-nuclear factor erythroid 2–related factor 2-antioxidant response element (Keap1-Nrf2-ARE) signaling pathway, a key axis in cellular antioxidant defense. Importantly, these findings may be particularly relevant for cancer patients undergoing chemo-radiotherapy, who face ovarian reserve decline as a major long-term complication. This study adds to the existing literature by providing preclinical evidence that warming needle moxibustion can attenuate oxidative stress and radiation-induced ovarian injury through regulation of the Keap1-Nrf2-ARE pathway. While related approaches have been studied, our work is among the first to explore this specific mechanism in a DOR rat model, thereby offering new insights into potential non-pharmacological strategies for fertility preservation.

MATERIALS AND METHODS

Experimental animals

Thirty-six specific pathogen-free (SPF) female Sprague-Dawley (SD) rats (8-10 weeks old, 180-220 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Shenyang, China). Animals were housed under controlled laboratory conditions (22 ± 2 °C, 50 ± 10% humidity, 12 h light/dark cycle) with ad libitum access to sterilized water and standard rodent chow. After a one-week acclimatization period, all experimental procedures were conducted in accordance with institutional guidelines and approved by the Animal Ethics Committee of Beidahuang Group General Hospital (Approval No. BGH-AEC-2024-016, approved March 2024).

Animal grouping and model induction

Rats were randomly assigned to four groups (n = 9 per group): control (AG), model (BG), warming needle moxibustion treatment (CG), and sham acupuncture (DG).

To establish the DOR model, animals received intraperitoneal injections of cyclophosphamide (50 mg/kg; Sigma-Aldrich, USA) and busulfan (10 mg/kg; MedChemExpress, USA) for five consecutive days, followed by a single localized pelvic X-ray irradiation (4 Gy; X-RAD 320, Precision X-Ray, USA). This regimen simulated the combined ovarian damage induced by chemotherapy and radiotherapy in clinical settings.

Intervention procedures

In the treatment group (CG), warming needle moxibustion was performed at Guanyuan (CV4) and Sanyinjiao (SP6) acupoints every other day for 4 weeks. Disposable sterile acupuncture needles (0.25 × 25 mm; Hwato, Suzhou Medical Appliance Factory, China) were inserted to a depth of 3-5 mm. A 1.5 cm moxa stick (Nanyang Hanyi Moxa Co., China) was affixed to the needle handle and ignited, producing mild, sustained heat until self-extinguishing (~10 min).

In the sham group (DG), superficial needling was performed at non-acupoint sites without moxibustion, serving as a placebo control. Control (AG) and model (BG) groups were handled but received no treatment.

Estrous cycle monitoring

Estrous cycle stages were monitored daily between 9:00–10:00 AM. Vaginal smears were obtained using saline-moistened cotton swabs, stained with Giemsa solution (Solarbio, Beijing, China), and examined under a light microscope (Olympus CX23, Japan). Cycle stage was determined based on the proportions of cornified epithelial cells, nucleated epithelial cells, and leukocytes (proestrus, estrus, metestrus, diestrus).

Measurement of oxidative stress markers

Ovarian tissues were homogenized in phosphate-buffered saline (PBS) and centrifuged at 3,000 rpm for 10 min. The supernatants were assayed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following manufacturer protocols:

Superoxide dismutase (SOD) by the xanthine oxidase method.

Glutathione peroxidase (GSH-Px) by the DTNB method.

Malondialdehyde (MDA) by thiobarbituric acid reactive substances (TBARS) assay. Absorbance was measured using a SpectraMax iD3 microplate reader (Molecular Devices, USA).

Serum sex hormone analysis

Orbital blood was collected under anesthesia, centrifuged at 3,000 rpm for 15 min, and serum was

[DOI: 10.61186/ijrr.24.2.20]

stored at −80 °C. Levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), and anti-Müllerian hormone (AMH) were determined by enzyme-linked immunosorbent assay (ELISA kits; R&D Systems, USA). Optical density was measured at 450 nm using the SpectraMax iD3 reader.

Detection of Keap1-Nrf2-ARE pathway proteins

Western blotting was used to measure protein expression of Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid 2–related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and glutamate-cysteine ligase catalytic subunit (GCLC). Ovarian tissues were lysed with RIPA buffer (Beyotime, Shanghai, China) containing protease inhibitors. Protein concentrations were determined with a BCA kit (Thermo Fisher Scientific, USA). Equal amounts (40 µg) were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Membranes were blocked with 5% skim milk and incubated with primary antibodies (Keap1 1:1000, Nrf2 1:800, HO-1 1:1000, GCLC 1:1500; Abcam, UK) overnight at 4 °C, followed by HRP-conjugated secondary antibodies (1:5000; Abcam, UK). Signals were visualized with ECL reagents (Bio-Rad, USA) and quantified using ImageJ software (NIH, USA). β-actin (1:2000; Abcam, UK) was used as the internal control.

Statistical analysis

Data were analyzed using SPSS version 22.0 (IBM, USA). Results are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by post hoc tests was applied to normally distributed data, while Mann–Whitney U test and chi-square test were used for non-normal and categorical variables, respectively. A p-value < 0.05 was considered statistically significant.

RESULTS

Estrous cycle regularity

Radiation and chemotherapy significantly disrupted estrous cycle regularity in the model group compared with controls ($P < 0.01$). Warming needle moxibustion improved cycle regularity compared with the model group ($P < 0.05$, figure 1; table 1). No significant difference was observed in the sham group.

Oxidative stress markers

The model group showed a marked reduction in SOD and GSH-Px activities, accompanied by elevated MDA levels, indicating oxidative damage. Treatment with warming needle moxibustion significantly restored antioxidant activity and reduced lipid peroxidation ($P < 0.05$, figure 2; table 1).

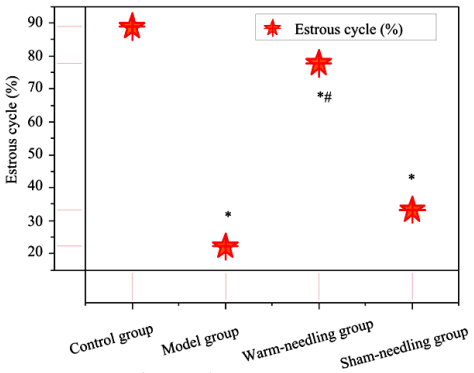

Figure 1. Estrous cycle regularity in rats. Y-axis: estrous cycle regularity (%). As against the AG, # as against the BG, $P < 0.05$.

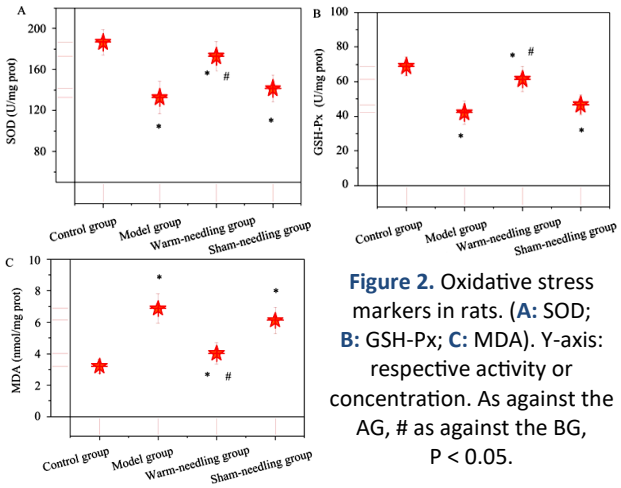

Figure 2. Oxidative stress markers in rats. (**A:** SOD; **B:** GSH-Px; **C:** MDA). Y-axis: respective activity or concentration. As against the AG, # as against the BG, $P < 0.05$.

Serum sex hormone levels

Chemotherapy and irradiation resulted in hormonal imbalance, with increased FSH and LH and decreased E2 and AMH. Warming needle moxibustion normalized these parameters compared with the model group ($P < 0.05$, figures 3–4; table 1).

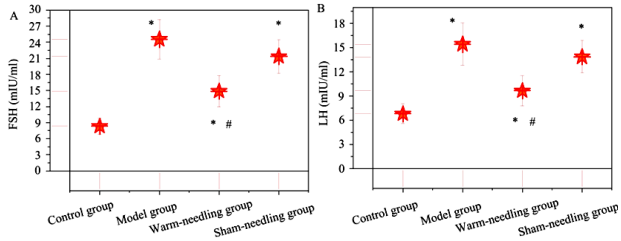

Figure 3. Serum FSH and LH levels. (**A:** FSH; **B:** LH). Y-axis: concentration (ng/mL).

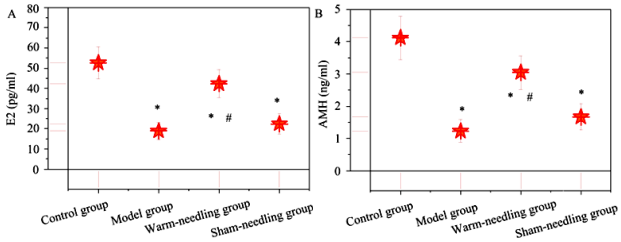

Figure 4. Serum E2 and AMH levels. (**A:** E2; **B:** AMH). Y-axis: concentration (pg/mL or ng/mL). As against the AG, # as against the BG, $P < 0.05$.

[DOI: 10.61186/ijrr.24.2.20]

Keap1-Nrf2-ARE pathway proteins

Radiation injury upregulated Keap1 expression while downregulating Nrf2, HO-1, and GCLC. Warming needle moxibustion reversed these changes, activating the antioxidant defense pathway ($P < 0.05$, figures 5 and 6; table 1).

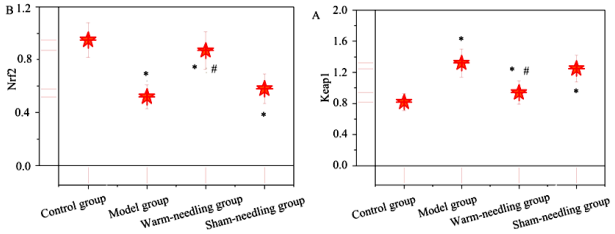

Figure 5. Expression of Keap1 and Nrf2 proteins. (**A:** Keap1; **B:** Nrf2). Y-axis: relative fold expression vs control.

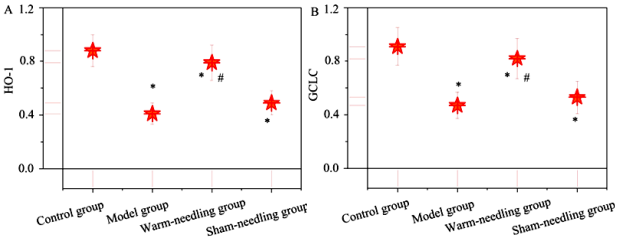

Figure 6. Expression of HO-1 and GCLC proteins. (**A:** HO-1; **B:** GCLC). Y-axis: relative fold expression vs control. As against the AG, # as against the BG, $P < 0.05$.

Effects of radiation and warming needle moxibustion on ovarian function

As summarized in table 1, combined chemotherapy and irradiation impaired ovarian function, as evidenced by disrupted estrous cycles, oxidative stress, hormonal imbalance, and suppressed antioxidant protein expression. Warming needle moxibustion significantly improved all parameters relative to the model group.

Table 1. Effects of radiation and warming needle moxibustion on ovarian function in DOR rats.

Parameter	Control (AG)	Model (BG)	Treatment (CG)	P (AG vs. BG)	P (BG vs. CG)
Estrous cycle regularity (%)	88.9	22.2	66.7	0.002	0.018
SOD (U/mg)	98.6± 6.1	62.3 ± 4.5	81.7 ± 5.2	<0.001	0.007
GSH-Px (U/mg)	245.4 ± 18.7	171.6 ± 15.2	209.3 ± 14.6	0.002	0.013
MDA (nmol/mg)	2.13 ± 0.24	3.74 ± 0.30	2.85 ± 0.27	0.003	0.009
FSH (ng/mL)	6.21 ± 0.53	9.87 ± 0.68	7.14 ± 0.59	0.001	0.011
LH (ng/mL)	3.45 ± 0.37	5.96 ± 0.41	4.31 ± 0.36	0.002	0.016
E2 (pg/mL)	51.6 ± 4.8	31.4 ± 3.6	43.2 ± 4.2	0.004	0.023
AMH (ng/mL)	3.94 ± 0.31	2.01 ± 0.25	3.12 ± 0.28	0.003	0.018
Keap1 (fold vs. AG)	1.00 ± 0.00	1.72 ± 0.15	1.18 ± 0.13	<0.001	0.002
Nrf2 (fold vs. AG)	1.00 ± 0.00	0.54 ± 0.07	0.91 ± 0.08	<0.001	0.005
HO-1 (fold vs. AG)	1.00 ± 0.00	0.49 ± 0.06	0.88 ± 0.07	<0.001	0.003
GCLC (fold vs. AG)	1.00 ± 0.00	0.52 ± 0.05	0.93 ± 0.06	<0.001	0.004

Note: Values are presented as mean ± standard deviation. Fold changes for protein expression are relative to the AG group (set as 1.00). $P < 0.05$ considered statistically significant.

Abbreviations: SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; AMH, anti-Müllerian hormone; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2–related factor 2; HO-1, heme oxygenase-1; GCLC, glutamate-cysteine ligase catalytic subunit.

DISCUSSION

Diminished ovarian reserve (DOR) is a pathological condition characterized by reduced follicle number and impaired oocyte quality, leading to hormonal imbalance, menstrual irregularities, and subfertility [12, 13]. A major contributor to its pathogenesis is oxidative stress (OS), in which excessive reactive oxygen species (ROS) promote granulosa cell apoptosis and accelerate follicular atresia [14]. In patients undergoing chemotherapy and radiotherapy for malignant diseases, ovarian injury is a frequent and serious complication, often resulting in premature ovarian failure and infertility [15].

In this study, we observed that warming needle moxibustion improved estrous cycle regularity, restored antioxidant enzyme activities, normalized serum hormone levels, and enhanced expression of antioxidant proteins in the Keap1-Nrf2-ARE pathway. These findings support the hypothesis that warming needle moxibustion exerts ovarian-protective effects through modulation of cellular redox homeostasis. Similar mechanisms have been suggested in related acupuncture and traditional Chinese medicine interventions, where activation of Nrf2 and downstream antioxidant enzymes was associated with improved ovarian function [16].

From a translational perspective, the relevance for cancer patients is noteworthy. Chemotherapy and radiation are widely recognized for their gonadotoxicity, leading to diminished ovarian reserve and compromised fertility. By enhancing intrinsic antioxidant defenses, warming needle moxibustion may offer a complementary, non-pharmacological approach to preserving ovarian function in women undergoing oncological treatment. While pharmacological antioxidants have been explored, their use can interfere with cancer therapy efficacy, whereas acupuncture-based approaches may avoid this limitation [17].

Our findings are consistent with reports that acupuncture can regulate the hypothalamic-pituitary-ovarian (HPO) axis, improve microcirculation, and reduce OS-related cellular injury [18]. In particular, stimulation of Guanyuan (CV4) and Sanyinjiao (SP6) has been linked with modulation of reproductive endocrine function and follicular development, aligning with both traditional meridian theory and modern biological evidence [19].

This study has several limitations. First, the

[DOI: 10.61186/ijrr.24.2.20]

experiments were conducted in rats, and results may not directly extrapolate to human physiology. Second, the radiation protocol was standardized to a single pelvic dose, which does not capture the variability seen in clinical oncology. Third, although the Keap1-Nrf2-ARE pathway was confirmed as a central mechanism, other potential pathways such as mitochondrial regulation or inflammatory signaling were not assessed. Lastly, long-term fertility outcomes were not evaluated, and future studies should include reproductive endpoints such as oocyte quality, fertilization rates, and offspring health.

In conclusion, our results provide preclinical evidence that warming needle moxibustion attenuates oxidative and radiation-induced ovarian injury in DOR through activation of the Keap1-Nrf2-ARE pathway. These findings highlight its potential role as a fertility-preserving adjunct for women at risk of ovarian damage due to chemo-radiotherapy. Further clinical studies are warranted to confirm safety, efficacy, and optimal treatment protocols.

CONCLUSION

Warming needle moxibustion attenuated oxidative stress and radiation-induced ovarian injury in diminished ovarian reserve rats by activating the Keap1-Nrf2-ARE pathway. The treatment restored antioxidant activity, improved hormonal balance, and enhanced ovarian function. These findings suggest a potential role for warming needle moxibustion as a fertility-preserving, non-pharmacological adjunct in patients exposed to chemotherapy and radiotherapy. Further clinical studies are needed to confirm its translational value.

***Acnowledgments*:** The authors thank the staff of the Animal Experimental Center of Beidahuang Group General Hospital for their technical assistance and animal care.

***Conflict of Interest*:** The authors declare no conflict of interest.

***Funding*:** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

***Ethical considerations*:** All experimental protocols were approved by the Animal Ethics Committee of Beidahuang Group General Hospital (Approval No. BGH-AEC-2024-016, approved March 2024). All procedures followed institutional and international guidelines for the care and use of laboratory animals.

***Authors' contributions*:** H.M. designed and performed the experiments. C.W. supervised the study and contributed to data interpretation. Both authors contributed to drafting and revising the manuscript and approved the final version.

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[DOI: 10.61186/ijrr.24.2.20]