Original Article

Hydrogen-rich Water Exerting a Protective Effect on Ovarian Reserve Function in a Mouse Model of Immune Premature Ovarian Failure Induced by Zona Pellucida 3

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Abstract

Background: Premature ovarian failure (POF) is a disease that affects female fertility but has few effective treatments. Ovarian reserve function plays an important role in female fertility. Recent studies have reported that hydrogen can protect male fertility. Therefore, we explored the potential protective effect of hydrogen-rich water on ovarian reserve function through a mouse immune POF model.

Methods: To set up immune POF model, fifty female BALB/c mice were randomly divided into four groups: Control (mice consumed normal water, $n = 10$), hydrogen (mice consumed hydrogen-rich water, $n = 10$), model (mice were immunized with zona pellucida glycoprotein 3 [ZP3] and consumed normal water, $n = 15$), and model-hydrogen (mice were immunized with ZP3 and consumed hydrogen-rich water, $n = 15$) groups. After 5 weeks, mice were sacrificed. Serum anti-Müllerian hormone (AMH) levels, granulosa cell (GC) apoptotic index (AI), B-cell leukemia/lymphoma 2 (Bcl-2), and BCL2-associated X protein (Bax) expression were examined. Analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software.

Results: Immune POF model, model group exhibited markedly reduced serum AMH levels compared with those of the control group (5.41 ± 0.91 ng/ml vs. 16.23 ± 1.97 ng/ml, $P = 0.033$) and the hydrogen group (19.65 ± 7.82 ng/ml, $P = 0.006$). The model-hydrogen group displayed significantly higher AMH concentrations compared with that of the model group (15.03 ± 2.75 ng/ml vs. 5.41 ± 0.91 ng/ml, $P = 0.021$). The GC AI was significantly higher in the model group (21.30 ± 1.74%) than those in the control (7.06 ± 0.27%), hydrogen (5.17 ± 0.41%), and model-hydrogen groups (11.24 ± 0.58%) (all $P < 0.001$). The GC AI was significantly higher in the model-hydrogen group compared with that of the hydrogen group (11.24 ± 0.58% vs. 5.17 ± 0.41%, $P = 0.021$). Compared with those of the model group, ovarian tissue Bcl-2 levels increased (2.18 ± 0.30 vs. 3.01 ± 0.33, $P = 0.045$) and the Bax/Bcl-2 ratio decreased in the model-hydrogen group.

Conclusions: Hydrogen-rich water may improve serum AMH levels and reduce ovarian GC apoptosis in a mouse immune POF model induced by ZP3.

Key words: Anti-Müllerian Hormone; Apoptotic Index; Granulosa Cells; Hydrogen-rich Water; Immune Premature Ovarian Failure; Ovarian Reserve Function

Introduction

In humans, premature ovarian failure (POF) is defined as a primary ovarian defect or arrested folliculogenesis/premature depletion of ovarian follicles before the age of 40 years. POF morbidity is approximately 0.01% by the age of 20 years and increases to 0.1% by the age of 30 years and 1% by the age of 40 years.[1] While the exact etiology of POF remains unclear, autoimmunity factors are responsible for approximately 4–30% of POF cases.[2] The zona pellucida (ZP) glycoprotein matrix consists primarily of three glycoproteins, ZP1, ZP2, and ZP3 that dictate the strong antigenic potency. Immunization with highly purified ZP protein can induce ovarian failure.[3,4] These glycoproteins are typically used

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to induce POF in an experimental mammalian model.[3,5,6] Therefore, we intended to establish an immune POF model using ZP3 to explore whether hydrogen-rich water has a protective effect on ovarian reserve function.

Anti-Müllerian hormone (AMH) is produced by granulosa cells (GCs) of preantral and small antral follicles as soon as follicles are recruited from birth up to menopause.[7,8] AMH levels are considered to be a reliable, sensitive, and accurate biomarker of ovarian reserve and function.[9,10] They are stable across the menstrual cycle and have an earlier predictive effect than other ovarian hormones.[11] They are usually used to predict diminished ovarian reserve in women with POF, hypogonadism, ovarian dysfunction, sub-clinical ovarian damage, and infertility.[10,12-16] Similar to previous studies,[17] we evaluated serum AMH levels to predict ovarian reserve function in this study.

Decreased ovarian reserve, such as during POF, may be due to ovarian cell apoptosis.[18] The oocyte-granulosa units are closely associated with ovarian reserve function. Exhaustion of oocytes or GCs can diminish ovarian reserve.[19] Ovarian GCs play a key role in maintaining ovarian physiological function, and the apoptosis of GCs can induce follicle loss before ovulation.[20] One of the primary mechanisms involved in ovarian apoptosis is mitochondrial apoptotic signaling cascades, which are mediated by mitochondria and B-cell leukemia/lymphoma 2 (Bcl-2) family members.[21] Hydrogen-rich saline exerts an anti-apoptotic effect by upregulating Bcl-2 levels and downregulating the BCL2-associated X protein (Bax) to Bcl-2 ratio.[22,23]

Hydrogen is the smallest molecule in the world. Hydrogen enters the digestive tract and diffuses into the blood rapidly. Hydrogen can penetrate biomembranes and diffuse into the cytosol, mitochondria, nuclei, and the blood-brain barrier. Thus, the tissue is able to absorb hydrogen extremely rapidly.[24] Hydrogen has not been reported to be toxic at high doses, because excess hydrogen is expired via the lungs into the air.[25] Since the first research was published in 2007, studies investigating the protective effect of hydrogen on various organs are gaining an increasing attention.[26] Recently, several studies have demonstrated beneficial effects against ischemia/reperfusion injury of several organs, such as liver, kidney, and skin.[22,23,27] Nakata *et al*.[28] revealed that hydrogen treatment stimulates low sperm motility in humans. He proposed hydrogen as a promising agent for male infertility treatment. However, there is still a lack of data regarding the beneficial effects of hydrogen on female infertility treatment.

To date, no effective treatment for POF has been developed. Therefore, we hypothesized that hydrogen-rich water may affect female infertility. We designed the present study to explore whether hydrogen-rich water can protect ovarian reserve function in a mouse model of immune POF.

Methods

Animals

Adult female BALB/c mice (weight: 15–18 g; age: 56–70 days) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China) and housed in the Animal Center of Capital Medical University (Beijing, China). All procedures and humane care were in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Mice were housed with standard cycles (12 h light; 12 h dark) under specific pathogen-free conditions with *ad libitum* access to food. Fifty mice with normal regular estrous cyclicity were included in the experiment. All animal experiments were performed at the Animal Laboratory of Capital Medical University and supervised by an experienced investigator. All protocols were in accordance with the approval of the Institutional Ethics Committee at the Capital Medical University.

Sample groups

Fifty female mice were randomly divided into four groups (using a random number table) and received subcutaneous injections into the abdomen and two hind footpads as indicated. In the control (n = 10) and hydrogen (n = 10) groups, each mouse received saline injections (0.15 ml). In the model (n = 15) and model-hydrogen (n = 15) groups, each mouse received ZP3 injections (75 μg, 0.15 ml). Control and model groups' mice consumed normal water until sacrifice, whereas hydrogen and model-hydrogen groups' mice consumed hydrogen-rich water until sacrifice. The immune-strengthening reagent was injected 2 and 4 weeks after the 1st day of immune reagent injection. For estrous cycle analysis, vaginal smears were obtained daily at 9:00 a.m. After 5 weeks, all mice were sacrificed.

Reagents and administration

The amino acid sequence of the mouse ZP3 330-342 peptides was NSSSSQFQIHGPR, purity ≥ 90% (Chinese Peptide Company, Hangzhou, China). ZP3 was solubilized in sterile double-distilled water at 1 mg/ml and then mixed with Freund complete adjuvant or Freund incomplete adjuvant (Sigma Chemical Co., St. Louis, MO, USA) in a proportion of 1:1 immediately before use as an immune reagent or immune-strengthening reagent. Two injections were administered at an interval of 14 days, three times in total. Each mouse received 0.15 ml by subcutaneous injection.[5,29]

Hydrogen-rich water production and management

A hydrogen generator was used to make high-purity hydrogen (Beijing Zhongke Huiheng Technology Development Co., Ltd., Beijing, China). Hydrogen was dissolved in 200 ml water for 20 min at a speed of 0.23 L/min to a supersaturated level. The concentration was evaluated by a needle-type H2 sensor (Unisense Co., Aarhus, Denmark) to ensure a concentration >0.8 mmol/L. Hydrogen-rich water was freshly prepared every time before use. Mice were administered water for every 4.5 h, four times a day. After 4.5 h without water, mice would immediately drink the water after feeding and finished drinking within 10 min.

The water was then removed. Hydrogen concentrations were re-analyzed and confirmed to be >0.8 mmol/L. In addition, water consumption was measured. On average, each mouse drank approximately 0.9 ml water every time they were fed.

Sample collection

Five weeks after the first injection, all mice were anesthetized by intraperitoneal injection of 3–4 μl/g 10% chloral hydrate. Blood samples were extracted from the retro-orbital plexus and centrifuged at 1000 r/min for 15 min to obtain serum samples. After mice were immobilized on a standard mouse surgery board, a ventral midline incision was made to expose the reproductive organs, and the ovaries were removed. For each mouse, one ovary was frozen and the other was fixed in 10% neutral-buffered formaldehyde solution. The fixed specimens were embedded in paraffin blocks sectioned at 5 μm thickness.

Serum anti-Müllerian hormone concentrations

Serum AMH levels were measured with the mouse AMH ELISA Kit (Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's instructions. The detection range of this assay was 0.375–150 ng/ml, and the minimum detectable dose of mouse AMH was typically <0.375 ng/ml. The optical density was determined using a Victor3™ 1420 multilabel counter (PerkinElmer Inc., Waltham, MA, USA) set to 450 nm.

Analysis of apoptosis

The *In Situ* Cell Death Detection POD kit (Hoffman-La Roche Ltd., Basel, Switzerland) was used to measure apoptosis according to the manufacturer's instructions. Terminal deoxynucleotidyl transferase dUTP nick end labeling-positive GCs were determined by counting the GCs with dark brown nuclear or apoptotic bodies. For each ovary, we randomly chose five inconsecutive slides for staining, and the antral follicles were evaluated. Five hundred GCs from five random areas (500 × 5 fields) were counted for each ovary (one random area per slide), and the average ratio was described as the apoptotic index (AI). This analysis was performed at ×400 original magnification by light microscopy (BX-71, Olympus Co., Tokyo, Japan) equipped with computer-controlled digital camera and imaging software DP2-BSW (Olympus Co.).[22]

Immunohistochemistry study

Paraffin sections were randomly selected from one ovary from each mouse for examination. Paraffin sections were heated for 30 min at 70°C, deparaffinized with xylene for 10 min twice, and rehydrated through a graded series of ethyl alcohol (70–100%) for 5 min each. After washing with distilled water for 1 min, the sections were incubated with 3% H_2O_2 to block nonspecific antibody binding for 10 min in dark and then washed with distilled water again for 2 min. For antigen retrieval, the sections were heated under high temperature with citrate buffer (pH 6.0, Sigma Chemical Co.). After cooled to room temperature, the tissues were incubated in goat serum for 30 min at 37°C, followed by incubation with rabbit polyclonal Bcl-2 (1:100 dilution; product No. ab59348; Abcam, Inc., Cambridge, UK) and monoclonal Bax (1:50 dilution; product No. 14796S; Cell Signaling Technology, Boston, MA, USA) primary antibodies for 2 h at 37°C. After three washes for 5 min with PBS, the tissues were incubated with secondary antibody (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) for 40 min and treated with diaminobenzidine (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.) for 10 s. The sections were then stained with hematoxylin and covered. After three washes with distilled water for 1 min, the tissues were stained with hematoxylin for 2 min, differentiated with 1% hydrochloric acid alcohol for 10 s, placed in tap water for 5 min twice, dehydrated through a graded series of ethyl alcohol (70–100%) for 5 min each, and transparentized with xylene for 20 min. Sections were sealed with neutral resin. The tissues were examined at ×400 original magnification by light microscopy (BX-71, Olympus Co.) equipped with computer-controlled digital camera and imaging software DP2-BSW (Olympus Co.).[22] Immunostaining was categorized as follows: Absence of staining (−), weak staining (+), moderate staining (++), or strong staining (+++).[30]

Western blotting analysis

Frozen ovaries were homogenized with the Cell Lysis Kit (Bio-Rad Laboratories, Hercules, CA, USA) in ice-cold lysis buffer with protease inhibitor, phosphatase inhibitor, and PMSF. Samples were centrifuged at 14,000 r/min at 4°C for 15 min, and the supernatant was removed. Equal amounts of supernatant protein (30 μg) were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes for immunoblotting. The membrane was blocked by blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1.5 h and incubated with anti-Bcl-2 (1:200 dilution; product No. ab59348; Abcam, Inc.), anti-Bax (1:200 dilution; product No. 14796S; Cell Signaling Technology), or mouse monoclonal anti-β-actin (1:1500 dilution; product No. TA-09; Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd.) primary antibodies for 14 h at 4°C. After three washes with PBS containing 0.05% Tween-20, the membranes were incubated with secondary antibodies (1:10000 dilution; product no. C00830-01; LI-COR Biosciences) in dark for 50 min at room temperature and then detected with a double color infrared laser imaging system (Odyssey, LI-COR Bioscience).[22] The results are normalized to β-actin, and the normalized ratio was used to generate data graphs.

Statistical analysis

If univariate quantitative data of the groups met the normal distribution and homogeneity of variance criterion, the one-way analysis of variance (ANOVA) would be used between the four groups, and Fisher's least significant difference method would be used for multiple comparisons. All statistical tests were performed using a two-sided test. $P \leq 0.05$ was considered to be statistically significant. Analyses were performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean ± standard error (SE).

Results

Estrous cyclicity

Most of the control and hydrogen groups' mice exhibited normal estrous cycles, with ordered proestrus, estrus, metestrus, and diestrous phases. All model groups' mice were entirely acyclic. We observed many leukocytes in the model groups' vaginal smear, which was consistent with the diestrous phase. Most model-hydrogen groups' mice were not entirely acyclic. There were still some cornified squamous epithelial cells in the model-hydrogen groups' vaginal smear. Although their estrous cycles were not completely regular, there was a tendency toward the estrus phase.

Serum anti-Müllerian hormone concentrations

The mean serum AMH concentrations were 16.23 ± 1.97 ng/ml, 19.65 ± 7.82 ng/ml, 5.41 ± 0.91 ng/ml, and 15.03 ± 2.75 ng/ml in the control, hydrogen, model, and model-hydrogen groups, respectively [Figure 1]. The mean serum AMH concentration in the model group was significantly lower than those in the control group ($P = 0.033$) and hydrogen group ($P = 0.006$). Notably, the model-hydrogen group displayed significantly higher AMH concentrations compared with those in the model group ($P = 0.021$). No significant differences were observed between the control and the hydrogen or model-hydrogen groups.

Granulosa cell apoptotic indexes

The GC AIs are shown in Figure 2, and the morphology analysis revealing apoptotic GCs is shown in Figure 3. The mean GC AIs were 7.06 ± 0.27%, 5.17 ± 0.41%, 21.30 ± 1.74%, and 11.24 ± 0.58% in the control, hydrogen, model, and model-hydrogen groups, respectively. The GC AI was significantly higher in the model group compared with those in the control, hydrogen, and model-hydrogen groups (all $P < 0.001$), respectively. The GC AI was significantly higher in the model-hydrogen group compared with that of the hydrogen group ($P = 0.021$). No significant difference was observed between the control and model-hydrogen groups.

B-cell leukemia/lymphoma 2 and BCL2-associated X protein expression

All the four groups displayed similar Bax immunostaining (+). Bax staining intensity in the control group was slightly stronger than those of the other three groups, among which no significant differences were observed. Bcl-2 immunostaining was moderate (++) in the control, hydrogen, and model-hydrogen groups and weak (+) in the model group [Figure 4].

We next evaluated and quantified Bax and Bcl-2 expression in the four groups by Western blotting analysis [Figure 5]. The mean Bax levels were 0.51 ± 0.04, 0.44 ± 0.06, 0.44 ± 0.04, and 0.44 ± 0.04 in the control, hydrogen, model, and model-hydrogen groups, respectively [Figure 5a]. Bax expression was slightly higher in the control group compared

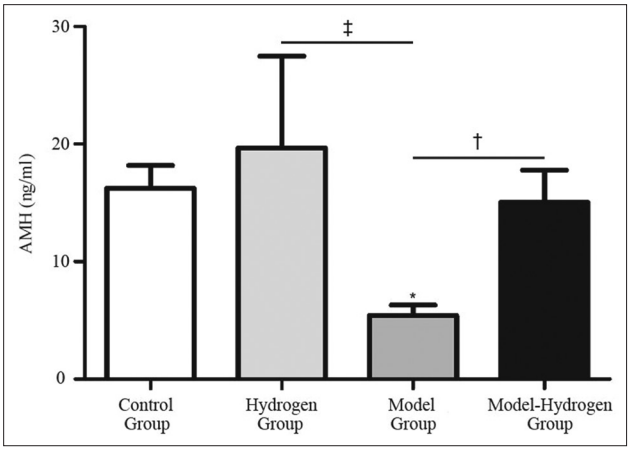

Figure 1: Effects of hydrogen-rich water on serum anti-Müllerian hormone (AMH) concentration. Data are presented as mean ± standard error. *$P < 0.05$ when compared with control; †$P < 0.05$; ‡$P < 0.01$.

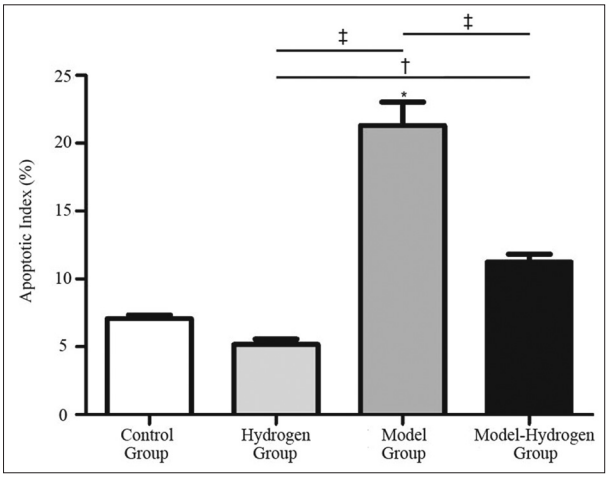

Figure 2: Effects of hydrogen-rich water on granulosa cell apoptotic index. Apoptosis was evaluated by TUNEL assay. Data represent the mean ± standard error. *$P < 0.001$ when compared with control; †$P < 0.05$; ‡$P < 0.001$. TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

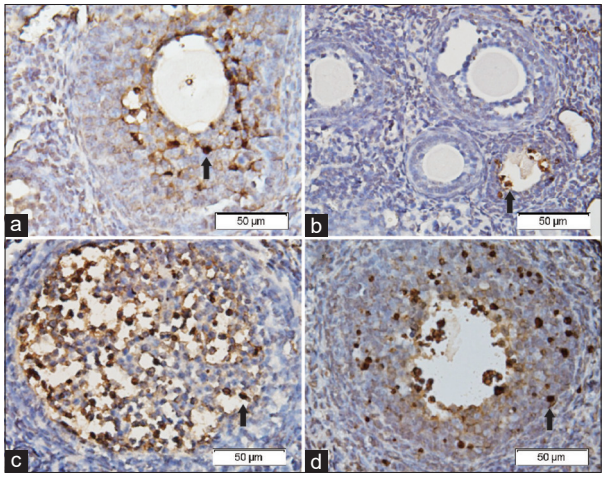

Figure 3: Morphology analysis of ovarian apoptotic granulosa cells. The TUNEL-positive granulosa cells in the (a) control, (b) hydrogen, (c) model, and (d) model–hydrogen groups. The dark brown dots (black arrowhead) indicate apoptotic granulosa cells. Bar = 50 μm. TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

with those of the other three groups. However, Bax levels were comparable in all the four groups.

The mean Bcl-2 levels were 2.61 ± 0.17, 2.71 ± 0.26, 2.18 ± 0.30, and 3.01 ± 0.33 in the control, hydrogen, model, and model-hydrogen groups, respectively [Figure 5b]. Model-hydrogen groups' mice exhibited significantly elevated Bcl-2 levels in ovarian tissue compared with that of the model group ($P = 0.045$).

The mean Bax/Bcl-2 ratios were 0.20 ± 0.02, 0.16 ± 0.01, 0.24 ± 0.06, and 0.17 ± 0.02 in the control, hydrogen, model, and model-hydrogen groups, respectively [Figure 5c]. The Bax/Bcl-2 ratio was higher in the model group compared with that of the model-hydrogen group, though the differences were not significant.

Discussion

Immune POF is a serious disease and currently lacks an effective treatment. As POF is both common and pervasive, there is an urgent need to develop new treatments. This is the first study to demonstrate the protective effect of hydrogen-rich water on ovarian reserve function in a ZP3-induced mouse immune POF model.

AMH is an indicator of ovarian reserve function. In our experimental study, we determined that serum AMH levels were markedly decreased in ZP3-treated mice compared with those of control mice. Following ZP3-induced immune ovarian injury, ovarian reserve function decreased notably. ZP3 immunization led to a marked increase in apoptotic GCs. GC apoptosis leads to a reduction in the available oocyte-GC units and results in ovarian function exhaustion and POF.[19] Thus, the increased number of apoptotic GCs accompanied by decreased AMH levels indicated compromised ovarian function and POF. These findings reflected the successful establishment of a mouse immune POF model.

The primary finding of this study was that following ZP3-induced immune ovarian injury, the use of hydrogen-rich water increased serum AMH levels dramatically and significantly compared with those of model mice alone. Furthermore, no significant difference was observed compared with mice without ovarian injury. Based on these results, we concluded that hydrogen-rich water improved ovarian reserve function in immune POF mice. Under the protection of hydrogen-rich water, ovarian reserve function in immune POF mice was comparable to ovarian function in healthy mice.

The protective effect on ovarian reserve function observed above is achieved by reducing GC apoptosis. Thus, preventing GC apoptosis is important to maintain ovarian function. In this study, under conditions of ovarian injury following ZP3 immunization, hydrogen-rich water significantly decreased GC AIs, restoring levels to those of mice without ovarian injury. This result indicated that hydrogen-rich water might offer protection from immune POF by inhibiting GC apoptosis.

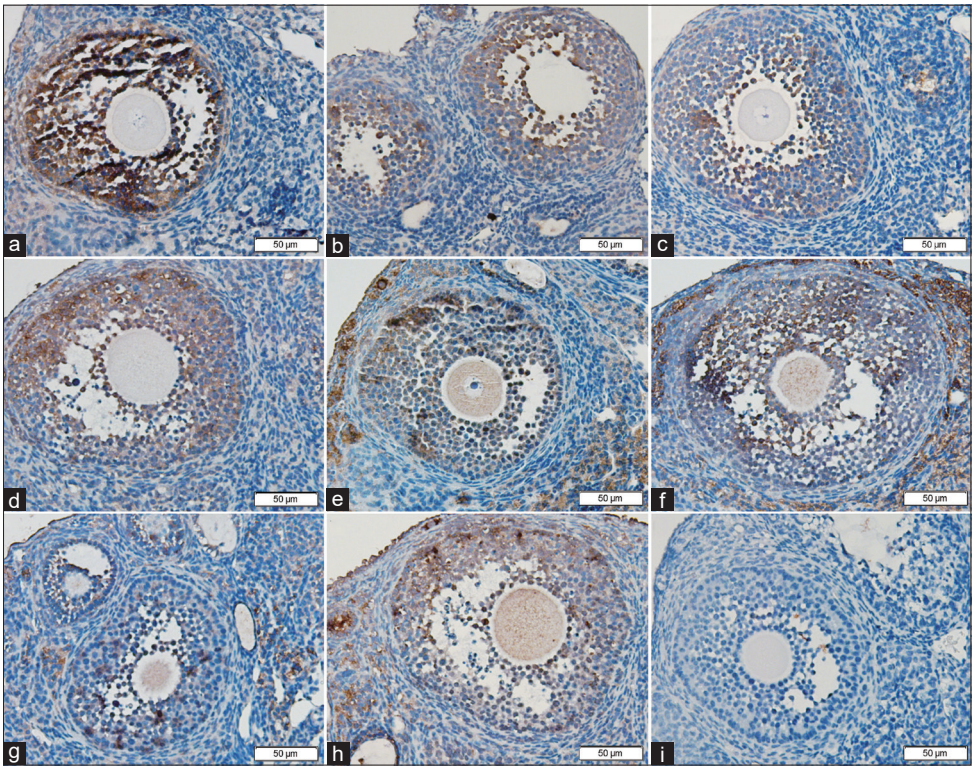

Figure 4: Immunohistochemical staining for Bax in the (a) control, (b) hydrogen, (c) model, and (d) model-hydrogen groups. Immunohistochemical staining for Bcl-2 in the (e) control, (f) hydrogen, (g) model, and (h) model-hydrogen groups. (i) Negative control. Brown staining represents Bax and Bcl-2 protein expression. Bar = 50 μm. Bcl-2: B-cell leukemia/lymphoma 2; Bax: BCL2-associated X protein.

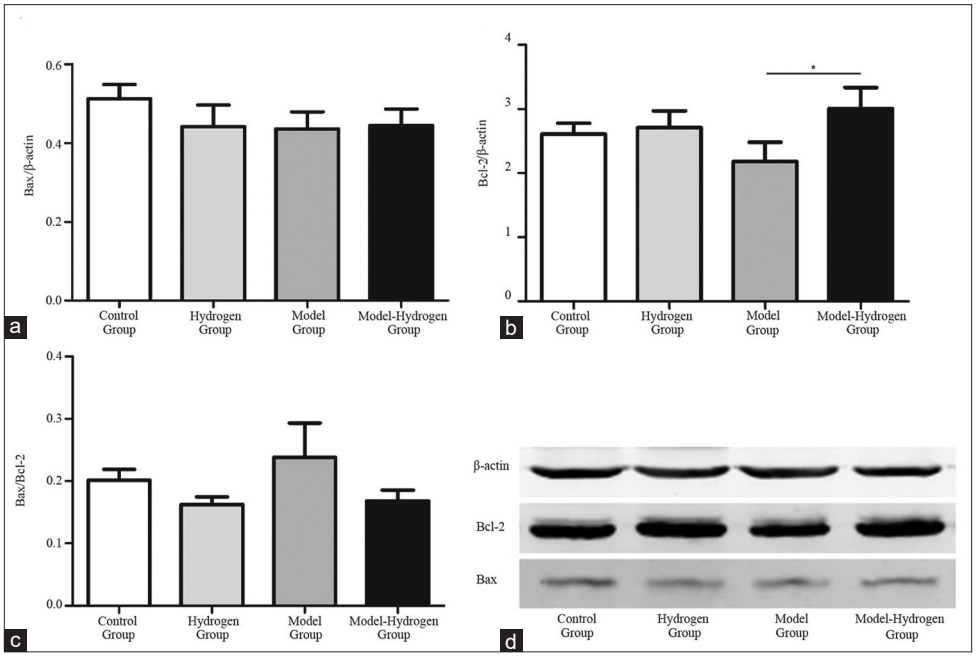

Figure 5: Bax and Bcl-2 expression determined by Western blotting analysis. Data are presented as mean ± standard error. (a) Bax levels as shown in panel D. (b) Bcl-2 levels as shown in panel D. *$P < 0.05$. (c) The Bax/Bcl-2 ratio. (d) The immunoblots. Bcl-2: B-cell leukemia/lymphoma 2; Bax: BCL2-associated X protein.

In addition, we explored the impact of Bcl-2 family members from the mitochondrial apoptotic signaling pathway on GC apoptosis. The Bcl-2 family can react to cell surface or internal cell death signals and affect the formation of the apoptosome and the activation of the caspase cascade.[31] Overexpression of the pro-survival molecule Bcl-2 inhibits apoptosis, while overexpression of the pro-apoptotic protein Bax promotes apoptosis.[32] With regard to the female reproductive system, Bax and Bcl-2 act as a "rheostat". During follicular maturation and atresia, the Bax/Bcl-2 "rheostat" plays an important role in regulating apoptosis, and the Bcl-2/Bax ratio can determine the fate of ovarian GCs.[33,34] It was reported that hydrogen-rich saline can operate this "rheostat" in the skin by upregulating Bcl-2 expression but does not affect Bax expression.[22] Thus, we speculated that hydrogen-rich water may regulate ovarian GC apoptosis through a similar pathway. In our study, we observed that hydrogen-rich water exhibited anti-apoptotic effects by directly inducing Bcl-2 expression and indirectly downregulating the Bax/Bcl-2 ratio. The immunohistochemistry and Western blotting results indicated that following ZP3-induced immune ovarian injury, hydrogen-rich water consumption can significantly increase Bcl-2 levels with almost no influence on Bax. This result was consistent with the research reported by Liu *et al*.[22] In addition, immune ovarian injury resulted in an increase in the Bax/Bcl-2 ratio, while hydrogen-rich water consumption restored this ratio to levels comparable to the control group. The small differences may be a result of the limited ovarian size and small sample numbers. These results indicated that hydrogen-rich water can protect against apoptosis by regulating the "rheostat" in ovaries and, specifically, upregulating Bcl-2 expression and then indirectly downregulating the Bax/Bcl-2 ratio.

This study had limitations yet. First, we did not explore whether the hydrogen-rich water reversed the POF phenotype or blocked the POF induction by ZP3. Second, the results presented here were obtained from an animal model. However, the current findings are preliminary. We are designing studies to explore the specific mechanism underlying the observed protective effect. In addition, the schedule, dose, and effect of hydrogen-rich water should be investigated in human ovaries in the future.

In conclusion, hydrogen-rich water may have a protective effect on ovarian reserve function in a mouse model of immune POF induced by ZP3 by improving serum AMH levels and reducing ovarian GC apoptosis. These results revealed a promising protective effect of hydrogen-rich water on female infertility.

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Conflicts of interest

There are no conflicts of interest.

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