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Icariin exerts a protective effect against D-galactose induced premature ovarian failure via promoting DNA damage repair

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ABSTRACT

Icariin is one of the most common active ingredients in traditional Chinese medicine, while its function against Premature ovarian failure (POF) has not been explored. POF animal model was induced by D-galactose, and icariin at different doses was administered. Ovarian structure and follicle counting were observed via hematoxylin and eosin staining. The levels of serum hormones were measured by ELISA. Primary ovarian granulosa cells were cultured to compare the protective effects of icariin on cell aging, and DNA damage markers including γH2AX and 53BP1 were assessed by Western Blot. Administration of icariin promoted ovary/body weight, follicles numbers and fertility outcomes. In addition, icariin downregulated the levels of follicle stimulating hormone and luteinizing hormone, and upregulated the levels of estradiol and anti-Müllerian hormone. Icariin protected ovarian granulosa cells from D-galactose induced aging, with increased cell viability and lower endogenous β-galactosidase activity. The alterations of expression level of γH2AX and 53BP1 by icariin indicated that the protection is via promoting DNA damage repair. In this study we tested the biological function of icariin against the D-galactose induced POF. Our results demonstrated that icariin effectively attenuated ovarian injury via promoting DNA damage repair, suggesting that icariin can be developed as a protective agent against POF.

1. Introduction

Premature ovarian failure (POF) is an ovarian disorder occurs in women, leading to amenorrhea, ovarian failure, or even infertility [1]. POF is featured by premature ovarian follicles depletion in approximately 1–3 % of women younger than 40 years old [2], and diagnosed by high levels of gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), and low levels of gonadal hormones including estrogens and inhibins [3]. The exact etiology of POF is unknown in the majority of women who have been diagnosed due to its multifactorial mechanisms which can be categorized into genetic and environmental factors [4]. In analyzing the genetic mutations related to POF, the abnormalities of the X chromosome and autosomes are highly associated with this disease [5–7]. In addition, autoimmunity and toxins have been reported as other important causes of the disease [8]. In terms of the treatment, the hormone replacement therapy (HRT) is one of the recommended therapeutic strategies for POF. Furthermore, estrogen replacement has been reported to decrease the risk of osteoporosis and cardiovascular disease for POF patients [9]. Stem cell therapy of POF has been gaining attention recent years as well. For instance, a mouse study using CD44+/CD105+ HuAFCs (human amniotic fluid cells) demonstrated that the cells were potential for stem cell transplantation of POF [10]. In spite of all these progresses, the treatment and management of POF remains challenging due to the limitation of current methods. As proof, researchers reported that the long-term use of hormone replacement therapy may increase the risk of breast and ovarian cancer, and thrombotic disorders [11]. The immunosuppressive therapy for POF such as the use of glucocorticoids is lack of convincing safety and efficacy evidences clinically [12]. Taken together, the further exploitation of effective treatments is particularly pivotal for the prevention and therapy of POF.

Because ovarian failure and menopause is highly associated with ovarian, tackling ageing and its sequential reactions would be an effective approach to overcome POF [13]. Icariin was isolated from the Chinese medicinal herb *Epimedium brevicornum*, which is one of the most common active ingredients in traditional Chinese medicine. Previous studies showed that icariin exerts a wide range of biological and pharmacological properties, including anti-inflammation, anti-depression, and anti-aging effects [14]. Icariin could improve the expression of SIRT6 histone deacetylase and reduce the expression of NF-κB

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protein and the inflammatory response of aged mice [15]. In the present study, we aimed to explore the protective role of icariin on POF using D-galactose induced POF mouse model. Since there is limited knowledge about the exact role of icariin on POF, the potential mechanisms will also be evaluated.

2. Materials and methods

2.1. Animals and experimental design

C57BL/6 female mice were housed in a pathogen-free animal facility under 12-h dark/light cycle, with food and water ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College. After one week's acclimatization, mice were randomly divided into five groups: Control group, mice were injected saline subcutaneously for 42 consecutive days; D-galactose group, mice were injected D-galactose (Sigma-Aldrich, Sunnyvale, CA, USA) at 200 mg/kg/day subcutaneously for 42 consecutive days; Three D-galactose + icariin groups, mice were injected with icariin at 10, 50 and 100 mg/kg via intraperitoneal injection daily following D-galactose injection at 200 mg/kg/day. The treated mice were mated with healthy C57BL/6 J male mice, and monitored daily for recording the number of pups for 6 consecutive months. In addition, mice were euthanized and ovaries were weighed to evaluate the ovary to body weight ratio.

2.2. Histological observation of ovary and follicle cells cocunting

For histological analysis, the ovaries were fixed in 4% paraformaldehyde overnight and embedded in paraffin. The ovarian tissues were cut into sections at 5-μm thickness, mounted on glass slides and stained with hematoxylin and eosin (H&E). Ovarian primordial, primary, secondary, antral and atretic follicles at every fifth section were counted. Briefly, only follicles with oocytes that contain clearly visible nuclei under a microscope were scored. The follicles were classified according to the number of granulosa cells layers surrounding the oocyte as described [16,17].

2.3. Serum hormones measurement by Enzyme-linked immunosorbent assays (ELISAs)

Blood samples were collected during diestrus period and stored at room temperature for 1 h, followed by a centrifugation at 1000 *g* for 15 min for serum harvesting. The bio-chemical parameters including serum estradiol (E2), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and anti-Müllerian hormone (AMH), were measured using commercially available ELISA kits: E2 (582251) from Cayman Chemicals (MI, USA), FSH (KA2330), LH (KA2332), AMH(NBP2-62674) from Novus Biologicals (CO, USA). The optical density value of each well was measured at 450 nm with a micro-plate reader (Molecular Device, Sunnyvale, CA, USA).

2.4. Primary ovarian granulosa cell culture and treatment

Immature C57BL/6 female mice (3 weeks) were injected with pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich, St. Louis, MO) subcutaneously at 50 IU. Forty-eight hours later, the mice were euthanized and ovaries were dissected immediately. Ovaries were rinsed with pre-chilled phosphate-buffered saline (PBS) twice and immersed in DMEM/F12 medium (Thermo Fisher Scientific, Waltham, MA USA). Granulosa cells of mice were isolated by needle puncture of ovarian follicles, and filtered with stainless steel mesh. The harvested cells were further centrifuged at 1000 × g for 5 min, and cultured in 96-well plates (1×10^5 cells/well) in DMEM/F12 medium supplemented with 15% fetal bovine serum, testosterone (1×10^{-7} M), 100 mg/mL streptomycin and 100 U/mL penicillin at 37 °C with 5% CO_2 in a cell incubator. Forty-eight hours later, the cells were treated with PBS, D-galactose, or D-galactose plus icariin at 100 nM, 1 μM, and 10 μM, and cultured for another 6 h.

2.5. Cell viability and endogenous β-galactosidase determination

The viabilities of treated primary ovarian granulosa cells were measured via the CCK-8 assay (Dojindo, Japan). Briefly, the medium in the 96-well plates were discarded, and replaced with 180 μL of DMEM/F12 medium. Twenty μL of CCK-8 reagent was added to the wells. After 3 h incubation at 37 °C, the optical density (OD) was measured at 450 nm using a microplate reader. The activity of endogenous β-galactosidase was assessed by Mammalian beta-Galactosidase Assay kit (Thermo-Fisher Scientific, Waltham, MA), and the OD was measured at 405 nm.

2.6. Western blot analysis

To investigate the molecular mechanism of action of icariin, primary ovarian granulosa cells in the presence of D-galactose and icariin were harvested and homogenized to release the protein. Equal amount (20 μg per lane) of total proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked by 5% non-fat milk in tris-buffered saline and Tween-20 (TBST) buffer for 2 h at room temperature, and incubated with primary antibodies against γH2AX and p53 binding protein 1 (53BP1) overnight at 4 °C. Membranes were washed five times with TBST, and were incubated with secondary antibodies for 1 h. The density of each band was measured by Amersham Imager 600 (GE Healthcare). The protein level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control in the assay.

2.7. Statistical analysis

Data was presented as mean ± SEM, and analyzed with Prism 6 software (GraphPad, CA, USA). All experiments were performed at least three times independently. One- and two-way ANOVA analysis was used for statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Icariin protected D-galactose induced premature ovarian failure in mice model

As shown in Fig. 1A, the histopathological examination of ovarian tissues revealed that the ovaries of control mice showed normal ovarian morphological characterizations including primordial and healthy-looking follicles with primary, secondary, and antral follicles. In contrary, the mice treated with D-galactose demonstrated significantly increased proportion of ovarian stroma, and massive atretic follicles. Notably, in the presence of icariin, the follicles structures were recovered obviously by different doses of icariin.

To observe the protective effect of icariin on ovary, we tracked the fertility outcomes via a breeding assay by mating D-galactose and icariin treated female mice with male mice of tested fertility for 6 months. As can be seen in Fig. 1B, continuous breeding assessment indicated that D-galactose treated mice had very low number of pups (< 10 pups), whereas co-administration of icariin showed significantly improved fertility in a dose-dependently manner. The pups in the D-galactose plus 100 mg/kg icariin group had nearly the same number of pups as saline control group (40 pups).

We also measured the size of the ovaries at different groups of mice from week 3 to week 8. As shown in Fig. 1C, the mean ovarian weight ratios of control mice were higher than those in the D-galactose group at

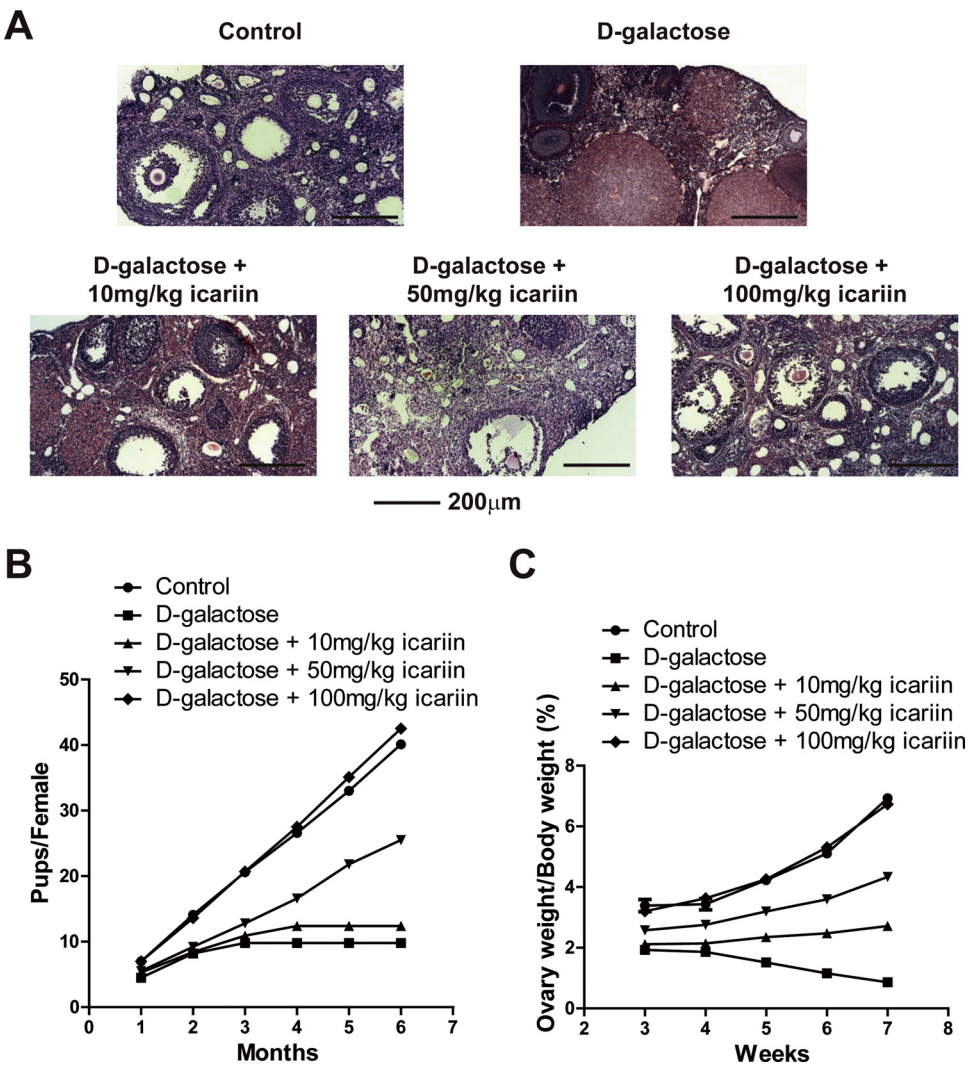

Fig. 1. Protective effects of icariin on the d-galactose induced premature ovarian failure mice model.

(A) Histopathological examination of ovarian tissues from different group as indicated. The mice in the D-galactose group were subcutaneously (s.c.) injected daily with the concentration of 200 mg/kg/day D-galactose for 42 days. The control group received an equal volume of saline (s.c. daily) for 42 days. The treatment group were injected with different dose of icariin via intraperitoneal injection following the injection of D-galactose injection for 42 days. N = 10 for each group. Photomicrographs (100×) showed hematoxylin and eosin stained ovaries and the scale bar was 200 μm.

(B) Comparison of the accumulative number of pups per female in each group as indicated 6 months. N = 10 for each group. Data were presented as the mean ± SEM.

(C) Ovary weight to body weight ratio of the mice in each group at 3, 4, 5, 6 and 7 weeks' birth. At the beginning of the experiment, there were 25 mice for each group, for each time point, three mice were sacrificed and were used for analysis. At the end of the whole experiment, there were 10 mice for each group. Data were presented as the mean ± SEM.

week 3, 4, 5, 6 and 7 respectively. Remarkably, co-administration of icariin significantly increased the ovary weight/body ratio across the entire monitoring window. Notably, the dose at 100 mg/kg icariin presented almost the same level as control group. Furthermore, we found icariin exerted commensurate protective results as estrogen, supported by the similar numbers of pups, and the ovary weight/body ratio (Fig. S1). All of these data revealed that D-galactose caused damage of ovaries in mice, which was significantly inversed by the administration of icariin, especially at 100 mg/kg dose.

3.2. Icariin protected follicular development in the D-galactose induced premature ovarian failure mice

Follicle numbers were counted after H&E staining (Fig. 2A–E). Quantitation of follicles showed that D-galactose treatment reduced the proportions of primordial, primary and secondary follicles when compared to those values in the control group ($P < 0.001$, Fig. 2A–C). Interestingly, co-administration of icariin at 50 mg/kg increased the numbers of follicles significantly ($P < 0.05$), while icariin at 100 mg/kg recovered the numbers of follicles to the same level of control mice.

3.3. Effects of icariin on the serum hormones in the D-galactose induced premature ovarian failure

The levels of the serum hormones in mice were detected by ELISAs. The mice group treated by D-galactose had elevated serum FSH and LH levels ($P < 0.05$ and $P < 0.01$) and lower E2 and AMH levels ($P < 0.01$) compared to those in the control group (Fig. 3A–D). Icariin treatment could significantly reverse the changes of serum hormones caused by D-galactose at the doses of 50 and 100 mg/kg.

3.4. Icariin protected cells from D-galactose induced aging

We further detected the effect of icariin on cell viability of cultured granulosa cells *in vitro*. In comparison with the control group, cell viability decreased by approximately 42% in the D-galactose group ($P < 0.001$). However, co-administration with icariin at various concentrations (100 nM, 1 μM and 10 μM) showed that icariin was able to recover the D-galactose -induced cell damage ($P < 0.001$, Fig. 4A), indicating that icariin possessed protective effects on cultured granulosa cells *in vitro*.

Further, we measured the activity of endogenous β-galactosidase, which was significantly increased by D-galactose treatment, and decreased by icariin at various concentrations in a dose-dependently manner (Fig. 4B). This result suggested that the *in vitro* protective effects of icariin on cultured granulosa cells were possibly relevant to cell aging.

3.5. Icariin protected cells from D-galactose induced aging via promoting damage repair

As shown in Fig. 5A and B, the levels of γH2AX and 53BP1, two

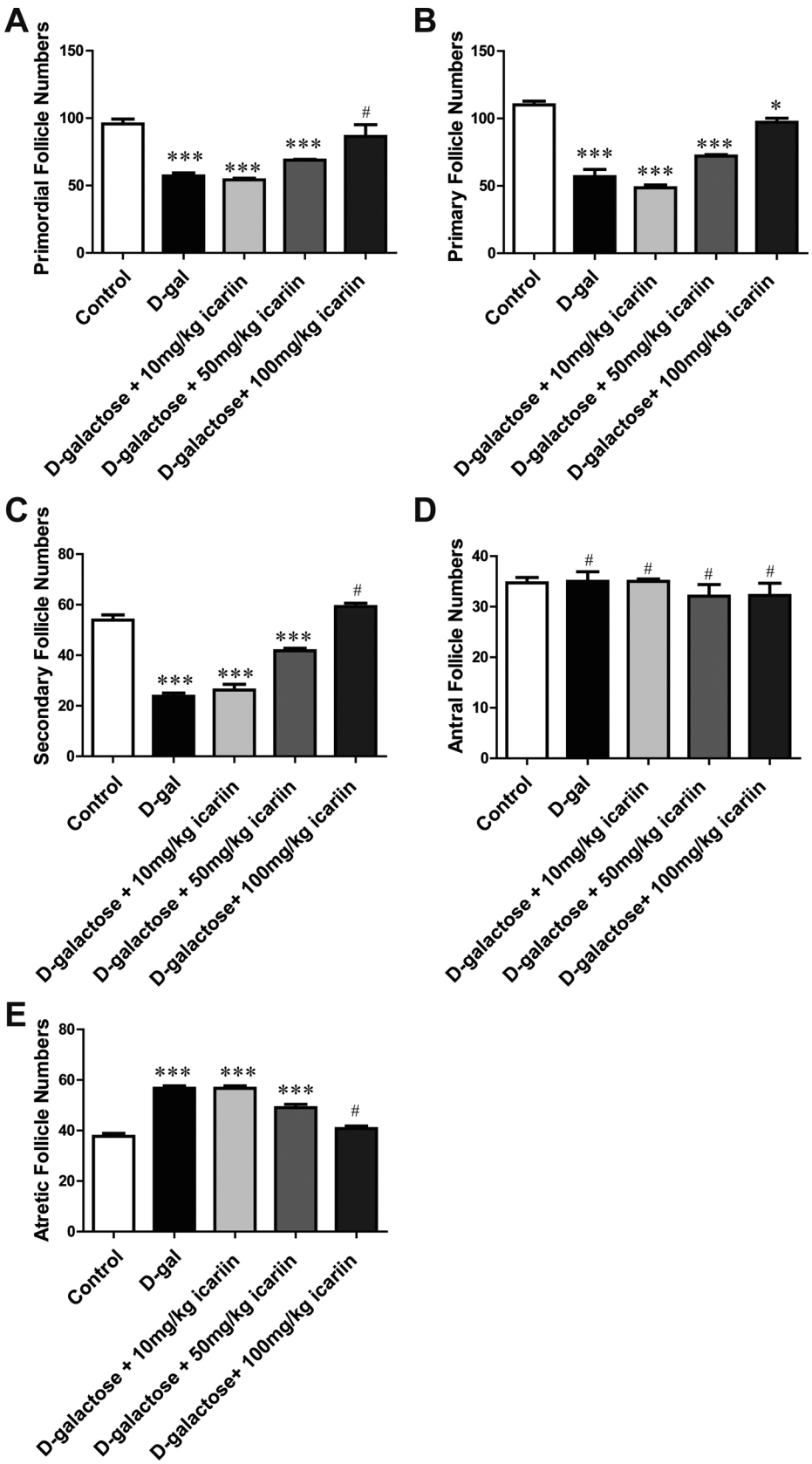

Fig. 2. Effect of icariin on follicular development in the d-galactose induced premature ovarian failure.
The numbers of Primordial Follicles (A), Primary Follicles (B), Secondary Follicle (C), Antral Follicle (D) and Atretic Follicles (E) were summarized. N = 10 for each group. Data were presented as the mean ± SEM. * indicated $p < 0.05$, *** indicated $P < 0.001$ and # indicated $P > 0.05$.

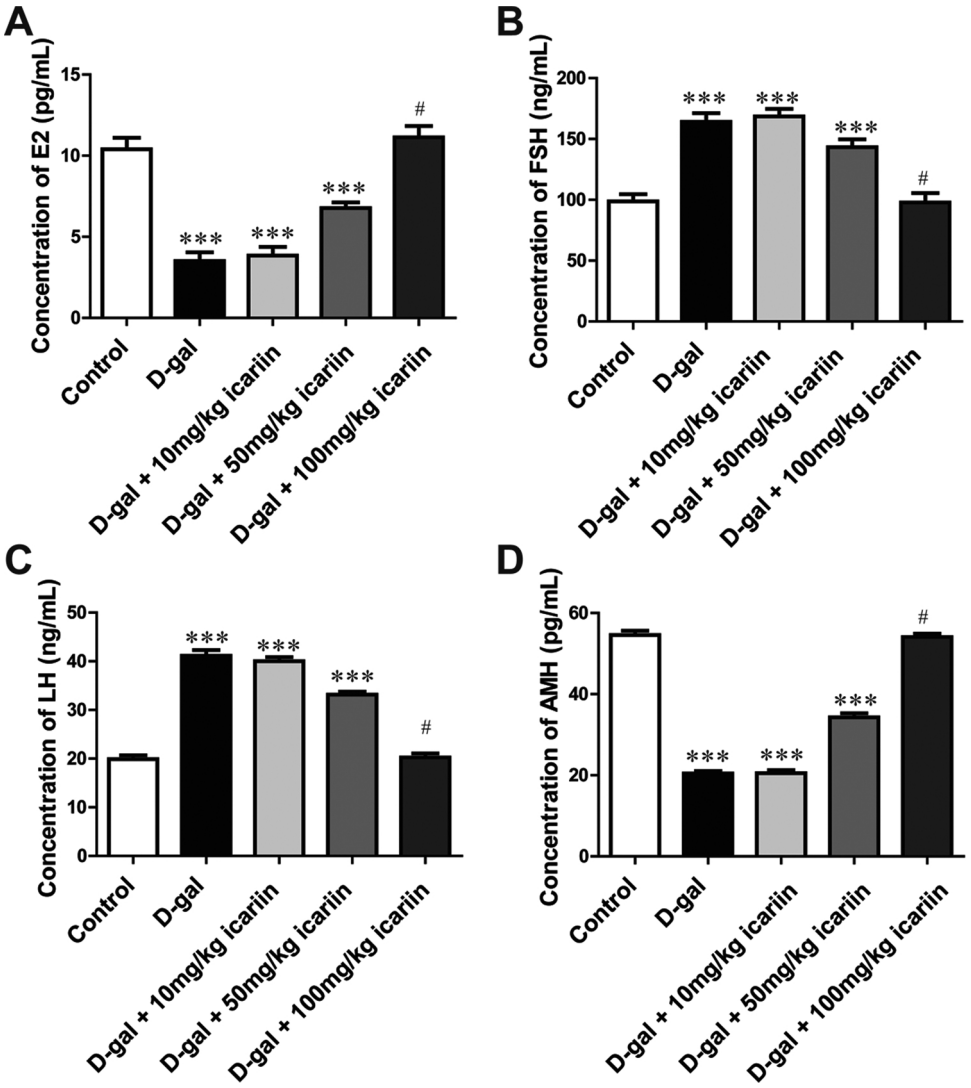

Fig. 3. Protective effects of icariin on the HPG axis and AMH in the d-galactose induced premature ovarian failure.
The serum E2 (A), FSH (B), LH (C) and AMH (D) levels were tested by ELISA. N = 10 for each group. Data were presented as the mean ± SEM. *** indicated $P < 0.001$ and # indicated $P > 0.05$.

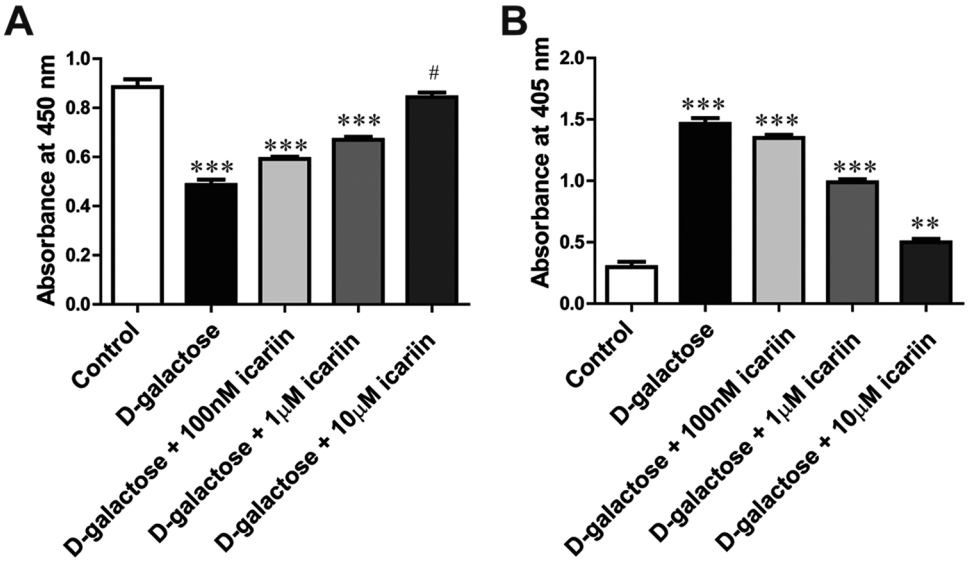

Fig. 4. Icariin protected cells from d-galactose induced aging.
(A) Cell viability for each group as indicated were determined by CCK-8 assay. D-galactose could reduce cell viability while high concentration of icariin dramatically recovered the cell viability. Data were represented as mean ± SEM; n = 6 for each group. # indicated $p > 0.05$ and *** indicated $p < 0.001$.
(B) The activity of endogenous β-galactosidase was assayed by Mammalian beta-Galactosidase Assay Kit. Data were represented as mean ± SEM; n = 6 for each group. ** indicated $p < 0.01$ and *** indicated $p < 0.001$.

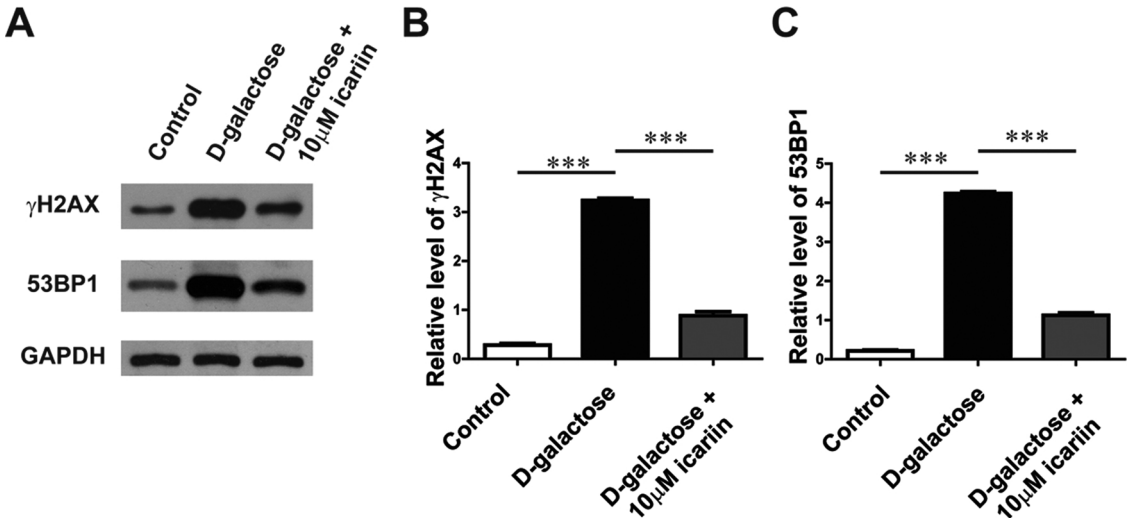

Fig. 5. Icariin protected cells from d-gal induced aging via promoting damage repair.
(A) Western bolt showing the protein expression level of γH2AX and 53BP1 in the treatment with D-gal in the presence or absence of icariin. n = 6 for each group and this panel presented one of these repeats.
(B) Statistic of the relative protein level of γH2AX in panel (A).
(C) Statistic of the relative protein level of 53BP1 in panel (A).
Data were represented as mean ± SEM; n = 6 for each group. *** indicated $p < 0.001$.

indicators for DNA damage, were significantly upregulated in ovaries of D-galactose treated mice, whereas the levels of these two factors were significantly downregulated by icariin at 10 μM, suggesting that DNA damage repair plays role in the protection of icariin on the damage of ovarian granulosa cells caused by D-galactose. In addition, the expression levels of γH2AX and 53BP1 were regulated by the administration of icariin in a dose-dependent manner, even when the concentration of icariin was as low as 100 nM (Fig. S2).

4. Discussion

POF is a cessation of normal ovary function before age 40, leading to insufficient hormone estrogen production and egg release, accounting for of the infertility. Currently hormone replacement therapy has been widely accepted by women with POF [18]. Unfortunately, adverse effects were observed in the estrogen therapy in young women with POI or early menopause [19]. A Women's Health Initiative (WHI) trial showed various kinds of health risks related to use of estrogen/progestin therapy, including cardiovascular disease breast cancer, and stroke [20]. Therefore, the development of safe and effective alternative treatments towards POF has been demanded. Icariin is one of the major bioactive compounds extracted from Epimedium, and has been mainly used for bone-protective and anti-aging agent [21]. In recent years, pharmacological studies suggest that icariin exerts various therapeutic potentials. For instance, icariin demonstrated antitumor activity in human esophageal squamous cell carcinoma (ESCC) via regulating endoplasmic reticulum activation [22]. Icariin also possesses cardio-protective effect against ischemia/reperfusion (I/R) injury through the activation of PI3K-Akt signaling pathway [23]. Icariin showed neuroprotective effect by significantly reducing microglia activation and attenuating lipopolysaccharides/6-hydroxydopamine-induced dopamine neuronal loss [24]. In terms of effects on reproductive functions, it has been reported that icariin at appropriate doses can increase testosterone production and affect spermatogenesis in male rats [25]. In this study we tested the biological function of icariin against the D-galactose induced premature ovarian failure mice model. Our results indicated that co-administration of icariin promoted ovary/body weight, follicles numbers and fertility outcomes, and this protection is relevant to the attenuation of ovary aging via promoting DNA damage repair. This finding revealed the novel pharmacological effect of icariin as a potential therapeutic agent for POF for the first time.

In order to explore the protective effects of icariin, we adopted *in vivo* mice model and *in vitro* cell culture model by co-administration of D-galactose and icariin at different doses. Although the precise mechanisms remain unknown, previous studies have shown that galactose toxicity directly increases serum levels of FSH and LH [26]. Early follicular phase serum levels of FSH and E2 are measured to assess patients' ovarian reserve, while E2 is produced by early antral follicles, in response to the classical feedback loop of the pituitary-gonadal axis to suppress the secretion of FSH [27]. In this study we found D-galactose treatment significantly increased serum FSH and LH, and decreased E2 levels compared to those in the control, indicating the successful establishment of the mice model. Remarkably, the treatment of icariin inversed all the deleterious alterations of these detected hormone levels. In addition, histological staining results showed that obvious aging characteristics were observed in the ovaries of galactose treated mice, including the significant increase of the ovarian stroma and massive atretic follicles. Our data showed that the quantities of healthy follicles in icariin treated mice were significantly improved from those in the galactose only group, whereas the numbers of atretic follicles were significantly decreased. AMH is expressed in granulosa cells of growing follicles in the ovary, serving as a pivotal early indicator of ovarian aging [28]. Lower AMH expression has been detected during the normal aging process in mice [29]. Our data showed that AMH expression in ovary was decreased by D-galactose, and increased by icariin significantly.

Granulosa cells or follicular cells are somatic cells of the sex cord that is closely associated with the developing oocyte in the mammalian ovary [30]. In line with previous study which showed that icariin at 10 μg/L significantly increased proliferation of cultured rat ovarian granulosa cells and secretion of estrogen and progesterone [31], we observed that the treatment of icariin at 10 μM inversed the viability of cultured mice granulosa cells insulted by the administration of D-galactose. Furthermore, it has been known that one characteristic of senescent cells is the increase in β-galactosidase activity, we biochemically measured the activity of β-galactosidase in cultured granulosa cells. Clearly, cells treated with D-galactose presented increased β-galactosidase activity, and icariin decreased the β-galactosidase activity in a dose-dependent manner, indicating the anti-aging effect of icariin on ovarian cells.

To this end, the results supported the protective effects of icariin on ovarian aging in a D-galactose induced POF mouse model and cultured granulosa cells. It has been well accepted that DNA damage responses (DDR) has a close association with the process of cell aging [32]. Moreover, previous report showed that chemotherapy and radiation therapies often lead to the depletion of the ovarian oocyte reserve and POF [33]. Herein we further detected the effects of icariin on the DNA damage markers including γH2AX and 53BP1 by western blot.

The phosphorylation of H2AX (γH2AX) is triggered at the very early phase of DNA damage and can be recruited to the chromatin regions of damaged DNA, followed by the loading of factors such as 53BP1after mitosis [34]. In this regard, we observed significantly up-regulation of γH2AX and 53BP1 expression levels caused by D-galactose in cultured granulosa cells, which were significantly down-regulated by icariin treatment. This result is consistent with previous study which showed that icariin protected against oxidative DNA damage induced by 2, 2′-azobis (2-amidinopropane) dihydrochloride [35].

As one of the most active ingredients from Traditional Chinese Medicine (TCM), icariin provided an interesting direction to explore towards the modulation on POF via its anti-aging effects. To be noted, there are lots of limitations affiliated to the clinical safety of these kinds of traditional medicine. In this present study, we used blank control and control with D-galactose only to compare the changes of the biochemical parameters. The comparison between the blank and D-galactose group indicated the impairment by D-galactose, which led to the pathological condition of ovary. The comparison between the D-galactose group and D-galactose plus Icariin supported the alleviation of detected features. To be noted, the control group with icariin only was not included, thus making it emphatically urgent to test the safety of icariin, especially at high dose of 100 mg/kg. Moreover, we should be aware of that the interactions between herbal medicines and prescribed medicines, which could potentially be harmful [36].

In conclusion, icariin effectively attenuated D-galactose -induced ovarian injury via promoting DNA damage repair. These results suggest that icariin can be developed as a protective agent against POF.

5. Conclusion

In this study we tested the biological function of icariin against the D-galactose induced POF. Our results demonstrated that icariin effectively attenuated ovarian injury via promoting DNA damage repair, suggesting that icariin can be developed as a protective agent against POF.

Funding

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Not applicable.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109218.

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