Quercetin Prevents Primordial Follicle Loss via Suppression of PI3K/Akt/Foxo3a Pathway Activation in Cyclophosphamide - Treated Mouse

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Research

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Abstract

Background: Chemotherapy improves survival rates but often causes some adverse effects associated with ovarian damage, characterized by a decreased of primordial follicle stockpiles. Recent studies reveal that chemotherapy may stimulate the PI3K signaling pathway, who has roles in manipulating the dormancy and activation of mammalian primordial follicles, resulting in accelerated primordial follicles activation followed by the loss of ovarian reserve. As an inhibitor of PI3K pathway, whether quercetin has protective properties against chemotherapy - induced follicle loss in mice is worth to be explored.

Methods: The effects of quercetin on the mouse model of cyclophosphamide-induced ovarian dysfunction were investigated. Paraffin sections of mouse ovary were stained with hematoxylin and eosin for differential follicles count and TUNEL assay for apoptosis detection. Immunohistochemistry stain with ki67 and Foxo3a were used to evaluate the activation of primordial follicles. The function of PI3K signaling pathway were assessed via the western blot of ovary.

Results: Quercetin cotreatment rescued the reduction number of dormant primordial follicles induced by cyclophosphamide. Moreover, analysis of the PI3K/Akt/Foxo3a pathway demonstrated that quercetin co-administration decreased phosphorylation of proteins that stimulate follicle activation in ovary induced by cyclophosphamide. Meanwhile, Quercetin prevents cyclophosphamide - induced apoptosis in early growing follicles and early antral follicles, maintaining AMH level secreted by these follicles, preserving the quiescence of the primordial follicle pool, characterized by the intranuclear staining of Foxo3a in primordial follicle.

Conclusions: Quercetin attenuates cyclophosphamide - induced follicle loss by preventing the phosphorylation of PI3K/Akt/Foxo3a pathway members and maintaining AMH level secreted by growing follicles.

Background

As an important means of anti-neoplastic therapy, chemotherapy improves survival rates but often causes some adverse effects related with ovarian damage in premenopausal oncological women patients, including ovarian endocrine dysfunction and an increased infertility rate(1). Nowadays, the cryopreservation of gametes makes it possible to preserve fertility in female cancer survivors(2), However, as a result of loss of ovarian function after chemotherapy, early menopause and its associated complications, have not been alleviated(3). Therefore, the development of effective methods to protect ovarian function during chemotherapy would be more advantage.

The extent of ovarian damage largely depends on chemotherapy regimen (drug family and dose) and the patient's age at treatment(4). Among of all the chemotherapy drug classes, alkylating agent (e.g. cyclophosphamide, Cy) causes the most damage to ovarian and it does so in a dose-dependent manner(5). Previous histological studies in human ovaries have shown that the end stage effects of chemotherapy treatment are ovarian atrophy and a depletion of the primordial follicle (PMF) stockpile(6).

The mechanisms that are responsible for chemotherapy-induced PMF loss were not fully elucidated. Recently, over activation of primordial follicles is considered as a possible mechanism of chemotherapy-induced primordial follicle loss(7).

The primordial follicle pool is formed during fetal in humans and a nonrenewable population representing the “ovarian reserve” of potential fertility available to each individual(8). To maintain the length of female reproductive life, the majority of primordial follicles in humans survive in the ovary for decades in a dormant state and only a few activated primordial follicles develop through primary and secondary stages before acquiring an antral stage(9). A number of molecules are indispensable for the maintenance of follicular quiescence and survival(10). Studies in genetically modified mouse models(11) showed that forkhead transcription factor class O (Foxo) 3a maintains the dormancy of primordial follicles and can be inactivated by phosphorylation, leading to follicular activation. Moreover, phosphatidylinositol 3 - kinase (PI3K) signaling pathway, which regulates Foxo3a expression, has roles in manipulating the survival of mammalian primordial follicles(10). Phosphorylation of protein members on this pathway, such as protein kinase B (Akt/PKB), mammalian target of rapamycin (mTOR), and ribosomal proteinS6 (rpS6), initiates follicle recruitment and cell growth. Balance the positive and negative regulation of PI3K pathways is important to keep primordial follicle in a quiescent state(10). Chemotherapy may disturb this balance, leading to a decrease in levels of inhibitory factors or an increase of stimulatory factors, followed by the activation of dormant primordial follicles(7). Based on the understanding of this mechanism, development of agents that regulate the normal expression of PI3K signaling pathway may prevent overactivation of PMF induced by chemotherapy.

Quercetin (Que), a flavonoid distributed widely in edible and medicinal plants, has various biological properties without any evidences of toxicity, carcinogenicity and genotoxicity related to consumption(12). For decades, quercetin has been studied with respect to its beneficial health effects and its possible use for cancer treatment and prevention, through its interaction with multiple cancer-related pathways(13), PI3K signaling pathway is one of that. In vitro, quercetin induces apoptosis and autophagy in primary effusion lymphoma cells by inhibiting PI3K / Akt / mTOR pathways(14) and restores nuclear Foxo3a and reduces chemokine expression partly by modulating epidermal growth factor receptor / PI3K / Akt activity(15). Therefore, whether quercetin has protective properties against chemotherapy - induced follicle loss in mice though regulation PI3K pathway is worth to be explored. In this study, we investigated the effects of quercetin administration on follicular development and the protein expression of PI3K/AKT/Foxo3a signaling pathway in a cyclophosphamide - induced mice model.

Methods

Mice

C57BL/6 female mice aged 5-6weeks were purchased from Jie Si Jie Laboratory Animal Co., Ltd. (Shanghai, China), and housed under specific pathogen-free conditions (temperatures at 22-24°C and a 12h light/12h dark photoperiod), with commercial rodent diet and deionized water available ad libitum.

All procedures were approved by the Ethic Committee of the Ninth People's Hospital of Shanghai (Approval No.: HKDL [2015]56).

After acclimatizing (5-7d), animals were given a single intraperitoneal injection of 100µl PBS or an equal volume containing 75mg/kg Cy (Sigma-Aldrich), a dose which destroys 50% of PMF reserve(16). Since the poor water solubility and low oral bioavailability(12), 20mg/kg(17, 18) or 40mg/kg quercetin (Sigma - Aldrich) was intraperitoneal. Moreover, in light of an overall follicle growth time of mouse(19, 20), the administration of quercetin was for 14 days beginning 1 week before Cy or PBS administration and ending 1 week.

Histological processing and follicle count

Mice were sacrificed 7d after Cy or PBS treatment, then ovaries were dissected and fixed in 4% paraformaldehyde. After paraffin-embedded, ovaries were serially sectioned at 5µm thickness and every fifth section was stained with hematoxylin and eosin for differential follicles count(16). Follicles that contained oocytes with clearly visible nuclei were scored in each section, as previously reported(21). Follicle stage was classified according to the well-accepted standards established by Pedersen and Peters(7, 22) and counted PMFs were defined as a nucleus surrounded by a single layer of flattened squamous follicular cells; primary follicles were defined as an enlarged oocyte surrounded by a single layer of cuboidal granulosa cells; secondary follicles had two or more layers of cuboidal granulosa cells, but no antrum; antral follicles were scored when containing several layers of granulosa cells, an oocyte with a clear nucleus, an antrum containing follicular fluid and a theca layer. For statistical analysis, primary follicles and secondary follicles were grouped as early growing follicles (EGFs). All follicle counts were performed by two experienced gynecologic pathologists who were blinded to the group assignment. By multiplying by a correction factor of 5, the final sum number of follicles in each stage was estimated to represent the whole ovary(23). Data are presented as number of follicles per developmental stage (n = 5 ovaries per group).

TUNEL assay

For apoptosis detection, ovarian tissue was obtained from mice sacrificed 24h after Cy or PBS treatment and serially sectioned (5-µm sections). An apoptosis detection kit (In Situ Cell Death, Detection Kit; Roche; Switzerland) was for in situ localization of nuclei exhibiting DNA fragmentation by the TUNEL technique according to the manufacturer's instruction, as previously described(24). Negative controls included terminal deoxynucleotidyl transferase (TdT) omission. Positive control was incubed with DNase I recombinant for 10 min at 15 - 25°C to induce DNA strand breaks, prior to labeling procedure. Sections were counterstained with hematoxylin. The number of apoptotic cells was determined by counting labelled cells from all follicle types in ×400 microscopic fields (four sections per ovary; five ovaries per group). Follicles presenting more than 5% labelled cells were considered unhealthy(25). The apoptotic index was calculated as a fraction of unhealthy/total follicles for each class(25).

Histochemistry and immunohistochemistry in ovarian tissues

For IHC, ovaries were also from mice sacrificed 24h after Cy or PBS treatment. After deparaffinized in xylene and rehydrated by graduated ethanol washes, endogenous peroxidase activity in sections was inactivated by 3% (vol/vol) hydrogen peroxide in PBS. After epitope retrieval treatment (at 98°C for 30min in 0.01M citrate buffer solution, pH6.0; Dako, Glostrup, Denmark), non-specific binding was blocked with 2% BSA for 1 h at room temperature. Then sections were incubated with primary antibody overnight at 4°C. The antibodies used were Foxo3a antibody [1:1000, from Cell Signaling Technology], ki67 antibody [1:400, from Cell Signaling Technology] and cleaved caspase-3 antibody [1:200, from Cell Signaling Technology]. After washing, slides were incubated with biotinylated anti-rabbit IgG (Dako, Glostrup, Denmark) for 1 h. Finally, protein signal was visualized with DAB (Dako, Glostrup, Denmark) staining. After stopping the reaction with distilled water, slides were counterstained with hematoxylin, dehydrated and mounted. Between each step, the sections were rinsed with PBS for 5-10 min three times. In all cases, negative controls were obtained in absence of primary antibody. The images were taken with a digital camera (olympus, Japan) mounted on a conventional light microscope (olympus, Japan), using a magnification of $\times 40(25)$ (n = 5 ovaries per group).

Western blot

After removed from mice sacrificed 24h after Cy or PBS treatment, ovaries placed on ice and extracted by radioimmunoprecipitation assay lysis buffer system (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with protease inhibitors (Santa Cruze, USA), then homogenized with an Ultra-Turrax homogenizer (IKA Werk, Germany). Samples were centrifuged at 4°C for 15min at 12000 g and the resulting pellets were discarded. Protein concentration in the supernatant was determined using a BCA protein assay kit (Thermo, USA). After boiling for 10min, 40µg of protein from each sample was loaded onto 8 - 12% sodium dodecyl sulfate - polyacrylamide gel and electrophoresis was performed at 80 - 120V for 1.5h. The resolved proteins were transferred onto polyvinylidene fluoride membranes (Merck Millipore, Germany). The membranes were then incubated in blocking buffer (5% non - fat milk, 0.1% tween - 20 in 20mM TBS pH 8.0) for 1 h at room temperature and probed with specific primary antibodies overnight at 4 °C. Primary antibodies against Akt, phosphor-Akt (Ser473), mTOR, phosphor - mTOR (Ser2448), rpS6, phosphor - rpS6 (Ser235/236), Foxo3a, phosphor - Foxo 3a (ser253) and β - actin were all rabbit monoclonal antibodies and purchased from Cell Signaling Technology. Horseradish peroxidase (HRP) - conjugated goat anti-rabbit IgG (MR-M100, MR Biotech, China) was used to determine the proteins and an ECL kit (Pierce, Thermo Scientific) was employed to visualization. Signals were scanned on Amersham600 imagers system (GE) and Integrated light intensity of each band was quantified with ImageJ (National Institutes of Health, Java image processing software) and used to compare treatment-induced changes in the concentration of phosphoproteins as well as calculated the ratio of phosphorylated proteins to their non - phosphorylated forms. β - actin expression was measured to verify

equal loading. Experiments were repeated three to five times with similar results, three to five ovaries were pooled per result, with a total number of animals of 45.

Serum anti-Müllerian hormone (AMH) measurements

24 hours after Cy or PBS treatment, blood was collected by cardiac puncture at the time of sacrificed and then separated by centrifugation at 1000g for 15min at 4°C and stored at -80°C until further analysis. Plasma AMH concentration was quantified with a mouse muellerian - inhibiting factor ELISA Kit (Signalway Antibody, USA) according to the manufacturer's instruction, as previous study(26) (n = 5 ovaries per group).

Statistical data analyses

The results are expressed as the mean $\pm$ standard error of the mean (SEM) and analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). The significant differences between groups were calculated with one-way analysis of variance (ANOVA) followed by Tukey's test in all cases. All samples were tested for normality before ANOVA. $P < 0.05$ were considered statistically significant.

Results

Quercetin reduces Cy - induced PMF loss

Two different dosage of quercetin (20 or 40 mg/kg per mouse) was given to adult female mice and two weeks later, the effect of quercetin alone on different stage of follicle development was first to investigate. The total number of follicles has no significant change after quercetin administration compared to control group. However, with the increase of quercetin dosage, the number of primordial follicles slightly increased, although there was no significant difference compared to control mice, and the EGFs count decreased gradually, especially in the high-dose quercetin group ($P < 0.05$ compared to control mice, Fig. 1A). This trend is apparently in a comparison of the ratio of EGFs versus PMF ($P < 0.05$ compared to control mice, Fig. 1A). These results suggest that quercetin may have the potential to inhibit the transition from primordial follicle to the next developmental stage.

To study the impact of quercetin cotreatment on Cy-induced follicle loss, adult female mice were treated with quercetin (20 or 40 mg/kg per mouse) every day, beginning 1 week before Cy treatment and ending 1 week. Histological analysis showed that Cy-treated ovaries are poor in follicles in comparison to control ones, presenting cortical fibrosis and altered stromal cells, while ovaries cotreatment with Cy and Que groups are similar to healthy, control ovaries (Fig. 1C). Furthermore, mice treated with Cy showed a dramatical reduction in the number of primordial follicles, early growing follicles and total follicles compared to control animals ($P < 0.001$, Fig. 1B). When quercetin co-administration, the decreased number of dormant primordial follicles induced by Cy was significantly recovered, especially those

received quercetin at 40 mg/kg retained an ovarian reserve equivalent to untreated controls (616.56 ± 55.41 vs. 554.00 ± 41.60 , $P = 0.275$). But unfortunately, both two dosage of quercetin cannot prevent the loss of EGFs caused by Cy (Fig. 1B). So, the ratio of EGFs to PMF was significantly decreased in mice after Cy and quercetin co-treatment, even lower than that in control. Finally, the follicle sum in Cy and quercetin co - treatment group obtained partially rescue, higher than that of Cy group ($P < 0.001$) but not yet returned to the level of control (Fig. 1B). Therefore, the quantification of different stage follicles revealed that quercetin in both dosages prevented the loss of primordial follicles induced by Cy but cannot provide protection against the damage of Cy to early growing follicles.

Quercetin prevents Cy - induced apoptosis in EGFs and AMH decrease

Apoptosis is one of the possible mechanisms of ovarian damage induced by chemotherapy(27), so whether quercetin prevents Cy-induced PMF loss through its anti-apoptotic effect was first to explore. However, neither cleaved caspase - 3 staining nor TUNEL staining showed any evidence of apoptosis in PMF after Cy treatment with or without quercetin, while following by the development of follicle, the apoptosis of granulosa cells induced by Cy gradually became obvious and cotreatment with quercetin may ameliorate this phenomenon (Fig. 2A).

To further verify the anti-apoptotic of quercetin in growing follicles, apoptotic index (TUNEL - positive follicles/total follicles) were quantified based on TUNEL sections (Fig. 2B). The results show that Cy treatment increased the apoptotic index in all growing follicles compared to control ovaries and quercetin coadministration protected follicles of preantral and early antral stages from apoptosis, since the apoptotic indexes in these two stages follicles after Que cotreatment were significantly fewer and similar to controls (Fig. 2B). However, this protective effect of quercetin was not obvious in granulosa cells of mature antral follicles.

AMH is expressed by granulosa cells and is produced by growing follicles ranging from the primary stage of development until selection for dominance(28). The study of *Amh*^{-/-} mice shows that AMH facilitates maintenance of primordial follicles in a dormant state(29). As an indirect indicator of follicle reserve, AMH was measured 24 h after Cy administration. Figure 2C shows that AMH concentration was significantly decreased with Cy alone (from 5.4 to 2.2 ng/ml, $P < 0.001$), but after coadministration with quercetin, the concentration of AMH remained to normal. Quercetin alone did not alter the concentration of serum AMH.

These results suggest that Cy-induced PMF loss may not directly induce apoptosis of primordial follicles, but indirectly via the acute reduction of growing follicles and the decrease of AMH result in increased recruitment of primordial follicles into the growing pool. Quercetin cotreatment may inhibit the apoptosis induced by Cy in growing follicles and maintain the AMH level secreted by these follicles, thus keeping the dormancy of primordial follicles.

Quercetin inhibits Cy - induced of PI3K/ AKT /Foxo3a signaling pathway activation

In addition to AMH, the PI3K signaling pathway is also principally responsible for manipulating the dormancy and activation of mammalian primordial follicles(10). Recent studies indicate that the mechanism in Cy-induced loss of ovarian reserve is related with the acceleration of primordial follicle activation via the activation of PI3K signaling pathway(7). Therefore, whether quercetin reduces Cy - induced PMF loss by inhibiting the activation of PI3K signaling pathway was then to investigate.

Foxo3a, a downstream effector of PI3K signaling pathway, is highly expressed in the nuclei of oocytes of primordial follicles and is important in maintaining primordial follicles in their dormant state(11). When it exports from nuclear, primordial follicles are activated and initial to development(30).

Immunohistochemical staining in this study conducted on ovaries showed that the expression of Foxo3a was absence in the primordial follicle population after Cy administration, but deeply dyeing in Que cotreatment group, similar to the control group (Fig. 3B). And the result of WB shows that the ratio of phosphorylated Foxo3a to their non-phosphorylated forms elevated 1.69-fold in Cy-treated ovary compared to control, which was recovery to control level after Que co-administration. However, quercetin alone did not significantly affect the expression of Foxo3a. (Fig. 3A)

Moreover, once the primordial follicles are activated, they enter the stage of growth and granulosa cells begin to proliferate. Immunostaining with the proliferation marker ki67 in control ovaries showed granulosa cell proliferation was obvious in large growing follicles and only occasional staining in small activated follicles. Of particular interest, there was the substantial staining for Ki67 in granulosa cells in transitional primordial and primary follicles on ovaries removed 24 hours after Cy administration compared with a fewer positive cell in the Que cotreatment group (Fig. 3B).

Except for the suppressor protein Foxo3a, the key activation proteins Akt, mTOR, and rpS6 were also detected. Analysis of Cy treated ovaries showed an increase in the phosphorylated forms of these proteins (1.3- to 1.6-fold increase compared to that in control group, Fig. 3A). This increased phosphorylation of these three proteins caused by Cy treatment was attenuated after cotreatment with quercetin, who suppresses phosphorylation of mTOR and rpS6 alone, especially in high dosage.

These results suggested that quercetin may prevent the activation of PI3K signaling pathway induced by Cy to preserve ovarian reserve.

Discussion

An unfortunate and devastating consequence of chemotherapy treatment is ovarian damage, which is characterized by ovarian atrophy with a mark loss of primordial follicles in histological studies(6). As an alkylating agent, Cy is widely used for various tumors and carry the greatest risk of premature ovarian failure in all the chemotherapy drug classes(5). Previous study has demonstrated 75 mg/kg Cy could

destroy 50% of PMF reserve in mice ovary(16). In this study, one week after 75 mg / kg Cy intraperitoneal injection, the number of primordial follicles in mouse ovary decreased to 1/3 of the control group.

The exact mechanisms of chemotherapy-induced ovarian damage remain ambiguous. The oocyte death by apoptosis has been identified as the main mechanism responsible for loss of germ cells and premature ovarian failure (POF) after chemotherapy(31–33). However, it is well known that chemotherapy drugs particularly target actively dividing cells(4), while primordial follicles are in their long dormancy, hence neither this study nor some previous in vivo studies(7) found any apoptosis in primordial follicles. Recently, accelerated primordial follicle activation is now considered as the mechanism of chemotherapy-induced ovarian damage(7). Chemotherapy may disrupt the function of PI3K/PTEN/Akt signaling pathway responsible for follicle quiescence and directly induce primordial follicle activation(7).

As the inhibitor of PI3K(13), quercetin was used in this study to explore the effect of quercetin cotreatment on Cy-induced follicle loss. Our results showed that after quercetin administration alone, along with the rise of quercetin dose, the number of primordial follicles increased slightly, whereas the number of EGFs decreased, especially in the high-dose quercetin group. When quercetin cotreatment with Cy, the number of dormant primordial follicles were rescued to that of control ovaries. And the results of WB also showed that the phosphorylation of Akt-mTOR and rpS6, which are involved in the regulation of primordial follicle activation, is decreased after quercetin addition. Meanwhile, the phosphorylation of Foxo3a, which represents the activation of primordial follicle, is also decreasing after quercetin addition. All these indicate that quercetin may inhibit the transformation from primordial follicle to primary follicle caused by Cy via inhibiting PI3K signaling pathway. However, PI3K signaling pathway is a “classic” network important for physiological processes including cell survival, growth, and migration(34). Whether quercetin negatively regulate the growth and development of normal cells via the inhibition of PI3K signaling pathway need to be explored in future study.

In addition to this direct mechanism described above, reduction of the primordial pool can arise indirectly via the loss of activated, growing follicles(4). With a high degree of proliferation, granulosa cells in rapidly growing follicles are more attacked by chemotherapy and showed extensive apoptosis(7). Our results showed that after chemotherapy, granulosa cells in growing follicles were apoptotic widely and the more mature the follicle is, the more obvious the apoptosis is. Accordingly, suppression of follicular activation by AMH from the granulosa cells of growing follicles is decreased and lead to accelerated depletion of the primordial follicle reserve(35). As a result of that, primordial follicles will be undergoing growth initiation to replace damaged growing follicles. In this study, Foxo3a staining showed that primordial follicles are activated after chemotherapy and ki67 staining showed that the proliferation of granulosa cells in transitional primordial and primary follicles is activity. However, quercetin coadministration protected follicles of preantral and early antral stages from apoptosis and maintained AMH level, consequently preserving the quiescence of the ovarian reserve. We speculate this phenomenon may also relate with the PI3K signaling pathway inhibition and anti - proliferation of quercetin, which weaken the sensitivity of preantral follicles and early antral follicles to the damage of Cy. However, there was no

protection of quercetin supplied for granulosa cells of mature antral follicles from damage induced by Cy, thus some anti apoptotic compounds can be considered to combined with quercetin in future research.

Quercetin has a variety of biological functions, such as anti-inflammatory, anti-oxidant and anti-cancer effects(36) and a reasonable dosage is important for its biological activity(37). Numerous in vitro studies reported that quercetin at high dosages promote cell to apoptosis and low concentrations of quercetin showed anti-oxidant effect(13). Previous study has demonstrated that 20 mg / kg quercetin intraperitoneal injection supply the protective effect against ovarian toxicity and POF induced by Cy without compromising its antitumor effect(17). But in xenograft tumor model, quercetin at 20 mg / kg may attenuate the therapeutic effects of chemotherapeutic drugs in ovarian cancer cells by reducing reactive oxygen species damage(18). Therefore, both 20 mg / kg and a relatively high dose of 40 mg / kg were chosen for this experimental dosage. Our results showed that both dosages of quercetin have the ability to inhibit the activation of primordial follicles induced by Cy, especially at high dosage. Moreover, an overall follicle growth time in mouse is 17 to 19 days(19) and the mouse follicle culture system showed that the duration from preantral follicle to ovulatory follicle last about 12 days(20), so the transformation from primordial follicles to primary follicle maybe within 7 days. Hence, the administration of quercetin for 14 days beginning 1 week before Cy or PBS administration and ending 1 week is to cover this process.

Conclusion

In summary, this in vivo mouse study first demonstrates that quercetin inhibited the activation of the dormant primordial follicles induced by Cy via regulating the activity of PI3K/ Akt / Foxo3a pathway and maintaining AMH level secreted by growing follicles. As an agent that can prevent the loss of follicles at the time of chemotherapy treatment, quercetin would provide significant advantages over existing fertility preservation techniques in that they would be suitable for patients of all ages and life stages, would not require invasive surgical procedures or subsequent use of assisted reproductive technologies, and would prevent the myriad endocrine related side effects of POF other than infertility in female cancer survivors. Besides of that, the result in our research also provides a new application field for quercetin.

Abbreviations

Cy: cyclophosphamide; PMF: primordial follicle; Foxo3a: forkhead transcription factor class O 3a; PI3K: phosphatidylinositol 3 – kinase; Akt/PKB: protein kinase B; mTOR: mammalian target of rapamycin; rpS6: ribosomal proteinS6; Que: Quercetin; EGFs: early growing follicles; AMH: anti-Müllerian hormone; POF: premature ovarian failure.

Declarations

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Authors' contributions

S.Y. and Y. P. K. conceived and supervised the study; J. H. L., H.L. and Y.Y.C performed animal breeding, histological analysis and biochemical analysis, respectively; S. Y., and H. Y. G. analyzed the data, and S. Y. wrote the manuscript with the help of Q. F. L.

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Availability of data and materials

The data and materials supporting the conclusions are included within the article.

Ethics approval and consent to participate

All methods applied in this study are in accordance with protocols approved

by the Ninth People's Hospital of Shanghai. All mice animal experiments

were approved and performed under the supervision of the Ethic Committee of the Ninth People's Hospital of Shanghai.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

A **B** **C** **Figure 1**

Quercetin reduces Cy - induced PMF loss. (A - B) Effect of PBS alone, quercetin alone, Cy alone or with quercetin on different stage of follicle development (n = 5/group). Data are expressed as the mean $\pm$ SEM. Statistical analyses were performed with one - way ANOVA followed by Tukey's test. (***) $P < 0.001$ compared with Cy group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with control group). (C) Representative images of H&E staining in the ovarian histological sections from all experimental groups. Scale bars represent 100μm (upper panel) and 20μm (lower panel). Black arrows indicate the primordial follicles.

A **B** **C** **Figure 2**

Quercetin prevents Cy - induced apoptosis in growing follicles and AMH decrease. (A) Representative images of mouse ovaries stained with cleaved caspase - 3 antibody (upper panel) and TUNEL (lower panel) are shown for each treatment condition. Bars represent 50µm. (B) TUNEL - based quantification of the apoptotic index per follicle class calculated as fraction apoptotic/total follicles for each type. n = 5 mice. (*P < 0.05, **P < 0.01, compared with Cy group; ## P < 0.01 compared with control group).

Primordial follicles did not present TUNEL - positive cells in any experimental group. Statistical analyses were performed with one - way ANOVA followed by Tukey's test. (C) AMH plasma concentrations were measured 24 hours after the administration of PBS or Cy treatment with or without quercetin (n = 5/group). Data represent means $\pm$ SEM. Statistical analyses were performed with one - way ANOVA followed by Tukey's test. (***) $P < 0.001$ compared with Cy group).

Figure 3

Quercetin reduce Cy-induced phosphorylation of PI3K/Akt/Foxo3a pathway proteins in the ovary. (A) Western blot analysis was conducted on ovaries from 8-week-old mice removed 24 hours after a single dose of Cy (75 mg/kg) treatment or the equivalent volume of PBS with or without cotreatment with

quercetin (Que1: 20mg/kg; Que2: 40mg/kg). Protein analysis comparing concentrations of phosphorylated and total Foxo3a, Akt, mTOR and rpS6, with β -actin used as a loading control. Fold change in this ratio for each protein is listed below each set of bands. (B) The ratio of the bands for phosphorylated/total protein was calculated and represented as a bar graph. Experiments were repeated three to five times with similar results; Three to five ovaries were pooled per result, with a total number of animals of 45. The data represent the mean values $\pm$ SEM. (**P < 0.001 compared with Cy group; # P < 0.05, ## P < 0.01, ### P < 0.001 compared with control group). Statistical analyses were performed by one - way ANOVA followed by Tukey's test. (C) Immunohistochemical staining of foxo3a and ki67 demonstrates quercetin coadministration inhibit Cy - induced primordial follicle overactive and short - term growth. A representative image from each group is shown. Upper: immunohistochemical staining of foxo3a show the localization of foxo3a in each group. Arrows indicate primordial follicles. Lower: ki67 staining for cell proliferation was conducted on ovary. Black frames indicate primary follicles. Scale bars, 20 μ m.