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Synergistic Effects of Capsaicin and Quercetin Improved Induced Premature Ovarian Failure in Rat

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Abstract

Objective: Premature ovarian failure (POF) is a heterogeneous disorder. POF is defined as hypergonadotropic hypoestrogenism in women under 40 years. There is no effective treatment to cure POF patients. Antioxidants prevent ovarian damage by reducing the lipid peroxidation cascades affecting folliculogenesis, meiosis and ovulation. Hence; the aim of present study was to investigate the effects of Capsaicin (CAP) and Quercetin (QUR) on cyclophosphamide (CYC)-induced POF in rat model.

Materials and Methods: In this experimental study, POF was induced by intraperitoneal injection of 200 mg/kg CYC on first day and then 8 mg/kg/day for the following 3 days. After 4 days of CYC administration, rats were randomly divided into five groups (n=6/group) as follows: POF, dimethyl sulfoxide (DMSO), CAP (0.5 mg/kg/day), QUR (100 mg/kg/day) and CAP+QUR. Biochemical, hormonal, gene expression, and histological evaluations were performed on blood serum and tissue samples after 14 days of treatment with the CAP and QUR.

Results: CAP, QUR and CAP+QUR groups showed signs of restored ovarian function in the form of a significant increase in serum total antioxidant capacity (TAC), estrogen, progesterone and anti-mullerian hormone (AMH) levels versus POF and DMSO groups and a significant improvement in histological parameters and follicle numbers in treatment groups compared to POF and DMSO groups. Polymerase chain reaction (PCR) analysis demonstrated that CAP and QUR upregulate the expression of *BAX* gene and decreased the expression of apoptosis inducing genes (*BCL-2* and *P53*).

Conclusion: CAP and QUR treatment of CYC-induced POF rats showed a positive effect on reducing ovarian damage by improving TAC levels, expression of apoptotic genes, levels of ovarian reserve markers, and histological parameters. Our results suggest that treatment with CAP or QUR may be a conservative treatment approach for CYC -induced POF.

Keywords: Capsaicin, Cyclophosphamide, Premature Ovarian Failure, Quercetin, Total Antioxidant Capacity

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Introduction

Decreased ovarian activity with increased levels of follicle-stimulating hormone (FSH) and decreased levels of estradiol (E2) in women under 40 years of age is defined as premature ovarian failure (POF) (1). These patients usually experience oligomenorrhea, amenorrhea, infertility, osteoporosis, cardiovascular and mental diseases showing long-term effects of hypoestrogenism (2). POF is one of the growing diseases affecting the life of 1% of women at the age of 40; although genetic, autoimmune, metabolic and infectious factors are related to the occurrence of POF, iatrogenic factors including chemotherapy is one of the most predictable reasons (3).

Recent studies have shown that the number of patients with POF caused by chemotherapy has been increased in recent years, and its incidence has reached 70-100% of people undergoing chemotherapy (4). Currently there is no effective treatment for POF, but hormone replacement therapy (HRT) including the use of both estrogen and progesterone, is used to reduce the symptoms of POF and preventing osteoporosis in these patients; new studies have clarified the relationship of this treatment method with the increased possibility of cardiovascular diseases (5). Cyclophosphamide (CYC), one of the most successful and widely used antineoplastic drugs, can increase the production of ROS via some signaling pathways such as the PI3K/Akt/mTOR pathway and some molecular complexes including the NADPH complex ($NADPH/NADP^+$) and the mitochondrial electron respiratory chain (6).

Duo to fewer side effects, recent studies focus on the use of natural phytochemicals and dietary supplements, including flavonoids and carotenoids (7, 8). One of these compounds is capsaicin (CAP), which is abundantly found

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in red pepper. CAP (8-methyl-N-vanillyl-6-nonenamide) is a phenolic compound reducing inflammation and oxidative stress because of its antioxidant, anti-inflammatory and anti-cancer properties that limits the negative effects of proteases and lysosomal enzymes (9, 10). CAP corrects the function of macrophages by reducing inflammatory mediators (11). Pretreatment with CAP before radiotherapy protects the primordial follicle reserve preventing ovarian that can be a plausible way to prevent radiation-induced POF (12).

Quercetin (QUR) is a natural product abundant in vegetables, fruits, tea, and olive oil that has antioxidant, anti-inflammatory, and anti-cancer properties (13). QUR (3, 3', 4', 5, 7-pentahydroxyflavanone) is one of the flavonoid compounds that play a role in reducing inflammation, oxidative stress, proteases, and lysosomal enzymes (9, 14) as well as in preventing and treating many chronic cardiac, nervous diseases and cancer (15). QUR's effect in preventing ovarian, breast, prostate, liver and lung cancers has been reported (16). In cases of ovarian cancer, it shows anti-cancer activity by controlling the cell cycle, preventing tumor growth and angiogenesis, inducing apoptosis (17) by activating Nrf2-ARE pathway that increase the expression level of anti-inflammatory enzymes. Phase II oxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH) and thioredoxin inhibit oxidative stress (18, 19) and its toxicity. Izaguirry et al. (20) used QUR as a natural antioxidant against the gonadotoxic effects of cadmium on the ovaries of rats and mice by scavenging free radicals and improving the activities of other antioxidants such as catalase, SOD, and GPx. Therefore, it improved ovarian health by increasing the secretion of E2, FSH, and luteinizing hormone (LH). Wang et al. (21) examined the effects of QUR on ovarian antioxidant capacity in menopausal mice showing no significant change in serum levels of total antioxidant capacity colorimetric (T-AOC), SOD, GSH, GSH-PX, and glutathione S-transferase (GST), but an increase in mRNA and protein expression of oxidative stress-related genes, including SOD -1, CAT, and glutathione synthetase (GSS). QUR prevents oxidative stress and histopathological changes in the ovaries at IR injury (ovarian ischemia-reperfusion injury) (22). There is evidence that QUR can decrease caspase 3 and TUNEL-positive cells in the ovaries, as well as the level of ischemia-modified albumin (IMA) in the serum of IR -injured rats. On the other hand, QUR also has pro-oxidant effects leading to genotoxicity and antiproliferative activity (23).

Endogenous antioxidant defense systems in humans are incomplete without exogenous reducing compounds such as ascorbic acid, tocopherols, carotenoids, phenolics- flavonoids, nonflavonoids, and polyphenols, which play an essential role in many antioxidant mechanisms in living organisms. Therefore, there is a constant need for exogenous antioxidants to prevent oxidative stress, representing a redox imbalance in favor of oxidation. Antioxidants can positively influence each other, such as the synergy of synthetic phenolic antioxidants or the regeneration of tocopherol from its oxidized form, the tocopheroxyl radical, by reduced coenzyme Q (24).

Therefore, considering the importance of POF in women of reproductive age and its high prevalence in women who are undergoing chemotherapy or radiation therapy and the side effects that chemotherapy drugs have as one of the inducing factors of this disease, the objective of current study was to investigate the synergistic effects of CAP and QUR on CYC-induced POF.

Materials and Methods

This *in vivo* animal experiment was performed according to the animal ethics guidelines of the Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1400.080). 36 healthy adult female Wistar albino rats (age: 3 to 4 months) (weight: 210 ± 10) obtained from central animal house and were housed in clean polypropylene rat cages under a 12/12-h light/dark cycle at an ambient temperature of 21-23°C. Food and water were provided ad libitum. Rats were given 3 days to adapt to the environmental conditions.

In the first phase of this study, the rats were randomly divided into POF (n=30) and control (n=6) groups. In order to establish the chemotherapy-induced POF model in rats after testing POF induction methods based on previous studies (25) and according to our pilots study, POF was induced by intraperitoneal injection of 200 mg/kg CYC (Endoxan -N, Batch No: BUX1035, India) on day 1 and then 8 mg/kg/day for the following 3 days.

In the second phase of the study, POF rats were randomly divided into five groups (n=6): POF, DMSO, CAP (0.5 mg/kg), QUR (100 mg/kg), and CAP+QUR. Rats received intraperitoneal injections for 14 consecutive days. The control group received no treatment. The doses of CAP and QUR were chosen based on previous studies (26). The weight of the rats was measured at four-day intervals and at the time of dissection of each rat. Twenty-four hours after the last injection of CAP / QUR or CAP+QUR, the animals were euthanized painlessly after deep anesthesia with 90 mg/kg Ketamine (Batch No: 1402053-01) and 10 mg/kg Xylazine (Batch No: 087238-4), and blood samples were collected for biochemical and hormonal studies. The weight of the ovaries was measured and used for histopathological and molecular examination.

Biochemical and hormonal assays

After clot formation, samples were centrifuged at 2500 g for 15 minutes to separate the serum (Froilabo SW14, France). serum anti-Mullerian hormone (AMH) was determined by the enzyme-linked immunosorbent assay (ELISA) method, and progesterone (P) and E2 were determined by the chemiluminescence immunoassay (CLIA) method. The concentration of these hormones was determined by measuring the absorbance at 450 nm using a spectrophotometer (Jenway 3620D, England).

Ferric reducing antioxidant power assay

The total antioxidant capacity (TAC) of the serum was evaluated by the Ferric Reducing Antioxidant Power Assay method (FRAP). Briefly, 150 µl of serum was mixed with 1.5 ml of fresh FRAP reagent (10 mM 2, 4, 6-tripyridyl-s-triazine, 20 mM $Fecl_3$, $6H_2O$ solution, and 300 mM acetate buffer pH=3.6) and incubated at 37°C. The incubation lasted for 10 minutes. Subsequently, the absorbance was measured at 593 nm using a spectrophotometer (Jenway 3620D, England) and compared to a standard curve obtained with known concentrations of $FeSO_4$ $7H_2O$ (27).

Histological examinations

Ovarian tissue was fixed in 10% formalin and embedded in paraffin wax, they were cut into 5 µm thick serial sections, mounted on slides, and stained with hematoxylin and eosin (H-E). Five larger and complete sections for each ovary were examined and the mean value of the number of primary, preantral, antral follicles and corpora lutea was determined using an optical light microscope (ZEISS Primostar 3, Germany).

Gene expression

The effects of CAP and QUR on the expression levels of *BAX*, *BCL-2* and *p53* genes were measured using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). In this method, Master Mix 2x SYBR Green I, High Rox (BioFACT, South Korea Cat. No. DQ385-40h) was used. Total mRNA was extracted from ovarian samples using Trizol reagent (Life Biolab, Germany, CatNO.LB38055) according to the manufacturer's instructions and then the quality and quantity of RNA was determined using a NanoDrop spectrophotometer (2000c, Thermo Scientific, Grand Island, New York, USA). cDNA was immediately synthesized using Two-step cDNA synthesis kit (BioFACT, South Korea) according to the instructions provided by the manufacturer. qRT-PCR was performed using a qRT-PCR system (Real-time PCR Applied Biosystems ™ Real-Time PCR, USA). The PCR primers are listed in the supplementary Table 1. *GAPDH* housekeeping gene was used as internal reference. The target gene expression was normalized to *GAPDH* and calculated using the comparative quantification method ($2^{-\Delta\Delta CT}$). Cycle conditions were as follows: 95°C for 15 minutes (denaturation) and was followed by 40 cycles (95°C for 15 seconds and 60°C for 60 seconds).

Statistical analysis

Results of this experiment were analyzed to identify the significant levels using One-way ANOVA in GraphPad Prism software (GraphPad Prism 9, Software Inc., USA) and presented as the mean ± SEM. $P<0.05$ was considered statistically significant.

Table 1: Primers and expected length of products

Gene	Gene ID	Primer sequence (5′-3′)	Amplicon length (bp)
BAX	24887	F: CCTGTGCACCAAGGTGCCGGAACT	99
		R: CCACCCTGGTCTTGGATCCAGCCC	
BCL-2	24224	F: TTGTGGCCTTCTTTGAGTTCGGTG	114
		R: GGTGCCGGTTCAGGTACTCAGTCA	
P53	24842	F: TAACAGTTCTGCATGGGCGGC	121
		R: AGGACAGGCACAAACACGCACC	
GAPDH	24383	F: GTCTCCTCTGACTTCAACAGCG	120
		R: ACCACCCTGTTGCTGTAGCCAA	

Results

Body and ovarian weight

CYC significantly decreased body weight in POF and dimethyl sulfoxide (DMSO) groups (187.5 vs. initial weight 218.75, 189.32 vs. initial weight 213.47, respectively) (P<0.0001). Treatment with CAP and QUR increase body weight in all groups, and there was a significant (P<0.01, P<0.001) increase body weight in QUR and CAP+QUR groups compared to POF group (Fig.1A). Furthermore, there was a non-significant decrease in ovarian weights of POF and DMSO groups compared to the control group. Ovarian weight of QUR and CAP+QUR was significantly increased after 14 days of treatment (Fig.1B).

FRAP assay

TAC levels were significantly decreased in POF and DMSO groups compared to the control group (P<0.0001). There was a significant increase in TAC level in CAP, QUR and CAP+QUR groups after receiving the treatment compared to the POF group (Fig.2A).

Hormonal assessment

CYC-induced POF decreased AMH level and treatment with CAP and QUR increased it. AMH level in CAP, QUR and CAP+QUR groups showed a significant improvement in comparison with POF and DMSO groups (Fig.2B). Estradiol and Progesterone level were also decreased in POF and DMSO groups. E2 level was significantly increased in QUR and CAP+QUR group (Fig.2C). Progesterone level was increased in CAP, QUR and CAP+QUR, while the biggest impact was observed in the CAP group (P<0.0001, Fig.2D).

Molecular assessment

The qRT-PCR results showed a significant increase of *BAX* and *P53* genes expression in POF group compared to the control group (P<0.001, P<0.0001, respectively). *BAX* gene expression in CAP, QUR and CAP+QUR groups were downregulated after 14 days of treatment. Expression level of *P53* gene was decreased significantly in all the treatment groups (P<0.0001); However, there were no significant differences between treated groups regarding the *BAX* and *P53* genes expression. Our result for *BCL-2* gene expression showed a significant increase in all the treatment groups (P<0.0001, Fig.3A-C).

Histological results

Histological parameters were significantly improved after administration of CAP and QUR. Our results showed a significant increase in the number of primary follicles in the CAP, QUR and CAP+QUR groups compared to the POF and DMSO groups (P<0.0001). There were no significant differences between CAP and QUR groups and a significant increase in the number of primary follicles was observed in CAP+QUR group compared to CAP and QUR groups (P<0.001). The number of preantral and antral follicles in the CAP, QUR and CAP+QUR groups was significantly increased compared to the POF and DMSO groups (P<0.05), and this increase was significantly higher in the CAP +QUR group than in the other treatment groups (P<0.001). Similarly, a significant increase in the number of Copora lutea was observed in the CAP and QUR groups compared to the POF and DMSO groups. An increase was observed in the CAP+QUR group, but it was not significant (Figs.4A-D, 5).

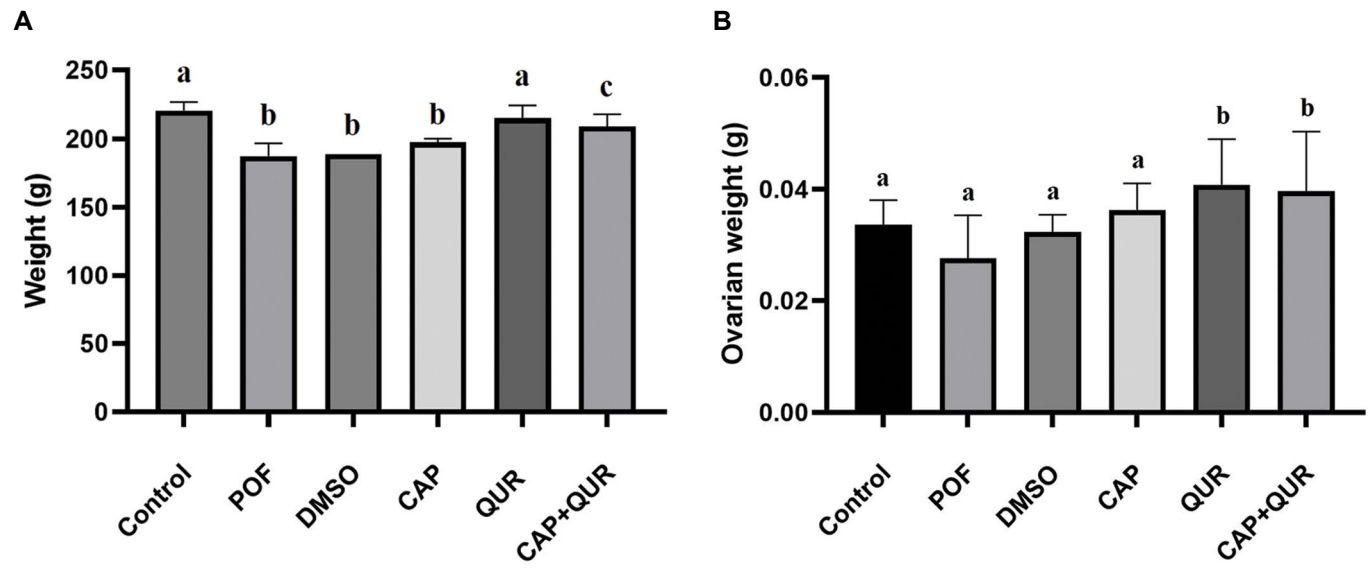

Fig.1: Changes in body and ovarian weight in CAP and/or QUR-treated POF rats compared to POF and DMSO groups. **A.** Body and **B.** Ovarian weight in different treatment groups (P<0.05). Data were represented as mean ± SEM (n=6). Different letters mean significant differences between groups. POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin.

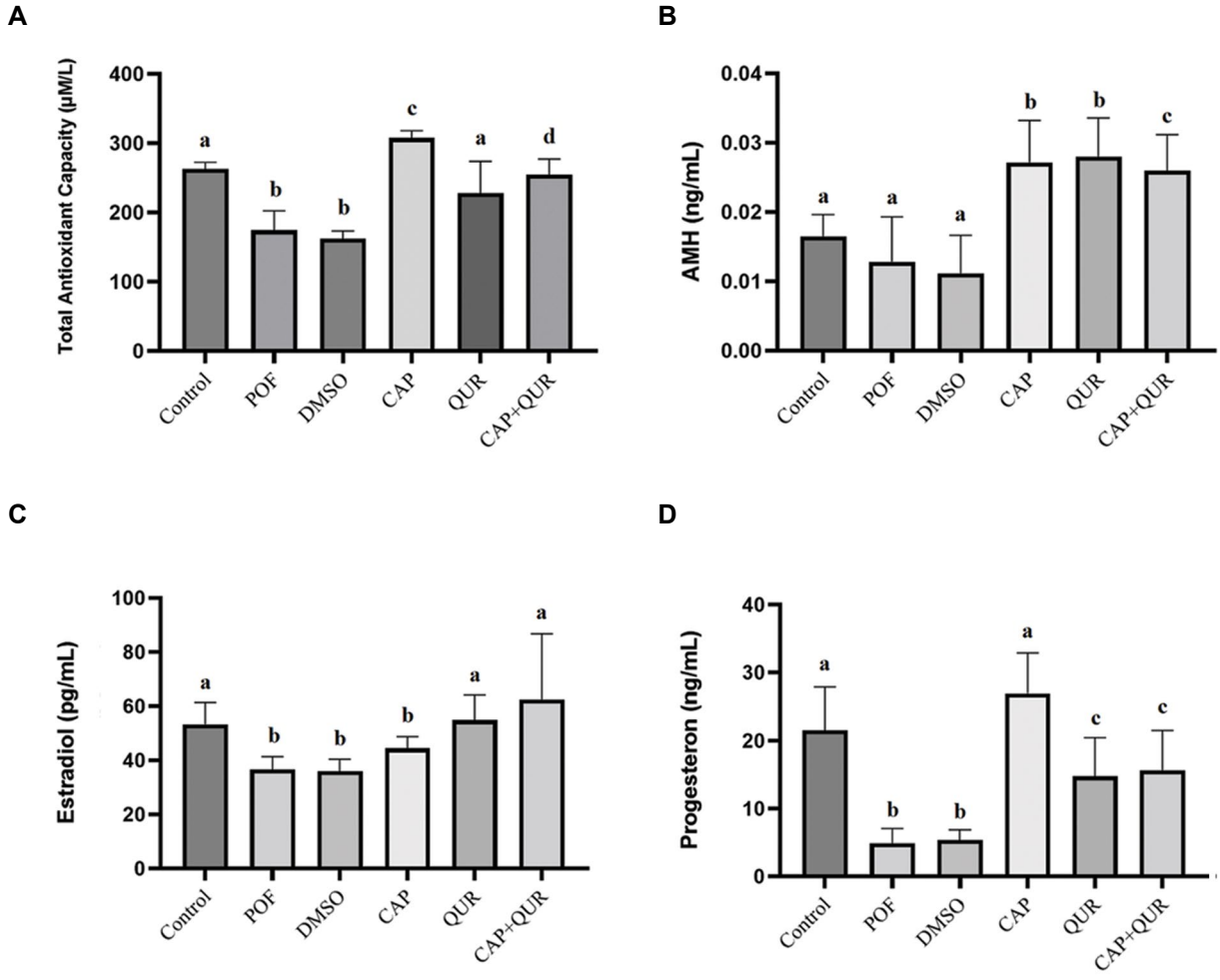

Fig.2: Changes in TAC level in CAP and/or QUR-treated POF rats compared to POF and DMSO groups. **A.** Changes in TAC level in CAP and/or QUR-treated POF rats compared to POF and DMSO groups (P<0.05). TAC increased in the groups treated with CAP and QUR (0.5, 100 mg/kg) compared to the POF and DMSO groups. **B.** AMH level was assessed in different groups by the enzyme-linked immunosorbent assay (ELISA). Data is expressed as AMH ng/ml of serum. **C, D.** CAP and QUR increased estradiol and progesterone concentrations in treatment groups by CLIA. Data are expressed as estradiol and progesterone pg/ml of serum. Data were represented as mean ± SEM (n=6). Different letters mean significant differences between groups. TAC; Total Antioxidant Capacity, POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, QUR; and Quercetin, AMH; Anti-Müllerian Hormone, and CLIA; Chemiluminescence immunoassay.

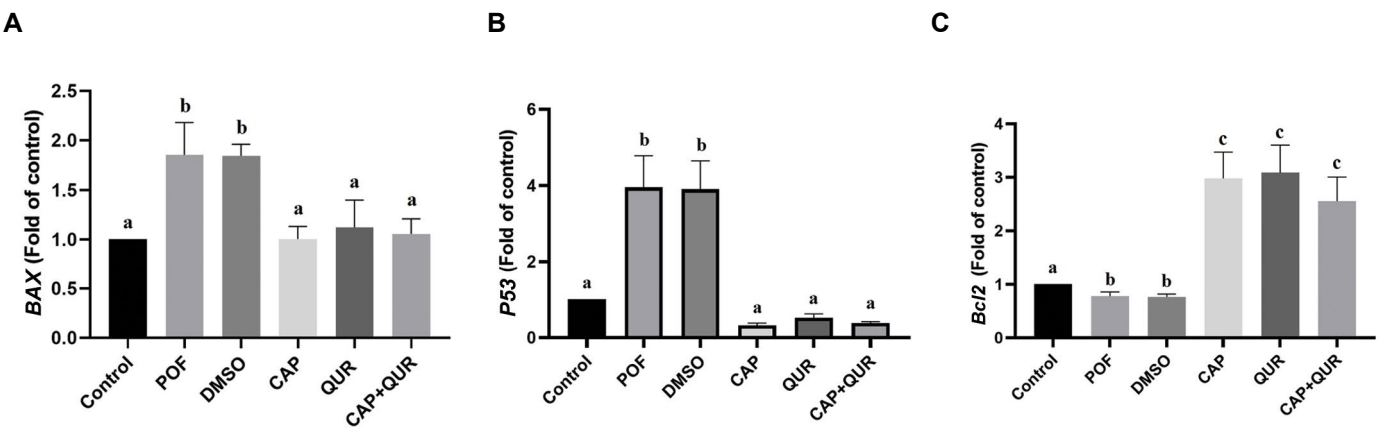

Fig.3: Genes expression levels in CAP and/or QUR-treated POF rats compared to POF and DMSO groups. **A**, **B**. *BAX* and *P53* expression significantly decreased in CAP and/or QUR-treated POF rats compared to POF group (P<0.05). **C**. *BCL-2* expression significantly increased in CAP and/or QUR-treated POF rats compared to POF Group (P<0.05). Data were represented as mean ± SEM (n=6). Different letters indicate significant differences between groups. POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin.

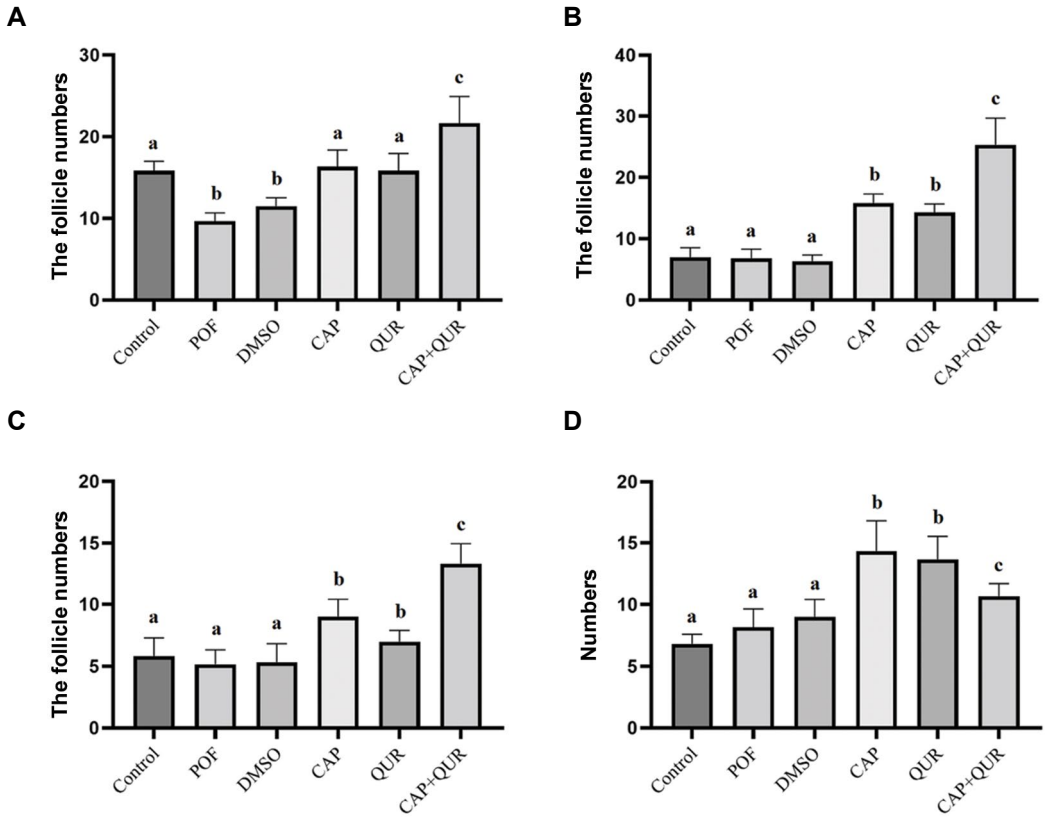

Fig.4: Comparison of the number of follicles in different groups. **A.** Primary follicles, **B.** Preantral follicle, **C.** Antral follicle and **D.** Corpora lutea. Different letters indicate significant differences between groups. Data are presented as mean ± SEM ($P<0.05$, n=6). Different letters mean significant differences between groups. POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin.

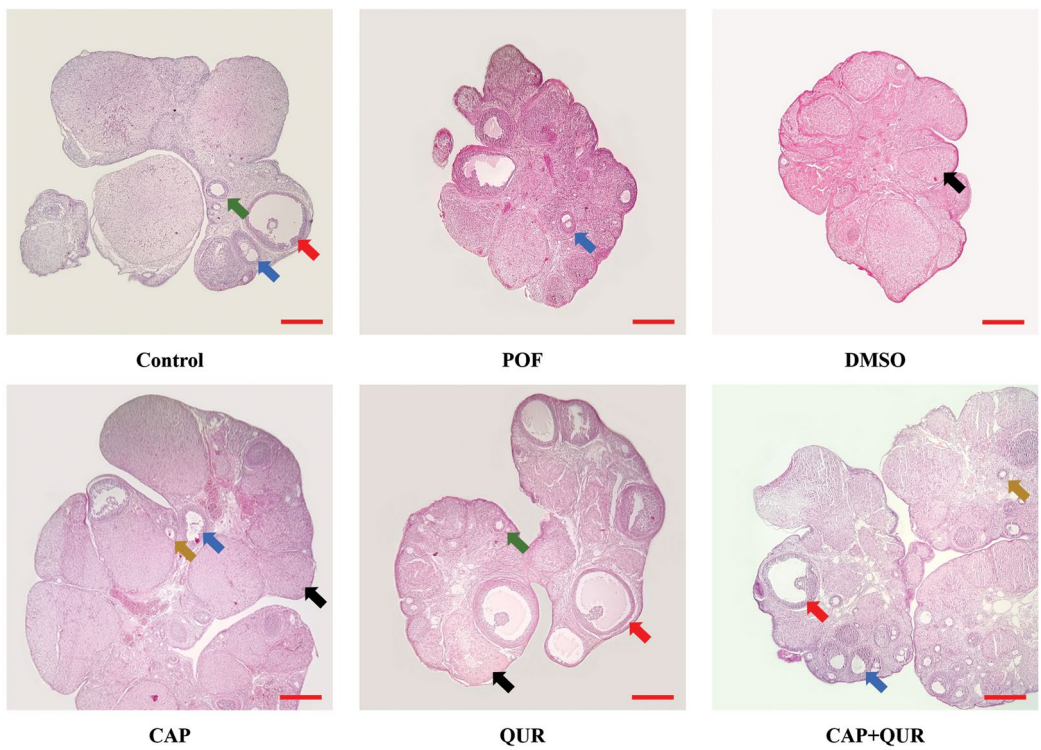

Fig.5: Photomicrograph of ovarian tissue (H&E staining; 10, scale bar: 200 µm). Primary follicles (yellow arrows), preantral follicles (green arrows), graafian follicles (red arrows), antral follicle (blue arrow), and \corpora lutea (black arrows). POF; Premature ovarian failure, DMSO; Dimethyl sulfoxide, CAP; Capsaicin, and QUR; Quercetin.

Discussion

In this study, the ovarian protective effects of CAP and QUR were investigated in a rat model of CYC -induced POF.

Chemotherapy played a causative role in ovarian reserve damage, which was associated with increased tissue oxidative stress, impaired hormone secretion and gene expression, and increased histological damage. In contrast, tissue oxidative stress parameters, ovarian reserve markers, and histopathological changes were significantly improved in rats receiving CAP and QUR alone or in combination after chemotherapy.

To the best of our knowledge, this is the first report to investigate the effects of CAP and QUR on the prevention of chemotherapy-induced ovarian damage. The results suggest that these two agents have a protective effect against CYC-induced ovarian failure. Ovarian weight of rats exposed to CYC was significantly reduced compared to the control group, which may be attributed to the ovarian toxicity of alkylating agents such as CYC. These agents generate DNA cross-links, which in turn induce DNA breaks and ultimately trigger apoptosis (28). In all treatment groups after being treated with CAP and QUR ovarian weight was significantly increased.

Roness et al investigated agents that prevent chemotherapy-induced ovarian damage and found that AS -101, AMH, imatinib, sphingosine-1-phosphate, granulocyte colony-stimulating factor, bortezomib, and multidrug resistance gene-1 play key roles in preventing chemotherapy-induced ovarian damage. They found several mechanisms of action associated with each protective agent, including prevention of follicle activation, anti-apoptosis effects, vascular effects, and upregulation of genes (29). CAP has been demonstrated to exert anti-apoptotic activity by over-activating TRPV1 (30). Tsuji and Aono (31) demonstrated that SA13353 (1-[2-(1-adamantyl)ethyl]-1-pentyl-3-[3-(4-pyridyl) propyl]urea), a novel TRPV1 agonist, inhibited tumor necrosis factor-α production by activating capsaicin-sensitive afferent neurons and reduced symptom severity in renal injury, lung inflammation, arthritis, and encephalomyelitis. These results suggest that TRPV1 agonists may act as anti-inflammatory *in vivo* in certain inflammatory and autoimmune diseases. In addition, Leonelli et al. (32) have shown that TRPV1 channels are involved in the control of early apoptosis during retinal development and that mitogen-activated protein kinase signaling may be involved in this process. Arzuman et al. (33) reported the beneficial effects of CAP and curcumin with monofunctional platinum (II) complex in platinum-resistant ovarian cancer cell lines.

In current experiment we demonstrated that CAP and QUR downregulate the expression of pro-apoptotic genes, *P53* and *BAX*, and upregulate *BCL-2* gene expression. Melekoglu et al. (34) showed that treatment with CAP and Curcumin can improve tissue oxidative stress markers. Oxidative stress leads to ovarian failure by inhibiting nuclear and cytoplasmic maturation of oocytes and inducing apoptosis (35). The mechanisms of antioxidant and anti-inflammatory effects of QUR have been reported to include alteration of Nrf2 expression at the gene and protein levels and induction of expression of phase II antioxidant enzymes. In addition, QUR can induce the Trx system and Trx/Txnip pathway by inducing the Nrf2/ARE pathway. They suggested that QUR could protect GCs from oxidative stress. CAP inhibits the activation of NF-κB and blocks the activation of Signal Transducer and Activator of Transcription 3 (induced by IL-6) in cancer cells (33).

The results of our study also confirmed the protective effects of CAP and QUR against CYC -induced ovarian failure in rat ovaries by demonstrating the antioxidant effects of CAP and QUR, including increased antioxidant activity and improved histological parameters. We demonstrated a significant improvement in TAC by CAP and QUR after chemotherapy-induced ovarian damage. Similarly, Chaudhary et al. investigated the protective effect of CAP against oxidative stress. They found that CAP had a significant protective effect against oxidative stress by increasing FRAP, GSH level and PMRS activity, and improving ROS, MDA, PCO and AOPP (36). In agreement with this study, Park et al. (37) demonstrated a protective effect of CAP against testicular injury induced by scrotal hyperthermia. They showed that pretreatment with CAP significantly suppressed oxidative stress malondialdehyde (MDA) level, phospholipid hydroperoxide GPx, heat shock 70-kDa protein 1, and manganese SOD) and heat stress- induced apoptosis in testes. The results of this study also showed that CAP and QUR improved the markers of ovarian reserve after CYC induced ovarian failure. Significant increase in AMH, E2 and progesterone levels were observed in POF+CAP, POF+QUR and POF+CAP+QUR groups compared to POF group.

Few experimental studies have examined the effects of chemotherapy-induced ovarian damage and antioxidants on markers of ovarian reserve. Özcan et al. (38) investigated the effect of resveratrol against cisplatin-induced oxidative damage to ovarian reserve in rats. They found that resveratrol significantly increased AMH levels compared to the control group. The improvements observed in ovarian reserve markers in their study suggest that resveratrol, a phenolic compound, has a beneficial effect on ovarian function recovery after chemotherapy exposure. In the present study, we demonstrated that treatments with CAP and QUR improved histological parameters in ovarian tissue subjected to treatment with CYC. The number of primary follicles, preantral follicles, antral follicles and corpus luteum was significantly reduced in POF rats compared to the control group. Similarly, Elkady et al. (39) and Melekoglu et al. (34) in separate studies showed a decrease in primordial follicles and an increase in atretic follicles in the POF model. The mechanism by which CAP and QUR protect ovarian tissue

may be related to reduced exposure to oxidative damage and reduced stimulation of TRPV receptors, which are thought to have antioxidant and anti-inflammatory activities (31).

Nowadays, using combination therapies is one of the most common strategies to achieve better clinical outcomes. Previous studies have reported the combined effects of natural compounds on altering the amount of apoptotic genes (27); therefore, due to resistance, toxicity and side effect of chemotherapeutic agents, the use of natural compounds as complementary therapies can be a promising alternative for current ineffective treatments.

Conclusion

Our results indicated that CAP and QUR show anti-apoptotic and anti-cancer effects, we confirmed that CAP and QUR alone, and in combination improved the TAC, hormone secretion, apoptosis regulatory genes expression and the number of follicles; CAP and QUR as natural occurrence antioxidants can be used to reduce and even prevent the adverse effects of chemotherapy on the ovaries in cancer patients.

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Authors' Contributions

Z.R., M.Kh.; Contributed to conception and study design. S.M., Z.R.; Contributed to all experimental work, data collection and evaluation, drafting and statistical analysis. All authors performed editing and approving the final version of this manuscript for submission, participated in the finalization of the manuscript and approved the final draft.

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