Fertility preservation in cancer patients: attenuation of chemotherapy-induced ovarian damage by oral administration of phytochemical carotenoids in an animal model

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Summary

We investigated the protective effects of a phytochemical carotenoid-rich extract obtained from Crocus sativus L., commonly known as saffron, against ovarian damage induced by cyclophosphamide (CPM). Mice received saffron extract (SE; 300 mg/kg) by oral administration for 15 days, followed by CPM treatment (100 mg/kg; SE+CPM group). The number of primordial and antral follicles decreased in CPM-mice when compared with control (p<0.001), whereas saffron administration prior to CPM partially prevented this loss. Protein expression of FOXO3a, a regulator of primordial follicle dormancy and adaptive response to oxidative stress, decreased in CPM-mice (p<0.05), whereas it was similar to control in the SE+CPM group (p=0.419). Levels of AGEs (Advanced-Glycation End-products), markers of oxidative stress and aging, were higher in CPM when compared to both control (p<0.05) and SE+CPM (p<0.05). Staining level for DNA double strand breaks increased in CPM ovaries (p<0.05), while it was similar to control in SE+CPM (p=0.194). These results provide evidence that oral administration of phytochemical carotenoids may counteract chemo-induced primordial follicle loss and oxidative damage. This approach may represent a pharmacological intervention to reduce the toxicity of alkylating agents so expanding options for fertility preservation in cancer patients.

KEY WORDS: cyclophosphamide (CPM), premature ovarian failure (POF), saffron extract, oxidative stress, FOXO3a.

Introduction

Improvements achieved in cancer treatments in the past decade have enabled cancer patients to survive at increasing rates. Nevertheless, along with their beneficial therapeutic effects, chemotherapy and radiotherapy can result in temporary or permanent loss of fertility in treated patients (1, 2). Hence, fertility planning and preservation has been integrated into oncology practice giving rise to a new discipline, the so-called oncofertility, that bridges oncology and reproductive research in order to explore and expand options for the reproductive future of cancer survivors (3). Although cryo-technologies have been providing increasing options for fertility preservation, they are suitable only for some categories of patients or are still experimental (4).
Alkylating agents, such as cyclophosphamide (CPM), are frequently administered as chemotherapeutic and immunosuppressive drugs in the treatment of a wide range of neoplastic diseases and some auto-immune diseases, such as systemic lupus erythematosus (5). CPM exerts beneficial therapeutic effects by forming covalent bonds with macromolecules, and particularly with DNA. The mechanism of action is thought to mainly involve adduct formation, as well as intrastrand and interstrand DNA cross-links, blocking the rapid replication machinery that permits the proliferation of cancer cells. Nevertheless, by disrupting antioxidant system and increasing oxidative stress, these drugs can also be harmful to quiescent cells (6). Recent studies in animal models have provided evidence that dietary antioxidant compounds during chemotherapy are efficient in reducing oxidative stress due to CPM in healthy tissues including testis (7). In the ovary, damage produced by anticancer treatments has been so far deeply characterized in terms of accelerated depletion of the primordial follicles (PF) pool leading to primary ovarian insufficiency (POI) or premature ovarian failure (POF) (8-10). Apart from follicle loss, chemotherapeutic drugs interfere with the orderly and intricate process of oocyte maturation, ovulation, fertilization, early embryonic development and implantation, thus culminating in poor reproductive health and infertility (11).

Alkylating agents are heavily toxic to the ovaries and are among the worst offenders with regard to the risk of ovarian failure (12). CPM toxicity in females has been correlated to dose, regimen and patient’s age (13), with progressively smaller doses producing ovarian failure as the patient’s age increases. In in vitro cultured mouse ovaries, CPM metabolites were found to increase double-strand DNA breaks in oocytes and granulosa cells (14) and to increase pyknosis (6, 15). Recently, it has been proposed that follicle “burnout” may be caused by increased follicle recruitment rather than PFs apoptosis. According to this model, CPM disrupts the regulatory mechanism underlying dormancy of PFs through inhibition of the transcription factor FOXO3a pathway, and stimulates follicle activation (16,17). Coadministration of AS101, an immunomodulator with chemopreventive effects in somatic tissues, promotes FOXO3a activity through downregulation of PI3K/PTEN/Akt pathway, thus reducing follicle recruitment and maintaining follicle reserve. Since FOXO3a is also involved in the activation of antioxidant genes, an imbalance of redox potential could be one of the factors contributing to CPM-induced ovarian damage (16).

Recent pharmacological studies have demonstrated that the aqueous extract of saffron (SE), rich in carotenoids, has antitumor effects and radical scavenger properties (18). Among SE constituents, crocin, crocetin and picrocrocin are the bioactive molecules mainly responsible for these pharmacological activities (19-22). By virtue of its antioxidant properties, we propose herein a possible role of SE in protecting ovary from chemotoxic effects in a mouse model. To this aim, mice received SE in combination to the alkylating agent CPM, administrated to mice by applying a single-dose protocol (100mg/Kg) in order to evaluate immediate effects of the drug (16).

Materials and methods

Saffron Spice Preparation
DOP certified Saffron (ISO 3632-1:1993) produced by Cooperativa Altopiano Navelli (Navelli, L’Aquila, Italy) was kindly provided by Agenzia per lo Sviluppo, Chamber of Commerce, L’Aquila, Italy. Briefly, 1 g of dried and ground stigma was extracted with 20 mL of water for 3 h in the dark (23). The extracts were filtered and concentrated under vacuum, and kept at 4°C until use.

Mice
Eighteen 4-to-8-week-old, body weight 20-25 g, young CD-1 female mice were obtained by Charles River Italia s.r.l. (Calco, Italy). The animals were kept under controlled conditions in accordance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH, 80-23, Tokyo) and allowed free access to standard laboratory pellet diet and water throughout the experimental period. Mice were randomly divided into three groups (6 for each):
- Group I - CTRL: normal control mice were maintained on standard laboratory pellet diet and water ad libitum, without administering medicine for 15 consecutive days. On the 15th day they received a single intraperitoneal injection of 100 µL of PBS.
- Group II - CPM: mice were maintained on standard laboratory pellet diet and water ad
libitum, without administering medicine for 15 consecutive days. On the 15th day they received a single intraperitoneal injection of CPM (100 mg/Kg).

- Group III - SE+CPM: mice received saffron extract (300mg/Kg) by using gastric gavage and allowed free access to standard laboratory pellet diet and water for 15 consecutive days. On the 15th day they received a single intraperitoneal injection of 100 µl of CPM (100 mg/Kg).

**Preparation of ovary samples**
At 24h after the administration of CPM, two mice of each group were sacrificed by cervical dislocation (in accordance with the provisions of the EEC regulation 86/609), and ovaries were immediately placed into liquid nitrogen and then stored at -80°C for further analysis. The remaining animals were sacrificed 7 day-post CPM. Ovaries were immediately placed into 4% paraformaldehyde (PFA) at 4°C overnight and subsequently dehydrated in a series of ethanol concentrations, cleared in xylene and embedded in paraffin. Ovarian sections of 5 μm were prepared for further analysis.

Considering that the period for maturation of primordial follicles to ovulatory oocyte in the mouse is 19 days, the activated PFs observed represent follicles which were previously exposed to SE and chemotherapy at the primordial stage (24).

**Hematoxylin and eosin staining and follicular classification**
Ovarian sections were deparaffinized in xylene, hydrated through a series of ethanol concentrations and stained with hematoxylin and eosin (H&E) using standard protocols and analysed under a light microscope. Blind count of number of follicles at each stage was performed in every fifth section, so that follicles would not be counted twice (24). Follicle stage was classified according to Pedersen and Peters (1968) (25), as follows: i) primordial follicle, when the oocyte was surrounded by a single layer of flattened squamous follicular cells; ii) primary follicle, when the oocyte was surrounded by a single layer of cuboidal granulosa cells; iii) secondary follicle, when there were two or three layers of cuboidal granulosa cells with no antral space; iv) antral follicle, when there were more than 4 layers of granulosa cells with one or more independent antral spaces, or when it was possible to observe the oocyte surrounded by cumulus cells.

The overall number of ovarian follicles per section was recorded. Follicles with eosinophilic (pyknotic) oocytes or granulosa cells were considered as degenerating or atretic in accordance with Desmeules and Devine (15), and were counted separately from healthy follicles.

**Immunohistochemistry analysis**
Ovarian sections were deparaffinized in xylene, hydrated through a series of ethanol concentrations and blocked with 2% BSA. Tissue sections were incubated with a primary antibody against rabbit anti-FOXO3A polyclonal antibody (Abcam, Cambridge, UK; 1:50), mouse anti-Methylglyoxal (MG)-AGE (Arg-Pyrimidine) monoclonal antibody (BioLogo, Kronshagen, Germany; 1:2000) or mouse anti-γH2AX (Abcam; 1:1000) overnight at 4 °C, and with a biotinylated anti-rabbit, anti-mouse or anti-goat IgG for 1 h at room temperature, and subsequently incubated in an ABC (avidin-biotinylated peroxidase complex) system by using the Vectastain, Elite ABC kit (Vector Laboratory, California, USA) for 30 min. The chromogenic reaction was developed by the incubation with a freshly prepared solution of amine nickel sulfate enhanced 3, 3′-diaminobenzidine (DAB) (Dako Italia, Milan, Italy) and hydrogen peroxide. Tissues were lightly counterstained with H&E and analyzed under light microscope. Negative controls were conducted on adjacent sections by omitting the primary antibodies.

In all cases, the immunohistochemical evaluations were independently performed by two researchers who were blinded to the experimental group. In all discordant cases, mutual agreement was reached. The immunoreactivity was obtained according to the formula: score = intensity x percentage of positive areas (26). The intensity was assessed as follows: very weak (1), weak (2), moderate (3), strong (4). The percentage was evaluated as follows: <10% (1), 10-30% (2), 31-60% (3), >61% (4) (27).

**Ovaries homogenization and western blotting**
Ovarian tissues were homogenized in RIPA buffer by repeated freeze/thaw cycles in liquid nitrogen. After centrifugation (33,000 rpm for
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1h at 4 °C), the supernatants were collected for protein analysis. Protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL). Protein samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Sigma-Aldrich, St. Louis, MO, USA). Non-specific binding sites were blocked overnight with 5% fat dry milk (Bio-Rad Laboratories, Italy) in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were incubated with polyclonal rabbit anti-FOXO3a (Abcam; 1:800) or mouse anti-βactin antibody (Abcam; 1:4000) for 1 h at room temperature, followed by incubation with horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (Abcam) for 1 h at room temperature. After washing, specific immunoreactive complexes were detected by ECL kit (Thermo Scientific, Waltham, MA, USA) and Uvitec Cambridge system (Alliance series, Cambridge, UK). FOXO3a bands were normalized for β-actin using ImageJ 1.44p software and values were given as relative units (RU). The experiment was performed in triplicate.

Statistical analysis

All data are presented as mean±SEM. Statistical analysis was assessed by OneWay ANOVA followed by Student-Newman-Keuls Multiple Comparison. Analyses were performed using the SigmaStat software (Jandel Scientific Corporation, San Rafael, CA, USA). P-value <0.05 was considered statistically significant.

Results

Effects of saffron administration on follicular development in ovaries of mice exposed to CPM

To assess the effect of CPM and the administration of saffron on ovarian follicle differentiation and development, we performed the histological analysis of mouse ovaries and counted the numbers of follicles at each stage after CPM or SE+CPM treatment. We identified ovarian follicles in the following developmental stages: primordial, primary, secondary, antral and atretic follicles. The mean number of follicles at each stage per ovarian section was counted and shown in Figure 1. The results showed that the CPM treatment induced a reduction of number of primordial follicles when compared to control mice (7.13±0.64 vs 21.65±1.71, P <0.001). Saffron administration prior to CPM showed to increase the number of primordial follicles in the SE+CPM group in comparison to CPM mice (14.52±1.57 vs 7.13±0.64, P <0.001), thus preserving follicle reserve. Nevertheless, the number of primordial follicles reported in the SE+CPM group was lower than that observed in the control group (14.52±1.57 vs 21.65±1.71, P <0.001).

As shown in Figure 1, CPM did not influence the number of primary follicles when compared with the control group (4.70±0.32 vs 4.95±0.63, not significant, NS). By contrast saffron administration prior to CPM promoted an increase in the number of primary follicles when compared with both CPM and control (SE+CPM vs CPM 7.00±0.55 vs 4.70±0.32, P=0.007; SE+CPM vs CTRL 7.00±0.55 vs 4.95±0.63, P=0.007).

The numbers of secondary follicles were not influenced by chemotherapy or saffron administration (CTRL 6.20±0.65, CPM 8.40±0.73, SE+CPM 7.25±0.80, NS).

Finally, the number of antral follicles was decreased in CPM ovaries when compared to control (2.00±0.21 vs 4.45±0.41, P <0.001). This reduction was prevented by SE administration prior to CPM, indeed the SE+CPM group presented values similar to the control (SE+CPM vs CPM 3.75±0.42 vs 2.00±0.21, P=0.001; SE+CPM vs CTRL 3.75±0.42 vs 4.45±0.41, NS).

Focusing on atretic follicles, we observed an increase of cells with pyknotic nuclei in ovarian section of CPM group when compared with control and SE+CPM group (CPM vs CTRL 0.46±0.09 vs 0.18±0.05, P=0.01; CPM vs SE+CPM 0.46±0.09 vs 0.25±0.07, P=0.31). Follicles with pyknotic nuclei were present in the ovaries of all three experimental classes although with significantly different frequency. In the primordial follicles, pyknosis was evident principally in the oocyte, where the nucleus appeared more intensely colored than the granulosa cells, where the nucleus-cytoplasm ratio resulted unitary. The number of granulosa cell layers in atretic antral follicles ranged from one to eight. Numerous pyknotic nuclei were mainly present in granulosa cell layers of antral follicles.
Results of immunohistochemistry

In order to identify possible beneficial effects of saffron on CPM injuries in ovaries, we performed immunohistochemical investigation of expression and distribution of FOXO3a, AGE and γH2AX.

Since activation/inhibition FOXO3a is known to suppress/activate follicle recruitment, we investigated its expression and distribution in mouse ovaries injured by chemotherapy and the beneficial effect of saffron administration. In addition, being a transcription factor, we assessed its nuclear localization as an evidence of its activated form in each samples. In all ovarian sections, FOXO3a signal was principally distributed in follicles. As shown in Figure 2, we observed a reduction of FOXO3A staining in ovaries of mice exposed to CPM when compared with control (3.45±0.59 vs 7.78±0.95, P=0.003). Moreover, saffron administration restored FOXO3a expression in the SE+CPM group when compared to the CPM group (6.30±0.82 vs 3.45±0.59, P=0.013) reaching a level similar to that observed in the control (6.30±0.82 vs 7.78±0.95, NS).

Positive effects of saffron administration was analysed by accumulation of AGEs in ovaries of mice receiving chemotherapy by MG-AGE staining. Immunohistochemical staining revealed that AGE were mainly distributed in granulosa cells of growing follicles. In addition, as shown in Figure 2, we observed that higher levels of MG-AGE adducts were produced in ovaries of mice exposed to CPM group when compared with control (11.20±0.91 vs 3.71±0.62, P<0.001). As expected, saffron administration counteracted CPM effects on MG-AGE accumulation in the SE+CPM group (CPM vs SE+CPM 11.20±0.91 vs 5.53±1.05, P<0.001). The level of MG-AGE adducts observed in the SE+CPM group was similar to the control (5.53±1.05 vs 3.71±0.62, NS).
The presence of double strand breaks to DNA was evaluated by the presence of γH2AX staining in the nucleus of ovarian cell. The γH2AX signal was predominantly observed in mature oocytes and in granulosa cells of antral follicles. As reported in Figure 2, immunoreactivity of γH2AX was found to be higher in the CPM group when compared to the control (11.40±2.27 vs 4.20±0.49, P=0.008). Saffron administration reduced γH2AX expression in the SE+CPM group in comparison to the CPM group (7.00±2.34 vs 11.40±2.27, P=0.046). γH2AX level in SE+CPM group was similar to that observed in the control (7.00±2.34 vs 4.20±0.49, NS).

**Saffron administration attenuated FOXO3a signalling in ovaries of mice exposed to CPM**

In order to evaluate whether the daily oral administration of saffron may have a protective role on follicle reserve of mouse ovaries injured by chemotherapy, we evaluated the protein expression level of FOXO3a. Results from Western Blotting analysis revealed that CPM induced a significant reduction of FOXO3a protein levels (Figure 3). As expected, saffron administration is shown to restored FOXO3a expression in the SE+CPM group, reaching the same level observed in the control.

**Discussion**

Chemotherapy affects germ cell survival and increases risk of infertility (28). Nowadays, increased rates of cure or remission obtained by anticancer treatments (29, 30) outlines the need for safe and efficient options for fertility preservation in cancer survivors. In this context, pharmacological interventions should rely on molecules that do not interfere with the efficacy of anticancer therapies. Therefore, since SE and its constituents have shown anti-proliferative ef-
Effects on numerous cancer types including those with high incidence in women of reproductive age, they may represent good candidates as protective compounds against chemoinduced gonadotoxicity (21, 31). In this context, present findings represent the first evidence for the chemoprotective potential of natural antioxidant compounds against ovarian cytotoxicity and are consistent with previous results in the male gonad and other organs in mice (21, 32, 33).

It is known that PFs are in a quiescent state by fine tuning of the PI3K/PTEN/Akt-FOXO3a pathway (17). Exposure to CPM disturbs this balance by up-regulating PI3K/PTEN/Akt axis, so leading to inactivation of FOXO3a and stimulation of follicle growth (16). According to our results, CPM not only suppresses FOXO3A activity through Akt signalling (16), but also promotes decay of FOXO3a protein levels. As expected, treatment with CPM induced strong cytotoxic effects in mouse ovaries with a drastic depletion of ovarian follicles. Seven days after CPM, primordial and antral follicles appeared to be significantly compromised, while primary and secondary follicles seemed to be less sensitive to chemotoxicity. Nevertheless, the presence of pyknotic nuclei suggests that they could be partially damaged and probably intended to undergo apoptosis. On the other hand, it is likely that the number of primary and secondary follicles also reflects the increased rate of PF activation induced by CPM. Indeed, our experiments demonstrated that SE protective effect on primordial and antral follicle pools was associated with the maintenance of FOXO3a protein at levels similar to control, suggesting that saffron components could counteract the activation of PI3K/Akt pathway induced by CPM.

Further evidence in this study demonstrated the efficacy of SE treatment in protecting the mice ovaries by CPM insult. Morphologically, in the SE group follicles showed a percentage of pyknosis lower than in the group treated with CPM alone. In addition, SE protective action against molecular injury was evidenced by the significant reduction of DNA damage, as demonstrated by decreased γH2AX signal, marker of early DNA double-strand break repair. Since this kind of DNA injury can result from ROS accumulation, the ability of SE to prevent this damage may reveal the role of oxidative stress as an effector of CPM ovarian toxicity. This is also the case of AGEs, that here we showed for the first time to be markers of CPM damage. Therefore, present data confirm that CPM accelerates the process of ovarian aging (34), and reveal that it
can be counteracted by SE. Although further research is needed, our results provide the first evidence for the efficacy of antioxidant molecules in preventing ovarian CPM-induced toxicity and for oxidative stress as one of the mechanisms by which alkylating drugs interfere with ovarian function. Overall, these findings may contribute to define biomolecular markers for evaluating safety of anticancer treatments and efficacy of chemopreventive strategies so opening new frontiers in the field of oncofertility.

References


