

Research Article

The Natural Carotenoid Crocetin and the Synthetic Tellurium Compound AS101 Protect the Ovary against Cyclophosphamide by Modulating SIRT1 and Mitochondrial Markers

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Received 28 July 2017; Accepted 1 October 2017; Published 15 November 2017

Academic Editor: Ryuichi Morishita

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Cancer therapies are associated with increased infertility risk due to accelerated reproductive aging. Oxidative stress (OS) is a potential mechanism behind ovarian toxicity by cyclophosphamide (CPM), the most ovotoxic anticancer drug. An important sensor of OS is SIRT1, a NAD⁺-dependent deacetylase which regulates cellular defence and cell fate. This study investigated whether the natural carotenoid crocetin and the synthetic compound AS101 protect the ovary against CPM by modulating SIRT1 and mitochondrial markers. We found that the number of primordial follicles of female CD1 mice receiving crocetin plus CPM increased when compared with CPM alone and similar to AS101, whose protective effects are known. SIRT1 increased in CPM mouse ovaries revealing the occurrence of OS. Similarly, mitochondrial SIRT3 rose, whilst SOD2 and the mitochondrial biogenesis activator PGC1- α decreased, suggesting the occurrence of mitochondrial damage. Crocetin and AS101 administration prevented SIRT1 burst suggesting that preservation of redox balance can help the ovary to counteract ovarian damage by CPM. Decreased SIRT3 and increased SOD2 and PGC1- α in mice receiving crocetin or AS101 prior to CPM provide evidence for mitochondrial protection. Present results improve the knowledge of ovarian damage by CPM and may help to develop interventions for preserving fertility in cancer patients.

1. Introduction

Novel management strategies have led to increased rates of cancer survivors throughout the past three decades highlighting the need of posttreatment care to improve the patient's quality of life [1]. For females, a serious long-term side effect of cancer therapies is the increased infertility risk due to

accelerated reproductive aging leading to premature ovarian failure (POF) [2]. Hence, fertility preservation has been integrated into oncology practice giving rise to oncofertility, a new discipline that bridges oncology and reproductive research [3]. Current strategies are based mainly on assisted reproductive technologies (i.e., oocyte-embryo cryopreservation and ovarian tissue cryopreservation/transplantation)

that are suitable only for few categories of patients or are still experimental [1]. Suppression of the pituitary-gonadal axis via GnRH analogue administration has been so far the most feasible intervention although results of recent clinical trials are contradictory [4–6]. One of the possible reasons for limited progress in the field is the partial understanding of the mechanistic events that could be targeted to provide protection or repair from ovotoxicity (reviewed by [7, 8]). Similar to women, studies in rodents revealed that the predominant effect of anticancer cytotoxic treatments is the total or partial loss of the finite pool of dormant oocytes in the primordial follicles at concentrations relevant to human exposures [9–11].

Clinically, the most ovotoxic drugs are the alkylating agents including cyclophosphamide (CPM). This drug is widely used for the treatment of cancers affecting females in their childhood or reproductive age, including breast cancer [5, 12]. It is also used as an immunosuppressant for autoimmune diseases and multiple sclerosis and preventing organ transplant rejection [13–15]. CPM requires hepatic bioactivation to form the active metabolite phosphoramidate mustard (PM) that covalently binds to DNA, inducing DNA-DNA, DNA-protein crosslinks, and DNA double-strand breaks (DSB). Both oocytes and granulosa cells show these types of DNA damage following *in vitro* exposure of ovaries and cells [16, 17]. The activation of an ovarian DNA damage repair response has been reported in terms of early upregulation of specific genes including ATM (ataxia telangiectasia mutated) in neonatal rat ovaries exposed to PM. This response was associated with increased levels of proapoptotic genes and follicle loss by apoptosis [18, 19]. In addition to apoptosis, CPM-induced DNA damage may also cause activation of follicle dormancy by stimulating the PI3K/PTEN/AKT signalling pathway. The upregulation of AKT signalling would lead to phosphorylation/inhibition of FOXO3a transcription factor in primordial follicles and subsequent disruption of the regulatory mechanism underlying dormancy of primordial follicles [20, 21]. *In vivo* administration of AS101 (ammonium trichloro(dioxoethylene-o,o')tellurate), an immunomodulator with antitumor effects [22], inhibits AKT phosphorylation/activation induced by CPM and prevents the loss of primordial follicles. Beneficial effects on growing follicles were also observed [20].

A further potential mechanism behind CPM ovarian toxicity is oxidative stress (OS) (reviewed by [23, 24]). In a human granulosa cell line, exposure to a pre-activated CPM metabolite results in depletion of glutathione (GSH), a crucial cellular antioxidant, a rise in reactive oxygen species (ROS) and apoptosis. Consistently, GSH exposure reduces CPM-induced granulosa cell toxicity [25]. Moreover, *in vivo* administration of CPM has been associated with low GSH content, reduced SOD2 (Superoxide dismutase 2) activity, and increased lipid peroxidation in rat ovaries [26, 27]. Oxidative stress is thought to arise from biotransformation/detoxification of PM as described by Madden and Keating [28] in *in vitro* ovarian models.

An important sensor of cell redox state is SIRT1, one of the seven members of the mammalian sirtuin family, NAD⁺-dependent enzymes with deacetylase and/or mono-

ADP-ribosyl transferase activity [29–33]. By its numerous targets, SIRT1 orchestrates cellular defence and repair mechanisms and controls cell fate avoiding survival of damaged cells [34]. Mouse oocytes upregulate SIRT1 gene to cope with OS supporting a pivotal role for this protein in the early adaptive response to OS [35, 36]. In many tissues, SIRT1 abundance can be regulated by modulating the mRNA stability by the RNA-binding protein HuR (Hu antigen R). Indeed, HuR stabilises SIRT1 transcripts and promotes their polyribosome engagement for active translation [37]. An important SIRT1 substrate is PGC1- α (peroxisome proliferator-activated receptor gamma coactivator 1- α), an activator of mitochondrial biogenesis and a key regulator of mitochondrial gene expression required to meet energetic demands during cellular stresses [38]. These activities under stress conditions are also regulated by the mitochondrial sirtuin, SIRT3, throughout a complex network [39–41].

Although OS has been proposed as an important mechanism involved in CPM ovarian toxicity, the efficacy of *in vivo* antioxidant interventions has been poorly investigated [26, 27]. Systemic treatment with some carotenoids has been largely demonstrated to protect nonmalignant tissues against CPM toxicity by promoting antioxidant and anti-inflammatory effects [42–46]. Crocetin (8,8'-diapocarotenoidic acid) belongs to the bioactive family of carotenoids derived from the stigmas of *Crocus sativus* (saffron spice) and well-known in traditional medicine [47, 48]. Crocetin is known to act as an effective free radical scavenger and lipid peroxidation inhibitor. Importantly, it effectively improved antioxidant biomarkers and attenuated inflammatory reaction [49–53]. Furthermore, crocetin is known to exert potent antitumour effects [54–56].

Based on the above observations, this work investigates whether (i) oral administration of the natural carotenoid crocetin prevents gonadotoxicity in female mice; (ii) SIRT1 is involved in the molecular pathways activated in the early ovarian response to CPM; and (iii) the protective effects of crocetin influence SIRT1 expression and mitochondrial toxicity induced by CPM. To address the first point, we tested the ability of crocetin to mitigate CPM-induced follicle loss, primordial follicle activation, and subfertility. To clarify SIRT1 involvement, we relied on a human granulosa cell line previously used to test CPM toxicity [25, 57], prior to investigation on the animal model. To gain knowledge about crocetin efficacy and mechanism of action, the fertoprotective agent AS101, known to protect the mouse ovary against CPM, was also tested [20].

2. Materials and Methods

2.1. Cyclophosphamide, Crocetin, and AS101 Preparation.

Cyclophosphamide (CPM) was obtained from Baxter, Rome, Italy. A solution of CPM at a concentration of 25 mg/mL in PBS (pH 7.4) was freshly prepared.

Crocetin isolation was performed by crocetin esters [56] and purified by an internal method of the Verdù Cantó Saffron Spain Company (Novelda, Alicante, Spain). Crocetin quantification was analysed by the reverse-phase HPLC technique. Twenty μ L of crocetin aqueous solution (252 mg/L)

was filtered through a 0.45 μm PTFE filter and injected into an Agilent 1200 chromatograph (Palo Alto, CA) operating with a 150 mm \times 4.6 mm i.d. and 5 μm Phenomenex (Le Pecq Cedex, France) Luna C18 chromatographic column, at 30°C. Eluents were water (A) and acetonitrile (B) with the following elution gradient: 20% B, 0–5 min; 20–80% B, 5–15 min; 80% B, 15–18 min; and 20%B, 18–30 min. The flow rate was 0.8 mL·min⁻¹, and the DAD detector (Hewlett Packard, Waldbronn, Germany) was set at 440 nm for the detection of *cis/trans* crocetin. Crocetin quantification was estimated using the method based on the extinction coefficient and the related area calculated according to [58, 59].

AS101 was obtained from Tocris Biosciences, Bristol, UK. A solution of AS101 at a concentration of 150 mg/mL in PBS (pH 7.4) was freshly prepared.

2.2. Mice and Study Design. A total of 69 young CD-1 female mice aged 4 to 8 weeks (Charles River Italia s.r.l., Calco, Italy) were used in the present study. All the experiments were carried out in accordance with the guidelines for the care and use of laboratory animals approved by the Animal Care Committee of the University of L'Aquila. Mice were randomly divided into four groups:

- (i) CTRL: normal control mice were maintained on a 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.
- (ii) CPM: mice were maintained on a 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).
- (iii) CRO + CPM: mice received crocetin extract (100 mg/kg, [56]) by using a gastric gavage and were allowed free access to a 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).
- (iv) AS101 + CPM: mice received AS101 (10 μg per mouse, [20]) by intraperitoneal injections on alternate days and were allowed free access to a 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).

At 12 h and 24 h after the administration of CPM, three 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 days post CPM, and ovaries were fixed in 4% paraformaldehyde (PFA) at 4°C overnight and paraffin-embedded. Ovarian sections of 5 μm were prepared for further analysis.

2.3. Haematoxylin and Eosin Staining and Follicular Classification. Ovarian sections were stained with haematoxylin and eosin (H&E) and analysed under a light microscope for differential follicle counts. Briefly, blind follicle counts were conducted on every fifth section of entire ovaries by two independent researchers. Follicle stage was classified according to [60]. The classification is based on (i) the size of the oocyte in follicles of different stages of development; (ii) the size of the follicle defined by the number of cells constituting the follicular envelope, and (iii) the morphology of the follicle. Primordial follicles are quiescent follicles characterized by a small oocyte, with a diameter of less than 20 μm , with up to 20 follicle cells attached to its surface on the largest cross-section. Growing follicles include primary follicles, characterized by one complete ring of follicle cells (21 to 60 cells on the largest cross-section) that surround a growing oocyte (diameter between 20 and 70 μm); secondary follicles, with two or three layers of follicle cells (61 to 200 cells on the largest cross section) surrounding a growing oocyte (diameter between 20 and 70 μm); and antral follicles, a fully grown oocyte (diameter 70 μm) surrounded by many layers of follicle cells separated by scattered areas or a cavity containing follicle fluid. Only those follicles in which the nucleus of the oocyte was clearly visible were considered and taken into account. The numbers were then multiplied by 5 in order to obtain an estimate of total follicle numbers per ovary [61].

2.4. Mating Protocol. The mating protocol proposed by Meirow et al. [10] in order to avoid the risk of CPM-related foetal malformations was selected. Briefly, twelve weeks after CPM, female mice from each experimental group were mated with untreated proven fertile males (two females to one male) for 1 week. Then, the females were separated for the duration of pregnancy (21 days) until 3 weeks after the birth of the litter. Females were remated every 8 weeks for a total of three successive mating rounds. The mean number of pups per mouse was counted after each mating round in all experimental groups.

2.5. In Vitro Culture of Granulosa Cells and Proliferation Analysis by BrdU Incorporation. COV434 cells are mitotic human granulosa cells used for *in vitro* studies of cytotoxic actions of chemotherapy drugs [25, 57, 62]. COV434 cells (Sigma-Aldrich, St. Louis, MO) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin and 2 mM L-glutamine. Cultures were maintained at 37°C in a CO₂ incubator with a controlled humidified atmosphere composed of 95% air and 5% CO₂. FBS, DMEM, penicillin/streptomycin, and all other reagents used for cell culture studies were purchased from Euroclone (Pero, Italy). Cells were seeded at a density of 2.5 \times 10⁴ cells per well in 96-well plates and exposed to the active CPM metabolite phosphoramidate mustard (PM, Niotech, Bielefeld, Germany) at concentrations ranging from 10 to 100 μM . The effects of PM on cell proliferation were assessed using the Cell Proliferation BrdU ELISA (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Briefly, after 60 h, 10 μL of BrdU reagent was

added to each well and the cells were cultured for 12 h at 37°C. After 72 h of culture, cells were fixed with Carnoy's fixative (3:1 methanol: glacial acetic acid) for 20 minutes at -20°C. DNA was partially digested with nucleases to allow the antibody to access BrdU; then cells were incubated with a monoclonal antibody to BrdU, followed by incubation with the anti-BrdU antibody labelled with peroxidase. Finally, the peroxidase substrate ABTS was added, in order to obtain a coloured reaction product. The absorbance of the samples was measured at approximately 405 nm with a standard microplate reader (Sunrise, Tecan, Männedorf, Switzerland).

2.6. Quantitative Real-Time PCR. Granulosa cells were exposed to 50 μ M PM for 3 h, 6 h, and 12 h, and total RNA was extracted using TRIZOL reagent (Life Technologies-Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. 1 μ g of total RNA was retro-transcribed in a final volume of 20 μ L using a cDNA Reverse Transcription kit (Life Technologies-Thermo Fisher Scientific). 2 μ L of cDNA was used for q-RT-PCRs using aCFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Milan, Italy). Primer sequences were the following: *SIRT1*: FW 5'-CAGTGTCATGGTTCCTTGC-3' and REV 5'-AGGACATCGAGGAACCTACCTG-3'; *HuR*: FW 5'-GCTATGGCTTTGTGAACCTACGTG-3' and REV 5'-TGATGTA CAAGTTGGCGTCTTTG-3'; and *RNA18S*: FW 5'-GCAGC TAGGAATAATGGAATAG-3' and REV 5'-TGGCAAAT GCTTTCGCTCTG-3'. mRNA levels were detected using a KAPA SYBR® FAST qPCR Master Mix (2X) Kit (KAPA Biosystems, Wilmington, MA, USA) according to the manufacturer's protocol. Gene expression was normalized to the housekeeping gene RNA 18S. Comparisons in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method. Two biological replicates were performed, each in technical triplicates.

2.7. Sample Preparation and Western Blot Analysis. Ovarian tissues were homogenized in RIPA buffer by repeated freeze/thaw cycles in liquid nitrogen. After centrifugation (33,000 rpm for 1 h at 4°C), the supernatants were collected for protein analysis. Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, USA). Protein samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Sigma-Aldrich). Nonspecific binding sites were blocked overnight with 5% not-fat dry milk (Bio-Rad Laboratories) in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were incubated with rabbit polyclonal anti-pAKT1 (SC-135650, Santa Cruz Biotechnology Inc., Dallas, TX, USA, 1:250), rabbit polyclonal anti-pFOXO3a (Ab47285, Abcam, Cambridge, UK, 1:700), rabbit polyclonal anti-SIRT1 (Ab12193, Abcam, 1:700), mouse monoclonal anti-HuR (SC-71290, Santa Cruz Biotechnology Inc., 1:250), rabbit polyclonal anti-SIRT3 (Ab86871, Abcam, Cambridge, UK, 1:700), rabbit polyclonal anti-SOD2 (Ab86087, Abcam, 1:1000), rabbit polyclonal anti-PGC1- α (SC-13067, Santa Cruz Biotechnology Inc., 1:500), or mouse monoclonal anti- β actin antibody (Ab8226, Abcam; 1:3000) for 1 h at room temperature, followed by incubation with horseradish peroxidase- (HRP)-

conjugated anti-rabbit (Ab6721, Abcam, 1:3000) or anti mouse (Ab6728, Abcam, 1:2000) secondary antibody for 1 h at room temperature. After washing, specific immunoreactive complexes were detected by an ECL kit (Life Technologies-Thermo Scientific) and Uvitec Cambridge system (Alliance series, Cambridge, UK). When membrane reprobing was necessary, stripping was performed by incubation in Tris-buffered saline (pH 6.7) containing 100 mM beta-mercaptoethanol, 2% SDS, at 54°C for 30 min.

Immunoreactive bands were normalized to β -actin levels using ImageJ 1.44p software. Values were given as relative units (RU). Each experiment was performed in triplicate.

2.8. Statistical Analysis. All data are presented as mean \pm SEM. Statistical analysis was assessed by one-way ANOVA followed by Holm-Sidak multiple comparison. Analyses were performed using the Sigma Stat software (Jandel Scientific Corporation, San Rafael, CA, USA). *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Pretreatment with Crocetin or AS101 Reduces CPM-Induced Follicle Loss. To assess the potential of crocetin as an attenuating agent against CPM toxicity, adult female mice were treated with a daily dose of 100 mg/kg crocetin for 15 days before administration of 100 mg/kg CPM. A group of adult female mice was treated with AS101 (10 μ g/mouse) on alternate days beginning 15 days before CPM treatment. Quantification of the different follicle populations was carried out in the ovaries 1 week after CPM administration. As shown in Figure 1(a), mice receiving crocetin prior to CPM retained a number of primordial follicles larger than CPM alone and not significantly different from untreated controls. Quantification of primordial follicles also revealed that the crocetin effect was similar to AS101. A number of growing follicles in mice receiving crocetin or AS101 prior to CPM was significantly larger than CPM alone reaching values similar to untreated controls.

3.2. Crocetin and AS101 Treatments Rescue Fertility in CPM-Treated Mice. To confirm that preservation of primordial follicles observed in CPM-treated mice receiving crocetin or AS101 was associated with increased fertility, mice from each experimental group were mated for three rounds. At the time of mating, all females had normal oestrous cycles, had similar weight, and appeared healthy. Our data show that a single dose of 100 mg/kg CPM did not affect the reproductive capability of mice until the third round of mating. At this time, we obtained no litter from the CPM mice since they were unable to get pregnant after one week of caging with males. By contrast, CPM-treated mice receiving crocetin or AS101 got pregnant after all mating rounds and presented a litter size not significantly different from the control group (Figure 2).

3.3. Effects of Crocetin and AS101 on PI3K/AKT/FOXO3A Pathway. To search for molecular mechanisms underlying protective effects of crocetin, we hypothesized a potential effect on the PI3K/AKT/FOXO3A pathway underlying

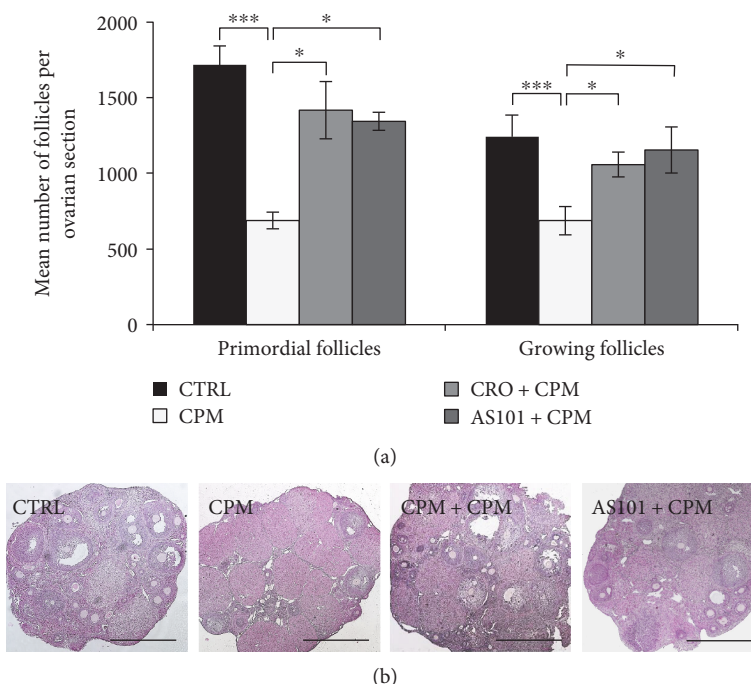


FIGURE 1: Crocetin reduces follicle loss after CPM treatment. (a) Differential follicle count was conducted on ovaries removed from adult (8 weeks old) female CD1 mice ($n=3$, per experimental group) 1 week after CPM treatment preceded or not by crocetin or AS101 pretreatment. Follicles were classified as quiescent primordial follicles or growing follicles. Data represent means \pm SEM. One-way ANOVA ($P < 0.001$), followed by multiple comparison by Holm-Sidak ($***P < 0.001$; $*P < 0.05$). (b) Representative histological sections of ovaries from the CTRL, CPM, CRO + CPM, and AS101 + CPM groups showing follicle reserve. Scale bars: 400 μ m.

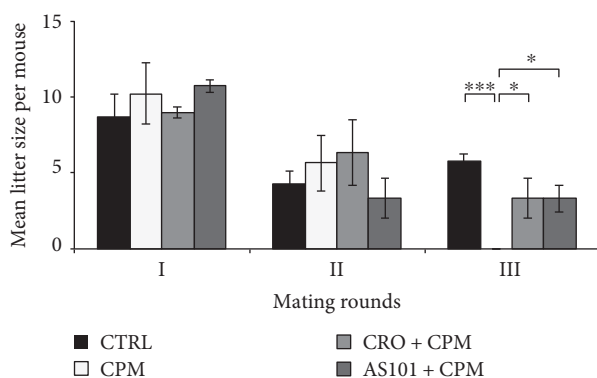


FIGURE 2: Crocetin and AS101 treatments rescue fertility in CPM-treated mice. Reproductive outcomes were assessed over three successive mating rounds in mice ($n=9$, per experimental group) that received CPM treatment preceded or not by crocetin or AS101 pretreatment. Litter size was counted after each mating round. Data represent means \pm SEM. One-way ANOVA ($P < 0.001$), followed by multiple comparison by Holm-Sidak ($***P < 0.001$; $*P < 0.05$).

primordial follicle activation. By looking at the effects at protein expression level, we found that CPM increased the activated form of AKT protein (pAKT) phosphorylated by PI3K and the inactive form of FOXO3a (pFOXO3a) phosphorylated by pAKT. By contrast, crocetin and AS101 treatment results in reduction of both pAKT and pFOXO3a

when compared with CPM alone (Figures 3(a) and 3(b)). However, crocetin effect was more pronounced than AS101 and similar to untreated control.

3.4. SIRT1 Protein and Its Regulator HuR Are Increased during the Early Response to CPM. To investigate the involvement of SIRT1 in the response to CPM, we conducted the first set of experiments in human granulosa cells aimed to analyse changes in mRNA levels of *SIRT1* and *HuR* after 3 h, 6 h, and 12 h of PM exposure. At first, we carried out experiments in order to identify the minimum effective dose of PM, the active metabolite of CPM. After 72 h of treatment, proliferation was significantly reduced in human granulosa cells cultured in the presence of 50 μ M PM. Since no further inhibition was observed at 100 μ M PM, ($P < 0.001$, Figure 4(a)), we employed the dose of 50 μ M PM in gene expression experiments. Then, we analysed the expression level of *SIRT1* and of *HuR* in COV434 cells exposed to 50 μ M PM for 3 h, 6 h, and 12 h. Both genes increased their expression level in a time-dependent manner, suggesting the activation of the OS response in this cell line (Figures 4(b) and 4(c)).

In the second set of experiment, mice received CPM and were sacrificed at 12 h and 24 h after CPM treatment. Our results show that SIRT1 protein level increased at 12 h and doubled its expression at 24 h after CPM treatment (Figure 5(a)). HuR protein increased at 12 h after CPM to decrease at 24 h (Figure 5(b)).

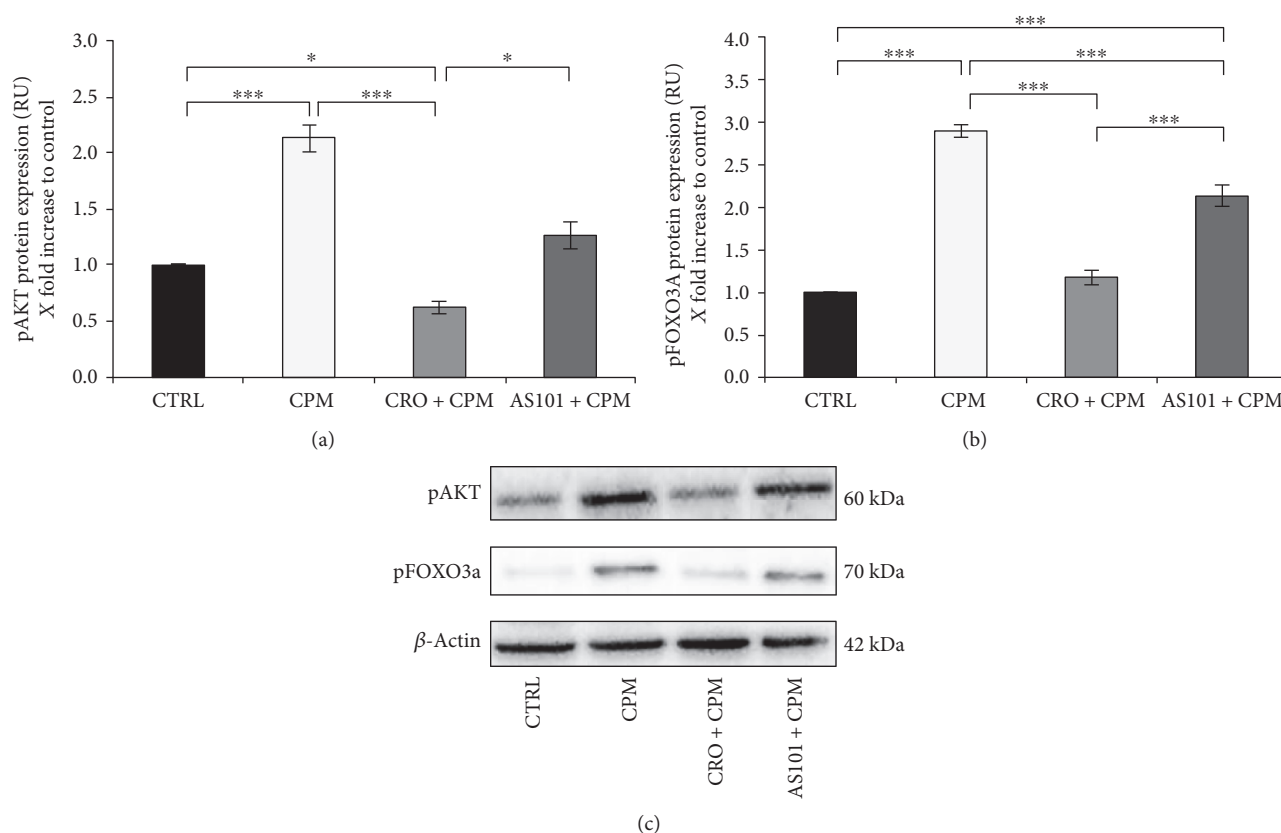


FIGURE 3: Crocetin reduces CPM-induced phosphorylation of key proteins in the PTEN/PI3K/Akt pathway. Protein analysis was conducted on ovaries from 8-week-old mice ($n = 3$, per experimental group) removed 24 hours after a single dose of CPM preceded or not by crocetin or AS101 pretreatment. Western blots of pAKT (a) and pFOXO3a (b) and representative images (c). Fold change is represented as a bar graph. Experiments were repeated three times with similar results. One-way ANOVA ($P < 0.001$), followed by multiple comparison by Holm-Sidak (** $P < 0.001$; * $P < 0.05$).

3.5. Crocetin and AS101 Prevent the SIRT1 Upregulation Induced by CPM. In the second set of experiments, we tested whether crocetin and AS101 act by modulating the early adaptive response regulated by SIRT1. Our data showed that crocetin treatment prevented the increased expression of SIRT1 induced by CPM treatment (Figure 6). Furthermore, mice receiving crocetin prior to CPM presented a SIRT1 expression level equivalent to untreated controls. AS101 induced a reduced activation of SIRT1 protein production in comparison to CPM treatment, but its levels were enhanced when compared to crocetin and untreated control mice.

3.6. Crocetin and AS101 Prevent CPM-Induced Changes in Mitochondrial Markers. To test whether the protective effect of crocetin was exerted throughout regulation of mitochondrial markers, we analysed the expression level of SIRT3, SOD2, and PCG1alpha. Similar to SIRT1, SIRT3 increased at 24 h following CPM (Figure 7(a)). Crocetin treatment prevented the SIRT3 increase induced by CPM although SIRT3 amount was lower than that observed in untreated controls. AS101 treatment promoted a similar effect. Moreover, CPM was found to significantly reduce SOD2 protein. Both crocetin and AS101 treatments attenuated this effect although

SOD2 level was lower than that observed in untreated controls (Figure 7(b)). Our results also showed that CPM mice presented lower levels of PCG1- α protein when compared with control whereas crocetin and AS101 induced a threefold increase of this protein in comparison to untreated controls (Figure 7(c)).

4. Discussion

Considering the increment in survival rates of cancer patients in their childhood or reproductive age, searching for fertoprotective agents is the main challenge in oncology practice. Here, we demonstrate for the first time that oral administration of crocetin, a natural carotenoid derivative and effective free radical scavenger, attenuates CPM-induced gonadotoxicity and modulates molecular pathways involved in the early ovarian response to this anticancer drug. These observations provide strong evidence that an imbalance of redox potential is the main factor underlying CPM-induced ovarian damage. Moreover, we outlined that crocetin effects at both molecular and biological levels resembled those by AS101, the most relevant candidate as a fertoprotective agent.

Crocetin, the main bioactive saffron compound [63, 64], is the hydrolysed active form of crocin, which is the most

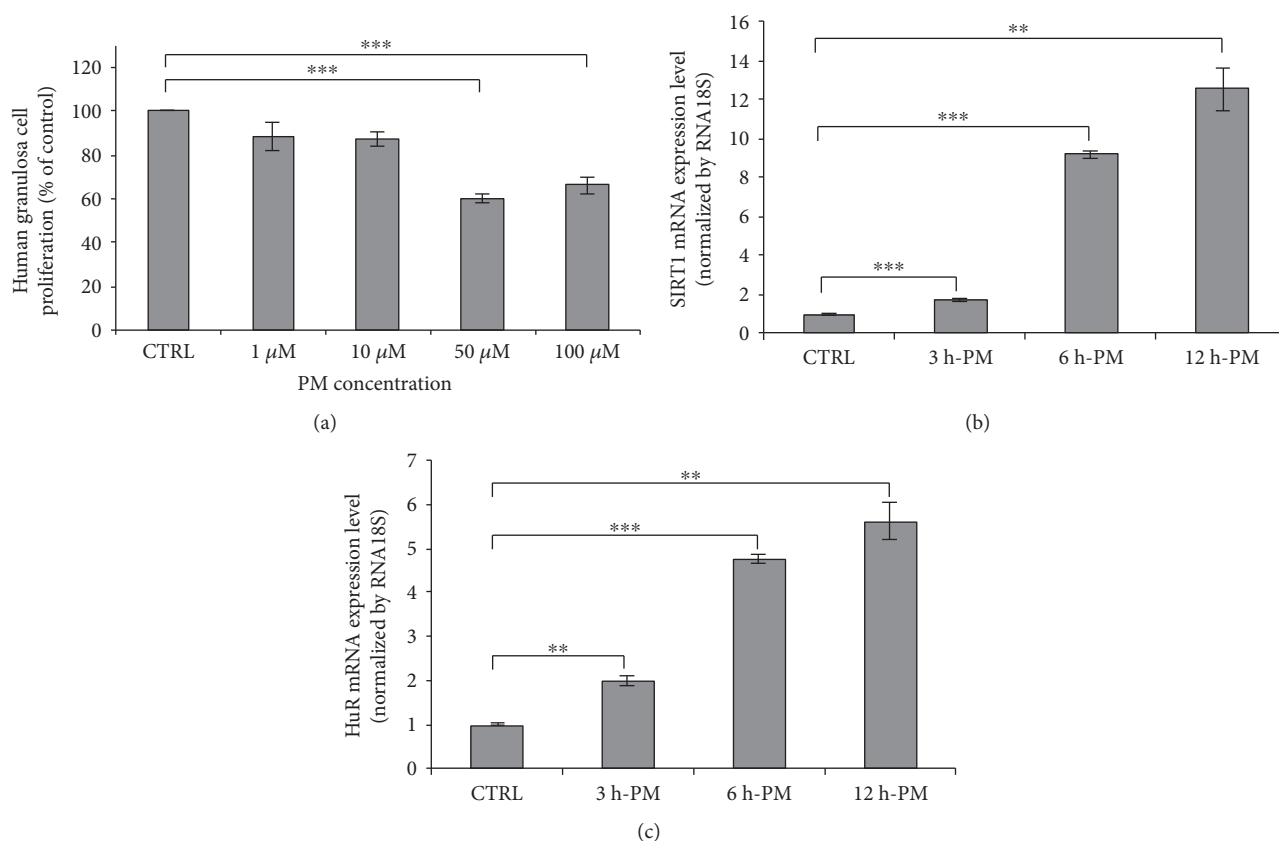


FIGURE 4: SIRT1 and HuR mRNA increase after a PM treatment in human granulosa cell. (a) After 72 h of treatment, human granulosa cells cultured in the presence of 50 μ M PM showed a significant reduction of proliferation. (b) SIRT1 and (c) HuR mRNA expression levels after 50 μ M PM treatment were evaluated by performing a q-RT PCR. RNA18S was used as an endogenous control. Data represent means \pm SEM. Experiments were done in triplicate ($n = 3$). ** $P < 0.01$, and *** $P < 0.001$.

investigated saffron carotenoid. In comparison with crocin, crocetin is more rapidly absorbed in the intestinal tract and exhibits greater efficacy [65]. It is well established that the therapeutic effects of crocetin against some types of cancers, including breast cancer, have been pointed out [47, 48, 54–56, 66–69]. Crocetin acts in a dose-dependent manner in *in vitro* models [47, 55, 67, 68]. Oral administration of crocetin has been employed in research on mice aimed to investigate its effects against retinal damage [70] and tumour growth [56, 69]. Crocetin has also been administrated to healthy adult human volunteers, demonstrating that it is beneficial also in humans [71, 72]. Although only one paper reported the prevention by crocetin of CPM side effects on the bladder and liver [73], a plethora of publications reveals that crocetin can provide protection against OS induced by toxicants or underlying disorders in numerous organs, tissues, and cells [49–52, 74–78].

Our data show that crocetin protects primordial and growing follicles from CPM injury and the mechanism underlying the protective action of crocetin is similar to that of AS101. Crocetin treatment results in the reduction of the phosphorylated/activated form of AKT protein (pAKT) and the phosphorylated/inactive form of FOXO3a (pFOXO3a) when compared with CPM. According to our results, crocetin treatment was more effective in reducing the activation

of the PI3K/AKT/FOXO3a pathway in response to CPM since pAKT and pFOXO3a levels were lower than that observed in AS101 although this difference did not result in higher efficacy in terms of follicle survival. Thus, it would be suggested that crocetin, similar to AS101, prevents dysregulation of follicle activation induced by CPM. Nevertheless, further investigation is needed to clarify whether crocetin, similar to AS101 [20], is able to reduce follicle apoptosis induced by CPM.

In addition to its role in follicle activation, FOXO3a is known to activate an antioxidant response when deacetylated by SIRT1. However, this function cannot be exerted when FOXO3a is phosphorylated by AKT. Thus, we can hypothesize that the increased levels of pFOXO3a in CPM ovaries may compromise the antioxidant response orchestrated by SIRT1.

SIRT1 plays a critical role in coordinating cellular response to stress, and SIRT1 levels are upregulated by cellular stressors, including metabolic, genotoxic, and oxidative stress [79]. Indeed, in our model of human granulosa cells sensitive to antiproliferative effects of CPM, Sirt1 and HuR transcription gradually increases with the same kinetics, demonstrating the involvement of SIRT1 in the early steps of cell response to CPM damage. This conclusion has been confirmed in the animal model where we observed that as

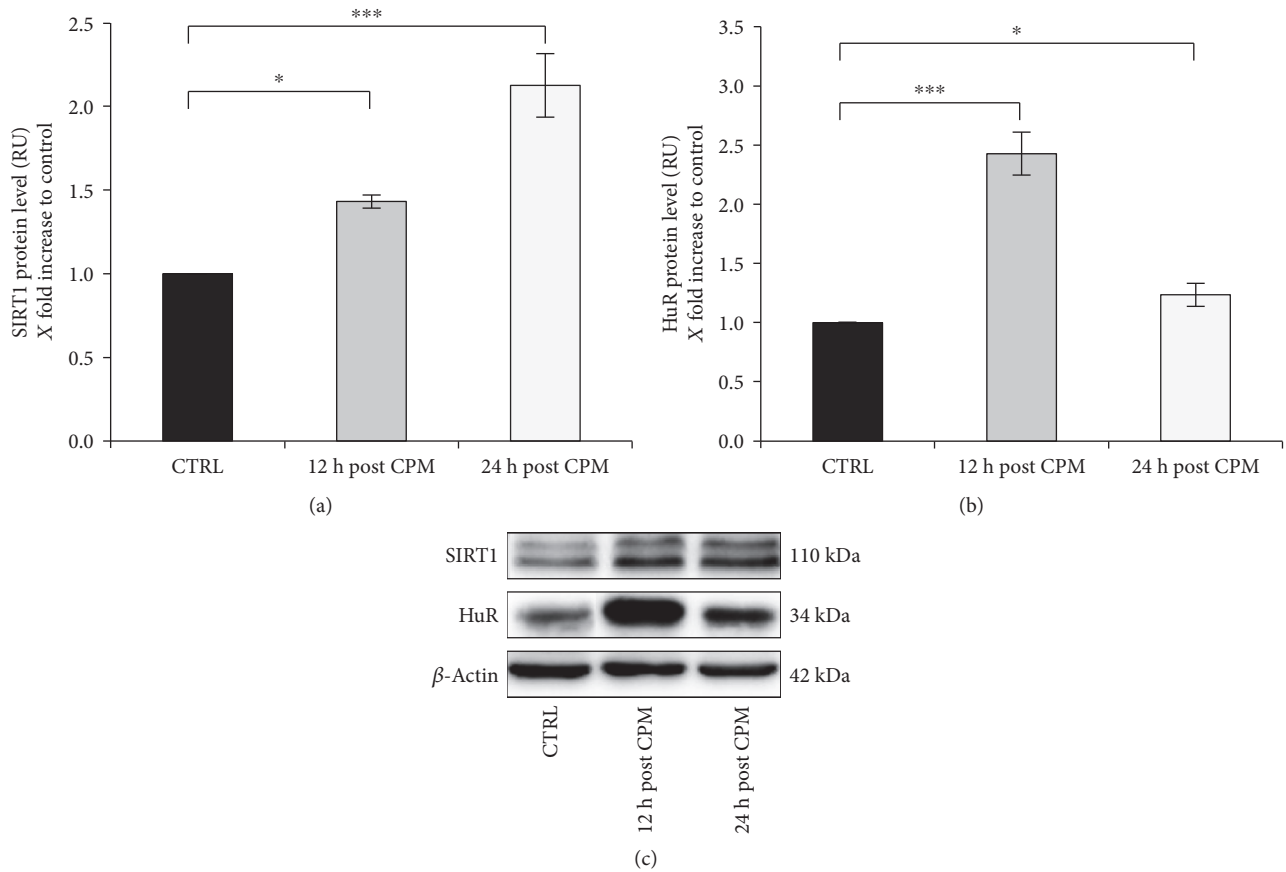


FIGURE 5: CPM treatment induces an increase of SIRT1 and HuR. Protein analysis was conducted on ovaries from 8-week-old mice ($n = 3$, per experimental group) removed 12 or 24 hours after a single dose of CPM. Western blots of SIRT1 (a) and HuR (b) and representative images (c). Fold change is represented as a bar graph. Experiments were repeated three times with similar results. One-way ANOVA ($P < 0.001$), followed by multiple comparison by Holm-Sidak (** $P < 0.001$; * $P < 0.05$).

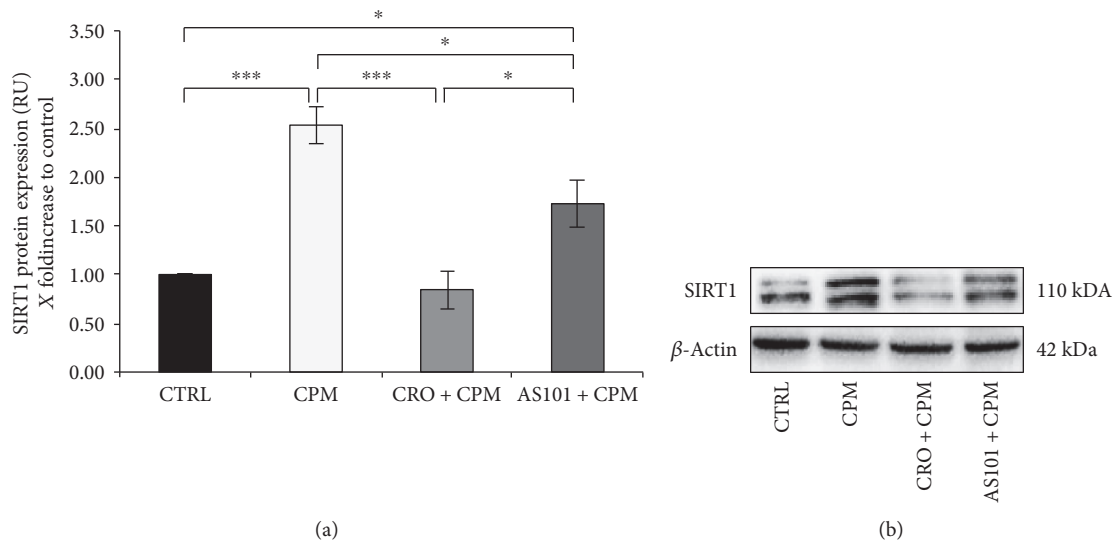


FIGURE 6: Crocetin and AS101 reduce CPM-induced activation of SIRT1 protein in the ovary. Protein analysis was conducted on ovaries from 8-week-old mice ($n = 3$, per experimental group) removed 24 hours after a single dose of CPM preceded or not by crocetin or AS101 pre-treatment. Western blots of SIRT1 (a) and representative images (b). Fold change is represented as a bar graph. Experiments were repeated three times with similar results. One Way ANOVA $P < 0.001$, followed by Multiple comparison by Holm-Sidak: *** $P < 0.001$; * $P < 0.05$.

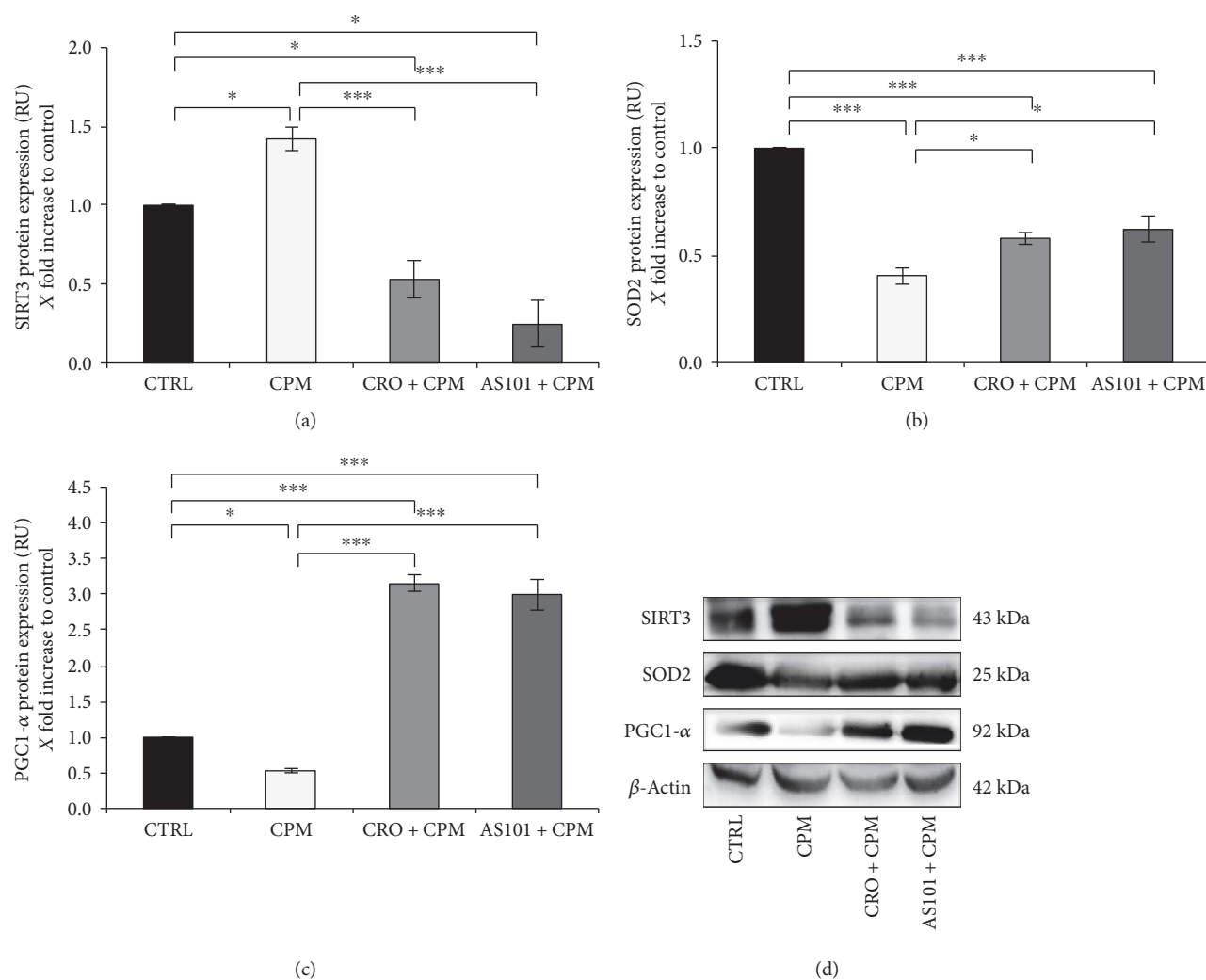


FIGURE 7: Crocetin and AS101 prevent CPM-induced mitochondrial damage assessed by SIRT3, SOD2, and PGC1- α protein levels in the ovary. Protein analysis was conducted on ovaries from 8-week-old mice ($n = 3$, per experimental group) removed 24 hours after a single dose of CPM preceded or not by crocetin or AS101 pretreatment. Western blots of SIRT3 (a), PGC1- α (b), and SOD2 (c) and representative images (d). Fold change is represented as a bar graph. Experiments were repeated three times with similar results. One-way ANOVA ($P < 0.001$), followed by multiple comparison by Holm-Sidak (** $P < 0.001$; * $P < 0.05$).

early as 12 h following CPM administration, the ovary activates an adaptive response based on upregulation of SIRT1 protein that peaks at 24 h. This pattern is preceded by upregulation of HuR which peaks at 12 h to decrease at 24 h indicating that in response to stress HuR may act transiently to stabilise SIRT1 transcripts prior to be degraded [80]. Nevertheless, other roles for HuR cannot be excluded. Posttranscriptional function of HuR has been described for a wide number of transcripts bearing AU-rich elements whose turnover is critical for cell fate [81–83].

Previous reports described a reduction of SIRT1 levels in rat ovaries in response to CPM and the protective role of caloric restriction associated with increased SIRT1 expression [84]. This is not in contrast with our results since we focused on the evaluation of the early response assessed before biological damage whereas the reduction of SIRT1 levels described elsewhere could be related to depletion of follicle population following CPM treatment.

Moreover, SIRT1 has been recently involved in the regulation of bioenergetics metabolism during folliculogenesis. In this regard, Cinco et al., [85] observed that oocyte expression of SIRT1 is increasing during primordial follicle awakening together with a significant increase of NAD^+ . This is related to the decreased $NADH/NAD^+$ levels resulting from the activation of oxidative phosphorylation for energy supply during growth. Thus, the increased levels of ovarian SIRT1 during the early phase of CPM damage may be the cause or the effect of primordial follicle activation leading to the burnout effect.

Our data showed that both crocetin and AS101 prevented the increased expression of SIRT1 induced by CPM. This could be ascribed to their ability to counteract CPM-induced changes in the redox potential leading to SIRT1 recruitment. However, the observation that levels of SIRT1 in the two groups are not the same would suggest that crocetin and AS101 have a different ability to modulate ovarian

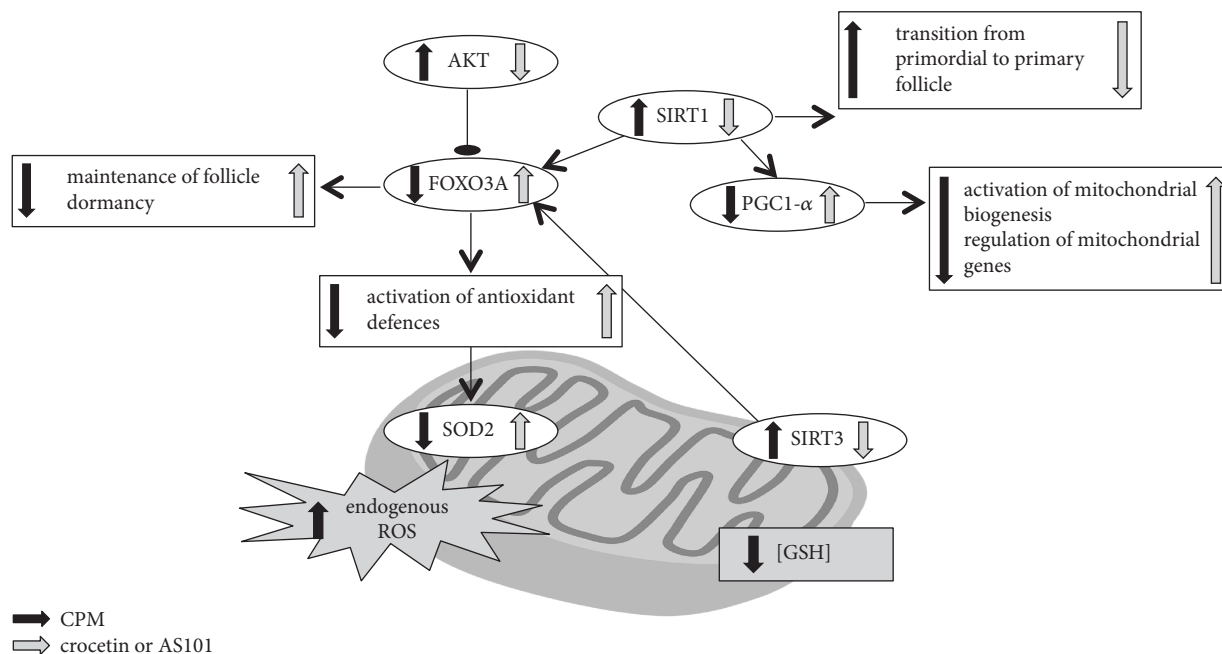


FIGURE 8: Possible mechanisms through which crocetin and AS101 reverse the early adaptive response of the ovary to CPM. Based on our results and current literature, CPM causes the activation of the PI3K/AKT/FOXO3A signalling pathway that ends with the activation of primordial follicles (“burnout” hypothesis). Increased oxidative stress, which probably arises from reduced GSH due to ovarian PM detoxification, is evidenced by reduced SOD2 levels and upregulation of the OS sensors SIRT1 and SIRT3. Upregulation of SIRT1 may contribute to CPM-induced follicle activation; upregulation of SIRT3 along with reduction of PGC1- α and SOD2 marks the CPM-induced mitochondrial damage. By contrast, the finding that crocetin and AS101 exert fertoprotective effects, prevent SIRT1 and SIRT3 rise, and maintain pFOXO3a, PGC1- α , and SOD2 levels provides evidence for a link between the burnout hypothesis, oxidative stress, and mitochondrial damage.

physiological environment, which influences SIRT1 expression. The hypothesis of the oxidative basis of CPM ovarian damage is supported by the finding that crocetin and AS101 prevented CPM-induced mitochondrial toxicity. In particular, crocetin and AS101 treatment counteracted the upregulation of the mitochondrial sirtuin SIRT3 induced by CPM. SIRT3 is considered a key coordinator of mitochondrial energy metabolism under stress conditions by directly targeting and modulating various processes [39–41] and downregulating mitochondrial protein synthesis [86]. Further evidence of OS and its mitochondrial basis are the downregulation of PGC1- α and SOD2 in CPM ovaries and the observation that these mitochondrial proteins severely increased their expression following crocetin and AS101 administration.

Therefore, it can be speculated that SIRT1 is recruited in order to regulate cell fate following CPM injury. In the experimental model proposed here, it seems to fail to orchestrate an efficient repair in concomitance with SIRT3, probably because its substrates, such as FOXO3a and PGC1- α , or downstream effectors, such as SOD2, are impaired. For these reasons, we propose that SIRT1 could be considered as a marker of CPM ovarian injury. Given its role as a sensor of damage and effector of cell fate, caution should be taken in proposing attenuating therapies based on SIRT1 targeting.

The inhibition of CPM-induced follicle loss by AS101 has been ascribed to its ability to inhibit AKT activation and reduce apoptosis in large growing follicles [20]. The finding

that a potent antioxidant such as crocetin exerts similar effects on the PI3K/AKT/FOXO3a pathway suggests that AS101 may also act upstream to this pathway by preventing the OS burst induced by CPM. This hypothesis is also supported by the observation that, similar to crocetin, AS101 prevents the activation of OS sensors and mitochondrial toxicity induced by CPM. It is also important to consider that beneficial effects by both crocetin and AS101 may be ascribed to their effects on the whole ovarian microenvironment. In this regard, crocetin’s ability to increase diffusion of oxygen through the plasma can contribute to counteract CPM-induced inhibition of follicular microvascularization [87]. Moreover, anti-inflammatory properties are known for both these agents [50, 53, 88, 89] although these effects in the ovary need to be investigated.

The present study represents a contribution to previous evidence in animal models about the use of saffron bioactive molecules in the fertility field. Recent investigations have revealed that crocetin and its derivative crocin have beneficial effects on *in vitro* oocyte maturation, sperm quality, and fertilization [90–94]. Moreover, crocin has been described to protect male gonad against CPM toxicity [46].

In conclusion, we speculate that the ovarian adaptive response to CPM consists in a complex network involving oxidative stress, SIRT1, and mitochondrial damage (Figure 8). Finally, due to its efficacy in the animal model and its anti-cancer properties and low toxicity in humans, this work demonstrates for the first time that crocetin presents all

the characteristics required for a natural fertoprotective agent to be included in future clinical studies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The funds for this work were provided by the Department of Life, Health and Environmental Sciences, University of L'Aquila (RIA 2013-2016).

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