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Cyanidin-3-*O*-glucoside promotes progesterone secretion by improving cells viability and mitochondrial function in cadmium-sulfate-damaged R2C cells

Xia Li[b,1], Jun-Li Lu[b,1], Jian-Xia Sun[a,**], Xin-Wei Jiang[b], Xu-Sheng Li[b], Ye Li[c], Rui Jiao[b], Ling-Min Tian[b], Wei-Bin Bai[b,*]

[a] Faculty of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou 510006, China
[b] Department of Food Science and Engineering, Jinan University, Guangzhou 510632, China
[c] Environment College, Shenyang University, Shenyang 110044, China

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ABSTRACT

Cadmium (Cd) is a poisonous metal that is toxic for male reproduction. Cyanidin-3-*O*-glucoside (C3G) as typical anthocyanin benefits many organs. In this study, we investigated the protective effects and associated underlying mechanisms of C3G against the toxicity of Cd on male reproduction in rat Leydig cell line R2C cells. Cells were pre-protected with C3G (5–160 μmol/L) for 2 h and then treated with cadmium sulfate ($CdSO_4$) (10–160 μmol/L) for 24 h. The results showed that cytotoxicity, mitochondrial damage, superoxide dismutase 2 (SOD2), and overproduction of reactive oxygen species (ROS) in $CdSO_4$-treated R2C cells were significantly reduced with C3G pre-treatment. Moreover, C3G pre-treatment led to upregulated expression of steroidogenic acute regulatory (StAR) protein and progesterone production. Our study suggests that C3G may be a potential therapeutic agent against Cd-induced reproductive toxicity.

1. Introduction

Cadmium (Cd) is an environmental pollutant, produced mainly by various industries. Sources of humans exposure to Cd may include industrial emissions, smoking, and dietary intake (Hogervorst et al., 2007). Cd has been identified as an essential items in the Food Contaminant Monitoring and Evaluation Program in the Global Environment Monitoring System (GEMS) (Jiang et al., 2007). A person can absorb about 1 μg Cd via food every day, and 1–3 μg of Cd from a daily pack of cigarettes (Waalkes, 2003). Recent epidemiological evidence suggests that increased environmental Cd exposure leads to increased overall mortality, and weekly Cd intake is close to weekly Cd tolerance for humans in Europe (Chain, 2011). The half-life of Cd ranges from 20 to 40 years *in vivo*, therefore Cd can accumulate in the body. Cd causes serious acute or chronic poisoning to the liver, kidney, lung, pancreas, bones, testes and ovaries (Matović et al., 2011; Thompson and Bannigan, 2008).

Recent studies illustrated that Leydig cells, which produce testosterone, are associated with reproductive toxicity of Cd in males (Ogawa et al., 2013). Cd stimulates reactive oxygen species (ROS) production, damages mitochondria, and decreases levels of cyclic adenosine monophosphate (cAMP) and steroidogenic acute regulatory (StAR) protein levels in Leydig cells. These effects cause steroidogenic disruption and impaired progesterone or testosterone synthesis in the testis (Siu et al., 2009; Zhang et al., 2011). Superoxide dismutase (SOD) can prevent toxicity and lipid peroxidation due to active oxygen species and thus reduces ROS effects (Nazima et al., 2016).

Anthocyanins are widely found in many fruits, vegetables, flowers, cereals and other plant-derived foods (Sun et al., 2009). Anthocyanin intake among Americans is about 12.5 mg/d (Wu et al., 2006), and anthocyanin daily consumption in adult Korean males can reach 23.58 mg/d (Kim et al., 2015). There is growing evidence that the

Abbreviations: AAPH, 2,2′-Azobis(2-amidinopropane)-dihydrochloride; ATP, adenosine triphosphate; C3G, cyanidin-3-*O*-glucoside; cAMP, cyclic adenosine monophosphate; CYP17, 17α-hydroxylase; CAT, chloramphenicol acetyltransferase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; MMP, mitochondrial membrane potential; P450scc/CYP11A1, cytochrome P 450 side-chain cleavage enzyme; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; StAR, steroidogenic acute regulatory protein; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17βHSD, 17β-hydroxysteroid dehydrogenase

* Corresponding author.
** Corresponding author.
E-mail addresses: jxsun1220@163.com (J.-X. Sun), baiweibin@163.com (W.-B. Bai).
[1] These authors contributed equally for this work.

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consumption of anthocyanin-rich fruits and vegetables is beneficial for human health with great biological effects (Burton-Freeman, 2010; Kalt, 2004; Zhang et al., 2018). Anthocyanins have antioxidant, anti-inflammatory, anti-apoptotic, anticancer (Darren et al., 2010; Gerd et al., 2006), and antibacterial activities (Mobin et al., 2018). They also play an important role in preventing skin photodamage, neurological and cardiovascular diseases, and they regulate lipid and glucose metabolism.Many *in vitro* and *in vivo* models show that anthocyanins are strong antioxidants that can: scavenge free radicals (Kähkönen and Marina, 2003; Kahle et al., 2010; Kulisic-Bilusic et al., 2009; Tsuda et al., 2003); reduce ROS production; reduce protein and lipid peroxidation; improve performance of the antioxidant defense system; stabilize DNA; improve mitochondrial function; and ultimately reduce oxidative stress (Alvarez-Suarez et al., 2017; Giampieri et al., 2014; Schantz et al., 2015). Anthocyanins downregulate mRNA and protein expression of inflammatory biomarkers (Cassidy et al., 2015; Cipriano et al., 2015). Cyanidin-3-*O*-glucoside (C3G), peonidin-3-glucoside, anthocyanin-rich Sveva strawberry polyphenol extract and acerola crude extract significantly increased the viability of endothelial cells or human dermal fibroblasts and reduced apoptosis (Alvarez-Suarez et al., 2017; Giampieri et al., 2014; Paixão et al., 2011).

Anthocyanins inhibited tumor cell growth, regulated immune cells, inhibited tumor development and tumor diversity, reduced self-renewal capacity, and induced apoptosis in a series of tumor cells (Bilal Bin et al., 2008; Charepalli et al., 2016; Cianciosi et al., 2018; Marko et al., 2004; Naomi et al., 2003; Olsson et al., 2004; Pan et al., 2018; Pu et al., 2008). Anthocyanins also: protected against hypercholesterolemia-induced lipid profile alterations (Olorunnisola et al., 2016); regulated postprandial lipemia, blood glucose/insulinemia (Burton-Freeman, 2010); prevented skin diseases (Alvarez-Suarez et al., 2017; Giampieri et al., 2014), atherosclerosis and cardiovascular disease (CVD) (Gaiz et al., 2018); and inhibited calcium cation (Ca^{2+}) elevation and neurodegeneration in prenatal rat hippocampal neurons (Paixão et al., 2011; Shah et al., 2013). Therefore, anthocyanins are excellent dietary supplements that can protect health and reduce the risk of disease.

C3G, one of the most widely distributed anthocyanins in nature, is the main pigment in blackberry, peach, elderberry and mulberry (Piotr et al., 2012), and also exists in gooseberry, black Aestivalis grape, nectarine, red pomegranate, and eggplant (Olivas-Aguirre et al., 2016). Several studies showed that C3G exhibits health-promoting effects including significant antioxidant, cardio-protective, anti-inflammatory, neuroprotective, and anticancer properties. C3G is an effective dietary supplement for inhibiting ultraviolet A (UVA) or UVB-induced skin injury (He et al., 2017a; Wu et al., 2018), regulating fatty acid metabolism in the liver (Guo et al., 2012), and improving diabetes and diabetic nephropathy (Qin et al., 2018; Rupasinghe et al., 2018). Bell et al. proved that C3G-rich haskap berries had cognitive benefits (Bell and Williams, 2018).

Interestingly, C3G has been reported to reduce testicular oxidative stress and apoptosis, protect the blood-testis barrier, and improve sperm quality (Jiang et al., 2018). Previous studies showed that flavocoxid, a flavonoid derivative, reduced the testicular injury caused by Cd (Minutoli et al., 2015). However, it is unknown whether anthocyanins have protective effects on Cd-caused reproductive injury. Herein, we investigated the effect of C3G on Cd-damaged R2C cells and evaluated the possible underlying mechanisms.

2. Materials and methods

2.1. Materials

Cadmium sulfate ($CdSO_4$), C3G (purity ≥ 98%), and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco (Rockville, MD, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from AMRESCO Inc. (Solon, OH, USA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) was obtained from Beyotime Institute of Biotechnology (Nantong, China). The radioimmunoassay kit was purchased from Beijing North Institute of Biological Technology (Beijing, China). The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) kit was purchased from MDBio (Taipei, Taiwan). The bicin-choninic acid protein assay (BCA) kit was purchased from Pierce (Rockford, IL, USA). Rabbit anti-SOD2 and anti-StAR antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Ultrapure water was supplied by a Milli-Q purification system (Millipore Co., Billerica, MA, USA). The Rat Leydig cell line (R2C cells) were provided by the Chinese Center of Typical Culture Conserve, Wuhan, P. R. China (CCTCC) and were cultured in DMEM-F12 medium containing sodium pyruvate, sodium bicarbonate ($NaHCO_3$), 15% HS, 3% FBS, and a 1% penicillin/streptomycin mixture with 5% carbon dioxide (CO_2) at 37 °C.

2.2. Cell viability assay and cell morphological observation

An MTT assay was performed to evaluate cell viability based on the method by Sun et al. (2013). R2C cells were seeded at 8×10^3/well in 96-well plates (Costar, Cambridge, MA, USA) and cultured with 5% CO_2 at 37 °C for 24 h. Then the cells were treated with different concentrations of $CdSO_4$ (10, 20, 40, 80, 160 μmol/L) for 24 h. A 20-μL volume of MTT (5 mg/mL in PBS) was added to each well and the cells were incubated at 37 °C for an additional 4 h. After removing the medium, 150 μL of DMSO was added at room temperature for 10 min. The absorbance was measured at 490 nm using a microplate reader (Thermo Scientific, Chantilly, VA, USA).

The MTT assay was also performed to detect C3G intervention against $CdSO_4$ in R2C cells. The treatment group was pre-treated with different concentrations of C3G (5, 20, 40, 80,160 μmol/L) for 2 h. Each group, except the control group, was supplemented with 44.80 μmol/L $CdSO_4$ for an additional 24 h.

For morphological observation, R2C cells were seeded at 1×10^4/well in 96-well plates for 24 h. These R2C cells were treated with C3G (10, 20, 40, 80 μmol/L) and 44.80 μmol/L $CdSO_4$, and then the cell shape was observed using a fluorescence microscope (Olympus IX-71, Tokyo, Japan).

2.3. Measurement of progesterone levels

A radioimmunoassay was used to evaluate progesterone levels according to the manufacturer's instructions. R2C cells were seeded at 4×10^5/well in 6-well plates and cultured for 24 h. After treatment, the culture medium was removed, and the cells were dissociated with trypsin and centrifuged at 250 g for 5 min. Progesterone content in the collected supernatant was measured using a radioimmunoassay kit.

2.4. Measurement of mitochondrial membrane potential (MMP)

The MMP of R2C cells was measured using JC-1 staining based on the method by Sun et al. (2013). R2C cells were seeded at 4×10^5/well in 6-well plates and cultured for 24 h. After treatment, the cells were dissociated with trypsin, centrifuged at 400 g for 5 min, and resuspended in medium containing JC-1 staining solution (5 μg/mL) for 20 min at 37 °C. Then, the cells were washed twice, resuspended with JC-1 staining buffer, and analyzed using a flow cytometer (BD FACSaria, USA).

2.5. Determination of ROS production

R2C cells were seeded at 1×10^4/well in 96-well plates and cultured for 24 h. After treatment, the cells were washed three times with serum-free medium and then fixed using 30 μL DCFH-DA diluted by

serum-free medium at a 1: 1000 ratio with incubation at 37 °C for 20 min. Serum-free medium was used to remove the excess DCFH-DA solution. Cellular fluorescence was detected using a fluorometric plate reader (excitation at 488 nm and emission at 525 nm) at 10, 20, 40, 80 and 160 min after $CdSO_4$ treatment.

2.6. Western blot

R2C cells were seeded at 4×10^5/well in 6-well plates for 24 h. After treatment, the cells were dissociated with trypsin, centrifuged at 250 g for 5 min, lysed with radioimmunoprecipitation assay (RIPA) lysis buffer on ice, and then centrifuged at 12,000 *g* for 30 min at 4 °C. Total protein content in the supernatants were quantified using the BCA assay, loaded to a 12% SDS-PAGE gel for electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h and incubated with rabbit anti-SOD2 and anti-StAR primary antibodies at 4 °C overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibodies at room temperature for 1 h, followed by washing and then imaging using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol.

2.7. Statistical analysis

Values were means of triplicate measurements for all experiments and all data were expressed as the mean ± standard deviation (SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA) with Graphpad Prism 5 software. $P < 0.05$ indicated that the results were statistically significant.

3. Results

3.1. C3G increased progesterone level in R2C cells exposed to $CdSO_4$

The effect of different concentrations of $CdSO_4$ on cell viability in R2C cells was explored. $CdSO_4$ decreased cell viability in a dose-dependent manner (Fig. 1A). At the 160 μmol/L dose, the cell inhibition rate increased to 95.26%, indicating the highly toxic effect of Cd on R2C cells; the $CdSO_4$ concentration used to treat R2C cells was 44.80 ± 0.0479 μmol/L. In the following experiments, this concentration was used to explore the protective effect of C3G on $CdSO_4$-treated R2C cells.

The ability of R2C cells to secrete progesterone was measured. Progesterone levels decreased significantly after $CdSO_4$ treatment (Fig. 1B). However, pre-treatment with C3G at the 80 μmol/L concentration increased the ability of the R2C cells to synthesize progesterone, suggesting that C3G had a protective effect on progesterone synthesis in R2C cells exposed to $CdSO_4$.

3.2. C3G improved cell viability and morphology of R2C cells exposed to $CdSO_4$

C3G intervention increased proliferation of R2C cells after treatment with $CdSO_4$ and C3G for 24 h, but proliferation levels in treatment groups were always lower than those in the control group (Fig. 2A). When the C3G concentration was 160 μmol/L, cell viability decreased.

R2C cells subjected to the control treatment grew more densely and adhered more firmly with irregular polygon morphology. When treated with $CdSO_4$ for 24 h, the cells were smaller and round with blurred contours and reduced transparency, and cell adherence was poor (Fig. 2B). Compared with the $CdSO_4$ control group (treatment with $CdSO_4$ only with no C3G pre-treatment), C3G pre-treatment at various concentrations improved the cell morphology.

3.3. C3G reduced ROS production and SOD2 expression in R2C cells exposed to $CdSO_4$

ROS are normally present at low levels in cells and participate in signaling processes. However, excess ROS can cause oxidative stress and destroy proteins and lipids in the cytoplasm or on the cell membrane, ultimately impairing cell function. Therefore, ROS production in $CdSO_4$-treated cells was measured in this study. The observations of this experiment were of such that the higher the fluorescence, the more ROS the cells had produced. R2C cells in the control group produced a stable amount of ROS that did not change significantly with time; but ROS generation increased with $CdSO_4$ concentration and with treatment duration (Fig. 3A–E). After 80 min and 160 min of treatment with $CdSO_4$, R2C cells demonstrated significantly increased ROS production. After C3G pre-treatment, especially at concentrations of 20–80 μmol/L, ROS production in R2C cells decreased at different time points, indicating that C3G scavenged the excess ROS in R2C cells exposed to $CdSO_4$.

SOD2 protein is an important enzyme associated with antioxidant activity. Changes in SOD2 expression could reflect the degree of

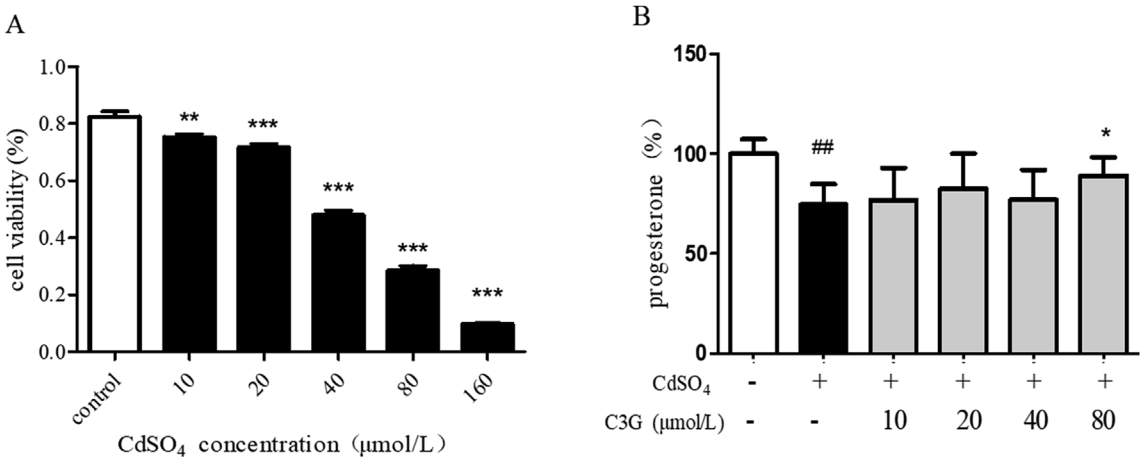

Fig. 1. Effects of different $CdSO_4$ concentrations on R2C cell viability and of different C3G concentrations on progesterone production in $CdSO_4$-treated R2C cells. (A) Inhibitory effect of $CdSO_4$ on the growth of R2C cells. Cells were treated with $CdSO_4$ (10–160 μmol/L) or a negative control for 24 h and cell viability was determined using the MTT assay. (B) C3G increased progesterone synthesis in R2C cells. Cells were pre-treated with C3G (10–80 μmol/L) or a negative control for 2 h and then treated with 44.80 μmol/L $CdSO_4$ or a negative control for 24 h. Progesterone levels were examined using radioimmunoassay. Mean ± SD, n = 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the $CdSO_4$-treated group, $^{\#\#}p < 0.01$ vs. control group.

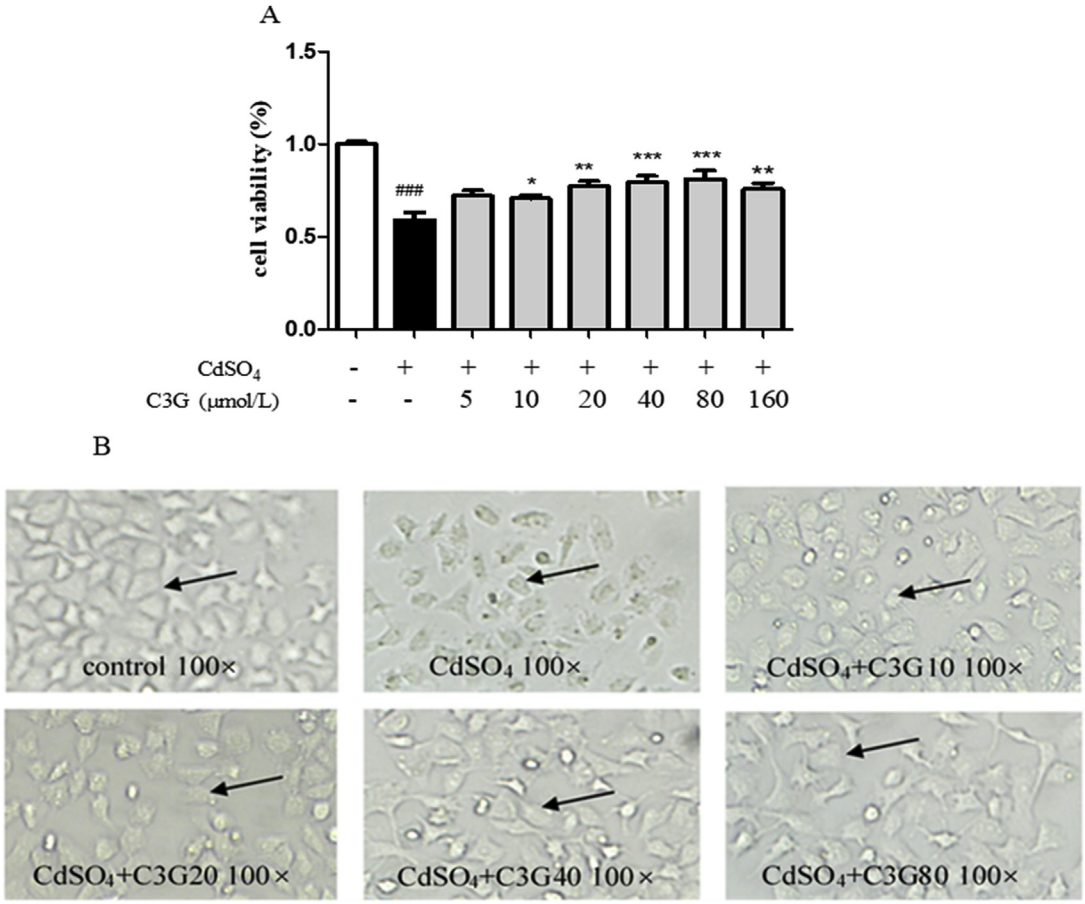

Fig. 2. Effects of various concentrations of C3G on cell viability and cell morphology in $CdSO_4$-treated R2C cells. (A) Cell viability of R2C cells treated with $CdSO_4$ and different C3G concentrations. Cells were pre-treated with C3G (5–160 μmol/L) or with a C3G negative control for 2 h and then treated with 44.80 μmol/L $CdSO_4$ or a $CdSO_4$ negative control for 24 h. (B) Cell morphology after treatment with $CdSO_4$ and various C3G concentrations. Cells were pre-treated with C3G (10–80 μmol/L) or with a C3G negative control for 2 h and then treated with 44.80 μmol/L $CdSO_4$ or a $CdSO_4$ negative control for 24 h, and observed using a fluorescence microscope (100×). Mean ± SD, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 versus $CdSO_4$-treated group, $^{###}$p < 0.001 when compared to the control group.

oxidative stress induced by $CdSO_4$ in R2C cells. SOD2 expression in R2C cells was up-regulated after treatment with $CdSO_4$ for 24 h (Fig. 3F–G). Compared with the $CdSO_4$-treated group, C3G pre-treatment, especially at a concentration of 80 μmol/L, significantly decreased SOD2 expression. This may be due to the fact that C3G may be partially fulfilling the role of SOD in reducing oxidative stress caused by antioxidant activity.

3.4. C3G decreased the MMP in $CdSO_4$-treated R2C cells

MMP is associated with oxidative stress, apoptosis, and progesterone formation. Decreased MMP reflects mitochondrial dysfunction, and the degree of cell injury increases with reduction in MMP. The proportion of mitochondrial depolarization was measured using the relative ratio of red and green fluorescence. The intensity of the green fluorescence was significantly increased in R2C cells after $CdSO_4$ treatment for 24 h, indicating increased mitochondrial membrane injury (Fig. 4A). Compared with the $CdSO_4$-treated group, different concentrations of C3G, especially the 80 μmol/L concentration, decreased the MMP and inhibited mitochondrial damage in R2C cells (Fig. 4B).

3.5. C3G upregulated StAR expression in R2C cells exposed to $CdSO_4$

In addition to the decrease in Leydig cells, progesterone levels are also impacted by enzymes related to progesterone secretion. The StAR protein is a critical rate point in the progesterone synthesis pathway (Sun et al., 2014). $CdSO_4$ down-regulated expression of StAR protein; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. However, different concentrations of C3G, ranging from 10 to 80 μmol/L, upregulated the expression of StAR protein in R2C cells exposed to $CdSO_4$ (Fig. 5A–B).

4. Discussion

Previous studies show that Cd damages the testis by inducing oxidative stress and disrupting the endocrine system (Siu et al., 2009). Anthocyanins can effectively scavenge free-radicals and inhibit Cd-induced reproductive toxicity in males (Hanukoglu et al., 1990). This study explored the protective effect of C3G against Cd toxicity in a Leydig cell model. C3G pre-treatment of Cd-treated R2C cells significantly reduced cytotoxicity; improved cell morphology and ability to proliferate; reduced SOD2 expression and ROS overproduction; and effectively increased StAR expression and progesterone production. The results indicated that anthocyanins can protect testosterone production from Cd cytotoxicity by preserving cell viability and increasing progesterone secretion.

The Leydig cell line R2C was used as a model of Cd-induced reproductive toxicity in males because primary rat Leydig cells are unable to continuously synthesize androgens and are difficult to isolate. R2C cells can proliferate continually *in vitro* and demonstrate physiological function that is highly consistent with that of Leydig cells. In addition, R2C cells can sustain secretion of progesterone (which is the precursor of testosterone) with high StAR protein expression in the absence of hormone stimulation in the mitochondria (Ferraro et al., 2010; Midzak

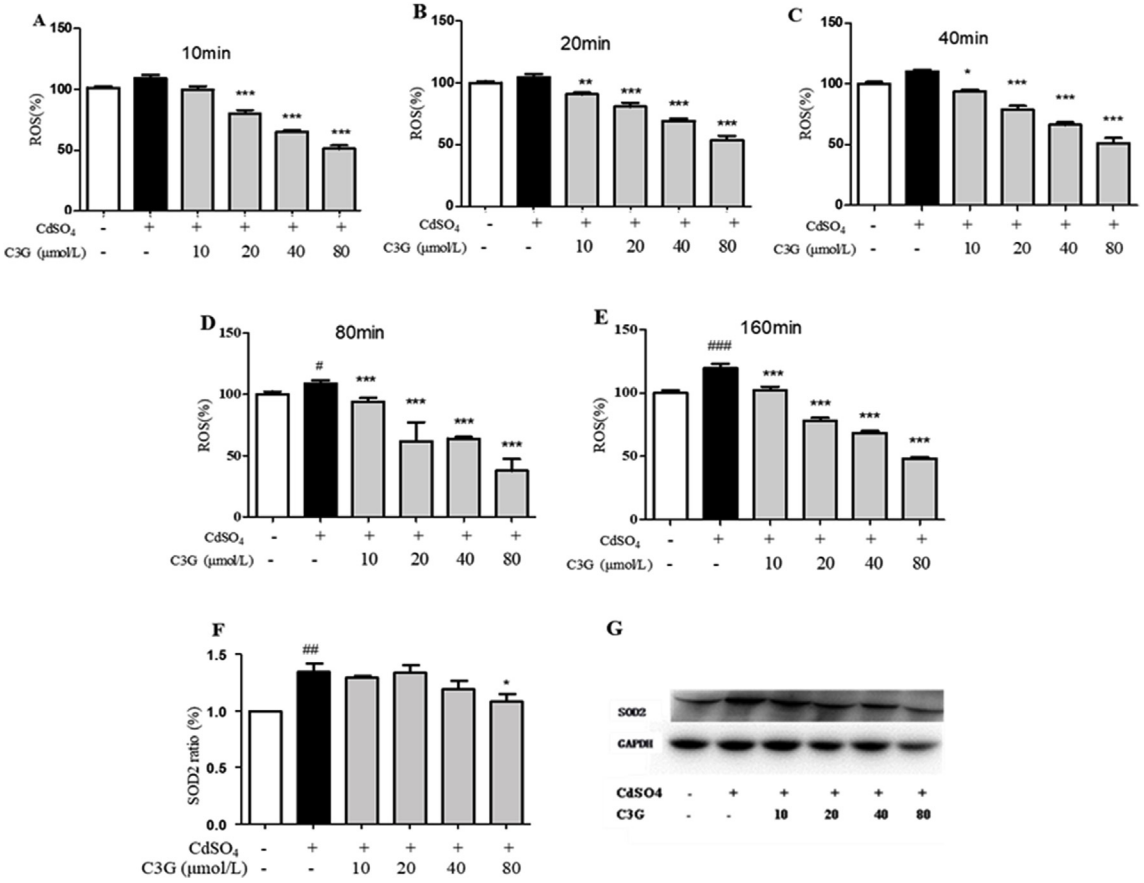

Fig. 3. Effects of $CdSO_4$ and various concentrations of C3G on ROS levels at different timepoints, and SOD2 levels. (A–E) C3G reduced ROS production in $CdSO_4$-treated R2C cells within 10–120 min. Cells were pre-treated with C3G (10–80 μmol/L), or with a C3G negative control, for 2 h. After treatment with 44.80 μmol/L $CdSO_4$, or with a $CDSO_4$ negative control, for 10, 20, 40, 80 and 160 min, R2C cells were stained with DCFH-DA and the resulting fluorescence detected using a fluorometric plate reader with excitation at 488 nm and emission at 525 nm. (F and G) C3G decreased SOD2 expression in R2C cells. Cells were pre-treated with C3G (10–80 μmol/L) or a C3G negative control for 2 h and then treated with 44.80 μmol/L $CdSO_4$, or with a $CdSO_4$ negative control, for 24 h. Expression levels of SOD2 were analyzed using Western blot. Mean ± SD, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 when compared to the $CdSO_4$-treated group, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 when compared to the control group.

et al., 2009). Therefore, R2C cells are a suitable model of Leydig cells for examining reproductive toxicity in males *in vitro*.

C3G administration downregulated levels of ROS and decreased the MMP damage ratio, indicating that C3G increased cell vitality by reducing $CdSO_4$-induced oxidative stress and increasing mitochondrial function. C3G decreased ROS levels by scavenging free-radicals in Cd-treated R2C cells. This observation was consistent with the effects of C3G treatment of cells from the HaCaT keratinocyte cell line (He et al., 2017b). Maintaining balanced levels of ROS contributes to the anti-bacterial and anti-inflammatory characteristics of cells, but excess ROS can destroy DNA, protein, and lipid, ultimately damaging cell function (Li et al., 2017).

Anthocyanins prevent damage to lipids, proteins and DNA in cells through their antioxidant properties and reductive impact on oxidative stress. Reports suggest that anthocyanin-rich blueberries can scavenge peroxyl radicals, and inhibit plasma lipid oxidation induced by oxidants such as peroxyl radicals, peroxynitrite, hypochlorite, 15-lipoxygenase (15-LOX), and singlet oxygen singlet oxygen (Morita et al., 2017). An anthocyanin rich bilberry extract reduced oxidative DNA damage (Acquaviva et al., 2003; Mas et al., 2000; Schantz et al., 2015). Previous reports show that anthocyanins in black rice (C3G and peonidin-3-O-glucoside) can scavenge oxygen free-radicals and hydroxyl radicals, and reduce ROS production *in vivo* (Chiang et al., 2006). Schantz et al. demonstrated that anthocyanin-rich cranberry extract reduced ROS in human colon tumor cell lines Caco-2 and HT-29, and protected the colon from oxidative stress (Schantz et al., 2015).

SOD, a member of the antioxidant systems, contributes to ROS elimination in the body and helps prevent oxidative stress (Eraslan et al., 2007). The anthocyanin-rich Sveva strawberry polyphenol extract and acerola crude extract protected against 2,2′-Azobis(2-amidinopropane)-dihydrochloride (AAPH)-induced oxidative stress by inhibiting ROS production and significantly increasing the activities of the antioxidant enzymes chloramphenicol acetyltransferase (CAT) and SOD. (Alvarez-Suarez et al., 2017; Giampieri et al., 2014). The increase in SOD levels observed in Cd-treated R2C cells could be a response to oxidative stress, as SOD may be enhancing antioxidant capacity (Vicente-Sánchez et al., 2008). The downregulation of SOD2 expression with C3G pre-treatment could result from functional contributions of C3G to the reduction of oxidative stress, since C3G has antioxidant properties.

Cd leads to the opening of mitochondrial permeability transition pore and the non-selective passage of macromolecules through the mitochondrial membrane, which causes depolarization of the mitochondrial membrane and a decrease in membrane potential. When the MMP is destroyed, the respiratory chain electrons in complexes I and III leak and ROS are generated (Qin, 2006; Santofimia-Castano et al., 2018). Cd induces mutation of mitochondrial DNA (mtDNA), which affects the synthesis of respiratory chain enzymes. Cd may also increase ROS production in mitochondria, eventually forming a vicious cycle of ROS activity and leading to cell death. Cd-induced impairment of the respiratory chain not only causes ROS accumulation, but also impairs mitochondrial adenosine triphosphate (ATP) synthesis, leading

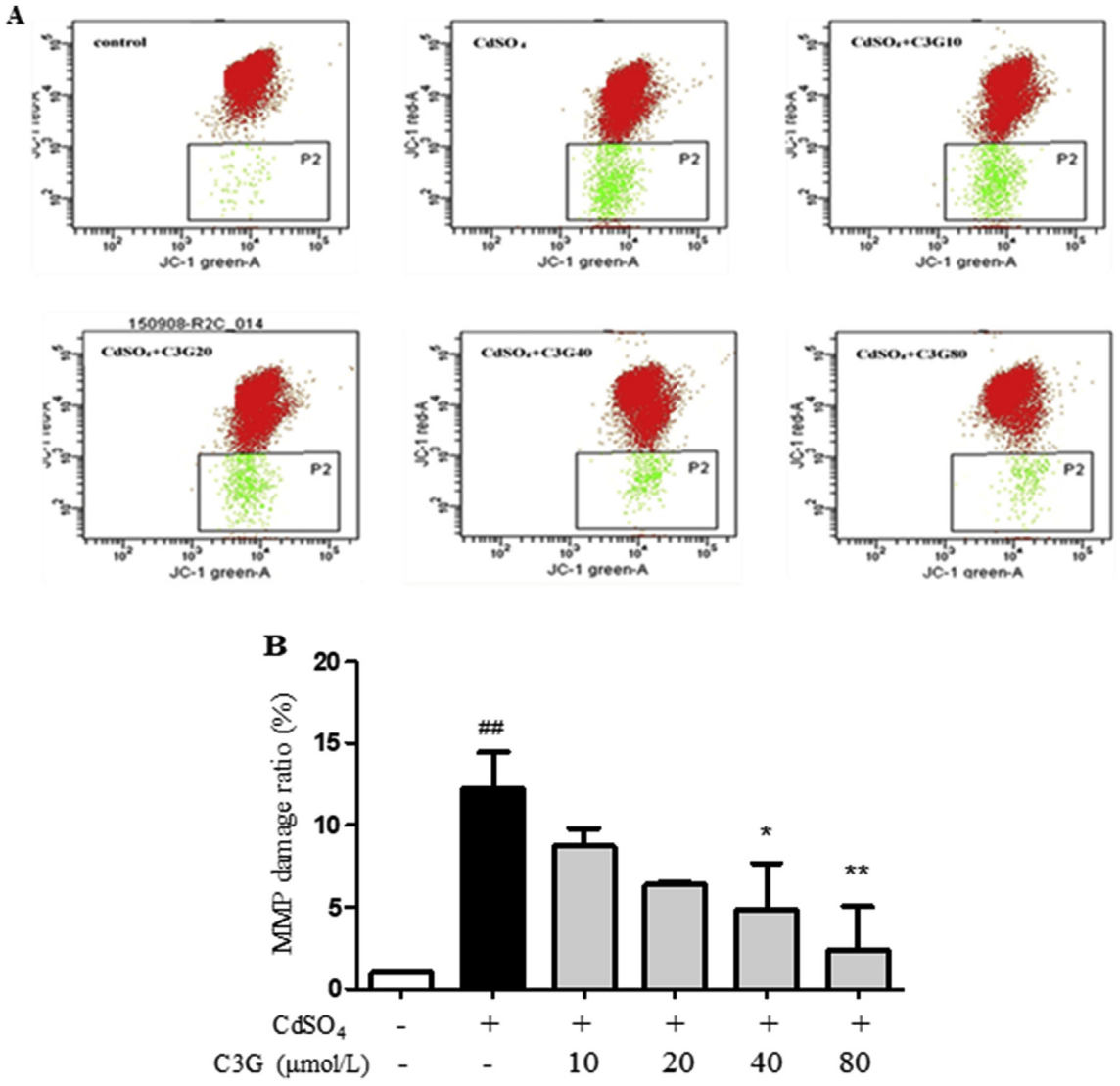

Fig. 4. Effects of C3G and $CdSO_4$ on MMP changes in R2C cells. (A and B) Cells were pre-treated with C3G (10–80 μmol/L), or with a C3G negative control, for 2 h, and then treated with 44.80 μmol/L $CdSO_4$, or with a CdSo4 negative control, for 24 h. Cells were stained with JC-1 and then analyzed using a flow cytometer. Mean ± SD, n = 3. *p < 0.05, **p < 0.01 when compared to the $CdSO_4$-treated group, ##p < 0.01 when compared to the control group.

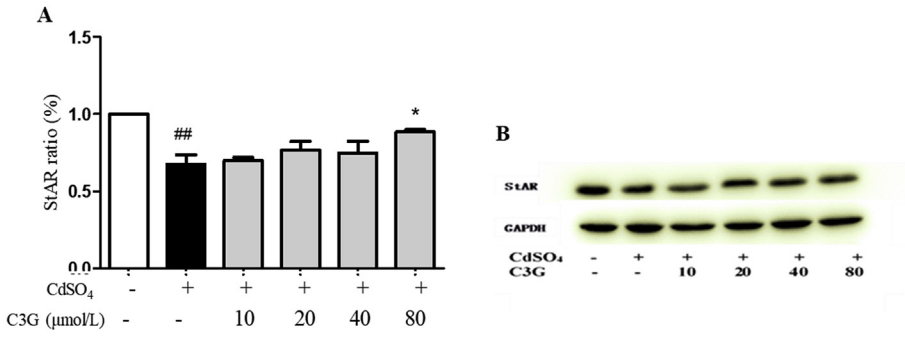

Fig. 5. Effects of $CdSO_4$ and various concentrations of C3G on StAR levels in R2C cells. (A and B) C3G upregulated the expression of StAR protein in R2C cells. Cells were pre-treated with C3G (10–80 μmol/L), or with a C3G negative control, for 2 h and then treated with 44.80 μmol/L $CdSO_4$, or with a $CdSO_4$ negative control, for 24 h. StAR expression levels were analyzed using Western blot. Mean ± SD, n = 3. *p < 0.05 when compared to the $CdSO_4$-treated group, ##p < 0.01 when compared to the control group.

to dysfunctional ATP-dependent processes (López et al., 2006; Zhang et al., 2008).

In this study, C3G reduced the MMP damage ratio induced by the rise of ROS *in vivo* and restored mitochondria to normal levels, thereby maintaining varies cell functions (Sun et al., 2016). MMP is a sensitive indicator of cellular energy and maintains oxidative phosphorylation and production of ATP precursors in mitochondria. Thus, the stability of MMP is conducive to maintenance of normal physiological cell function. Similarly, anthocyanins were previously reported to reduce amyloid beta-induced neurotoxicity in the hippocampal neuronal cell line HT22, and increase cell viability of human dermal fibroblasts (Alvarez-Suarez et al., 2017; Giampieri et al., 2014) by improving mitochondrial function (Badshah et al., 2015). C3G and Pg-3-glucoside inhibited peroxynitrite-induced MMP dissipation and intracellular ROS, ultimately ensuring mitochondrial function in endothelial cells (Paixão et al., 2011).

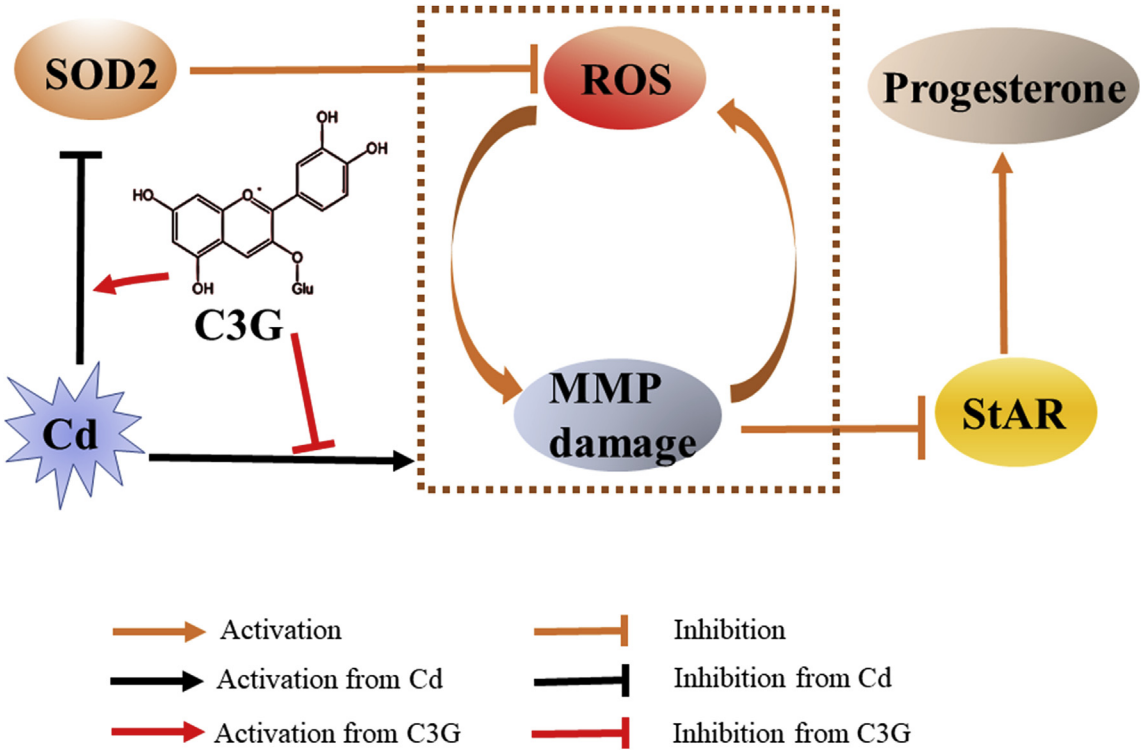

Fig. 6. C3G increased progesterone secretion by improving cell viability and mitochondrial function, decreasing SOD2 expression and ROS production, and increasing StAR protein levels in $CdSO_4$-treated R2C cells.

C3G enhanced progesterone or testosterone synthesis by reducing the MMP damage ratio and upregulating StAR protein expression in R2C cells. The mitochondria in Leydig cells play an important role in testosterone synthesis (Chen et al., 2016). The recovery of mitochondrial function resulting from C3G administration is beneficial for ATP production, which helps improve StAR protein phosphorylation and thus its activity (Hales et al., 2005). In addition, C3G reduced mitochondrial membrane lipid oxidation induced by ROS overproduction (Zhang et al., 2017) and alleviated the decrease in membrane fluidity, thereby contributing to cholesterol transport and progesterone synthesis (Sun et al., 2016).

The StAR protein is the key protein in the progesterone synthesis pathway and transfers cholesterol, an important precursor of steroid hormones, from the matrix into the mitochondrial inner membrane in R2C cells (Christenson and Strauss, 2001). Previous studies showed that some environmental pollutants and toxic substances caused reproductive toxicity, mostly via an impact to StAR protein expression, which led to significant reduction in testosterone synthesis (Yang et al., 2003). Activation of StAR protein is affected by mitochondrial damage, including inhibition of protein phosphorylation, protein instability, promotion of protein degradation, inhibition of gene transcription level, etc. Cholesterol on the inner mitochondrial membrane is converted into pregnenolone by the cytochrome P450 side-chain cleavage enzyme (P450scc/CYP11A1) (Christenson and Strauss, 2001; Sun et al., 2013). Pregnenolone further enters the endoplasmic reticulum by diffusion and is subsequently used to synthesize progesterone via 3β-hydroxysteroid dehydrogenase (3β-HSD).

Pre-treatment of R2C cells exposed to $CdSO_4$ with various concentrations of C3G enhanced progesterone synthesis, indicating that C3G may have a protective effect on testosterone synthesis because progesterone can be converted to testosterone by 17α-hydroxylase (CYP17) and 17β-HSD (Payne and Hales, 2004). Taken together, these results suggest that C3G reduced membrane lipid oxidation and MMP damage ratio, upregulated StAR protein expression, promoted cholesterol transport, and eventually enhanced progesterone or testosterone synthesis in R2C cells (Fig. 6).

5. Conclusions

In summary, the present study indicated that C3G increased proliferation, and reduced SOD2 expression and ROS production in R2C cells treated with the injurious $CdSO_4$. In addition, C3G pre-treatment increased progesterone synthesis in $CdSO_4$-treated R2C cells by decreasing MMP damage ratio and improving StAR protein expression. Therefore, dietary C3G could be a potential therapeutic or preventive agent against Cd-caused reproductive injury.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.040.

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