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Cyanidin-3-*O*-glucoside protects against cadmium-induced dysfunction of sex hormone secretion *via* the regulation of hypothalamus-pituitary-gonadal axis in male pubertal mice

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ABSTRACT

Cadmium (Cd) has been generally recognized as an endocrine-disrupting chemical for its toxic effects on the hypothalamus-pituitary-gonadal (HPG) axis accompanied by dysfunction in sex hormone secretion. Particularly, exposure to Cd during puberty versus post-puberty exhibits differing age-dependent effects that require further examination. This study sought to determine if cyanidin-3-O-glucoside (C3G), a typical anthocyanin with neuroprotective bioactivity, could protect against Cd-induced sex hormone-disorder in Pubertal male mice. C3G treatment reversed the disruption of hormone levels and increased Gnrh1 gene expression in the hypothalamus. In addition, the levels of gonadotropins, including luteinizing hormone (LH) and follicle stimulating hormone (FSH), were reversed by C3G. Interestingly, C3G improved the expression of LH and FSH receptor in the testis in mice exposed to Cd. Furthermore, C3G activated the signaling pathway related to the synthesis of testosterone processing. In conclusion, C3G protected against Cd-induced dysfunction of sex hormone secretion through the regulation of the HPG axis in male mice during puberty. The results of this study suggest that consumption of anthocyanins can be protective against metal-induced male reproductive dysfunction.

1. Introduction

Cadmium (Cd) is a toxic metal that accumulates in the environment due to its wide range of applications in industry and agriculture and has been considered a neuroendocrine disruptor (Cobbina et al., 2015; Rodriguez-Barranco et al., 2013; Zhai et al., 2015). Diet, water supply, and cigarette smoking are the main routes of Cd exposure to the general population (Guo et al., 2019; Milnerowicz et al., 2015; Wang et al., 2018). Numerous studies have demonstrated that long-term Cd exposure can cause toxic effects in multiple cells, tissues, and organs due to its long biological half-life (approximately 20–30 years in humans) and low rate of excretion (Shen et al., 2017; Wang and Du, 2013). Moreover, the endocrine alterations produced by Cd exposure can cause various types of damage to other physiological systems such as the reproductive system, which then can give rise to male infertility (Lafuente, 2013).

The steady state of the reproductive system is regulated by the hypothalamus-pituitary-gonadal (HPG) axis *via* a negative feedback loop (Chimento et al., 2014; Zhou et al., 2018). Gonadotropin-releasing hormone (GnRH) is released from GnRH nerve cells in the hypothalamus under the regulation of central neurons (Liu and Tang, 2017; Mao et al., 2017; Qian et al., 2018). GnRH then reaches the pituitary *via* the portal circulation and binds to the GnRH receptor (GnRHR) of the pituitary gland, causing gonadotropin cells to secrete gonadotropins, including luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Allan et al., 2004). The released LH and FSH can promote the

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synthesis of inhibin B (INH−B) and testosterone, respectively, which is necessary for the differentiation of spermatogonia and sperm maturation (Oczkowski et al., 2014). Cd exposure significantly affects the HPG axis by acting as a neuroendocrine disruptor and causing a hormonal imbalance by altering the effective concentrations of GnRH, LH, FSH, testosterone, and others steroid hormones, which in turn contributes to neurotoxicity (Ciarrocca et al., 2013; de Angelis et al., 2017; Lafuente, 2013; Lafuente et al., 2004). Previous studies have supported the notion that hormone dysregulation is a result of the negative effects of oxidative stress that Cd exerts on the hypothalamus and the pituitary (Cabilla et al., 2016; de Angelis et al., 2017; Poliandri et al., 2006; Togno-Peirce et al., 2018). It is worth noting that the age at which exposure to Cd occurs is also a decisive factor contributing to hormone dysfunction. For example, one study showed that the plasma FSH levels were decreased in adult rats treated subcutaneously with cadmium chloride ($CdCl_2$) but was not altered in rats during puberty under the same treatment conditions. Plasma levels of testosterone increased in pubertal rats but decreased in adult rats (Lafuente et al., 2000). Also, metal accumulation in the hypothalamus and pituitary was higher when exposure occurred postpubertal compared to prepubertal metal exposure (Lafuente et al., 2001). Altogether, Cd accumulation disrupts the regulatory mechanisms of the HPG axis and exerts age-dependent effects.

Anthocyanins are a type of flavonoid pigment found in various fruits, vegetables, and grains, such as strawberry, purple potato, and black soybean (Sandoval-Ramirez et al., 2018; Sun et al., 2018; Wu et al., 2018). As an important family of phytochemicals, anthocyanins have the potential to be a bioactive compound with anti-oxidant and anti-inflammatory properties that can protect against Cd damage (Jiang et al., 2018a,b; Li et al., 2017; Sandoval-Ramirez et al., 2018). Importantly, it has been reported that the 3,4-ortho-dihydroxy on the B ring structure of anthocyanins is able to chelate with heavy metals and reduce metal ion concentrations, thus further alleviating heavy metal-induced toxicity (Li et al., 2017). Cyanidin-3-*O*-glucoside (C3G) is the most ubiquitous and plentiful anthocyanin in the plant world constitutes a large proportion of phytonutrient intake in the average in the average daily dietary intake (Raj et al., 2017; Sun et al., 2018). Our previous studies have revealed the protection of C3G against various contaminant-induced reproductive toxicities *in vivo* and *in vitro* (Jiang et al., 2018a,b; Li et al., 2019; Sun et al., 2018; Wen et al., 2018). However, to date, little is known about the effects of C3G on disruption of the HPG axis under heavy metal exposure. The aim of this study was to investigate whether C3G has protective effects against Cd-induced dysfunction of the HPG axis in male mice during puberty.

2. Experimental

2.1. Preparation of C3G

Following our previous methods, black soybean peels were extracted with 65% ethanol containing 0.1% trifluoroacetic acid under dark conditions at 4 °C for 8 h. The extraction was repeated 3 times and combined, then subsequently one-third of the volume was added to petroleum ether to remove lipids. Finally, the C3G samples were purified using macroporous resin and preparative medium pressure liquid chromatography (Jiang et al., 2018a,b). The purity of C3G was examined by liquid chromatography-mass spectrometry (LC-MS) and was higher than 91.46%.

2.2. Reagents and antibodies

Antibodies for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cholesterol side-chain cleavage enzyme (P450scc), steroidogenic acute regulatory protein (StAR), and protein kinase A (PKA) were obtained from Cell Signaling Technology (Boston, USA). 3β-Hydroxysteroid dehydrogenase (HSD3B) and cyclic adenosine monophosphate (cAMP) responsive element modulator (CREM) were purchased from Abcam (Cambridge, UK). The secondary anti-rabbit IgG antibody was purchased from Cell Signaling Technology. The analytical grade of cadmium chloride was from Sigma-Aldrich (MO, USA). All other chemicals in this study were of analytical grade unless otherwise noted.

Table 1
Diet composition of the control, Cd, and Cd + C3G group, respectively.

Ingredients (g/kg diet)	Control	Cd	Cd + C3G
Casein, 30 mesh	200	200	200
L-Cystine	3	3	3
Corn starch	397.49	397.49	397.49
Sucrose	100	100	100
Lodex 10	132	132	132
Soybean Oil	70	70	70
Solka Floc, FCC200	50	50	50
Mineral mixture, S10022G	35	35	35
Vitamin mixture, V10037	10	10	10
Choline Bitartrate	2.5	2.5	2.5
tert-Butylhydroquinone (tBHQ)	0.01	0.01	0.01
Cyanidin-3-O-glucoside	0	0	0.5

2.3. Animals and treatment

The animal study was approved by the Animal Care and Protection Committee of Jinan University. Ninety-six male Kunming mice aged 25 days were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The animals were allowed access to food and water *ad libitum* and maintained at 23 °C with 12/12-h light-dark cycle. All mice were randomly divided into three groups after acclimatization: the control group (standard chow), the cadmium chloride (5 mg/kg/day via gavage) group, and the cadmium chloride plus C3G (500 mg/kg with chow) group (See Table 1). Eight, eight, and twelve mice of each group were sacrificed at days 10, 20 and 30, respectively, and the remaining 4 mice in each group were sacrificed without any treatment at the beginning of the experiment period as the basic control. After the mice were anesthetized and sacrificed, the serum was separated via centrifugation of the blood samples at 3000 g for 15 min at 4 °C, and was kept frozen at −80 °C until analysis. The hypothalamus and pituitary tissues were removed and then stored in Trizol. Testes were excised and separated into two parts: one part frozen immediately in liquid nitrogen and kept in −80 °C for protein isolation, and the other part was stored in Trizol for RNA extraction and RT-PCR.

2.4. Hormone determination

The level of sex hormones in the serum was measured using a commercialized enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's instructions. The ELISA kits for testosterone, INH−B, DHT, and LH were purchased from CUSABIO (Wuhan, China). The commercial kit for FSH was from LIUHEBIO (Wuhan, China).

2.5. RNA extraction and quantitative real-time PCR

Total RNA was extracted by Trizol reagent according to the manufacturer protocol and converted to complementary DNA (cDNA) by using a high capacity cDNA reverse transcription kit (TaKaRa, RR047A). Gene expression was measured by quantitative polymerase chain reaction (qPCR) using TB Green Premix Ex Taq II (TaKaRa, RR820A) and a CFX96 real-time PCR detection system (Bio-Rad). The total volume of each reaction was 20 μL and the amplification cycles were 95 °C for 30 s, 95 °C for 5 s (40 cycles), and then 60 °C for 30 s. The sequences of primer used for RT-qPCR analysis are listed in Table 2.

Table 2
Quantitative real-time PCR primers used for gene expression analysis in the study.

Gene	Accession No.	Forward primer (5′-3′)	
Avp	NM_009732	Forward	GCCAGGATGCTCAACACTACG
		Reverse	TCTCAGCTCCATGTCAGAGATG
Kiss1	NM_178260	Forward	CTCTGTGTCGCCACCTATGG
		Reverse	AGGCTTGCTCTCTGCATACC
Gnrh1	NM_008145	Forward	GAACCCCAGCACTTCGAATGTA
		Reverse	TGGCTTCCTCTTCAATCAGACTTT
Lhcgr	NM_013582	Forward	CTCGCCCGACTATCTCTCAC
		Reverse	ACGACCTCATTAAGTCCCCTG
Fshr	NM_013523	Forward	TGCTCTAACAGGGTCTTCCTC
		Reverse	TCTCAGTTCAATGGCGTTCCG
Gnrhr	NM_010323	Forward	TGCTCGGCCATCAACAACA
		Reverse	AGGAAGCATTGAAGGCAGTAGA
beta-actin	NM_007393	Forward	GAGCGCAAGTACTCTGTGTG
		Reverse	AACGCAGCTCAGTAACAGTC

Genes that were examined include *Avp*, *Kiss1*, *Gnrh1*, *Fshr*, *Gnrhr,* and *Lhr*. Fold expression relative to the *beta-actin* internal control genes was calculated according to the $2^{-\Delta\Delta Ct}$ methods.

2.6. Protein extraction and western blotting

The western blotting analysis was performed to measure protein expression. Briefly, according to weight, frozen testes samples were added to cold lysis buffer comprised of a cocktail of protease inhibitors and phenylmethanesulfonyl fluoride (PMSF) and homogenized. The mixture was centrifuged at 14000 g for 10 min at 4 °C, and the supernatant was collected. The protein concentration was determined using a BCA Protein Assay Kit. Afterward, 20 μg of proteins were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 45 V for 45 min, then transferred to 110 V for 70 min. The separated proteins were electrophoretically transferred onto a PVDF membrane at 200 mA for 1 h. The PVDF membranes were washed with TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and blocked in TBS containing 5% non-fat milk powder for 1.5 h at room temperature. After that, the membrane was incubated overnight at 4 °C in the same solution consisting of anti-P450scc (1:1000), anti-HSD3B (1:1000), anti-StAR (1:1000), anti-CREM (1:1000), and anti-PKA (1:1000). Subsequently, the membrane was incubated with peroxidase-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Protein expression was detected with chemiluminescence (ECL) after being washed with TBST. Protein bands were analyzed using Image J Software. The protein concentrations of P450scc, HSD3B, StAR, CREM, and PKA were normalized to GAPDH.

2.7. Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (version 7.0). All data were expressed as means ± standard deviation (SD). The significance of difference was evaluated by one-way analysis of variance (ANOVA), and the differences between two groups were analyzed by Bonferroni *post hoc* test. The value of $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. Body weight and food intake

Over the course of the animal experiment, the exposed group treated with $CdCl_2$ exhibited decreased physical and mental conditions in addition to a reduction in food intake (Fig. 1A). Moreover, the body weight was significantly reduced in the Cd group compared with the control group after 20 days (Fig. 1B). Interestingly treatment with C3G improved the mental state, while there was no significant difference observed between the Cd group and Cd + C3G group with regard to body weight or food intake.

3.2. C3G reversed the FSH and testosterone levels in serum

The hormonal regulation of spermatogenesis involves a complex interplay within the HPG axis, which commences before birth with male sexual development and continues through puberty and into adulthood (McLachlan, 2000). To evaluate the sex hormone secretion during the developmental process in puberty, we measured testosterone, FSH, LH, INH−B, and DHT in the serum at different treatment time points by ELISA. As shown in Fig. 2A the FSH level in the Cd group was increased at 20 and 30 days when compared to the control group and interestingly, the Cd + C3G group presented a normal level that is consistent with the control group. FSH is a glycoprotein polypeptide hormone that can stimulate the release of INH−B from the Sertoli cells in the testes while INH−B levels can modulate simultaneously the FSH secretion by feedback regulation. According to our results, the C3G restored the Cd-induced decrease in the INH−B concentration in serum at 30 days, although this was not significant (Fig. 2D). LH, another sex hormone secreted from the pituitary, is the precursor for testosterone secretion located in the Leydig cells. There was a modest increase in serum LH after C3G feeding (Fig. 2B). On the contrary, the presence of C3G prevented the Cd-mediated increase in serum testosterone levels (Fig. 2C). Moreover, the DHT derived from testosterone by 5α-reductase did not change in either of the three groups (Fig. 2E). In short, C3G treatment switched the sex hormone secretion from a dysfunction condition caused by Cd exposure back to a normal state.

3.3. C3G attenuated the Gnrh1 expression in the hypothalamus

Hypothalamic GnRH is the master regulator of the HPG axis (Luo et al., 2018). GnRH is a trophic peptide hormone, which is synthesized and released from GnRH neurons within the hypothalamus and is responsible for the release of FSH and LH from the anterior pituitary (Ojeda and Lomniczi, 2014). Since GnRH levels in the serum are unstable, the *Gnrh1* mRNA expression in the hypothalamus is a suitable alternative for assessment of GnRH secretion. Gene expression analysis revealed that treatment with C3G lowered the over-expression of *Gnrh1* caused by Cd exposure in mice hypothalamus at the end of the experiment (Fig. 3). This means the GnRH secretion was under normal functional regulation with the help of C3G.

3.4. C3G ameliorated the Avp and Kiss1 in the hypothalamus

Kisspeptin (*Kiss1*), the gene encoding kisspeptin peptide, is expressed in the anteroventral periventricular (AVPV) and plays a critical role in positively regulating the HPG axis (Williams et al., 2011). In addition, the biosynthesis and release of GnRH in the hypothalamus are directly regulated by the arginine vasopressin (AVP) from the suprachiasmatic nucleus (SCN) and the kisspeptin from AVPV. Therefore, we also considered whether the *Avp* and *Kiss1* in the hypothalamus were expressed. As shown in Fig. 4, the C3G attenuated the Cd-induced increase in mRNA expression of *Avp* and *Kiss1*, followed by a regular secretion of GnRH. These results revealed the possibility of C3G interfering with sex hormone secretion through hypothalamic interactions.

3.5. C3G improved the expression of sex hormone receptor in pituitary and testis

Encoded by the *Gnrhr* gene and expressed on the surface of pituitary gonadotropic cells, the GnRH receptor (GnRHR) can be combined with and activated by GnRH released from the hypothalamus, and ultimately cause the release of LH and FSH. Since we saw an alteration of *Gnrh1* gene and FSH concentration in the serum, as shown above, we first

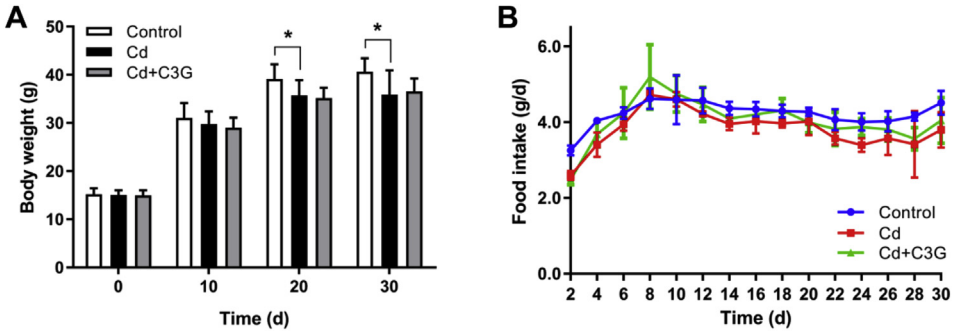

Fig. 1. Body weight and food intake at different treatment time points.

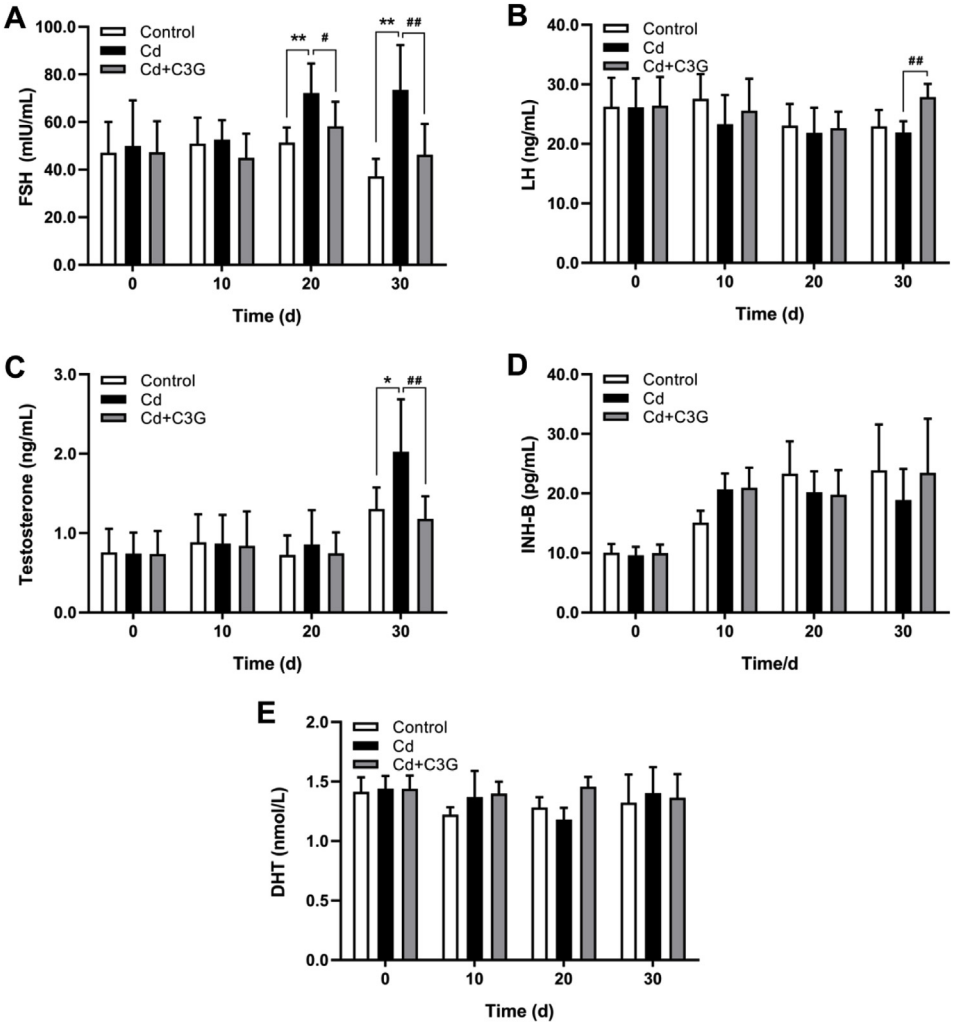

Fig. 2. Hormone levels in the serum after Cd and C3G treatment. Levels of follicle-stimulating hormone (FSH) (A), luteinizing hormone (LH) (B), testosterone (C), inhibition B (INH−B) (D), dihydrotestosterone (DHT) (E) in the serum at different treatment times were measured with the ELISA kit. Data are shown as means ± SD (Day 0, n = 4; day 10 & 20, n = 8; day 30, n ≥ 9). Comparison between all groups was evaluated through One-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with Cd group.

examined the mRNA expression of *Gnrhr* in the pituitary. Interestingly, *the Gnrhr* mRNA levels were consistent across all groups (Fig. 5A). Furthermore, the *Lhr* gene that translated to LH receptor in Leydig cells and the *Fshr* gene that responded to FSH receptor in Sertoli cells *in vivo* was determined to explain the corresponding change of sex hormone. It was demonstrated that the gene expression of both *Lhr* and *Fshr* were dramatically suppressed after exposure to Cd, whereas C3G partly restored expression, suggesting the C3G has as protective bioactivity for the male reproductive system (Fig. 5B and C). The restoration of normal expression as a result of C3G treatment suggests that the Leydig cells and Sertoli cells received the appropriate signaling from the upstream hormone to keep an integrated feedback function at corresponding checkpoints.

3.6. C3G normalized the cAMP-CREB/CREM pathway and activated the PKA system

After the LH and LH receptors combine to form a complex, cAMP

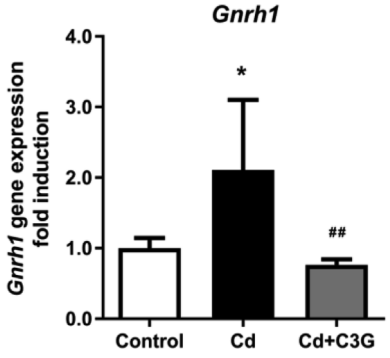

Fig. 3. The *Gnrh1* gene expression in the hypothalamus. The *Gnrh1* mRNA expression in the hypothalamus after treating with Cd and C3G for 30 days was conducted by qPCR and normalized to *β-actin*. Data are shown as means ± SD (n = 4). Comparison between all groups was evaluated through One-way ANOVA. $^{*}p < 0.05$, compared with control group. $^{\#\#}p < 0.01$, compared with Cd group.

expression in the cells increase and the PKA system is activated, regulating the testosterone synthesis in Sertoli cells. Because we observed a change in testosterone after treating with C3G, we analyzed the impact of Cd and C3G on the cAMP-cAMP-response element binding protein (CREB)/CREM signaling pathway. Importantly, Cd notably repressed the expression of CREM in the testis as seen by western blot analysis, but the C3G treatment restored the expression back to normal (Fig. 6A). Meanwhile, PKA protein expression was upregulated, as expected, in the presence of C3G (Fig. 6B). The restoration of the protein expression as a result of C3G treatment may aid in the reestablishment of normal hormonal signaling pathways and testosterone synthesis.

3.7. C3G interfered with the testosterone synthesis pathway

The synthesis and secretion of testosterone in testis are crucial for maintaining a stable microenvironment system with a reasonable and moderate concentration of testosterone. This is important for spermatogenesis which promotes sperm cells maturation. We measured the expression of critical proteins involved in the testosterone synthesis pathway. The protein expression of StAR, P450scc, and HSD3B was severely reduced, which is a consequence of the downregulation of CREM and PKA. With the supplementation of C3G, the levels of StAR, P450scc, and HSD3B recovered (Fig. 7). Interestingly, the testosterone concentration in the serum was increased after exposure to Cd, while the expression of the interrelated protein was decreased. This contrary response may be the result of negative feedback regulation in puberty and is something that needs to be investigated in a future study. These results suggest that C3G intervention can protect the testosterone synthesis pathway from the detrimental effects of Cd exposure.

4. Discussion

In recent years, anthocyanins, some of the most recognized bioactive compounds from fruits, vegetables, and grains, have been regarded as effective nutritional components for the treatment or prevention of several diseases(Sandoval-Ramirez et al., 2018). C3G used in this study is a typical and widespread anthocyanin, which has been revealed as a beneficial compound for the reproductive system (Jiang et al., 2018a,b), the nervous system (Milbury and Kalt, 2010), and other organs and cells (Jiang et al., 2018a,b) under particular conditions. C3G is easily accessible as it can be obtained from a variety of foods and has great potential to be widely used in the future. Drawing from the experience from our previous study, the dose of C3G at 500 mg/kg in the diet was considered efficient for this treatment and correlates with the actual human consumption of C3G in the daily diet. Cd is a neurotoxic heavy metal, and the exposure to Cd has been considered a harmful factor for male infertility according to its neurotoxicity to the HPG axis. Considering the different steps of age-dependent hormone regulation in puberty and sexual maturity, we evaluated the protective effect of C3G on Cd-induced dysfunction of sex hormone secretion in pubertal male mice. As expected, the C3G exerted pronounced benefits on the male reproductive system (See Fig. 8).

Male fertility, and hence its reproductive potential, is a result of a complex and intricate biological system under fine neuroendocrine control. The hypothalamus, pituitary, and testis, known as the HPG axis, are in charge of regulating the release of gonadotropins and androgens through a negative feedback loop (Chimento et al., 2014). The pulsatile release of GnRH from the hypothalamus stimulates the pituitary gonadotropin cells to release FSH and LH at same intervals, followed by the synthesis and secretion of INH−B and androgen-binding protein (ABP) in Sertoli cells and testosterone in Leydig cells. The secreted testosterone combines with the ABP and is recognized by the androgen receptor where it enters the Sertoli cells, an optimal environment for spermatogenesis. In this study, the concentration of testosterone, FSH, LH, INH−B, and DHT in the serum and the mRNA expression of *Gnrh1* in the hypothalamus were investigated. After treatment with C3G, the Cd-induced increase in the expression of testosterone, FSH, and *Gnrh1* genes was downregulated significantly, and the Cd-induced decrease in INH−B was attenuated, although the results were not statistically significant. According to published research, the exposure to Cd in maturity can reduce testosterone levels and give rise to spermatogenesis failure (de Angelis et al., 2017). However, Lafuente et al. reported a higher testosterone level after treatment with cadmium in mice during puberty, which is consistent with the conclusions of this study (Lafuente et al., 2000). This suggests that Cd exerts age-dependent effects on the HPG axis function, and disrupts the regulatory mechanisms of the HPG axis. In short, treatment with C3G can reorganize the dysfunction of sex hormone secretion caused by Cd exposure during puberty.

GnRH is a decapeptide only encoded by *Gnrh1* gene in mammals. It is reported that, as the upstream neuropeptide of GnRH neuron, the

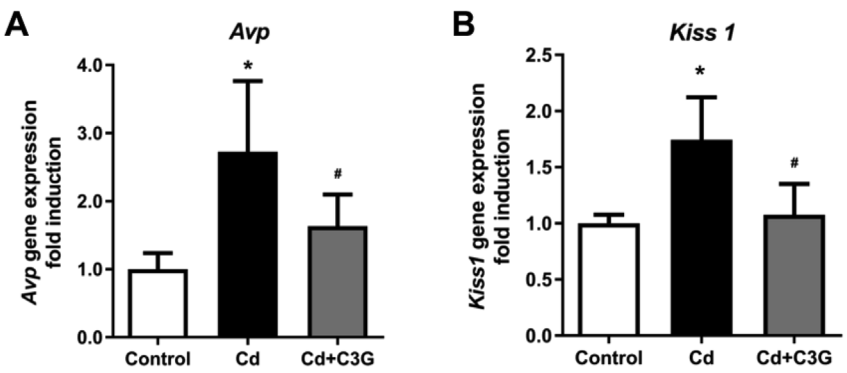

Fig. 4. The *Avp* and *Kiss1* gene expression in the hypothalamus. The *Avp* and *Kiss1* mRNA expression in the hypothalamus after treating with Cd and C3G for 30 days was conducted by qPCR and normalized to *β-actin*. Data are shown as means ± SD (n = 4). Comparison between all groups was evaluated through One-way ANOVA. $^{*}p < 0.05$, compared with control group. $^{\#}p < 0.05$, compared with Cd group.

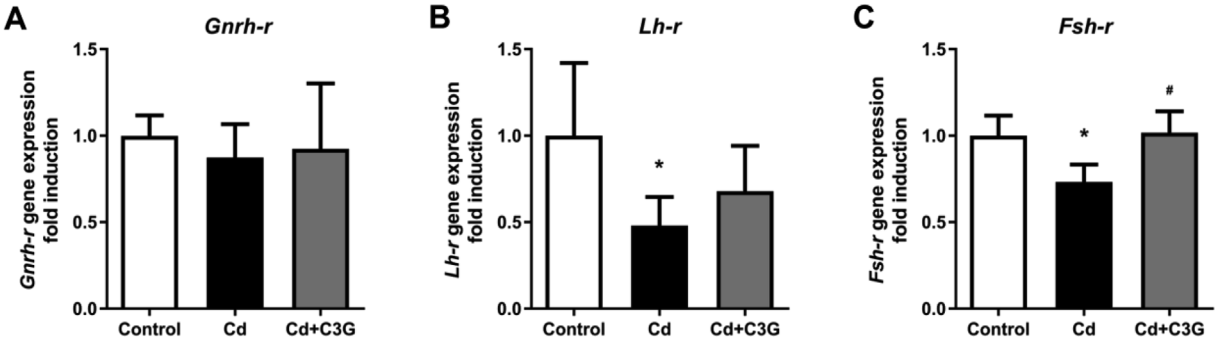

Fig. 5. The *Gnrh-r* gene expression in the pituitary, and *Lh-r* and *Fsh-r* gene expression in the testes. The *Gnrh-r* gene expression (A) in the pituitary, and *Lh-r* (B) and *Fsh-r* (C) gene expression in the testes of mice treated with Cd and C3G for 30 days was conducted by qPCR and normalized to *β-actin*. Data are shown as means ± SD (n = 4). Comparison between all groups was evaluated through One-way ANOVA. $^{*}p < 0.05$, compared with control group. $^{\#}p < 0.05$, compared with Cd group.

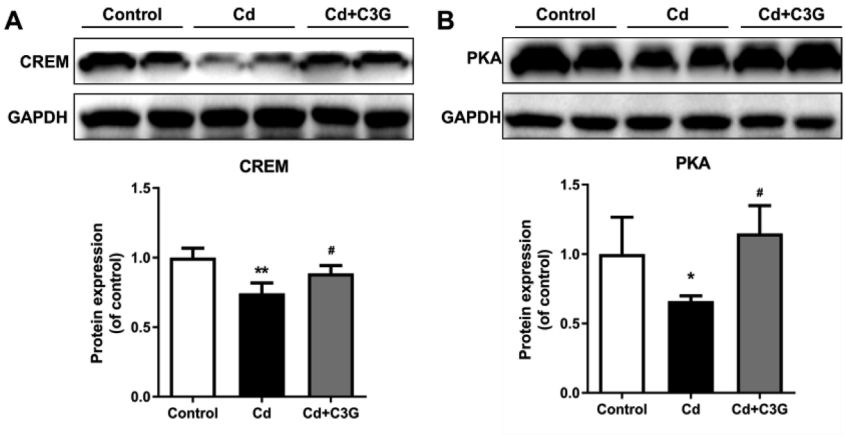

Fig. 6. The protein expression of CREM and PKA. The CREM protein expression (A) and PKA protein expression (B) in the testes of mice treated with Cd and C3G for 30 days was analyzed by western blotting and normalized to GAPDH. Data are shown as means ± SD (n = 4). Comparison between all groups was evaluated through One-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control group. $^{\#}p < 0.05$, compared with Cd group.

kisspeptin from AVPV plays a critical role in the regulation of GnRH secretion and maintains the normal function of the HPG axis (d'Anglemont de Tassigny et al., 2007; Navarro et al., 2015; Ruka et al., 2013). Meanwhile, suprachiasmatic nucleus cells targeting the AVPV can express AVP, which has a positive correlation with kisspeptin (Owen et al., 2013; Williams et al., 2011). Oxidative stress, interference with calcium and zinc-dependent processes, and the induction of apoptosis are the main processes involved in Cd neurotoxicity. This is an outcome of Cd crossing the blood-brain barrier (BBB) by selective permeability, which can disrupt the functional balance of the hypothalamus(Mendez-Armenta and Rios, 2007). Most importantly, anthocyanin, a red flavonoid pigment found in plant-derived foods, has been shown to exhibit similar capabilities of crossing the BBB and has been demonstrated to have notable neuroprotective effects (Milbury and Kalt, 2010; Strathearn et al., 2014). Therefore, we isolated the hypothalamic RNA for the determination of the mRNA expression level of *Avp* and *Kiss1*. As our present research showed, the *Avp* and *Kiss1* were increased significantly due to Cd exposure, which induced higher gene expression of *Gnrh1*. Meaningfully, the C3G treatment lowered these abnormal gene expression levels and was beneficial to the nervous

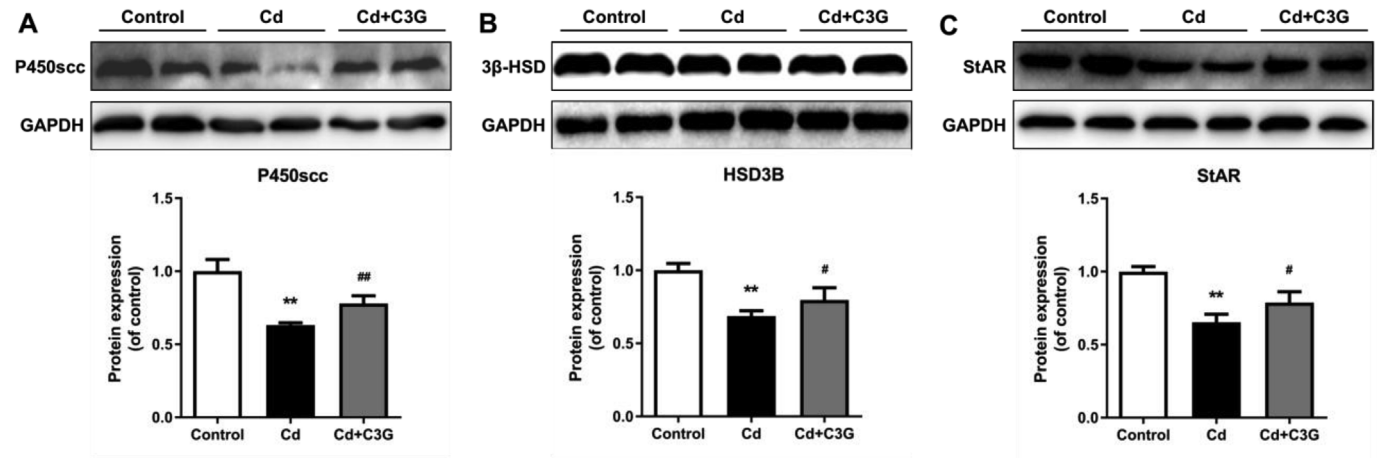

Fig. 7. The expression of proteins in the testes related to the testosterone synthesis pathway. The P450scc (A), 3β-HSD (B), and StAR (C) protein expression in the testes of mice treated with Cd and C3G for 30 days was conducted by western blotting and normalized to GAPDH. Data were shown as mean ± SD (n = 4). Comparison between all groups was evaluated through One-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with Cd group.

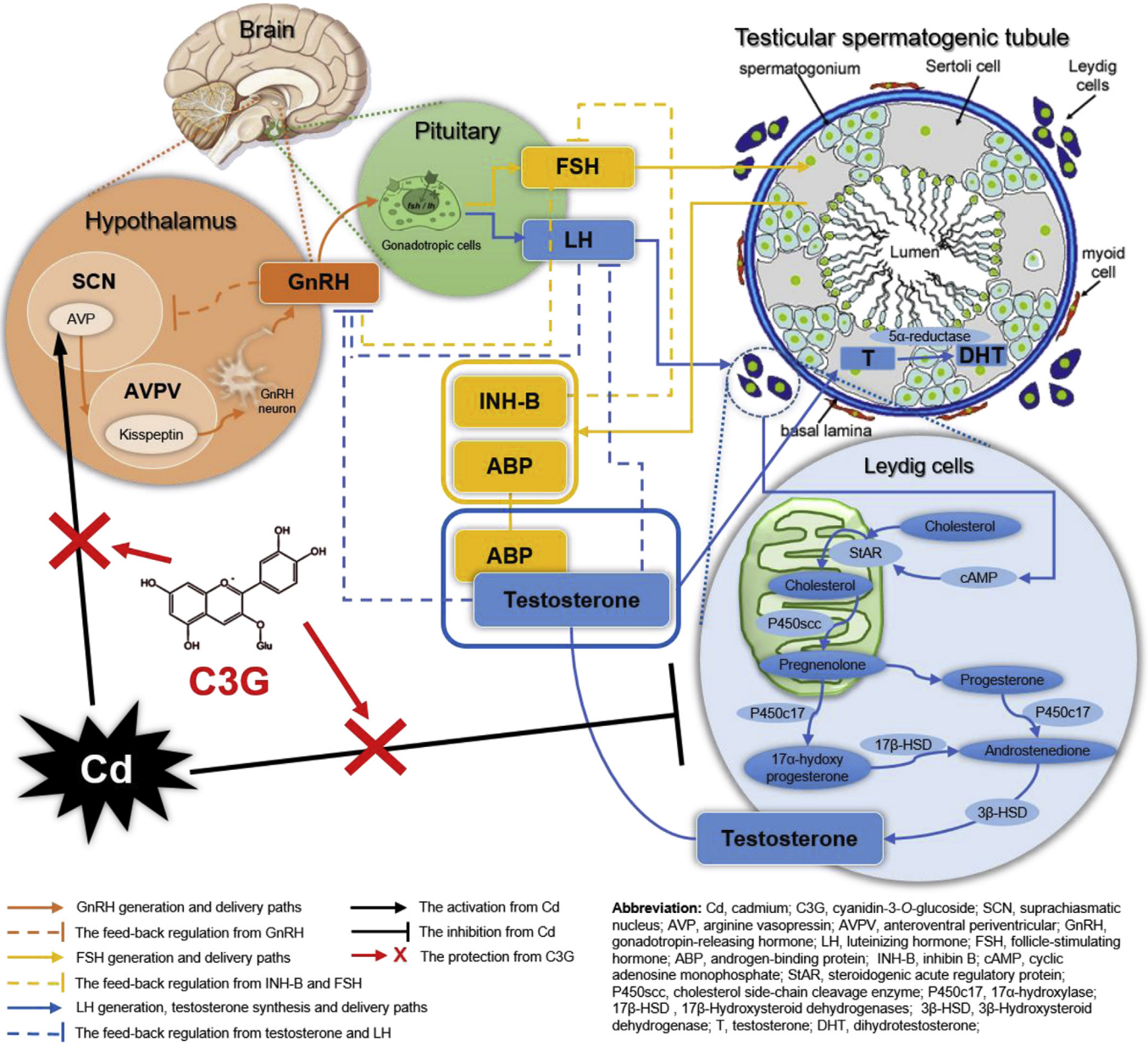

Fig. 8. The mechanism of C3G against the damage to the hypothalamus-pituitary-gonadal axis in male mice caused by Cd exposure.

system. However, it is still ambiguous as to whether it is the C3G or metabolites of C3G that show direct biological effects at the level of the hypothalamus.

Cd exposure resulted in GnRH being released in large quantities, an unchanged *Gnrhr* gene expression level, and greater FSH production from the pituitary. Hence, the *Fshr* in Sertoli cells was downregulated which allowed for a normal synthesis and secretion of INH−B at the days of 20 and 30. Following C3G intervention, there was a well-organized secretion of FSH, and the FSH receptor (FSHR) and INH−B were unaffected. On the contrary, exposure to Cd exerted an obvious decrease in LH and LH receptor (LHR) as a response to the increased testosterone after 30 day treatment through a negative feedback mechanism. This process differed from the influence of Cd during sexual maturity. This discrepancy may be due to the overwhelming release of GnRH and/or biological changes during puberty, which needs to be considered in a future study.

Pre-synthesis of testosterone depends on the coupling of LHR with the guanine nucleotide-binding proteins (G protein). The α-subunit free from G protein combines with adenosine triphosphate sulfurylase, and then the adenosine triphosphate is transformed into cAMP. With the increase of cAMP in cells, the PKA system is activated for further regulation of Leydig cell function (Zhang et al., 1998). In this research, caused by the excessive synthesis of testosterone, the *Lhr* gene expression was suppressed under stress and gave rise to restraint in the cAMP-CREB/CREM signaling pathway that is associated with spermatogenesis (Delmas and Sassonecorsi, 1994). Thereafter, the production of testosterone was reduced along with the suppression of the steroidogenic acute regulatory protein (StAR) (McGee and Narayan, 2013). Dietary exposure to C3G normalized the cAMP-CREB/CREM pathway outcome *via* upregulating the expression of *Lhr* gene and CREM to a normal level. Moreover, the increase in StAR expression allowed cholesterol to be transferred to the cholesterol side-chain cleavage enzyme that is commonly referred to as P450scc, which also supports the synthesis of progesterone in Leydig cells. Additionally, the C3G treatment also raised the HSD3B protein expression in the testis to induce the final crucial step of testosterone production, i.e., from androstenedione to testosterone.

5. Conclusion

It has been documented that Cd exerts neurotoxicity on the HPG axis and this is accompanied by dysfunction of hormone secretion. However, few studies have focused on the effects of Cd exposure in males during puberty. Moreover, as potentially targeted compounds, the protective effects of C3G on Cd-induced dysfunction of sex hormone secretion remains uncertain. In this study, we fed Cd-exposed male mice during puberty with C3G to investigate the specific mechanism by which C3G can cause protective properties. In conclusion, C3G ameliorated the Cd-induced changes in sex hormone levels in the serum to levels similar to the untreated mice. The protective effects are due to the controlling of Avp and Kisspeptin genes in the hypothalamus, thus mediating the level of LH and FSH receptor, and maintaining the testosterone synthesis pathway in the testes. This suggests a significant interaction of C3G with the HPG axis. Thus, this research provides new data that consumption of C3G can protect against Cd-induced sex hormone secretion dysfunction in pubertal males. Further investigation to examine the dose-dependent effects and also the effect on humans should be conducted in the future.

Conflict of interest

The authors declare that there is no conflict of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

Cd	cadmium
HPG	hypothalamus-pituitary-gonad
C3G	cyaniding-3-O-glucoside
GnRH	gonadotropin-releasing hormone
LH	luteinizing hormone
FSH	follicle stimulating hormone
INH−B	inhibin B
$CdCl_2$	cadmium chloride
LC-MS	liquid chromatography-mass spectrometry
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
P450scc	cholesterol side-chain cleavage enzyme
StAR	steroidogenic acute regulatory protein
PKA	protein kinase A
HSD3B	3β-Hydroxysteroid dehydrogenase
cAMP	cyclic adenosine monophosphate
CREM	cAMP responsive element modulator
DHT	dihydrotestosterone
ELISA	enzyme-linked immunosorbent assay
AVPV	anteroventral periventricular
AVP	arginine vasopressin
SCN	suprachiasmatic nucleus
GnRHR	GnRH receptor
CREB	cAMP-response element binding protein
ABP	androgen-binding protein
BBB	blood-brain-barrier
FSHR	FSH receptor
LHR	LH receptor
G protein	guanine nucleotide-binding protein

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