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Cyanidin-3-O-glucoside promotes the biosynthesis of progesterone through the protection of mitochondrial function in Pb-exposed rat leydig cells

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ABSTRACT

Cyanidin-3-O-glucoside (C3G) is an anthocyanin that has been reported to reduce the toxicity of heavy metals. In the present study, the protection effects of C3G on the biosynthesis of progesterone, the precursor of testosterone, against lead (Pb) in R2C rat Leydig cells were examined. Treatment of R2C cells with 100 μM Pb resulted in a significant decrease in progesterone production. After being cultured in a medium containing C3G and Pb, R2C cells exhibited an increase in progesterone concentration compared with the Pb treatment, as a result of up-regulation of the expression of the steroidogenic enzymes steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450 enzyme cholesterol side chain cleavage enzyme (CYP11A1). Pb-induced inhibition of extracellular regulated Kinase 1/2 (ERK 1/2) phosphorylation and down-regulation of protein kinase A (PKA) expression were alleviated by C3G. In addition, Pb-induced reactive oxygen species (ROS) overproduction led to mitochondrial depolarization, resulting in a decrease in progesterone biosynthesis, while C3G intervention reduced the ROS level and increased progesterone production. In conclusion, C3G may alleviate the Pb-induced decrease of progesterone biosynthesis by modulating the dysfunction of mitochondria, including decreasing oxidative stress and regulating expression of steroidogenic enzyme proteins.

1. Introduction

Lead (Pb) is a ubiquitous heavy metal which has been widely used in the production of, inter alia, electricity, cosmetics, chemicals, paint pigments and printing. It has been reported that Pb is associated with gastrointestinal disturbances (Kalahasthi et al., 2014), kidney dysfunction (Fels et al., 1998), impaired hemoglobin synthesis (Dai et al., 2017) and nervous system damage (Buchner, 2009). As one of the most well-known reproductive toxicants, Pb has inhibitory effects on the hypothalamic-pituitary-testis axis, which plays key roles in male reproductive function (Gandhi et al., 2017; Rana, 2014). Pb disrupts endocrine systems, including the activity of Leydig cells which secrete testosterone in males (Iavicoli et al., 2009). Testosterone is produced from cholesterol in leydig cells. Cyclic Adenosine 3,5-Monophosphate (cAMP) accumulates in response to the stimulation of gonadotropin luteinizing hormone (LH). Then steroidogenic acute regulatory protein (StAR) is activated and cholesterol is transported into the inner mitochondrial membrane by StAR (Miller and Bose, 2011). Next, cholesterol is converted to pregnenolone by the cytochrome P450 enzyme cholesterol side chain cleavage enzyme (CYP11A1). Pregnenolone is converted to testosterone in the presence of steroidogenic enzymes in the smooth endoplasmic reticulum (Rone et al., 2009). It has been reported that Pb inhibits the production of testosterone in Leydig cells (Ji et al., 2015; Liu et al., 2001; Thoreux-Manlay et al., 1995). A Pb-induced decrease in the protein expression level of StAR, the steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and CYP11A1 was found to lead to decreased steroidogenesis (Ji et al., 2015).

It has been reported that Pb-induced decreases in testosterone production are associated with oxidative stress, including increased production of reactive oxygen species (ROS) and decreased antioxidant enzyme activity (Pandya et al., 2012). ROS have been shown to inhibit

Abbreviations: cAMP, Cyclic Adenosine 3,5-Monophosphate; CAT, Catalase; CYP11A1, Cytochrome P450 Enzyme Cholesterol Side Chain Cleavage Enzyme; C3G, Cyanidin-3-O-glucoside; ERK 1/2, Extracellular Regulated Kinase 1/2; LH, Luteinizing Hormone; PKA, Protein Kinase A; p-ERK 1/2, phosphorylated ERK 1/2; ROS, Reactive Oxygen Species; SOD2, Superoxide Dismutase 2; StAR, Steroidogenic Enzymes Steroidogenic Acute Regulatory Protein; 3β-HSD, 3β-Hydroxysteroid Dehydrogenase

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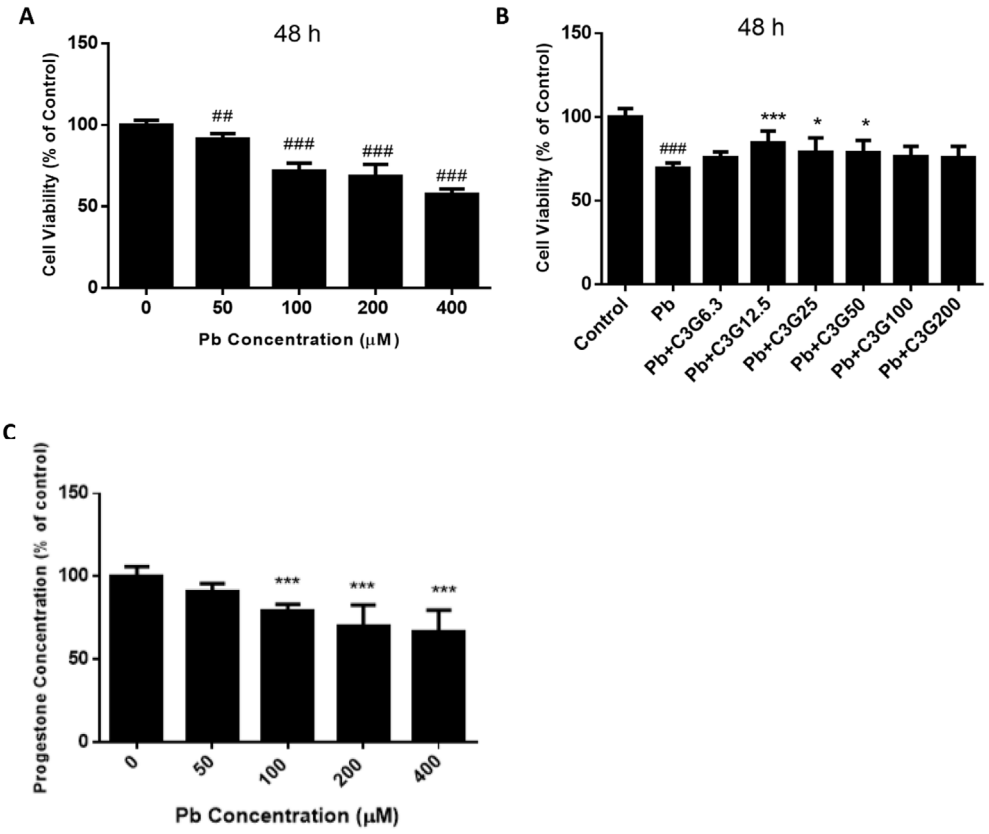

Fig. 1. (A) Cell viability of R2C cells treated with Pb for 48 h. (B) Cell viability of R2C cells treated with Pb and C3G for 48 h. (C) Progesterone concentration of R2C cells treated with Pb for 24 h. Mean ± SD, n = 3. $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$ vs. control; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. Pb-treated group.

cholesterol transfer and steroidogenesis in MA-10 tumor Leydig cells (Diemer et al., 2003). Mitochondria are central to the regulation of steroid hormone biosynthesis (Hales et al., 2005). Excessive production of ROS induces a decrease in mitochondrial membrane potential (MMP), which further leads to a decrease in protein expression of StAR (Diemer et al., 2003).

Anthocyanins are antioxidants abundant in fruits and vegetables, and are responsible for the red, blue, or purple color of plants (Fernandes et al., 2014). Anthocyanins extracted from blueberries have been reported to confer protective effects against cadmium-induced hepatotoxicity in mice due to their antioxidant and anti-inflammatory properties (Gong et al., 2014). Cyanidin-3-O-β- glucoside (C3G) is the most abundant anthocyanin in nature (Kamiloglu et al., 2015). Several studies have found that C3G may decrease oxidative damage through scavenging free radicals and regulating the activity of reductase (Jiang et al., 2014; Sun et al., 2016; Zhang et al., 2010). Moreover, The 3, 4 ortho-dihydroxy on the B ring of anthocyanin is able to chelate with heavy metals and reduce metal ion concentrations, and further alleviate heavy metal-induced toxicity (Li et al., 2017). Thus, C3G may be able to alleviate Pb-induced decreases in steroid hormone biosynthesis.

R2C is a rat Leydig tumor cell line, it secretes large amounts of progesterone in the absence of hormonal stimulation (SI et al., 1968). The progesterone production in R2C cells is in a cycloheximide-sensitive manner instead of a cAMP-dependent manner (Freeman, 1987, 1996). Progesterone is the precursor of testosterone and is regarded as the key evaluation index with respect to reproductive function (Rao et al., 2003). In this study, R2C cells were cultured in medium containing Pb with or without C3G, and possible protective effects of C3G against Pb-induced decrease in progesterone biosynthesis were studied.

2. Materials and methods

2.1. Cell culture

R2C was purchased from ATCC (Manassas, VA, USA). Cells were cultured in Ham's F12 nutrient medium supplemented with 2.5% fetal bovine serum (Gibco, Rockville, MD, USA), 15% horse serum (Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 g/mL) in a humidified atmosphere of 5% CO_2 at 34 °C.

2.2. Cell viability test

Cells were seeded at a density of 4000 cells per well in 96-well plates and treated with 50, 100, 200 or 400 μM Pb (lead acetate, purity > 99%, Sigma–Aldrich, St. Louis, MO, USA) for 48 h. The medium was then replaced by 200 μL CCK8 solution (Beyotime Biotech, Nantong, China) diluted at 1: 10 with F12 medium per well, and cells were incubated at 34 °C for 2 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Chantilly, VA, USA). Relative cell viability of treated cells was expressed as a percentage of the control (which represented 100%). In addition, a cell viability test was also performed on the R2C cells treated with 100 μM Pb and 12.5, 25 or 50 μM C3G (purity > 98%, J & K Scientific, Beijing, China).

2.3. Progesterone measurement

Cells were cultured in 96-well plates at a density of 10^4 cells per well and treated with 50, 100, 200 or 400 μM Pb for 24 h. The culture medium was collected and centrifuged at 400 g for 5 min at 4 °C. Progesterone concentrations in the supernatants were measured using a radioimmunoassay kit (Beijing North Institute of Biological Technology, Beijing, China) according to the manufacturer's instructions. GC-1200γ γ-radioactive immunoassay count machine (Anhui