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Ovario-protective effects of genistein against cyclophosphamide toxicity in rats: Role of anti-müllerian hormone and oestradiol

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ABSTRACT

Cyclophosphamide (CP), the commonly used chemotherapeutic agent in cancer treatment, is proven to cause ovarian toxicity and infertility in women. In the present study, we investigated the protective effect of genistein (GEN), a phytoestrogen found in the soy protein, against CP-induced ovarian toxicity in rats. Forty female adult rats were allocated into five groups. A normal control group received the vehicle; another group was injected with a single acute intraperitoneal dose of CP (200 mg/kg). Three other groups were pretreated with GEN (0.5, 1 or 2 mg/kg; s.c.) for 14 days. Sera and ovaries were obtained 48 h after CP treatment. Serum levels of anti-müllerian hormone (AMH) and oestradiol (E_2) were detected as well as the ovarian level of reduced glutathione (GSH), activity of superoxide dismutase (SOD), level of malondialdehyde (MDA) and interleukin 1β (IL-1β) were evaluated. Histopathological examination and immunohistochemical detection of inducible nitric oxide synthetase (iNOS) were conducted. Results of the present study revealed that CP-induced severe ovarian toxicity via decreasing serum levels of AMH and E_2 and elevating oxidative stress and inflammation in ovarian tissues. Histologically, CP caused increase in primordial follicles with less graafian follicles and corpora lutea in ovarian tissues as well as severe induction of iNOS. GEN inhibited the severe decrease in serum AMH and E_2 with alleviation of oxidative stress and inflammation significantly compared to CP-treated group. GEN improved ovarian histology and immunostaining of ovarian iNOS disrupted by CP. Finally, it can be concluded that GEN exerted protective effects against CP-induced ovarian toxicity.

1. Introduction

Cyclophosphamide (CP) is the most effective alkylating anticancer drug widely used in the treatment of chronic and acute leukemia, multiple myeloma, and lymphomas (Zaki et al., 2003). CP is associated with a great risk of infertility in females (Lee et al., 2006; Tomao et al., 2010). CP itself, phosphoramide mustard, and other metabolites are detoxified by conjugation with GSH (Gamcsik et al., 1999) with subsequent production of intracellular reactive oxygen species (Ghosh et al., 2002; Manda and Bhatia, 2003) and interfere greatly with the antioxidant defense of the ovary (Tsai-Turton et al., 2007). Not only the ovaries, CP affects major organs such as heart and kidney through elevation of oxidative stress markers and depletion of antioxidant defense mechanisms (Alhumaidha et al., 2015). CP exerts cytotoxicity by changing the activities of enzymes and levels of non-enzymic antioxidants (Lopez and Luderer, 2004; Selvakumar et al., 2006). Superoxide dismutase (SOD), an important enzymatic antioxidant (Fujii et al., 2005), suppression of this antioxidant enzyme due to CP or its metabolites (Yener et al., 2013) promoted oxidation by overproduction of the free radicals which covalently bind to DNA and increase the pro-apoptotic signals (Lopez and Luderer, 2004). MDA levels, as a measure of lipid peroxidation, increase in ischemia (Isaoglu et al., 2012) and ischemia/reperfusion (I/R) injuries in rat ovaries (Bayir et al., 2012). Acrolein, a metabolic product of CP, has been identified as an initiator of lipid peroxidation (Adams and Klaidman, 1993). Nevertheless, MDA was elevated significantly in CP-induced ovarian toxicity (Yener et al., 2013).

Linking cytokine production to reactive oxygen species generation; cytokines act normally to stimulate the generation of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) in their target inflammatory cells (Wang et al., 1999). Being the most potent molecules of the innate immune system, IL-1 family members (Sims and Smith, 2010), specially, IL-1β is generated upon inflammatory signals (Dinarello, 2011). Studies reported various inflammatory cytokines, including TNF-α and IL-1β, implicated in the pathogenesis of CP-induced hemorrhagic cystitis through the induction of iNOS and NO production (Linares-Fernandez and Alfieri, 2007; Ribeiro et al., 2002).

Cyclophosphamide causes ovarian failure by modification of rat

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ovaries structure and function along with modification of granulosa cell proliferation and ovarian hormone (oestradiol) secretion (Ataya et al., 1989). Moreover, ovarian toxicity-induced by CP, in a dose dependent manner, reported widespread loss of primordial, preantral, and healthy antral follicles associated with reduction in serum progesterone and estradiol (Jarrell et al., 1987).

Being exclusive to the ovary, anti-müllerian hormone (AMH), a dimeric glycoprotein which belongs to the TGF-β family are essential for tissue growth and differentiation (La Marca et al., 2005). It is well established that AMH is the more accurate marker of the ovarian reserve (de Vet et al., 2002) than follicle stimulating hormone (FSH), luteinizing hormone (LH), oestradiol (E_2), and inhibin B because their levels vary widely by assay, laboratory, population, and reproductive aging (Dayal et al., 2014). AMH is undetected in serum 3–5 days after bilateral ovariectomy (La Marca et al., 2005). As depletion of primordial follicles is rapid and drastic in animals treated with CP (Ataya et al., 1990; Ramahi-Ataya et al., 1988), one study reported a deep AMH decrease in CP-treated mice concurrent with histological follicular depletion (Detti et al., 2013).

Oxidative stress is a well-established mediator of CP-induced granulosa cell apoptosis (Tsai-Turton et al., 2007). Thus, compounds with antioxidant activity largely decreased atresia of antral follicles (Tilly and Tilly, 1995). Application of natural components and plant extracts has been evolved worldwide to minimize the harmful effects of CP (Yener et al., 2013). Most soybeans products contain large amounts of isoflavones called soy phytoestrogens. Genistein (GEN) is the major phytoestrogen (60%) found in soybean (Lee et al., 2012). Phytoestrogens function as natural selective estrogen receptor (ER) modulators, depending on the tissue and the presence of co-regulator proteins (Pike et al., 1999). Most studies focused on the pharmacological activities of GEN due to its chemoprotective activities against cancers and cardiovascular disease, and its antioxidant and phytoestrogen activity (Dixon and Ferreira, 2002). Furthermore, numerous studies have shown that GEN has protective effects against ovarian carcinogenesis (Andres et al., 2011; Kim et al., 2011). However, the beneficial effect of GEN on ovarian follicular development, which may prolong ovarian reproductive life has been shown in mature (Zhuang et al., 2010) and immature rats (Medigovic et al., 2012).

Women subjected to chemotherapeutic drugs progress amenorrhea and/or infertility, therefore, additional information is required to establish whether phytoestrogens can prevent gonadotoxicity induced by chemotherapeutic drugs. To our knowledge, this is the first study to investigate the effects of GEN on the prevention of ovarian damage induced by CP not only through its antioxidant effect but also its possible anti-inflammatory, anti-apoptotic effects with specific emphasis on the hormonal status during GEN administration.

2. Material and method

2.1. Drugs and chemicals

Genistein (LC Laboratories, Woburn, Massachusetts, USA; purity $>99\%$) and cyclophosphamide (CP, Endoxan®, Baxter Oncology GmbH, Germany) were used in the study. GEN was dissolved in 5 ml DMSO (1.25%) and divided into equal aliquots (Vera et al., 2005). Every day, an aliquot of the drug was freshly diluted by distilled water and injected subcutaneously. The concentration was adjusted so that each 100 g animal body weight received 0.25 ml, 0.5 ml and 1 ml for doses 0.5, 1 and 2 mg/kg, respectively. All of the other chemicals were of highest analytical grade available.

2.2. Experimental animals

The study included a total of 40 female Wistar Albino rats, weighing 200–250 g, were obtained from the animal house at the National Research Centre (Giza, Egypt). The animals were kept under suitable laboratory conditions throughout the period of investigation. They were kept in specially prepared cages in a room that had daylight for 12 h. They were fed standard pellet chow, provided by the animal house at the National Research Centre, and allowed free access to water. Water was given through special dropper-tipped bottles placed specially in the cages.

The experiment was conducted in accordance with ethical rules for standard experimental animal studies and the Medical Research Ethics Committee (MREC) of the National Research Centre under approval number 15021.

2.3. Treatment regimen

The rats were equally allocated to 5 groups. A group was injected with distilled water subcutaneously for 14 days, on the 15th the rats were administered a single-intraperitoneal injection CP at a dose 200 mg/kg in saline (Korach, 1998; Oktem and Oktay, 2007). Three other groups were injected subcutaneously with GEN at doses 0.5, 1 and 2 mg/kg (Saleh et al., 2014), respectively, daily for 14 consecutive days, on the 15th the rats were administered a single injection of CP (200 mg/kg, i.p). In addition to, a normal control group that received the corresponding vehicles.

Forty-eight hours after CP injection, blood samples were withdrawn via retro-orbital sinus, and sera samples were extracted and kept at -20 °C until use. The whole left ovarian tissues were collected, homogenized and supernatants were used for biochemical analysis. The right ovary was entirely removed, weighed, fixed in 10% formaldehyde, and processed for histological and immunohistochemical evaluations.

2.4. Biochemical analysis

2.4.1. Measurement of ovarian oxidative stress biomarkers

Total glutathione (GSH/GSSG) assay was performed with a spectrophotometric assay kit (Catalog number STA-312, Cell Biolabs, Inc., USA). Assay was based on the presence of glutathione reductase which reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH. Subsequently, the chromogen reacts with the thiol group of GSH to produce a colored compound that absorbs at 405 nm. Data were expressed as μmol of GSH per 1 g of ovary tissue.

Malondialdehyde (MDA) assay was performed with a spectrophotometric assay (Catalog number STA-330, Cell Biolabs, Inc., USA). Assay was based on the reaction of MDA with thiobarbituric acid (TBA) at 95 °C forming an MDA-TBA complex which absorbs light strongly at 532 nm. The absorbance was directly proportional to the MDA concentration. Data were expressed as nmol of MDA per 1 g of ovary tissue.

Superoxide dismutase (SOD) activity was measured immediately by a colorimetric assay of SOD (Catalog number 7500-100-K, Trevigen, Inc, USA). SOD assay kits based on generation of ions from the conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxidase, converts NBT to NBT-diformazan. NBT-diformazan absorbs light at 550 nm. SOD reduces superoxide ion concentrations and thereby lowers the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity present in experimental sample. Data were expressed as U of SOD per 1 g of ovary.

2.4.2. Measurement of ovarian inflammatory cytokine

Interlukin-1beta (IL-1β) measurement was performed using