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Pro/antioxidant status in young healthy women using oral contraceptives

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ABSTRACT

The aim of the study was to analyze the effects of oral contraceptives (OCs) on pro/antioxidant status in the blood of healthy women aged 20–25 years.

Individuals were divided into OCs users and OCs nonusers. Markers of oxidative stress in the blood such as Cu, Cu/Zn ratio, malondialdehyde (MDA), glutathione oxidized (GSSG), and gamma-glutamyl transpeptidase (GGT) were determined. Antioxidants such as glutathione reduced (GSH), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), and superoxide dismutase (SOD) were estimated.

Higher Cu concentrations, Cu/Zn ratio and GGT activity in women taking OCs were noted. A significant increase in MDA concentrations in oral OCs users was observed. Heightened activity of CAT in plasma was observed in OCs users, whereas SOD activity remained unchanged in plasma and erythrocyte lysate. A decline of GSH and GSSG in whole blood and glutathiono-dependent enzymes (GPx in plasma, GR in plasma and GST in lysate) was shown.

Use of OCs leads to a pro/antioxidant imbalance. The results in the present study confirmed that GGT is an early marker of oxidative stress. Catalase is the main antioxidant, involved in the removal of free radicals in OCs users.

1. Introduction

Oral contraceptives (OCs) are now commonly used by millions of women worldwide as a methods for preventing pregnancy; they work primarily by suppressing ovulation. OCs contain two active components: estrogen and progestin (Oesterheld et al., 2008).

Use of contraceptive pills brings with it numerous side effects. Depending on their estrogenic and progestogenic components, they can exert a variety of effects on the body. One of these effects is alterations in hepatic enzyme levels related to the synthesis and/or turnover of lipids and lipoproteins (Inal et al., 2008).

The liver plays a key role in the metabolism of oestrogens and progestogens, which can act directly or indirectly on the liver to produce a variety of biological effects of both physiological and pathological significance. One of the enzymes which indicates the degree of liver damage caused by contraceptive pills is gamma-glutamyl transpeptidase, GGT. This enzyme is measured in the most commonly-used tests for qualitative assessment of liver disease (Daniel et al., 2000). The function of GGT is to metabolize extracellular reduced glutathione, the main antioxidant in the body, allowing for precursor amino acids to be assimilated and reutilized for intracellular GSH (Lim et al., 2004). Experimental studies indicate that presence of the membrane protein GGT in serum might be an early and sensitive marker for oxidative stress (Lim et al., 2004) associated with increased levels of transition metals in body. The oxidative properties of GGT escalate in the presence of *inter alia* copper (Stark et al., 1993). Hormones contained in OCs impact levels of copper, zinc and iron in the blood of women (Lopes et al., 2004). An imbalance between transition metal ions, such as copper and zinc, could lead to oxidative stress. Transition metal ions play an important role in initiating lipid peroxidation. Iron and copper can also initiate the peroxidation of membrane lipids. Peroxidation of membrane structures leads to the functional impairment of membrane-bound proteins. Side products of lipid peroxidation can also lead to greater toxicity, even at sites more distant from the primary peroxidation event. Therefore, synthetic sex steroids may increase concentrations of free radicals ($O_2^{\bullet -}$, $^{\bullet}OH$), and in consequence boost oxidative stress (Chen and Kotani, 2012; Kose et al., 1993).

In order to prevent excessive production of free radicals, antioxidants systems, both enzymatic and non-enzymatic, are generated

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in the body. The primary, ubiquitous nonenzymatic regulator of intracellular redox homeostasis is γ-glutamylcysteinylglycine. This tripeptide exists in reduced (GSH) or oxidized (GSSG) form, and participates in redox reactions by the reversible oxidation of its active thiol (Masella et al., 2005). In physiological status, GSH concentrations are greater than its oxdized species. GSSG is produced by the catalysis of glutathione peroxidase (GPx) influenced by ROS. GPx constitutes a family of enzymes which are capable of reducing hydrogen peroxide (H_2O_2) (previously transformed by superoxide dismutase, SOD) as well as converted or organic hydroperoxides to water (H_2O) or the corresponding alcohols, respectively, utilizing GSH as a reductant (Ursini et al., 1995). GSSG may then be converted back to its reduced form by glutathione reductase (GR) (Valko et al., 2007). GSH is also a substrate for glutathione S-transferase (GST) in reactions contributing to the transformation of a wide range compounds including products of oxidative stress, therapeutic drugs and carcinogens (Valko et al., 2007). Similarly to GPx, catalase (CAT) also converts hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) (Zal et al., 2012). In physiological status, these enzymes interact and complement each other in order to maintain the pro/antioxidant balance (Valko et al., 2007).

The aim of the present study was to examine the influence of OCs on pro/antioxidant balance in young healthy women. This was achieved by attaining the following sub-goals: (1) Determination of oxidative stress levels in OCs users (MDA, GSSG and Cu concentrations, GGT activity and Cu/Zn ratio); (2) Determination of antioxidant status in women taking OCs (GSH concentration, SOD, CAT, GPx, GST, GR activities).

2. Materials and methods

2.1. Patients

Blood samples were collected from 120 healthy students of Wroclaw Medical University examined by a doctor of occupational medicine. The study protocol was approved by the Local Bioethics Committee of Wroclaw Medical University (KB-141/2015). All participants were of a similar age (22.6 ± 1.0 years) and similar BMI (20.71 ± 2.20 kg/m^2). A personal interview about their lifestyle was carried out. Participants were asked about their health and nutritional habits, and the use of dietary supplements/medications. Subjects who did not report any diseases or special diet (e.g. vegetarian, Mediterranean), and were not taking any drugs nor dietary supplements which could disrupt the homeostasis of the body, were admitted to the study. In order to excluded alcohol abusers from the study, carbohydrate-deficient transferrin (CDT) (CEofix CDT kit for Beckman Coulter P/ACE MDQ Series; Ref. No.: 844111036), a biomarker for long-term alcohol consumption, was estimated. Furthermore, cotinine (Cotinine ELISA test; Ref. No.: EIA-3242, DRG International, Inc. USA), a metabolite of nicotine, was measured to eliminate smokers. The results in all the patients were within the normal range, and based on these tests only non-smokers and non-abusers of alcohol were examined. Samples were divided into 2 groups: A and B. Group A consisted of 74 females who do not take OCs. Group B consisted of 46 females taking OCs for a period of at least one year (monophasic pills containing 0.02 mg ethinyl estradiol and 3 mg drospirenone).

2.2. Sample preparation

Venous blood was collected in the morning, after 8 h of fasting. Plasma and erythrocyte pellet were obtained according to the standard procedure by taking venous blood into tubes free of trace elements and containing heparin (ref. No.: 04.1931.001, Sarstedt, Germany). Whole blood samples were centrifuged at $2500 \times g$ for 15 min to separate the plasma and buffy coat from the erythrocyte pellet. The washed cells were lysed by the addition of ice-cold double distilled water (1:1.4). The pellet was washed in an equal volume of ice-cold 0.9% NaCl. This process was repeated twice.

Serum was obtained according to the standard procedure by drawing venous blood into tubes free of trace elements and with a serum clotting activator (ref. No.: 03.1524.001, Sarsted, Germany), left at 25 °C to complete thrombosis, and centrifuged at $1200 \times g$ for 20 min.

In order to obtain samples for analysis of GSH and GSSG, 1050 μl of distilled water was added to 150 μl of whole blood, then mixed and incubated for 10 min. Next, 300 μl of 25% metaphosphoric acid (MPA) was added (ref. No.: 253-433-4, Sigma-Aldrich, Germany), mixed and centrifuged for 10 min at $3000 \times g$. Then the supernatant was collected.

The obtained serum, plasma, erythrocyte lysate and whole blood treated with MPA were portioned and stored in sealed tubes (No. Cat. 0030101.002, Eppendorf, Germany). Blood samples were stored at −80 °C until analysis.

2.3. GSH, GSSG and MDA concentrations assay

GSH and GSSG concentrations were measured by the capillary electrophoresis (CE) method described in Kowalska et al. (2015a). CE was performed on a PA 800 Plus Pharmaceutical Analysis System equipped with an ultraviolet detector set at 200 nm. The separations were performed on a fused-silica capillary purchased from Beckman Coulter. GSH and GSSG measurements were conducted using the determination of GSSG and GSH in the whole blood analysis kit from Analis (ref. No.: 10-004770, CEofix^{TM}, Belgium).

The concentration of MDA in plasma was measured by a tiobarbituric acid (TBA) assay, in which MDA present in plasma forms a red adduct with two molecules of TBA (Nielsen et al., 1997).

Values of GSH, GSSG and MDA were expressed in μM.

2.4. GGT estimation

GGT activity in serum was measured manually using a reagent for quantitative determination of GGT activity (ref. No.: 1-228-0060, BioMaxima, Poland). GGT is the enzyme which catalyzes the transfer of the γ-glutamyl group of γ-glutamyl-3-carboxy-4-nitroanilide to glycyl-glycine, and produces L-γ-glutamyl-glycylglycine and 5-amino-2-nitrobenzoate. The rate of formation of 5-amino-2-nitrobenzoate is directly proportional to GGT activity at $\lambda = 405$ nm. One unit of GGT is the amount of the enzyme which catalyzes the transfer of 1.0 μmol of the γ-glutamyl group from γ-glutamyl-3-carboxy-4-nitranilide to glycylglycine per 1 min at 37 °C.

Values of GGT activities were shown in U/l.

2.5. Estimation of GPx, GST, GR, SOD and CAT activities

The assay of GPx activity was performed with a Glutathione Peroxide Assay Kit (ref. No.: 703102, Cayman Chemicals, USA), which measures GPx activity indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at $\lambda = 340$ nm. One unit of GPx is defined as the amount of enzyme which oxidizes 1.0 nmol of NADPH to NADP$^+$ per 1 min at 25 °C (ref. No.: 703102, Cayman Chemicals, USA Kit).

GST activity was measured by a Glutathione S-Transferase Assay Kit (ref. No.: 703302, Cayman Chemicals, USA). This test determines total GST activity by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at $\lambda = 340$ nm. One