In Vivo Effects of Oral Contraceptives on Paraoxonase, Catalase and Carbonic Anhydrase Enzyme Activities on Mouse

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Many effects that oestrogens and progestrogens used in oral contraceptive (OC) have on enzyme physiology are of importance on homeostasis. This study was carried out in order to determine the *in vivo* effect of three oral contraceptives containing ethinyl estradiol in combination with desogestrel and levonorgestrel on the paraoxonase (PON), catalase (CAT) and carbonic anhydrase (CA) activities in mice, which are model organisms for humans. Serum and liver paraoxonase activities were determined spectrophotometrically by using paraoxan as a substrate according to the methods of Gan *et al.* and Gil *et al.*, respectively. Catalase and carbonic anhydrase activities were determined from erythrocytes used Aebi and Maren methods, respectively. For these studies, a group of ten mice (25±2 g) was selected for oral administration for 21 d of each drug (0.15 mg desogestrel+0.03 mg ethinylestradiol (A); 0.15 mg levanogestrel+0.03 mg ethinylestradiol (B) and 0.15 mg desogestrel+0.02 mg ethinylestradiol (C)). A group of ten mice was included in the study for a control group, which were not subject to drug administration. For each drug, a mean of the serum and liver paraxonase activity and erythrocytes catalase and carbonic anhydrase activities were determined and compared to the control groups. While mouse liver PON activity showed a statistically significant decrease for all three drugs, serum PON activity increased. Erythrocytes catalase activity was significantly decreased by all contraceptives used. On the other hand, these contraceptives did not change the erythrocytes carbonic anhydrase activity.

Key words contraceptive; paraoxonase; catalase; carbonic anhydrase; *in vivo*; mice

Almost 40 years of clinical experience with birth control pills established their role not only as the most reliable method of contraception but also for a variety of therapeutic indications. Over the intervening decades a remarkable change in the hormone content of the various formulations has been seen: hormone quantity has decreased; multiphase formulations and different progestogens have been introduced. These changes were necessary after case reports on serious side effects during oral contraceptive (OC) use had been published.[1—3]

Progestogens, are known to have various metabolic effects, including effects on lipid metabolism and, therefore, are potentially on the risk for cardiovascular disease.[4] It has been suggested that contraceptive steroids might exert their metabolic effects by changing hepatic enzyme levels related to the synthesis and/or turnover of lipids and lipoproteins.[5] A study has demonstrated that there is a close relationship between women taking third generation OCs, and increased atherothrombotic risk.[1] OCs undoubtedly affect carbohydrate metabolism and this effect relates to deterioration in glucose tolerance and increase of peripheral insulin resistance.[6] Yager *et al*. demonstrated a correlation between the prolonged use of oral contraceptives and the development of liver cancer in rats.[7] Although over 60 million women use contraceptives like A, B and C worldwide, the exact effects of these oral contraceptives on paraoxonase, catalase and carbonic anhydrase have been unknown.

Paraoxonase (PON) (aryldialkyl phosphatase, E.C.3.1.8.1) is a calcium dependent serum esterase that is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high-density lipoproteins (HDL).[8] PON1 received its name from paraoxon, the toxic metabolite of the insecticide parathion, which is one of its most studied substrates. PON1 hydrolyzes the active metabolites of several other organophosphorus insecticides (*e.g.*, chlorpyrifos oxon, diazoxon), as well as nerve agents such as sarin, soman and VX.[9—11] One natural physiological function of PON1 appears to be the metabolism of toxic oxidized lipids of both low-density lipoprotein (LDL) particles as well as HDL particles.[12] Catalase (CAT) plays a major role in the protection of tissues from the toxic effects of H_2O_2 and partially reduced oxygen species. Catalase, iron-containing enzyme (oxidoreductase, E.C.1.16.1.6) which catalyses the breakdown of H_2O_2 is a potentially destructive agent in cells.[13] Carbonic anhydrase (CA) (Carbonate hydrolysis, E.C.4.2.1.1), which reversibly catalyses the hydration of carbondioxide to bicarbonate and hydrogen ions, is widely distributed in mammalian tissues and has an important role in gas transport, acid/base regulation, calcification, and various secretory functions in tissues.[14—17] Therefore, the aim of this study was to determine the *in vivo* effects of some contraceptives, containing ethinyl estradiol in combination with desogestrel and levonorgestrel on PON, CAT and CA activities because of their physiological importance. These oral contraceptives were chosen because they are very common in our country.

MATERIALS AND METHODS

Materials All chemicals used in this study were obtained from Sigma Chem. Co. and Merck (Germany) and they were analytical grade. Contraceptives and CO_2 that were used are commercially available.

Methods Ten female mice (*Mus musculus diolecticus*, white type) (25±6 g) were selected for oral administration of each contraceptive. Mice used for *in vivo* studies were under special conditions (in a windowless room, 22 °C, with light on for 12 h and 65% humidity) for 1 month. A group of ten mice were included in the study for a control group, which were not subject to any drug administration. These contraceptives were orally given to mice through 21 d. Drug dosage

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Table 1. Experiment Design of Study Groups

Names of contraceptives used	Names of components	Amounts of components (μg/tablet)	Dose treated (μg/d)
Control	Water	No component	50 μl
A	Desogestrel+ethinyl estradiol	7.1+1.4	$3.2\times10^{-3}+6.4\times10^{-4}$
B	Desogestrel+ethinyl estradiol	7.1+1.0	$3.2\times10^{-3}+4.3\times10^{-4}$
C	Levonorgestrel+ethinyl estradiol	7.1+1.4	$3.2\times10^{-3}+6.4\times10^{-4}$

Ten mice (*Mus musculus diolecticus*, white type) (25±6 g) were selected for oral administration of each contraceptive. A group of ten mice were included in the study for a control group, which are not subject to any drug administration. These contraceptives were orally given to mice through 21 d. Drug dosage for mice was calculated from suggested dose for humans. A, C and B were selected because of used frequently among young women.

for mice was calculated from suggested dose for humans (Table 1). For each drug mice were sacrificed by using cervical dislocation method. Blood and liver samples were taken from each mouse. Once liver samples were taken, they were kept at −80 °C until analysis. Blood was collected in dry tubes and serum was seperated by centrifugation. Serum and erythrocytes were stored at −80°C until analysis. Enzyme activities were not affected by freezing and storage at −80°C.

Preparation of the Microsomal Fraction Mice livers were removed and then placed in beakers on ice, rinsed with ice-cold homogenization buffer (5 mM Tris–HCl buffer, pH 7.4 containing 0.25 M sucrose), minced with scissors and placed in 4 vol. of ice-cold homogenization buffer. They were then homogenized (6 strokes at 1100 rpm) using a homogenizer. After diluting the homogenate to % 10 (w/v) with homogenization buffer, nuclei and mitochondria were removed by successive centrifugation at 460 ***g*** for 10 min. The post-mitochondrial supernatant fraction was then centrifuged at 105000 ***g*** for 60 min. The microsomal pellet derived from 10 g of liver tissue was suspended in 20 ml of 5 mM Tris–HCl buffer, pH 7.4. The microsomal fraction was adjusted to 0.75% Triton X-100, vortexed, stored at 4 °C for 30 min and then centrifuged at 105000 ***g*** for 60 min. The resultant supernatant fraction was used for enzyme activity assay.[18,19]

Enzyme Assay. Measurement of PON1 Activity Paraoxonase activity was quantified spectrophotometrically using 100 mM Tris–HCl buffer, pH 8.0 containing 2 mM $CaCl_2$. Reaction was initiated by the addition of 50 μl of serum or 100 μl of microsomal fraction and was followed for 2 min at 37 °C by monitoring the appearance of *p*-nitrophenol at 412 nm in a Biotek automatic recording spectrophotometer. All rates were determined in dublicate and corrected for the non-enzymic hydrolysis. The final substrate concentrations during enzyme assay were 2 mM and 1.5 mM for microsomal fraction and serum, respectively.[20]

Measurement of CAT Activity The catalase activity was measured by the Aebi method. In this method, 20 μl enzyme solution was added to the 1 ml 10 mM H_2O_2 in 20 mM potassium phosphate buffer (pH 7.0) and incubated at 25°C for 1 min. Initial reaction rate was measured from the decrease in absorbance at 240 nm.[21]

Measurement of CA Activity Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson and modified by Maren *et al*. CO_2-hydratase activity as an enzyme unit (EU) was used the equation $(t_o - t_c/t_c)$ where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.[22]

Statistical Analysis Statistical analysis was performed by using Minitab program for Windows, version 10.02. Analysis of variance, ANOVA, was used when more than two groups were compared. Data are presented as mean±S.D. The values $p<0.05$ were considered significant.

Table 2. *In Vivo* Serum and Liver Paraxonase Activities

Contraceptives	Serum PON1		Liver PON1	
	Mean±S.D.	p	Mean±S.D.	p
Control	53.4±11.2	-	33.8±4.2	—
A	125.2±17.1	$p<0.05$	18.9±3.7	$p<0.05$
B	71.5±16.6	0.247	20.2±3.1	$p<0.05$
C	105.8±8.2	$p<0.05$	12.3±2.5	$p<0.05$

For each drug mice were sacrificed by using cervical dislocation method. Blood and liver samples were taken from each mouse at these points after injection. Blood was collected in dry tubes and serum was seperated by centrifugation. Serum and erythrocytes were stored at −80 °C until analysis. Paraoxonase activity was quantified spectrophotometrically using 100 mM Tris–HCl buffer, pH 8.0 containing 2 mM $CaCl_2$. Reaction was initiated by the addition of 50 μl of serum or 100 μl of microsomal fraction and was followed for 2 min at 37 °C by monitoring the appearance of *p*-nitrophenol at 412 nm in a Biotek automatic recording spectrophotometer. All rates were determined in dublicate and corrected for the non-enzymic hydrolysis. The final substrate concentrations during enzyme assay were 2 mM and 1.5 mM for microsomal fraction and serum, respectively. Comparison between means of the control groups and drug administration revealed significant differences on A (*p*: 0.006), C (*p*: 0.000) on serum, A (*p*: 0.000), C (*p*: 0.000) and B (*p*: 0.000) on liver for Paraoxonase (ANOVA $P<0.05$).

RESULTS

In the present study, investigation of effects of OCs on mice serum and liver PON and erythrocytes CAT and CA was proposed. In order to make this study, three drugs which ratio of chemicals showed in Table1 were selected for administration. Drug dosage for mice was calculated from suggested dose for humans (Table 1). The calculations of used dosage were determined by comparison of human and mice weight.

The results of *in vivo* effects of the OCs on serum and liver PON activity are presented in Table 2. It was observed that, the activity of the control of serum and liver, which did not contain any drug, were determined as 53.4±11.2 EU and 33.8±4.2 EU, respectively. As seen in Table 2, while liver PON activity showed a statistically significant decrease for all three drugs, serum PON activity levels of study groups treated with A and C statistically significant increased. However, the increase of activity of other drugs (B) on serum PON activity was not statistically significant ($p>0.05$). The mean±S.D. values of liver and serum paraoxonase activities in the test group and control group were compared in Fig. 1. Oestrogens increase the synthesis of key enzymes of lipoprotein metabolism such as paraoxonase, hepatic and lipoprotein lipase and synthesis of the principal apoprotein of HDL, apoAI.[23]

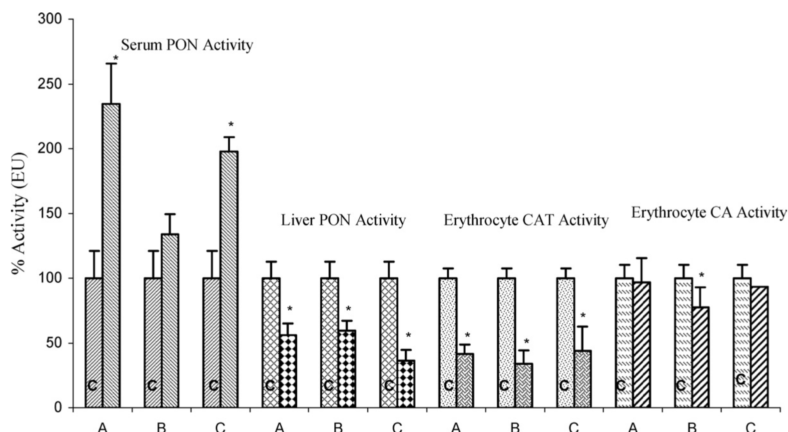

Fig. 1. *In Vivo* Effects of OCs (A, C and B) on Paraoxonase, Catalase and Carbonic Anhydrase Activity

PON activity determined from mouse serum and liver. CA and CAT activity determined from mouse erythrocytes. Values are mean±S.D. for $n=3$ independent experiments per group of PON, CA and CAT activity measured serum, liver and erythrocytes after the drug administration. Analysis of variance, ANOVA, was used when more than two groups were compared. The values $P<0.05$ were considered significant. (C, control group; PON, CA and CAT activity % after the drug administration; * the statistically significant data [Desolet ($p<0.05$), C ($p<0.001$) for serum PON activity and A ($p<0.001$), B ($p<0.001$) and C ($p<0.001$) for liver PON activity. A ($p<0.001$), B ($p<0.001$) and C ($p<0.001$) for CAT and B ($p<0.05$) for CA].

Table 3. *In Vivo* Catalase and Carbonic Anhydrase Activities

Contraceptives	Erythrocyte			
	Catalase		Carbonic anhydrase	
	Mean±S.D.	p	Mean±S.D.	p
Control	$79.7\times10^4\pm5.9\times10^4$	—	30.3±3.2	—
A	$33.0\times10^4\pm6.4\times10^4$	$p<0.05$	29.3±5.7	0.726
B	$27.0\times10^4\pm5.8\times10^4$	$p<0.05$	23.5±5.6	$p<0.05$
C	$34.8\times10^4\pm8.1\times10^4$	$p<0.05$	28.4±4.7	0.402

The catalase activity was measured by the Aebi method. In this method, 20 μl enzyme solution was added to the 1 ml 10 mM H_2O_2 in 20 mM potassium phosphate buffer (pH 7.0) and incubated at 25 °C for 1 min. Initial reaction rate was measured from the decrease in absorbance at 240 nm. Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson and modified by Maren *et al*. CO_2-hydratase activity as an enzyme unit (EU) was used the equation (t_o-t_c/t_c) where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively. Comparison between means of the control groups and drug administration revealed significant differences on A ($p<0.001$), C ($p<0.001$) and B ($p<0.001$) for Catalase and B ($p<0.05$) (ANOVA $P<0.05$).

The results of CA and CAT activity were shown in Table 3. While erythrocyte CA activity showed a statistically significant decrease only one drug (B; $p<0.05$), the effects of other drugs was not statistically significant. On the other hand, some *in vitro* and *in vivo* studies show that some antibiotics, drugs, chemicals and pesticides inhibit CA enzyme activity at to a wide range of degrees.[16,17] The CAT activity was significantly inhibited by all three drugs at the rate of 40% ($p<0.05$; Table 3).

DISCUSSION

Many drugs and chemicals, at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of specific enzyme. The effects could be dramatic and systemic.[24] Although oral contraceptives are used in birth control, there is no report related with PON, CAT and CA activity for OCs used in our study.

The present results show that these contraceptives are effective inhibitors only liver PON activity, but playing a part in activator for serum PON activity, *in vivo*. It has been reported that PON has an important role of lipoprotein metabolism and primary physiological role is to protect low-density lipoproteins (LDL) from oxidative modification. This is important early step in the pathogenesis of arteriosclerosis and cancer.[25] Oestrogens increase the synthesis of key enzymes of lipoprotein metabolism, hepatic and lipoprotein lipase and synthesis of the principal apoprotein of HDL, apoAI.[23] For this reason, our serum PON activity may be increased. Similar result was found in rat serums by Vincent-Viry *et al*. who stated that women taking oral contraceptive exhibited higher basal paraoxanase, salt-stimulated paraoxanase and arylesterase activities than women who were not taking oral contraceptives. At the same time, they also determined found a relationship between current smoking and PON1 activity.[26]

CA is generally recognized that it controls the bulk of carbon dioxide exchange between blood and tissues as well as the regulation of proton and other ion movements between cells and extra cellular fluids. All CA izoenzymes are also deeply involved in a great number of secretory activities including fluid movements.[17] The presence of several CA isoenzymes has been reported in human placenta and reproductive tract. The concentrations of CA isoenzymes have shown to be influenced by hormones, particularly steroids, in a number of tissues in various species.[27]

Several studies reported that hydrogen peroxide (H_2O_2) is one of the reactive oxygen species which can cause widespread damage to biological macromolecules leading to lipid peroxidation, protein oxidation, enzyme inactivation, DNA base modifications and DNA strand breaks.[28] CAT catalyses

the breakdown of H_2O_2 and so plays a major role in the protection of tissues from the toxic effects of H_2O_2 and partially reduce oxygen species.[13,29] Decreased CAT activity may be due to enzyme protein oxidation as a result of accumulation of H_2O_2 and other radicals. The observed decrease in CAT activity after administration of contraceptives may be related to oxidative inactivation of enzyme protein.[30] However, our results are in contrast with those of Massafra *et al.*,[31] who demonstrated that the use of OCs leads to an increase in antioxidant defenses.

There are many drugs and chemicals, which are known to have adverse or beneficial effects on human enzymes and metabolic events. Inhibition of some important enzymes, which play a key role in a metabolic pathway, may lead to pathologic conditions or disorders. Also PON, CAT and CA have a vital function in many kinds of tissues and play an important role in metabolism.

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