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PLASMA LIPID PEROXIDATION, BLOOD GSH CONCENTRATION AND ERYTHROCYTE ANTIOXIDANT ENZYMES IN MENSTRUATING FEMALES WITH OVULATORY AND ANOVULATORY CYCLES COMPARED WITH MALES

G.Lutosławska[1], J.Tkaczyk[2], E.Hübner-Woźniak[1], B.Panczenko-Kresowska[4], E.Skierska[2], A.K.Gajewski[3]

Depts. of [1]Biochemistry, of [2]Biology, of [3]Sport Theory, Józef Piłsudski Academy of Physical Education, Warsaw, Poland; [4]Dept. of Biochemistry and Nutrition, Institute of Food and Nutrition, Warsaw, Poland

Abstract. This study was undertaken to evaluate plasma TBARS and blood GSH concentration and erythrocyte antioxidant enzymes (glutathione peroxidase, catalase and superoxide dismutase) in active, regularly menstruating female physical education students with ovulatory and anovulatory menstrual cycles and in their male counterparts. A total of 27 subjects (12 males and 15 females) volunteered to participate in the study. All females were regularly menstruating with cycle length between 26-31 days. Plasma progesterone and 17-β-estradiol concentrations were assayed during the 7th-9th and 22nd-25th day of the menstrual cycle. Women with plasma progesterone concentration exceeding 19 $nmol \cdot l^{-1}$ during the 22nd-25th day were referred to as ovulatory (Group OV; n=7). Women without a peak plasma progesterone concentration were referred to as anovulatory (Group AN; n=8). Blood from male subjects was withdrawn twice - two weeks apart, at their convenience. It was found that the menstrual cycle phases did not affect plasma TBARS and blood glutathione concentration and erythrocyte GPX, CAT and SOD activity. However, erythrocyte GPX activity either in ovulatory or anovulatory women was by about 30% higher than in male subjects. Erythrocyte SOD activity in ovulatory women both in follicular and luteal phase of the menstrual cycle (1557 U/g Hb and 1394.6 U/g Hb, respectively) was markedly lower than in men (1951.8 and 1937.7 U/g Hb for blood sampling I and II, respectively). In contrast, erythrocyte SOD activity in anovulatory women (1855.5 U/g Hb and 1745.7 U/g Hb in the follicular and luteal phases, respectively) was similar to that found in men. The above data indicated that erythrocyte GPX and SOD activities are sensitive to plasma ovarian hormone concentration. In addition, they suggested that due to higher erythrocyte GPX activity females even with anovulatory menstrual cycles are protected better than males against hydrogen

Reprint request to: Grażyna Lutosławska, PhD, Dept. of Biochemistry, Józef Piłsudski Academy of Physical Education, 01-968 Warsaw 45, Box 55, Poland
e-mail: grazyna.lutoslawska@awf.edu.pl

peroxide action. However, lower superoxide production and consequent lower erythrocyte SOD activity was solely observed in females with ovulatory menstrual cycles and normal ovarian hormone plasma levels.

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Key words: Ovulatory cycles - Anovulatory cycles - Lipid peroxidation - Blood antioxidants

Introduction

It has been documented that ovarian hormones affect many physiological and metabolic processes. There is ample evidence suggesting that progesterone and estradiol play a substantial role in stimulation of the minute ventilation [4,20]. 17-β-estradiol has been demonstrated to increase insulin sensitivity, glucose tolerance and adipose tissue and muscle lipoprotein lipase (LPL) activity [8,14]. Furthermore, it has been reported that estrogens attenuate exercise-induced muscle damage in vivo [2,3,33]. Recently it has been noted that 17-β-estradiol-3-benzoate diminishes post-exercise skeletal muscle leukocyte infiltration and inflammatory response in both male and female skeletal muscle [34].

In vitro estrogens have been shown to destroy oxygen free radicals and to protect lipid and DNA against oxidative damage [28,31,32]. In rats estradiol has exerted protective effects against spontaneous lipid peroxidation in liver cells [16,27]. On the contrary, contraceptive steroids were found to increase erythrocyte lipid peroxidation in female rats [17]. Recently it has been postulated that in male rats estrogen administration contributes to a decline in overall antioxidant protection by inducing losses in tissue vitamin C content [35]. Data concerning estrogen action on lipid peroxidation and antioxidant defense in humans in vivo are scarce. An early study of Capel *et al.* [9] has revealed that women using oral contraceptives for longer than 7 months have higher erythrocyte GPX activity than either premenopausal or post-menopausal non-oral contraceptive users. Kanaley and Ji [18] have found that in eumenorrheic females the resting GPX activity in the early follicular phase (day 3 to 5) was lower than in their amenorrheic counterparts. On the other hand no sex-related differences in glutathione peroxidase and superoxide dismutase erythrocyte activity have been noted [10]. These data suggest that major ovarian hormone fluctuations might affect blood antioxidant status in vivo. However, there is lack of studies regarding the influence of more subtle anomalies in ovarian hormone secretion such as anovulation in regularly menstruating women on blood antioxidant status. Hence, this study was

undertaken to examine blood glutathione concentration, antioxidant enzyme activity (catalase, superoxide dismutase, glutathione peroxidase glutathione reductase,) and plasma TBARS concentration in physically active regularly menstruating women with ovulatory and anovulatory menstrual cycles. Moreover, to establish sex-related differences in antioxidant erythocyte status and plasma lipid peroxidation the above-mentioned indices were also tested in male physical education students.

Material and Methods

Subjects: A total of 27 subjects (12 males and 15 females) volunteered to participate in the study after giving their written consent. All were physical education students but none of them were high-performance athletes. Their physical activity due to the obligatory physical education program (swimming, games, dancing, track and field) was 8.5 h weekly. They were healthy non-smokers and not taking any medications. All procedures of this study were approved by the Ethics Commission of the Academy of Physical Education.

Prospective female subjects monitored their cycle 3 months prior to the study according to the procedure described by Hackney *et al.* [15]. The cycle days from the onset of menses to ovulation were considered follicular, the days from the ovulation to the next menses were considered luteal. All women were regularly menstruating with cycle length between 26-31 days. No cycle disturbances were reported throughout the study. Menstrual cycles of 7 subjects were referred to as ovulatory, those of 9 subjects as anovulatory. Plasma 17-β-estradiol and progesterone concentrations were determined twice per cycle - between the 7th and 9th day of the cycle and again between the 22nd -25th day of the cycle. In 7 women plasma progesterone concentration between the 22th-25th day of the cycle exceeded 19 $nmol \cdot l^{-1}$ and they were reffered to as ovulatory (Group OV), in 9 women plasma progesterone concentration between the 22nd and 25th of the cycle did not differ from that determined between the 7th-9th days of the cycle, and they were referred to as anovulatory (Group AN) [6]. Subject characteristics are given in Table 1.

Biochemical analysis: In all subjects blood sampling was performed twice. In females - between the 7th-9th days and 22nd-25th days of the menstrual cycle, in males - two weeks apart, at their convenience.

Blood was withdrawn from the antecubital vein from sitting subjects under fasting conditions between 8:00-8:30 a.m. into heparinized tubes. Blood for ovary hormone and TBARS determination was centrifuged 20 min at 3000 rpm, frozen

and stored until analysed (at -20° C and at -70° C for hormones and TBARS determination, respectively).

Glutathione (GSH) concentration was determined colorimetrically at 412 nm in whole fresh blood using DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) reagent according to Beutler [5]. Whole blood for glutathione peroxidase (GPX; EC 1.11.1.9) determination was immediately frozen and stored at - 70° C until analysed. GPX activity was assayed in the hemolysate of whole blood (obtained by thawing of frozen specimens) at 340 nm using Randox Laboratories commercial kits (United Kingdom). Blood for catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD: EC 1.15.1.1) determination was washed three times with cold saline and erythrocytes were frozen at - 70°C untill analysed. CAT and SOD were assayed in the hemolysate of erythrocytes obtained by the thawing of frozen specimens. SOD was assayed colorimetrically at 480 nm according to Misra *et al.* [22] and one unit of enzyme activity was defined as the amount of SOD required to inhibit adrenaline autooxidation by 50% at 25° C and pH 10.2. CAT was assayed spectrophotometically at 240 nm according to Aebi [1] using hydroxyperoxide as the substrate. Blood hemoglobin was determined using the Drabkin reagent.

Plasma TBARS concentration was assayed colorimetrically with thiobarbituric acid using double wavelength measurements (at 535 nm and 520 nm), free malonyldialdehyde (MDA) standard curve, and extraction of the chromogen with n-butanol to increase the assay specificity [7,25]. To prevent polyunsaturated fatty acid oxidation butylated hydroxytoluene (BHT e.i. 2,[6]-Di-tetr-butyl-p-cresol) was added to plasma specimens for TBARS determination.

Coefficient of variation for GSH and TBARS concentrations and antioxidant enzyme activity did not exceed 5.7 %.

Plasma 17-β-estradiol and progesterone concentrations were determined in duplicate using RIA methods and Orion Diagnostica (Finland) commercial kits. Within and between variations for each hormone were below 6% CV.

Statistics: All data are presented as the mean ±SD. Statistical analysis was carried out using analysis of variance (ANOVA), and Tukey post-hoc test. The level of significance was set at $P<0.05$.

Results: Physical characteristics of the subjects are presented in Table 1. Their age and BMI did not differ significantly. Cycle length did not differ in ovulatory and anovulatory females (Table 2). Plasma 17--β-estradiol concentration between the 7th-9th and 22nd-25th day of the cycle in ovulatory women (245.0 and 431.8 $pmol \cdot l^{-1}$) was markedly higher than in their anovulatory counterparts (90.1 and 243.7 $pmol \cdot l^{-1}$). Plasma progesterone concentration between the 7th-9th day of the

cycle was similar in both groups (2.5 vs 2.2 nmol·l^{-1} in ovulatory and anovulatory women, respectively). In ovulatory females plasma progesterone concentration increased up to 37.4 nmol·l^{-1} during the 22nd-25th day of the menstrual cycle. In anovulatory women plasma progesterone concentration on the same days of the menstrual cycle increased, but reached only 4.3 nmol·l^{-1}.

Table 1
Characteristics of subjects (means ±SD)

	Women Group OV (n=7)	Women Group AN (n=8)	Men (n=12)
Age (years)	21.5±1.5	19.9±0.6	21.5±1.2
Weight (kg)	60.3±2.6	59.9±6.9	72.4±9.0
Height (cm)	166.6±5.8	164.5±6.9	178.0±7.0
BMI	21.7±1.1	22.5±3.8	23.6±3.9

Values are means ±SD; Group OV - ovulatory menstrual cycles; Group AN - anovulatory menstrual cycles

Table 2
Cycle length and ovary hormone concentrations in plasma in ovulatory and anovulatory women

	Women Group OV (n=7)	Women Group AN (n=8)
Cycle length (days)	28.0±0.9	28.7±3.1
E_2 (pmol·l^{-1})		
day 7-9	245.0±154.9	90.1±37.6[b]
day 22-25	431.8±185.7[a]	243.7±125.6[a,b]
P (nmol·l^{-1})		
day 7-9	2.5±0.8	2.2±0.9
day 22-25	37.4±12.6[a]	4.3±3.2[b]

Values are means ±SD; Group OV - ovulatory cycles; Group AN - anovulatory cycles; E_2 - 17-β-estradiol; P – progesterone; a-significantly different vs.

respective value between the 7th-9th day of the cycle; E_2: Group AN -P<0.05; Group OV - P<0.03; P: P<0.0002; b- significantly different vs. respective value in Group OV - E_2: day 7-9 - P<0.05; day 22-25 - P<0.05; P : P<0.001

In males no significant differences in plasma TBARS, blood GSH concentration, erythrocyte GPX, CAT and SOD activities were noted when above variables were tested two weeks apart (Table 3).

Table 3
Plasma TBARS and blood GSH concentrations and erythrocyte CAT, GPX and SOD activity in males determined 2 weeks apart

	Males	
	Blood sampling I	Blood sampling II
TBARS (μmol/l)	2.73±0.29	2.54±0.27
GSH (μmol/mg Hb)	2.42±0.46	2.55±0.21
CAT (U/g Hb)	89.6±13.5	97.5±8.2
GPX (U/g Hb)	29.7±7.1	31.6±7.2
SOD (U/g Hb)	1951.8±171.2	1937.7±174.1

Values are means ±SD

Table 4
Plasma TBARS and blood GSH concentration, erythrocyte CAT, GPX and SOD activity in regularly menstruating women with ovulatory (Group OV) and anovulatory (Group AN) menstrual cycles

	Group OV		Group AN	
	Day 7-9	Day 22-25	Day 7-9	Day 22-25
TBARS (μmol/l)	3.07±0.29	2.92±0.63	2.57±0.48	2.85±0.43
GSH (μmol/mg Hb)	2.09±0.35	2.22±0.57	2.38±0.64	2.31±0.65
CAT (U/g Hb)	102.9±11.3	103.1±14.9	96.8±14.8	96.1±12.1
GPX (U/g Hb)	43.3±15.3[b]	39.2±18.2[c]	43.4±8.6[b]	43.6±15.6[b]
SOD (U/g Hb)	1557.0±366.2[a]	1394.6±268.8[a]	1855.5±156.6	1745.7±78.0

Values are means ±SD

a - significantly lower in comparison with respective values in men ($P<0.006$)
b,c- significantly higher in comparison with men (b -$P<0.0005$; c -$P<0.052$)

Plasma TBARS and blood GSH concentrations, erythrocyte GPX, CAT and SOD activities did not differ with respect to the phases of the menstrual cycle neither in ovulatory nor in anovulatory women (Table 4). However, in ovulatory and anovulatory women erythrocyte GPX activity both in the follicular and luteal phase of the menstrual cycle was significantly and by about 33% higher than in men. In addition, erythrocyte SOD activity in ovulatory women both in follicular and luteal phase (1557 and 1394.6 U/g Hb) was markedly lower than in men (1951.8 and 1937.7 U/g Hb in blood sampling I and II, respectively). In contrast, in anovulatory women erythrocyte SOD activity did not differ from that found in men.

Plasma TBARS concentration was affected neither by sex nor by the phase of the menstrual cycle (Tables 3 and 4).

Discussion

According to our knowledge the present study is the first which examined plasma TBARS concentration and blood antioxidant status in active, regularly menstruating women with subtle hormonal disturbances and in their male counterparts.

It should be stressed that anovulatory females differed from ovulatory ones not only with respect to plasma progesterone concentration during the 22nd-25th day of the cycle, but also with respect to plasma 17-β-estradiol level which was markedly lower in anovulatory females than in their ovulatory counterparts on the same cycle days. However, the above disturbances did not affect plasma TBARS concentration indicating similar plasma lipid peroxidation in both ovulatory and anovulatory women. Additionally, the lack of differences in plasma TBARS concentration in males and females of the present study suggested that ovarian hormones played a minor role in the protection of plasma lipids against oxidative damage. It seems feasible, since many other antioxidants including vitamines C, A and E, and uric acid are present in concentration higher than ovarian hormone [21,37].

Significantly higher erythrocyte GPX activity in females than in males suggested that ovarian hormones affected enzyme activity. This is in agreement with the study of Kanaley and Li [18] which indicated higher erythrocyte GPX activity in eumenorrheic than in amenorrheic women. Erythrocyte GPX is known

to be a the main erythrocyte enzyme involved in protecting the cell membrane from hydrogen peroxide action [12]. Assuming positive ovarian hormone effect on GPX activity, it could be postulated that women's erythrocytes are better protected against hydrogen peroxide action than those of men. On the other hand, our data demonstrated that subtle disturbances in ovarian hormone levels and subsequent anovulation did not affect erythrocyte GPX activity, which was similar in ovulatory and anovulatory women.

It is well known that SOD serves as a major antioxidant enzyme in erythocytes, where superoxide radicals are continuously generated by the autooxidation of hemoglobin [23]. In addition, it is well recognized that SOD and superoxide formation plays an important role in the female reproductive system affecting luteal cell regression and luteal function during pregnancy [29,30,36]. However, according to our knowledge the data concerning the effect of ovarian hormones on erythrocyte SOD activity in humans are lacking. In the present study erythrocyte SOD activity in ovulatory women it was markedly lower than in men. In contrast, in anovulatory women was similar to that determined in men. It is well documented that SOD undergo adaptational changes parallel to the magnitude of the oxidative stress [13,19,24]. Therefore, lower erythrocyte SOD activity in ovulatory women than in men may indicate lesser superoxide formation in the former than in the latter. However, other factors also have to be considered. Erythrocyte SOD is a copper- zinc enzyme the activity of which decreases in response to copper-deficient diet [26]. Unfortunately, the subjects dietary status was not evaluated in the present study.

It should be stressed that erythrocyte SOD activity in anovulatory women did not differ in comparison with men. Therefore we tentatively postulate that for efficient erythrocyte protection against superoxide production high ovarian hormone plasma levels, characteristic for normal ovulatory cycles are indispensable.

The lack of the effect of the ovarian hormone fluctuation due to the menstrual cycle phases on the indices of oxidative stress is in agreement with Chung *et al* [11]. They indicated that neither plasma MDA and TBARS concentration nor blood GSH concentration was affected by menstrual cycle phase in healthy women.

In conclusion, the current study indicated that in physically active females erythrocyte GPX and SOD activities are sensitive to plasma ovarian hormone concentration. Erythrocyte GPX activity is higher in women than in men, even when plasma ovarian hormone concentration is depressed as in anovulatory subjects of the present study. In consequence, female erythrocytes are probably

better protected against hydrogen peroxide damage. On the other hand, erythrocyte SOD activity is similar in anovulatory women and in men, and markedly lower in ovulatory women indicating that enzyme activity is very sensitive to ovarian hormone plasma levels. Assuming adaptational increase of SOD activity in response to superoxide production it could not be excluded that in women with normal ovarian hormone plasma levels superoxide-induced oxidative stress in erythrocytes is less than in anovulatory ones and in males.

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