Changes in the oxidant/antioxidant balance in rats with pharmacologically induced ovarian failure

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1 RENATA LACRIMIOARA NICULA, DARIA MARIA POP, RAZVAN CIORTEA, ANDREI MALUTAN, CARMEN ELENA BUCURI, DORU DICLESCU, DAN MIHU

“IULIU HATIEGANU” University of Medicine and Pharmacology Cluj-Napoca, 2nd Department of Obstetrics and Gynaecology, Cluj-Napoca, Romania

*Address for correspondence to: dariamariapop@icloud.com

Abstract
Reactive oxygen species are constantly produced in all aerobic organisms, following intracellular metabolism, in response to environmental stimuli. With a composition including free oxygen radicals, they are incriminated in inflammatory, cardiovascular diseases, playing a role in the damage and loss of function of various tissues and organs. The aim of the present work was to assess the effect of different cyclophosphamide doses onto the serum oxidant/antioxidant balance in female rats with ovarian failure. We also monitored the effect after the administration of an antioxidant complex (Sel-E-Vit). Oxidative stress was measured based on the serum levels of malondialdehyde and protein carbonyls; antioxidant defence was quantified based on the serum levels of hydrogen donors and reduced glutathione. Sel-E-Vit treatment causes changes in the oxidant/antioxidant balance of animals with cyclophosphamide-induced ovarian failure. Our results demonstrate the presence of oxidative stress in menopause and recommend non-hormonal antioxidant therapy during perimenopause.

Keywords: reactive oxygen species, cyclophosphamide, oxidant/antioxidant balance, antioxidants.

1. Introduction
ROS are constantly produced in all aerobic organisms, following intracellular metabolism, in response to environmental stimuli. They are incriminated in inflammatory, cardiovascular diseases, playing a role in the damage and loss of function of various tissues and organs due to free oxygen radicals: peroxyl, alkoxyl, hydroxyl and superoxide, the (HU & al [1]). Starting from this premise, research has been conducted in order to prevent these negative effects and to study the antioxidant (AO) action of different substances (MORALES [2]). Thus, animal models have allowed the study of the implication of OS in ovarian failure induced pharmacologically, surgically or by radiation, as well as the concomitant biochemical and histological analysis of the affected ovarian and endometrial tissues. CFA, a cytotoxic alkylating agent, has been frequently used for the experimental induction of OS in animals, mice or rats, and selenium is the best element with cytoprotective action, extremely useful in some physiological processes, an important AO factor. The role of selenium in fertility is extensively studied, its decrease in the follicular fluid being associated with infertility.
(PASZKOWSKI [3]), and selenium deficiency is frequently found in idiopathic abortions (BARRINGTON & al [4]). Experimental studies have shown that selenium is able to improve the growth and maturation of ovarian follicles in mice, and sodium selenite reduces ROS levels, increasing antioxidant defence and glutathione activity (ABEDELAHI & al [5]). In vivo, the action of sodium selenite at cellular level is not clearly understood, but its positive action on folliculogenesis is certain.

The aim of the present work was to assess the effect of different cyclophosphamide (CFA) doses in female rats with CFA-induced ovarian failure and the effect of co-administration of an AO complex (Sel-E-Vit).

2. Materials and Methods
The research was approved by the Ethics Commission of the” Iuliu Hațieganu” University of Medicine and Pharmacy Cluj-Napoca, in accordance with international norms regarding studies on animals for scientific and experimental purposes.

In the present study 6 month - old female Wistar rats (200-220g) were divided in four experimental group: control group (group I); group II: CFA treated group (30 mg/kg i.p. for 5 weeks); group III: CFA treated group (15 mg/kg i.p. for 5 weeks); group IV: CFA treated group (15 mg/kg for 5 weeks) followed by Sel-E-Vit (SC Pasteur) administration (15 mg/kg for 3 weeks). Blood samples were collected for group II after week 4, for group III after week 5, and for group IV after week 8, O/AO balance were determined. OS was measured based on the serum levels of: malondialdehyde (MDA), using Conti’s method; protein carbonyls (PC), according to the method of Resnick and Packer (REZNICK [7]). AO defence was quantified based on the serum levels of: hydrogen donors (HD), by the method of Janazsewska and Bartasz (JANAZSEWSKA [8]); reduced glutathione (GSH), via Hu’s method (HU [9]).

Statistical analysis
Descriptive statistics analysis were performed; normal distribution were assessed using Shapiro-Wilk test, the variance was tested with the F or Levene’s and/or Bartlett’s tests.

To compare unpaired samples, the t (Student) test in the case of normal distribution data and the non-parametric Mann-Whitney (U) test for values with non-uniform distribution or ranks were used. For the analysis of three or more samples, the ANOVA test for normal distribution data and the non-parametric Kruskal-Wallis test for values with non-uniform distribution or ranks were used. The correlation between two continuous quantitative variables with normal (uniform) distribution were identified using Pearson’s (r) correlation coefficient. In the case of variables with non-uniform distribution, Spearman’s (p) rank correlation coefficient was used. The correlation coefficients were analysed using Colton’s rule. Thus, starting from the properties of the correlation coefficient, which indicate that this is a number between -1 and 1 and that the” intensity” of the linear relation between the two variables will be higher as the absolute value of the correlation coefficient comes close to 1, Colton (1974) suggested the following empirical rules regarding the interpretation of the correlation coefficient: a weak/null correlation if – marked by *; an acceptable correlation if – marked by **; a good correlation if – marked by ***; a very good correlation if – marked by ****.

Polynomial regression was the method employed to obtain the mathematical equation of dependence of a continuous variable on another variable. Statistical processing was performed with the Excel application (Microsoft Office 2007) and StatsDirect v.2.7.2 software. The results were graphically represented using the Excel application (Microsoft Office 2007).
3. Results and discussion

Comparative analysis of OS indicators

The statistical analysis of MDA values, considering all four groups, showed no statistically significant differences between any of the groups (p = 0.8584). As expected, the statistical analysis of MDA values for unpaired samples evidenced no statistically significant differences between any of the groups (p > 0.05) (table1, fig.1).

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Group</th>
<th>Mean</th>
<th>SE</th>
<th>Median</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>I</td>
<td>2.64</td>
<td>0.1892</td>
<td>2.77</td>
<td>0.5983</td>
<td>1.65</td>
<td>3.49</td>
<td>I-II-III-IV</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>2.69</td>
<td>0.1395</td>
<td>2.76</td>
<td>0.4412</td>
<td>2.02</td>
<td>3.35</td>
<td>II-III</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>2.74</td>
<td>0.2585</td>
<td>2.74</td>
<td>0.8175</td>
<td>1.68</td>
<td>4.43</td>
<td>I-II</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>2.49</td>
<td>0.2344</td>
<td>2.25</td>
<td>0.7412</td>
<td>1.63</td>
<td>3.70</td>
<td>I-III-IV</td>
</tr>
</tbody>
</table>

Table 1. Comparative analysis of MDA values (nmol/ml) in the studied groups and statistical significance

![Figure 1. MDA (nmol/ml) in the studied groups](image)

The statistical analysis of PC values, considering all four groups, showed statistically significant differences between at least two of the groups (p = 0.027). As expected, the statistical analysis of PC values for unpaired samples revealed statistically significant differences between groups I-II and II-III (p < 0.05) (Table 2, Fig.2).

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Group</th>
<th>Mean</th>
<th>SE</th>
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<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>PC</td>
<td>I</td>
<td>3.36</td>
<td>0.1359</td>
<td>3.40</td>
<td>0.4296</td>
<td>2.45</td>
<td>3.79</td>
<td>I-II-III-IV</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>2.65</td>
<td>0.2353</td>
<td>2.20</td>
<td>0.7440</td>
<td>1.99</td>
<td>3.87</td>
<td>II-III</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>3.94</td>
<td>0.3839</td>
<td>3.87</td>
<td>1.2141</td>
<td>1.79</td>
<td>5.87</td>
<td>I-II</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>2.99</td>
<td>0.2642</td>
<td>2.92</td>
<td>0.8354</td>
<td>1.86</td>
<td>4.49</td>
<td>I-III-IV</td>
</tr>
</tbody>
</table>

Table 2. Comparative analysis of PC values (nmol/mg protein) in the studied groups and statistical significance
Comparative analysis of AO defence indicators

The statistical analysis of HD values, considering all four groups, showed highly statistically significant differences between at least two of the groups ($p = 1.56 \times 10^{-11}$). As expected, the statistical analysis of HD values for unpaired samples showed highly statistically significant differences between groups I-II, II-IV and III-IV ($p < 0.001$) and very statistically significant differences between groups I-III and I-IV ($p < 0.01$) (Table 3, Fig. 3).

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Group</th>
<th>Mean</th>
<th>SE</th>
<th>Median</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HD</strong></td>
<td>I</td>
<td>29.30</td>
<td>1.90</td>
<td>29.25</td>
<td>6.01</td>
<td>18.98</td>
<td>37.18</td>
<td>1.56 x $10^{-11}$</td>
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<tr>
<td></td>
<td>II</td>
<td>11.56</td>
<td>1.57</td>
<td>10.24</td>
<td>4.98</td>
<td>6.28</td>
<td>19.19</td>
<td>0.0597</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>17.56</td>
<td>2.49</td>
<td>18.42</td>
<td>7.86</td>
<td>5.89</td>
<td>31.73</td>
<td>1.53 x $10^{-6}$</td>
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<tr>
<td></td>
<td>IV</td>
<td>36.31</td>
<td>0.52</td>
<td>36.20</td>
<td>1.65</td>
<td>32.47</td>
<td>38.39</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

Table 3. Comparative analysis of HD values (inhibition %) in the studied groups and statistical significance

The statistical analysis of GSH values, considering all four groups, showed highly statistically significant differences between at least two of the groups ($p = 0.0004$). As
expected, the statistical analysis of **GSH** values for unpaired samples indicated highly statistically significant differences between groups II-IV and III-IV (p < 0.001) and statistically significant differences between groups I-IV (p < 0.05) (Table 4, Fig.4).

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Group</th>
<th>Mean</th>
<th>SE</th>
<th>Median</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>I</td>
<td>3.45</td>
<td>0.401</td>
<td>2.87</td>
<td>1.268</td>
<td>2.60</td>
<td>5.89</td>
<td>0.0185</td>
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<tr>
<td></td>
<td>II</td>
<td>2.84</td>
<td>0.329</td>
<td>2.48</td>
<td>1.042</td>
<td>1.65</td>
<td>4.51</td>
<td>0.0004</td>
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<tr>
<td></td>
<td>III</td>
<td>2.94</td>
<td>0.261</td>
<td>2.78</td>
<td>0.827</td>
<td>1.85</td>
<td>4.92</td>
<td>0.1655</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>5.24</td>
<td>0.149</td>
<td>5.33</td>
<td>0.473</td>
<td>4.29</td>
<td>5.89</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 4. Comparative analysis of GSH values (nmol/ml) in the studied groups and statistical significance

![Figure 4. GSH (nmol/ml) in the studied groups](image)

**Correlation analysis between the O/AO balance indicators**

The statistical correlation analysis between the values of the studied indicators in group I showed a good negative correlation between MDA-PC, PC-HD and an acceptable positive correlation between HD-GSH; in group II a good positive correlation between MDA-PC, PC-HD and an acceptable negative correlation between MDA-GSH; in group III an acceptable positive correlation between PC-HD and in group IV showed no correlation between the studied indicators (Table 5, Fig. 5, 6).

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-PC</td>
<td>-0.5460</td>
<td>0.5636</td>
<td>0.2355</td>
<td>-0.0239</td>
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<tr>
<td>MDA-HD</td>
<td>0.1634</td>
<td>0.0186</td>
<td>0.1548</td>
<td>-0.1609</td>
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<tr>
<td>MDA-GSH</td>
<td>-0.1394</td>
<td>-0.3827</td>
<td>0.0667</td>
<td>0.0030</td>
</tr>
<tr>
<td>PC-HD</td>
<td>-0.6140</td>
<td>0.5879</td>
<td>0.4811</td>
<td>-0.1893</td>
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<tr>
<td>PC-GSH</td>
<td>-0.0182</td>
<td>-0.2364</td>
<td>-0.1879</td>
<td>0.1016</td>
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<tr>
<td>HD-GSH</td>
<td>0.3697</td>
<td>-0.1821</td>
<td>0.0061</td>
<td>-0.2430</td>
</tr>
</tbody>
</table>

Table 5. Statistical correlation analysis between the values of the O/AO balance indicators for the four groups
Redox homeostasis in ovarian failure

Biochemical oxy-nitrosamine stress (ONS) is caused by an excessive endogenous production of oxygen and nitrogen metabolites (ROS, RNS), with the disturbance of redox O/AO homeostasis. ROS and RNS are classified into the following derivatives: radicals: hydroxyl \( \text{OH}^- \), hydrodioxyl \( \text{HO}_2^- \), alkoxyl \( \text{R-O}^- \), hydroperoxide \( \text{R-OOH}^- \), alkyl peroxy \( \text{R-O}_2^- \) or \( \text{R-OO}^- \), nitric oxide \( \text{NO}^- \), superoxide \( \text{O}_2^- \) and non-radicals: ozone \( \text{O}_3 \), triplet oxygen \( 3\text{O}_2 \), singlet oxygen \( \text{O}_2^* \), hydrogen peroxide \( \text{H}_2\text{O}_2 \), hypochlorite \( \text{ClO}^- \), peroxynitrite \( \text{ONOO}^- \) (TACHE [10]). RONS formation can occur in extreme physiological conditions (pregnancy,
new born), aging, physical exercise, hypo/hyperbaric, under the action of air pollutants, exposure to extreme temperature conditions, radiation, as well as in pathological conditions, being currently involved in the etiopathology of more than 100 diseases. The general effects of RONS are paradoxical, both favourable and unfavourable. The presence of ROS and RNS has been evidenced in many animals, as well as in humans, including in the female genital tract, ovaries (BEHRMAN [11], fallopian tubes (EL MOUATASSIM [12], embryo (GUERIN [13])). Reactive oxygen species have been involved in (AGARWAL [14]) modulation of physiological reproductive functions, as signal key molecules for oocyte maturation, ovarian steroidogenesis, corpus luteum functions and luteolysis (CONTI [6], ISHIKAWA [15]), fertility and normal pregnancy, nidation (BEDAJWY [16]) and formation of the blastocyst (MOCATTÁ [17]), normal delivery and menopause (de BRUIN [18]). ROS also have been involved in pathological processes in the female genital tract such as pre-eclampsia and eclampsia (TAKAGI & al [19]), hydatidiform mole (HARMA [20]), delivery disorders (LOEKEN [21]), endometriosis (FOYOUZI [22]), tubal, peritoneal and idiopathic sterility (DUCKITT [23]), fetal embryopathy (DENNERY [24]), pregnancy diabetes, abortion (LAGOD [25]).

AO defence is achieved by endogenous AOs and when these mechanisms are insufficient, by exogenous AOs. An AO is a substance which, present in small concentrations compared to the concentration of an oxidable substrate, prevents or significantly delays the oxidative degradation of that substrate. These are compounds that protect biological systems from the negative effects of processes or reactions that cause excessive oxidation. AOs play a role in neutralizing or removing ROS, RNS and the oxidation products of organic molecules. Also, there are some primary, prophylactic AOs, which decrease the frequency of reactions leading to the formation of free radicals and inhibit the propagation of already formed free radicals. AOs are substances that under normal conditions act to prevent ROS and RNS production. There are two types of AOs, depending on the mechanisms of action: enzymatic, which are natural AOs that neutralize the excess of reactive species and prevent cell lesion and non-enzymatic – which are mainly known as synthetic AOs or dietary supplements.

Enzymatic AOs include: the family of superoxide dismutase (SOD), the family of catalases (CAT), the family of glutathione enzymes – glutathione peroxidase (GSH-PX), glutathione reductase (GSH-R), glutathione transferase (GSH-S-T); the family of peroxiredoxins (PRDx) – peroxiredoxin 6 (PRDx6), and the family of heme oxygenases (HO).

Natural non-enzymatic AOs include: vitamins (A, E, C); hormones (melatonin, estrogen, cortisol, prolactin, choriogonadotropin hormone, serotonin); metal ion chelators (uric acid, albumin, haptoglobin, hemopexin, transferrin, lactoferrin, ceruloplasmin, metallothioneins), bio thiols (thioredoxin (TRX), glutathione (GSH), lipoate) and other substances (taurine, selenium).

In the female genital tract, the presence of AOs including the following was evidenced taurine, hyotaurine and transferrin in the tubal and follicular fluid (GUERIN [13]); GSH in oocytes (de MATOS [26]); CuZnSOD and MnSOD in the granulosa and theca cells of the growing follicle; SeGSH-PX in the follicular fluid (EL MOUATASSIM [12]); estrogen hormones in ovarian follicles. The maintenance of intracellular redox homeostasis or redox buffering is
ensured by major redox buffering systems of GSH/GSSG and TRX, which are controlled by GSH reductase and TRX reductase, respectively; these systems are also involved in cell signaling; low molecular weight AOs, which in high concentrations play a scavenging role – amino acids, peptides and proteins; and hemoxygenase – 1 (HO-1).

The presence of OS in menopause has been attributed to a decrease of oestrogen hormones, considered to be a better antioxidant than GSH (KAUR & al [27]).

The Sel-E-Vit AO complex contains selenium as a trace element and liposoluble vitamin E. Selenium acts as an antioxidant, being a cofactor of the enzyme glutathione peroxidase (Se-6SH-PX), it has a synergistic effect with vitamins A and E and is a component of selenoprotein P, a plasma transport protein. Se-6SH-PX catalyses the reduction of lipid peroxides and H2O2. Se influences vitamin E by 3 mechanisms: it maintains normal vitamin absorption, reduces the amount of vitamin E required for the maintenance of lipoprotein membrane integrity via 6SH-PX, and facilitates the maintenance of vitamin E in the plasma (TACHE [28]).

Vitamin E (α-tocopherol) acts as an AO through several mechanisms, being the best membrane AO; it is a scavenger of O2⁻, OH⁻ and a membrane stabilizer; it inhibits lipid peroxidation and LDL oxidation; it accelerates the breakdown of lipid peroxides and the fragmentation of the radical chain; it reduces xanthine oxidase synthesis; in enhances the AO effect of selenium, maintaining the amount of selenium in the body in an active form and preventing losses.

Our results show changes in the O/AO balance after the induction of ovarian failure in animals. In group II, chronic CFA administration was followed by significant decreases of PC and HD and insignificant changes in MDA and GSH compared to the control group.

In group III, chronic CFA administration was followed by significant decreases of HD, insignificant increases of MDA and PC and an insignificant decrease of GSH compared to the control group; significant increases of PC and insignificant increases of MDA, HD and GSH compared to group II.

In group IV, chronic Sel-E-Vit administration after induction of ovarian failure by CFA was followed by significant increases of HD and GSH, insignificant decreases of MDA and PC compared to the control group, and significant increases of HD and GSH, insignificant decreases of MDA and PC compared to group III, which received the same CFA dose.

Our results indicate changes in redox homeostasis, with significant decreases of AO defence on account of HD, and an insignificant decrease on account of GSH following CFA administration in groups II and III, compared to the control group. The decreases were more pronounced after CFA administration in doses of 30 mg/kg compared to doses of 15 mg/kg.

The administration of the AO complex (selenium + vitamin E) to animals with induced ovarian failure, group IV, caused significant increases of AO defence on account of HD and GSH both compared to the control group and the group in which ovarian failure was induced with the same CFA dose (group III); in group IV, there was a decrease of OS indicators – MDA and PC, compared to the other groups for MDA and compared to groups I and III for PC.

The correlation analysis indicates a good negative correlation for PC-MDA and PC-GSH in group I and a good positive correlation for HD-MDA and HD-GSH.

Our results are in agreement with experimental data regarding the O/AO balance in female rats ovariectomized for the induction of menopause, which were administered oestrogen-
progesterone replacement treatment (FLORESCU [29]).
The pro-oxidant OS-inducing effect of CFA was demonstrated by other authors in experimental studies on mice (LATA [30], WEI [31], ROY [32]), who also studied the protective AO effects of some preparations: ginsenoside Rg3 in mice (WEI [31]), Phyllanthus fraternus (LATA [30]), flavonyl-thiazolidinedione based organoselenocyanate compound (ROY [32]), tacotrienol (SALEH [33]), vitamin E (CUCE [34]).

4. Conclusion
MDA and PC, as indicators of oxidative stress, are increased in the serum of animals with pharmacologically induced ovarian failure. HD and GSH, as indicators of antioxidant defence, are decreased in the serum of animals with pharmacologically induced ovarian failure. The oxidant/antioxidant balance changes are more expressed after CFA administration in doses of 30 mg/kg, with significant decreases of antioxidant defence on account of HD and GSH.

Sel-E-Vit treatment causes changes in the O/AO balance of animals with CFA-induced ovarian failure, in which oxidative stress decreases on account of MDA and PC, and antioxidant defence increases significantly on account of HD and GSH.

Our results demonstrate the presence of oxidative stress in menopause and we recommended non-hormonal AO therapy during perimenopause.

References
5. ABDELHAI A, SALEHNIA M, ALLAMEH AA, DAVOODI D. Sodium selenite improves the in vitro follicular development by reducing the reactive oxygen species level and increasing the total antioxidant capacity and glutathione peroxide activity. *Hum Reprod*, 25: 977–985 (2010).


