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HISTOPATHOLOGICAL CHANGES IN OVARIAN FAILURE: A RESEARCH MODEL IN FEMALE RATS WITH PHARMACOLOGICALLY INDUCED OVARIAN FAILURE

MODIFICĂRI HISTOPATOLOGICE ÎN INSUFICIENȚA OVARIANĂ: UN MODEL DE CERCETARE LA ȘOBOLANI DE SEX FEMININ CU INSUFICIENȚĂ OVARIANĂ INDUSĂ FARMACOLOGIC

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ABSTRACT | REZUMAT

Perimenopause is characterised by clinical manifestations based on endocrinological and histological changes in the female genital system. The progressive reduction of the endogenous level of ovarian steroids, as well as the cytotoxic implication of oxidative stress (OS), induces both uterine and ovarian alterations. The aim of this research was to assess ovarian follicular and uterine horn histological changes in animals with chemically induced ovarian failure. Also, we monitored the effect on the structure of the ovarian and uterine horns after administration of an antioxidant complex of selenium and vitamin E. The experiments were performed on white female Wistar rats of the *Rattus norvegicus* species, aged 20 weeks, with a weight between 200 and 220g. The animals were assigned to 4 groups (n=10 animals/group): group I- control group, group II: animals that were administered for 5 weeks Cyclophosphamide (CFA) iv, in a dose of 30 mg/kgbw, every 3 days, group III – animals that were administered for 5 weeks CFA iv, in a dose of 15 mg/kgbw, every 3 days, group IV –animals that were administered for 5 weeks CFA iv, in a dose of 15 mg/kgbw, followed by administration of Sel-E-Vit in a dose of 15 mg/kgbw for 3 weeks. At the end of the experiment, the animals were euthanized, after which ovary and uterine horn samples were collected for histopathological examination. In the current study, we assessed histopathological sections obtained from the ovaries and uterine horns of female rats treated with CFA, CFA+Sel-E-Vit respectively. The histopathological data were correlated with the biochemical indicators investigated in study III (groups I, III, IV). Cyclophosphamide administration induces ovarian failure with inhibition of folliculogenesis and uterine atrophy. Administration of an ovo- and utero-toxic chemical agent and post-administration of an antioxidant complex have a favourable effect on ovarian and uterine morphology in vivo.

Keywords: perimenopause, ovarian failure, cyclophosphamide, antioxidant complex

Perimenopauza este caracterizată de manifestări clinice bazate pe modificări endocrinologice și histologice în sistemul genital feminin. Reducerea progresivă a nivelului endogen de steroizi ovarieni, precum și implicația citotoxică a stresului oxidativ (OS), induc modificări atât uterine cât și ovariene. Scopul acestei cercetări a fost de a evalua modificările histologice foliculare ovariene și ale cornului uterin la animale cu insuficiență ovariană indusă chimic. De asemenea, s-a monitorizat efectul asupra structurii ovariene și asupra cornului uterin după administrarea unui complex antioxidant de seleniu și vitamina E. Experimentele au fost efectuate pe șobolani albi de sex feminin din specia *Rattus norvegicus*, în vârstă de 20 de săptămâni, cu o greutate între 200 și 220g. Animalele au fost repartizate în 4 grupuri (n=10 animale/grup): grupul I - grupul de control, grupul II: animale cărora li s-a administrat Ciclofosfamidă (CFA) iv timp de 5 săptămâni în doză de 30 mg/kg corp la fiecare 3 zile, grupul III - animale cărora li s-a administrat CFA iv timp de 5 săptămâni în doză de 15 mg/kg corp la fiecare 3 zile, grupul IV - animale cărora li s-a administrat CFA iv timp de 5 săptămâni în doză de 15 mg/kg corp urmată de administrarea Sel-E-Vit în doză de 15 mg/kg corp timp de 3 săptămâni. La sfârșitul experimentului, animalele au fost eutanasiate, după care s-au colectat probe de ovar și corn uterin pentru examinare histopatologică.S-au analizat secțiunile histopatologice obținute de la nivelul ovarului și de la nivelul cornului uterin la femelele tratate cu Ciclofosfamidă și respectiv Ciclofosfamidă si SEl-E-Vit. Datele histopatologice au fost corelate cu indicatorii biochimici investigați anterior. Administrarea de Ciclofosfamidă induce insuficiență ovariană cu inhibarea foliculogenezei și atrofie uterină. Administrarea unui agent chimic ovo- și utero-toxic și post-administrarea unui complex antioxidant au un efect favorabil asupra morfologiei ovariene și uterine in vivo.

Cuvinte cheie: perimenopauză, insuficiență ovariană, ciclofosfamidă, complex antioxidant

Perimenopause is characterised by clinical manifestations based on endocrinological and histological changes in the female genital system. The progressive reduction of the endogenous level of ovarian steroids, as well as the cytotoxic implication of oxidative stress (OS), induces both uterine and ovarian alterations (6, 20, 23). The substrate of clinical processes is represented by the histopathological transformation of the uterus and ovarian tissue, which can be studied in perimenopausal animal models that are similar to human model in terms of tissue changes. The most frequently used perimenopausal animal model is induced by treatment with cyclophosphamide (CFA) (8, 5, 13, 27), a pharmacological agent that causes cytotoxic effects on most of the internal organs of rats and mice (25).

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In human subjects, cyclophosphamide, used as an antitumoral agent, induces a number of toxic, atrophic effects on all tissues [8], not just on target tissues. In this sense, cyclophosphamide, which is very frequently used in the treatment of haematological neoplasms, causes severe uterine and ovarian changes (2,3,7).

The purpose of the study was to monitor the following changes and effects induced to animal subjects: the ovarian histopathological, ovarian follicular and uterine horn changes in animals with chemically induced ovarian failure by cyclophosphamide administration, but also to assess the effect of administering an antioxidant complex of selenium and vitamin E on ovarian and uterine horn changes in female rats with chemically induced ovarian failure. At the same time, we aim to evaluate the correlation between the number of primordial, primary, and mature ovarian follicles and the indicators of the oxidative/AO balance previously investigated and the correlation between the values of uterine horn wall thickness and the O/AO balance investigated by our team in previous studies.

MATERIALS AND METHODS

The research was conducted with the approval of the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, in accordance with international norms regarding studies on animals with a scientific and experimental purpose.

The experiments were performed on white female Wistar rats of the Rattus norvegicus species, aged 20 weeks, with a weight between 200 and 220 g, from the Animal Facility of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca.

The studies were conducted in the Experimental Laboratory of the Physiology Department, where the animals were kept under adequate vivarium conditions.

The animals were assigned to 4 groups (n=10 animals/group) as follows:

- group I – control group, healthy, fertile animals, injected with physiological serum iv;
- group II – animals that were administered for 5 weeks CFA iv, in a dose of 30 mg/kgbw, every 3 days.

Regarding this group, it was excluded because of the toxicity of the dose which led to the death of the animals before sample collection for histopathological examination:

- group III – animals that were administered for 5 weeks CFA iv, in a dose of 15 mg/kgbw, every 3 days;
- group IV – animals that were administered for 5 weeks CFA iv, in a dose of 15 mg/kgbw, followed by administration of Sel-E-Vit in a dose of 15 mg/kgbw for 3 weeks, every 2 days.

Cyclophosphamide (Endoxan), 200 mg/powder vial for injectable solution from Sindan Pharma, was administered in a dose of 15 mg/kgbw for 30 days, every 3 days. Administration of Selenium and vitamin E (Sel-E-Vit), injectable solution for veterinary use from Pasteur Pharmavet, was in a dose of 15 mg/kgbw ip, for 30 days, every 2 days. At the end of the experiment, the animals were euthanized, after which ovary and uterine horn samples were collected for histopathological examination. The monitored indicators were:

- the number of ovarian follicles: primordial, primary, and mature
- uterine horn wall thickness.

The samples were collected for groups II and III after week 5, and for group IV after week 8. At the end of the experiment, the animals were and sacrificed.

Histopathological examination was performed in the Pathomorphology Laboratory of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. The collected samples were placed in 10% neutral buffered formol solution for fixation. After fixation, the samples were processed using the paraffin technique.

Serial sections were cut at a thickness of 4 micrometres with a Leica RM 2125 RT microtome. The sections were displayed on routine histological slides and stained by haematoxylin-eosin. The preparations were examined using an Olympus BX 51 microscope; the images were recorded with an Olympus UC 30 digital camera and processed using the Olympus Stream Basic software for image acquisition and processing. A morphometric examination was also performed by measuring uterine horn wall thickness in three separate points, for each section. The results of histopathological examination correlated with the values of the O/AO balance indicators presented in study III (groups I, III, IV). Statistical analysis was performed.

RESULTS AND DISCUSSION

In the current study, we assessed histopathological sections obtained from the ovaries and uterine horns of female rats treated with CFA, and CFA + Sel-E-Vit respectively. Also, histopathological examination of samples from untreated female rats of the same age as the animals of the experimental group, which represented the control group, was performed. The histopathological data were correlated with the biochemical indicators investigated in study III (groups I, III, IV).

Analysis of ovarian follicles

Regarding the ovarian sections, the control group showed a normal histological appearance, with the presence of many primordial, primary and even mature follicles, while in the group treated with CFA, a severe reduction in the number of primordial and primary follicles, as well as ovarian atrophy was observed. Treatment by Sel-E-Vit administration partially corrected the atrophy induced by CFA, and an increase in the number of ovarian follicles was seen (Fig. 1).

The statistical analysis of the values of primordial follicles, considering all three groups, showed highly statistically significant differences between at least two of the groups ($p = 0.0005$). As expected, the statistical analysis of the values of primordial follicles for unpaired samples revealed very statistically significant differences between groups I-III and III-IV ($p < 0.01$) (Table 1).

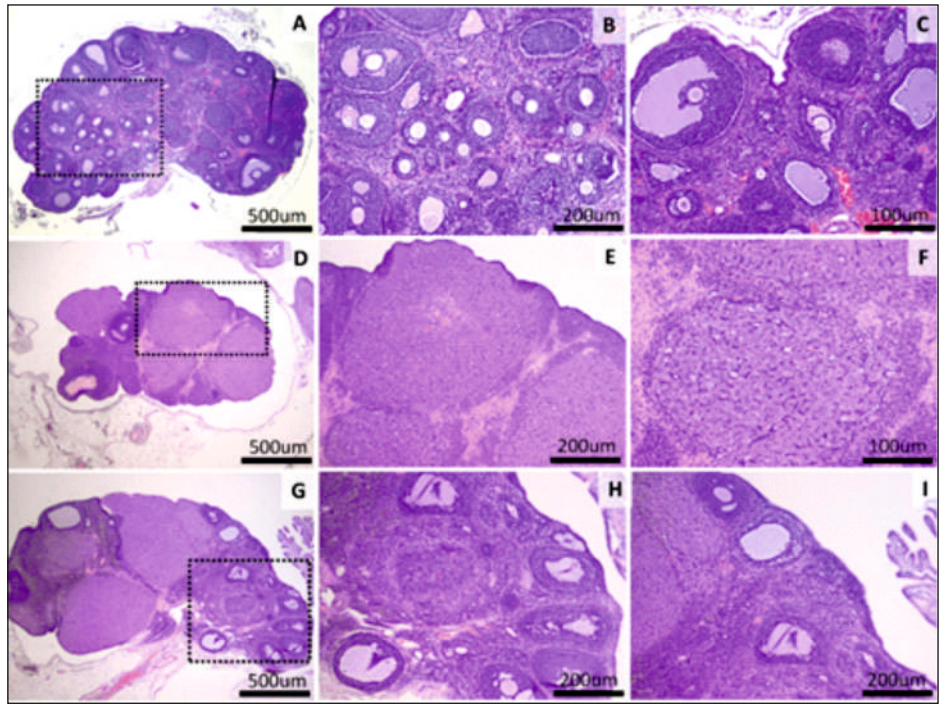

Fig. 1. Morphology of the ovary in the absolute control group (A,B,C), in the group treated with CFA (D,E,F), and in the group treated with CFA + vitamin E and Se (G, H, I). A dramatic reduction in the number of follicles in the animals treated with CFA, and an increase in the number of follicles in the group treated with CFA + vitamin E and Se are observed, HE stain, ob X40 (A, D, G), X100 (B, E, H), X200 (C, F, I)

Table 1

Comparative analysis for the values of primordial ovarian follicles (number) in the studied groups and statistical significance

Indicator	Group	Mean	SE	Median	SD	Minimum	Maximum		*p*	
Primordial follicles	**I**	13.4	1.7205	15.5	5.4406	5	19	**I-III-IV**	**I-III**	**0.0017**
	III	5.8	0.8667	5.5	2.7406	3	11	**0.0005**	**I-IV**	0.0537
	IV	9.4	0.7333	8.5	2.3190	7	14		**III-IV**	**0.0053**

Table 2

Comparative analysis for the values of primary ovarian follicles (number) in the studied groups and statistical significance

Indicator	Group	Mean	SE	Median	SD	Minimum	Maximum		*P*	
Primary follicles	**I**	5.8	1.0832	4.5	3.4254	2	12	**I-III-IV**	**I-III**	0.3305
	III	4.6	0.4761	4.5	1.5055	2	7	0.487	**I-IV**	0.8011
	IV	5.5	0.4282	5.5	1.3540	4	8		**III-IV**	0.1769

The statistical analysis of the values of primary follicles, considering all three groups, showed no statistically significant differences between any of the groups (p= 0.487) (Fig. 2). As expected, the statistical analysis of the values of primary follicles for unpaired samples evidenced no statistically significant differences between the groups (p > 0.05) (Table 2).

The statistical analysis of the values of mature follicles, considering all three groups, showed no statistically significant differences between any of the groups (p= 0.5863) (Table 3). As expected, the statistical analysis of the values of mature follicles for unpaired samples

Table 3

Comparative analysis for the values of primordial ovarian follicles (number) in the studied groups and statistical significance

Indicator	Group	Mean	SE	Median	SD	Minimum	Maximum	p		
Mature follicles	I	4.8	0.9043	4.0	2.8597	1	10	I-III-IV	I-III	0.3474
	III	3.8	0.4899	3.5	1.5492	2	7	0.5863	I-IV	0.7150
	IV	4.4	0.5812	4.5	1.8379	2	7		III-IV	0.4408

Table 4

Comparative analysis for the values of uterine wall thickness in the studied groups and statistical significance

Indicator	Group	Mean	SE	Median	SD	Minimum	Maximum	p		
Uterine horn wall thickness	I	654.75	56.595	574.38	178.969	476.18	965.12	I-III-IV	I-III	**0.0147**
	III	424.24	54.573	424.62	172.575	182.55	679.16	**0.0249**	I-IV	0.6305
	IV	607.01	47.431	608.68	149.990	379.60	794.59		III-IV	**0.0211**

indicated no statistically significant differences between the groups ($p > 0.05$) (Table 3, Fig. 3).

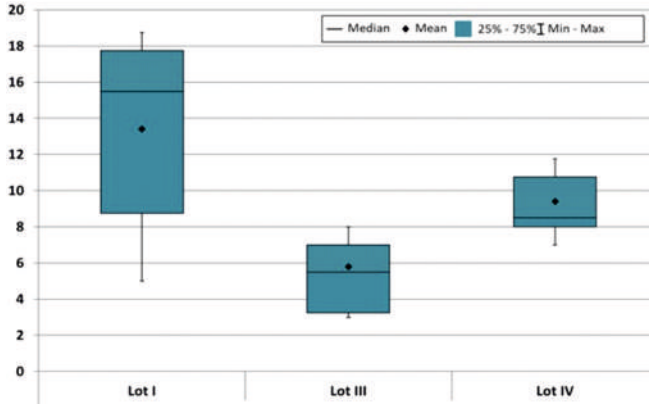

Fig. 2. Primordial ovarian follicles in studied groups

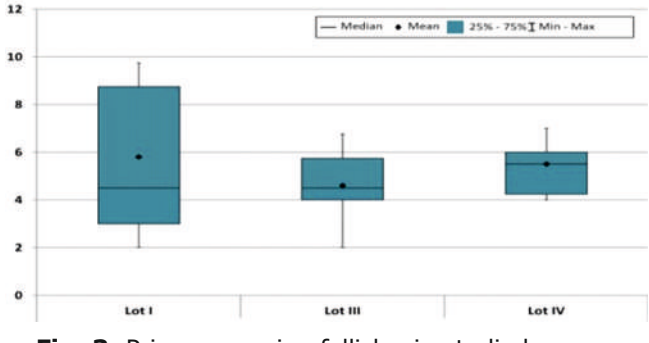

Fig. 3. Primary ovarian follicles in studied groups

Analysis of ovarian uterine horn thickness

The changes in uterine horn sections were evident. If in the absolute control group, the morphology of the horns was normal, in the animals of the group treated with CFA there was mild epithelial and endometrial gland atrophy. The myometrium was also atrophied, and at this level, some sections showed many macrophages loaded with ceroids. In this case, ceroids resulted from the necrosis/destruction of muscle cells and endometrial structures following ovarian failure. Also, in the chorion, there were numerous eosinophils and macrophages. In our study, administration of Sel-E-Vit, after ovarian failure induced by CFA administration, determined an increase in uterine wall thickness (Fig. 3), thus correcting the atrophy caused by CFA administration (Fig. 4, Fig. 5). Marked atrophy of the uterine horn walls in animals treated with CFA and correction of induced atrophy in the group treated with CFA + vitamin E and Se are observed, HE stain, ob X40 (A, D, G), X100 (B, E, H), X200 (C, F, I). The statistical analysis of the values of uterine horn wall thickness, considering all three groups, evidenced statistically significant differences between at least two of the groups ($p = 0.0249$) (Fig. 5, Table 4). As expected, the statistical analysis of the values of uterine wall thickness for unpaired samples revealed statistically significant differences between groups I-III and III-IV ($p < 0.05$) (Fig. 6).

Correlation analysis between the number of ovarian follicles and the O/AO balance indicators

The statistical correlation analysis between the values of uterine horn wall thickness and ovarian follicles showed (Table 5, Fig. 7):

- in group I: a good negative correlation with primary and mature follicles and an acceptable negative correlation with primordial follicles
- in group III: no correlations between the studied indicators

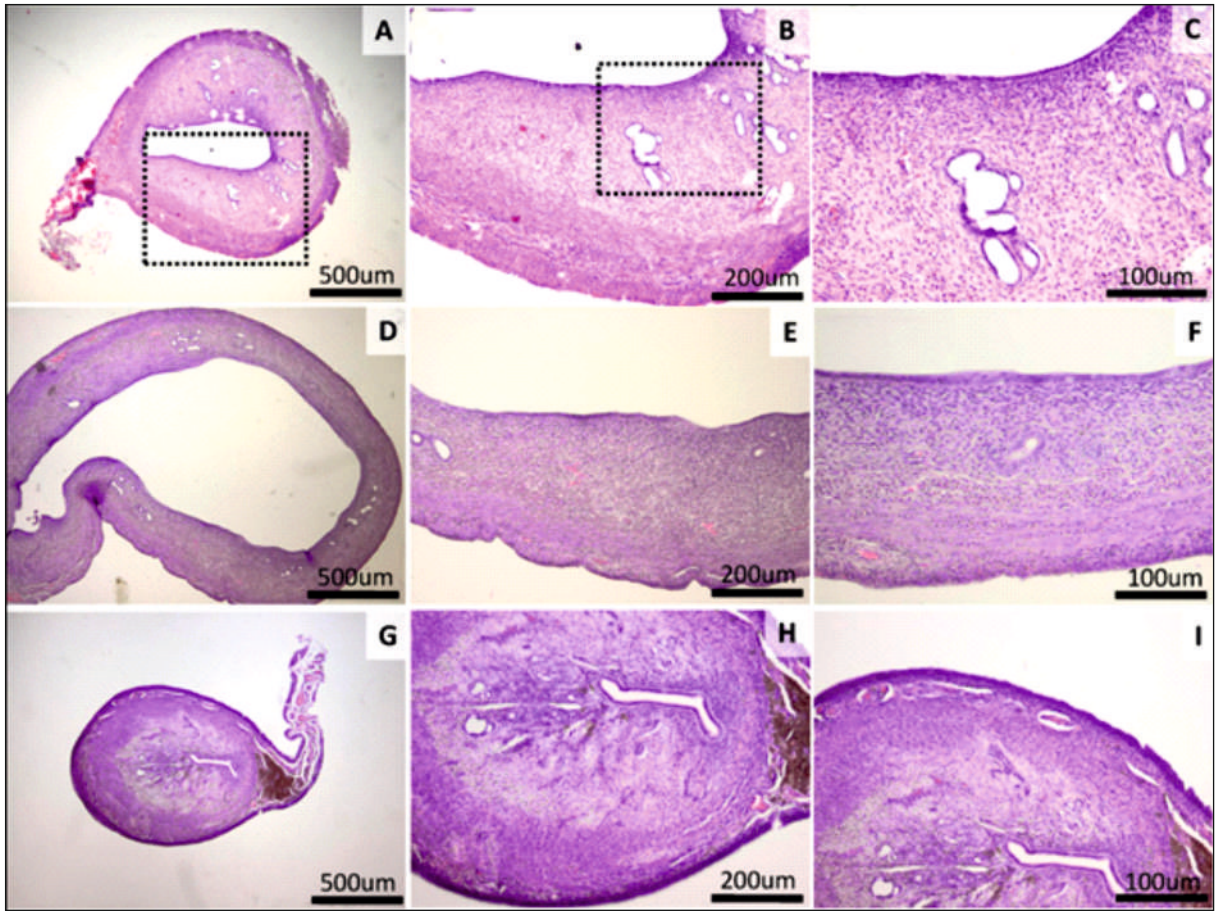

Fig. 4. Morphology of the uterine horn wall in the absolute control group (A, B, C), in the group treated with CFA (D, E, F), and in the group treated with CFA + vitamin E and Se (G, H, I)

Table 5

Statistical correlation analysis between uterine wall thickness and ovarian follicles for the three groups

Indicator		Group I	Group III	Group IV
Uterine horn wall thickness	Primordial follicles	-0.2675 **	0.1780 *	0.3875 **
	Primary follicles	-0.5662 ***	-0.0317 *	0.1057 *
	Mature follicles	-0.7378 ***	0.2378 *	0.4371 **

- in group IV: an acceptable positive correlation between primordial and mature follicles.

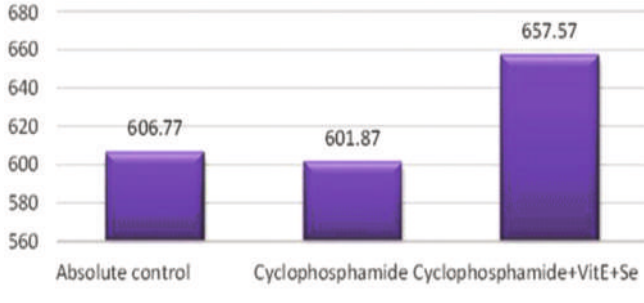

Fig. 5. Graphic representation of the variation in uterine horn wall thickness in different experimental groups

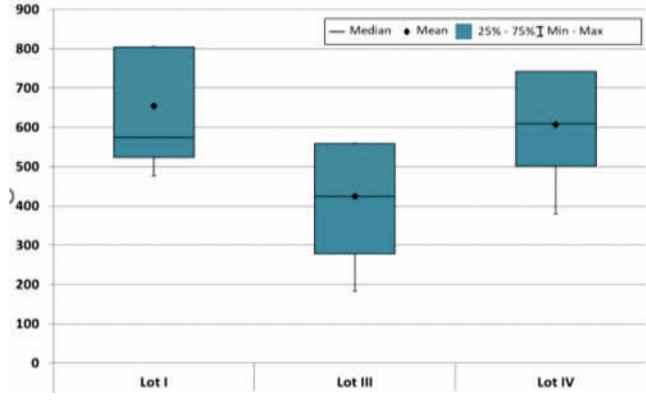

Fig. 6. Uterine wall thickness in studied groups

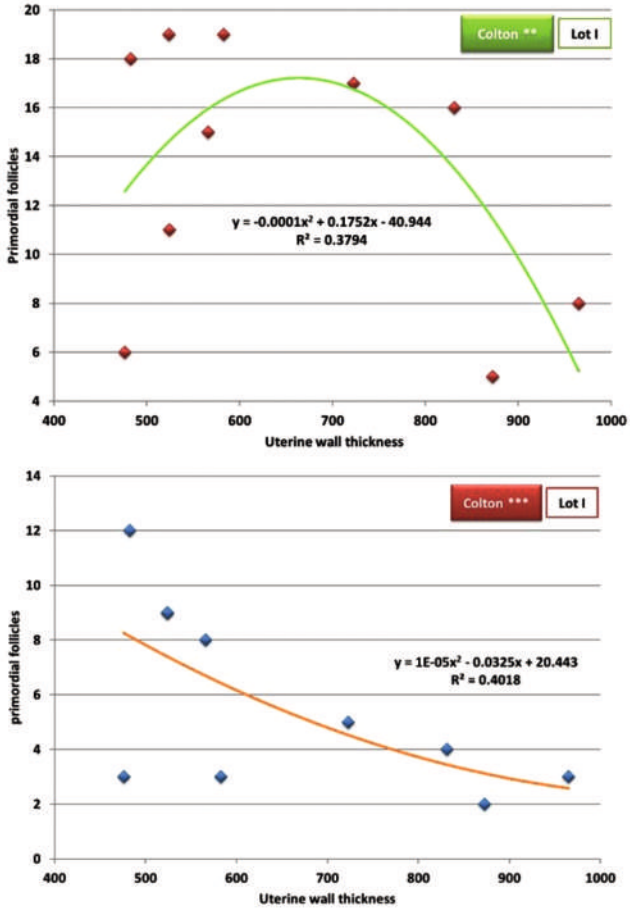

Fig. 7. Statistical analysis of correlation between uterine wall thickness and ovarian follicles

Correlation analysis between the number of ovarian follicles and the O/AO balance indicators

Statistical correlation analysis between the values of primordial ovarian follicles and the O/AO balance indicators showed (Table 6, Fig. 8):

- in group I: a good positive correlation with PC and a good negative correlation with MDA
- in group III: no correlations between the studied indicators
- in group IV: a good positive correlation with GSH and a good negative correlation with DH and an acceptable positive correlation with MDA.

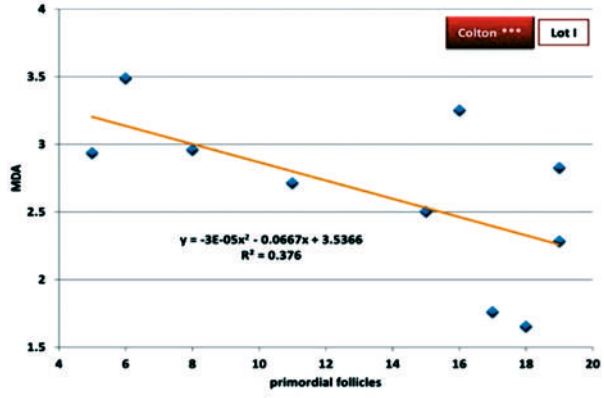

Fig. 8. Statistical correlation analysis between the values of the primordial ovarian follicles and the O/AO balance indicators groups

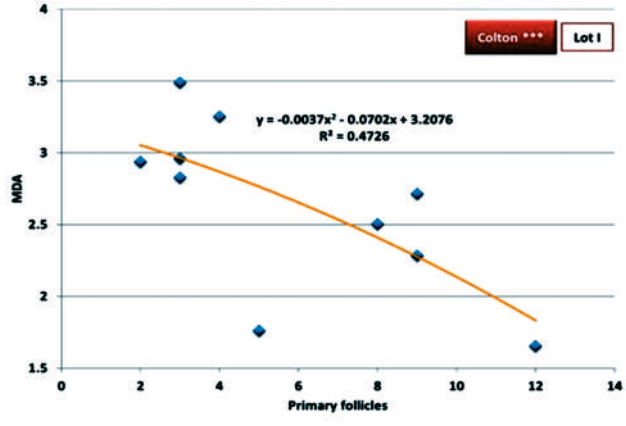

Fig. 9. Statistical correlation analysis between primary ovarian follicles and O/AO balance indicators

Statistical correlation analysis between the values of primary ovarian follicles and the O/AO balance indicators showed (Table 7, Fig. 9):

- in group I: a good negative correlation with MDA

Table 6

Statistical correlation analysis between primordial ovarian follicles and O/AO balance indicators for the three groups

Indicator		Group I		Group III		Group IV	
Primordial follicles	MDA	-0.6132	***	-0.1572	*	0.3339	**
	PC	0.7383	***	-0.0108	*	0.1517	*
	DH	-0.0276	*	0.0874	*	-0.7111	***
	GSH	-0.0304	*	-0.2400	*	0.6656	***

Table 7

Statistical correlation analysis between primary ovarian follicles and O/AO balance indicators for the three groups

Indicator		Group I		Group III		Group IV	
Primary follicles -	MDA	-0.6854	***	-0.3846	**	0.1497	*
	PC	0.4939	**	0.1811	*	0.0338	*
	DH	-0.3584	**	-0.0524	*	-0.6572	***
	GSH	0.1046	*	-0.1914	*	-0.1299	*

Table 8

Statistical correlation analysis between mature ovarian follicles and O/AO balance indicators for the three groups

Indicator		Group I		Group III		Group IV	
Mature follicles -	MDA	-0.4247	**	-0.4259	**	0.1530	*
	PC	0.4513	**	-0.1146	*	-0.1110	*
	DH	-0.4332	**	0.0189	*	-0.1906	*
	GSH	-0.3232	**	-0.1796	*	0.8628	****

Table 9

Statistical correlation analysis between uterine wall thickness and O/AO balance indicators for the three groups

Indicator		Group I		Group III		Group IV	
Uterine horn wall thickness	MDA	0.2848	**	-0.2531	**	0.0435	*
	PC	-0.5515	***	-0.0339	*	0.3718	**
	DH	0.7212	***	0.2919	**	-0.0996	*
	GSH	0.2606	**	-0.4061	**	0.3199	**

and an acceptable positive correlation with PC and an acceptable negative correlation with DH
- in group III: an acceptable negative correlation with MDA
- in group IV: a good negative correlation with DH.

Statistical correlation analysis between the values of mature ovarian follicles and the O/AO balance indicators showed (Table 8, Fig. 10):
- in group I: an acceptable positive correlation with PC and an acceptable negative correlation with MDA, DH and GSH
- in group III: an acceptable negative correlation with MDA
- in group IV: a very good positive correlation with GSH.

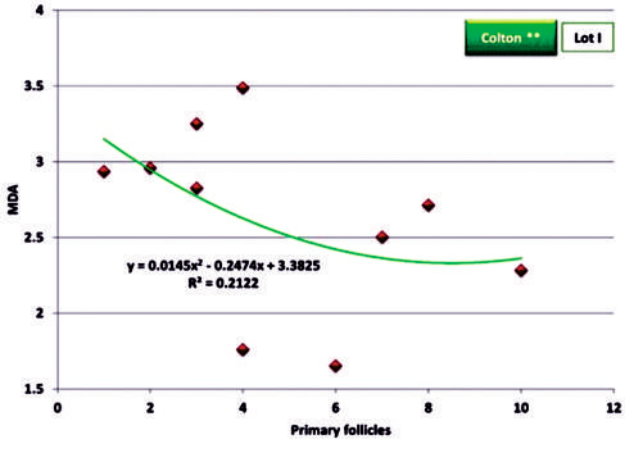

Fig. 10. Statistical correlation analysis between mature ovarian follicles and O/AO balance indicators

Correlation analysis between the values of uterine horn wall thickness and the O/AO balance indicators

Statistical correlation analysis between the values of uterine wall thickness and the O/AO balance indicators showed (Table 9):
- in group I: a good positive correlation with DH, a good negative correlation with PC and an acceptable positive correlation with MDA and GSH
- in group III: an acceptable positive correlation with DH and an acceptable negative correlation with MDA and GSH
- in group IV: an acceptable positive correlation with PC and GSH.

Discussion

Cyclophosphamide is an alkylating agent from the family of oxazaphosphorines which acts by blocking DNA replication, by forming both intrastrand and interstrand crosslinks (21, 24, 28).

In medicine, CFA is used in the treatment of neoplastic processes (e.g., lymphoma, leukaemia, astrocytoma, glioblastoma, meningioma) and various autoimmune diseases due to its potent immunosuppressive effect (e.g., lupus erythematosus, rheumatoid polyarthritis) (12,15,19). Because of its toxicity, its therapeutic use requires continuous monitoring of the renal function, as well as screening to evaluate its side effects on the bone marrow.

The side effects of cyclophosphamide administration on the body are: vomiting, suppression of the bone marrow function, abdominal colic, haemorrhagic cystitis, diarrhoea, alopecia and lethargy, and ovarian failure (4, 16). Cyclophosphamide itself may have carcinogenic properties, so that its use for a longer time period will increase the risk of neoplastic processes such as lymphoma, leukaemia, skin carcinoma, and urinary bladder tumours (11,22).

Experimental studies on animals have shown that the most severe toxic effect of cyclophosphamide is on the ovary, but concomitant uterine wall atrophy also occurs (1,14). In the ovary, the primordial effect manifests through a reduction in the number of follicles; primordial and primary follicles are severely affected, while secondary and antral follicles are less affected. The size of the uterus and ovaries was also reduced to about 2/3 of control values.

Another side effect of cyclophosphamide is neutropenia, which may predispose patients to bacterial, mycotic complications, and various infections caused by opportunistic germs (17,18).

In the current study, cyclophosphamide administration induced ovarian failure characterised by a dramatic reduction in the number of primordial and primary follicles, and uterine atrophy following ovarian failure. The treatment applied, consisting of Sel-E-Vit administration, partially corrected cyclophosphamide-induced ovarian failure, which was demonstrated by an increase in the number of ovarian follicles and an increase in uterine horn wall thickness (1,17,18).

Our results regarding the number of ovarian follicles show for:

primordial follicles:

- ✓ a significant decrease in group III and group IV compared to controls
- ✓ a significant increase in group IV compared to group III

primary and mature follicles

- ✓ no numerical changes

The correlation between the number of ovarian follicles and uterine horn wall thickness is acceptable in group IV for primordial and mature follicles.

The correlation between the number of ovarian follicles and the O/AO balance indicators is for:

primordial follicles:

- ✓ absent in group III
- ✓ good, positive for GSH and negative for DH in group IV
- ✓ acceptable positive for MDA in group III

primary follicles:

- ✓ acceptable negative for MDA in group III
- ✓ good negative for DH in group IV

mature follicles:

- ✓ acceptable negative for MDA in group III
- ✓ very good positive for GSH in group IV

The correlation between uterine horn wall thickness and the O/AO balance indicators is for:

group III – acceptable positive for DH and acceptable negative for MDA and GSH

group IV – acceptable positive for PC and GSH

Our results regarding the effect of iv CFA administration are in accordance with the findings of other authors related to the marked decrease in the number of primordial and primary follicles in female mice, an effect attributed to the ovo-toxic action of the preparation, also evidenced by the decrease in plasma oestrogen and progesterone levels. The effect of cyclophosphamide as an alkylating agent is considered to be irreversible in the ovaries, particularly on oocytes and follicular depletion (26, 29). In the administered doses, the cytotoxicity of the preparation may be lower, which could explain the favourable, reversible effect of Sel-E-Vit, reported by other authors as well (9, 18, 26, 9).

The model used by us can be useful for studying the dynamics of the decrease in ovarian follicles in women with premature ovarian failure. The administration of the Sel-E-Vit complex to animals, after induction of ovarian failure, shows its favourable AO effect, also confirmed by the biochemical determinations performed in study 3, with the diminution of OS and the increase of AO defence. Our results are supported by some studies regarding the increase of folliculogenesis after treatment with Sel-E-Vit in women with ovarian failure induced after irradiation [10].

CONCLUSIONS

Cyclophosphamide administration induces ovarian failure with inhibition of folliculogenesis and uterine atrophy. Administration of an ovo- and utero-toxic chemical agent and post-administration of an antioxidant complex have a favourable effect on ovarian and uterine morphology in vivo.

The pro-oxidant effect of CFA is reduced by administration of the AO complex. Cy-cyclophosphamide treatment in female rats can be an inexpensive, sensible, and valid alter-native method for the accelerated study of ovarian aging, which can be transposed to human subjects.

Institutional Review Board Statement:

The study was conducted according to the guidelines of the Declarations of Helsinky and approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj Napoca, in accordance with international norms regarding studies on animals with scientific and experimental purpose.

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