

## ORIGINAL ARTICLE

## ASSISTED REPRODUCTIVE TECHNOLOGY EFFICIENCY IMPROVEMENT OF TUBAL-PERITONEAL INFERTILITY

10.36740/WLek202011109

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### ABSTRACT

**The aim:** This study aims to develop and implement a complex of pregravid preparation based on endogenous melatonin level, development of oxidative stress and violation of apoptosis regulation in women with tubal-peritoneal infertility.

**Materials and methods:** We have examined 100 women of reproductive age, including 65 women with tubal-peritoneal infertility treated by ART. All studied women were defined levels of endogenous melatonin, oxidative status and level of apoptosis in the endometrium and in follicular fluid (infertile women only). As a powerful free radical neutralizer, we used melatonin to improve the microenvironment of maturing oocytes and create favourable conditions for implantation in the endometrium.

**Results:** Treatment by exogenous melatonin showed better results than standard therapy without antioxidants: the quality of oocytes and embryos, the level of ongoing pregnancies and childbirth was significantly higher.

**Conclusions:** We achieved such results due to restoring the balance in pro / antioxidant system and apoptosis in female reproductive tract.

**KEY WORDS:** infertility, melatonin, oxidative stress, apoptosis, endometrium, follicular fluid

Wiad Lek. 2020;73(11):2370-2377

### INTRODUCTION

The problem of infertility is an actual question of the world medicine, despite the intensive development of reproductive medicine and embryology. According to the Research by the World Health Organization (WHO) in 2010, 48.5 million couples worldwide were unable to have a child, and about 1 million in Ukraine. In our country, every fifth family is infertile (Kulakov, 2009). In the structure of infertility, the female factor is 30%. Tubal-peritoneal factor is the main cause of female infertility, which reaches 72% of prevalence all forms of reproduction impairment [1] and has no tendency to decrease. Despite is the achievements of modern medicine, the tubal-peritoneal infertility remains one of the most serious pathology, considering the complexity of its diagnosis and treatment, and possibility of restoration of reproductive function.

The most effective way to achieve pregnancy in tubal-peritoneal infertility is use of assisted reproductive technologies (ART). At the same time, the effectiveness of this method does not exceed 40%. The main goal of ART is the birth of a healthy child, so the issue of improving ART and studying new factors that positively influence on implantation with normal pregnancy is the main question of the modern world reproduction. Considerable number of studies are devoted to factors affecting the results of ART [2-8].

Today, the negative influence of oxidative stress (OS) and dysregulation of apoptosis on the reproductive function has been confirmed. According to researches, tubal-peritoneal infertility is characterized by high levels of reactive oxygen species (ROS) in the peritoneal environment, which is responsible for numerous damages of embryo and oocytes [9]. It is known that the adverse effects of ROS include DNA damage, lipid peroxidation and protein damage. Oxidative stress is one of the factors that affect regulation of apoptosis [10,11]. Therefore, development of OS and activation of apoptosis is associated with poor quality oocytes, low fertilization and implantation, a violation of embryos, which significantly reduces the pregnancy rate [12-15]. Thus, the study of OS and the regulation of apoptosis in reproductive tract as potential predictors of ART failures is important.

Currently, interest in antioxidant protection, based on the natural metabolites of cells, is increasing. In this aspect attention is paid to the role of melatonin. It is established that it is effective at system, tissue, cellular and subcellular levels by inhibiting the production of oxygen free radicals and antioxidant protection by activation [16].

Melatonin is not only a regulator of repro and reproduction, but also a powerful antioxidant that controls the antioxidant defence by activating SOD and catalase, binding free radicals of oxygen [17, 18]. Thus, the determination of the level of melatonin, markers of oxidative stress and proapoptotic indices

**Table I.** Demographic, laboratory and anamnestic patient data, characteristics of IVF / ICSI program.

Groups Characteristics	First group (n=33)	Second group (n=32)	Control group (n=35)
Age*, years	33,00±1,61	33,00±1,21	30±1,29
BMI*, kg/m <sup>2</sup>	21,60±1,26	23,40±1,39	22,9±0,69
Duration of infertility*, years	6,48±1,68	7,03±1,19	
Basal melatonin level**, пг/мл	20,69±2,44	21,72±4,33	30,4±1,1
Basal FSH level *, mIU / ml	7,30 ± 0,63	7,11 ± 0,58	6,8±0,49
Basal LH level *, mIU / ml	6,30± 0,98	6,02 ± 1,010	6,6±0,39
Basal estradiol level *, pg/ml	60,40±11,17	67,70±14,68	54,90±4,73
No. of antral follicles *	15,40 ± 2,45	14,10 ± 3,14	16,50±1,45
Duration of stimulation *, days	10,55 ± 0,20	10,37 ± 0,30	
Average dose of gonadotropins *, IU	2059,0 ± 99,7	2345,0±89,0	
No. of retrieved oocytes *	12,80±4,77	13,50±5,71	
In vitro fertilisation*, %	48	46,8	
ICSI*, %	94	96,8	
Fertilization rate*, %	85,7	88,4	
No. of transferred embryos *	1,80±1,32	1,08±1,36	

\* - p < 0.05 reliability results of groups relative to each other

\*\* - p = 0.05 reliability results of group 1, 2 relatives to control group

**Table II.** Correlation analysis between the content of antioxidants and the level of f-DNA in follicular fluid and endometrium in group 2.

Correlation pairs	Correlation coefficient, r
SOD in the endometrium - SOD in the follicular fluid	0,608*
Catalase in the endometrium - Catalase in the follicular fluid	0,698*
TBA-products in the endometrium - TBA-products in the follicular fluid	0,548*
APH in the endometrium - APH in the follicular fluid	0,657*
KPH in the endometrium - KPH in the follicular fluid	0,578*
f-DNA in the endometrium - f-DNA in the follicular fluid	0,538*

\*for a given level of significance p = 0,05, n = 32, critical r = 0.349

in the reproductive tract of women with infertility of tubal peritoneal genesis will allow them to evaluate their fertility, and correction of antioxidant balance in the stage of pregravid preparation will improve the results of ART.

Melatonin is not only a regulator of reproductive maturation and reproduction, but also a powerful antioxidant that controls the antioxidant defence by activating SOD and catalase, binding free radicals of oxygen [17, 18]. Thus, the determination of the level of melatonin, markers of oxidative stress and proapoptotic indices in the reproductive tract of women with tubal-peritoneal infertility will allow to evaluate their fertility, and correction of pro/antioxidant balance in the stage of preparation for pregnancy will improve the results of ART.

## THE AIM

Develop and implement a complex of pregravid preparation based on the level of endogenous melatonin, the development of oxidative stress and the violation of the regulation of apoptosis in women with tubal peritoneal infertility.

## MATERIALS AND METHODS

### STUDY POPULATION

We have examined 100 women of reproductive age, including 65 women with tubal-peritoneal infertility treated by ART in Clinic of reproductive technologies by Ukrainian State Institute of Reproduction, Kiev. They were divided into two groups, depending on the medical preparation for ART (in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI)). The first group included 33 women who received antioxidant therapy "Vita-melatonin" (JSC "Kyiv Vitamin Factory", Ukraine) at a dose of 30 mg inside for 3 months before and during 2 weeks of ovarian stimulation in the IVF program. [19] Approval for this study was obtained from the local Ethics Committee of NMAPE and expressed informed consent was obtained from women before IVF-ET treatment. The second group included 32 women who received treatment according to the traditional regimen of controlled ovarian stimulation (COS) with gonadotropins.

65 candidates with tubal-peritoneal infertility in reproductive age, with body mass index (BMI) not more than

30 kg / m<sup>2</sup>, with normal level of reproductive hormones, regular menstrual cycle (25 – 35 days), normal responders with more than 6 antral follicles on ultrasound and FSH less than 10 mIU/ml and normozoospermia were included in study. Exclusion criteria were patients with structure disorders of reproductive system, male and another female factor of infertility. The control group consisted of 35 conditionally healthy women of reproductive age with implemented reproductive function.

## ART

COS by gonadotropins was performed according to the standard long-term protocol with gonadotropin-releasing hormone agonist (a-GnRH). Patients were administered the a-GnRH triptorelin (S.R. liof. do inj. 3,75 mg, Ipsen Pharma) starting in the mid-luteal phase of the preceding cycle. On 2-3 days of menstruation started stimulation with gonadotropins (follitropin alfa or follitropin beta). Doses of gonadotrophins were selected individually, considering the hormonal status, age, and ultrasound monitoring parameters follicular growth and endometrial thickness. As the trigger final oocyte maturation, we used human chorionic gonadotropin (hCG) in ovulatory dose (Horahon, Ferring GmbH, Germany, 10000 units) which were administered an average of 11 days of stimulation in both groups.

Oocyte retrieval was performed by transvaginal aspiration 35-36 hours after hCG injection with needles Luer, 17G (Henke, Germany). The assessment of the quality of oocytes was carried out in terms of maturity: mature – MII (oocytes in the stage of metaphase 2 meiosis) and immature: MI (in metaphase I meiosis), Gv (oocytes at the stage of the germinal vesicle), Atr (atretic oocytes that are not capable of fertilization). The fertility of spermatozoa was evaluated in accordance with WHO 2010 recommendations (WHO., 2010). Fertilization of mature oocytes in vitro or ICSI was carried out in embryological laboratory by biologists according to the standard method. The quality of embryos was estimated at 5th days of cultivation according to Gardner's classification (Gardner et al., 2000). According to this classification, we compiled an integral table by which embryos were divided into 3 types: high-quality, average and low quality (1, 2 and 3). The embryo transfer carried on 5th day of cultivation under transabdominal ultrasound control. 1-2 embryos of high or average quality were transferred.

Luteal phase support was provided by micronized progesterone 600 mg per day vaginally daily, or hydroxyprogesterone caproate 12.5% intramuscularly once every 3 days. To detect implantation, serum  $\beta$ -subunit of hCG was measured on 12-14 day after embryo transfer. An increase  $\beta$ -hCG levels greater than 50 mIU / ml was considered a biochemical pregnancy. Absence of  $\beta$ -hCG increase was classified as fail IVF cycle. Visualization of a fetal egg in uterus by ultrasound in the fifth week was defined as a clinical pregnancy. Loss of pregnancy till 12 weeks was considered as miscarriage. Progressive pregnancy after 12 weeks was defined as ongoing pregnancy.

For a comprehensive assessment of the reproductive system, all patients with infertility were examined according to the Ministry of Health of Ukraine 787 of 09.09.2013 on approval of the use of ART in Ukraine.

## OXIDATIVE STRESS AND APOPTOSIS BIOMARKERS

All studied women were defined levels of endogenous melatonin, oxidative status and level of apoptosis in the endometrium and in follicular fluid (infertile women only). To assess the oxidative status and apoptosis level in the endometrium and the follicular fluid, the prooxidant markers were determined: proteins modification (PM) intensity, thiobarbituric acid (TBA) active products content, and antioxidant markers: superoxide dismutase (SOD) and catalase activity.

Apoptosis level was estimated by content of DNA – fragmentation (f – DNA) in the sample. Endometrial secretion aspiration was in second phase of the menstrual cycle (18-21 days) by catheter. Follicular fluid for research was received during oocyte retrieval after oocyte extraction.

Markers of PM – aldehyde phenylhydrazones (APH) and carboxy phenylhydrazones (CPH) was determined by the method of Dubinina (Dubinina et al., 1995). TBA- active products – by conventional method, which is based on ability of thiobarbituric acid to react with malondialdehyde (Orekhovich, V. N. 1977). SOD activity was determined by Chevari (Chevari, Chaba & Sekei, 1985), activity of catalase – according to the method Korolyuk (Korolyuk, Ivanova, Mayorova & Tokarev, 1988), the content of fragmented DNA was evaluated by the Barton method of diphenylamine test (Barton. K., 1956).

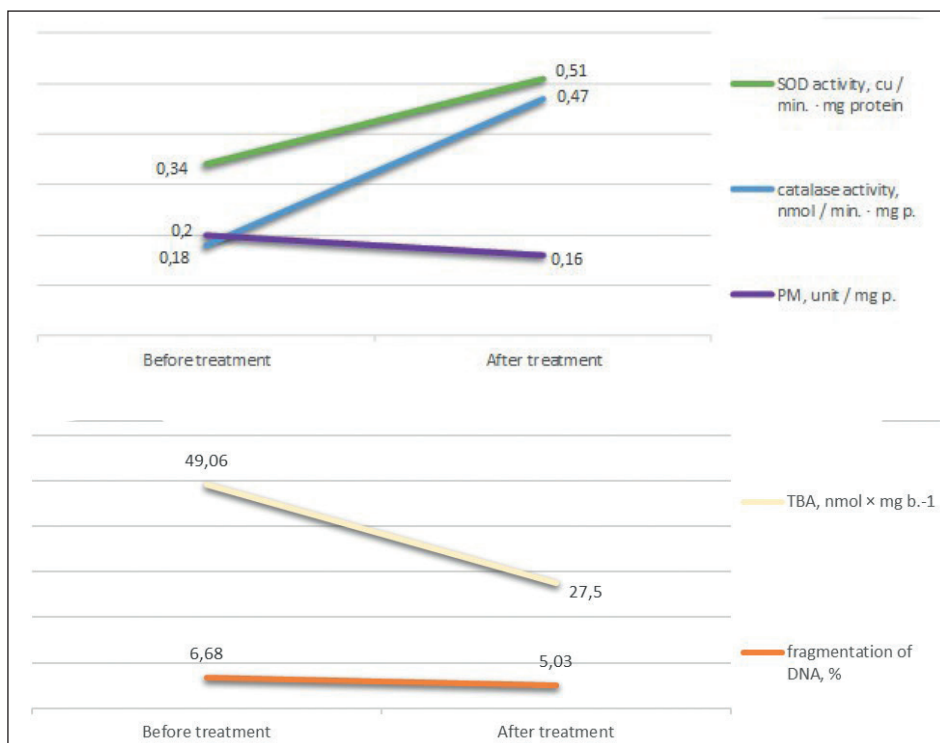
## STATISTICAL ANALYSIS

Statistical data was performed using Microsoft Office Excel program, using the non-parametric T-Wilcoxon T-criteria, and Spirman-based application rank-correlation software. Data are presented as mean (M)  $\pm$  standard deviation (SD) values. Statistically significant differences and correlation bonds were considered at  $p = 0.05$ .

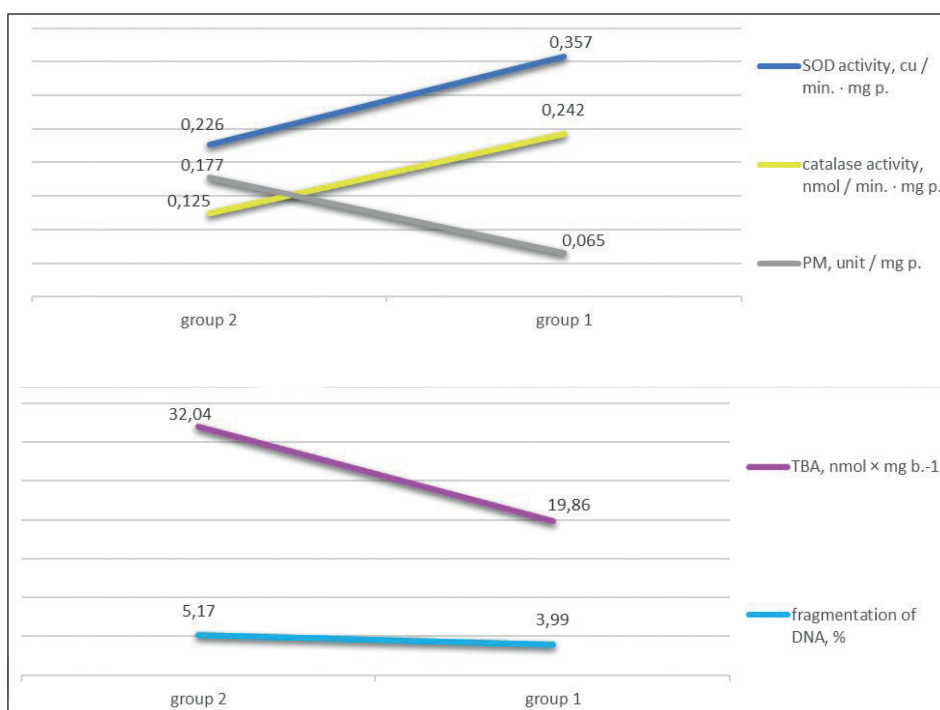
## RESULTS

Comparison of three groups by age, BMI, FSH level, LH, oestradiol and number of antral follicles, and two groups by duration of infertility, duration of stimulation, average dose of gonadotropins, number of received oocytes, fertilization rate (IVF / ICSI) and the number of transferred embryos in the cavity of uterus showed no statistical difference ( $P > 0.05$ ). Statistically lower endogenous melatonin level in infertile group in comparison with healthy women group were determined (Tab. I).

The first stage of the study we determined biochemical markers in the studied group of women before the treatment. Comparative analysis showed disbalance between pro- and antioxidants with the trend of oxidative stress and



**Fig. 1.** State of antioxidant system and apoptosis activity in endometrium of women from first group before and after receiving antioxidant therapy.

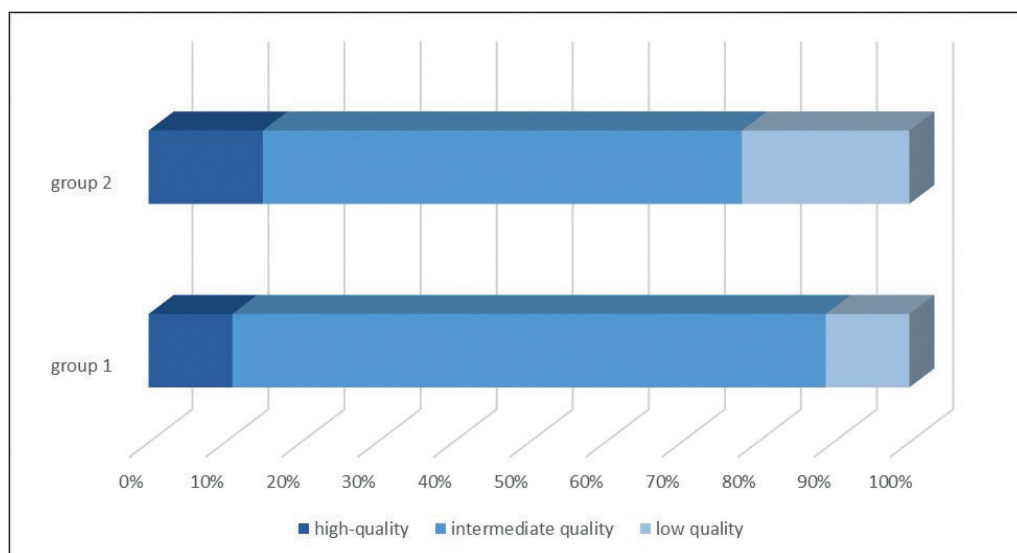


**Fig. 2.** Status pro / antioxidant system and apoptosis activity in follicular fluid of women from 1 and 2 groups.

increased apoptosis in the endometrium of women with tubal-peritoneal infertility as opposed to healthy women [20, 21]. A similar trend was identified in follicular fluid in group of patients who did not receive the antioxidant therapy. We conducted a correlation analysis between pro / antioxidants and f-DNA in the biomaterial of the reproductive tract and found middle direct correlation in the group of women who did not receive treatment with melatonin (Tab. II).

As a result of antioxidant treatment with exogenous melatonin, the level of lipid peroxidation and oxidative modification of proteins decreased by an average of 44% and 24.3%, respectively, while the activity of SOD and catalase increased by 1.5 and 2.6 times respectively; the level of f – DNA decreased by 1.3 times (Fig. 1).

In the follicular fluid of women after antioxidant therapy, there was also a tendency towards a decrease in the level of prooxidants, fragmented DNA and an increase in anti-



**Fig. 3.** Distribution of embryo quality in study groups.



**Fig. 4.** Structure of results IVF program for women with infertility in first and second groups.

oxidant defence: the activity of enzymes SOD and catalase in group 1 was higher in 1.5 and 2 times, compared with the second group, and the levels of prooxidants – lower in 1,7 times. The f-DNA content in the exogenous melatonin group was lower on 23% than in the standard treatment group (Fig. 2). Analysis of these data in both groups showed a significant difference at  $p < 0.05$ .

Correlation analysis of markers pro / antioxidant system and biochemical indicator of apoptosis showed the relationship between the products of lipid and protein peroxidation and activity of antioxidant enzymes, while the content f-DNA levels correlated only with pro-oxidant. In the follicular fluid, a strong negative correlation was

found between catalase activity and f-DNA content ( $r = -0.57$ ) [21].

Evaluation of the quality of oocytes in comparative groups showed a higher percentage of mature oocytes in group 1 relative to the group of women who did not receive the antioxidant: 74% vs. 69%, which is statistically significant. The quality of the oocytes depends on the microenvironment in which they develop, therefore, we analysed the correlation between the prooxidants and the MII oocytes and determined the dependence of number of mature oocytes to a greater extent of protein peroxidation and to a lesser extent of lipid peroxidation and f-DNA in the follicular fluid [22].

Analysis of the embryos of women in both groups showed statistically significant differences: the first group was 25% good quality embryos (1 and 2 quality group) more and in 2 times less embryos of poor quality, but in the second group the number of high-quality embryos in 4% higher ( $p \leq 0.05$ ) (Fig. 3). The higher content of high-quality embryos in the second group was obtained by several women with relatively low content of lipid and protein peroxidation products, DNA fragmentation, high enzyme activity of antioxidant defence and increased levels of endogenous melatonin.

Correlation analysis of high and average quality embryos showed a negative dependence on prooxidants and f-DNA in the endometrium of both groups [22].

Results of ART program infertile women showed a higher percentage of pregnancies in the first group than in the second group – 51.51% vs. 37.51%, respectively. Note the low number of miscarriages in the group that received antioxidant therapy: 3% versus 12.5% in the second group. The level of biochemical pregnancies was 18.18% and 9%, respectively, in groups 1 and 2, which coincides with the average population level (13-22%). Data on the development of pregnancies of the studied groups are presented in Fig. 4.

According to the correlation analysis, pregnancy is associated with the processes of peroxidation of proteins (PM,  $r = -0,84$ ;  $r = -0,85$ ) and lipids (TBA,  $r = -0,71$ ,  $r = -0,85$ ) in the endometrium and follicular fluid and activation of apoptosis (f-DNA,  $r = -0,86$ ,  $p = 0.05$ ) and the activity of enzymes catalase and SOD ( $r = 0,82$ ;  $r = 0,84$ ,  $p = 0, 05$ ) in follicular fluid. It was determined that the pregnancy rate correlates with the number of mature oocytes (MII,  $r = 0,86$ ,  $p = 0.05$ ).

## DISCUSSION

Our study showed that the level of TBA-active products and PM markers in endometrium and follicular fluid of women with tubal-peritoneal infertility are elevated and the activity of antioxidant defence enzymes is not sufficient to neutralize free radicals, thus, there is a disturbance in balance between pro- and antioxidants with a tendency to develop oxidative stress. The imbalance in pro / antioxidant system accompanied by increased DNA fragmentation, which also indicates a dysregulation of apoptosis in reproductive tract. Thus, oxidative stress and apoptosis activation in the follicular fluid, which is a microenvironment of maturing oocytes, and in the endometrium, which is a substrate for implantation, can be factors that affect the quality of oocytes, embryos and endometrial susceptibility to these embryos [23]. It was detected an increase of SOD and catalase antioxidants activity and reduction of free radicals under the influence of exogenous melatonin correlate with the successful outcome of ART.

It is believed that in vitro fertilization disturbs the oxidative balance in the culture medium, which makes them less protected from free radicals, and the addition of antioxidants plays an important role in the efficacy of

IVF [24]. Some studies confirm the link between development of oxidative stress in the endometrium and female infertility [25]. Consequently, the use of ART in infertility is accompanied by the risk of oxidative stress, so treatment by powerful antioxidants is desirable to provide favourable conditions for in vitro fertilization.

The maturity of the oocytes is associated with a low level of free radicals and enough quantity of unmodified (native) proteins [26]. The imbalance in the system pro / antioxidants in favour of oxidation products and abnormal regulation of apoptosis in the reproductive tract of women is negatively affects the quality of embryos [27]. According to our previous study, we found strong correlation between the quality of oocytes and embryos, that is, a sufficient number of mature oocytes contributes to increase the number of quality embryos [28], which we observed in a group of women who received antioxidant therapy. Thus, the normalization of antioxidant defence contributes improvement of oocyte and embryos quality (due to increase of number of intermediate quality embryos), similar results were obtained by T. Nishihara et al., studying the effect of exogenous melatonin on fertilization rate and quality of blastocyst in infertile women [28].

Pregnancy – is a complex process in which there is a close relationship between embryo and endometrium, it depends on the state of pro / antioxidant system, balance in pro / antiapoptotic factors in the reproductive tract, but the impact of oxidative stress on endometrial receptivity is insufficiently explored [29]. According to the authors [30], the level of SOD in the endometrium secretion was significantly higher in the group with progressive pregnancy, compared with the group with failed IVF cycles. Registration of systemic increase of antioxidants in serum also positively influenced the onset of clinical pregnancy [31]. Revealed correlation dependencies in our study indicate that the normalization of the balance in the system “ROS – antioxidants” promotes pregnancy. Reduction of fragmented DNA is accompanied by a sufficient quality and quality of endometrial cells to ensure its implantation function. So, we found a positive effect of exogenous melatonin on the ratio of pro / antioxidants and apoptosis regulation, as in the endometrium and in follicular fluid, which is closely correlated with the quality of oocytes, embryos, and as a result – improving endometrial implantation ability and pregnancy.

## CONCLUSIONS

Despite the important role of free radicals in the physiological functioning of the reproductive system of women, their excessive content adversely affects fertilization, implantation and pregnancy. Lack of antioxidant enzymes activity also contributes to OS and as a consequence to disruption of apoptosis in the reproductive tract of women with tubal-peritoneal infertility. It was established that correction of this condition by using exogenous melatonin for 3.5 months increases the effectiveness of ART by 14%.

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**Conflict of interest:**

*The Authors declare no conflict of interest.*

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**Received:** 11.11.2019

**Accepted:** 17.07.2020

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**A** – Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis,

**D** – Writing the article, **E** – Critical review, **F** – Final approval of the article