Melatonin protects against experimental immune ovarian failure in mice

Tatyana Voznesenskaya¹, Nataliya Makogon, Tatyana Bryzgina, Vera Sukhina, Nataliya Grushka and Irina Alexeyeva
Bogomoletz Institute of Physiology,
Ukrainian Academy of Sciences, Kyiv, Ukraine

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SUMMARY

Experimental immune ovarian failure induced in CBA mice by either administration of xenogenic anti-ovarian antibodies or immunization with allogenic ovarian extracts impaired the meiotic maturation of oocytes and increased apoptosis of follicular cells. Immunization was accompanied with the inflammation and active immune reaction, as shown by the enlargement of regional lymph nodes, the increase of apoptosis in cultured lymph node cells and the increase of band and segmented neutrophil percentage in the blood. Triple injections of melatonin (5 mg/kg of the body weight) an hour before antibodies administration restored the meiotic maturation of oocytes and supported the survival of follicular and lymph node cells. In contrast, melatonin application upon immunization was not effective to prevent the ovary impairment and cell death. It is concluded that melatonin protects against immune ovary failure induced by xenogenic anti-ovarian antibodies. Reproductive Biology 2007 7 3: 207-220.

Key words: melatonin, immune ovarian failure, oocytes, follicular cells, lymphocytes, apoptosis, mice

¹Corresponding author: Department of Immunology, Bogomoletz Institute of Physiology, 4, Bogomoletz str., 01024 Kyiv, Ukraine; voz@biph.kiev.ua

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INTRODUCTION

Several ovarian disorders in women including premature ovarian failure [8, 17, 18], polycystic ovary syndrome (in which immunological disturbances may be secondary; [8, 20]), some types of oophoritis [20] or unexplained infertility [8, 17] are associated with autoimmune factors. They have been reported to depend on anti-ovarian autoantibodies against granulosa cells, thecal cells as well as zone pellucida [18, 20, 21] and to be T-cell-mediated [20, 22, 26]. However, the effects of anti-ovarian antibodies on oocyte maturation have not been studied in detail.

Follicular cells play an important role in oocyte maturation and follicular atresia. A number of studies provide evidence that follicular cells’ death during atresia occurs via apoptosis [12, 14, 33]. The apoptotic death of large number of follicular cells may be the reason for functional ovary failure. The early steps of both apoptosis and necrosis result in increased mitochondrial membrane permeability and enable the release of pro-apoptotic factors [14]. It was shown that melatonin, the major secretory product of the pineal gland, inhibited the permeability transition pore activity directly [1] resulting in anti-apoptotic effect [4, 19, 23, 29].

Melatonin is a recognized free radical scavenger and an indirect antioxidant which influences anti-oxidative enzymes [19, 23, 24, 27]. Its immunomodulating effects are widely known as well [4, 28, 31, 34]. Numerous organs and tissues, such as the ovary, testis, vascular system, intestines, smooth muscles and some immune cells possess membrane and nuclear melatonin receptors [6, 31]. Moreover, a direct effect of melatonin on the oocytes was observed [5]. Exogenous melatonin was protective against the follicular degeneration caused by irradiation [13]. Therapeutic intervention with melatonin as the antioxidant and inhibitor of the mitochondrial permeability transition pore may provide beneficial clinical applications for the treatment of some disorders since melatonin is safe and non-toxic [1]. The aim of our study was to investigate the effect of melatonin on the meiotic maturation of oocytes and follicular cell death under the experimental immune ovarian failure induced by either xenogenic anti-ovarian antibodies or immunization with the allogenic ovarian extracts.
MATERIALS AND METHODS

Experiments were carried out on mature CBA female mice (18-20 g) in accordance with the International Principles of the European Convention concerning the protection of vertebrates. Mice were kept on a 12-12 hour light-dark cycle with free access to food and tap water.

Xenogenic anti-ovarian antibodies

Xenogenic anti-ovarian antibodies were generated by immunizing rabbits with mouse ovarian extracts. To prepare the extract, ovaries of CBA mice were homogenized with a glass homogenizer in ice-cold normal saline. The homogenate was centrifuged at 1000 g for 7 min and the supernatant was used for immunization. Protein concentration in the supernatant was determined by Lowry’s procedure [16].

Chinchilla rabbits were immunized with the ovarian extracts intravenously four times within two weeks. Immunizations were made with gradually increasing quantities of protein (7.5, 10.5, 14.0 and 14.0 mg per kg of body weight). The sera were pooled from the blood of two or three rabbits on the 9th day after the last immunization. The ovary-specific antibodies were characterized by a titer of 1:400 [9], while the titers of antibodies against liver, kidney or testis extracts (obtained in a similar way) ranged from 1:20 to 1:40. Gamma-globulin fraction extracted from the sera by ammonium sulfate precipitation [9] was further used as xenogenic anti-ovarian antibodies (xAoAB). The latter were injected intravenously into mice (0.01 mg of protein per g of body weight) once a day for three days. Twenty four hours after the last injection mice were killed with an overdose of nembutal anaesthesia. Their blood, ovaries and groin lymph nodes were taken for examination.

Immunization of mice with allogenic ovarian extracts

The CBA mice were immunized five times with the ovarian extracts from BALB/c mice. The first immunization (0.1 mg of protein per g of body
weight) was made subcutaneously in complete Freund’s adjuvant. A week later, intravenous immunizations were started. Mice were injected four times within two weeks with 0.025, 0.0375, 0.05 and 0.07 mg of protein per g of body weight, respectively, without an adjuvant. Six days after the last injection, the mice were killed with an overdose of nembutal, and their blood, ovaries and groin lymph nodes were sampled. The control mice received equivalent volumes of normal saline instead of the antibody solution or ovarian extract and were treated similarly to those of experimental groups.

**Melatonin injection**

The mice were injected with melatonin (Sigma, USA) intravenously (5 mg/kg of the body weight; [4, 7, 13, 15, 27]) at the same time of day (3-5 p.m.). Melatonin was injected 1/ three times (one hour before the xAoAB administration), or 2/ seven times when mice were immunized with allogenic ovary extracts, always one hour before antigen administration (five times within two weeks) plus between the 1st and the 2nd immunizations (one time) plus on the 3rd day after the last immunization (one time).

**Determination of the oocyte meiotic maturation**

Follicles were separated from ovaries and then counted, cumulus-oocyte cellular complexes were extracted mechanically. The cumulus-oocyte cellular complexes were cultured for 20 hours at 37°C in DMEM (Sigma, USA), supplemented with 5% fetal bovine serum and antibiotics. The number of oocytes with germinal vesicle breakdown (metaphase I) was counted after four hours, while the number of oocytes forming the first polar body (metaphase II) was estimated by light microscopy after 20 hours of culture.

**Determination of the death of follicular and lymph node cells**

The percentage of viable, apoptotic and necrotic cells within follicular and lymph node cells (obtained by a standard procedure) was determined by
their vital staining with fluorescent dyes Hoechst 33342 and propidium iodide [30]. Propidium iodide penetrates only into necrotic cells with leaky plasma membranes and stains their nuclei in red. Hoechst 33342 penetrates non-injured membranes and stains the nuclei of live cells in blue. Being bound to chromatin, these dyes provide a possibility to follow the apoptotic changes in the nuclear material i.e. peripheral localization of chromatin, chromatin condensation, fragmentation of the nuclei and disintegration of the cells into apoptotic bodies. Staining was conducted in phosphate buffered saline (PBS) using the dyes’ final concentration of 10 µmol/l; the ovarian cells were treated for 10 minutes in darkness, while staining of the lymph node cells required 25 minutes. Then, the cells were washed with PBS by centrifugation, fixed by 5% formalin in PBS for two minutes, washed again and examined under a fluorescent microscope (×700). Not less than 200 cells were counted in each preparation; the ratio (%) of live, apoptotic and necrotic cells to the total amount of cells was calculated. We estimated also the percentage of cells possessing morphological features of secondary (or postapoptotic) necrosis i.e. those with apoptotic nuclear morphology and the damaged plasma membrane integrity. The number of viable, apoptotic and necrotic follicular cells was counted not later than two hours after their mechanical separation from the cumulus-oocyte cellular complexes. The number of viable, apoptotic and necrotic cells, isolated from groin lymph nodes was counted after 16-hour culture in RPMI 1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum at 37 °C. To induce apoptosis experimentally, we used etoposide (“Ebewe”, Austria) in the final concentration of 80 µmol/l.

The percentage of blood leukocytes were counted in the blood smears stained according to the Pappenheim method. The numbers of lymph node cells were counted in hemocytometer. Statistical analyses of data were carried out by one way ANOVA followed by Fisher’s LSD post hoc test using the STATISTICA-6 program. All percentage values were subjected to arc-sin transformation before statistical analysis. Data are presented as means ± SEM and p<0.05 was considered significant.
RESULTS

An immune ovary failure was evoked experimentally by administration of the xenogenic anti-ovarian antibodies (xAoAB) or immunization with the allogenic ovarian extracts. Both treatments impaired the meiotic maturation of mouse oocytes: the number of oocytes at metaphase I and metaphase II decreased compared to that of control mice (fig. 1). In both experimental procedures, the impaired oogenesis was accompanied by a reduction in the number of viable follicular cells and an increase in the number of cells.

*Figure 1.* The effect of melatonin on the percentage (mean±SEM) of oocytes with germinal vesicle breakdown (metaphase I) and oocytes forming the first polar body (metaphase II) in A/ mice treated with xenogenic anti-ovarian antibodies, or B/ immunized with allogenic ovarian extracts. Control mice received equivalent volumes of normal saline and were treated similarly to those of experimental groups. Different superscripts indicate significant differences (p<0.05)
with morphological signs of apoptosis (fig. 2). The number of necrotic follicular cells decreased after the xAoAB administration, but increased upon the immunization with ovarian extracts (fig. 2).

**Figure 2.** The effect of melatonin on the percentage of viable, necrotic or apoptotic follicular cells (mean±SEM) in A/ mice treated with xenogenic anti-ovarian antibodies, or B/ immunized with allogenic ovarian extracts. Control mice received equivalent volumes of normal saline and were treated similarly to those of experimental groups. Different superscripts indicate significant differences (p<0.05).
The experimental immune ovarian failure was also accompanied by changes in the content of white blood cells (tab. 1). In both experiments, we found a decrease in the lymphocyte numbers, while the numbers of neutrophils with segmented nuclei increased. The numbers of neutrophils with banded nuclei increased only in animals immunized with the allogenic ovarian extracts indicating that this treatment provoked stronger inflammatory reaction. Regional lymph nodes responded by increasing their mass and cell numbers in mice immunized with the allogenic ovarian extracts.

The immunization with the allogenic ovarian extracts resulted in an increase of apoptosis in lymph node cells cultured in the absence (spontaneous apoptosis) or in the presence of etoposide (induced apoptosis; tab. 2), and the number of cultured live cells decreased (data not shown). The xAoAB administration increased only the etoposide-induced apoptosis in lymph node cells. In addition, the immunization with the allogenic ovarian extracts was accompanied by an increase in the number of cultured lymph node cells with fragmented apoptotic nuclei and damaged plasma membranes indicating the development of secondary necrosis (tab. 2). In contrast, no such changes were found after the xAoAB administration.

Injection of melatonin alone did not result in any changes in the ovaries of control mice; and it did not significantly influence the blood leukocyte content or mass, quantity and viability of the cells in the lymph nodes (tabs. 1 and 2). However, when injected into the mice administered with the xAoAB, melatonin reduced the adverse effect of xenogenic antibodies. It improved the meiotic maturation of oocytes by increasing the number of cells at metaphase I and metaphase II (fig. 1) and favored follicular cell survival by decreasing the number of apoptotic cells (fig. 2). At the same time, an increase in the number of cells dying through the necrotic pathway was observed. Melatonin did not affect the blood leukocyte content, as well as the mass and quantity of the lymph node cells in the xAoAB-treated mice (tab. 1). On the other hand, it decreased significantly the number of apoptotic lymph node cells, both spontaneous and induced with etoposide in these animals (tab. 2).

In contrast, melatonin injections to the mice immunized with the allogenic ovary extracts did not improve the meiotic maturation of oocytes
at any stage of meiosis (fig. 1) and did not change the ratio of live, apoptotic and necrotic follicular cells (fig. 2). It also did not affect the number of apoptotic cells (both spontaneous and induced) in the cultured lymph node cells (tab. 2). On the other hand, melatonin increased the number of cells with the signs of secondary post-apoptotic necrosis in the immunized mice. Interestingly, melatonin reduced the percentage of blood banded neutrophils which increased after immunization (tab. 1).

Table 1. Blood leukocytes, mass of two groin lymph nodes and number of lymph node cells (mean±SEM) in the studied groups of mice

<table>
<thead>
<tr>
<th>Groups of mice</th>
<th>Blood</th>
<th>Lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total leukocytes (10³/mm³)</td>
<td>Banded neutrophils (%)</td>
</tr>
<tr>
<td>Controls (n=9)</td>
<td>5.1±0.3a</td>
<td>1.3±0.4a</td>
</tr>
<tr>
<td>Melatonin-treated (n=6)</td>
<td>5.1±0.4ab</td>
<td>1.3±0.7a</td>
</tr>
<tr>
<td>xAoAB-treated (n=6)</td>
<td>3.4±0.4c</td>
<td>3.0±1.1ab</td>
</tr>
<tr>
<td>Melatonin+ xAoAB treated (n=7)</td>
<td>3.6±0.5bc</td>
<td>4.3±1.1bd</td>
</tr>
<tr>
<td>Immunized with ovarian extracts (n=7)</td>
<td>4.7±0.7ac</td>
<td>6.3 ±0.9d</td>
</tr>
<tr>
<td>Melatonin-treated and immunized with ovarian extracts (n=7)</td>
<td>3.4±0.4c</td>
<td>1.1±0.6a</td>
</tr>
</tbody>
</table>

xAoAB: xenogenic anti-ovarian antibodies; *mg/g of body weight; control mice received equivalent volumes of normal saline and were treated similarly to those of experimental groups; different superscripts in the same column indicate significant differences (p<0.05)
Two experimental models of autoimmune ovary failure have been employed in the present study. In the first model, xenogenic anti-ovarian antibodies were passively transferred to the recipient mice, while in the second one the immune response was stimulated in mice by injecting the allogenic ovarian extracts. Both experimental approaches resulted in similar ovary impairment characterized by failed meiotic oocyte maturation and death of follicular cells. This means that immunization of mice with the allogenic

Table 2. The percentage (%) of apoptotic and secondary necrotic lymph node cells (mean±SEM) cultured in the absence (spontaneous apoptosis) or in the presence of etoposide (induced apoptosis) in the studied groups of mice

<table>
<thead>
<tr>
<th>Groups of mice</th>
<th>Spontaneous apoptosis</th>
<th>Induced apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>apoptotic cells</td>
<td>secondary necrotic cells</td>
</tr>
<tr>
<td>Controls (n=13)</td>
<td>35.6±3.4^{bc}</td>
<td>8.5±1.6^{a}</td>
</tr>
<tr>
<td>Melatonin treated (n=6)</td>
<td>27.4±2.1^{ab}</td>
<td>13.2±1.9^{abc}</td>
</tr>
<tr>
<td>xAoAB treated (n=11)</td>
<td>39.8±4.6^{bd}</td>
<td>10.9±2.4^{ad}</td>
</tr>
<tr>
<td>Melatonin+xAoAB treated (n=6)</td>
<td>20.4±0.8^{a}</td>
<td>10.3±1.9^{ae}</td>
</tr>
<tr>
<td>Immunized with ovarian extracts (n=14)</td>
<td>49.7±5.8^{d}</td>
<td>14.2±1.5^{bde}</td>
</tr>
<tr>
<td>Melatonin treated and immunized with ovarian extracts (n=7)</td>
<td>46.8±3.5^{cd}</td>
<td>22.5±2.5^{c}</td>
</tr>
</tbody>
</table>

xAoAB: xenogenic anti-ovarian antibodies; control mice received equivalent volumes of normal saline and were treated similarly to those of experimental groups; different superscripts in the same column indicate significant differences (p<0.05)
ovary tissue also resulted in the generation of antibodies. The presence of anti-ovarian antibodies in the immunized mice was confirmed in the antibody fixation test (data not shown).

The pathways of follicular cell death observed in these two experimental approaches, however, were different. In both cases the ovary impairment was accompanied by a significant increase in the numbers of apoptotic cells, but the numbers of cells dying through a necrotic pathway decreased after the xAoAB injection, and increased following the immunization with allogenic ovary extracts. This difference may be due to some active immune processes occurring during immunization.

Immunization with the ovarian extracts resulted in increased mass and cell number in the lymph nodes indicating the development of the immune response. The study of cell death in cultured lymph node cells demonstrated the intensification of spontaneous apoptosis (typical for immune cells activation) after immunization with the allogenic ovarian extracts. This treatment also was accompanied by the increase in the number of secondary necrotic lymph node cells (with apoptotic nuclei and disrupted plasma membrane). It is well known, that the release of intracellular content after cell membrane damage provokes and facilitates the inflammation. The inflammatory reaction in mice immunized with the allogenic ovarian extracts with complete Freund’s adjuvant was confirmed by the significant increase in the percentage of banded and segmented neutrophils. As a whole, in contrast to the passive xenogenic antibody transfer, immunization with the ovarian extracts was accompanied by inflammation and immune system activation.

Melatonin did not affect the ovaries, lymph nodes and blood leukocytes of the control mice. When applied to the mice administered with the xAoAB, melatonin reversed the adverse effect of anti-ovarian antibodies on oogenesis and increased the quantity of viable follicular cells. Surprisingly, no such melatonin effects on the oocyte meiotic maturation and the survival of follicular and lymph node cells were found in mice immunized with allogenic ovarian extracts. Moreover, an increase in the secondary (or post-apoptotic) necrosis of the lymph node cells was observed. These data indicate that in spite of similar consequences, the mechanisms of the ovary impairment underlying passive transfer of anti-ovarian antibodies and
active immunization with ovarian extracts are different. The differences may be due to inflammation or T lymphocyte involvement during immunization. Melatonin neutralized the short-term effect of xenogenic antibodies, but was not effective against the systemic immune reaction.

We demonstrated the protective effect of melatonin on the follicular cells of xAoAB administered mice and the absence of such an effect in mice immunized with the ovarian extracts. Our finding is in accordance with other data indicating that in some experimental models melatonin did not protect cells from apoptosis; in cancer cells it even enhanced apoptosis [1, 10, 11, 25, 29]. It was also shown that the effect of exogenous melatonin on the ovarian follicles depended on the follicular development [13]. Melatonin could also influence the immune cells, the effect varying dependently on dose, way of application and the way of immune system activation [2, 32]. It could by either anti- or pro-inflammatory by modulating the release of pro-/anti-inflammatory cytokines [4]. In our studies, melatonin seemed to be anti-inflammatory by decreasing the band neutrophils numbers in the blood of immunized mice.

In the current paper, melatonin either restored meiotic maturation and survival of the oocytes (when xenogenic anti-ovarian antibodies were passively transferred to mice) or did not affect these parameters (when immune response was stimulated in mice by injecting the allogenic ovarian extracts). Favoring follicular cell survival may be a mechanism serving to recover the meiotic maturation of the oocytes. We suggest that in addition to the known anti-oxidant [19, 23, 24, 27] and immunomodulating effects [3, 4, 28, 31, 34], a positive melatonin influence on the ovary may be caused by its anti-apoptotic effect. The presented results indicate that melatonin may exert beneficial action on immune-mediated ovarian pathology, although the understanding of the detailed mechanism requires further examination.

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