## EXPERIMENTAL RESEARCHES

# THE EFFECT OF MELATONIN ON THE BCL-2 AND BAD PROTEINS EXPRESSION IN OVARIAN CORPUS LUTEUM CELLS AFTER EXPOSURE TO EXPERIMENTAL HYPERTHERMIA

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## ABSTRACT

***Background.*** *There is growing interest in determining the role of melatonin in the regulation of proliferation and apoptosis of ovarian cells at various diseases and destabilizing influences. It is believed that the choice between the implementation of a cell death or survival program determines the ratio of anti-apoptotic and pro-apoptotic proteins.*
***The aim.*** *To identify the effect of melatonin on the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bad and the Bcl-2/Bad ratio in the ovarian luteocytes of Wistar rats in the acute (day 3) and recovery (days 7 and 14) periods after a single exposure to experimental hyperthermia.*
***Materials and methods.*** *Warming up took no more than 17 minutes. Melatonin was injected subcutaneously (0.1 mg in 0.2 ml of physiological solution) for 3 days after experimental hyperthermia. Comparison groups included rats with physiological solution injection (control) and animals after experimental hyperthermia + physiological solution injection. The Bad and Bcl-2 expression was determined immunohistochemically on days 3, 7 and 14 after experimental hyperthermia + physiological solution or melatonin injection.*
***Results.*** *On the day 3 after experimental hyperthermia, the effect of the hormone was not detected. A week after experimental hyperthermia + melatonin injection, the Bad expression area decreased more significantly than in rats after experimental hyperthermia + physiological solution injection, which led to an increase in Bcl-2/Bad ratio. This indicated an increase in anti-apoptotic protection, blocking the development of the internal apoptosis pathway at this time. 2 weeks after experimental hyperthermia + physiological solution injection, the Bcl-2 area decreased more significantly than the Bad area. As a result, the Bcl-2/ Bad ratio decreased almost 2-fold compared to the control group. This indicated the activation of the "mitochondrial branch" of luteocyte apoptosis. Two weeks after experimental hyperthermia + melatonin injection, the Bad and Bcl-2 areas decreased synchronously, which restored Bcl-2/Bad to control values.*
***Conclusion.*** *The melatonin injection after experimental hyperthermia shifts the ratio of Bcl-2/Bad expression areas towards an increase in anti-apoptotic Bcl-2 already a week after the recovery period and promotes earlier normalization of Bcl-2/Bad to physiological levels (as early as 2 weeks after experimental hyperthermia + melatonin injection).*

***Key words:*** *melatonin, apoptosis, experimental hyperthermia, rat ovaries, corpus luteum, luteocytes, Bad, Bcl-2, Bcl-2/Bad*

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# ВЛИЯНИЕ МЕЛАТОНИНА НА ЭКСПРЕССИЮ БЕЛКОВ BCL-2 И BAD В КЛЕТКАХ ЖЁЛТЫХ ТЕЛ ЯИЧНИКОВ ПОСЛЕ ВОЗДЕЙСТВИЯ ЭКСПЕРИМЕНТАЛЬНОЙ ГИПЕРТЕРМИИ

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## РЕЗЮМЕ

*Растёт интерес к выяснению роли мелатонина (МТ) в регуляции пролиферации и апоптоза клеток яичников при различных заболеваниях и дестабилизирующих воздействиях. Считается, что выбор между реализацией программы гибели или выживания клетки определяет соотношение антиапоптотических и проапоптотических белков.*

***Цель.*** *Выявить влияние мелатонина на экспрессию антиапоптотического Bcl-2 и проапоптотического Bad и соотношение Bcl-2/Bad в лютеоцитах яичников крыс Вистар в острый (3-и сутки) и восстановительный (7-е и 14-е сутки) периоды после однократного воздействия экспериментальной гипертермии (ЭГ).*

***Методы.*** *Разогревание составляло не более 17 минут. МТ вводили подкожно (0,1 мг в 0,2 мл физиологического раствора (ФР)) в течение 3 суток после ЭГ. Группы сравнения – крысы с введением ФР (контроль) и животные после ЭГ и ФР. Экспрессию Bad и Bcl-2 определяли иммуногистохимически на 3-и, 7-е и 14-е сутки после ЭГ и введения МТ/ФР.*

***Результаты.*** *На 3-и сутки после ЭГ эффект гормона не выявлялся. Через неделю после ЭГ + МТ площадь экспрессии Bad уменьшалась значительнее, чем у крыс после ЭГ + ФР, что приводило к росту Bcl-2/Bad. Это свидетельствовало об увеличении антиапоптотической защиты, блокирующей развитие внутреннего пути апоптоза на данном сроке. Через 2 недели после ЭГ + ФР площадь Bcl-2 уменьшалась значительнее, чем площадь Bad. В результате Bcl-2/Bad практически в 2 раза снижался по сравнению с контролем. Это свидетельствовало об активации «митохондриальной ветви» апоптоза лютеоцитов. Через 2 недели после ЭГ + МТ площади Bad и Bcl-2 уменьшались синхронно, что восстанавливало Bcl-2/Bad до контроля.*

***Заключение.*** *Введение МТ после ЭГ сдвигает соотношение площадей экспрессии Bcl-2/Bad в сторону увеличения антиапоптотического Bcl-2 уже через неделю восстановительного периода и способствует более ранней нормализации Bcl-2/Bad до физиологического уровня (уже через 2 недели после ЭГ + МТ).*

***Ключевые слова:*** *мелатонин, апоптоз, экспериментальная гипертермия, яичники крыс, жёлтые тела, лютеоциты, Bad, Bcl-2, Bcl-2/Bad*

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## INTRODUCTION

In modern conditions, the impact of high temperature to the human body is increasing as a result of global climate change, the development of regions with hot climate, the development of tourism and migration, as well as work in a number of industries (metallurgical, coal, mining, etc.). Meanwhile, to date, extensive scientific material has been accumulated about the use of hyperthermia for the therapy of oncological, infectious, parasitic diseases, drug-dependent conditions, AIDS. General hyperthermia in the temperature range from 42.5° to 44.0 °C is an extreme factor of the environment, to the action of which the body responds with a combination of complex changes that lead to profound disorders of cellular and extracellular relationships in biological structures and significant disorders of blood circulation and lymph flow, the development of hypoxia and stimulation of apoptosis. The reproductive organs, in particular the ovaries, which are the centre of the female reproductive system, are under considerable strain in these conditions [1].

Crucially, stress-induced regulated cell death also represents a strategy for preserving biological equilibrium, resembling an adaptive response to stress.

Two main pathways of apoptosis signal transduction are distinguished: receptor-dependent (external) signalling pathway involving cell death receptors expressed on the cell membrane surface, and mitochondrial (internal or intrinsic) pathway. The intrinsic apoptosis pathway is a form of regulated cell death triggered by various changes in the microenvironment, including DNA damage, endoplasmic reticulum stress, excess reactive oxygen species, etc. The mitochondrial signalling pathway of apoptosis is activated as a result of increased permeability of the mitochondrial outer membrane, release of apoptogenic proteins from the mitochondrial intermembrane space into the cell cytoplasm and subsequent triggering of a whole cascade of reactions leading to the development of programmed cell death. An important role in the mechanisms of regulation of programmed cell death is assigned to the inhibitor of apoptosis – Bcl-2 protein, which prevents translocation of Bcl-2-associated cell death promoters Bax and Bad by oligomerisation with these proteins and thus blocks the release of proapoptotic small molecules from mitochondria [2]. The ratio of active forms of apoptosis inhibitors and inducers determines the choice between the implementation of the cell death programme and cell survival and is informative in determining the degree of apoptosis inhibition [3]. The ability to influence the interaction between anti-apoptotic and pro-apoptotic members of the BCL-2 family through either pharmacological or genetic interventions is of great importance in medicine for the treatment of various (cancer, autoimmune, neurodegenerative, etc.) diseases [3].

Any stresses and extreme exposures, including hyperthermia, lead to disturbances in the body's detoxification and adaptation systems. The epiphysis hormone melatonin (MT) is involved in restoring disturbed homeostasis and optimising the functions of various organs and systems. This hormone, which has a wide range of properties, is the main synchronizer of the body's endogenous rhythms, as well as a powerful antioxidant. Moreover, MT is an antioxidant that is found in the mitochondria of cells, and at higher concentrations than in other organelles or subcellular sites. It is believed that MT can even be synthesized in mitochondria [4]. The ability to regulate cell proliferation and apoptosis is one of the most significant physiological properties of epiphyseal MT [5, 6].

Epiphyseal MT is involved in the regulation of sex hormone secretion and puberty processes, thus ensuring the full functioning of the reproductive system. MT deficiency in mice leads to follicle atresia and accelerates age-related fertility decline [7]. This hormone is a key regulator of human reproductive functions [8].

The corpus luteum serves as a temporarily functioning organ and defines a crucial role in the regulation of the estrous cycle and maintenance of pregnancy. The luteal function is largely performed by progesterone, the main steroid hormone synthesised by this gland. MT has been found to play a key role in reproductive physiology by regulating the production of prolactin, follicle-stimulating and luteinising hormones. MT synthesis in the ovaries and testes reflects the auto- and paracrine regulation of reproductive physiology, ensuring high quality ova and sperm. The hormone is produced in the cells of the epithelium, stroma, and myometrium and is involved in maintaining the homeostasis of the organ by regulating multiple pathways associated with the processes of decidualisation and implantation [9]. MT functions as an important regulator in the ovary, as indicated by the expression of melatonin receptors MT1 and MT2 in different compartments of the ovary [10]. The presence of MT1 and MT2 in luteocytes and the regulatory role of MT in the endocrine function of the ovarian luteal bodies of horses [11], pigs, and mice [10, 12] have been confirmed. It has been revealed that this hormone is able to increase progesterone release by corpus luteum in gestating sows [10], and to induce progesterone production by granulosa and luteal cells in humans [13]. MT activates a set of genes expressed in the sows' and mice corpus luteum associated with progesterone synthesis, including cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*), aldo-keto reductase family 1, member C18 (*Akr1c18*), isopentenyl diphosphate delta isomerase 1 (*Idi1*) and luteinising hormone/choriogonadotropin receptor (LHCGR), and consequently increases progesterone production in sows [14].

There is now increased interest in elucidating the role of MT in the regulation of cell proliferation and apoptosis in a number of different ovarian cells. The aspects concerning the role of MT and its effect on the «mitochondrial branch» of apoptosis in luteocytes of ovarian luteal bodies in experimental models of overheating are, however, insufficiently covered in scientific publications. This determined our interest in studying the effect of MT

on programmed cell death processes in ovarian luteal cells when exposed to high temperature.

## THE AIM OF THE STUDY

To analyse the effect of melatonin after a single exposure to experimental hyperthermia (EH) against the expression of apoptosis inhibitor Bcl-2 and inducer of programmed cell death protein Bad and against the Bcl-2/Bad ratio in luteocytes of Wistar rats in acute (3rd day) and recovery (7th and 14th day) periods.

## MATERIALS AND METHODS

The study was conducted on 3-month-old female Wistar rats with body weight 180-200 g. The animals were kept in a certified vivarium of the Central Research Laboratory of the Novosibirsk State Medical University of the Ministry of Health of Russia at an air temperature of 20–22 °C on a standard dietary intake and with free access to water. Experiments were performed in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union governing the protection of animals used for scientific purposes and the rules of good laboratory practice. The study was approved by the Ethical Committee of the Research Institute of Clinical and Experimental Lymphology – branch of the Federal Research Centre of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Minutes No. 128 dated March 15, 2017). Sexual cycle phases were determined using the vaginal swab method [15]. Rats in the diestrus phase of the sexual cycle were exposed once to EH in accordance with the «Method of experimental modelling of general hyperthermia in small laboratory animals» [16]. According to the method of modelling general hyperthermia, animals were warmed up in the tank of a standard TB-110 thermobath when immersed in hot water up to the neck level. The thermobath design provides for automatic maintenance of water heating temperature and uniform mixing of its layers, that allows to consider the temperature of the coolant as a constant value in the experiment. The advantage of modelling general hyperthermia in an aqueous environment over an air environment is that uniform, deep and rapid heating of the animal body is achieved. The heating temperature regime of hot water – warm carrier was selected experimentally and equalled 45 °C. The time of warming up of each individual to the level of rectal temperature of 43.5 °C (heat shock stage) was no more than 17 minutes and was individual. No deaths of rats from hyperthermia have been reported.

Three groups were formed: 1st (control) – rats without EH exposure, which were subcutaneously injected with 0.2 ml of physiological solution (PS); 2nd (EH + PS) – animals exposed to EH and received 0.2 ml of PS; 3rd (EH + MT) – animals exposed to EH and received MT. MT (ICN Biomedicals Inc., USA) was administered subcutaneously at a dose of 0.1 mg in 0.2 ml of PS. The first MT injection was administered on the day of EH, then – in the following 2 days (in the evening, after sunset, once a day). Animals were removed from the experiment under ether anaesthesia 3 (acute period), 7 and 14 days (recovery period) after exposure to EH and MT, that corresponded to the ideas of phasicity in the course of the posthyperthermic period. There were 5 individuals from each group for each point of withdrawal from the experiment.

Ovaries were fixed in 10 % neutral formalin solution, then dehydrated in a series of alcohols of increasing concentration and encapsulated in paraffin. Immunohistochemical study of Bcl-2 and Bad protein expression was performed on 3 μm thick paraffin sections of ovaries by indirect two-step streptavidin-biotin method using Novostain 500 kit (NCL-RTU-D, Novocastra, UK), mouse monoclonal antibodies to anti-apoptotic protein Bcl-2 (IgG, No. 610538; BD Biosciences, USA) and to the proapoptotic protein Bad (IgG, No. 610392; BD Biosciences, USA). In the last step, immunohistochemical staining was performed in a chromogenic substrate containing diaminobenzidine. Sections were examined and microphotographs were obtained using an AxioImager M2 motorised microscope (Carl Zeiss, Germany) with an AxioCam HRc camera (Carl Zeiss, Germany) at a final magnification of 630x. Quantitative assessment over the relative areas of stained sections was performed using the computer programme Axio Vision 4.7.1 (Carl Zeiss, Germany) and an automatic measurement unit (NEXIV AutoMeasure; Nikon, Japan). Bcl-2/Bad area ratios were calculated.

Statistical processing of the obtained data was performed in Statistica 6.1 program (StatSoft Inc.; serial number AXXR101E832903FA). As a baseline for each marker, the differences in mean visual field within a group were compared. Each sample included up to 50 fields of view per group for each marker. The samples corresponded to a normal distribution. The values of arithmetic mean and standard error of the mean were calculated. Statistical significance of the differences between the compared values was determined using the parametric Student's criterion. The differences were considered statistically significant at $p < 0.05$. Median, first and third quartile values were determined for the Bcl-2/Bad ratio. Statistical significance of the differences between the compared values was evaluated using the nonparametric Mann – Whitney U-criterion. Differences were considered statistically significant at $p < 0,05$.

## RESULTS

In corpus luteum cells on the 3rd day of the experiment, when both placebo and MT were administered after EH, the expression areas of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bad increased synchronously (Fig. 2). Along with this, there was an increase in the intensity of luteocyte staining for both proteins (Fig. 1). Consequently, in animals of both groups, the Bcl-2/Bad ratio remained at the control level (Fig. 3). This is an evidence

that the intensity of apoptosis in ovarian luteal cells during the acute period after EH remains within the physiological values, and at this stage MT has no statistically significant effect on the development of cell death.

One week after EH and PS administration, the areas of Bcl-2 and Bad expression in luteocytes and the Bcl-2/Bad ratio were persisted at 3 days (Fig. 2, 3). MT administration after hyperthermia contributed to a decrease in staining intensity (Fig. 1, 2) and decreased values for both proteins compared to all groups (Fig. 2). The expression area of the pro-apoptotic protein Bad, however, decreased more significantly than that of the anti-apoptotic protein Bcl-2 (Fig. 2). In consequence, the Bcl-2/Bad ratio was statistically significantly increased at this recovery period compared to all groups (Fig. 3). This is an evidence of earlier establishment of anti-apoptotic defence blocking the development of the intrinsic pathway of apoptosis of ovarian luteal cells already on the 7th day after hyperthermia and MT administration compared to animals without MT treatment.

On the 14th day of the experiment in rats receiving PS after EH, the area of Bcl-2 expression in ovarian luteal cells decreased, more significantly than for Bad (Fig. 2).

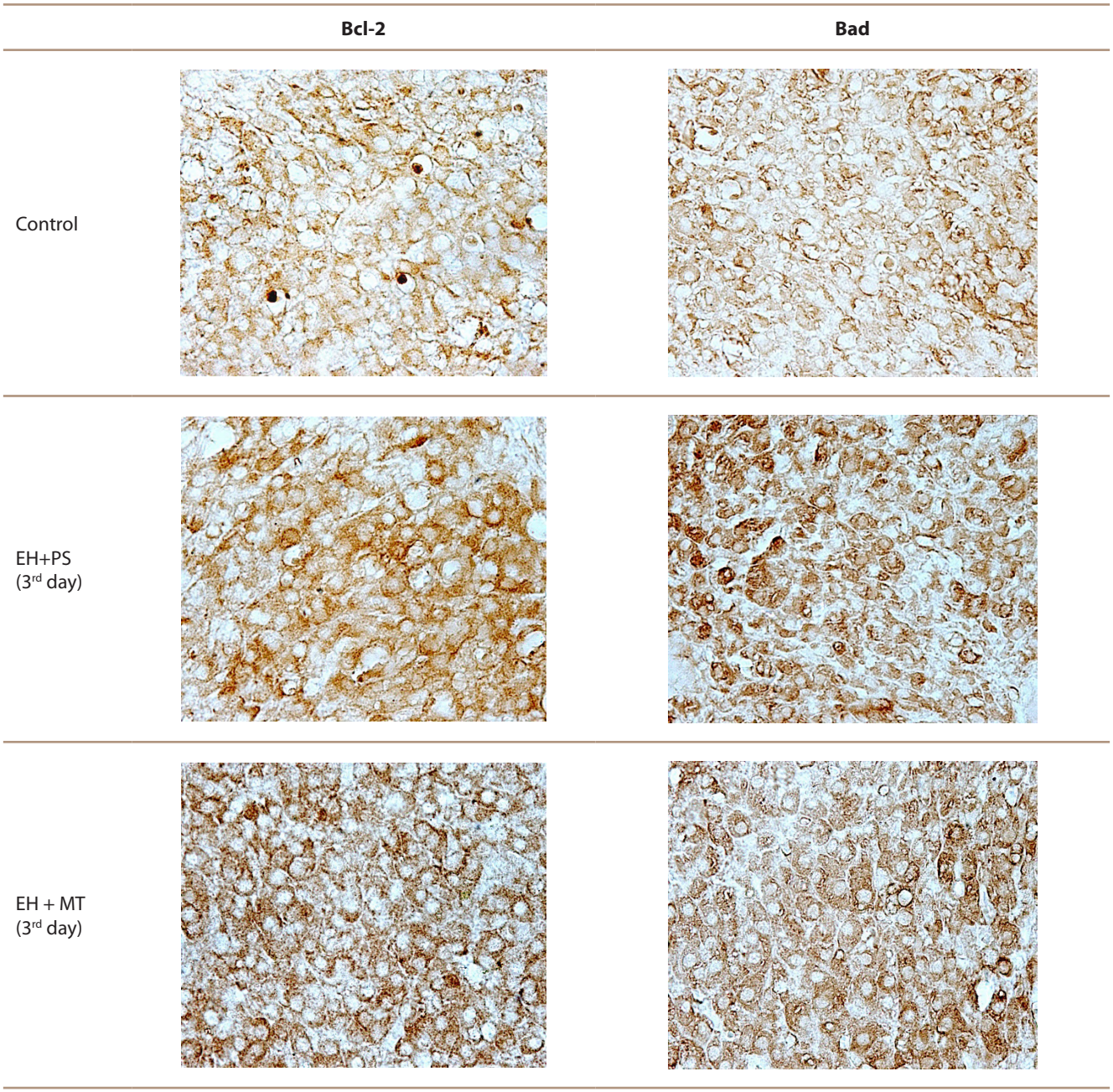
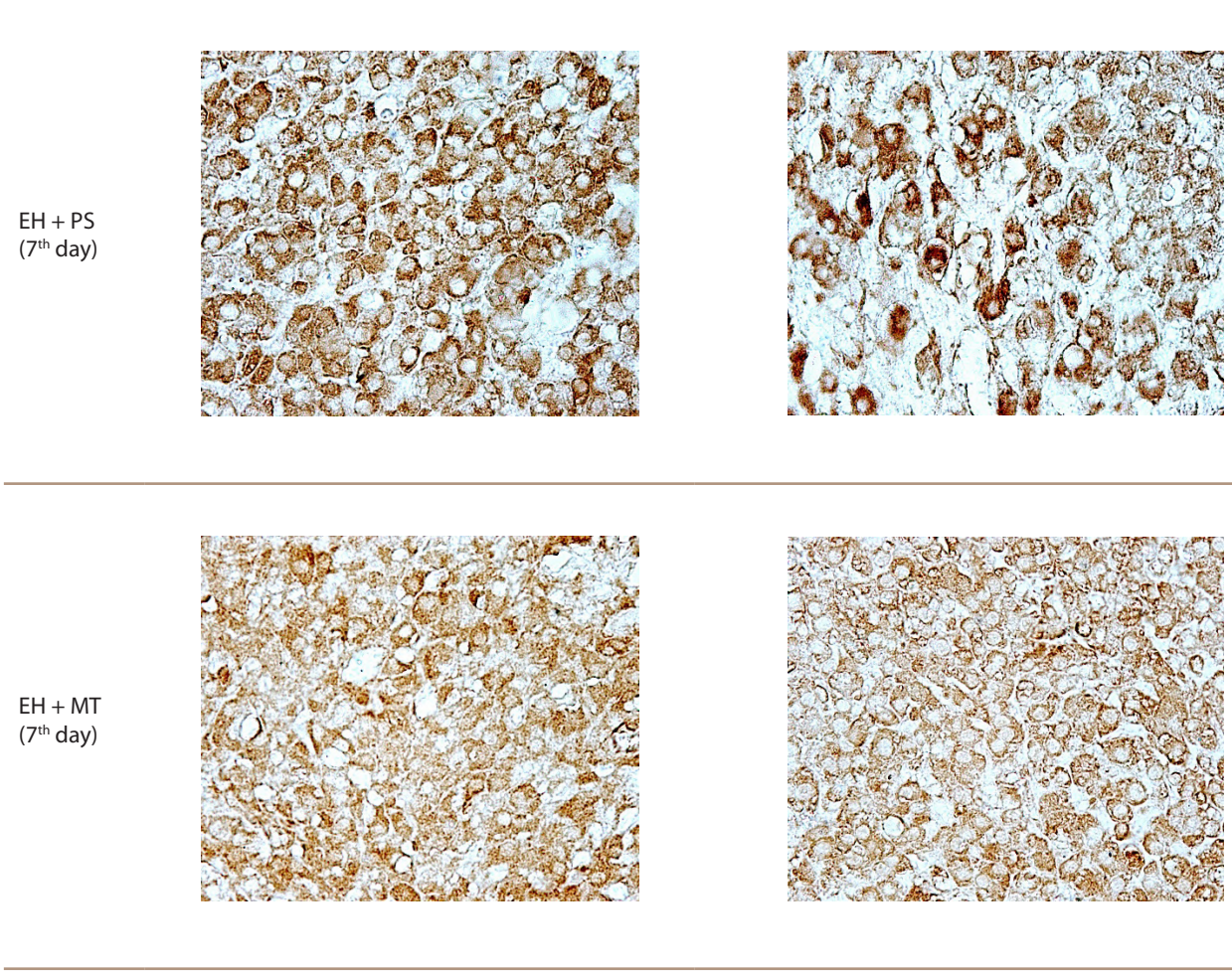
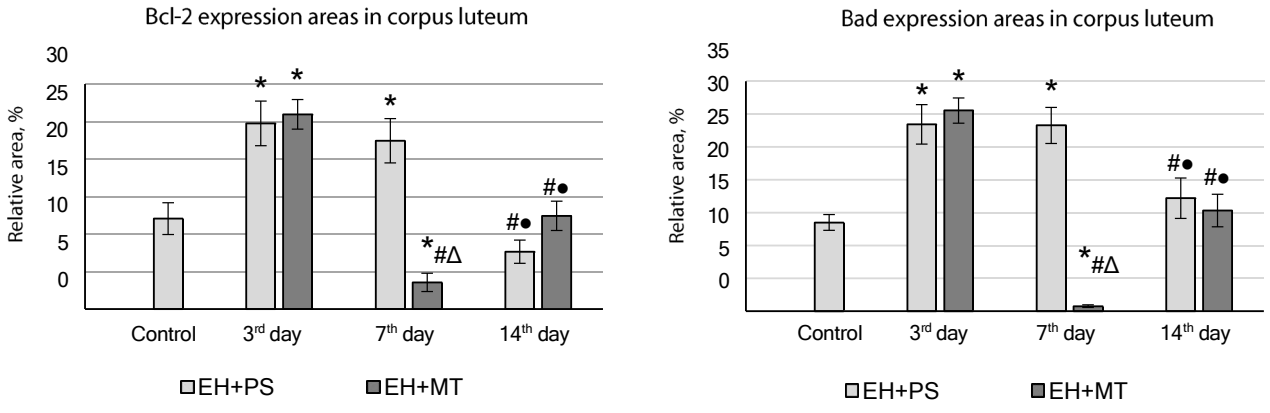



***FIG. 1.***
*Microphotographs of rat ovarian corpora lutea on the 3rd day after exposure to experimental hyperthermia (EH) and administration of physiological saline (PS) or melatonin (MT). Immunohistochemical staining by indirect streptavidin-biotin method for anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bad in corpus luteum cells; magnification × 400*




*a*




*b*

***FIG. 2.***
*a – microphotographs of rat ovarian corpora lutea on the 7th day after exposure to EH+PS and EH+MT; immunohistochemical staining by indirect streptavidin-biotin method for Bcl-2 and Bad in the cells of corpora lutea; magnification × 400. б – graphs of the expression areas of the studied proteins in different terms of the experiment ($p < 0.05$): * – compared to control; # – compared to 3rd day; ● – compared to 7th day; Δ – intergroup comparison of the same term*

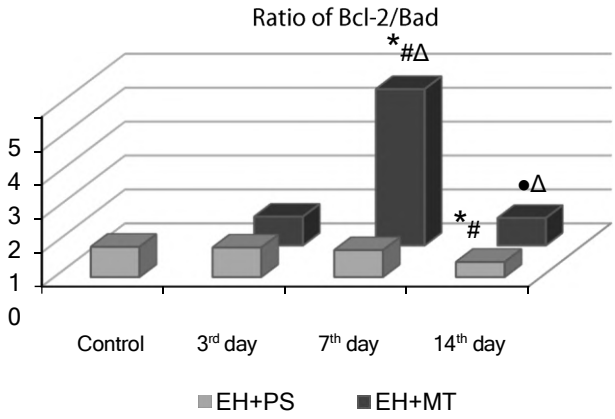



***FIG. 3.***
*Ratio of Bcl-2/Bad expression areas at different experimental time intervals ($p < 0.05$): * – compared to control; # – compared to 3rd day; ● – compared to 7th day; Δ – intergroup comparison of the same time period*

As a result, the Bcl-2/Bad area ratio decreased almost 2-fold compared to the control (Fig. 3). It evidences the activation of the «mitochondrial branch» of programmed cell death of luteocytes at this stage of the recovery period. MT administration to hyperthermia-treated animals promoted a decrease in staining intensity (Fig. 4) and a more pronounced decrease in Bad compared to the decrease in Bcl-2, leading to the restoration of the Bcl-2/Bad index to control levels (Fig. 2, 3). The obtained results indicate the cytoprotective effect of MT administration to animals after hyperthermia, which as early as on the 14th day of the recovery period ensures the development of mitochondrial pathway of luteocyte apoptosis within the physiological norm.

## DISCUSSION

Heat stress is a known promoter of the reactive oxygen species (ROS) formation that can jeopardise pregnancy and fetal development. Overheating / thermal stress has deleterious effects against oocyte development potential in pigs, mice, and cattle, including adverse effects

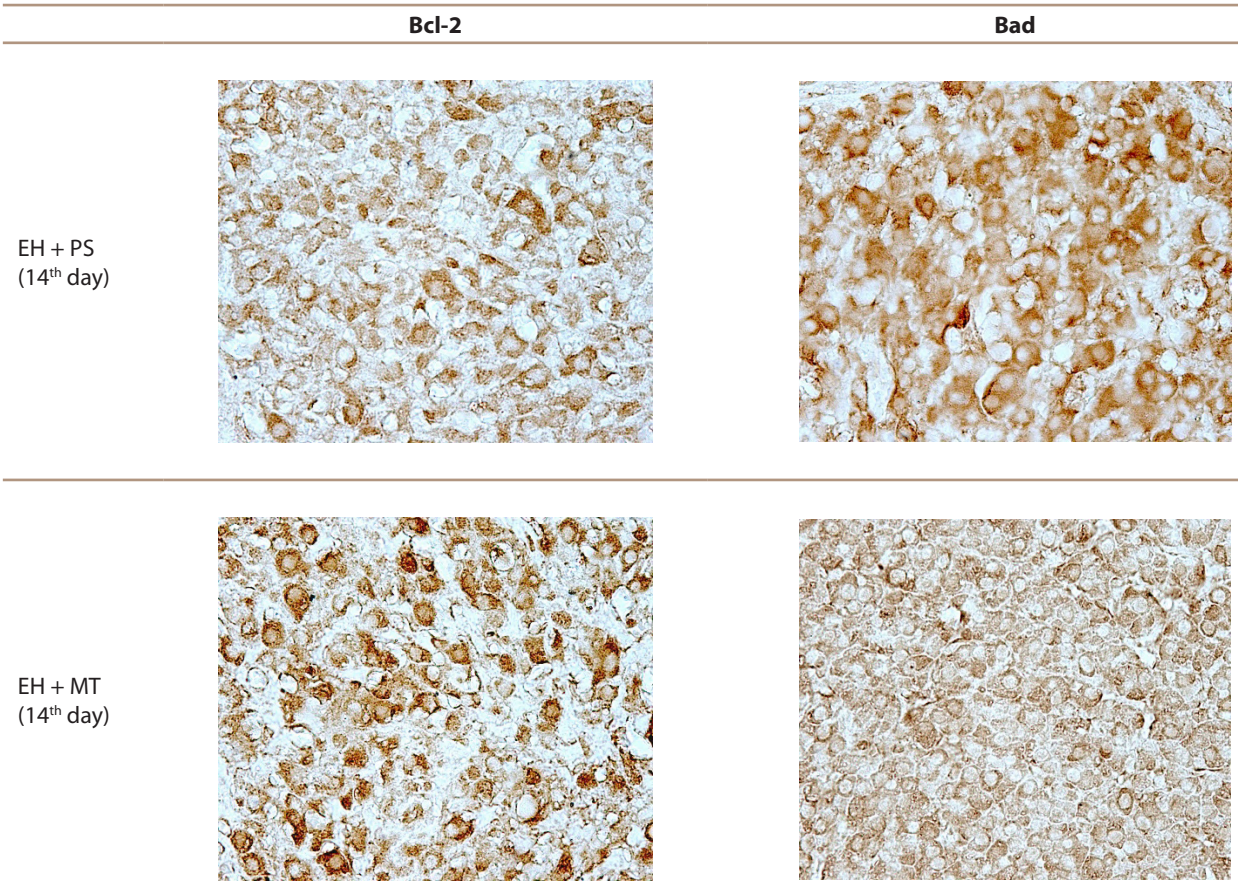



***FIG. 4.***
*Microphotographs of rat ovarian corpora lutea on the 14th day after exposure to EH+PS and EH+MT. Immunohistochemical staining by indirect streptavidin-biotin method for Bcl-2 and Bad in ovarian luteocytes; magnification × 400*

against survivability, maturation rate, and meiotic competence of oocytes, the formation of F-actin and α-tubulin in them, and oocyte expression of the NRF2, CDK1, and GDF9 genes [17].

MT is a pleiotropic molecule that regulates various processes including pregnancy. MT usage under heat stress in pigs improves the quality and rate of oocyte maturation (partly by restoring the distribution of F-actin) [17], and has a positive effect on the duration of the estrous period and embryo survival [18]. The use of MT reduces ROS production in maturing cattle oocytes and increases the ability to develop embryos from heat shocked oocytes [17]. MT administration throughout pregnancy in heat stressed sheep improves their redox status and leads to an increase in the average number of offspring, lamb weight, and milk production [19]. Under heat stress, this hormone was observed to suppress p53 expression and increase luteinising hormone level and *Bcl-2* gene expression in sheep granulosa cells [20].

Our studies revealed that the effect of high external temperature led to a change in the ratio of apoptosis regulator proteins (predominance of pro-apoptotic protein Bad over anti-apoptotic protein Bcl-2) in ovarian luteal 2 weeks after EH. Of significance, the expression area of the anti-apoptotic protein Bcl-2 decreased significantly more than the expression area of the pro-apoptotic protein Bad, resulting in a 2-fold decrease in the Bcl-2/Bad ratio index compared to control. It evidenced the insufficiency of anti-apoptotic protection and activation of the «mitochondrial branch» of luteocyte apoptosis. Exposure to hyperthermia promotes to the occurrence of state of oxidative stress. The damaging effects of such exposures lead to mitochondrial dysfunction and excessive accumulation of ROS in them, that promotes cell apoptosis in various ovary compartments. In consequence, due to its antioxidant and anti-apoptotic effects, MT can significantly reduce oxidative stress and restrain apoptosis [20-22]. At the same time, the ratio of active forms of pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-2, Bcl-xL) proteins will determine the choice between the implementation of the cell survival or cell death programme [3]. Specifically, Bcl-2 can prevent translocation of apoptosis inducers Bax and Bad by oligomerising with these proteins and thereby block the release of these molecules from mitochondria, that in turn will restrain apoptosis. Bcl-2 has been found to bind more strongly to Bad than to Bax [2].

MT has been demonstrated to significantly inhibit apoptosis of thecal cells in sheep reducing the expression of the pro-apoptotic protein Bax and increasing the expression of the anti-apoptotic protein Bcl-2, that has implications for delaying ovarian atresia and aging [23]. MT has been found to inhibit the mitochondrial apoptosis pathway of granulosa cells in ovaries of cyclophosphamide chemotherapy-treated mice – reducing the increased expression levels of cleaved caspase 3, Bax, cytoplasmic Cyt-c and increasing the decreased Bcl-2 expression in ovaries [24]. MT can protect mouse ovaries from premature deficiency caused by trypterygium glycosides by reducing apoptotic damage – the hormone reduces caspase 3, Bax expression and increases Bcl-2 expression [25].

As a consequence of the present study, we found that MT administration to rats after hyperthermia shifts the ratio of Bcl-2/Bad expression areas in ovarian corpora lutea towards an increase in anti-apoptotic Bcl-2 as early as on the 7$^{th}$ day of the recovery period, in contrast to animals not treated with this hormone, which contributes to the normalisation of Bcl-2/Bad to physiological levels (as early as on the 14th day). In an earlier study, we also revealed that MT could inhibit the mitochondrial apoptosis pathway in rat ovarian follicles as early as day 7 post-hyperthermia [5].

MT, by reducing oxidative stress and apoptotic damage, protects corpus luteum cells in the ovaries. The hormone restores the number of corpora lutea and follicle density in mice and rats reduced by toxic effects [25, 26], and improves luteinising hormone levels, corpus luteum function and survival of sheep and goat embryos [19]. MT protects the corpus luteum from ROS and plays a key role in sustaining its function in women [19]. Treatment with this hormone of infertile women with luteal phase defect increases intrafollicular MT concentration, reduces intrafollicular oxidative damage, improves progesterone production by the corpus luteum and increases fertilisation and pregnancy levels [27].

MT and its MT1 receptor play an important role in luteinization. Melatonin/MT1 signalling was revealed to markedly improve the expression of corpus luteum marker genes. High-throughput sequencing results revealed that interaction with the extracellular matrix receptor, focal adhesion and activation of the PI3K/Akt pathway, which are involved in luteinisation of granulosa cells, can mediate the effects of melatonin/MT1 signalling [28]. MT increases progesterone production in the corpus luteum of gestating sows and increases the expression of both P450scc and StAR, resulting in increased luteal cell viability. Epiphyseal hormone exerts its regulatory role in luteocyte function through signalling pathways mediated by the MT1 and MT2 melatonin receptors, the presence of which has been confirmed in corpus luteum cells [10, 12].

MT-mediated signalling mechanisms through receptors for this hormone are very complex and vary depending on the type and kind of ovary cells. They mainly include the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway, the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, the phosphatidylinositol-3-kinase/apoptosis signal-regulated kinase (PI3K/AKT) pathway, and the calcium-regulated pathway [23, 29]. More specifically, MT activates the PI3K/Akt pathway, mediating progesterone synthesis and secretion by thecal cells. In addition to its antioxidant properties, MT can activate the SIRT1/PGC-1α pathway, which promotes mitochondrial biogenesis disrupted by environmental toxins [20].

Counteraction to oxidative stress and reprogramming of impaired metabolism in cells is provided by MT synthesised in mitochondria, where its distribution is much

higher than in other subcellular organelles and has no circadian rhythm. MT has been confirmed to be released from mitochondria and then controls cytochrome *c* release via the MT1 receptor on the membrane, i.e. provides automitocrine regulation. MT is present in mitochondrial membranes and is transported into mitochondria by the membrane-bound oligopeptide transporters PEPT1 and PEPT2. The high concentrations of MT and its multiple antioxidant actions provide a powerful defence for these organelles exposed to free radicals. Within mitochondria, MT acts as a direct scavenger of free radicals and associated non-radical products and stimulates antioxidant enzymes including superoxide dismutase 2, catalase and glutathione reductase, while inhibiting pro-oxidant enzymes. MT, by regulating lipoxygenase activity, protects cells from hydroperoxidation of polyunsaturated fatty acids. It modulates endoplasmic reticulum responses to stress, sirtuin activity, mitophagy and autophagy processes. MT by direct capture of ROS in mitochondria, activation of antioxidant defence and preservation of membrane integrity plays a crucial role in maintaining normal mitochondrial functions and energy metabolism in cells [9]. In summary, melatonin exposure under conditions of hyperthermia leads to a decrease in apoptotic death of corpus luteum cells and, as a consequence, to a reduction in the damaging effect of overheating on the morphological organisation of the organ.

## CONCLUSION

MT administration after EH shifts the Bcl-2/Bad expression area ratio towards an increase in the anti-apoptotic Bcl-2 protein as the 7$^{th}$ day of the recovery period. Administration of this hormone after hyperthermia promotes earlier normalisation of Bcl-2/Bad to physiological level – already on the 14$^{th}$ day after EH and MT exposure.

**Conflict of interest**

The authors declare no conflict of interest.

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