Influence of melatonin on plasma glucose-regulated protein 78 levels in ovariectomized female rats

Abstract

In rats with surgically induced primary ovarian failure, melatonin may influence intra-retroperitoneal fat and a number of metabolic factors, which are risk factors for a number of disorders, including endometrial cancer (EC). The aim of this study is to investigate the influence of melatonin treatment in female rats with surgically induced menopause on the plasma glucose-regulated protein 78 (GRP78) levels as a risk factor of EC. In the current study 40 mice were included. At 14 days post-ovariectomy the animals were exposed to estrogen replacement treatment and combined treatment of estrogen and melatonin. The treatment duration, with products and doses recommended for veterinary use, was 12 consecutive weeks. The plasma estrogen and GRP78 level was measured. Groups which received estrogen associated with melatonin had a lower level of GRP78 compared to the control group. This study supports the idea that melatonin influences plasma estrogen level, which is directly, correlated with plasma GRP78 level, suggesting the implication of GRP78 in the pathogenesis of EC.
Keywords: *melatonin, endometrial cancer, GRP78*

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Introduction

Estrogen is the main factor in the development and progression of endometrial cancer (EC). Nocturnal melatonin release normally suppresses ovarian estrogen production. In persons who work on night shifts, melatonin suppression following nocturnal exposure to artificial light determines the loss of inhibition of ovarian estrogen secretion, with the appearance of high estrogen levels that are directly correlated with the incidence of breast and endometrial cancer[1].

The hypothesis of an alteration of the estrogen-progesterone balance is used to support the relationship between obesity, endogenous steroid hormones and the risk of EC, but this cannot explain the effects on the entire population, given that not all obese women develop endometrial abnormalities.

The identification of biological markers in obese women at high risk for the development or recurrence of EC might be useful for decreasing the mortality and morbidity of EC.

In rats with surgically induced primary ovarian failure, melatonin may influence intra-retroperitoneal fat and a number of metabolic factors, which are risk factors for a number of disorders, including EC[2]. Blood melatonin levels are indirectly correlated with the tumor proliferation index in patients with EC[3].

The strong association between obesity and EC might be due to the development of endoplasmic reticulum stress in adipocytes. Endoplasmic reticulum stress is an adaptive reaction, which normally occurs in any cell that is required to process more molecules than usual per time unit[4].

Glucose-regulated protein 78 (GRP78) is a protein that is ubiquitously expressed in normal cells and a marker for endoplasmic reticulum stress. The expressed GRP78 amount is directly correlated with tumor invasiveness and can be used as a biomarker for cancer[5]. GRP78 levels in visceral adipocytes are correlated with the stage of EC as well as with the survival of these patients, and might be clinically useful as a predictor of EC[6].

The aim of this study is to investigate the influence of melatonin treatment in female rats with surgically induced menopause on the plasma GRP78 levels as a risk factor of EC.

Methods

The research was performed in white female Wistar rats, with a weight of 160-200 g, from the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca. The animals were kept under adequate standard zoo-hygienic vivarium conditions: at an environmental temperature of 22±1°C, with standard food (Forti-Diet Mouse and Rat Food mixed grains) and water *ad libitum*, with a 12 hour light-dark cycle. The Ethics Commission of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca approved the experiment.

The animals underwent bilateral adnexectomy by applying the adnexectomy technique used in humans. At 14 days post-ovariectomy, a time period required for the postoperative validation of ovarian failure, with the experimental induction of artificial primary ovarian failure in the studied animals, estrogen replacement treatment (estrogen monotherapy) and combi-

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ned estrogen and melatonin treatment were initiated. The duration of the administered treatment, with the products and the doses recommended for veterinary use, was 12 consecutive weeks.

The ovariectomized animals were assigned to 4 groups of 10 animals each:

Group I: control group - healthy ovariectomized animals (surgically induced primary ovarian failure), without estrogen or melatonin administration

Group II: healthy ovariectomized animals, receiving estrogen replacement monotherapy (estradiol benzoate (E2b) 10 μg/day intramuscular injections for 12 weeks, 5 days/week)

Group III: healthy ovariectomized animals, receiving estrogen therapy (E2b - 10 μg/day intramuscular injections for 12 weeks, 5 days/week) supplemented with melatonin (dissolved in 100% ethanol, added to the drinking water at a concentration of 25 μg/mL ethanol concentration 0.01%). Melatonin was dissolved in ethanol in order to increase its solubility in water. The bottles with water were covered with aluminium foil in order to prevent photodegradation. The solution was prepared three times a week.

Group IV: healthy ovariectomized animals, receiving estrogen therapy (E2b 10 μg/day intramuscular injections for 12 weeks, 5 days/week) supplemented with melatonin (dissolved in 100% ethanol, added to the drinking water at a concentration of 50 μg/mL ethanol concentration 0.01%)

At the beginning of the study, as well as one day after the administration of the last drug dose, blood samples were collected from all animal groups by orbital sinus puncture, and plasma estrogen and GRP78 levels were determined. The serum obtained by centrifugation was divided and stored in 600 μl freezing tubes at a temperature of -60°C until the tests were performed, in order to avoid repeated freezing-thawing cycles. The serum GRP78 concentration was determined by the sandwich ELISA technique using the Rat GRP78 Immunoassay MBS031039 kit, R&D Systems USA. The sensitivity of the kit was 10 ug/ml and 50 ug/ml for the detection rate. No significant cross reactivity or interference between human GRP78 and analogues was found.

Statistical analysis in the case of the comparison of two means for independent samples used the Student t test or the Mann-Whitney test for rank comparison. The ANOVA test or the Kruskal-Wallis test was used for the comparison of the means for independent samples. For the comparison of the means for paired samples, the Student t test was used. Statistical calculations were performed using the applications SPSS 15.0.

Results

1. Plasma estradiol levels in the four studied groups

Before the ovarectomy there was no significant difference in plasma estrogen levels between the four groups.

After the intervention, group I showed lower estradiol levels than mice receiving estrogen replacement monotherapy (group II), (17.9 vs. 35.6, p <0.0001), than mice receiving estrogen therapy supplemented with melatonin (group III) (17.90 vs. 35.8, p<0.0001) and mice receiving estrogen therapy supplemented with melatonin in a double dose (group IV) (17.90 vs. 36, p<0.0001).

No difference was found between groups II, III and IV. Melatonin administration in groups III (35.8 pmol/L) and IV (36 pmol/L) induced slight changes compared to group II (35.6 pmol/L) (Figure 1).

2. Plasma GRP78 levels in the four studied groups

Group I showed higher plasma GRP78 levels (0.24 pg/ml) than group II [0.24 (0.22-0.27) vs. 0.16 (0.12-0.17), p <0.0001] , group III [0.24 (0.22-0.27) vs. 0.13(0.10-0.14), p <0.0001]or than group IV mice [0.24 (0.22-0.27) vs. 0.10 (0.09-0.11), p <0.0001].

Goup II mice showed statistically significant higher plasma GRP78 levels than group IV [0.16(0.12-0.17) vs. 0.10(0.09-0.11), p=0.02].

The mice from group IV which received a double dose of melatonin had a lower plasma GRP78 level compared to group III, but with no statistical difference between the two groups [0.10(0.09-0.11) vs 0.13(0.10-0.14), p = 0.06) (Figure 2).

3. Correlation between estrogen and GRP78 levels in plasma of the four studied groups

Our results showed that estrogens and GRP78 were correlated only in group III, while in the other groups the correlation between the two parameters was not significant (Table 1).

Discussion

Melatonin secretion decreases in postmenopausal women, who have a high incidence of EC. Diabetes, obesity and hypertension are recognized as the main risk factors for both EC and atherosclerosis. Atherosclerotic changes in the capillary network supplying the pineal gland reduce the production and release of melatonin, thus favoring the development of EC[7]. Clinical studies have reported that patients with EC have a much lower serum melatonin concentration compared to the healthy population[8].

Studies performed on cell cultures have showed that melatonin has a different anti-proliferative effect on the types of cancer cells, which differ between them by the status of estrogen receptors. Melatonin had no anti-proliferative action on SNG-II cell lines (without estrogen receptors), but had a significant anti-proliferative effect on Ishikawa cells (with estrogen receptors) at different cellular densities and different incubation times[9].

There are two possibilities by which estrogen blocks the antiproliferative effect of melatonin: estrogen may compete with melatonin for binding to a common receptor, or each ligand has its own receptor interacting with one another by different mechanisms than those

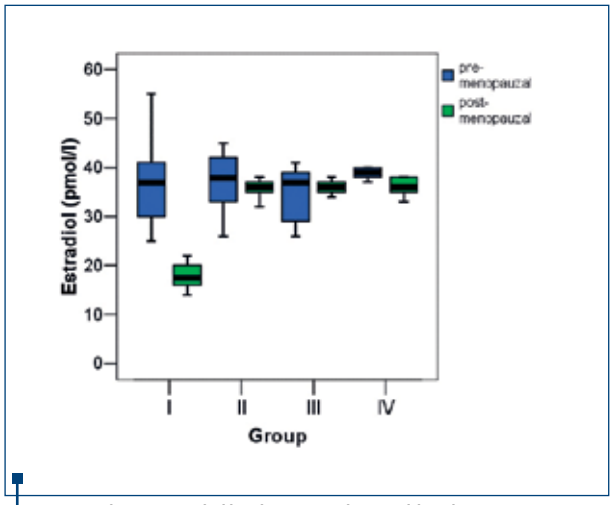

Figure 1. Plasma estradiol levels expressed in pmol/l (ordinate) were represented in the four studied groups (abscissa): I: pre and post-ovarectomised mice withoud treatment; II: pre and postovarectomised mice with estradiol replacement therapy; III: pre- and post-ovarectomised mice with estrogen therapy supplemented with melatonin; and IV: pre- and post-ovarectomised mice with estrogen therapy supplemented with melatonin in a double dose

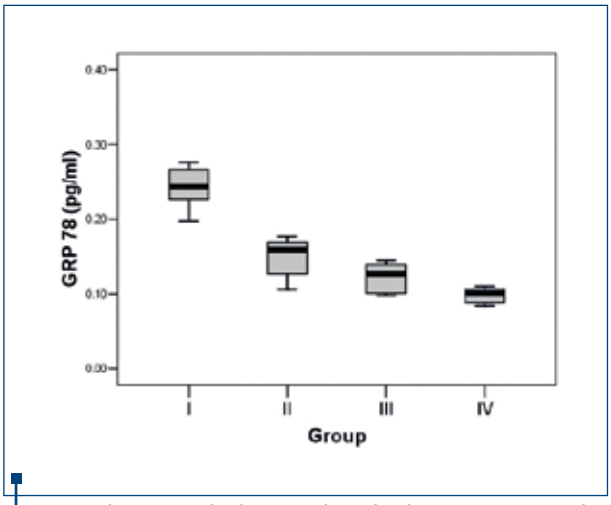

Figure 2. Plasma GRP78 levels expressed in pg/l (ordinate) were represented in the four studied groups (abscissa): I: pre- and post-ovarectomised mice withoud treatment; II: pre- and postovarectomised mice with estradiol replacement therapy; III: pre- and post-ovarectomised mice with estrogen therapy supplemented with melatonin; and IV: pre- and post-ovarectomised mice with estrogen therapy supplemented with melatonin in a double dose

Table 1 Spearmann correlation between estradiol and GRP78 levels in the 4 studied groups before and after ovarectomy

Estradiol vs. GRP78	Group 1	Group 2	Group 3	Group 4
Pre-menopausal				
r*	-.21	0.45	0.67	-0.15
p**	0.54	0.25	0.03	0.68
Post-menopausal				
r	0.23	-0.15	-0.33	-0.26
p	0.51	0.68	0.34	0.47

**r=Spearman correlation coefficient; **p=statistical significance*

related to the ligand's own receptor.There are also additional estrogen-independent mechanisms, which may involve melatonin receptors. Melatonin receptors have been identified in the membrane of estrogen-responsive tumor cell lines and it has been demonstrated that the antiproliferative effect of melatonin is mediated by these receptors[9].

In this study, the plasma estrogen level was influenced by the administration of melatonin, dependant of the dose, but in not a significant way.

The reduction of melatonin production, secondary to aging or to prolonged night shift work in artificially environments, induces insulin resistance, glucose intolerance and sleep disorders as well as circadian rhythm disturbance. All these changes cause metabolic dysfunction that induces obesity[10].

Melatonin is a key mediator molecule for the integration of physiological and behavioral processes in a cyclic environment and a circadian distribution pattern, as well as for the optimization of energy balance and the regulation of body weight, which are essential for healthy metabolism[11].

The disturbance of metabolism caused by the absence of melatonin in animals having undergone pinealectomy has been characterized as a diabetogenic syndrome that includes glucose intolerance and peripheral insulin resistance (in the liver, adipose tissue and skeletal muscles) and central insulin resistance.

These changes can be prevented by melatonin replacement therapy[12,13].

There are data supporting that melatonin plays a role in the biology of adipocyets, influencing energy metabolism, lipemia and body weight. Melatonin also plays a role in lipolysis and lipogenesis, adipocyte differentiation and fatty acid absorption[14,15].

GRP78 is a marker[16] and at the same time, a central regulator of ER stress, due to its major anti-apoptotic role, as well as to its capacity to control the activation of ER transmembrane stress sensors[17,18]. Recent studies have demonstrated that GRP78 plays an important role in tumor development, progression and chemoresistance[19]. The expressed GRP78 amount is directly correlated with tumor invasiveness and can be used as a biomarker for cancer[20,21].

The anti-obesity effect of melatonin supplementation therapy is validated by the significant reduction of body mass and intra-abdominal visceral fat[22,23].

Insulin and insulin-like growth factor 1 stimulate protein synthesis, anti-apoptotic proliferation and signaling by the induction of mitogen activated protein kinase. GRP78 expression is a target downstream of insulin, recent evidence suggesting that GRP78 and the overall protein balance of the endoplasmic reticulum can regulate the insulin sensitivity of the organism and can protect cells during acute stress. Obesity and type 2 diabetes mellitus are metabolic disorders characterized by insulin resistance and hepatic steatosis.

The presence of endoplasmic reticulum stress in the context of metabolic syndrome has been documented[24,25] and the presence of chaperones might be a key in the regulation of insulin sensitivity and glucose homeostasis.

This study supports the idea that plasma GRP78 levels are decreased by the administration of estrogen and all the more so, by the association of estrogen and melatonin. Thus, melatonin administration reduces adipose tissue and visceral fat, regulates insulin sensitivity, and decreases plasma GRP78 levels, all of which are risk factors for EC.

Conclusions

This study supports the idea that melatonin influences plasma estrogen level, which is positively correlated with plasma GRP78 level, suggesting the implication of GRP78 in the pathogenesis of EC. ■

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