Effect of prolonged psychoemotional stress and melatonin on experimental mammary carcinogenesis in female rats

M. KASSAYOVÁ*, L. FRIEDMANOVÁ, P. ORENDÁŠ, P. KUBATKA[1], B. BOJKOVÁ, N. BOBROV[2], E. AHLERSOVÁ, I. AHLERS

Department of Animal Physiology, Institute of Biological and Ecological Sciences, P.J. Šafárik University – Faculty of Science, Moyzesova 11, 040 01 Košice, e-mail: monika.kassayova@upjs.sk; [1]Institute of Pharmacology, Jessenius Faculty of Medicine, Comenius University, Záborského 2, 036 45 Martin; [2]Institute of Forensic Medicine, P.J. Šafárik University – Faculty of Medicine, Šrobárova 2, 040 01 Košice

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The aim of the present study was to determine whether prolonged stress – repeated immobilization in boxes during the period of 18 weeks (IMS) influenced development and progression of N-methyl-N-nitrosourea (NMU)-induced mammary tumors in female Sprague-Dawley rats and whether long-term MEL application affected changes caused by stress. NMU was applied intraperitoneally in two doses each of 50 mg/kg b.w. between 40 – 50 postnatal days. Melatonin (MEL) was administered in drinking water in a concentration of 4 μg/ml (daily from 3 p.m to 8 a.m), application was initiated 3 days prior to first NMU dose and lasted until the end of the experiment. Immobilization (2 h/day) began on the fifth day after second carcinogen application, animals were immobilized three times a week.

Repeated immobilization of rats during 18 weeks decreased tumor frequency per group and per animal by 30% and tumor volume gain by 16% as opposed to control (NMU) animals. Combination of repeated immobilization and a long-term MEL application lowered incidence by 13% when compared to control, prolonged latency by 13%, decreased tumor frequency per group (by 44%) and per animal (by 35%). Tumor volume gain increased by 35% but their cumulative volume prominently decreased by 74% as opposed to control. Tumor volume was the most markedly influenced by MEL, induced tumors developed more rapidly – tumor volume gain increased by 61%. However, their cumulative volume markedly decreased by 75% when compared to immobilized group drinking water.

Prolonged stress inhibited development and progression of NMU-induced mammary gland tumors in female rats and this effect was enhanced by long-term melatonin administration.

Key words: prolonged immobilization stress, mammary carcinogenesis, melatonin, rat

Breast cancer is the most frequently occurred tumor in women. According to International Agency for Research on Cancer 1.2 million new cases were diagnosed in 2002 and more than 400 000 women died of breast cancer [1]. Several biological factors have been identified representing risk in breast cancer etiology and these are related to life style, e.g. dietary fat intake, alcohol consumption**,** physical activity. The role of psychosocial factors has not been sufficiently explained. Although a great number of studies investigating the effect of psychoemotional stress on development and progression of mammary tumors in humans appear annually, their results are inconsistent due to certain degree of subjectivity associated with filling in questionnaires, which are fundamental for these studies as well as objectionable proof of direct causal relation between stress and cancer development. In connection with the aforementioned experimental animal models the studies carried out under controlled conditions, with exactly defined kind, duration and intensity of stressor are expected to reveal more valuable results [2].

Melatonin, a pineal gland hormone, displays a wide range of beneficial effects in organism; its receptors were described in numerous peripheral tissues. Melatonin synthesis has been proved, apart from pineal gland, in gastrointestinal tract, Harderian glands, retina, gonads and other organs. MEL has also been shown to have immunostimulating and tumorsuppressive effect [3].

The aim of our study was to determine the extent to what prolonged stress – repeated immobilization in boxes influences development and progression of chemically induced mammary tumors in female rats and the effect of long-term MEL application on stress-induced changes.

* Corresponding author

Material and methods

Female Sprague-Dawley rats (Central vivarium, Faculty of Medicine, P. J. Šafárik University, Košice, Slovak Republic) aged 35±2 days, weighing 105±1.4 g were adapted to standard vivarium conditions (temperature 23±2 °C, relative humidity 60-70%), artificial regimen light:dark – 12:12 h (light on from 7 a.m. to 7 p.m., intensity 150 lux per cage). Animals were fed standard MP diet (Top-Dovo, Dobrá Voda, Slovakia) and drank tap water ad libitum.

Mammary carcinogenesis was induced by freshly prepared N-methyl-N-nitrosourea (NMU, Sigma, Diesenhofen, Germany) dissolved in saline solution. NMU was administered intraperitoneally (0.5 ml per animal) in two doses each of 50 mg/kg b.w. between 40 – 50 postnatal days. Immobilization as a psychoemotional stress model began five days after second chemocarcinogen application. The animals were repeatedly immobilized in special boxes for 2 hours starting at 2 p.m. three times a week (Monday, Wednesday, Friday) for the period of 18 weeks. Melatonin (MEL) was administered in drinking water in a concentration of 4 μg/ml daily from 3 p.m to 8 a.m (from 8 a.m to 3 p.m. animals drank tap water only). MEL solution was made up fresh three times a week. Twenty mg of MEL were diluted in 0.4 ml of 30% ethanol and mixed up with tap water to the desired volume. The animals were divided into 3 groups: 1. NMU – control group without immobilization, 2. NMU+IMS- immobilized group, 3. NMU+IMS+MEL- immobilized group and drinking MEL until the end of the experiment. The experiment was carried out for 20 weeks (from October to February) with 15 animals in each group at the beginning of the experiment. All animals were weekly weighed and palpated, and the location and size of tumors were registered. In the last week of the experiment the animals were quickly decapitated and mammary tumors were excised and measured. Tumor incidence, latency, frequency per group and animal, average tumor volume gain and cumulative tumor gain were evaluated. The tissue samples from mammary tumors were fixed in 4% buffered solution of formol for histological examination. Tumors were classified as adenocarcinomas or fibroadenomas according to the criteria for tumor classification in rats [4].

Tumor incidence was evaluated by Mann-Whitney U-test and one-way analysis of variance and Kruskal-Wallis test was used to compare other parameters according to the value of Bartlett number. Tumor volumes were calculated according to formula $V = \pi.(S_1)^2. S_2 /12$; where S_1 and S_2 are tumor diameters measured perpendicularly; $S_1 < S_2$. The differences among the groups were expressed in percentage.

Results

Prolonged immobilization of rats during 18 weeks lengthened latency period by 5%, decreased tumor frequency per group and animal by 30% when compared to control group (NMU). No influence on incidence was found. Tumor volume gain in immobilized animals reduced by 16%.

Combination of repeated immobilization and long-term melatonin application decreased incidence by 13% as opposed to control (NMU) and immobilized (NMU+IMS) group, prolonged latency period by 13%, decreased tumor frequency per group (by 44%) and per animal (by 35%). Tumor volume gain increased by 35%, but their cumulative volume markedly decreased by 74% when compared to control.

The effect of MEL was not statistically significant in either of two stressed groups. MEL prolonged latency by 7%, decreased tumor frequency per group (by 20%) and per animal (by 7%) as opposed to immobilized group drinking water. Tumor volume was the most markedly influenced by MEL, induced tumors developed more rapidly – tumor volume gain increased by 61%. However, their cumulative volume prominently decreased by (75%) when compared to immobilized group drinking water.

Body weight gain decreased by 18% in immobilized animals, MEL administration enhanced this decrease to 23% as opposed to control. Melatonin induced insignificant decrease in body weight in immobilized animals as opposed to stressed group drinking water (by 6%) (Tab. 1). Food and water intake was influenced neither by immobilization nor by MEL administration (data not shown).

The overall number of histopathologically evaluated mammary tumors was 154, and out of these 145 (94%) were identified as adenocarcinomas. Fifty-six adenocarcinomas and six fibroadenomas (90:10%) were identified in NMU group, 49 adenocarcinomas and 3 fibroadenomas (94:6% in NMU+IMS group, and all 40 tumors were classified as adenocarcinoma in NMU+IMS+MEL group (100%).

Discussion

Not only kind of stress but also length of its application represents one of the key factors when investigating effects of stress on the development and progression of experimental mammary gland tumors. Effects of acute, repeated, prolonged or chronic stress may differ. Timing of stressor application is suggested to play an important role in relation to carcinogenesis induction (i.e. to chemocarcinogen administration).

The effect of acute stress on development and progression of mammary gland tumors was investigated by Adámeková et al. Single resp. repeated immobilization during 2 hours in boxes was used as a stress in female Sprague-Dawley rats and was applied before or after chemically (NMU) induced mammary carcinogenesis. Single stress application before [5] or after [6] chemocarcinogen administration did not result in significant alterations. Repeated immobilization (during 7 days) after chemocarcinogen application markedly stimulated development and progression of NMU-induced mammary neoplasia in female rats: incidence increased by 57%, frequency per group by 153%, per animal by 61% and latency

Tab.1. Effect of prolonged psychoemotional stress and melatonin on experimental mammary carcinogenesis in female rats

GROUP	NMU	NMU+IMS	NMU+IMS+MEL
Number of animals at the end of experiment	8	10	10
Incidence (%)	100.00 ± 0	100.00 ± 0	86.67 ± 9.09 (- 22%) [-13%]
Latency (days)[a]	66.00 ± 2.91	69.50 ± 4.05 (+5%)	74.67 ± 4.30 (+ 13%) [+7%]
Frequency per group[a]	8.07 ± 1.05	5.64 ± 1.17 (-30%)	4.53 ± 0.94 * (- 44%) [-20%]
Frequency per animal[a]	8.07 ± 1.05	5.64 ± 1.17 (-30%)	5.23 ± 0.93 (- 35%) [-7%]
Tumor volume gain (cm^3)[a,b]	1.31 ± 0.32	1.10 ± 0.32 * (-16%)	1.77 ± 1.11 ** (+35%) [+61%]
Cumulative tumor volume (cm^3)[c]	66.64	67.67 (+2%)	17.26 (- 74%) [-75%]
Body weight gain (g)	97.00 ± 5.70	79.15 ± 4.48 * (-18%)	74.71 ± 5.12 ** (- 23%) [-6%]

NMU – control rats injected with NMU
NMU + IMS – rats immobilized three times a week for the period of 18 weeks
NMU + IMS + MEL – group immobilized for the period of 18 weeks and drinking MEL
NMU – N-methyl-N-nitrosourea, IMS – immobilization stress, MEL – melatonin
[a] Data are expressed as means ± SEM, [b] data are expressed as means of tumor volume gains evaluated during the last 8 weeks of experiment (only palpable/palpated tumors that appeared until week 12 were taken into account), [c] data are expressed as sums of tumor volumes.
Values in round brackets are calculated as %-ual deviation compared to control with NMU (100%), values in square brackets are calculated as %-ual deviation of NMU+IMS+MEL vs NMU+IMS (100%), Significantly different: *P < 0.05 vs NMU, ** P < 0.01 vs NMU.

period shortened by 7 days when compared to control group without immobilization [6].

Newberry et al. [7] examined the effects of prolonged stress on DMBA-induced mammary tumors in female Sprague-Dawley rats. When the animals were immobilized in pre-inductive period (prior to carcinogen administration) during 20 days, 14 hours per day or during 20 days in the period of tumor induction (from the first carcinogen administration to its elimination from the organism), development and progression of mammary gland tumors were not influenced by immobilization. Stress application during 50 days in post-inductive period (after carcinogen elimination from the organism) has had significant inhibitory effect on mammary carcinogenesis and reduced significantly the number of tumors. The rats exposed to immobilization stress daily for 5 of 10 hours during 73 days had significantly reduced number of tumors when compared to controls [8]. Tumor-inhibitory effect of immobilization stress was recorded also in our experiments, prolonged repeated immobilization during 18 weeks (3 times a week for 2 hours) insignificantly lengthened latency period, decreased frequency per animal and group while tumor volume gain decreased significantly. Inhibition of tumorigenesis may result from adaptation of experimental animals to regularly repeated stress at the same time during a long period. Similar effect of another stressor – chronic isolation was reported in mice with DMBA-induced mammary tumors: the authors of the study documented decreased tumor volume in animals from day 10 to day 52 of the isolation period. The observation began on the day of first tumor appearance. Simultaneous application of antagonist – α_1-adrenergic receptors – prazosin to stressed animals did not influence the observed parameters of tumorigenesis [9]. This finding points to lacking participation of α_1-adrenergic receptors in stress effect mediation in the above experiments. Longer survival after inoculation of tumor cells as opposed to control animals without social confrontation was reported in rats affected by social confrontation (12 short confrontations between a resident, male rat originally housed in a cage, and an intruder) [10]. Contrastingly, other authors [11] recorded stimulation of mammary tumor metastases after single social confrontation lasting for 7 hours. Simultaneous administration of ß-adrenergic antagonist butoxamin reduced this effect by 50%. More rapid tumor growth was observed in mice influenced by psychosocial stress – alterations in housing (high density in a cage, isolation, groups of identical sex) but was completely inhibited after application of non-selective ß-adrenergic antagonist – propranolol [12]. The above results point to a possible role of adrenergic receptors in stimulation of tumor growth influenced by stress.

The effect of psychosocial factors on risk of breast cancer in humans has been studied for several decades with inconsistent results. Jacobs and Bovasso [13] investigated effect of acute and chronic stress in 1213 women and their findings suggest that significant risk of breast cancer may be linked with the death of mother in childhood (acute stress) and chronic depression. Meta-analysis of the studies published from 1966 to 2002 was printed in 2003 and did not confirm unambiguous relation between stressful life events and risk

of mammary neoplasia; significantly increased risk of mammary tumors was proved only in connection with partner's death [14]. In contrast, recent prospective studies carried out in the northern European countries reported positive link between stress and breast cancer [15, 16]. The probable risk factors are associated with well-defined psychosocial factors [17], e.g. two-fold increase of cancer risk has been associated with divorce and husband's death and approximately 1.5-fold increase with death of a relative or a friend [18].

Acute stress – specific adverse life events (divorce, disasters, family death) prospectively differ from chronic stress by their physiological effect on humans. Acute stress may activate while chronic one may suppress activity of hypothalamic-pituitary-gonadal axis thus decreasing synthesis of sex hormones [2]. Daily chronic persisting stress may result in stimulation of synthesis and secretion of stress hormones with a subsequently decreased estrogen synthesis lowering risk of mammary gland tumors [19]. Prospective cohort study including 6689 women observed during 18 years demonstrated that a high level of daily stress is connected with a decreased risk of breast cancer (each increase in stress level by 6 points of stress scale declared in questionnaires was associated with the decrease in the risk of primary mammary tumors by 8 %) [20]. Nurses providing prolonged care to an ill adult or to a child did not appear to increase incidence of mammary tumors but revealed significantly decreased levels of serum estradiol [21]. Regarding the fact that level of endogenous estrogen is a key factor in mammary tumor development and NMU-induced tumors are estrogen-responsive, modulation effect of estrogens may have played role in tumorsuppressive impact of stress in our experiment. Further studies are needed to determine their role in the above processes in relation to the animal models having proved increased estrogen synthesis due to chronic stress [22].

Stress is hypothesized to affect mammary carcinogenesis by different mechanisms of actions. The role of immune system, apart from that of nervous and endocrine, is most frequently being discussed as its activity is suppressed by elevated glucocorticoid and catecholamine levels occurring under exposure to chronic stress [23]. Recent research suggests that chronic stress and depression may actually enhance certain immune responses and inhibit other events by disrupting balanced immune responses existing under physiological conditions [24]. Chronic action of glucocorticoids and catecholamines impairs the rate of Th1 and Th2 lymphocytes and results in dysbalanced synthesis of proinflammatory and antiinflammatory cytokines [25]. Direct link between immunosuppression and increased risk of mammary neoplasia has not been demonstrated in two studies in immunosuppressed patients: a decreased incidence of breast tumors was reported in women observed 1-11 years after organ transplantation [26] and a lowered risk of mammary tumors was also recorded in women suffering from AIDS [27].

Melatonin is well-known for its pleiotropic effects, it has immunostimulating [28] and antistress properties [29, 30]. In vivo animal model studies with chemically induced mammary tumors have proved experimental manipulations as pineal gland stimulation and exogenous MEL administration prolonged latency, decreased incidence and growth of mammary tumors while pinealectomy had the opposite effect [31]. Direct oncostatic properties of MEL were described also in in vitro experiments with estrogen-responsive human mammary tumor MCF-7 cell line. Physiological MEL concentration (1 nM) proved to be the most effective [32]. Antiestrogen activity of MEL is supposed to be a key mechanism of tumorsuppressive effect of MEL [33]. In our experiment MEL potentiated tumor-inhibitory effects of stress. Stressed animals with continuous administration of MEL in low doses showed insignificant incidence decrease, prolonged latency period, a remarkable decrease in tumor volumes (by 74%) and significant decrease in tumor frequency per group as opposed to control group without immobilization and melatonin.

Available bibliographic data suggest that it is impossible to predict the role of stress on a stimulation or inhibition of tumor growth. Future experimental and clinical studies are required to find a definite solution of the problem. It seems probable that despite generally accepted negative stress impact on the organism it may display even tumor-suppressive effects under certain conditions. Chronic stress inhibited development and progression of NMU-induced mammary gland tumors in female Sprague-Dawley rats in our study and this effect of stress was enhanced by long-term melatonin administration.

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