Effect of a Short-Term and Long-Term Melatonin Administration on Mammary Carcinogenesis in Female Sprague-Dawley Rats Influenced by Repeated Psychoemotional Stress

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Abstract

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The aim of this study was to evaluate the effect of melatonin (MEL) on N-methyl-N-nitrosourea (NMU)-induced mammary carcinogenesis in female Sprague-Dawley rats exposed to repeated psychoemotional stress - immobilization in boxes. NMU was applied intraperitoneally in two doses each of 50 mg/kg b.w. between 40 - 50 postnatal days. Melatonin was administered in drinking water at a concentration of 4 µg/ml daily from 15:00 h to 8:00 h. The application was initiated 5 days prior to the first NMU dose and lasted 15 days, i.e. during the promotion phase of tumour development, or long-term until the end of the experiment (week 20). Immobilization (2 h per day) began on the third day after the second carcinogen application and lasted for 7 consecutive days.

Short-term MEL administration to immobilized animals increased incidence by 22%, decreased tumour frequency per animal by 26% and reduced tumour volume gain (by 21%) when compared to the immobilized group without MEL application. Decreased frequency per animal by 28% and more than a 40% decrease in tumour volume gain and cumulative volume were the most pronounced changes in the animals drinking MEL until the end of the experiment.

Long-term MEL administration reduced the number and size of mammary tumours more markedly than its short-term administration. Melatonin decreased certain attributes of mammary carcinogenesis in female rats influenced by psychoemotional stress.

Melatonin, mammary carcinogenesis, psychoemotional stress - restraint, rat

Breast cancer is the most frequently diagnosed tumour in women world-wide. In most European countries the incidence is increasing; 350 000 new cases are recorded annually and annual mortality rate is 130 000 (Tyczynski et al. 2004). Chemically induced mammary carcinogenesis in rats is a widely used model to study preventive and therapeutic effects of various substances on tumour development. Melatonin (MEL) has been one of the most discussed substances since the 1970's. Melatonin is an endogenous pineal gland hormone and its oncostatic properties were proved mainly in hormone-sensitive mammary tumour *in vivo* and *in vitro*.

Tumour-suppressive effects of MEL are exerted by different mechanisms of action. Indirect effect of MEL is produced through its inhibition of the activity of neuroendocrine-reproductive axis with a consequent decrease in circulating levels of hormones that stimulate mammary tissue proliferation, mainly estrogens (Sánchez-Barceló et al. 2005) and prolactin (Tamarkin et al. 1981). MEL is capable of direct interaction with estrogen receptors resulting in their expression decrease and modulation of estrogen signal transfer

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into a cell (García Rato et al. 1999). In *in vivo* and *in vitro* conditions MEL inhibits the activity of telomerase: an enzyme that ensures the lengthening of telomeres and stability of chromosome structure in eucaryotic cells; its activity is regarded as a ubiquitous tumour marker (Leon-Blanco et al. 2003). It has been proved that MEL inhibits proliferation and invasion of tumour cells (Sánchez-Barceló et al. 2003), enhances the protein p53 and p21WAF1 expression (Mediavilla et al. 1999), lengthens the cell cycle by blocking the G1 transition to the S phase (Cos et al. 1996a) and lowers DNA synthesis in MCF-7 human mammary gland tumour cell line (Cos et al. 1996b). MEL has also been shown to have immunoenhancing properties (Carrillo-Vico et al. 2005), its efficient scavenger effect of free radicals has been found (Reiter et al. 2000), it demonstrates antiangiogenic and proapoptotic activity (Anisimov 2003), and displays minimal side effects. All the aforementioned facts predetermine MEL to be applied in clinical oncology.

The role of stress in mammary carcinogenesis has not been sufficiently explained. Experimental studies carried out on animals proved tumour-inhibitory (Newberry et al. 1972, 1976) as well as tumour-stimulatory effect (Tejwani et al. 1991; Kavetsky et al. 1966) of psychosocial factors on mammary tumourigenesis. Activation of the hypothalamic-pituitary-adrenal system and sympathetic nervous system due to stress results in an increased production of catecholamines and glucocorticoids and consequent negative effects on functions of the immune system. An imbalance between Th1 and Th2 lymphocytes is produced; synthesis of proinflammatory and antiinflammatory cytokines is disordered, which together with decreased activity of NK cells may lead to tumour development and progression (Moynihan 2003; Ben-Eliyahu and Shakhar 2001). MEL has a potential to eliminate stress-induced immunodepression probably through the mechanism of a direct effect on lymphocytic functions, especially cytokine synthesis (Maestroni 2001).

The aim of this study was to evaluate chemopreventive and therapeutic effect of exogenous MEL on mammary carcinogenesis in rats repeatedly exposed to psychoemotional stress.

Materials and Methods

Female Sprague-Dawley rats (Anlab, Prague, Czech Republic) aged 35 ± 2 days, weighing 136 ± 14 g were adapted to standard vivarium conditions (temperature 23 ± 2 °C, relative humidity 60 - 70%, artificial regimen light : dark – 12 : 12 h, light at 7:00 h, intensity 150 lux per cage. Animals were fed MP diet (Top-Dovo, Dobrá voda, Slovakia) and drank tap water *ad libitum*. The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic by accreditation No. 431/03-220.

Mammary carcinogenesis was induced by freshly prepared N-methyl-N-nitrosourea (NMU, Sigma, Diesenhofen, Germany) dissolved in isotonic saline solution. NMU was administered intraperitoneally (0.5 ml per animal) in two doses each of 50 mg/kg b.w. on postnatal days 42 and 48 between 15:00 and 16:00 h. Immobilization as a psychoemotional stress model began three days after the second chemocarcinogen application. The animals were immobilized in special boxes for 2 hours during 7 consecutive days. Melatonin (MEL) was administered in drinking water at a concentration of 4 μg/ml daily from 15:00 h to 8:00 h; from 8:00 h to 15:00 h the animals drank tap water only. Melatonin chemoprevention was initiated 5 days prior to the first NMU dose. The animals were divided into 4 groups: 1. NMU - control group without immobilization, 2. NMU + 7IMS - group immobilized seven times (during 7 consecutive days), 3. NMU + 7IMS + MEL(S) - group immobilized 7 times and drinking MEL for a short term, 15 days after second NMU-dose administration, i.e. in the promotion phase of tumour development, 4. NMU + 7IMS + MEL(L) - group immobilized 7 times and drinking MEL for a long term, until the end of the experiment. The experiment was carried out for 20 weeks (from June to November) with 20 animals in each group at the beginning of the experiment. The fifth, intact group (10 animals) was used to compare the weight gain. All animals were weekly weighed and palpated, and the number, localization and size of tumours were recorded. In the last week of the experiment the animals were sacrificed by quick decapitation and mammary tumours were excised and measured. Tumour incidence (per cent proportion of tumour-bearing animals from the total number of animals in the experimental group), latency (time period from the second chemocarcinogen application to the first tumour appearance), frequency per group (frequency per animal using all animals in a group - tumour-bearing and tumour-non-bearing) and animal (frequency per tumour-bearing animal), tumour volume gain and cumulative tumour gain were evaluated.

The number of animals decreased at the end of the experiment to 14 (NMU), 18 (NMU + 7IMS), 19 (NMU + 7IMS + MEL(S)) and 16 (NMU + 7IMS + MEL(L)) due to tumour development. Sick animals with the exulcerated tumours or with the tumour bleeding were sacrificed; tumours were excised, measured and included to the tumour evaluation in the appropriate week (the data from each of weeks are not shown).

Tumour incidence was evaluated by Mann-Whitney U-test and one-way analysis of variance and Kruskal-Wallis test was used to compare other indicators according to the value of Bartlet number. Tumour volumes were calculated according to formula $V = \pi.(S_1)^2. S_2 /12$; where S_1 and S_2 are tumour diameters measured perpendicularly; $S_1 < S_2$.

Results

Short-term MEL administration during 15 days in the group NMU + 7IMS + MEL(S) increased incidence by 22% when compared to the group NMU + 7IMS, but did not affect latency period of the first tumour (+ 1%), decreased tumour frequency per group by 11% and frequency per animal by 26%. Tumour volume gain reduced by 21% and cumulative volume by 1%. Long-term MEL application in the group NMU + 7IMS + MEL(L) during 20 weeks increased incidence by 13%, lengthened the latency period by 10%, decreased tumour frequency per group by 19% and frequency per animal by 28% when compared to the control group without MEL administration (NMU + 7IMS). The most prominent effect of long-term MEL administration was observed in the tumour volume, volume gain decreased by 45% and the total cumulative volume by 42% in comparison with immobilized animals without MEL (Table 1). Body weight gain, food and water intake were influenced neither by immobilization nor by MEL administration (data not shown).

Discussion

The results of this study showed that a short-term MEL administration to repeatedly stressed rats merely in the promotion phase of tumour development has a less remarkable oncostatic effect in comparison with a long-term MEL application, although the differences are not statistically significant. A fifteen-day-administration of MEL induced a less pronounced decrease in tumour frequency per animal and tumour volume gain when compared to the group with a 20-week-application of MEL, where MEL was administered 5 days prior to first chemocarcinogen application. Saez et al. (2005a) administered MEL (5 mg/ml/rat/day) to female rats preventively 1 month prior to first carcinogen (DMBA) administration until decapitation (22 weeks) and reported survival time lengthening, increase in latency period and decrease in tumour volume. Their results correspond with our findings. In another study the above authors applied MEL (at the same dose) during 1 month in the phase of tumour growth progression, i.e. with the onset of administration from the first tumour appearance (Saez et al. 2005b) and they found out the lengthening of survival time, increase in the latency period without influencing tumour growth indicators, frequency and tumour volumes remained unchanged. The oncostatic properties of MEL on DMBA-induced tumour growth in rats were also observed by Lenoir et al. (2005). The authors compared chemopreventive effect of MEL (10 mg/kg daily) administered 15 days prior to chemocarcinogen application with the therapeutic effect of long-term MEL application during 6 months (with the onset from DMBA administration). The animals were observed for one year after DMBA administration. The study did not prove differences between preventive and therapeutic effects of MEL in mammary tumour growth. Further experiments are required in order to determine optimal period of MEL administration before chemocarcinogen application and definite evaluation of its chemopreventive effect on experimental mammary carcinogenesis.

The most remarkable change recorded in this experiment was a decrease in the tumour volume by 45% in animals with a long-term MEL application. Blask et al. (1999) proved in experimental estrogen-responded liver adenocarcinoma *in vivo* that tumour tissue growth is stimulated by the scavenging of fatty acids from circulation. Chronic MEL administration (50 ng, 500 ng or 5 µg/rat/day) significantly decreased hepatoma growth via inhibition of fatty acid scavenging by tumour tissue and their

Table 1. Effect of a short-term and long-term melatonin administration on mammary carcinogenesis in female rats influenced by immobilization stress

GROUP	NMU	NMU+7IMS	NMU+7IMS+ MEL(S)	NMU+7IMS+ MEL(L)
Number of animals	14	18	19	16
Incidence (%)	50.00	38.89	47.37 $p = 0.62$ (+ 22%)	43.75 $p = 0.79$ (+ 13%)
Latency (days)[a]	74.00 ± 8.76	85.43 ± 12.65	84.22 ± 8.49 $p = 0.94$ (- 1%)	94.14 ± 10.53 $p = 0.61$ (+ 10%)
Frequency per group[a]	1.36 ± 0.50	1.00 ± 0.42	0.89 ± 0.30 $p = 0.84$ (- 11%)	0.81 ± 0.28 $p = 0.72$ (- 19%)
Frequency per animal[a]	2.71 ± 0.68	2.57 ± 0.78	1.89 ± 0.34 $p = 0.44$ (- 26%)	1.86 ± 0.34 $p = 0.43$ (- 28%)
Tumour volume gain (cm^3)[a,b]	6.43 ± 1.74	3.86 ± 0.93	3.04 ± 0.77 $p = 0.58$ (- 21%)	2.14 ± 0.45 $p = 0.29$ (- 45%)
Cumulative tumour volume (cm^3)[c]	74.14	36.63	36.14 $p = 0.96$ (- 1%)	21.16 $p = 0.22$ (- 42%)

NMU – control rats injected with NMU
NMU + 7IMS – rats immobilized seven times (during 7 consecutive days)
NMU + 7IMS + MEL(S) – rats immobilized 7 times and drinking MEL for a short-term, 15 days after second NMU-dose administration
NMU + 7IMS + MEL(L) – group immobilized 7 times and drinking MEL for a long term, until the end of experiment (20th week)
NMU – N-methyl-N-nitrosourea, IMS – immobilization stress, MEL - melatonin
[a] Data are expressed as means ± SEM
[c] Data are expressed as sums of tumour volumes
[b] Data are expressed as means of tumour volume gains evaluated during the last 8 weeks of experiment (only palpable/palpated tumours that appeared until week 12 were taken into account, number of evaluated tumours in week 12 was in the group NMU – 10, NMU + 7IMS – 7, NMU + 7IMS + MEL(S) – 6, NMU + 7IMS + MEL(L) – 3)
Values in brackets are calculated as % deviation from the group NMU + 7IMS (100%)
p – significance level

metabolism (Blask et al. 2004). It is supposed that this mechanism also acts in other tumour types and could have influenced decrease in tumour volume gain and their cumulative volumes in our experiment.

MEL dosage, i.e. the amount of MEL administered to one animal per day is together with the time of day during MEL application a key factor of the MEL oncostatic effect. In our previous experiment (DMBA-induced carcinogenesis, Ahlersová et al. 2000) MEL was administered at a concentration of 100 µg/ml continuously for 24 hours (the daily intake of MEL was 2500 µg/day/animal) and isolated decrease in tumour frequency was observed while other observed indicators were not influenced. The reduction of MEL concentrations to 20 µg/ml and its administration during late afternoon and night, i.e. in the period before endogenous MEL synthesis increases and the tissues are most sensitive to its effect, was more effective in chemoprevention of tumour growth, led to decreased tumour incidence and significantly prolonged latency period (NMU-induced carcinogenesis, Bojková et al. 2000), or prominent decrease in

tumour volume (DMBA-induced carcinogenesis, Kubatka et al. 2001). In this study even lower concentration of MEL solution - 4 μg/ml (total daily MEL intake was about 100 μg/day/animal) was applied. However, the observed changes in indicators were not significant. It may be explained by a modified hormonal state of the animals repeatedly exposed to immobilization stress. The highest oncostatic activity of MEL was recorded when its concentration was similar to the physiological range i.e. 1 nM (1 nM = 0.232 μg MEL) in MCF-7 cell line *in vitro* (Blask and Hill 1986).

The effect of immobilization stress on mammary tumour development and progression in the present experiment was rather inhibitory, when compared to our previous experiments. We found it surprising although this experiment was not aimed at the observation of the stress impact. Thus, the statistic evaluation of the comparison of controls without MEL application was not included. Stimulation of tumour growth due to stress was documented by significantly increased tumour incidence and frequency per group 20 weeks after NMU application and consecutive immobilization (7 consecutive days, immobilization 120 minutes a day) (Adámeková et al. 2003). We assumed that contradictory results of these two experiments may be at least partially due to different origins of SD rats - from two different farms; the second reason may be the different season when studies were performed: winter-spring (previous experiment) vs. summer-autumn (present study). The season could play a prominent role in the neuroendocrine reactions of the rat (Ahlers et al. 1990) or mouse (Meyer et al. 2006). Seasonal and strain dependence of chemically induced mammary carcinogenesis was demonstrated by our research team (Kubatka et al. 2002). Higher tumour incidence during long days in comparison with short winter days was found in Sprague-Dawley rats; Wistar:Han rats were far less sensitive. For an explanation of the contradictions, we plan to repeat the present experiment with a higher number of animals in each group, from the same farm, in two different seasons (summer, winter). An experimental animal may be stressed by the tumour presence itself. Female rats with DMBA-induced mammary tumours had significantly increased circulating norepinephrine and prolactin concentrations together with mildly increased epinephrine in comparison with non-tumour-bearing animals and MEL administration returned levels of these hormones to those of healthy animals (Saez et al. 2005b). Increased circulating levels of prolactin were described in stressed non-tumour-bearing rats after acute and repeated (7 days) immobilization (Zelena et al. 2003).

Certain authors at present regard MEL as naturally occurring selective modulator of estrogen receptors. Antiestrogenous effects of MEL, which are essential in its antineoplastic activity, are exerted by three fundamental mechanisms: MEL suppresses gonadal steroid synthesis with a consequent decrease of their circulating levels, directly interacts with estrogen receptors and simultaneously is able to lower activity of enzymes participating in peripheral synthesis of estrogens from androgens, e.g. aromatase (Sánchez-Barceló et al. 2005). MEL inhibits the aromatase activity in the MCF-7 neoplastic cell line *in vitro* (Cos et al. 2005). The above cited effect was recently confirmed also in DMBA-induced mammary rat tumours *in vivo* directly in tumour tissue (Cos et al. 2006). Aromatase inhibitors are widely used in human breast cancer treatment (Miller 2004). MEL reduces toxicity and increases efficacy of some cytostatic drugs (Reiter et al. 2002), which together with its inhibitory effect on local estrogen synthesis in tumour tissue as well as other beneficiary effects could make this hormone attractive for a wide use in clinical practice.

It may be concluded that in our experiment MEL decreased certain attributes of mammary carcinogenesis influenced by psychoemotional stress in female rats. MEL administration limited to the promotion phase of tumour development was less effective than its long-term application.

Vplyv krátkodobého a dlhodobého podávania melatonínu na vznik mamárnych tumorov u samíc potkanov opakovane vystavených psychoemočnému stresu

Cieľom štúdie bolo otestovať účinok melatonínu (MEL) na N-metyl-N-nitrozoureou (NMU)-indukovanú mamárnu karcinogenézu u samíc potkanov kmeňa Sprague-Dawley, opakovane vystavených psychoemočnému stresu - imobilizácii v boxoch. NMU bola podaná v dvoch dávkach po 50 mg/kg i.p. medzi 40. - 50. postnatálnym dňom. MEL bol aplikovaný v pitnej vode v koncentrácii 4 ųg/ml (od 15.00 do 08.00 nasledujúceho dňa) so začiatkom podávania od 5. dňa pred prvou dávkou NMU krátkodobo - 15 dní, t.j. v priebehu promočnej fázy vývoja nádorov resp. dlhodobo - až do ukončenia pokusu (20. týždeň). Imobilizácia (2 h/deň) sa začala na 3. deň po podaní 2. dávky karcinogénu a trvala 7 po sebe nasledujúcich dní.

Krátkodobé podávanie MEL imobilizovaným zvieratám spôsobilo oproti imobilizovanej skupine bez aplikácie MEL 22 %né zvýšenie incidencie, pokles frekvencie nádorov na zviera o 26 % a znížený bol aj priemerný prírastok objemov nádorov (o 21 %). U zvierat pijúcich MEL až do ukončenia pokusu boli navýraznejšími zmenami zníženie frekvencie na zviera o 28 % a viac ako 40 %ný pokles prírastku objemov nádorov a ich kumulatívneho objemu.

Dlhodobé podávanie MEL redukovalo počet a veľkosť mamárnych nádorov výraznejšie ako jeho krátkodobé podávanie. Melatonín znížil niektoré charakteristiky mamárnej karcinogenézy pri pôsobení psychoemočného stresu u samíc potkanov.

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