Effects of Retinyl Acetate and Melatonin on N-methyl-N-nitrosourea-Induced Mammary Carcinogenesis in Rats. A Preliminary Report

(breast cancer / rats / retinyl acetate / melatonin)

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Abstract. The aim of this project was to evaluate the effect of retinyl acetate (RA), melatonin (Mel) and their combination on N-methyl-N-nitrosourea (NMU)-induced rat mammary carcinogenesis. Female Sprague-Dawley rats were given two intraperitoneal doses of NMU, each of 50 mg/kg of b.w. between 43th to 57th postnatal day. The administration of RA started 11 days and the administration of Mel 12 days before the first dose of NMU. RA was given daily in a dose of 8.2 mg per animal and day at the base of the tongue. Mel was given as a solution (20 μg/ml of tap water) between 3 p.m. and 8 a.m., from 8 a.m. to 3 p.m. the animals were drinking tap water only. The experiment was finished 22 weeks after the first administration of the carcinogen. The tumour incidence in the control group was 88%, in the group treated with RA 80% and in the group treated with Mel 61%. A substantial decrease in tumour incidence to 37% was noted in the group treated with RA plus Mel. Significant differences in incidence were noted in the group treated with the combination of RA and Mel as compared to the control group and the group treated with RA. Chemoprevention lengthened the latency significantly in the group treated with Mel and with the combination of RA and Mel. The decrease in tumour frequency per group was confirmed in the group treated with the combination of RA and Mel; differences between groups in the frequency per tumour-bearing animal were not observed. The volume of mammary tumours in the groups treated with chemopreventive agents was not changed.

The occurrence of breast cancer as the most common cancer disease in women is still rising. This fact indicates an urgent need for improving not only the treatment but also the prevention of this disease. A wide range of substances acting in various stages of tumour progression is already known. The mechanism of effects of chemopreventive agents consists in blocking the carcinogen activity (calcium ion, polyphenols, non-steroidal anti-inflammatory drugs (NSAID), tamoxifen, melatonin etc.), in antioxidative activity (polyphenols, α-tocopherol, NSAID, tamoxifen, melatonin etc.), or chemopreventives can reduce proliferation and progression (NSAID, antioestrogens, calcium ion, selenium, α-tocopherol, retinoids, carotenoids etc.) (Kelloff et al., 1997). Concerning retinoids, retinyl acetate (RA) and N-(4-hydroxyphenyl)retinamide (4-HPR) proved to be most effective in prevention of rat mammary carcinogenesis. Their administration reduces the incidence of chemically induced mammary tumours and increases their latency (Moon et al., 1976; Moon and Sporn, 1977; Mc Cormick et al., 1980; Moon and Mehta, 1989). However, long-term administration of high doses of RA results in an accumulation of retinyl esters in the liver. As to toxicity, 4-HPR is more suitable to be chronically administered (Moon and Mehta, 1989). Presently, the chemopreventive effect of retinyl-substituted benzyl ethers to chemically induced mammary carcinogenesis is under investigation. Retinyl-2-propynyl ether (RPE) and 3,4,5-trimethoxybenzyl ether (RTMBE) with lower toxicity in comparison with RA has shown to be effective. Besides, the latter does not cause significant increase in retinyl palmitate concentration in the liver (Shealy et al., 1998).

Melatonin (Mel) as the main hormone of mammalian pineal gland possesses many favourable properties including an oncostatic effect, noticed already in the 80-ties. In 1986 Blask and Hill reported an antiproliferative effect of physiological concentration of Mel on MCF-7 human breast cancer cell sublines. Other authors (Panzer et al., 1998; Papazisis et al., 1998) did not acknowledge this effect. Summarizing, an inhibitory action of melatonin on the growth of non-differentiated neoplasia, sarcomas and carcinomas, especially hepatocarcinoma, Lewis lung carcinoma, cervical and mammary carcinoma has been observed experimentally. Mel was most effective on hormone-dependent tumours such as tumours of mammary gland and prostate (Kothari, 1988).

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Abbreviations : DMBA – 7,12-dimethylbenz(a)anthracene, 4-HPR – N-(4-hydroxyphenyl)retinamide, Mel – melatonin, NMU – N-methyl-N-nitrosourea, RA – retinyl acetate.

The question whether Mel acts as an anti-initiating agent and/or anti-promoting agent was investigated by the group of Blask et al. (1991). Daily late-afternoon injections of Mel (500 μg/day) restricted to the initiation phase of NMU-induced rat mammary carcinogenesis were ineffective. When Mel treatment began 4 weeks after NMU administration and continued through the remainder of the promotion phase, the number of tumours but not tumour incidence was significantly lower. When Mel was given through the entire promotion phase, the tumour number and tumour incidence were significantly decreased. According to these results, Mel appeared to act as an anti-promoting rather than anti-initiating agent (Blask et al., 1991).

We assumed that the combination of retinyl acetate and melatonin would connect the induction of apoptosis and direct influence on the mitotic cycle (mechanism of retinyl acetate action) with the inhibition of mitogenic-active hormones (oestrogens, prolactin) and with immunostimulative and antioxidative activities (of melatonin).

Material and Methods

Female Sprague-Dawley rats of SPF breeding (AnLab, Prague, Czech Republic) were used in this experiment. All animals were housed in the vivarium under controlled conditions of temperature (23 ± 2°C), humidity (60–70%) and light regimen (light : dark – 12 : 12 h), with the onset of light at 7 a.m.). Rats were fed PM diet (Top-Dovo, Dobrá Voda, Slovakia) and they drank tap water ad libitum. The animals were divided into four experimental groups:

1. NMU
2. NMU + RA
3. NMU + Mel
4. NMU + RA + Mel

N-methyl-N-nitrosourea (NMU, Sigma, Deisenhofen, Germany) was given to all experimental groups intraperitoneally (i. p.) in two doses each of 50 mg/kg per body weight between 43^{th}–57^{th} postnatal day; the latter dose of NMU was given 7 days after the first one.

The treatment with RA began 11 days and the treatment with Mel 12 days before the first dose of NMU. The dose of RA and the way of its administration in groups number 2 and 4 were the same. RA was given daily from Monday to Friday at 2 p.m. at the base of the tongue by a pipette. The dose per animal and rat was 8.2 mg of pure RA in 20 μl of oil concentrate (Fluka, Buchs, Switzerland) (1 mg of oil concentrate contains 1500 I.U. of pure RA, 1 I.U. = 0.34 μg RA.) The dose and administration of Mel in groups 3 and 4 were the same. Mel was given in tap water in a concentration 20 μg/ml (for preparation of 1 l of solution, 20 mg of Mel were dissolved in 4 ml of ethanol and mixed up with tap water to the desired volume). The solution of Mel was changed three times a week. Mel (Biosynth AG, Staad, Switzerland) was given from 3 p.m. to 8 a.m.; from 8 a.m. to 3 p.m. the animals were drinking tap water. All animals were weighed and palpated weekly and the incidence, size and location of each tumour were registered. The experiment was carried out from November 1998 to April 1999 and lasted 22 weeks from the first administration of the chemocarcinogen. All rats were sacrificed by decapitation, mammary tumours were removed, weighed, measured and fixed in formalin.

Statistical significance of the differences in the final incidence of tumours was evaluated by Mann-Whitney test, and that of the latency period was evaluated by one-way analysis of variance; for evaluation of differences in frequency and volume of tumours at the end of the experiment, Kruskal-Wallis test was used.

Results

The incidence, latency period, frequency and volume of mammary tumours at the end of the experiment are given in Table 1.

The incidence of mammary tumours in the control group (without chemoprevention) was 88%, in the group with RA 80%, in the group with Mel 61%, and the incidence in the group treated with the combination of RA and Mel was significantly decreased to 37%. The incidence in the group receiving the combination of RA and Mel was significantly lower than that in the group treated with RA. The latency period was increased in all groups subjected to chemoprevention; in groups treated

Table 1. The effect of retinyl acetate and melatonin on the incidence, latency, frequency and volume of mammary tumours induced by NMU

	Incidence	Latency	Frequency per group	Frequency per animal	Tumour volume (cm^3)
NMU (n=17)	88%	91.67 ± 3.96	2.29 ± 0.51	2.60 ± 0.53	1.20 ± 0.23
NMU+RA (n=20)	80%	107.0 ± 6.45	2.40 ± 0.64	3.00 ± 0.73	1.41 ± 0.36
NMU+Mel (n=18)	61%	114.5 ± 5.40+	1.72 ± 0.62	2.82 ± 0.87	1.76 ± 0.75
NMU+RA+Mel (n=17)	37%+x	119.7 ± 7.14+	0.59 ± 0.21+	1.43 ± 0.30	1.43 ± 0.48

Experimental groups: NMU – control group treated only with NMU, NMU+RA – group treated with retinyl acetate, NMU+Mel – group treated with melatonin, NMU+RA+Mel – group treated with retinyl acetate and melatonin, n = number of animals. Values of individual parameters are given as means ± S.E.M, significance of differences between the control group and other groups for $P < 0.05$ is expressed as +, significance of differences between NMU+RA and NMU+RA+Mel for $P < 0.05$ is expressed as x.

with Mel and with the combination of RA and Mel this increase was statistically significant. Tumour frequency per group was significantly lower in the group treated with RA plus Mel. Tumour frequency per animal and volume of tumours were not significantly changed.

Discussion

Retinoids belong to the agents widely used in cancer chemoprevention. They can act both as anti-initiators and anti-promoters, alter carcinogen metabolism, cell division and differentiation, as well as expression of oncogenes and biosynthesis of polyamines and prostaglandins (El-Bayoumy, 1994). Retinoids can induce apoptosis. Carcinogenesis could be influenced by their immunostimulative activities – they activate cell-mediated cytotoxicity and increase activation of natural killers (Hill and Grubbs, 1992).

The positive effect of retinoids on chemically induced mammary carcinogenesis was reported by Moon et al. already in 1976. RA significantly decreased the incidence of DMBA-induced mammary tumours in rats (Moon et al., 1976). McCormick et al. (1980) pointed out that a positive effect can be reached also by short-term application of RA. In this study the treatment with RA two weeks before and one week after administration of DMBA showed similar positive effect as did the 30-week administration. Inhibition of chemically induced mammary carcinogenesis was observed by delayed administration of RA, too, as late as 16 weeks after chemocarcinogen application (McCormick and Moon, 1982).

Another effective retinoid that reduces incidence and extends the latency period is N-(4-hydroxyphenyl)retinamide (4-HPR). RA and 4-HPR are active in chemoprevention of other types of tumours, too, e.g. tumours of urinary bladder and lung (Moon and Mehta, 1989). Concerning toxicity, 4-HPR is more suitable for long-term administration – chronic administration of RA increases the accumulation of retinyl esters in the liver and its subsequent damage. In experiments 4-HPR was observed to be highly concentrated in the mammary gland, but its concentration in the liver was low (Moon and Mehta, 1989).

Two experiments investigating the effect of RA and another chemopreventive agent, Mel, as wel as the effect of their combination were performed at our laboratory. The first was carried out from January to June 1998, the second from November 1998 to april 1999. In both of them, female Sprague-Dawley rats bred in the same conditions were used, but in the first experiment (not described in this paper) mammary carcinogenesis was induced by DMBA in a dose of 3 × 10 mg per animal during 10 days. The dose and administration of RA were the same in both experiments, but its effect differed. In the first experiment RA reduced the incidence, tumour frequency and lengthened the latency period in comparison with the control group (without chemoprevention); the volume of tumours was not affected (Ahlersová et al., 2000). In the experiment described in this paper RA did not reduce the incidence and volume of tumours significantly, the latency period was non-significantly lengthened, the tumour frequency per group was not changed and the frequency per tumour-bearing animal was even non-significantly higher.

The inhibitory effect of Mel on tumour growth was originally reported in the 80-ies. Lower incidence and longer latency period were observed after Mel administration or after activation of pineal gland function by blindness connected with induction of anosmia, hypocaloric diet or after exposure to cold. The incidence of mammary tumours usually increased after pinealectomy or exposure of experimental animals to continous light (functional pinealectomy) (Tamarkin et al., 1981; Shad et al., 1984; Sánchez-Barceló et al., 1988). Female rats of the Holtzman strain were given DMBA intragastrically between 50^{th}–60^{th} postnatal day; the incidence of mammary gland tumours was reduced after administration of Mel in drinking water in a dose of 200 µl per rat and day between 48^{th}–58^{th} postnatal day (Kothari 1987; Subramanian and Kothari, 1991). Physiological concentrations of Mel inhibited the growth of MCF-7 human breast cancer cell sublines. It was established that this effect of Mel was due to the prolongation of cell cycle and subsequent delayed entrance of MCF-7 cells into mitosis (Mollis et al., 1983; Blask and Hill, 1986). On the other hand there are studies that denied the effect of Mel in physiological concentrations on growth, morphology and cell cycle of not only MCF-7 cells, but also cervical cancer cell lines (HeLa), osteosarcoma cells (MG-63) and lymphoblastoid cells (TK-6). Panzer et al. (1998) explained the different effect of Mel by a supposed usage of melatonin-sensitive MCF-7 cell clone in other papers. Similarly, Papazisis et al. (1998) did not observe any inhibitory effect of MEL in physiological concentrations on MCF-7 and T47D human breast cancer cell lines.

In our previous experiment (Ahlersová et al., 2000), Mel was given continuously in a dose of 100 µl/ml of tap water and in this way significantly reduced tumour frequency. The latency period was not affected and the tumour volume was even higher than in the control group. In the present experiment Mel was given in a dose of 20 µl/ml; the tumour incidence was reduced and the latency period was significantly lengthened. The tumour frequency per group was non-significantly decreased, the frequency per tumour-bearing animal was not affected and the volume of tumours was even non-significantly higher. It is not clear whether the difference in the effect of Mel is caused by its dose, mode of administration or by other factors, e.g. effect of different season.

The complexity in the activities of both chemopreventives could be assumed for the successful decrease in the incidence and frequency in NMU-induced mammary tumours. The initiating effect of the selected

chemocarcinogen is of substantial importance; Ahlersová et al. (2000) recorded, except of the evaluation of tumour incidence, other results with the same combination of chemopreventive agents in DMBA-induced mammary carcinogenesis. To explain the effect of this combination of agents, further experiments are needed.

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