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Variability of Mammary Carcinogenesis Induction in Female Sprague-Dawley and Wistar:Han Rats: the Effect of Season and Age

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Summary

It is important to determine and clarify the variability of mammary carcinogenesis induction in animal experimental studies particularly in connection with chemoprevention projects. The circannual seasonal rhythms of hormone levels or various parameters within the immune system may involve factors participating in mammary gland carcinogenesis. In our study, 19 experiments were conducted and all of them lasted for about 25 weeks after chemical carcinogen administration (DMBA or NMU) under standard laboratory conditions. Females of two rat strains – a medium susceptible Sprague-Dawley strain and a very low susceptible Wistar:Han were used. We observed not only the effect of seasonal changes but also the effect of age after single or repeated carcinogen administration. The seasonal dependence of mammary carcinogenesis with higher tumor incidence during long days in comparison with winter short days has been demonstrated in Sprague-Dawley rats. In experiments on the Wistar:Han strain, certain features of seasonal character were recorded, although the very low susceptibility of this strain to mammary carcinogenesis might have influenced the results. A limited period of carcinogen administration in early puberty around postnatal days 43-46 (higher susceptibility), when compared to the period after postnatal day 50, is the factor significantly increasing incidence and frequency of mammary carcinogenesis in the Sprague-Dawley strain. Our results indicate the need to consider the effect of season and age of animals at the time of carcinogen administration on rat mammary carcinogenesis induction. However, the application of the results obtained in one strain of experimental animals may only lead to misleading conclusions.

Key words

Mammary carcinogenesis • Seasons • Critical periods • Rat strains • Susceptibility

Introduction

A number of factors influence the induction of experimental mammary carcinogenesis in female rats, including age, strain, dose, time of the day and year of the carcinogen administration, immune system patterns, endocrine system status or diet. In addition, some still unknown factors may cause changes in tumorigenesis induction in experiments performed under identical conditions.

Löscher *et al.* (1997) found apparent seasonal variability in the incidence of induced mammary gland

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tumors in female Sprague-Dawley rats kept under constant laboratory conditions, with a maximum in April to July and a minimum in September to December. Some studies have indicated an obvious seasonal character of the incidence of first clinical symptoms of breast carcinoma in women with the maximum between late spring and early summer when compared to the minimum in late autumn and early winter (Mason *et al.* 1985, Kirkham *et al.* 1985). Bartsch *et al.* (1994) found seasonal rhythmicity in pineal gland function when they observed urinary 6-sulphoxymelatonin excretion by female rats in the dark. The variability of values was recorded despite constant laboratory conditions including the light regimen and this may affect the variability of mammary carcinogenesis induced in rats during the year. In this context, the review of Cos and Sánchez-Barceló (2000) summarized the role of the main pineal hormone, melatonin, as an anti-tumor substance in mammary gland carcinogenesis. The administration of chemical carcinogens to rats with changed pineal gland function and consequently with altered levels of melatonin due to various photoperiods were the objects of many studies. Exposure of female rats to constant light since birth increased the incidence and shortened the latency of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary fibroadenomas in comparison with animals exposed to a light regimen L:D = 10:14 h (Kothari *et al.* 1982). The effect of melatonin seems to be dependent not only on the photoperiod, but also on the time of the day when the substance was administered. Melatonin applied in the morning supported tumor growth in mice. In contrast, its administration in the evening slowed down tumor growth (Bartsch and Bartsch 1981), while the experiments conducted under a light regimen L:D = 12:12 h or L:D = 8:16 h did not differ significantly. Halberg (1964) pointed out the significance of the time of DMBA administration in mammary carcinogenesis induction in mice. The administration of DMBA in the late afternoon increased tumor incidence when compared to that in the morning.

Susceptibility of the mammary gland to the carcinogen in mammary carcinogenesis depends on the age of animals. High susceptibility of female rats can be observed in carcinogen application between postnatal days 40-60 in early puberty with highly proliferating terminal end buds (TEBs) in the mammary gland (Huggins *et al.* 1959). Carcinogen administration to virgin rats induces the largest number of transformed foci in the mammary gland when the number of TEBs decreases due to their differentiation into alveolar lobules. The highest number of tumors per animal was observed when the carcinogen was given to animals aged between 40 and 46 days. This corresponds to the period when mammary gland exhibits the highest density of extensively proliferating and differentiating TEBs (Russo *et al.* 1979).

Isaacs (1986) divided rat strains according to their susceptibility to carcinogens into four groups. Besides very sensitive strains, he distinguished strains with medium sensitivity, e.g. outbred Sprague-Dawley; Copenhagen or Wistar-Kyoto strains were completely resistant. Our group (Ahlers *et al.* 1998) observed very low susceptibility of the Wistar:Han strain to induction of mammary carcinogenesis by a single dose of DMBA or N-methyl-N-nitrosourea (NMU). The repeated administration of NMU in critical periods of mammary gland development on postnatal days 3-4, 21 and 50-55 considerably increased the susceptibility of female Wistar:Han rats to the induction of mammary tumors (Bojková *et al.* 2000).

Variability of the susceptibility of mammary carcinogenesis with regard to the season, rat strain, age, carcinogen type and route of its administration in our experiments resulted in evaluation of 19 experiments. The data of individual experiments were compared with respect to a single selected parameter mentioned above as the simultaneous evaluation of variations in more parameters may be misleading.

Methods

Female outbred Sprague-Dawley rats (AnLab Prague, Czech Republic), Wistar:Han (Velaz, Prague, or Research Institute for Pharmacy and Biochemistry – RIPB, Pardubice, Czech Republic) aged 35-41 days were adapted to standard vivarium conditions (temperature 23±2 ^{0}C, relative humidity 60-70 %, artificial regimen light 12:12 h, light on at 07:00 h, with an intensity of 150 lux). The animals were fed the MP diet (Top-Dovo, Dobrá Voda, Slovak Republic) and drank tap water *ad libitum*. Mammary carcinogenesis was induced by NMU (Sigma, Deisenhofen, Germany) applied in a single dose of 30 mg/kg of body weight on postnatal day 50 (Experiments 17, 18), in three doses of 30 mg/kg b.w. between postnatal days 53-63 (Experiment 19) or in two doses of 50 mg/kg b.w. between postnatal days 43-58 (Experiments 6-10). DMBA (Sigma, Deisenhofen, Germany) was administered in a single dose of 20 mg/rat on postnatal days 50-53 (Experiments 1, 2 and 11-13) or in three doses of 10 mg/animal between postnatal days 43-63 (Experiments 3-5 and 14-16). NMU was

administered intraperitoneally and was freshly prepared by dissolving in 1 ml of saline solution. DMBA (dissolved in 0.5 or 1 ml corn oil) was administered intragastrically. The carcinogen was applied between 14:00 to 15:00. The animals were manually palpated and weighed each week. The incidence, number, size and localization of tumors were recorded. Around week 25 (except for Experiment 3, which lasted for 13 weeks) the animals were killed by rapid decapitation, mammary tumors were excised, measured and weighed. Tumor incidence was evaluated by the Mann-Whitney U-test; one-way analysis of variance or Kruskal-Wallis test were used to evaluate the differences in tumor frequency per group and animal.

All experiments were conducted according to the principles provided by the Law No. 115/1995 §24 of Slovak Republic for the Care and Use of Laboratory Animals.

Table 1. Mammary tumor incidence and frequency in female Sprague-Dawley rats (AnLab, Prague) induced by a single dose or by repeated doses of DMBA.

	DMBA 20 mg		**DMBA 3x10 mg**		
Experiment	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Season	Dec-May	Jun-Nov	Apr-Jul	Nov-Jun	Jun-Dec
Carcinogen application*	52	52	43-57	45-55	50-60
Number of animals	24	24	24	15	16
Tumor-bearing animals	11	16	23	15	13
Tumor incidence %	45.8	66.7	95.8	100.0	81.3
Tumor frequency per group[#]	1.13	1.54	4.33	3.40	5.81
Tumor frequency per animal[#]	2.45	2.31	4.52	3.40	7.15

**Postnatal days, [#] data are expressed as means*

Table 2. Mammary tumor incidence and frequency in female Sprague-Dawley rats (AnLab, Prague) induced by repeated doses of NMU.

	NMU (2x50 mg/kg)				
Experiment	Exp. 6	Exp. 7	Exp. 8	Exp. 9	Exp. 10
Season	Apr-Oct	Nov-Apr	Nov-May	Oct-Apr	Oct-May
Carcinogen application*	43-54	43-57	46-57	48-55	51-58
Number of animals	20	17	20	20	20
Tumor-bearing animals	16	15	16	14	17
Tumor incidence %	80.0	88.2	80.0	70.0	85.0
Tumor frequency per group[#]	3.65	2.29	2.20	2.10	1.30
Tumor frequency per animal[#]	4.56	2.60	2.75	3.00	1.53

**Postnatal days, [#] data are expressed as means*

Results

Tables 1-4 show the rat strains, their origin, season, time of carcinogen administration, number of animals, number of tumor-bearing animals, incidence and frequency of tumors per group or animal induced by DMBA or NMU in two strains – Sprague-Dawley and Wistar:Han rats.

Sprague-Dawley rats. In Experiments 1 and 2 (each performed in different season) a pronounced difference in the incidence (45.8 % *vs* 66.7 %, P=0.15) was found after a single standard dose of DMBA (Table 1). After repeated administration of DMBA in

Experiments 3-5 (Table 1) there were no significant differences in incidence; on the other hand, tumor frequency per animal significantly differ in Experiment 4 (started in late November) and Experiment 5 (started in June) (3.40 and 7.15, respectively; $P<0.05$). High values of tumor frequency per animal (7.15) in Experiment 5 were reached despite the shift of carcinogen application to a less sensitive period (postnatal days 50-60) in comparison with Experiment 4 (postnatal days 45-55). In Experiment 3, regarding its duration (lasted only 13 weeks during long days), very high values of incidence (95.8 %) as well as the incidence of tumors per group and animal (4.33 and 4.52, respectively) were recorded. In Experiments 6-10 (Table 2) with NMU applied in two doses, the incidence of mammary tumors was from 70.0 to 88.2 %. In Experiments 7-10 (carried out in the same season), a markedly increased tumor frequency per group was observed and that might have been the consequence of differences in the age of animals at the time of first carcinogen administration (frequency: 2.29 – 2.20 – 2.10 – 1.30; age: 43 – 46 – 48 – 51 postnatal days). The comparison of Experiments 6 and 7 (with the same age of carcinogen application), carried out in summer and winter, respectively, showed a substantial increase in tumor frequency per animal during the long days (4.56 and 2.60, respectively).

Wistar:Han rats. After a single dose of DMBA (Table 3) in Experiments 11-13, the average incidence under 10 % and average frequency per group 0.13 were observed. Comparison of Experiments 14 and 15 (started at the beginning of winter) with Experiment 16 (initiated in June) revealed the decrease in incidence by 48 % ($P=0.07$) and 49 % ($P=0.08$), respectively, and in the frequency per group in both experiments by 79.5 % ($P<0.01$) (Table 3). As is shown in Table 4, after a single dose of NMU in Experiments 17 and 18, the incidence of tumors was 10.0 % and their frequency per group was 0.10. After repeated carcinogen administration, the incidence of mammary tumors increased to 30.4 % and frequency per group to 0.43 (Experiment 19).

Table 3. Mammary tumor incidence and frequency in female Wistar:Han rats from two breeding farms (Velaz Prague and RIPB Pardubice) induced by a single dose or by repeated doses of DMBA.

	DMBA 20 mg			**DMBA 3x10 mg**		
Experiment	Exp. 11	Exp. 12	Exp. 13	Exp. 14	Exp. 15	Exp. 16
Breeder	Velaz	RIPB	Velaz	Velaz	Velaz	Velaz
Season	Oct-Mar	Oct-Mar	Nov-Apr	Nov-Apr	Dec-Apr	Jun-Dec
Carcinogen application*	50	50	53	51-62	51-62	50-60
Number of animals	19	19	20	17	14	30
Tumor-bearing animals	2	1	2	5	4	17
Incidence %	10.5	5.3	10.0	29.4	28.6	56.7
Tumor frequency per group[#]	0.21	0.05	0.10	0.29	0.29	0.80
Tumor frequency per animal[#]	2.00	1.00	1.00	1.00	1.00	1.41

**Postnatal days, [#] data are expressed as means*

Discussion

The results obtained from 10 experiments in female Sprague-Dawley rats (Tables 1 and 2) point to the seasonal variability of induction of mammary tumors by chemical carcinogens DMBA and NMU. In order to avoid the variability in susceptibility of mammary carcinogenesis due to different photoperiods (natural, artificial light) and administration of the carcinogen at different times of the day, the same light regimen L:D = 12:12 h was used and the carcinogen was applied between 14:00 and 15:00 h in all the experiments. The markedly lower incidence of mammary tumors observed in Experiment 1 (initiated in December) in comparison with Experiment 2 (initiated in June), with the same postnatal period of DMBA application, confirmed the hypothesis of seasonal variability in mammary gland cancer induction (Table l). The seasonal effect was observed when comparing Experiments 3 and 4, where an increase in the frequency of tumors per animal was found in Experiment 3 (carried out predominantly during long days) in comparison with Experiment 4 (carried out predominantly in winter) (4.52 *vs* 3.40). The high incidence and high tumor frequency per group and animal

were found (Table 1) regarding the duration of Experiment 3 (which lasted only half of the period of Experiment 4).

Table 4. Mammary tumor incidence and frequency in female Wistar:Han rats from two breeding farms (Velaz Prague and RIPB Pardubice) induced by a single dose or by repeated doses of NMU.

	NMU		
	30 mg/kg		**3x30 mg/kg**
Experiment	Exp. 17	Exp. 18	Exp. 19
Breeder	Velaz	RIPB	Velaz
Season	Nov-Apr	Oct-Apr	Apr-Sep
Carcinogen application*	50	50	53-63
Number of animals	20	20	23
Tumor-bearing animals	2	2	7
Tumor incidence %	10.0	10.0	30.4
Tumor frequency per group#	0.10	0.10	0.43
Tumor frequency per animal#	1.00	1.00	1.43

**Postnatal days, # data are expressed as means*

Experiment 5 with mammary carcinogenesis initiated during the longest days of the year attained high values of tumor frequency per animal (7.15), despite the shift of carcinogen application to the less sensitive period (postnatal days 50-60) in comparison with postnatal days 40-46 (Russo *et al.* 1979). Comparison of Experiments 6 and 7 (also with the same postnatal day of NMU administration), which were performed mainly in the summer or winter, showed a 43 % decrease in the frequency of mammary tumors per animal during the short days (Table 2). The data correspond with those of Löscher *et al.* (1997), who observed a seasonal variability in the incidence of DMBA-induced mammary carcinogenesis in female Sprague-Dawley rats: autumn values (34 %) differed from those in the spring (62 %). Nelson and Blom (1994) supposed that both people and animals increase the "adjustment" of the immune system during the short day, thus helping in the protection against "winter" stress factors. This statement was documented by a significant decrease in the incidence of tumors in DMBA-induced mammary carcinogenesis in female mice during the short day in comparison with the long day. Bartsch *et al.* (1994) observed seasonal changes in the pineal melatonin synthesis in females of the Fischer 344 rat strain despite a standard light regimen. They found that the horizontal component H of the geomagnetic field (GF) showed seasonal oscillations opposite to melatonin levels. The mean monthly values of the horizontal component of the GF showed the highest values in summer and the lowest ones in autumn and winter. Reiter (1993) pointed out the fact that GF decreases the synthesis of pineal melatonin. Similarly, Löscher *et al.* (1993) indicated in experiments on rats that the potential effect of GF on melatonin formation decreased. Bartsch and Bartsch (1981) observed a prolongation of the mammary tumor latency after administration of DMBA in the females of Fischer 344 strain performed in January in comparison with the experiment performed in July. Moreover, they recorded a shift to a lower number of malignant tumors after administration of the carcinogen in the winter (H. and C. Bartsch – personal communication). It follows that a short day in late autumn and winter accompanied with reduced intensity of GF and higher formation of pineal melatonin may explain the lower incidence and frequency of mammary tumors in our experiments despite the regulated light regimen (12:12 h). A seasonal character of the incidence and frequency of mammary tumors can also be observed in the Wistar:Han strain (Table 3) when comparing the experimental groups 14, 15 with the group 16 (incidence 29.4 %, 28.6 %, frequency per group 0.29, frequency per animal 1.00 *vs* 56.7 %, 0.80, 1.41). A single standard dose of DMBA and NMU administered to the Wistar:Han strain from a different breeding station (Velaz Prague or RIPB Pardubice – Experiments 11-13 and 17-18) showed a low incidence (maximum 10.5 %) as well as frequency of mammary tumors (Tables 3 and 4). The same rat strain was obtained from the second breeder (RIPB Pardubice) in order to confirm the low susceptibility to chemical carcinogens that was originally found in rats from the Velaz (Prague) breeder (Ahlers *et al.* 1998).

Our results have confirmed the fact that Wistar:Han rats are considerably resistant to "classical" carcinogens involved in mammary carcinogenesis initiation. In contrast, the experiments 1-10 with Sprague-Dawley strain using both single and repeated doses have confirmed high susceptibility of this strain to the carcinogen. Based on the classification of Isaacs (1986) we can confirm the classification of the Wistar:Han rats as a group with "very low susceptibility" to mammary carcinogens (Ahlers *et al.* 1998). Thus, the Wistar:Han strain is not suitable for experiments with carcinogen-induced mammary tumorigenesis. From this point of view the studies with the resistant Copenhagen strain

appear to be of interest with regard to the peroral and locally intraductal application of DMBA and intravenous administration of NMU (Isaacs 1986). The presence of Mcs gene (mammary carcinoma suppressor) – a single autosomal dominant gene localized in the centromere region of chromosome 2 (Hsu *et al.* 1994) – is considered to be the cause for the resistance of this strain to mammary carcinogenesis. Lu *et al.* (1992) showed the possibility of breaking the resistance to chemically induced mammary carcinogenesis in the Copenhagen rat strain by NMU administration on postnatal days 2 and 3. The administration of NMU in critical periods of mammary gland development on postnatal days 3-4, 21 and 50-55 considerably increased the sensitivity of female Wistar:Han rats to the induction of mammary tumors (Bojková *et al.* 2000). Similarly, repeated administration of DMBA in three consecutive intervals between postnatal days 50-60 (Experiment 16; Table 3) restored the sensitivity of Wistar: Han females to DMBA induction of mammary tumors to the range of "high" sensitivity. The possibility of restoring mammary carcinogenesis initiation revealed rather a non-genetic background of Wistar:Han animals with an originally "very low" sensitivity. Shepel *et al.* (1998) confirmed that the resistance of the Copenhagen strain is polygenic in character and includes genes suppressing tumorigenesis (Mcs1, Mcs2, Mcs3), or a gene increasing the sensitivity to the chemical carcinogen (Mcs4), respectively. The sensitivity genes were found in highly sensitive Wistar:Furth and Sprague-Dawley strains. In the Fischer 344 strain (possessing a medium sensitivity) the resistance or sensitivity genes were not found. The onset and development of mammary tumors do not require the sensitivity genes and the loss of resistance genes appears to be important, but not necessary for carcinogenesis induction (Shepel and Gould 1999). This fact was also confirmed by the results of Harvell *et al.* (2000), who compared the specificity of the effect of 17β-estradiol in mammary gland tissue in the genetically closely related rat strains ACI and Copenhagen, finding significantly higher proliferative activity in the ACI strain. Festing (1995) postulated the necessity to use more than one rat strain for the analysis of xenobiotic effects in the organism, including induction of neoplasia, otherwise leading to incorrect conclusions. We decided to use females of the less sensitive Wistar:Han strain and of the more sensitive outbred Sprague-Dawley strain for mammary carcinogenesis induction, whereby we partially imitated the situation in the human populations (not only in relation to the appearance of neoplasia) divided into more and less sensitive individuals.

The mammary gland sensitivity in female rats to DMBA- or NMU-induced carcinogenesis is considerably dependent on age. Huggins *et al.* (1959) defined the period of high sensitivity to chemical carcinogen in early puberty between postnatal days 40-60. In our opinion, the highest incidence and frequency of tumors per animal may be attained by application of the carcinogen between postnatal days 40-46, in the period of maximal density of highly proliferating TEBs (Russo *et al.* 1979). This assumption was confirmed by an apparent increase in the frequency of tumors per group in Experiments 7-10 which were inversely dependent on postnatal period, in which the first dose of the carcinogen was administered (Table 2). In Experiment 6, a pronouncedly higher frequency in comparison with other groups was observed, probably due to combination of the first dose of the carcinogen applied in the most sensitive period on postnatal day 43 and long days, when the experiment was carried out. In Experiment 3, the first dose of the carcinogen was applied on postnatal day 43 and high values of the incidence and frequency of mammary tumors were recorded despite the fact that the experiment lasted only 13 weeks. These results have clearly confirmed the importance of the animals` age at the time when experimental mammary cancer is induced.

Neoplasia of the mammary gland in experimental models is always a result of the binding of the carcinogen to the target tissue. NMU and DMBA are the most frequently used chemical substances for mammary tumor induction in rats. DMBA interacts with the DNA *via* epoxides, arising in the course of its metabolism. The effectiveness of DMBA in mammary carcinogenesis may be to a certain extent unpredictable; in contrast to NMU, which represents an alkylating substance binding itself directly to DNA (Russo and Russo 1996). Comparison of the incidence and frequency of tumors in rats with single intragastric administration of 20 mg of DMBA (Experiments 1 and 2) with those when NMU was administered intraperitoneally in two doses (Experiments 6-10) by 50 mg/kg (total dose about 15 mg of NMU) has confirmed the higher efficiency of NMU to induce mammary cancer in our experiments. This corresponds with the results of McCormick *et al.* (1981).

Results of the experiments in the sensitive Sprague-Dawley strain suggest that season may play a substantial role in experimental mammary carcinogenesis in female rats. Circannual oscillations in the pineal melatonin production might be a possible explanation for

this phenomenon, despite the fact that animals were housed under standard laboratory conditions. A key factor influencing the mammary carcinogenesis in laboratory rats is the limited period of carcinogen application. The optimal intervention is in early puberty around postnatal day 43, at the time of the highest density in extensively proliferating TEBs. The experiments for mammary tumorigenesis using Wistar:Han strain have confirmed a very low sensitivity to the carcinogen (Ahlers *et al.* 1998). Twofold intraperitoneal application of NMU using the dose of 50 mg/kg or triple intragastric administration of DMBA in the critical period of mammary gland development after postnatal day 43 provided good reproducibility of our results on experimental mammary carcinogenesis in rats. The results based on one strain of experimental animals only might lead to biased conclusions. Generally, we may agree with the principle of "multi-strain assay" (Festing 1995), where the results obtained from two or more strains are compared.

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