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THE LACK OF A MODIFYING EFFECT OF TERBUTHYLAZINE ON MAMMARY CARCINOGENESIS INDUCED BY POLYCYCLIC HYDROCARBON — 7,12-DIMETHYL- BENZ[A]ANTHRACENE IN RATS

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ABSTRACT. Objective*. To evaluate the possibility of promoter Terbuthylazine action on mammary carcinogenesis induced by polycyclic hydrocarbon — 7,12-dibenz[a]anthracene (DMBA) in female Wistar Han rats.*
Materials and Methods. *The experiments were performed in 60 Wistar Han female rats with a body weight of 105–125 g, 45 of which were initiated by DMBA, and 15 served as a control for study of the effect of Terbuthylazine. Initiation was performed in accordance with the protocol of Anderson L.E., 1999. DMBA was administered intragastrically as a 2.5 % oil solution in a dose of 50 mg/kg of body weight daily for 4 weeks. After a one-week break, Terbuthylazine was administered intragastrically on a daily basis in the doses of 0.3 mg/kg and 30.0 mg/kg of body weight, corresponding to the inactive and active level by a carcinogenic effect. In the control group, Terbuthylazine was administered in the dose of 30 mg/kg for DMBA effect. Terbuthylazine administration duration — 16 weeks. During the experiment, clinical studies were conducted. The general condition of animals, their body weight and weight gain, the time of tumour nodes appearance, their number and area were estimated. After necropsy, a macroscopic and a histological examination was performed. Tumours number, their sizes, histological type and metastases presence were determined.*
Results*. Terbuthylazine had no toxic effect on the body of rats, which had not been initiated by DMBA. In groups of rats initiated by DMBA, equal mortality of animals (13 %) was observed before tumours appearance. Terbuthylazine at a dose of 30 mg/kg caused higher mortality (14 % higher) of animals with tumours and reduced their lifetime in comparison with the control and a low dose. There was no difference in the overall dynamics of changes in the mean group parameters of body weight, body weight gain, and its cumulative gain in rats on Terbuthylazine compared to the control. Statistically significant data on changes in tumour incidence, duration of the latent period of their appearance, the number of tumour nodes, their growth, localisation, and degree of malignancy, indicative of a promoter Terbuthylazine action on mammary carcinogenesis, were not observed.*
Conclusion*. Terbuthylazine has no promoter action on DMBA-induced mammary carcinogenesis in rats.*
Key words: *carcinogenesis, mammary gland, polycyclic hydrocarbons, 7,12-dibenz[a]anthracene, DMBA, pesticides, Terbuthylazine, promoter action, Wistar Han female rats.*

Since 1967, pesticides based on Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine, IUPAC), pre- and post-emergence herbicides were used for the first time. Currently, they are used in different countries. The mode of action of this substance on plants is to inhibit photosynthesis processes. Its toxicological properties have been well studied. Terbuthylazine is a moderately hazardous substance. Long-term experiments in two animal species — rats and mice — resulted only in limited evidence of its carcinogenic activity, namely: an increase in the incidence of mammary tumours and Leydig tumours in rats. Based on this data, EPA Carcinogenicity Peer Review Committee has classified Terbuthylazine as a group D carcinogen — one for which these is inadequate evidence to determine carcinogenicity in humans. EU experts classified Terbuthylazine as a group R40 — Limited evidence of carcinogenic effect. In accordance with the current hygienic classification of pesticide hazard in Ukraine, this substance belongs to the third hazard class [1–3]. Terbuthylazine causes dysfunction of the endocrine system and reproductive function [1, 2, 4].

According to its chemical structure, Terbuthylazine belongs to the chemical class of chlorotriazines. The most studied representative of this class of compounds is atrazine, which also induced mammary tumours in Sprague-Dawley rats [5]. A careful examination of the mechanism of mammary carcinogenesis in these rats has shown that it is associated with a species-specific hormonal imbalance. IARC experts came to the conclusion that the identified oncogenic effect is not relevant to the human body [6]. However, endocrine system dysfunction caused by these

compounds [7] may significantly influence the development of mammary cancer induced by other factors. Among such factors, carcinogenic polycyclic hydrocarbons are prevailing in the environment. It is shown that atrazine accelerates the development and growth of mammary tumours induced by 7,12-dibenz[a]anthracene. The authors note the possibility of atrazine effect on other branches of the endocrine system [8].

This experimental model in various modifications is widely used both to determine the factors impeding the development of mammary tumours [9] and as a medium-term test to identify potential carcinogens which are promoters of mammary cancer, especially those that act through endocrine system disorders [10–13]. Given that mammary cancer is a prevailing type of cancer in women, studying of endocrine disruptor substances effect on mammary carcinogenesis is the priority both in experimental oncology and in toxicology. The results of these studies allow to specify the identification of carcinogenic hazard in the evaluation of the risk of using such compounds in human activities [12,13].

The objective of this paper was to examine the possibility of promoter Terbuthylazine action on mammary carcinogenesis induced by polycyclic hydrocarbon — 7,12-dibenz[a]anthracene (DMBA) in female Wistar Han rats.

Materials and Methods. The programme of the experimental studies above fulfils the requirements of the Bioethics and Animal Welfare Committee [14].

In this model, Sprague-Dawley rats are usually used [8, 10–13], but Wistar rats are also acceptable [9]. Wistar Han rats were obtained from SPF nursery of small laboratory animals of the SI "L. I. Medved's Research Center of Preventive Toxicology, Food and Chemical Safety, Ministry of Health of Ukraine". 60 animals with an average body weight of 105–125 g and placed to a clear area of the vivarium. After 7-day acclimatisation, randomization was performed. According to veterinary examination, all animals were healthy.

After randomization, rats were allocated to 4 groups: Group 1 — control (15 rats), Group 2, 3 and 4 — experimental (15 rats per each group).

Animals were placed to a clear area of the barrier-type vivarium. Experimental animals were under the same conditions, 5 rats per a cage. Sterilised non-chlorinated food paper was used as a litter. The room was equipped with a forced ventilation (12 volumes per an hour) with a prepared air. Temperature and relative humidity were registered on a daily basis, temperature fluctuations: 20–21 °C, humidity fluctuations: 40–50 %. Lighting in the rooms: Fluorescent lamps (12 hours of light, 12 hours of darkness). During the experiment, rats received balanced granulated diet Altromin (Germany) and disinfected filtered water.

In the experiments for carcinogenesis initiation, 7,12-dibenz[a]anthracene (DMBA), 95%, was used. D3254-1G. CAS 57-97-6) manufactured by Sigma-Aldrich Co., USA. DMBA was dissolved in sesame oil. Initiation was performed in accordance with the protocol [10]. DMBA was administered intragastrically on a daily basis as a 2.5 % oil solution in a dose of 50 mg/kg of body weight for 4 weeks. After a 7-day break, study factor was initiated.

95 % technical Terbuthylazinel corresponding to the standards of FAO and EU and containing no genotoxic and carcinogenic impurities was used [2, 15]. Terbuthylazine solutions were prepared ex tempore with drinking water for animals with OP-10 emulsifier. Aquatic solution with OP-10 equalled 0.05 %.

The doses of Terbuthylazine selected for the experiment at the level of 0.3 mg/kg and 30.0 mg/kg of body weight correspond to the inactive and active level established in the toxicological evaluation of its effect on the body [1, 2]. Concentrations of the solutions: for a low dose — 0.003 % solution, for a high dose — 0.3 % solution. Administration method: Terbuthylazine was injected under fasting conditions intragastrically by means of a syringe with a metal probe in an acceptable volume for small laboratory animals [16]. Control animals (Group 1) received drinking water with OP-10 under the same conditions as experimental ones.

Animals of Group 2 and 3 received study product for 16 weeks — on a daily basis for 5 consecutive days per week. Duration of exposure at the level of 16 weeks (and for the whole experiment — 20 weeks) was defined on the basis of literature data — tumours in animals begin to appear at Week 6 and reach significant size for the remaining time or become a reason of animals death [10–13].

Animals of Group 4 received Terbuthylazine for 20 weeks — on the daily basis for 5 consecutive days a week. This group was used as a control for the toxic effect of DMBA. Duration of the experiment at the level of 20 weeks for this group included both the period of potential carcinogenesis initiation with Terbuthylazine and the period of promotion.

During the experiment, a daily clinical examination of animals was conducted to identify any abnormalities associated with the action of the substance, namely: changes in behaviour, mobility, appetite, condition of hair, skin and mucous membranes of animals. Death of animals was also registered. For 21 weeks, the body weight of animals and its gain was monitored every 7 days. 4 weeks after the last dosing of DMBA and until the death of the animal, daily palpation of a rat was performed to detect mammary swelling and/or tumour nodes in the mammary. Each week, sizes of detected tumour nodes were measured and their areas were determined during weighing and examination of animals. Anatomically, female rats have six pairs of mammary glands and twelve nipples, respectively. Therefore, node location, its mobility and external condition of the tumour were determined.

Animals were sacrificed on the next day after the end of the exposure (Day 142) in a special chamber by the inhalation intake of carbon dioxide, in accordance with the rules of animal welfare, in accordance with the requirements of the European Convention for the Protection of Experimental Animals 86/609 EC [14].

An autopsy was performed in all animals. Before the necropsy, examination of the corpse was performed and all abnormalities were registered. During the necropsy, macroscopic examination of all organs and tissues of animals was performed according to the guidelines [17]. Location, size, shape, and colour of tumour nodes were recorded. Material for histological processing was sampled. All organs and tissues with visual changes observed were also subjected to histological processing, which was performed according to the generally accepted method [18].

Histological diagnostics of changes in the selected samples was performed with due account for core parameters, namely: degree and nature of lesions, the expressiveness of compensatory changes, and the presence of pretumour and tumour changes were taken into account. Pretumour and tumour changes in mammary tissues were diagnosed in accordance with the recommendations of the IARC monograph [19].

Quantitative parameters of a carcinogenic effect were the following: an increase in the tumour incidence compared with the effective number (EN), an increase in the multiplicity factor, a reduction in the latent period of tumour appearance and the degree of their malignancy, tumour growth rate, and mortality of animals with tumours [20, 21]. Promoter Terbuthylazine effect was evaluated on the basis of changes of these carcinogenesis parameters.

Statistical evaluation of Terbuthylazine on the body and carcinogenesis was performed by comparing the parameters of experimental group subjects and the control ones using different methodological approaches. The precision of the data distribution to the normal one was defined using Kolmogorov-Smirnov and the Shapiro-Wilk tests. In the case of normal distribution, a parametric, two-sided, t-test for independent samples was used. In the case of abnormal distribution, the non-parametric Pearson $\chi 2$ test or Mann-Whitney U test [20, 21] was used.

Univariable analysis of variances (ANOVA) was used to objectively assess the statistical significance of the differences between the parameters. The homogeneity of variables was evaluated using Cochran, Hartley, Bartlett tests. ANCOVA was used to correct the results with respect to changes in the indices depending on the tumour growth period. The period from Week 6 to Week 16 (until sacrification due to humane consideration) was used as a covariance variable. Tumours incidence in the experimental and control groups was also evaluated considering the effective number, using Kaplan-Meier method. Significance of differences was evaluated using Log-rank test and Peto test [20, 21].

Statistical calculations were performed using a package of the software: Excel 2010, Statistic 6.0, Stat Soft. The product licensed to XP Game 2007 Russia 31415926535897.

Results and Discussion. Terbuthylazine at a dose of 30 mg/kg had no toxic effect on the body which would lead to the death of animals. As it can be seen in Fig. 1, one rat in Group 4 died at Week 6 of the experiment. The reason of death was mechanical asphyxia.

In the group of rats having injections of the initiator — DMBA carcinogen (Fig. 10), the part of animals died after the 4th injection (from Week 4 to Week 10). It confirms the data on the toxic dose of this substance [20, 23, 24]. Therefore, 2 rats in each group died during the period of initiation (until Week 5). Hereinafter, as the tumour process develops, mortality of rats had been increasing. During this period, 3 rats more died in the control group (Group 1), 5 rats — in Group 2 and 7 rats — in Group 3. Despite the fact that the data presented have no statistical difference ($p \geq 0.05$), here we can suggest the increased mortality of rats receiving a high dose of the study product. However, in the control group on high Terbuthylazine dose without DMBA initiation, only one death was observed. This fact indicates that the cause of animal death is the toxic effect of DMBA and the development of tumours. During the period of manifestation and growth of tumours (from Week 7 to Week 14 of the experiment), the mortality of animals in all groups being initiated was the same, 5 rats died in each group. In subsequent stages of tumour growth, animal death was observed only in the experimental groups. Among the animals receiving Terbuthylazine at a dose of 0.3 mg/kg, 2 deaths were observed, at a dose of 30 mg/kg — 4. By the end of the experiment, 5 animals of the control group died, 2 of them had the tumour (40 %), 7 animals receiving the dose of 0.3 mg/kg of Group 2 died, 3 of them had the tumour (33 %). In Group 3, 9 animals receiving the dose of 30 mg/kg died, 6 of them had the tumour (66 %). The average life span of animals with tumours was the following: Group 1 — 124.4 days, Group 2 — 126.7 days, Group 3 — 116.4 days. Therefore, Terbuthylazine at a dose of 30 mg/kg caused higher mortality in animals with tumours. However, statistical data processing revealed no significant differences in mortality rates of control and experimental animals.

Clinical examinations of Group 4 animals revealed no toxic effect of Terbuthylazine. During the experiment in all experimental groups, animals which did not have tumours or ulcerated tumours willingly ate food and consumed water. Their behaviour and motor activity had no differences. At the same time, as mammary tumours grow, especially in case of their ulceration, rats started to lose weight, weakened, and became apathetic. No other clinical symptoms associated with Terbuthylazine were observed.

Body weight and its gain were statistically significantly higher in animals have hot been initiated with DMBA and receiving only Terbuthylazine at a dose of 30 mg/kg, in comparison with animals of other groups (Fig. 2).

In animals being initiated with DMBA, no statistically significant body weight changes and body weight gain were observed under the influence of Terbuthylazine, in comparison with the control group. The overall dynamics of the mean group parameters of the body weight in rats on Terbuthylazine at a dose of 0.3 mg/kg was 4 % higher than in rats on 30.0 mg/kg, and 5 % lower than in animals of the control group ($p \geq 0.05$).

In animals receiving DMBA and Terbuthylazine, statistically significant short-term changes in the body weight gain were

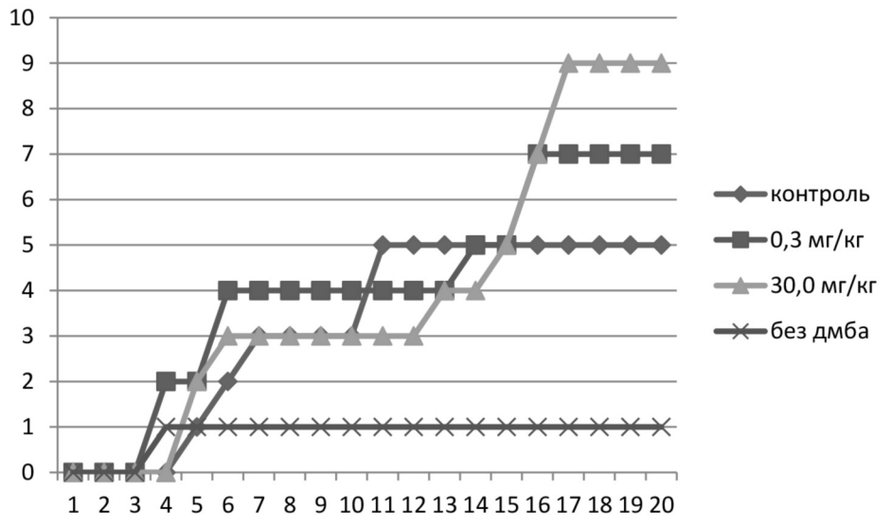

Fig. 1 Cumulative mortality of animals during DMBA-induced mammary carcinogenesis and Terbuthylazine exposure.

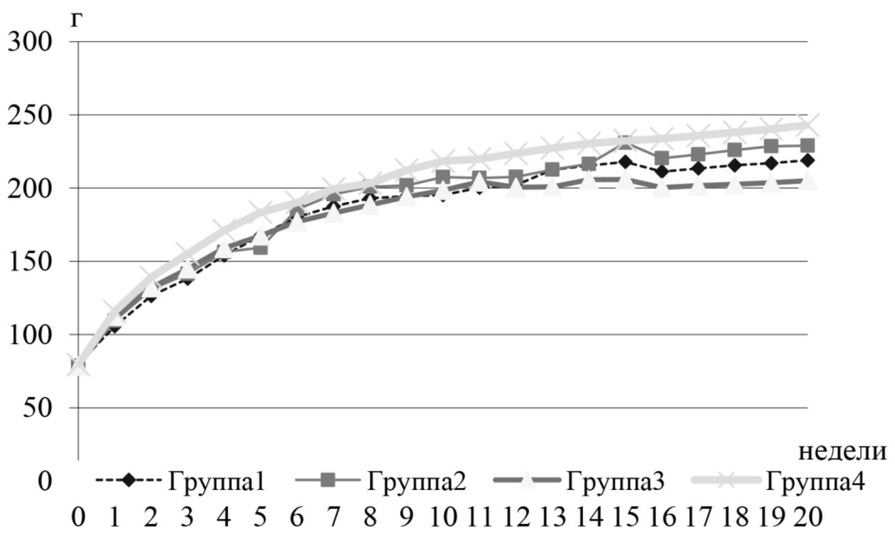

Fig. 2. Change in body weight of animals receiving Terbuthylazine. Group 1 — DMBA and water. Group 2 — DMBA and Terbuthylazine at a dose of 0.3 mg/kg. Group 3 — DMBA and Terbuthylazine at a dose of 30 mg/kg. Group 4 — Terbuthylazine at a dose of 30 mg/kg.

observed, in comparison with the control group. These changes had no toxicological significance. There was no difference in the overall dynamics of changes in the mean group parameters of body weight gain and the cumulative gain in rats on Terbuthylazine compared to the control ($p \geq 0.05$). No general toxic effect of Terbuthylazine according to body weight gain data was defined.

Palpation of animals enabled us to control the appearance of mammary tumours at an early stage and trace the dynamics of their growth. Mammary neoplasms were observed only in rats initiated with DMBA (Group 1, 2, and 3). First tumours were observed at Week 6 of the experiment corresponding to the 3rd week following the last DMBA administration.

At this stage of the process, palpation of rats' mammary revealed a small solitary node of a soft, doughy consistency, which subsequently consolidated, increased in its size and often became knobby. Further development of the process led to the rigidity of the tumour and tension of the skin covering the neoplasm. Some of the tumours observed were tightly fixed to the adjacent tissues. By the end of the experiment, many tumours became ulcerous with a further formation of the fistula with necrotic degradation. The average number of nodes observed in the animals of the control and experimental groups increased with the advancement of the experiment.

With the advancement of the experiment, mammary neoplasms were characterised by multiplicity and several neoplasms were palpable as a rule on one or another side of one or another pair of mammarys. The most common locations of mammary tumours were the following: infraclavicular, inguinal, or abdominal region. The following numbers of neoplasms were observed in rats of different groups according to their localisation: the third pair of mammarys — 8 to 9 nodes, the fifth and the sixth pair — 3 to 9 nodes. Less often, the second and the fourth pair were affected.

The number and the rate of the development of these nodes characterises the intensity of neoplastic transformation of mammary tissues and is an indicator of carcinogenesis promotion. Fig. 3 shows the dynamics of tumour nodes detection in the mammary of rats.

As it can be seen from the figure, the shape of curves showing the detection of tumour nodes in rats from the moment of Terbuthylazine exposure does not differ from the control.

Therefore, Terbuthylazine had no effect on the common pattern of DMBA-induced carcinogenesis.

At Weeks 8, 9 and 10, the average number of tumour nodes in rats of Group 2 exceeded this parameter in control animals by 28.6 %, 22.7 % and 12.9 %, respectively, and in rats of Group 3 — by 78.5 %, 59.1 % and 25.8 %, respectively. As it can be seen from the data presented, such stimulation of tumour development is dose-dependent, although it is not statistically significant. As time passed, the number of tumour nodes in the control group increased with a higher rate reached the values of the experimental group and even exceeded them.

In the period of tumour growth, no statisti-

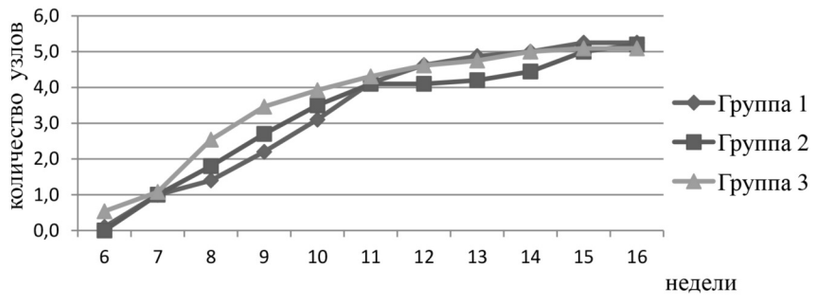

Fig. 3. The dynamics of tumour nodes detection in the mammary of rats. Group 1 — control, DMBA and water. Group 2 — DMBA and Terbuthylazine at a dose of 0.3 mg/kg. Group 3 — DMBA and Terbuthylazine at a dose of 30 mg/kg.

cally significant difference in the integral area of nodes in all groups was not observed and had the same principle (Fig. 4).

Visual examination of internal organs and tissues during the autopsy of rats of different groups reveals no statistically significant difference in localisation between groups being initiated ($p \geq 0.05$). This difference is observed only in animals not being initiated and receiving high Terbuthylazine dose. In these group of rats, no visible pathological changes of non-tumour and tumour aetiology were observed.

Mammary tumours observed in rats were often located directly under the skin or shallowly in the loose subcutaneous tissue. Size and consistency of tumour mammary nodes observed during palpation did not always correspond to the description obtained by visual examination of dissected rats.

By the end of the experiment, mammary neoplasms were observed in 10 females of Group 1 and 2 and in 12 females of Group 3. Ulceration was observed in 60 % of animals in Group 1 and 2, and in 75 % in Group 3.

Therefore, an average area of a node per an animal having mammary tumours was 3.6 cm^2 in Group 1, 2.5 cm^2 in Group 2 and 2.6 cm2 in Group 3. Histological examination showed that all mammary tumours detected at autopsy of dead and sacrificed animals were malignant. Diagnosed mammary tumours were confirmed as luminal-like mammary cancer with a predominance of cribriform and solid structures. These results are consistent with the literature data, according to which DMBA-induced tumours were represented by multifocal ductal carcinomas or papillary cancers in the majority of cases [11, 20].

Metastases of detected tumours were observed in several cases — in the thymus (invasion of the vessel) in one animal in Group 1 and in one in Group 2. Metastases in the liver were observed in one animal in Group 2 and in one animal in Group 3.

Therefore, Terbuthylazine had no influence on the incidence of metastatic spreading of DMBA-induced mammary tumours during the experiment.

Summarised evaluation results of a carcinogenic effect in rats receiving DMBA and Terbuthylazine is presented in Table 1. As it can be seen from the table, the incidence of tumour detection in rats receiving Terbuthylazine at a dose of 30 mg/kg is 17 % higher in comparison with the control one.

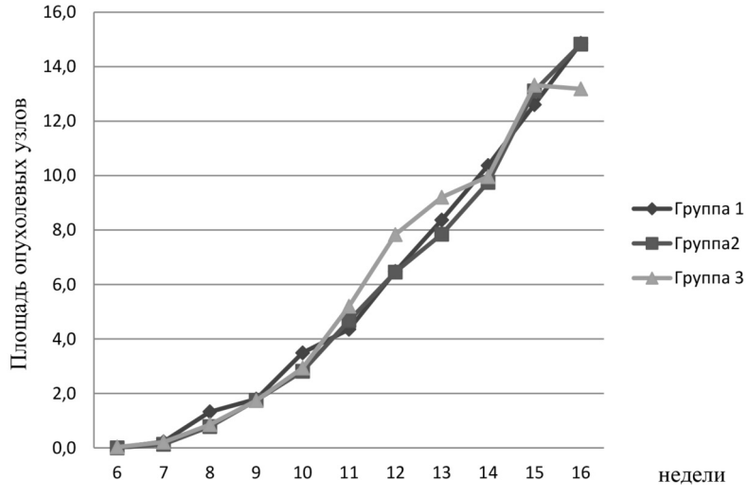

Fig. 4 Change in the average total area of mammary tumours in rats induced by DMBA under the influence of Terbuthylazine

However, this increase was not statistically significant when evaluated using different statistical methods of evaluation. No difference was also observed in the latent period of the appearance of these tumours. The first tumours were observed almost at the same time in all groups, the difference was several days. The duration of an average latent period of animals receiving DMBA and Terbuthylazine was higher (5–8 days more) in comparison with the control. All studied tumours were malignant. The number of metastatic tumours in all groups was low. No statistical difference in this parameter between groups was observed. The total number of mammary tumour nodes of various locations was 27 % higher in animals on low Terbuthylazine dose. In animals on high Terbuthylazine dose, this parameter was at the level of the control one. Analysing the dynamics of the increase in tumour nodes in this group, their appearance in the later stages of the experiment should be noted. The small size of these nodes significantly reduced the average area of tumour nodes in this group, which was statistically significantly lower by 31 % in comparison with the control. The average area of the tumour node was lower in comparison with the control and the animals receiving Terbuthylazine at a high dose by 28 % ($p < 0.05$).

The small size of these nodes significantly reduced the average area of tumour nodes in this group, which was statistically significantly lower by 31 % in comparison with the control. The average area of the tumour node was lower in comparison with the control and the ani-

Table 1

Carcinogenic effect parameters

Parameters	Group 1 – 0.0 mg/kg	Group 2 – 0.3 mg/kg	Group 3 – 30.0 mg/kg	Group 4 – 30.0 mg/kg (without DMBA initiation)
n	15	15	15	15
Effective number, abs. (%)	12 (80)	11 (73)	12 (80)	—
Number of animals with tumours, abs. (%)	10 (83)	10 (91)	12 (100)	н/о
p - χ^2 with Yates correction	—	1,0	0,46	—
p — Fisher exact test (unilateral)		0,50	0,24	—
p — Peto method	—	1,00	0,58	—
Latent period of the 1st tumour, weeks (days)	6 (45)	7 (49)	6 (45)	—
Average latent period of tumours, weeks (days)	11 (75)	12 (83)	11 (80)	—
Histological type of the 1st tumour	carcinoma	carcinoma	carcinoma	—
Multiplicity factor, R	4,7	6,0	4,8	—
Number of malignant tumours, abs. (%)	10 (100)	10 (100)	12 (100)	—
The ratio of benign/malignant tumours abs. (%)	0/10 (0/100)	0/10 (0/100)	0/12 (0/100)	—
Number of animals with metastatic tumours	1	2	1	
Size of mammary tumours, cm^2	3,6±1,3	2,5*±1,4	2,6±1,7	—
p/t	—	0,04	0,11	—
Died animals, abs. (%)	5 (33)	6 (40)	9 (60)	1 (7)**
Cox F-test		0,41	0,35	
Died animals with tumours compared to the effective number abs. (%)	2 (17)	3 (27)	6 (50)	н/о
			0,19	
Average life expectancy of dead animals, weeks (days)	11 (77)	8 (60)	11 (76)	6 (28)**

Note: * — $p<0.05$; ** — accidental death

mals receiving Terbuthylazine at a high dose by 28 % ($p < 0.05$).

The average life span of dead animals of the control group and the group of animals on Terbuthylazine are the same, which makes it possible to compare the number of dead animals with tumours. In the group of animals on high Terbuthylazine dose, a proportion of such animals was 50 % in comparison with 17 % of the control ($p \geq 0.05$).

Since the changes revealed are not confirmed statistically, they cannot be considered sufficient evidence of the Terbuthylazine influence on the course of DMBA-induced carcinogenesis in Wistar Han female rats. However, it should be noted that the revealed tendencies of the positive influence of Terbuthylazine at a dose of 30.0 mg /kg on mammary carcinogenesis may be regarded as a weak one due to the toxic effect of the substance on animals with tumours. According to available literature data, long-term experiments also showed no clear carcinogenic effect [1].

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