INTRODUCTION
A primary prophylaxis of ecodependent forms of the malignant tumors among population is known to imply the timely revealing of carcinogenic factors and development of the measures for man’s protection from their adverse effect. According to the USA National Toxicological Program a total number of chemical substances in a man’s life, industrial medium and environment exceeds 100 thousand [1]. 5-10% of them are supposed to be the carcinogens [2]. Special urgency of this problem is illustrated by the fact that only nearly 3000 chemical compounds were studied from the whole number of chemical substances. Carcinogenicity was demonstrated in the experiments on the animals and in the epidemiological investigations for almost 1000 of them. At present time 113 agents which contain chemical substances, including medicinal, and also biological, household, and physical factors, industrial processes are considered by the experts of the International Agency for Cancer Research as the human carcinogenic agents and 66 are probably carcinogenic [3]. At the same time their control and assessment become complicated because of the absence of correspondent hygienic standards in the Ukrainian base. By present time hygienic standards have been adopted only for 17 compounds and only for 5 taking into account the carcinogenic criteria of damage [4].

Mentioned circumstances tell about permanent necessity of the intensification of this process. In this connection we propose one of possible approaches which must promote a solution of the issue of the accelerated hygienic setting. For the determination of such an approach we performed experimental investigations, including a study of the complex of joint immunological reactions of the organism, pathomorphological and genotoxic changes in the organs of the experimental animals at different ways of entry (skin application and peroral administration) of carcinogenic and toxic substance.

THE AIM
The aim of the work was assessment of the criterial significance of the complex of early immunological reactions of the organism, pathomorphological and genotoxic changes in the organs for the acceleration of testing and hygienic assessment of carcinogenic danger of chemical substances.

MATERIALS AND METHODS
Experiment was carried out on the white random-bred male mice with a body weight of 15–20 g at the beginning of the experiment, purchased in the nursery “Phoenix” (Kiev) and included 2 series of research: the 1st – application of benz(a)pyrene on the skin; the 2nd – peroral administration.
of benz(a)pyrene (BP) and phenol. For administration we used the substances (BP, phenol, acetone) produced by SIGMA-ALDRICH, Germany.

For application we took 160 mice divided into 5 groups. Application of BP in the form of acetone solution (in the volume of 0.1 ml) was carried out with the help of pipette-doser on the areas of the skin on interscapular part of previously sheared back. Single doses of BP made up 0.21 µg; 2.1 µg; 10.4 µg. Two groups of mice were the control ones: mice of the first group were put on the applications of the solutions (acetone) in the same volume, the second group was an intact control. Substances were applied 5 times a week during 11 months.

The second series of the experiment was performed on 195 mice divided into 4 groups which contained intact control, control of the solvent – triethylene glycol (TEG) and 2 groups of animals taking BP or phenol isolatedly in the equal single doses of 0.1 mg. Substances in the volume of 0.2 ml were administrated intragastrically on an empty stomach by the probe once a week up to the end of the experiment lasting for 14 months.

Periodically in a day after last exposure of the substance, on the 8th, 22nd, 90th days and after 6, 11 months from the beginning of the experiment at the skin applications, 9th, 31st, 95th, days and 6, 11, 14 months at peroral administration, 6 animals from each group were killed by means of dislocation of the vertebrae of the cervical part of the spinal column and took the material for study. The work with the animals was performed in accordance with the rules of local Committee for Bioethics [5].

Mutagenic effect was determined with the help of micronuclear (MN) test [6, 7] taking into account literary data about its advantages [8]. Method is a useful and informative enough short-term test (STT) for the identification of genotoxicity, it is relatively simple, available, economically profitable, it allows to explore a large number of chemical substances for a short period of time. Taking into account a coincidence of organospecificity of MN test with the localization of carcinogenic effect [9], on the one hand, and an advantage of local character of the BP exposure, on the other hand, mice’s skin and forestomach were taken for the study of the mutagenic effect and the morphological changes.

ZEISS and BIOLAM light-optical microscopes at the magnifications ×1000 and ×900, respectively, were used for the analysis of the number of the cells with MN. Identification of MN was performed by the criteria presented in literature [10].

Immunological disorders were determined simultaneously with the determination of genotoxic effect by means of the determination of the content of leucocytes in the peripheral blood and their cell composition; amount of T- and B– lymphocytes, natural cells-killers, and the reactions as well: degranulation of basophils (after Shelly), inhibition of the spreading of macrophages, phagocytosis, precipitation of the circulating immune complexes by the solution of polyethylene glycol.

Pathomorphological investigations of skin and forestomach were performed with the use of pathohistological technique of the production of histologic preparations by means of the paraffin embedding of the organs after their fixation in the 10% stabilized neutral formalin. Histological sections were stained with hematoxylin-eosine.

Statistical analysis of the obtained data was performed using Student’s t-test. Values are reported as mean ± standard error. Significance level was set at P < 0.05. Existence of the connections between the parameters of the frequency of the cells with MN and immunosuppression was determined after Pirson correlational analysis.

RESULTS AND DISCUSSION
By the results of the analysis of all organism’s reactions we revealed the most informative indices, their character and level under exposure of the carcinogen (BP) and toxic compounds (phenol, acetone) in the generalized form are presented in the Table I.

At both ways of BP administration we identified the following general regularities.

Thus genotoxic effect (existence of the cells with MN) in the experimental animals of all groups, received the applications of the substances, is registered in the first days of the experiments (the 8th day); number of the cells with MN depends upon a single dose of carcinogen and is increased during the first month of exposure; maximum effect is observed at the application of the largest does of BP (10.5 µg), while minimum dose (0.21 µg) didn’t have any effect: frequency of the cells with MN didn’t significantly differ from the level in the animals of intact control. Genotoxic effect wasn’t revealed also in the mice receiving acetone applications. Frequency of the cells with MN didn’t differ from the observed one in the intact mice. It agrees with the literary data that acetone is not a mutagen [11].

Henceforth, in the period between the first and the third months we revealed a stabilization of the number of the cells with MN even just at the lower levels in comparison with the previous period. This may be connected with the existence of a definite critical value of cytogenetic effect for each dose.

A similar dynamics of the exhibition of the mutagenic effect depending on the total dose and time of exposure took place also at peroral administration of BP.

Dose dependence of genotoxic effect, established by us, correlates with the results of other authors, obtained at the study of carcinogenic compounds of different chemical classes [11-13], and it is a reflection of the general regularity of the effect of mutagenic agents [14-15]. Similar peculiarity of the dynamics of genotoxic effect – increase of the number of the cells with MN and further stabilization of the indices after reaching a definite level was observed in the cells of thyroid gland (TG) and polychromatophil erythrocytes (PCE) of the rat’s bone marrow after repeated administrations of N-nitro-N-methyl urea [16]. A level of the frequency of the cells with MN was different in the cells TG and PCE at the moment of stabilization. Definite reasons of this phenomenon are unknown. However, taking into account the fact that levels of stabilization of the cells with
MN are unequal depending on the size of mutagen dose and also examined tissues, we can assume that it is connected both with a different degree of caused injuries and functional state of the protection mechanisms of the organism and organ-tissue-specific peculiarities of these processes. At the analysis of immunologic reactions we revealed that in the early period of the exposure of carcinogen (end of the first month) T-cell chain of the immunity was the most sensitive early parameter, it revealed by its suppression (a decrease of the relative number of T-lymphocytes). With an increase of the time of BP exposure up to three months we noted an increase of immunosuppression at the expense of the addition of the suppression of humoral chain of the immunity (a decrease of the relative number of B-lymphocytes). Immunotoxicity by the type of immunosuppression is inherent in the majority of chemical carcinogens [17-18].

According to the retrospective studies, the compounds - immunotoxicants for the rodents, are concurrently carcinogenic for them. At the same time the issues what particularly immunotoxic parameters are connected with the potential carcinogenicity is unsettled in full [19].

Literary data on the BP immunotoxicity concerning its exhibition have not a uniform character and are concerned both with cell and with humoral chains of immunity and the factors of unspecific resistance as well [17, 20]. It may be connected with the different conditions of the performance of the experiments and with a use of different doses and time of carcinogen exposure in particular, and also methods and indices characterizing a state of immune system.

According to the modern points of view, there are two main mechanisms in the basis of immunosuppression induced with the polycyclic aromatic hydrocarbons (PAH) including BP. One of them is realized by means of the activation of aryl hydricarbon receptor (AhR), another one is connected with an capacity of the carcinogens to increase the intracellular calcium concentration in the immune cells, possibly due to protein – tyrosine – kinase activation by PAH. In any case, antigen and mitogen receptor signaling pathways are altered leading to proliferation and/or apoptosis of immune cells [17, 21-23].

At the comparison of the revealed indices one can see (Table 1) that increase of mutagenic effect (number of the cells with MN) was accompanied by the parallel development of immunosuppression during the first month. That is, these two phenomena, typical for the effect of chemical carcinogens, are intercommunicated between each other and have a unidirected character relative to carcinogenesis. This is proved by the results of performed correlative analysis after the Pison method they indicate the reliable reverse correlative connection between them. Coefficients of correlation between parameters of mutagenic effect and immunosuppression made up: for BP -10.5µg \( r = (-0.87) \), \( p < 0.01 \); 2.1 µg \( r = (-0.89) \), \( p < 0.01 \) (skin application); for 0/1 mg \( r = (-0.80) \), \( p < 0.05 \) (peroral administration).

According to the present formed point of view there are mechanisms in the organism, controlling integrity of genome at different levels – from molecular up to organism, reparative processes and immune supervision, in particular, fall into them [11].

According to the modern point of view on the immunobiology of the tumor there are three phases of interconnection of the tumor and the immune system (phase of immunological supervision, phase of equilibrium and phase of slipping off), each of them correlates with a definite stage of disease [24]. The early period of the exposure of carcinogens falls in the first phase, it is the beginning of single mutant and tumor cells which are revealed and eliminated from the organism with the help of the components of congenial and adaptive immunity; T- and B-lymphocytes fall into the last one as well. From the point of view of anti-tumor protection a cell link is assessed as the most significant one in this phase.

Obviously that violation of reparation and suppression of the function of immune system as one of the key factors of genetic instability may lead to the accumulation of genetically injured cells, including mutated ones which contain MN, in the tissues of organism, that is observed at tumor growth [11].

It is known that carcinogenic PAH even in the early terms of the exposure cause injuries of the cells, accompanied with

### Table 1. Comparative characteristics of appearing of the genotoxic and immunological changes in random-bred white male mice after benzo(a)pyrene and toxic compounds (acetone, phenol) administration

<table>
<thead>
<tr>
<th>Rout of administration</th>
<th>Substances</th>
<th>Single dose</th>
<th>Effects</th>
<th>Number of micronucleated cells, %&lt;sub&gt;0&lt;/sub&gt;</th>
<th>T-cells, %&lt;sub&gt;0&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 8; 9</td>
<td>Day 22; 31</td>
<td>Day 90; 95</td>
</tr>
<tr>
<td>Dermal</td>
<td>Benzo(a)pyrene</td>
<td>0.21 µg</td>
<td>0.50±0.29</td>
<td>0.50±0.34</td>
<td>0.60±0.40</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>2.1 µg</td>
<td>1.50±0.29&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.00±0.26&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.67±0.21&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>10.5 µg</td>
<td>2.5±0.29&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5.83±0.31&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.20±0.58&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td>0.2 ml</td>
<td>0.25±0.25</td>
<td>0.40±0.24</td>
<td>0.50±0.22</td>
</tr>
<tr>
<td></td>
<td>Intact control</td>
<td></td>
<td>0.25±0.25</td>
<td>0.40±0.24</td>
<td>0.50±0.22</td>
</tr>
<tr>
<td>Peroral</td>
<td>Benzo(a)pyrene</td>
<td>0.1 mg</td>
<td>1.33±0.21&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.83±0.31&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.33±0.33&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Phenol</td>
<td>0.1 mg</td>
<td>0.33±0.21</td>
<td>0.50±0.22</td>
<td>0.33±0.21</td>
</tr>
<tr>
<td></td>
<td>Triethyleneglycol</td>
<td>0.2 ml</td>
<td>0.50±0.022</td>
<td>0.50±0.022</td>
<td>0.67±0.21</td>
</tr>
<tr>
<td></td>
<td>Intact control</td>
<td></td>
<td>0.33±0.21</td>
<td>0.33±0.21</td>
<td>0.50±0.22</td>
</tr>
</tbody>
</table>

Notes: * – significant change of indexes of compared to the control (\( p<0.05 \)).
a suppression of reparative processes, and as a result of this the injured cells are preserved in the tissues for a long time.

The pathomorphologic research, performed by us in the early period, point to this possibility, they indicate changes in the organs of mainly dystrophic and atrophic character with the separate small foci of epithelium proliferation.

Taking into account mentioned literary data and results of our pathomorphological and immunological investigations we consider that an increase of the number of cells with MN during the first month is connected with a violation of the processes of reparation and suppression of T-cell chain of the immunity.

It should be mentioned that there are no many investigations devoted to the simultaneous study of joint changes of genotoxic effect by the indices of MN-test, in particular, and immunological reactions of the organism and their interconnection both in the experiment and on a man.

Thus it is informed about interconnection between frequency of the cells with MN and immunosuppression among population: the persons with a stable high level of the number of the cells with MN in the erythrocytes of peripheral blood had simultaneously an immunosuppression [11, 25]. Correlation between individual levels of the number of the cells with MN and specific immunological indices was revealed also in the workers of rubber industry contacting with many carcinogenic compounds [26].

In comparison of the results of genotoxic and pathomorphologic research were carried out an increase of the number of the cells with MN in the late period of the experiment (the sixth month) after application of BP in the animals with pathomorphological diagnostics of the development of proliferative-hyperplastic changes and skin tumors.

A similar character of the changes of genotoxic effect was determined also at the peroral administration of BP in the period of the beginning of pretumor changes and tumors in the mice's forestomach in eleven and fourteen months from the beginning of the experiment. That is, at this time a growth of the indices of genotoxicity was connected not with a dose but with the morphological pretumor changes and tumors, that was connected with a growth of the unbalance between processes of proliferation and apoptosis, violation of differentiation of the cells and high level of instability of genome in the process of carcinogenesis [27, 28].

It is important to mention that increase of the frequency of the cells with MN in exfoliative epithelium in connection with proliferative-hyperplastic changes in mucous membrane of oral cavity (hyperplasia, metaplasis, dysplasia), considered as the pretumor states, were determined in the people as well [29].

As for hematologic and immunologic indices, they didn’t differ from the indices revealed in the mice of intact control in the late period.

Unlike carcinogen, under exposure of toxic substances (phenol and acetone) the mutagenic and carcinogenic effects were not observed, and suppression of T- and B-chains of the immunity and factors of unspecific resistance, noted in the early period, had a transient character and didn’t appear at the end of the 3d month.

Thus we consider that the obtained results – parallelism of development and unidirection relative to carcinogenesis of the changes of the indices of mutagenic effect and immunologic reactions and also a presence of the reliable correlative link between them in early period (during 1 month) only under exposure of BP doses which induced the tumors of skin and forestomach is an evidence of a possibility of the use of the complex of these indices as an early criteria of the carcinogenicity of genotoxic chemical compounds. It is expediently to perform the experiment during 3 months. It ensures, on the one hand, a determination of the typical features of chemical carcinogenesis - mutagenic effects and immunosuppression, and, on the other hand, differentiation with toxic substances.

Materials of the performed research became a basis for the development of the methodic scheme of accelerated testing of the chemical substances for carcinogenicity, studied for the first time, and regulation of chemical genotoxic carcinogens at the presence of the data on carcinogenic potential (Figure 1). As we can see, a developed scheme includes a performance of the experiment for the revealing of dose-effect dependences of the manifestation of the complex of early changes of immune system by the type of suppression and mutagenic effect by polyorgan micro-nuclear method with a further assessment of carcinogenic properties of the substances and their doses.

Unlike other systems for prediction of the carcinogenicity of chemical substances by genotoxic indices proposed by other authors and also with a use of the latter in the complex with the medium-term tests [30] our scheme includes an additional early attributive index (immunological) encouraging more exact identification of carcinogenic effect.

We consider a use of the foregoing complex allows to move to a new level of the quality of the data of the experimental research both from the point of view of acceleration and increase of the reliability of testing and screening of carcinogenic chemical compounds with genotoxic mechanisms of effect and from the accelerated assessment of carcinogenic activity of their doses for hygienic setting.

CONCLUSIONS

In the experiments on mice at different ways of administration (application on skin, peroral administration) of benzopyrene and toxic compounds (acetone, phenol) we revealed definite regularities of the changes of genotoxic and immunologic indices which manifestations depended upon dose, duration of exposure and character of affecting substances.

Under effect of carcinogen, we determined an increase of mutagenic effect (frequency of the cells with micronuclei) and suppression of T-link of immune system during the first month which were characterized by a parallelism of development, unidirection relative to carcinogenesis and presence of reliable reverse correlative connection between them. In the period between the 1t and the 3d months we observed a stabilization of the number of cells with micronuclei and deepening of immunosuppression at the expense of the suppression of humoral chain of the immunity.
Dynamics of the revealed changes under effect of toxic compounds (acetone, phenol) has another regularities of manifestations of genotoxicity and immunologic reactions. There was no genotoxic effect and a number of the cells with micronuclei didn’t differ significantly from the quantities of a spontaneous level in the intact animals, and indices of suppression of T-chain and unspecific resistance of the organism, observed during the first month, had a transitory character and regenerated up to the levels noticed in the intact animals for 3 months.

Revealed differences in the effect of carcinogen and toxic substances allow to consider a complex of the indices of genotoxicity and immunosuppression as possible early criteria of carcinogenicity of chemical substances. Use of this complex ensures a possibility of the determination of carcinogenic properties of chemical compounds, their differentiation from toxic compounds in the experiment during 3 months.

The obtained data became a basis for the development of methodic scheme of accelerated testing of the chemical substances under investigations for carcinogenicity and hygienic setting of genotoxic carcinogens.

REFERENCES


Authors' contributions: According to the order of the Authorship.

Conflict of interest: The Authors declare no conflict of interest.