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Criteria for Revealing the Early Preclinical Signs of Benzene Intoxication

Ragim Almamed Orujov, Rana Anver Jafarova
Azerbaijan Medical University
Baku, Azerbaijan, Bakichanov St. 23., AZ 1022

Abstract.

The object of the present study is to substantiate the possibility to use the dynamics of leukocyte response as a criterion for estimating the changes in organisms of people working under the conditions of permanent benzene effect. We have conducted the study of inhalation poisoning of rabbits that received benzene 4 hours per day, 6 times per week during 4 months. An average benzene concentration was 1240 ± 82 mg/m^3. The experimental animals demonstrated a decrease in the amount of hemoglobin and erythrocytes and an increase in the amount of reticulocytes. A double-vertex erythrogram, a shift of the acidic erythrogram to the right and a shift of the erythrocyte sedimentation velocity curve to the left were observed. The level of adrenalin in daily urine was higher during the whole study period, while the amount of noradrenalin decreased by the end of the experiment. The amount of acetylcholine and true cholinesterase arose while the activity of false cholinesterase decreased. At the same time, the amount of intracellular potassium increased, while the content of potassium in plasma decreased to some extent. The sodium exchange varied insignificantly. Succinate dehydrogenase started to increase during the first month and the acid phosphatase went higher during the second month of poisoning. The amount of succinate dehydrogenase has reduced by the end of the experiment. Alkaline phosphatase didn't change noticeably. It is precisely at these times that the changes in blood that appeared during the experiment under the influence of benzene intoxication can be detected: leucopenia, neutropenia, lympho- and monocytopenia, eosinophilic-basophilic dissociation and an increase in the entropy index. These changes are more clearly expressed in varying concentrations. The conducted research made it possible to theoretically substantiate and to criterially develop the significant tests for early revealing of prepathological changes in health condition of the stuff employees.

Keywords: benzene intoxication, experiment, reticulocytes, eosinophilic granulocytes (EOS), lymphocytes, alkaline phosphatase (ALP), adrenaline, dopamine, allergic response.

INTRODUCTION.

There is no sector of national economy where people can avoid using the oil products. They are used in every sector starting from the consumer goods industry and finishing with all branches of heavy industry. Nowadays, several thousand different products are produced from oil, and the number of these products constantly increases. Throughout Azerbaijan, at least 2.5 million people have a contact with hydrocarbons of gasoline, benzene and other light oil products in connection with their industrial production and use, and this determines their hygienic value [1, 4]. The development of the chemical industry on the basis of modern achievements in science and technology has almost eliminated the cases of acute intoxication; however, another problem of weak chemicals concentration effect arose, and here the problem is that there can be no clinical manifestations of chronic poisoning, or they can be so weak that it is quite difficult to detect them. Meanwhile, the nonspecific effect of small irritant agents of chemical etiology is far from indifferent to our organism, as they change the general, allergic and immunological reactivity which increases the disease incidence among the population, which is usually not classified as occupational. At the same time, the changes in the human body occur on a phase-by-phase base and have defensive and adaptive responses. The lack of manifestations of changes in the body is considered as habituation to the changed conditions of the external industry environment. However, the studies have shown that the habituation is not an indicator of the prosperity and safety in the body, and it should be considered as the first phase of intoxication, as a signal on the need for preventive measures since the adaptive response as a general biological problem to a certain extent applies to the field of pathology as well [3, 6].

One of the main sections of the complex measures for preventing the occupational diseases are the organization and smooth implementation of preliminary and periodic medical examinations of workers whose labor activity is connected with the possibility of adverse effects of industry environmental factors onto their health. Only the properly organized preliminary medical examinations, which every new employee with the presence of certain professional factors must go through, can prevent the above-named problem.

The development and implementation of simple, easy-to-perform tests generally intended for the early detection of employees' health disorders – this is a very important task for the progress of occupational hygiene. In this research paper, we have made attempts to find the criteria for revealing the initial changes in the organism under the influence of low-intensity occupational irritant agents.

MATERIALS AND METHODS OF STUDY.

The experiments were carried out on male rabbits with the weigh at the beginning of the experiment from 2.1 kg to 2.3 kg divided into three groups: the 1st group was poisoned with increasing benzene concentrations, the 2nd – with fluctuate benzene concentrations, and the 3rd group was represented by the control animals not affected by benzene. Beyond the experiments, all animals were under the same conditions in terms of maintenance and nutrition. All experiments on animals were carried out according to the

"European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, March 18, 1986).

Chronic poisonings were carried out during 4 months on a daily base, 4 hours per day with one day per week free from intoxication. In setting up the experiment, we tried to maximally simulate the conditions of industry environment where workers are the most frequently exposed to chemicals, the concentration of which is constantly changing during the working day; therefore, the experiments were carried out in two versions. In the first version of the experiment, the concentrations were gradually increased during each day and in the second version they were sharply fluctuated, while the average concentration during the period of poisoning did not exceed the threshold concentrations by calculation. In fact, according to analyses, the average benzene concentration was within 1240 + 82 mg/m^3. The animals (rabbits) were examined before the beginning of poisoning during 4 months on a monthly base, and then a month after the end of the experiment. Monthly, the experimental and control rabbits were tested to determine the number of leukocytes and the leukocyte formula by the standard conventional method [7, 10]. The blood for study was taken in the morning before feeding from the ear marginal vein. The number of reticulocytes was counted in preparations colored by brilliant cresyl blue Pappenheim method. For chamber counting of eosinophilic granulocytes, the blood was diluted with a liquid suggested by I.S. Piralashvili [12], while the chamber counting of basophiles was made according to M.P. Vilchinsky's modification [7]. The acid resistance of erythrocytes was determined according to I.A. Terskov's and I.I. Gitelzon's method in A.I. Vorobiev's modification [8]. The activity of alkaline and acid phosphatase (ACP) was determined in neutrophils by the azo-coupling method according to L.S. Kaplov [7]. The activity of succinate dehydrogenase (SDG) of lymphocytes was determined according to Narcissov [7]. Acetylcholine (ACh) was determined by the method of Hestrin [2], cholinesterase (ChE) – by the method of [11]. We used a trioxyindole fluorimetric method to determine the level of adrenaline (A), noradrenaline (NAD), dopamine (DA) and dihydroxyphenylalanine (DOPA) in urine [5, 10]. To find out the allergic response, we have used the Wanye reaction in N.N. Klemparskaya's and N.V. Raeva's modification [9]. To define the parameters of blood coagulation system, we used the standard methods of the study [10].

The state of the blood system reflects the general immunobiological response of the body under the conditions of production factors effect. This is an extraordinary mobile system that delicately reacts to physiological and pathological changes. In some cases, it deepens the idea of pathogenesis of changes in intoxication and expands the possibilities for diagnosis and prevention [3].

The statistical processing of the obtained results was carried out using the parametric method with the implementation of Student's t-test and nonparametric determination of **U**-values for the Mann–Whitney U-test.

OBTAINED RESULTS.

The results of the studies are given below in Table 1. As it can be seen from the table, as a result of the chronic effect of benzene, a significant decrease in both erythrocytes ($4.28 \pm 0.28 \cdot 10^{12}$ w/l) and hemoglobin (111 ± 0.72 g/l) could be noted by the end of surveys in the first group of animals. The content of reticulocytes in the peripheral blood of control rabbits (3rd group) did not undergo the significant changes in the dynamics of surveys. Among the experimental rabbits, an increase in the number of reticulocytes was observed. The maximum significant increase in the 1st group of animals was noted only by the end of surveys (21.7 ± 1.45), while in the 2nd group a true increase in the number of reticulocytes was noted starting from the 3rd month of the experiment.

If for 1 hour the sedimentation of erythrocytes of control animals was 3.08 mm, the sedimentation of erythrocytes of experimental animals for the same period was 9.3 mm, while the maximum sedimentation from the third stage of registration had moved to the first stage. Both these facts should be considered as pathological changes caused by a three-month chronic benzene effect.

The acid resistance of erythrocytes among the control rabbits has insignificantly changed in the dynamics of surveys. The period of maximum hemolysis varied from 7.2 ± 1.0 min up to 8.2 ± 0.8 min, while the period of complete hemolysis varied from 9.4 ± 0.9 min up to 11.2 ± 0.7 min. Among the experimental rabbits both the period of maximum and the period of complete hemolysis were a little bit greater than the same periods among the control rabbits. However, these differences were noted only during the 2nd month of poisoning when the period of maximum hemolysis among the experimental rabbits was 9.5 ± 0.7 min and the period of complete hemolysis was 12.3 ± 0.45 min. The number of double-vertex erythrograms during all periods of the study was greater among the experimental animals than among the control animals, and especially by the 4th month of poisoning when this criteria among experimental rabbits was 77.8 ± 14.8%, while the same criteria among the control animals was 50.0 ± 16.0% ($p < 0.005$).

One month after the beginning of the experiment, the entropy index increased up to 101% which indicated a relatively small decrease in the organization of the studied system. This was expressed in leukocytosis and eosinophilia (156.9%). Two months later, as it could be seen from the increase in the entropy index (103.7), the system organization had reduced. The total number of leukocytes and eosinophils continued to increase. In the neutrophils formula, a shift to the left was noted. In separate smears, new forms of cells were found. The number of segmented neutrophils decreased in comparison with the initial data. Three months later, it increased (93.9%) due to the defense reactions. However, the continuing effect of the toxic factor required a new defense reaction which was reflected in a new decrease in the system organization (PE – 102%). By the end of the experiment, leucopoiesis was depressed: the total amount of leukocytes decreased and became lower than the initial and the control numbers.

Table 1. Changes in the functional state of the rabbits' body under the effect of weak benzene concentration

Criteria		Primary reaction		By the end of the experiment	Current criteria	Primary reaction		By the end of the experiment
		Present term	Direction			Present term	Direction	
	Body weight			–*)	Potassium in erythrocytes	2	+	–
	Erythrocytes	1	–*)	–	Sodium in Erythrocytes	0	0	0
	Hemoglobin	1	–*)	0	Potassium in erythrocytes Potassium in plasma	1	+	–*)
	Erythrocytes in total	1	+*)	–*)	Sodium in erythrocytes Sodium in plasma	2	+	–*)
Leukocyte formula (abs)	Banded neutrophils (abs)	1	+	–*	Acetylcholine in blood	1	+*)	+*)
	Segmentonuclear neutrophils (abs)	1	+*)	–*)	False cholinesterase	1	+	–*)
	Eosinophilic granulocytes (abs)	1	+	0	True cholinesterase	1	–	+*)
	Lymphocytes (abs)	1	+*)	–*)	Vitamin C in blood	0	0	0
	Monocytes (abs)	1	+*)	–*	Vitamin C in urine	0	0	0
	NPAI	2	+*)	+	Blood aldolases	2	–*)	–*)
	Neurocyte AHC	2	+	0	Blood clotting time	2	+*)	+*)
	Lymphocytes AHC	2	+	0	Prothrombin time	2	–	–
	Lymphocytes SDG	1	+*)	–*)	Prothrombin index	2	+	+
	Erythrocyte acid stability	2	+	+	Blood clot retraction of	2	+*)	+*)
	Adrenaline in urine	1	+*)	+*)	Time of plasma recalcification	2	–	–
	Noradrenaline in urine	1	+	+*)	Acid phosphatase	2	–	–
	Dopamine in urine	1	+*)	–*)	Fibrinolytic activity	2	–	–*)
	DOPA in urine	1	+*)	0	Plasma tolerance to heparin	2	–	
	Potassium in plasma	1	–	0	Eosinophilic granulocytes and basophilic cells	2	+	+
	Sodium in plasma	0	0	0				

Note: *) – statistical significance with control

In the dynamics of the experiments, the neutrophils phosphatase activity index (NPAI) among the control rabbits has changed insignificantly. The primary reaction to the effects of benzene among the experimental rabbits appeared only during the 2nd month of poisoning and was expressed by an increase in NPAI up to 283 ± 3.0, which was significantly higher than among the control animals (237 ± 9.0) during the same period.

During this period, SDG among the experimental animals was determined in the amount of 13.4 ± 0.73 which was significantly higher than among the control rabbits during the same period (9.6 ± 0.59, $p < 0.005$). By the end of the experiment, the SDG among the experimental rabbits was significantly smaller (7.1 ± 0.33) than among the control rabbits (9.3 ± 0.48, $p < 0.005$).

The level of adrenaline in the daily urine of experimental animals was significantly higher during all periods of the study. Especially significant these differences were in 4 months ($P < 0.005$) after the beginning of poisoning. The release of adrenaline with the daily urine of control rabbits in the dynamics of the experiments has slightly decreased. Thus, in the 1st month of experiment it was 0.4 ± 0.13 μg per day, and by the 4th month of the experiment is was 0.088 ± 02 μg per day. The amount of NAD among the experimental animals significantly increased already after 2.5 months of the experiment (from 0.596 ± 0.28 to 1.074 ± 0.4 μg/day). Such dynamics of A and NAD are confirmed by the changes in the A/NAD coefficient. This ratio among the control rabbits decreased from 2.2 to 0.38, while the ratio among the experimental animals decreased from 1.3 to 0.28. The greatest amount of DA was determined in urine of experimental rabbits in a month after the beginning of poisoning. During this period, the amount of DA in urine of experimental animals reached 3.93 ± 1.63 μg/day, and by the end of the experiments, this index decreased to 0.7 ± 0.4 μg/day. This indicates a significant mobilization of catecholamines precursors among the experimental animals. This circumstance is also confirmed by the value

of the NAD/DA ratio. Among the control rabbits, this coefficient by the end of the experiment was 4 times greater than at its beginning, and among the experimental animals – 17 times greater. The maximum number of DOPA was determined among the experimental rabbits in a month after the beginning of poisoning and was 9.96 ± 2.6 μg/day.

The content of ACh in blood of control animals and the activity of the true and false ACh did not change during the experiments. The amount of ACh in the blood of experimental animals increased from 0.22 ± 0.03 mg% in the initial period and up to 0.46 ± 0.04 mg% by the 4th month of poisoning, $p < 0.001$. The activity of false ACh among the experimental rabbits decreased after a certain increase during the 1st month of poisoning (from 0.27 ± 0.03 to 0.44 ± 0.12% &) and reached (0.18 ± 0.01 &, $p < 0.05$) by the 4th month of poisoning. The changes in the activity of true ACh had a slightly different directivity. Thus, after the initial decrease of poisoning during the 1st month of the experiment (from 0.94 ± 0.14 to 0.69 ± 0.09 &), the activity of true ACh increased to (1.26 ± 0.08 &, $p < 0.001$) by the 3rd month of intoxication.

The maximum amount of potassium in erythrocytes of experimental animals by the 3rd month of poisoning was (104.5 ± 3.79 mmol/l), and by 4th month this indicator was slightly higher than during the 1st month (80.44 ± 2.24 mmol/l). The ratio of intracellular sodium to plasma among the experimental and control animals significant differed only by 4th month of poisoning ($p < 0.05$). Thus, this coefficient slightly decreased among the control animals (from 11.25 ± 0.45 to 10.58 ± 0.37), and among the experimental animals it, vice versa, slightly increased (from 10.47 ± 1.28 to 12.62 ± 0.63).

The activity of aldolases in blood plasma and the content of vitamin C in the blood and urine of experimental animals have decreased in the dynamics of surveys.

DISCUSSION OF RESULTS.

The analysis of the obtained results has shown the following: when studying the acid resistance of erythrocytes, it was found out that the hemolysis period of experimental animals was considerably extended and the hemolysis proceeded unevenly, the acid erythrogram had two peaks, wherein the first peak accrued to the 2nd month and the second peak – to the 4th month of poisoning. Apparently, in this case we can speak about the nonspecific changes in the acid resistance of erythrocytes of animals when exposed to weak benzene concentrations. However, the most frequent shift of the acid erythrogram to the right indicates the appearance of erythrocytes in the blood of experimental animals with altered physical and chemical properties.

To analyze the leukocyte response, it is necessary to combine the differentiated function of leukocytes with the phase neurohumoral and humoral changes. The leukocyte response reflects the violation of correlation between two parts of autonomic nervous system. An increase in the tone of the sympathetic divisions increases the number of leukocytes due to eosinophilic granulocytes, neutrophils and monocytes. The same changes are caused by mineralocorticoids, which affects the ratio of neutrophils to lymphocytes, while the excitation of the thyroid gland reduces the number of basophilic cells. When the parasympathetic part of the autonomic nervous system is excited, the number of leukocytes decreases due to eosinophilic granulocytes, neutrophils and monocytes. The same leukocyte response is caused by glucocorticoids of the adrenal cortex, which functionally corrects the excitation of the thyroid gland and increases the number of basophilic cells. Hence, it can be concluded that two associations of cells – eosinophils-basophils and neutrophils-lymphocytes - have a leading role in the leukocyte response. In this case, the association of eosinophils-basophils responds more quickly to any nonspecific irritation than the association of neutrophils-lymphocytes. This is explained by the fact that the sympathetic nervous system reacts quickly to any irritation. Not coincidentally the adrenaline is called an "emergency hormone". Observing the dynamics in the number of basophilic cells and eosinophilic granulocytes, it is possible to predict the dangerous changes for the organism. The indicator of the resulting changes and the leukocyte response can be the indicator of entropy.

The conducted experiment has shown the high sensitivity of the studied criteria of peripheral blood as to the effect of benzene. At the same time, the maximum voltage of compensatory-adaptive reactions occurred during the 3rd month of the experiment, and after that we got the appearing of depletion and inhibition of compensatory mechanisms. The change in indicators in the 2nd group was of earlier and more pronounced nature. Toxic cytoplasmic granularity appeared in a number of cells. A month after the termination of contact with benzene, not all indicators have returned to the level of the initial values which indicates an insufficient recovery period for this poisoning regime.

The imbalance in the activity of oxidative and catabolic enzymes, expressed in the decrease of SDG activity and in the increase of ACP activity in the cell, is considered as an unfavorable response. NPAI among the control rabbits has changed insignificantly in the dynamics of experiments.

The activity of neutrophils and lymphocytes ACP in the dynamics of surveys did not change significantly among the control animals. The average histochemical coefficient (AHC) of neutrophils acid phosphatase was slightly larger than the AHC of lymphocyte acid phosphatase. The greatest changes in SDG were also observed by the 2nd month of poisoning. Thus, the intoxication with weak benzene concentrations caused an increase in the activity of oxidation-reduction enzymes during the 1st month and catabolic enzymes during the 2nd month of experiments. By the end of poisoning, the activity of oxidation-reduction enzymes slightly decreased, while the phosphatase activity of granulocytes and lymphocytes was not significantly different from the control one.

The sympathetic-adrenal system has a great importance in the studied problem [5]. The intoxication with weak benzene concentrations causes stimulation of the sympathetic-adrenal system of experimental rabbits and mobilization of its reserves starting from the very 1st month

of experiments. This is also confirmed by the change in the ratio of amount of catecholamines as to their predecessor.

ACh is considered to be a factor that changes the functional state of the central nervous system [2]. Intoxication with weak benzene concentrations causes an increase in the amount of acetylcholine in the blood of experimental rabbits with a simultaneous decrease in the activity of false cholinesterases and an increase in the activity of true cholinesterases.

Electrolytes of sodium and potassium play an important role in the formation of noradrenaline-protein complexes. In this case, potassium promotes the recovery of noradrenaline, while sodium promotes their strong binding. The studies have shown that the experimental animals in contradistinction from animals in the intact state have more significant changes in potassium metabolism. The amount of intracellular potassium has increased, while the content of potassium in plasma has, vice versa, slightly decreased.

The activity of aldolases in blood plasma and the content of vitamin C in the blood and urine of experimental animals have decreased in the dynamics of surveys.

The essence of the Wanye method is to determine the turbidity of plasma which occurs as a result of reaction between the allergen and antibodies in the plasma or serum of sensitized persons [9]. In 1:480 dilution zone of experimental animals we can observe a delay in optical density and even some increase which forms somewhat like "plateau". This type of reaction has unfavorable prognostic value. The blood plasma of control animals does not react with the lysate of erythrocytes; only initial indicators of optical density are changed with a tendency to decrease.

FINDINGS.

1. The reaction of the blood system onto the effects of benzene was revealed. The studies have shown that changes in the leukocyte response can serve as a simple criterion which reflects the functional interrelationships of all parts of the body's defense reactions. The information-entropy analysis of the leukocyte response is an objective criterion for indicator of reactive state of the organism and necessitates a wide application in such studies. The leukocyte system was more organized during the poisoning period of up to 1 month than during the period of 3 and 4 months of poisoning. There were qualitative changes in erythrocytes which were characterized by a shift of acid erythrograms to the right and by an increase in the number of double-vertex erythrograms.

2. Weak benzene concentrations have caused phase changes in the neuro-humoral regulation which ensures the adaptation of the organism to the active toxic factor.

CONCLUSION.

Thus, the result of the conducted experimental studies has clearly shown that the effect of small benzene doses during 2-3 months and more leads to chronic intoxication of the organism with a change in the pattern of blood and neuro-humoral regulation of the organism. Moreover, not all changes disappear with the termination of benzene toxic effect.

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