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THE ROLE OF MULTIVARIATE ANALYSIS IN THE STUDY OF THE CHANGES IN ANIMAL ODOR AFTER HEPOTOCELLULAR CARCINOMA TRANSPLANTATION

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Abstract

Previously we demonstrated that mice biosensors, with no prior training, accurately distinguish the odor of hepatocarcinoma animal urine from urine of healthy animals [5]. However, we were not sure about the nature of the odor, whether it was related to the development of tumors, inflammation or immune reaction of the body or something else. Especially, considering the fact that the odor is a complex mixture, and we are looking for clear differences between patients and healthy models. However, these evident differences may be due to different factors, and the animals are guided by those differences that are understandable and useful to them, and not by those that are easier to determine. In this study, we considered the key factors influencing the olfactory pattern; in our opinion, these factors are the main guidelines in working with model patients and model cancer.

Keywords: disease odor, database, hepotocelullar carcinoma, animal biosensors

Introduction. The subject of this work is the ability to macrosmatic animals (dogs and mice) to distinguish mice with hepotocelullar carcinoma from healthy mice by the odor of urine. Microsmatic animal have been used for the diagnosis of cancer for several decades [1, 2, 7, 8]. However, the question

still remains: what exactly animal biosensors are sensitive to, especially considering the fact that the odor of urine contains the information not only about the donor's health, but also about his diet, age, possible assident stress and much more [6].

Despite the high sensitivity of modern analytical instrumentation, the comparison of mixtures and identification of the components associated with the disease remains very challenging. However, this task is successfully fulfilled by microsmatic animals. We cannot get the information on the composition of the mixtures from the animals, but, in our opinion, it is possible to train the animal, or set an experiment in such a way that an animal could point to the mixtures that contain patterns of volatile organic compounds which are associated with the disease. Meanwhile, it is important to take into consideration that it is not always clear what the odor “keys" the animal uses in order to distinguish a healthy animal from a sick one, and whether these "keys" are related to the disease.

The specific character of this issue lies in the fact that it is difficult to get a definite answer (yes / no), i.e. the researcher has to make the decision based on the data received implicitly (based on indirect signs). It is also necessary to remember that for the reliability and repeatability of the results similar conditions and composition of the experiment are required, however, there are many factors which cannot be aligned or eliminated, and which may affect the response of biosensors. For example, age and condition of the sensor; age and "biography" of the donor; the sequence of injections, their types and the time after the latest injection. As one can see that a lot of interfering factors may cause the so-called "noise". To remove the effect of this noise it is necessary to average the results, which will certainly lead to a large number of experiments, and as a consequence a large data set. On the basis of data analysis and the selection of the factors influencing the result of the experiment, the issue of experiment design is raised.

In order to analyze the effects of quantitative and qualitative features of samples and the experiment conditions impacting the choice of the biosensor animal the structure that combines the information about donors, samples, sensors, conditions and results of the experiments is required. ODD (Odor of Disease) database has been developed, the main components and the structure of which have been described previously [3]. The basis of the ODD database included the information gathered as a result of multi-purpose behavior experiments carried out by the employees of the Institute for Information Transmission problems within a period of 2012-2015. The received data is partially published [biobul]. A separate component of the database is a form called “ODD_Mouse_Exp”, which anables to simplify and automate logging the data on sensor reactions within the experiments in accordance with the modified method scheme "habituation-generalization".

We have identified a group of factors that biosensors could respond to. In our opinion, the greatest contribution to the creation of an olfactory pattern similar to the one of the disease is made by the injection of healthy liver tissue, as the latter does not only cause similar injury, stress and inflammation, but also triggers the immune response to the foreign tissue, with a similar reaction to the implantation of the tumor tissue, at least in the first few days after the injection. We believe in significant contribution of the donor age, though it is a much less significant factor. The fact that a series of experiments which are so called "on itself" should have at least two weeks between the injections of healthy liver tissue and tumor tissue, requires additional control of biosensors' reactions. In this study, behavioral experiments with biosensor animals were designed to detect the presence of difference in the composition of VOCs (volatile organic compounds) in the urine of healthy mice with different types of injections and of different ages. In order to test our hypothesis on the influence of additional factors, we conducted additional experiments with biosensor mice.

Method. *The experimental model.* The experimental model was male hybrid mice BDF1-f1 (DBA2 x C57Bl/6). We used a strain of mice transplanted liver tumor H33, which was received by the Research Carcinogenesis Instituted, Russian Cancer Research Center named after N. Blokhin, RAMS, from from hepatocellular carcinoma induced earlier in the same hybrid male mice BDF1 by a single intraperitoneal injection of 90 mg / kg diethylnitrosamine [4].

Types of injections and collection of urine samples. All hypodermal injections were made in the scapular region. For tumor tissue implantation 100 mg of minced tumor tissue in 0.5 ml of 0.9% physiological saline was injected subcutaneously. Mice of the control group had two types of injections: normal liver tissue (50 units) was implanted as well as the tumor tissue with physiological saline (30 units). During experiments the donor had no more than 5 injections with an interval of at least one month.

Donor mice were kept by 10 in polypropylene cages (36 cm x 24.5 cm x 10.5 cm). Urine samples were collected from the mice within 1 to 13 days after injection. Urine was collected into performed plastic jars. Donar mice were put into the plastic jars with perforated bottoms. These jars were placed in whole plastic jars where animal urine was dripping to. Urine was dispensed into plastic test tubes of 60 mcl, therefore several identical samples were obtained from a single donor. All jars were labeled and each donor had his own set of jars. After urine collection the jars were washed with hot water and aired for 24 hours. Each tube with a urine sample was labeled, so that one could always identify the sample matching a certain animal and a sample collection time. After selection the urine samples were frozen at -23 C and defrosted right before the experiment.

Animal biosensors. CBA male mice aged 2-7 months were used as biosensors. For 10 days before the beginning of the experiments the animals were put by one into standard polypropylene cages with net cover (25 cm X 12 cm X 10 cm).
All mice were fed a laboratory rodent diet, Laboratorkorm (Moscow, Russia). All animals were kept in similar conditions in a room with controlled temperature (21 ° C) and at 12x12-hour light-dark cycle. Care was carried out in accordance with the Guide for the Care and Use of Laboratory Animals and Experimental Protocols, " OECD. Principles of Good Laboratory Practice.

Experiments with biosensor mice on identification of a specific urine odor, which is associated with the transplantation of foreign tissue. In this case, we used the method of the protocol of sequential presentations or «habituation-dishabituation» (Fig. 1). Each of the samples was presented with three repetitions, i.g. the entire experiment consisted of 12 presentations of olfactory stimuli. For each of the next presentation a new nozzle was used. Each presentation lasted 3 minutes starting with the sample sniffing. We conducted 21 experiments like this in total. Eleven experiments with the following sequence: water, donor urine after saline injection, donor urine after saline injection, but collected into another test tube, and donor urine after trasplatation of healthy liver tissue (line 1 in Chart 1). Ten experiments where the samples were presented as follows: water, donor urine in 24 hours after the injection of normal liver tissue, a sample from the same donor, but from another test tube, and the urine sample of the same donor in 24 hours after saline injection (line 2 in Chart 1).

All three urine samples which were used in the experiment were obtained from a male. In all cases the time of sniffing odors by males was recorded with a stopwatch with an accuracy of up to 0.1 seconds. The urine of one and the same animal was not shown twice to the animal. In each series of experiments at least three urine donors of every possible option were used. In the end of the experiment the chamber was thoroughly washed with hot water and aired for at least 24 hours. The information on the presented odors, series of experiments, and the reliability of differences received during the study of olfactory signals are demonstrated in Table 4. The results were processed using a paired nonparametric Wilcoxon test for dependent variables with the help of statistical software package SigmaPlot 12.5.

Such an experiment design allowed us to identify the presence of significant differences in the odor of mice after the injection of normal liver tissue and saline in 24 hours after injection. In addition, we were able to verify if the change of test tubes was significant for mice biosensors, as sample aliquots were collected and stored in different test tubes.

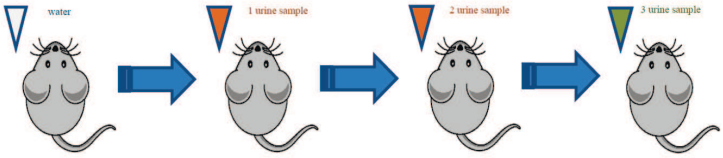

Fig 1. Sample presentation scheme in «habituation-dishabituation» test.

Experiments with mice biosensors for the identification of a specific urine odor associated with donor age. It is obvious that with age VOC urine composition changes. However, does it change significantly for our biosensors if only donors of a certain age participate in the experiments, and the interval between injections is about 30 days? Experiments on the identification of whether donor age in "on itself" experiments affects the reaction of biosensors were carried out with mice biosensors in accordance with the modified scheme «habituation-generalization». At the habituation stage the odor of donor urine was presented after the injection of a healthy liver tissue, but at the recognition stage the donor was given a urine sample taken at the same time, which was identical to the first one, and a urine sample from the same donor after another injection of healthy liver tissue, but which was done in a month time (Fig. 2).

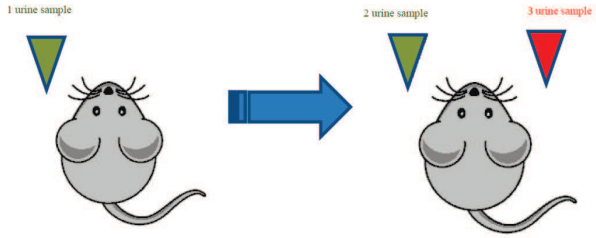

Fig 2. Sample presentation scheme in «habituation-generalization» test

In all cases the time of sniffing odors by males was registered in ODD_Mouse_Exp form. The registry of the start and the end of the sample sniffing was carried out by pressing a key on a computer. Thanks to the automatic registration not only the total time of sample "sniffing" can be recorded, but also a detailed timing for a unique pattern of biosensor behavior. By the end of the experiment the results recorded are automatically added to the corresponding database table. The animals were not shown the urine of the same animal twice. In each series of experiments at least three urine donors of every possible option were used. In the end of the experiment the chamber was thoroughly washed with hot water and aired for at least 24 hours. The information on the presented odors, series of experiments, and the reliability of

differences received during the study of olfactory signals are demonstrated in Table 4. The results were processed using a paired nonparametric Wilcoxon test (Wilcoxon — Mann — Whitney test) for dependent variables using a statistical software package MatLab R2013a.

Results and discussion. Chart 1 demonstrates the total time of sniffing water and urine samples which were obtained after saline injection and healthy liver tissue injection.

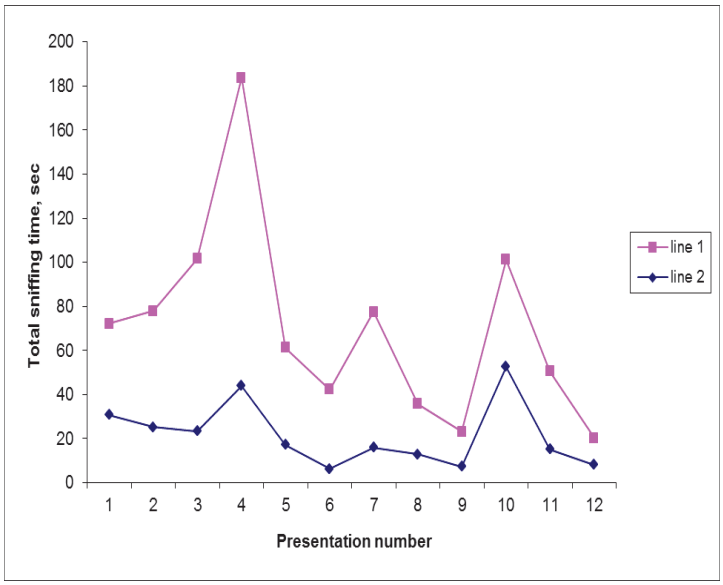

Chart 1. Recognition of male BDF1 by the urine odor after healthy liver tissue injection and after saline injection by mice biosensors.

Table 1 represents the results of the experiment on the recognition of male BDF1 by the urine odor after the healthy liver tissue injection and after saline injection by mice biosensors. In order to see if mice biosensors will identify the urine of donors with various injections, we compared the sniffing time of 3 samples (3 presentation) and the sniffing time of the 4th sample (1 presentation). In order to check if the test tube replacement is significant in this experiment design, we compared the sniffing time of the 2d sample (3 presentation) with the sniffing time of the 3d sample (1 presentation), the data are in Table 2.

Table 1. Recognition by urine odor of male BDF1 after healthy liver tissue injection and after saline injection by mice biosensors.

Line 1 on Chart 1 and the compared samples	Sample description	Study time of the compared odors total (min – max), sec	Reliability of differences by Wilcoxon criterion for the ajoint pairs
Line 1 Samples 9 and 10	Male urine after saline injection. Test tube 2. Presentation 9	23,2 (0 – 5,2)	n=11 T (ranksum) = 87.5 h = 1.0 zval = - 2.53 p = 0,01
	Male urine after healthy liver tissue injection. Presentation 10	101,2 (0,3 – 26,3)	
Line 2 Samples 9 and 10	Male urine after healthy liver tissue injection. Test tube 2. Presentation 9	7,3 (0 – 1,5)	n=10 T (ranksum) = 61.0 h = 1.0 zval = - 3.29 p = 0,001
	Male urine after saline injection. Presentation 10	52,6 (0,8 – 23,3)	

Table 2. Recognition of the identical BDF1 males' urine samples which were collected into different test tubes based on the urine odor.

Line 1 on Chart 1 and the compared samples	Sample description	Study time of the compared odors total (min – max) sec	Reliability of differences by Wilcoxon criterion for the ajoint pairs **
Line 1 Samples 6 and 7	Male urine after saline injection test tube 1. Sample 6	42,2 (0,2 – 15,2)	n=11 T (ranksum) = 122.0 h = 0.0 zval = - 0.26 p = 0,8
	Male urine after saline injection test tube 2. Presentation 7	77,5 (0,2 – 33,7)	
Line 2 Samples 6 and 7	Male urine after healthy liver tissue injection Test tube 2. Presentation 6	6,4 (0,2 – 1,5)	n=10 T (ranksum) = 82.0 h = 0.0 zval = - 1.70 p = 0,1
	Male urine after healthy liver tissue injection test tube 2. Presentation 7	16,0 (0,2 – 3,5)	

Table 3 represents the experiment results on recognition by urine odor of BDF1 males of different ages after health liver tissue injection by mice biosensors. While the recognition of donor urine odors after health liver tissue injection and after the second similar injection 30 days after the first one, mice biosensors have not reliably studied neither of the other groups of samples. One and the same donor urine was used in every experiment. It demonstrated the fact that 30 days difference in age is not significant for the biosensor animals. It is important because from 14 to 30 days pass between the injections to one and the same donor.

Table 3. Recognition by urine odor of BDF1 males of different ages after health liver tissue injection by mice biosensors.

Starting odor *	Odor represented at the recognition stage *	Time of odor study at the recognition stage total (min – max) sec	Reliability of differences Wilcoxon criterion for the ajoint pairs
Urine of male 1 after healthy liver tissue injection	Urine of male 1 after healthy liver tissue injection	52,9 (0,3 – 13,5)	n = 20 T (ranksum) = 86.0 h = 0.0 zval = - 0.73 p = 0,1
	Urine of male 1 (+ 30 days) after one more healthy liver tissue injection	53,9 (0,0 – 6,8)	

Conclusion. At this point of time we were unable to identify the VOC which are specific for cancers in general or for certain types of tumors with the sufficient level of confidence. Apparently, failures are due to the fact that the search among a large quantity of volatile substances in body wastes is similar to looking for a needle in a haystack; and it is not clear in which part of the spectrum of VOCs associated with the disease should be looked for. Moreover VOC complex which is associated with tumor development in body wastes is rather complicated. In addition, tumor that develops in the body causes a reaction of the immune system. Cell work of this system is also changing, and their metabolites are also present in wastes. It is possible that certain VOC marking some type of cancer has not yet been identified because different researchers specify substances from different sectors of VOC "spectrum", which are associated with the development of disease, and the correlation of "spectrum sectors" varies from patient to patient.

On the one hand, work with animals is being criticized because the design of experiments is very different in all the undertaken studies, and in our opinion, it is partly due to the fact that it is not always clear what olfactory key is used by the animal to distinguish between the healthy from the sick, and whether exactly these keys are associated with the disease. From our point of view, the use of the "real nose" combined with the various " patient models" and the chemical analysis of the VOC is very promising for identifying the volatile substances which are related only to the disease. Furthermore, the use of macrosmatic animals can be helpful for indentifying the pathophysiological mechanisms which underlay the development of various VOCs; it will lead to new therapeutic approaches of various tumor types.

We have previously shown that mice biosensors, without prior training, reliably distinguish between the wastes of animals with hepatocellular carcinoma from those of healthy animal by odor [5]. However, we were not clear about the nature of the odor, whether it is associated with the development of tumors, inflammation or immune reaction of the body or something else.

The results of this study prove that animal biosensors are able to distinguish between the animals with various types of injections, whereas model patient age deference of up to 30 days and test tubes changes are not highly important. It has strengthened our assurance in the fact that animals are able to identify the urine VOCs associated with cancer. Also is has pointed out our next step in the studies of disease odor.

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