pH Monitoring of Tumor Microenvironment and Low Volume of Urine in Experimental Rats

Terezia Kiskova[1,*], Steffekova Zuzana[2], Karasova Martina[2] and Kokosova Natalia[1]

[1]*P.J. Šafárik University in Košice, Faculty of Sciences, Moyzesova 11, 040 01, Košice, Slovakia*

[2]*University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia*

Abstract: The pH monitoring of the tumor microenvironment *in vivo* seems to be in fact complicated and technically quite challenging nowadays. Also the strategy of measuring urine pH of a little amount is not fully solved. Thus, the aim of our study was to monitor pH of urine samples (< 0.1 ml) and of tumor microenvironment of anesthetized rats in a minimal invasive way. The small urine volumes of rats or mice make pH measurements difficult, as standard pH electrodes usually need a minimal volume of several milliliters to function. The manual micromanipulator together with a needle-type housed pH microsensor offers a simple and effective way to do so. Our results show that pH of urine and tumor microenvironment was lower in tumor bearing rats compared to healthy subjects. The unique technology of pH microsensors could be a promising way to monitor the pH in many experimental designs and clinical praxis.

Keywords: pH, tumor microenvironment, urine, monitoring, *in vivo*, rats.

INTRODUCTION

Solid tumors contain various cell types, such as cancer, stromal, or immune cells, including a plenty of proteins and chemical signals [1]. The interactions between these cells form the unique tumor microenvironment and regulate tumor growth and development [2]. However, only recently specific alterations of the microenvironment have emerged as driving causes of malignancy and/or as critical steps during disease progression. The extracellular pH of malignant solid tumors is acidic, in the range of 6.5 to 6.9, whereas the pH of normal tissues is significantly more alkaline, 7.2 to 7.5 [3].

Systemic acid-base balance is maintained in a large part by renal excretion of excess ions in urine. Diet composition, body weight as well as pathological state, such as cancer, may influence urine pH through the production of certain ions and organic acids [4].

MATERIALS AND METHODS

Female Sprague-Dawley rats (n = 20) aged 31 days and weighing 100–130 g were obtained from Velaz (Únetice, Czech Republic). They were adapted to standard vivarium conditions with a temperature of 21–24°C, and a relative humidity of 50–65% and to an artificial 12:12-h light:dark regimen. They were fed standard pellets (Peter Miško, Snina, Slovakia) and drank only tap water *ad libitum*.

*Address correspondence to this author at the P.J. Šafárik University in Košice, Faculty of Sciences, Moyzesova 11, 040 01 Košice, Slovakia; Tel: +421 55 234 1216; Fax: +421 55 622 2124; E-mail: terezia.kiskova@gmail.com

Breast cancer was induced to the half animals with two intraperitoneal doses (50 mg/kg body weight) of N-methyl-N-nitrosourea (NMU, Sigma, Deisenhofen, Germany) on the 43rd and the 50th postnatal days. The rest of animals, so called intact group (INT), consisted of healthy control animals.

After the tumor formation (from the 10th to 13th experimental week), five random tumor bearing rats were anesthetized (isoflurane with prescribing dosage) to monitor the pH of tumor microenvironment (manual micromanipulator together with a needle-type housed pH microsensor, PreSens, Regensburg, Germany) as seen on Figure **1**. Briefly, a pH microsensor was connected to the pH-1 micro transmitter, and a PC with the pH-1 view software to control the measurements [5]. The pH microsensor was inserted into the tumor microenvironment with a manual micromanipulator for exact localization.

Anesthesia was provided by isoflurane in an induction chamber of 1000 ml capacity. A 4% concentration of isoflurane gas was adequate (0,2ml/L chamber volume) for short-term anesthesia. The gas in liquid phase was applied to a cotton ball below the false floor of the chamber.

At the final week of the experiment (14 weeks), urine samples from each rat were obtained. During handling on a sterile underlay, the rat started to urinate spontaneously. The urine was immediately collected into prepared sterile microtubes. pH was measured using pH microsensor (manual micromanipulator together with a needle-type housed pH microsensor PreSens, Regensburg, Germany) after urine collecting.

ISSN: 1927-7210 / E-ISSN: 1927-7229/15

The measuring latency for one sample was not longer than 10 minutes.

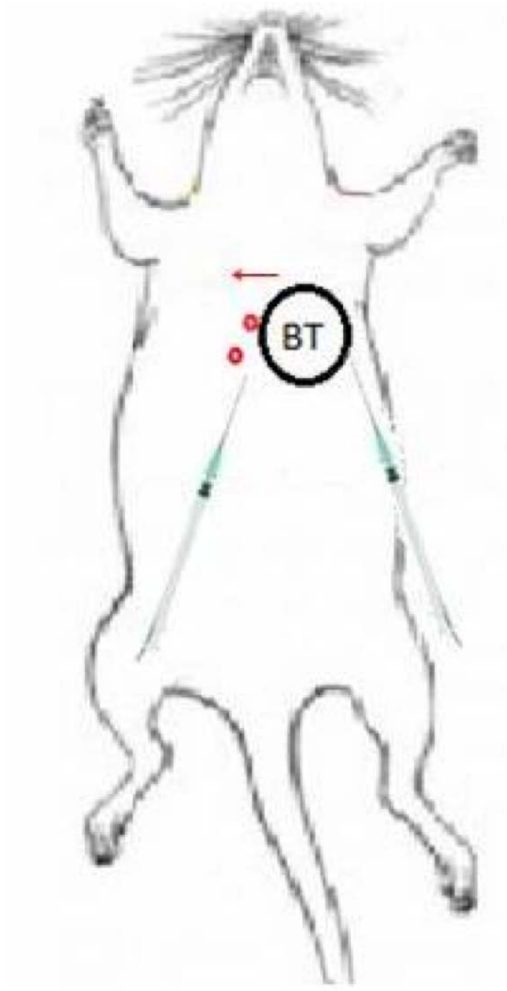

Figure 1: Schema of the experimental pH monitoring in tumor microenvironment after the anesthesia in tumor bearing female rat.

The anesthetized rat was lying on the underlay. The unique system of micro fiber optic pH transmitter with needle-type housing pH microsensor (PreSens, Regensburg, Germany) was used to monitor the pH of the tumor microenvironment. The needle was able to monitor the pH of the tissue each two seconds to give the proper pH of the microenvironment. The dots near breast are representative injection places. BT-breast tumor.

The data were evaluated using GraphPad Prism 4.0 statistical software (GraphPad Software, Inc., San Diego, CA, USA). The results were analyzed using Mann-Whitney test. Significance levels are indicated in the legend of each figure.

RESULTS AND DISCUSSION

With the aim to monitor the pH of formatting tumor in time, we anesthetized the experimental rats once a week in order to see the exact place of the tumor. The tumor microenvironment of the formatting tumor is characterized as acidic. Nevertheless, we found out that the pH of tumor microenvironment seems to be only slightly decreasing (7.33-7.24). Thus, the border of tumor and the surrounding tissue seems to play the crucial role in the monitoring. As seen on Figure **2**, the growing tumor spreads uncontrolled into many sides. Unfortunately, the uncontrolled growth and spread of the tumor tissue made a repeated monitoring over several weeks of a spatially defined tumor region difficult. The pH microsensor was inserted into the tumor microenvironment with a manual micromanipulator for exact localization. However, the needle with the pH transmitter reached not the same place in the same depth during the monitoring period over four weeks. That is why our data could not give conclusive results about how pH alters during the tumor formation. However, the monitoring protocol of pH measurement in tumor microenvironment may have a crucial clinical significance in the future because in cancer therapy, the tumor microenvironment is an important area which is studied to design new therapies [6]. The interactions between the tumor cells and tumor vasculature results in three recognized microenvironmental hallmarks of solid tumors: low extracellular pH, low oxygen tension or hypoxia, and high interstitial fluid pressure [7]. Although tumor pH may vary according to the tumor area, average extracellular tumor pH is between 6.0 and 7.0 whereas in normal tissues, the extracellular pH of is more than

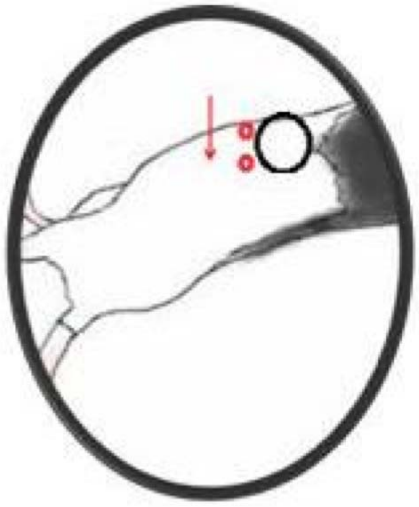

Figure 2: The mistakes in the methodic of pH monitoring of tumor microenvironment *in vivo*.

The microenvironment is not clearly horizontal and vertical bordered. That's why the same monitoring place could not be found in following experimental weeks. The dots near breast are representative injection places.

7.4. Low pH and low pO2 are intimately linked and a variety of insights now support their roles in the prediction of the progression of tumor from *in situ* to invasive cancer. In a low pH extracellular environment, the uncharged fraction of a weak acid increases and such a drug can thus more easily diffuse through the cell membrane [6]. So, pH monitoring could represent a prediction way of the therapy in cancer patients. Nowadays, there is some evidence, dealing with *in vivo* pH measuring of tumor microenvironment using magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), and positron emission tomography (PET) imaging methods (reviewed in [8]). However, these methods are predominantly not used to evaluate the pH of the tumor microenvironment as well as to monitor the changes of tumor pH in time. Using a specific microelectrode with a silver needle, the precise protocol to evaluate tumor pH was developed in mice [9]. Though, in this study the tumor volume reached a volume > 800 mm^3. However, in our present study, the volume of tumors changed in time and usually reached smaller volume with the depth not up to 1.3 cm as used in the previous study. In addition, no tumor microenvironment was evaluated. Nevertheless, there is no evidence about the experimental protocols for application of the pH monitoring in tumor microenvironment in clinical trials and therefore it needs to be precisely described in the future.

Some studies indicate that urine pH could change during cancer development, despite the acid-base balance in the organism [10]. And indeed, our results clearly show the pH changing (P<0.001) in urine of tumor bearing animals (see Table **1**). In order to see whether the pH value of urine correlates with the amount of tumors in the rat, we used the online Pearson Correlation Coefficient Calculator [11]. Our data showed only very weak positive correlation with the R value 0.21. The weak positive correlation could be caused by low replication of the experimental protocol (n = 10). When working with clinical samples, the protocol is straight forward. Since, some experimental protocols cannot be conducted on humans due to bioethics, experimental animals are necessary for such experiments. Nevertheless, the main problem when working with rats or mice is that the urine volume is often under measurement limits. Standard pH meters usually need the minimal volume of milliliters; however, the amount of rat urine is frequently under 100µl. Even so, our samples did mostly contain the urine volume under 100 µl. So, the unique technology of manual micromanipulator together with a needle-type housed pH microsensor allows measuring the fluid volume under 100 µl precisely.

Table 1: Urine pH in the final experimental week in tumor bearing (NMU) and healthy (INT) rats

	pH	pH range
NMU	7.04±0.35 ***	6.42 -7.37
INT	7.56±0.16	7.54 -7.88

The data are expressed as means ± standard deviation (SD) or as the range values. Significance vs. INT is by P<0.001.

CONCLUSION

pH monitoring with micro-invasive, precise pH microsensors could be used for many experimental designs, including *in vivo* pH measurements of tumor tissue and low volumes of urine or other body fluids. The application of the manual micromanipulator with its µm reading accuracy allowed us safe insertion and precise localization of microsensors into the tissue. However, during *in vivo* pH monitoring of growing tumor tissue over several weeks it was difficult to determine a spatially defined measurement region. With a more precise protocol we hope to evaluate pH changes of tumor formation in the future.

ACKNOWLEDGEMENT

The authors declare that they have no conflict of interests.

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Received on 19-09-2015 Accepted on 09-11-2015 Published on 11-12-2015

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