EFFECT OF LONG-TERM EXPOSURE TO NON-THERMAL PLASMA ACTIVATED WATER ON METHEMOGLOBIN IN MICE

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Abstract

Non-thermal plasma activated water (PAW) is described as a potent antimicrobial agent, but although it has numerous bio-medical applications, there is a lack of toxicity studies in living organisms. Thus, as a main objective, we aimed to evaluate the *in vivo* methemoglobin sizing potential of non-thermal plasma activated water (PAW) in CD-1 mice. The device used in our experiment is based on the GlidArc principle, with the advantage of adjusting the values of the current in the circuit on account of a special power supply, which works with magnetic scattering fluxes. A daily volume of 300 ml of PAW was prepared daily with this reactor with the following physico-chemical parameters: conductivity 446 ± 25 μS / cm, pH 2.78 ± 0.12, ORP + 1.06 V, NO2-192 ± 10 mg / L, NO3-1550 ± 95 mg / L, H2O2 2.6 ± 0.12 mg / L, O3 1.08 ± 0.07 mg / L, peroxynitrite - ONOO-. After analysis and interpretation of the data, it was found that methemoglobinemia did not differ significantly in the groups treated with PAW ($p < 0.05$) compared to the control group ($p = 0.8076$). Thus, long-term consumption of PAW has no detrimental effects on the health status of CD-1 mice.

Key words: PAW, mice, methemoglobin

INTRODUCTION

In physics, the term "plasma" refers to the fourth state of aggregation of matter that exists in nature in a wide variety of forms and can be artificially created in different ways, known in technology as thermal plasma and non - thermal type (the gas used remains at a low temperature).

Due to the diversity of fields of application (industrial, bio-medical) the generation of a certain type of plasma and the specific requirements of each application (temperature, potential, chemical composition, flow, etc.) depend on the selection of the source.

Most plasma sources cannot operate in a wide range of parameters, so the operating conditions must be adapted to each application. Technologically, the most commonly used method of generating plasma at low temperatures is to apply an electric field to a neutral gas (Conrads H., Schimidt M., 2000).

An increasing number of original articles discuss numerous applications of non-thermal plasma in biology and medicine. For example, this type of plasma has been used with very good results in decontamination and sterilization of medical devices (Weinstein R.A., 2011), in the treatment of periodontal disease (Sun S.Y. et al, 2016), dermatology (Bogle M.A. et al., 2007), microbiology and many other fields (Laheij A.M.G.A. et al., 2012; Fridman G. et al., 2007).

In dentistry, treatment with cold plasma as a source of reactive species was superior to that with chlorhexidine in the removal of periodontal biofilm (Nayansi Jha, et al., 2017; Sun S.Y. et al., 2016).

Particular attention was paid to the use of non-thermal plasma in contact with liquids (PAL-Plasma activated liquid, including PAW-Plasma activated Water) (Brisset J.L., et al., 2008).

Water treatment with non-thermal plasma induces structural changes (water becomes mono-molecular by breaking hydrogen bonds) and generates numerous reactive species of oxygen and nitrogen, the most important being hydrogen peroxide and peroxynitrite, both showing a strong antimicrobial effect, with technological implications in the medical field, especially decontamination and sanitation.

In our study we aimed to evaluate the *in vivo* methemoglobin sizing potential of non-thermal plasma activated water (PAW) in CD-1 mice, watered 4 hours daily for 90 days.

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MATERIAL AND METHOD

The device used in our experiment is based on the GlidArc principle. PAW preparation requires the blowing of atmospheric air at high speed (10 m/s), supplied by an air compressor and injected through a stainless-steel nozzle into the plasma area produced by the electric discharge in order to maintain the character of cold plasma and to create a turbulent regime on the water surface, to have an efficient mixing of reactive species in water.

The operating conditions of this cold plasma generator have been previously described by Hnatiuc et al., 2014. PAW was prepared in a cylindrical, electro-insulated glass device, in which two metal electrodes with divergent profiles are placed, between which the useful electric discharge can be manifested.

Cold plasma activated water was prepared in the parameters: 446 ± 25 µS / cm, pH 2.78 ± 0.12, ORP +1.06 V, NO2-192 ± 10 mg / L, NO3-1550 ± 95 mg / L, H2O2 2.6 ± 0.12 mg / L, O3 1.08 ± 0.07 mg / L. The average value of the power used to prepare the water was calculated at 111.6 W.

The scheme of the plasma generator is shown in Figure 1.

Fig. 1. Configuration of the non-thermal plasma generator based on a gliding arc discharge

In our study were used 100 CD-1 mice, purchased 2 weeks before the start of the experiment at the Cantacuzino Institute Bucharest, Baneasa resort, 10-week-old nulliparous females, with a mean weight at the beginning of the experiment of 28.85 ± 5.12 grams. The acclimatization of the mice was done under identical conditions of temperature (22 ± 0.7^{o}C) and humidity (50 ± 10%), and the light/dark cycle provided was 12 hours.

Each experimental group was housed in autoclavable polycarbonate cages of 1500 cm2, approximately 300 cm^2/mouse. The animals had a permanent access to water (ad libitum) (autoclavable bottles with drip system) and standardized food at Cantacuzino Institute, with the following composition: 23% protein, 10% fat, 50% carbohydrates, 8% crude fiber and 9 % vitamin-mineral premix, calcium carbonate and phosphate, amino acids.

Mice were divided into two groups, namely the control group (M) watered with drinking water (n = 5) and the experimental group (E), watered with PAW (n = 5). The experiment took place over 90 days, during which time drinking water was replaced daily, at the same time, with PAW prepared under the same conditions. Thus, a volume of 300 ml of distilled water was treated every morning at 8 o'clock for 10 minutes, after which the treated water was passed into borosilicate bottles used for watering all animals. In order to guarantee that the mice consumed this water, they were deprived of running water 6 hours before the administration of PAW. Their watering with PAW was done only for 4 hours, daily, throughout the experiment.

The physico-chemical parameters of the distilled water used in our experiment in the preparation of PAW are the following: conductivity 5 ± 0.3 µS / cm, pH 6.5 ± 0.16, NO2 - undetectable, NO3- undetectable. The physico-chemical parameters for PAW are: conductivity 446 ± 25 µS / cm, pH 2.78 ± 0.12, ORP + 1.06 V, NO2-192 ± 10 mg / L, NO3-1550 ± 95 mg / L, H2O2 2.6 ± 0.12 mg / L, O3 1.08 ± 0.07 mg / L, peroxynitrite - ONOO-.

The two experimental groups were monitored daily, in the same time interval, for 90 days, clinically following: signs of general toxicity (hepatic, cardiac, pulmonary or renal adverse effects) and behavioral changes (reduced food intake, depression, and abnormal body posture).

The determination of the interaction between nitrates and hemoglobin (methemoglobinemia) was evaluated by spectrophotometry. To determine the degree of methemoglobinemia, each mouse was harvested by puncturing the submandibular vein 0.2 ml of blood. Then, 100 µl of blood was mixed with 100 µl of 1% saponin and homogenized to hemolyze the red blood cells, after which 6 ml of phosphate buffer M / 60 pH-6.8 was added.

The relative absorbance measurement was performed with a Boeco UV-vis UV spectrophotometer at 630 nm. The percentage of methemoglobin is calculated according to the formula% MtHb = absorbance x 100. If the MtHb level was below 10%, it was considered safe for mouse and human health.

Statistical analysis

Statistical comparisons between two groups were evaluated using unpaired T TEST. All numerical data were presented as Mean ± SD. Significance was accepted at P> 0.05.

Ethical implications

The study was conducted in accordance with national and international legal regulations on animal welfare, identification, control and elimination of factors causing physiological and behavioral disorders: Directive EC86 / 609 EU.

RESULTS AND DISCUSSION

The data recorded in the two groups were 3,631 ± 0.46 in the control group (M) watered with drinking water and 3,695 ± 0.33 in the experimental group watered with PAW (p = 0.8076) (Table 1).

Table 1

Methemoglobin variations (%)

GROUP	MtHg (%) (M±SD)
Martor	3.631±0.46
PAW	3.695±0.33
p > 0.05	p=0.8076

Methemoglobin levels recorded in the experimental group (PAW) were not significantly different from those recorded in the control group (p> 0.05). The averages of these measurements are below the clinically tolerated human minimum of 10%, a level from which adverse health effects can be observed. No adverse effects were observed in mice during the 90 days of the experiment.

During the 90 days of adaptation of mice with PAW, no mortality or morbidity, adverse effects or other clinical and behavioral changes occurred, especially in terms of food and water consumption, they remained at the same level throughout the experiment.

The effect of PAW is determined by the type and concentration of reactive oxygen species (ROS) and nitrogen (RONS), which are generated depending on the chemical environment (gases and liquids used), the stress exerted and the method of preparation. The use of atmospheric air and distilled water in the production of PAW, leads to the formation of primary species (atomic oxygen, oxygen, superoxide, ozone, hydroxyl radicals and nitrogen atoms), which will then continue to interact and form secondary species (hydrogen peroxide , peroxynitrite, nitric oxide, nitrates and nitrite ions), species that will be distributed in the liquid mass, thus enriching the water in reactive species with antimicrobial role, especially peroxynitrite (Brisset J.L., Hnatiuc E., 2012; Brisset J. L., & Pawlat J., 2016).

Methemoglobin is formed by the oxidation of iron from hemoglobin. The oxidation reaction affects the ability of hemoglobin to carry oxygen, leading to tissue hypoxia and possibly death (Umbreit J., 2007). A small percentage of methemoglobin (1-2%) is commonly found in human and animal blood, but does not produce clinical signs. A level higher than 10% causes mucosal cyanosis, dyspnea, anxiety, fatigue, confusion, dizziness, tachypnea, convulsions and even death when the proportion of total hemoglobin is > 50. The causes are various, but among the most involved substances in the development of this condition we note nitrites and nitrates.

Due to the generous nitrite content of PAW, we expect the percentage of methemoglobinemia to vary significantly in mice given this water daily for 90 days. However, the methemoglobin levels recorded in the experimental group did not differ significantly from those recorded in the control group (p> 0.05). Moreover, the averages of these measurements are below the clinically tolerated human minimum of 10%, a level from which adverse health effects can be observed. No adverse effects were observed in mice during the 90 days of the experiment. The presence of an approximately similar percentage in the control group of methemoglobinemia can be explained by the interaction of chlorides in drinking water with hemoglobin. Balalau et al., 2005, stated that chlorations in mouthwashes and toothpastes induce a certain level of methemoglobinemia.

The absorption of the ingested nitrate ion is usually achieved in the upper segment of the small intestine, with a bioavailability of 100%, being quickly distributed in the body. A probable explanation for this low percentage of methemoglobin in the mice of the experimental group, adapted with PAW (<4%) is related to the interactions between reactive nitrogen species

(SRON) with the organic material in the digestive tract, thus neutralizing at this level.

According to Zhou et al., 2018, nitrite can be converted to unstable HNO_2 under acidic conditions and subsequently decomposed into other nitric oxide species. These reactions can occur at the acidic pH of gastric juice in the stomach (forestomach) of mice (Zhou, R. et al., 2018).

CONCLUSIONS

Prolonged consumption of PAW does not induce methemoglobinemia ($p> 0.05$) and does not affect the health of mice.

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