MODIFICATION OF THE INCUBATION MEDIUM OXIDATIVE ACTIVITY WITH ELECTROCHEMICALLY REDUCED WATER

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Received by editor: 30.06.2018

Abstract. It has been shown that Dulbecco's incubation medium prepared with electrochemically reduced water compensates for oxidative stress induced by hydrogen peroxide addition (H_2O_2, 0.2 mM) to the incubation medium. The incubation medium modified in this way does not affect the H_2O_2 induced apoptosis in an early mouse embryo. The embryonic cell in the experimental model of apoptosis shows the presence of characteristic morphological changes and decreased cell volume. These apoptosis-related changes were detected using laser-scanning microtomography.
Key words*: electrochemically reduced water, redox potential, early mouse embryo, apoptosis, hydrogen peroxide, quantitative laser microtomography.*

Upon water electrolysis, a metastable fraction, called the catholyte or electrochemically reduced water (ERW), is accumulated at the cathode cell. This fraction is characterized by alkaline pH and a negative redox potential [1, 2]. Comprehensive information on the problem of electrochemical activation of water has been presented in the current literature by V.M. Bakhir [3]. Clinical trials of the catholyte have shown its therapeutic properties, as manifested in improving the state of the gastrointestinal tract [4, 5] and in positive effects on diabetes [6], oncology [7], neurodegenerative changes [8], and multiple sclerosis [9]. The fact that curative waters of natural origins, for example, Tenryosui Hita (Japan), Nordenau (Germany), or Arkhyz spring water (Russia) have similar physical and chemical properties is intriguing. What is the nature of these unique properties of the catholyte? The ERW fraction has a negative redox potential and an antioxidant activity [10]. The simulation of the catholyte properties by saturation of the aqueous solution at a given alkaline pH with the molecular hydrogen had no physiological effect. The antioxidant features are possibly due to the presence of nanoparticles, which serve as a depot and a generator of atomic hydrogen and/or hydrogen-anion [1]. It has been assumed in the cited paper that in the catholyte these may be nanoparticles of platinum from the electrode material or mineral nanoparticles in the natural water.

It has been proposed that the antioxidant activity determines the ability of the catholyte to compensate for age-related accumulation of reactive oxygen species (ROS) in the body [11]. Despite the attractiveness of the proposed hypotheses, it is worth taking the following consideration into account. The catholyte, before it enters the tissue, interacts in the lumen of the gastrointestinal tract with secreted f luid, which can change its properties unpredictably. In other words, the antioxidant capacity of the original ERW fraction may be significantly mitigated in the conditions of the experimental environment. A similar situation occurs in cellular biotechnology, where incubation media with stable physical−chemical parameters are used. Therefore, the aim of this work was to explore the extent to which replacement of ordinary water by the catholyte modifies the redox potential of the physiological medium. A model of apoptosis induced in vitro in early mouse embryos by hydrogen peroxide has been chosen as a biological test for the antioxidant properties of the final solution [12, 13].

MATERIALS AND METHODS

The result of the replacement of the ordinary water to the catholyte in the physiological solution was studied with three well-known media: Dulbecco's Modified Eagle's medium (phosphate buffer), M 16 (phos-phate-carbonate buffer), and Tyrode's medium (phosphate-carbonate buffer). Dulbecco's medium is used to conduct short-term experiments that do not involve prolonged incubation of early mouse embryos.

The M 16 medium is widely used for culturing the early embryo of mammals [13]. Tyrode's solution is recommended for experiments, for example, on isolated heart with perfusion according to Langendorf. The electrolyte composition of the tested physiological f luids is shown in Table 1. Bidistilled water or its ERW fraction (the catholyte) was used for the preparation of the physiological media (Table 1). The pH and redox potential of the aqueous solution was measured using an Ecotest-120 pH-meter-ionometer (EKONIKS, Moscow). The redox potential was recorded with an EPV-1 platinum electrode; pH was determined by an ion-selective glass electrode; a silver chloride electrode was used as a reference electrode. We note that the reduction kinetics of the ERW fraction of bidistilled water are relatively fast; the redox potential increases from –800 mV during the first 15–20 min to a quasi-steady state close to –200 mV. However, in this experimental model, this time factor is moderated by the duration of the preparation of physiological solution and the subsequent procedure of apoptosis induction.

Early NMRI mouse embryos were used in the test on the biological activity, which was incubated in physiological Dulbecco's medium under conditions of apoptosis induced by hydrogen peroxide. The samples of isolated zygotes and two-cell embryos were prepared according to a method described previously in [15, 16]. Embryos were isolated from the oviduct according to a published procedure [17, 18]. Apoptosis was induced by incubation (40 min) of isolated embryos in Dulbecco's medium containing hydrogen peroxide at a concentration of 0.2 mM [12, 13, 19].

Table 1. The composition of salts (in mM) in the three physiological media, whose properties were tested in the experiment with the replacement of bidistilled water to the catholyte

Salt	Medium		
	Dulbecco's	M 16	Tyrode's
NaCl	136.8	94.7	136.9
KCl	2.68	4.78	5.0
KH2PO4	1.47	1.19	–
MgSO4	–	1.19	–
MgCl2 · 6H2O	0.49	–	0.6
CaCl2	0.9	1.71	2.5
NaH2CO3	–	25	7.7
Na2HPO4 · 12H2O	7.0	–	–
NaH2PO4 · 2H2O	1.25	–	1.3

The principles and details of the sample preparation based on the ultra-fast cryofixation of biological tissue were discussed in [20, 21]. The initial step is the cryofixation of the embryos in liquid propane (-188 °C). Frozen embryos were lyophilized under vacuum (~ 10^{-3} Pa) at low temperature (-100 °C) using an MBA 5 apparatus (Balzers, Liechtenstein). Upon completion of the low-tempera-ture dehydration of the object, the sample was immersed in embedding medium prepared on the basis of Epon 812 epoxy resin. The use of cryogenic approaches enables one to preserve the shape, size, and structure of the cells close to the native state.

The distinctive morphological characteristics and a decrease in cell volume, which were recorded by quantitative laser microtomography, served as a criterion of apoptosis. The volume of the zygote or two-cell embryo was measured by quantitative three-dimen-sional reconstruction [17, 18, 22, 23]. The samples were examined in a Leica TCS SPE laser scanning microscope (Leica, Austria) in the transmitted light mode of a green laser with a wavelength of 532 nm. For this, a series of successive optical slices in the vertical direction with a step of 2 μm was obtained. Given the low contrast of the obtained digital image, each slice was further treated according to a standardized algorithm [24]. An edge contour of a cell has been drawn on the obtained micrographs in the plane of a slice and a three-dimensional computer model of the object has been obtained using a series of the successive contours [25].

RESULTS AND DISCUSSION

Modification of the Incubation Medium by the Catholyte. Comparative data on pH and redox potential in the experiment on the bidistilled water replacement by the catholyte in the preparation of the physiological solution are shown in Table 2.

The analysis of data presented in Table 2 shows that the acidity of the physiological medium does not depend on whether water or the ERW fraction were used in the preparation of the buffer solution, but rather the choice of the solvent affects the redox potential. The salt composition of the buffer after dissolution causes opposite effects on the redox potential of the original water and the catholyte. A redox potential decrease in water solution and the redox potential increase in the solution prepared with the ERW faction have been observed. In the case of the catholyte, the redox potential of Dulbecco's medium even changes sign to a positive value, but still remains much below the level of the buffer solution in water. These results demonstrate the relative reduction of the antioxidant activity of the catholyte in the physiological medium, but do not allow evaluation of its oxidative activity. For this, a unified algorithm of comparison has been used, which is based on the concentration of molecular hydrogen in an aqueous solution [2].

Table 2. The pH and redox potential of the physiological media prepared with bidistilled water or the catholyte

Physiological medium	pH (22 °C)		Redox potential, mV	
	bidistilled water (5.6) *	ERW (10.5) *	bidistilled water (470)	ERW (-800)
Dulbecco's	7.4	7.4	300	60
M 16	7.7	7.7	320	-140
Tyrode's	7.5	7.5	330	-100

* - bidistilled water: the physiological medium was prepared with bidistilled water, ERW: the physiological medium was prepared with the catholyte obtained from the original bidistilled water. The acidity or redox potential values of the original bidistilled water and its ERW fraction are in parentheses.

Table 3. The oxidative activity index rH_2 of the physiological media prepared with bidistilled water or the catholyte

Solvent	Mater medium				
	bidistilled water	ERW faction	Dulbecco's	M 16	Tyrode's
Bidistilled water	28.6	–	27.8	29.1	28.8
ERW faction	–	5.4	21.6	17.2	17.6

In the cited work, the following expression is obtained after transformations:

$$rH_2 = A(E_h + \Delta) + 2pH, \quad (1)$$

where rH_2 is an index of the oxidative activity of an aqueous solution; A is the constant of proportionality (mV^{-1}); E_h is the oxidation-reduction potential (mV) measured with a platinum electrode; Δ is a correction (200 mV) for a silver chloride reference electrode with saturated KCl at room temperature (~ 22 °C); pH is the acidity of the medium.

The state where $rH_2 \approx 28$ is considered as a redox-neutral (pH 7.2, E_h = 320 mV). The coefficient A is 0.026 mV^{-1} as determined by substituting these parameters into the expression (1). Table 3 shows the rH_2 index values for the original bidistilled water and the ERW fraction, as well as for Dulbecco's, M 16 and Tyrode's physiological media. The rH_2 values were computed on the basis of the experimental data (Table 2) using expression (1).

According to the rH_2 index scale, a solution is consider as being antioxidant in the range $0 < rH_2 < 28$ and as prooxidant in the range $28 < rH_2 < 42$ [2]. The data comparison shows (Table 3) that the index of oxidative activity of double-distilled water and physiological solutions on its basis is close to a neutral state ($rH_2 \approx 28$). The replacement of water by the ERW fraction changes the status of the solution in the direction of the antioxidant state that to a lesser extent is expressed for Dulbecco's medium. Thus, the incubation medium modified by the catholyte has the potential to compensate for the ROS. Is this actually true? Table 4 shows the values of pH, redox potential, and the rH_2 index for Dulbecco's medium into which hydrogen peroxide is added to stimulate apoptosis in the early mouse embryo [12, 13, 19].

Comparison of the rH_2 index before and after the addition of hydrogen peroxide (Tables 3 and 4) shows the oppositely directed effects of ROS upon Dul-becco's solution. The modeling of the oxidative stress reduces the normal incubation medium (from 27.8 to 27.0) and oxidizes that prepared with the catholyte (from 17.2 to 23.1). It should be noted that in both cases, the addition of hydrogen peroxide leaves the physiological solution in the range of antioxidant status ($rH_2 < 28$).

The Biological Activity of the Catholyte in a Model of Induced Apoptosis. The biological effect of the ERW fraction was studied in a model of apoptosis induced by the hydrogen peroxide (0.2 mM, 40 min) in Dulbecco's medium in which early mouse embryos were incubated. It was assumed that the antioxidant status of the physiological medium prepared with the catholyte will arrest the apoptotic action of ROS. The expected effect should prevent the occurrence of morphological changes specific to apoptosis. Figure 1 shows photomicrographs of an intact mice zygote (fig. 1a) and mouse zygotes after natural (fig. 1b) and induced (fig. 1c) apoptosis and after apoptosis induced in Dulbecco's medium prepared with the catholyte (fig. 1d).

It can be seen in the photomicrographs that, in contrast to a healthy embryo (fig. 1a), there are cytoplasmic outgrowths, which is a characteristic sign of apoptosis, in other cases (figs. 1b-1d). In other words, in spite of the antioxidant status of the physiological solution with the catholyte, apoptosis occurred in the presence of ROS (40 min, 0.2 mM H_2O_2). Thus, the antioxidant balance of the catholyte compensates for oxidative stress but does not prevent the development of the induced apoptosis.

Table 4. pH, redox potential and index of oxidative activity (rH_2) of physiological Dulbecco's medium prepared with bidistilled water or the catholyte and the hydrogen peroxide addition

	Parameters of Dulbecco's medium containing 0.2 mM H2O2		
	pH	redox potential, mV	*r*H2 index
Bidistilled water + H2O2	7.4	270	27.0
ERW faction + H2O2	7.4	120	23.1

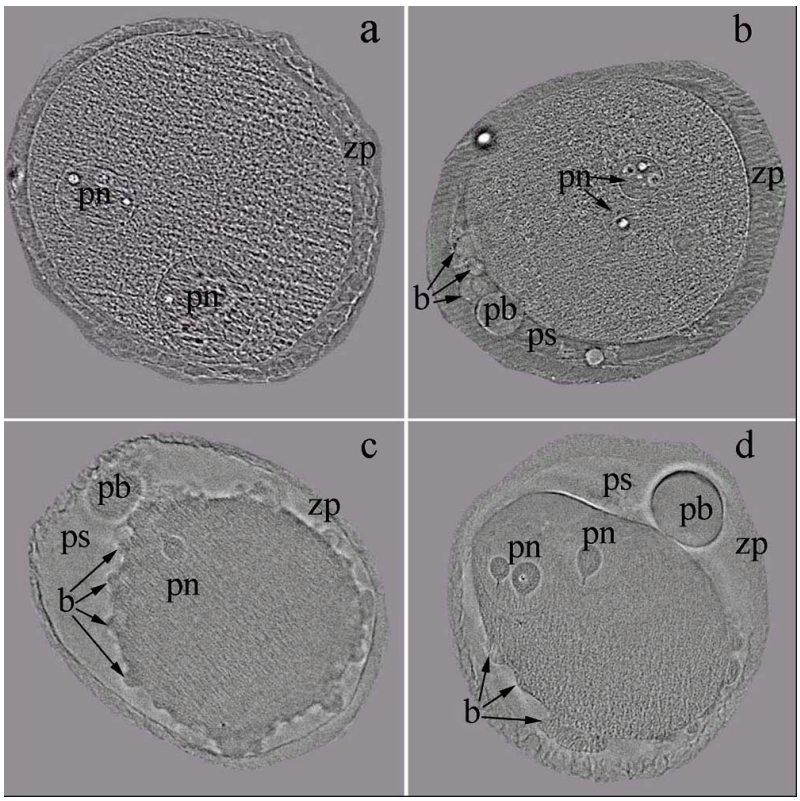

Figure 1. The image of a single-cell NMRI mouse embryo in an optical slice plane, obtained by the quantitative laser microtomography. (a) Control; (b) natural apoptosis; (c) experimental apoptosis caused by the addition of ROS (0.2 mM H_2O_2, 40 min) to the ordinary Dulbecco's medium; (d) experimental apoptosis caused by the addition of ROS (0.2 mM H_2O_2, 40 min) to Dulbecco's medium with the catholyte. Designations: b, protrusions; pb, polar body; pn, pronucleus; ps, the space between the envelope and the cell; zp, the envelope of the embryo

The cascade of events, their sequence and causes, as well as different scenarios of activation of apoptosis are highly debated topics [26-28]. It has been shown in two recent papers that shrinkage of cells, which is considered as a sign of the early stage of apoptosis, precedes the appearance of known morphological and biochemical signs [29]. The decreased cell volume could be due, for example, to potassium ion outf low via the K_{2p} channel, one of the members of the potassium channel family [30, 31]. The effect of mouse embryo shrinkage in natural apoptosis is illustrated by photomicrographs in figure 2.

The volume change of embryonic cells in vivo (fig. 2) is more pronounced than in laboratory models of apoptosis (fig. 1). However, in the latter case, as well, the apoptotic shrinkage is visually determined as the increase in extracellular space between the zygote and the envelope. For the purpose of assessing the statistical significance of the reduction in size of the embryo, its volume was measured in an experiment with induced apoptosis by the method of quantitative laser microtomography (fig. 3).

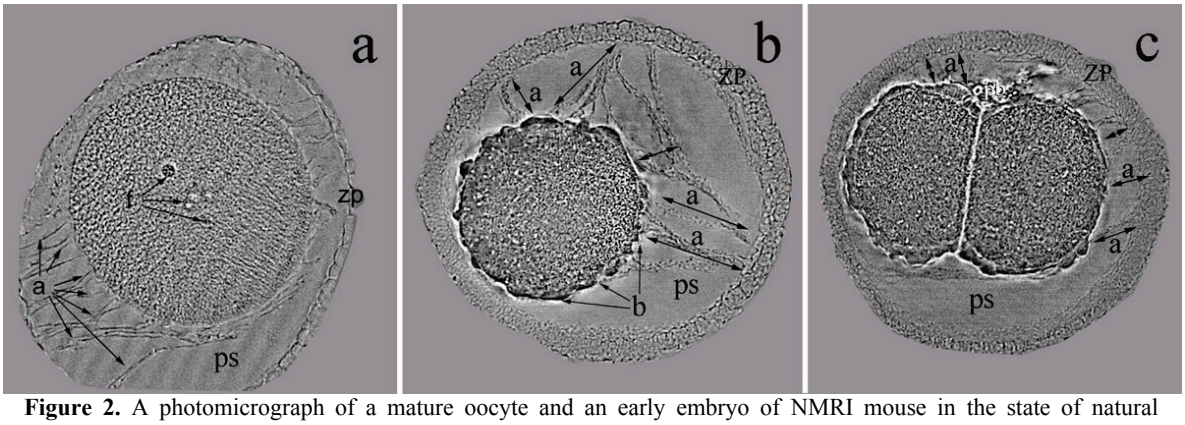

Figure 2. A photomicrograph of a mature oocyte and an early embryo of NMRI mouse in the state of natural apoptosis; the image has been obtained in the plane of an optical slice. (a) The shrunken oocyte; (b) the shrunken single-cell embryo; (b) the shrunken two-celled embryo. Designations: a, filaments connecting the cell and the envelope of the embryo; b, protrusions; pb, polar body; ps, the space between the envelope and the cell; zp, the envelope of the embryo

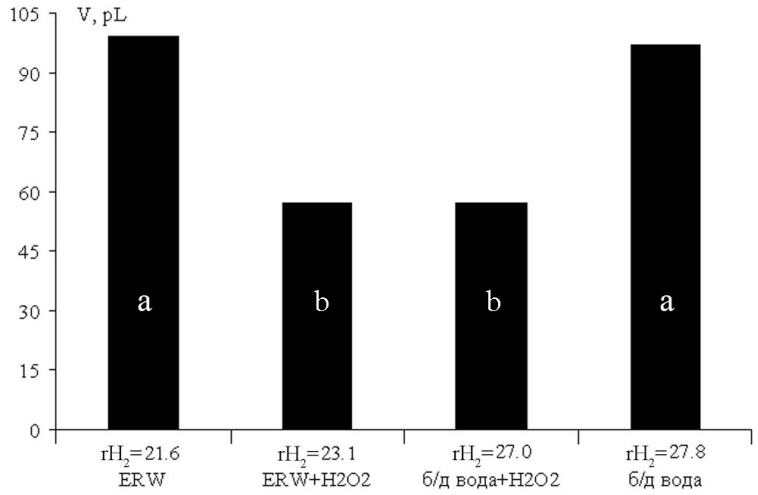

Figure 3. The volume of a two-cell mouse embryo in the experiment with induced apoptosis (0.2 mM H_2O_2, 40 min). The data are arranged according to the magnitude of the index of oxidative activity (rH_2). Designations: ERW, Dulbecco's medium prepared with the catholyte, b/d water, Dulbecco's medium prepared with bidistilled water; $+H_2O_2$, hydrogen peroxide added to Dulbecco's medium at a concentration of 0.2 mM; *V*, the volume of the embryo in picolitre. The data in the columns with different marking letters vary with the significance level $p < 0.05$. The significance of differences was assessed by Stu-dent's test, when the number of embryos was no less than 20 in each group

Figure 3 shows comparative data of the measurements of the two-cell mouse embryo volume relative to the oxidative activity index at different variants of the Dulbecco's medium. Note that apoptosis develops in the antioxidant region of the rH_2 index (~ 23-27), which in itself is unexpected. It is interesting that outside of this limited range, as in the usual medium (rH_2 = 27.8) and in the medium with the catholyte (rH_2 = 21.6) the signs of apoptosis are not observed. If such an effect of submillimolar concentration of hydrogen peroxide is to be discussed in terms of a redox-sensitive mechanism, it is necessary to note two limiting values. The value of rH_2 close to 23 can be regarded as the sensitivity, i.e., the level below which the effect does not occur. If the rH_2 value is above 27, the induced apoptosis is prevented, which may mean the desensitization of the mechanism. One target of the exogenous ROS molecules can be the thiol group in, for example, Cys-loop receptors [18]. In discussing these results it should be remembered that they were obtained in the experimental model with a relatively short interval of exposure. Perhaps the outcome of the catholyte action would be different during its long-term (chronic) consumption, for example, in the conditions of the lumen of the small intestine with active mechanisms of homeostasis stabilization.

Summarizing these results, we can conclude the following that Dulbecco's medium with the catholyte compensates for oxidative stress but does not prevent apoptosis induced by hydrogen peroxide (0.2 mM) in the early mouse embryo. The development of apoptosis in a limited range of rH_2 suggests the action of exogenous H_2O_2 molecules via a redox-sensitive mechanism. In this case, the targets for the H_2O_2 molecule are probably the thiol groups of Cys-loop receptors, K_{2p} potassium channel or VSOAC-like transporters of the Cl^- anion, which are responsible for the compensatory cell volume decrease [32].

The authors are grateful to V.M. Bakhir for the opportunity to use the setting he developed for bidistilled water electrolysis in the laboratory conditions. This work was supported by Russian Science Foundation, project no. 16-16-00020.

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