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COMPENSATORY RESPONSE OF 2-CELL MOUSE EMBRYO TO HYPOOSMOTIC STRESS

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Abstract. Compensatory response of blastomeres in two-cell mouse embryo to hypoosmotic stress was studied employing the direct measurement of cellular volume with Laser Scanning Microtomography followed by 3-D reconstruction (QLSM). It was shown that embryos exposed to hypotonicity first swelled and then returned to the initial size. Swelling phase was defined by a water permeability coefficient (Lp) of 0.4 micron/(min·atm). The next compensatory phase of regulatory volume decrease (RVD) in embryonic cells was not dependent on Na^+/K^+-ATPase inhibition or low pH (6.4), but RVD was abolished by Cytochalasine B (Cyto B) treatment.

Key words: two-cell mouse embryo, regulatory volume decrease, Na^+/K^+-ATPase, Cytochalasine B, laser scanning microscopy, 3D-reconstruction.

The Nernst-Plank model considers three sources for the solvent transport across membrane. The van`t Hoff osmosis, due to diffusion, results from concentration gradient. The electroosmosis represents the water flux indirectly caused by an electric field [1]. The anomalous osmosis, the convection factor, is a term distinctly unique to ion-exchange membranes [2]. The electroosmosis and anomalous osmosis are osmotic phenomena which induce the "abnormal" liquid flow via a membrane contrary to van`t Hoff concept of normal osmosis. Two are intrinsic to these osmotic phenomena - an electrolyte solution and a charged membrane [3, 4]. The above factors reside commonly in a living cell.

It is likely that electroosmosis and anomalous osmosis can be involved in adaptive reaction of cell subjected to osmotic shock. These species of osmosis seem to equilibrate at least partially the van`t Hoff osmosis. This offers the additional basis for an explanation of cellular volume recovery following, for instance, hypotonic stress [5-7]. Cited investigations were performed for the culture of differentiated cells. At what manner cellular volume of early mammalian embryo is appreciably altered by the anisotonic extracellular solution is not understood. Originally, a little attention was paid to this direction since there were no effective ways to determine the volume of embryonic cell (blastomere). Even

with the well characterized developmental system of mice, the major impediment to studies is the limited availability of ~30 embryos/female (10^5-10^6 less than that for cell culture) and the small size of ~60 mkm in diameter (10^1-10^2 times smaller than for flies, fishes or amphibian). These limitations do not allow us to employ *on mass* techniques and get to develop the quantitation of volumetric parameters of the single blastomere. Applying the LSM approach followed with 3-DR this work is aimed to measure the kinetics of a cellular volume in 2-cell mouse embryo subjected to hypoosmotic shock.

Experimental.

All the studies were carried out under the supervision of the ITEB RAS ethics committee that approved the experimental protocols. We used two-cell embryos that were taken as rapidly as possible from the mouse oviduct. Samples obtained immediately after flushing were defined as the control. The hypotonicity was created by reducing 140mM NaCl in Dulbecco`s solution. The details of the techniques for embryo sources, treatments and volume quantitation can be found in the previous paper [8-11]. The used embryo preparation preserved the volumetric characteristic in life like state. Briefly, specimens were frozen in liquid propane at -188ºC, freeze-dried in vacuum at -80ºC and then warmed embryos were immersed immediately in Epon mixture. After epoxy resin was polymerized, prepared specimens were examined using Leica TCS SPE (Leica, Austria) microscope with 532 nm laser in the mode of transmitted light.

Results and discussion.

Several optical parameters should be provided to use specimens for a laser scanning microscope. In the first place, the sample substances should be transparent for laser light. Second, the embryo deepness in sample block should be no more 0.2 mm. Finally, sample thickness is limited to 3 mm and planes of the top and bottom surfaces should be parallel. LSM images show poor contrast for embedded, unstained embryos (Fig.1A). Therefore the optical section picture recorded digitally is processed using conventional graphic software [12]. The embryo 2D image treated with software graphics is sufficiently high to form morphological resolution necessary for observation of embryonic compartments (Fig.1B). The volume quantitation following the software treatment of optical sections is based on 3-DR technique [11]. Standard graphic editors enable this strategy to be realized when LSM Z-stack of optical sections is applied for a 3-D reconstruction in 3ds max medium (Fig.1C).

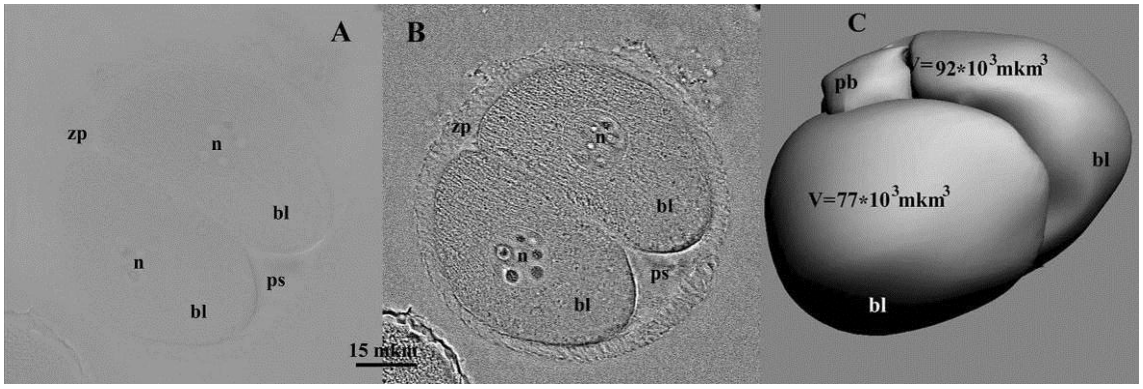

Figure 1 – Optical section and 3-DR images of freeze-dried, embedded embryo. (A) Unstained mouse embryo picture in LSM, (B) the same after processing with graphic editor, (C) two-cell mouse embryo image after 3-DR; bl – blastomere, n – nucleus, ps – perivitelline space, zp – zona pellucida, V – blastomere volume value, scale bar – 15 mkm

Originally, a little attention was paid to the influence of physiological medium on embryonic cell volume since qualitative observations showed no visible changes in volumetric parameters. Our finding suggests that the volume decrease seems to be initiated at initial phase of "physiological" exposure [11]. It should be emphasized that even brief exposure to hypertonicity impaired the embryo development, for instance, embryos subjected to hypertonic shock were shown to increase p38 MAPK activity in conjunction with elevated CCM2 levels [13]. Unlike hypertonic conditions, lower osmolarity is beneficial to early embryo development [14]. Volumetric data obtained for blastomeres of two-cell embryo subjected to hypotonic stress are summarized in Fig.2.

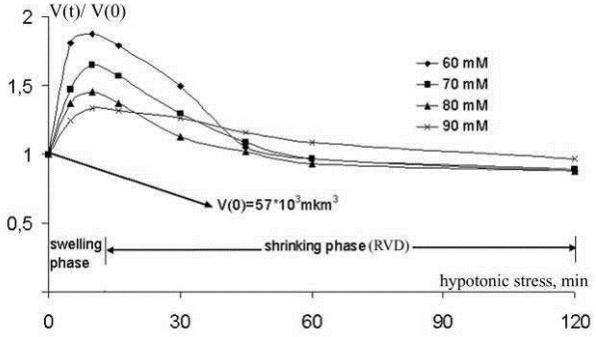

Figure 2 – Relative volume response upon exposure of embryonic cells in the Dulbecco`s at hypotonic condition created by replacing 140 mM NaCl with 60 mM NaCl, 70mM NaCl, 80mM NaCl or 90 mM NaCl. V(t) – blastomere volume in hypotonic conditions, V_o – initial cellular volume

Figure 2 represents, in term of normalized embryonic cells volume, the rapid swelling and subsequent RVD in response to hypotonic stress. Embryonic cells subjected to hypotonic stress are initially seen to act as nearly perfect

osmometers ideally being defined by van't Hoff equation. Ten minutes after beginning the hypotonic exposition the swollen blastomere are approximately at their maximally volumes that changes dependent linearly on NaCl concentrations in the Dulbecco`s (Fig.3).

The line shown in Fig. 3 is crossed with axis ΔmM (NaCl) in near 30 mM. This fact may indicate that no volume alteration would be induced in the Dulbecco`s containing approximately 110 mM NaCl. This suggestion will be under investigation but the diapason of NaCl content (100 mM – 140 mM) predicted above for intact state includes the value of 110 mM. Based on the assumptions: (1) the rapid swelling phase is provided only by the osmotic water entry, (2) the amount of aquaporines controlling water flow trough membrane remains constant during osmotic shock and (3) osmotic coefficient for Na^+ and Cl^- in the Dulbecco's is taken to be 1.0 plasma membrane water permeability can be estimated from Eq.1

$$Lp = (\Delta Vt / \Delta P) / (S * t), \quad (1)$$

where Lp - effective hydraulic conductivity; ΔV – difference between blastomere volume for interval "t" (5 minutes) and its initial volume; ΔP – difference between the initial osmotic pressure and hypotonic osmotic pressure; S – original area of cellular surface.

The value of ΔV/ΔP slope to be obtained from Fig.3 is equal to $0{,}5*10^3$ mkm^3/mosm. The initial surface area for blastomere of two-cell mouse embryo was determined as approximatelly 10^4 mkm^2 [11]. According to Eq.1 Lp for embryonic cell membrane is equal to 0,01 mkm/(min∗mosm) or 0,4 mkm/(min∗atm) at 26 degrees C. This is only rough calculation, but the determined coefficient of water permeability is close to the value measured for MII mouse oocytes [15-17].

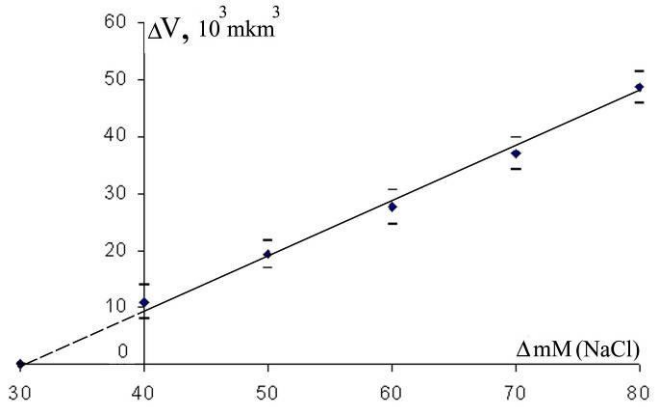

Figure 3 – Changes of volume in blastomere subjected to hypotonic exposure. ΔV – difference between the maximum blastomere volume for interval of 10 minutes and its initial volume, ΔmM (NaCl) – difference between the conventional (140 mM) and hypotonic NaCl concentrations in the Dulbecco`s. The solid line represents the experimental results (mean ± sem). The broken line represents the expected linier extrapolation

The previous observations suggest that Na,K pump is required for RVD occurring in many kinds of somatic cells [6, 7]. Employing K^+-free solution as a specific inhibitor of Na^+/K^+-ATPase, the experimental was designed to test the possible contribution of electrogenic forces to the shrinking phase in blastomere of two-cell mouse embryo. Our results from such experiments are shown in Table 1 along with data on the pH-sensitive anomalous osmotic response phase.

Table 1 – Kinetics of blastomere volume in two-cell mouse embryos determined by Q LSM during exposure to the hypotonic Dulbecco`s (70мМ NaCl) with and without K^+ at pH 7,4 or pH 6,4 *

15 minutes pretreatment in conventional Dulbecco`s solution	0 min **	15 min	30 min	60 min
	volume (x10^3 μm^3) of blastomere subjected to hypotonic stress			
4,2 mM K^+, pH 7.4	56±4, n=22	88±10, n=14	77±10, n=18	53±4, n=14
K^+-free, pH 7.4	54±3, n=22	86±8, n=24	78±12, n=18	54±6, n=36
K^+-free, pH 6.4	55±4, n=22	74±10, n=28	79±6, n=24	54±4, n=22

(*) data are presented as mean ± s. d., n – number of embryos measured.
(**) "0 min" – embryos cryofixed immediately after 15 minutes pretreatment.

The pump inhibition is seen in Table 1 not to influence significantly RVD phase in embryonic cells. This finding can be explained by the low Na^+/K^+-ATPase activity shown for early mouse embryo [18, 19] and initially low level of the membrane potential which is a characteristic attributed to the early embryo [20]. Additionally, this enzyme is also inhibited by taurin, an amino acid that abounds in the mouse oviduct. Therefore the active Na,K transport allows the negligible contribution to volume regulation of blastomeres *in vivo*. In the absence of elctroosmosis canceled with Na^+/K^+-ATPase inhibition the pH-sensitive anomalous osmosis seems to decrease the swelling phase (Table). There are at least two possible events which can explain this phenomenon. One is that changes in isoelectric state are followed by the modification of aquaporin function [21, 22]. Water transport was suggested to depend on aquaporin regulation appearing at low pH [23, 24]. Another possibility is that external acidification activates Na^+/H^+ exchanger counteracting water

uptake into cell. Mouse embryos at two-cell stage, however, do not seem to posses specific pH-regulatory mechanisms [25].

The possible role of cytoskeleton during RVD was reviewed [26, 27]. Cyto B for differentiated cells in the culture and suspension was discussed to inhibit RVD suggesting that an intact microfilament network is prerequisite for an "anomalous" osmotic behaviour [28]. Our data (not shwn) confirm the validity of this proposition also for cells of early mouse embryo. On isotonic exposure to Cyto B the blastomere exhibits vigorous blebbing activity (Fig.4). Blebs are developed to a maximum after 5 minutes exposure to the chemical and they are subsided 10-15 minutes later, restoring a smooth surface to the cell. The principal modes by which ions move trough cellular membrane are ATP-dependent transport (pumps) and gradient-dependent transport (diffusion, ion-exchangers, anti- and co-porters). There is a surprising diversity between different cell types and, especially, between differentiated cells and blastomeres. In contrast to the most cell kinds, the high concentrations of both potassium and sodium were reported inside embryos during early development [20].

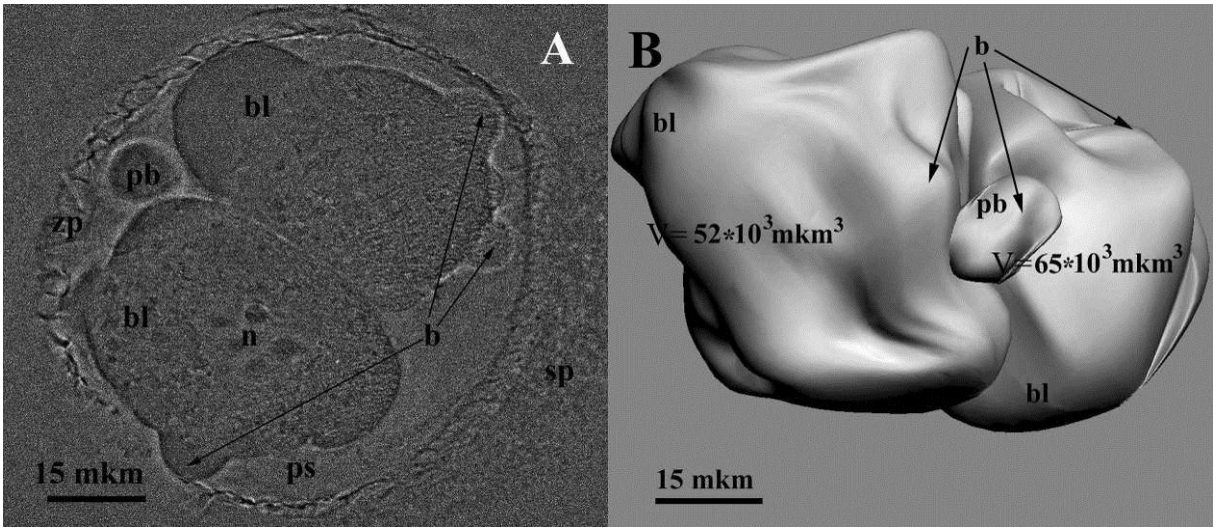

Figure 4 – Typical image of two-cell mouse embryo exposed for 5 minutes to conventional Dulbecco's containing Cytochalasin B [from: 29]. (A) Embryo picture on optical section from LSM stack and (B) the same embryo after 3-DR processing; bl – blastomere, b – blebs, n - nucleus, pb – polar body; ps – perivitelline space, sp – salts powdered from Dulbecco`s solution during freeze-drying, zp – zona pellucida, V – blastomere volume value, scale bar –15 mkm

The nature of the regulatory mechanisms governing the volume changes in blastomere during RVD is, as yet, totally unclear. Activation of the net Na^+-K^+ flux by membrane stretch and actine cytoskeleton seems to be possible. Note, the equimolare replacement of potassium on extracellular sodium ions changes thermodynamic state of cellular liquid. For instance, water may be transformed into more viscous substance and, as a result, less osmoticaly active fluid because of positive hydration caused by sodium ions [30]. This is the additional mechanism of volume regulation which requires the approach to measure the cytoplasmic Na^+ and K^+ content *in vivo*.

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