JOURNAL OF SURGICAL RESEARCH **60,** 122–128 (1996)
Article No. 0020

The Effects of Hyperosmolarity on the Viability and Function of Endothelial Cells[1]

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Submitted for publication July 6, 1994

Hypertonic solutions have been demonstrated to be efficacious in the treatment of hypovolemic shock. Their continued use when serum osmolarity is elevated may be harmful because they induce cellular dehydration. Because the hyperosmotic tolerance of cells is largely unknown, we determined the effects of increased media osmolarity on *in vitro* endothelial cell viability and function following periods of normoxia, anoxia, and anoxia with reoxygenation. Bovine aortic endothelial cells were exposed to hypertonic media of 330–570 mOsm/liter for 6–30 hr. Cell viability and function were ascertained utilizing trypan blue exclusion, lactate dehydrogenase (LDH) enzyme release, and cell replating assays. Endothelial cells exposed to media of 460 mOsm/liter demonstrated no significant decrease in the percentage of viable cells (69.81 ± 6.03 vs 70.64 ± 4.62% for controls), LDH activity (334.67 ± 7.91 vs 228.03 ± 191.28 Berger–Broida U/ml), and replating efficiency (58.27 ± 42.07 vs 59.10 ± 5.79%) after 30 hr of normoxic incubation. Hypertonic media up to 570 mOsm/liter did not adversely affect cell viability following a 6-hr anoxic insult. A 6-hr anoxic insult followed by 24 hr of reoxygenation in media of 530 and 570 mOsm/liter resulted in significantly increased viability and replating efficiency compared to 30 hr of normoxia. Our data demonstrate that *in vitro* endothelial cells tolerate media osmolarity of up to 460 mOsm/liter without apparent decrement in viability or replating efficiency even in adverse conditions of anoxia and reoxygenation. Our data also suggest that exposure to anoxia may induce tolerance of endothelial cells to hyperosmotic media.

Hemorrhagic shock is the second leading cause of traumatic death in the United States [1]. Resuscitation from hemorrhagic shock requires expansion of the vascular volume by the administration of blood and asanguineous fluids [2]. Of the available asanguineous fluids, hypertonic solutions have been shown to significantly improve hemodynamic parameters and to be more efficacious than isotonic fluids for the treatment of hemorrhagic shock [3–8].

Although effective in small volumes, excessive administration of hypertonic fluid could lead to intracellular dehydration and cellular dysfunction [9]. Because of the risk of intracellular dehydration due to serum hypertonicity, current clinical recommendations are to stop the infusion of hypertonic fluids when a serum osmolarity (SOsm) of 350 mOsm/liter is exceeded [4, 5]. Although a few prospective controlled studies have shown that serum osmolarities of 320–330 mOsm/liter after hypertonic resuscitation are well tolerated [6, 7], there are no data to support the presumption that exceeding a SOsm of 350 mOsm/liter will result in cellular dysfunction. In fact, the upper limit of osmolarity which can be tolerated by cells is unknown.

It was the intent of this study to ascertain the upper limit of media osmolarity which could be tolerated by bovine aortic endothelium cells (BAEC) after prolonged periods of normoxia in hypertonic media. Additionally, we sought to simulate ischemia in endothelial cells, as it might occur during hemorrhagic shock and resuscitation, by exposing BAEC to prolonged periods of anoxia and anoxia followed by reoxygenation. We then introduced hypertonic media into this system and determined the viability and function of the exposed cells.

MATERIALS AND METHODS

Endothelial cells. Primary cultures of BAEC were isolated and grown to confluence using conventional protocols [10, 11]. Following isolation, cells were seeded onto 24-well untreated petri dishes (well surface area: 4.9 cm^2) at a seeding density of 75,000–100,000 cells/ml. Cells were then grown to confluency using complete MM 199 medium supplemented with 20% heat-inactivated fetal bovine serum, 100 μg/ml neomycin, 20 units/ml nystatin, and 2 m*M* L-glutamine at 37°C under routine culture conditions. All cells used were between the 10th and 20th cell population doubling life (CPDL). BAEC are stable and retain functional and morphologic characteristics of endothelial cells and, thus, are suitable for *in vitro* studies for up to 100 CPDLs [12, 13].

Hypertonic media. Stock serum-free MM 199 media (Gibco/BRL Industries, Grand Island, NY) with an osmolarity of 330 mOsm/liter was the control. Media osmolarity was increased by adding sodium chloride (Sigma Chemical Corp., St. Louis, MO) to the standard stock media. The amount of added sodium chloride was determined using stoichiometric calculations to yield solutions ranging in osmolarity from 330 to 570 mOsm/liter. All solutions were filtered and allowed

[1] Supported in part by Grant NINDS P20 NS30324-03 from the National Institutes of Health. Winner of the Association for Academic Surgery Student Research Award.

0022-4804/96 $12.00

to equilibrate at room temperature for a minimum of 2 hr prior to use. Final osmolarity was determined using freezing point depression (Osmette A, Automatic Osmometer, Precision Systems Corp., Newton, MA). A pH between 7.3 and 7.5 was maintained by controlling the CO_2 level in the incubator (95% air:5% CO_2).

Experimental conditions. It was desirable to perform these studies in a serum-free medium system, thus avoiding serum-induced artifacts. Prior to exposure to the media, and prior to anoxia and reoxygenation conditions, the BAEC were rinsed with Hank's buffered salt solution (HBSS) and then refed with serum-free MM 199 of the appropriate osmolarity. Control and experimental cells remained in serum-free medium throughout the course of the experiment.

Normoxic incubation. Media were removed from confluent cultures and cells were rinsed with HBSS. Each cell culture was then refed with 1.0 ml of serum-free MM 199 of the appropriate osmolarity and incubated under normoxic conditions (95% air:5% CO2) for periods of 6, 12, 24, and 30 hr.

Anoxia/reoxygenation. We sought to simulate reperfusion by exposing BAEC to anoxia (95% N_2:5% CO_2) followed by reoxygenation (95% air:5% CO_2) as described by Shatos *et al.* [14]. Anoxia was maintained using an oxyreducer (Model 311, Reming Bioinstruments, Redfield, NY) [15] designed to maintain low levels of oxygen within the incubator chamber. The oxyreducer consisted of an oxygen sensor and a nitrogen infusion tube placed inside the incubator attached to a digital monitoring device located outside of the chamber. This apparatus operates by sensing and displacing oxygen with nitrogen. Nitrogen was infused for approximately 1 hr prior to the experiment. A level between 0 and 1% oxygen was maintained throughout the experiment.

Endothelial cells were exposed to anoxia for 6 hr. Shatos and coworkers [14] have shown that 6 hr of anoxic conditions produce an increase in intracellular and extracellular lactate levels, indicative of an ischemic insult. Following anoxia, a subset of cells was reoxygenated under normoxic conditions of 95% air:5% CO_2 for 24 hr. This anoxia/reoxygenation model results in the production of superoxide radicals and can induce sublethal cellular injury [14, 16].

Trypan blue dye exclusion. Trypan blue is an acid dye which stains nonviable cells [17]. Following incubation the supernatant of each sample was removed from the tissue wells and replaced with 0.5 ml of PBS. Trypan blue was then added to cultures of endothelial cells and allowed to remain for 3 min. Three representative fields were examined using a Zeiss light microscope (Carl Zeiss, Inc., West Germany) at 400× magnification. A total of 300 cells per culture were counted. Viability was expressed as the percentage of cells excluding trypan blue dye.

Lactate dehydrogenase (LDH) release assay. Following incubation, the media were analyzed for LDH activity using a modification of the calorimetric method of Cabaud and Wroblewski [18]. Each cell culture supernate was added to fresh assay mixture (pyruvate substrate, NADH, Sigma color reagent, and 8 *N* NaOH) and absorbance at 450 n*M* was recorded. LDH activity is expressed as Berger–Broida units per milliliter (B-B units/ml) and directly off a calibration curve prepared in accordance with the assay protocol.

Cell detachment/plating efficiency. After the specified periods of incubation as described previously, anisotonic medium containing the nonadherent/nonfunctional cells was removed and versene (0.25 ml) and trypsin (0.25 ml) were added and allowed to stand 5–15 min to detach adherent cells from the culture wells. Complete MM 199 (0.5 ml) was then added to neutralize the proteolytic activity of trypsin, followed by removal of an aliquot of the cells for the purpose of counting using a hemocytometer. These cells represented the original number of cells which were functional immediately following exposure to anisotonic media and the stress of prolonged periods of normoxia, anoxia, or anoxia/reoxygenation. In order to evaluate subsequent cellular dysfunction due to the above perturbations, the cells were then replated into fresh, untreated culture wells as described. After 10 hr incubation, each culture well was rinsed with complete MM 199 and total cell yield was again determined. Plating efficiency is expressed as a percentage of cells which reattached. This methodology has been used previously as an indicator of endothelial cell viability and function [19, 20].

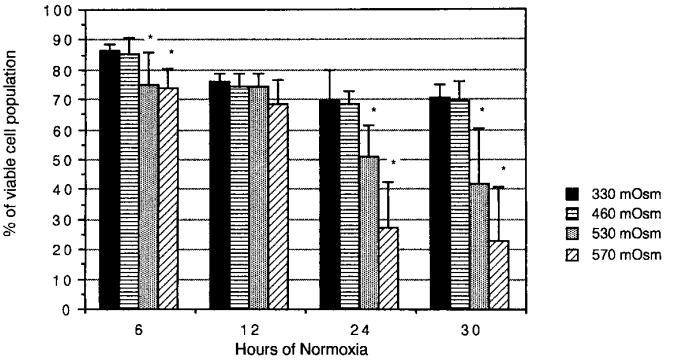

FIG. 1. Percentage of cells viable determined by trypan blue exclusion following normoxic exposure to hypertonic media for various periods. Cells exposed to 530 and 570 mOsm/liter had significantly lower viability than controls (330 mOsm/liter) at 6, 24, and 30 hr. $n = 9$, $^*P < 0.05$ vs control.

Statistical analysis. Each experiment was performed three times on three separate cultures of similar passage level and seeding density ($n = 9$). Data are expressed as mean ± standard deviation. Nonpaired two-tailed Student's *t* test was used for between-group comparisons. Paired Student's *t* test was used for within-group comparisons. Significance was attributed to a *P* value of ≤ 0.05.

RESULTS

Cell Viability

Trypan blue vital dye. Following 6 hr of normoxia, 86.6 ± 2.2% of control cells (330 mOsm/liter) remained viable. This decreased to 70.6 ± 4.6% of viable control cells after 30 hr of normoxic incubation. The observed decrease in viability of control cells over the time course described is within the range expected for our serum-free system.

Cells exposed to an osmolarity of 460 mOsm/liter had survival similar to controls. When the media osmolarity was increased to 530 and 570 mOsm/liter viability was significantly lower than controls at 6, 24, and 30 hr of normoxic incubation (Fig. 1).

A 6-hr anoxic insult did not significantly decrease the percentage viability of any of the cultures when compared to 6 hr of normoxia (Table 1). Six hours of anoxia significantly decreased the viability of cells exposed to a media of 570 mOsm/liter compared to controls (330 mOsm/liter; Fig. 2). Six hours of anoxia followed by 24 hr of reoxygenation significantly reduced the viability of cells in media of 530 and 570 mOsm/liter compared to controls. There was no significant difference in viability between controls and cells exposed to media of 460 mOsm/liter after either 6 hr of anoxia or 6 hr of anoxia followed by 24 hr of reoxygenation (Fig. 2).

We compared viability of the cultures after 6 hr of anoxia followed by 24 hr of reoxygenation (total 30 hr) to 30 hr of normoxia. Viability in control cells and cells exposed to 460 mOsm/liter was similar under both conditions and there was no significant difference between them. Viability of cells in media of 530 and 570 mOsm/liter was significantly greater after 6 hr of anoxia fol-

TABLE 1

Percentage Viability and LDH Activity after 6 hr: Normoxia vs Anoxia

Media osmolarity (mOsm/liter)	Normoxia		Anoxia	
	Percentage viability	LDH activity	Percentage viability	LDH activity
330	86.6 ± 2.2	147.9 ± 20.5	89.2 ± 5.1	235.5 ± 28.6*
460	85.4 ± 5.6	227.4 ± 14.8†	84.7 ± 3.0	221.4 ± 29.8
530	74.9 ± 10.7	265.3 ± 9.9†	79.9 ± 14.6	184.2 ± 13.3*
570	73.9 ± 6.5	206.7 ± 52.5	74.7 ± 8.7	202.9 ± 26.2

* $P < 0.05$ vs normoxia.
† $P < 0.05$ vs controls (330 mOsm/liter).

lowed by 24 hr of reoxygenation than it was after 30 hr of normoxia (Fig. 3).

Lactate dehydrogenase release. Following 6 hr of normoxia, the LDH activity in the supernatant from the control cells (330 mOsm/liter) was 147.9 ± 22.5 B-B U/ml. This increased to 329.7 ± 288.9 B-B U/ml after 24 hr. LDH activity was significantly increased after 6 hr of normoxic incubation in 460 and 530 mOsm/liter media compared to controls (Table 1). Thereafter (12–30 hr), LDH activity increased slightly in all groups but there was no significant difference among them and no significant difference from the 6-hr activity (data not shown).

When compared to 6 hr of normoxia, 6 hr of anoxia significantly increased LDH activity in the supernatant of the controls and significantly reduced LDH activity in the supernatant of cells exposed to 530 mOsm/liter (Table 1). Following 6 hr of anoxia and 24 hr of reoxygenation, LDH activity in the supernatant of all cultures was significantly greater than after 6 hr of anoxia alone (Fig. 4).

Cell Function

Cell detachment/plating efficiency. Following 6 hr of normoxia, 70.4 ± 4.3% of control cells were capable

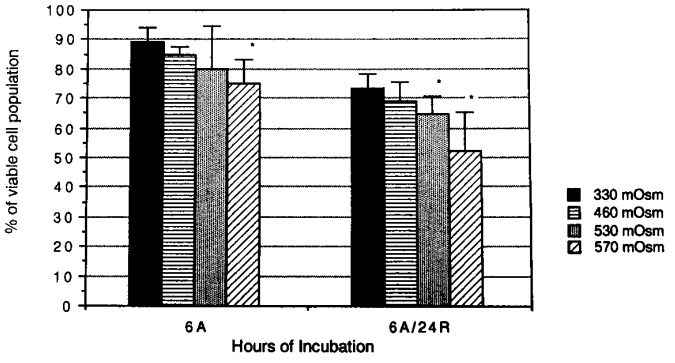

FIG. 2. Percentage of cells viable determined by trypan blue exclusion following exposure to hypertonic media and anoxia or anoxia/reoxygenation. Compared to controls, viability after 6 hr of anoxia was significantly lower in cells exposed to 570 mOsm/liter. Following 6 hr of anoxia and 24 hr of reoxygenation, viability was significantly lower in cells exposed to 530 and 570 mOsm/liter. $n = 9$, $^*P < 0.05$ vs controls; 6A, 6 hr of anoxia; 6A/24R, 6 hr of anoxia followed by 24 hr of reoxygenation.

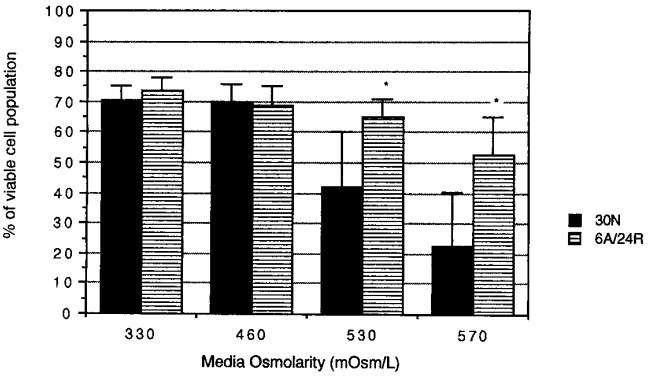

FIG. 3. Percentage of cells viable determined by trypan blue exclusion following a 6-hr anoxic/24-hr reoxygenation insult compared to 30 hr of normoxic conditions. In cells exposed to 530 and 570 mOsm/liter viability was significantly greater after conditions of anoxia/reoxygenation than it was after normoxic conditions. $n = 9$, $^*P < 0.05$ vs normoxia; 30N, 30 hr of normoxia; 6A/24R, 6 hr of anoxia followed by 24 hr of reoxygenation.

of reattaching to fresh culture plates. This number decreased significantly to 59.1 ± 5.8% after 30 hr of normoxic incubation. Plating efficiency was not affected by a medium osmolarity of 460 mOsm/liter as there was no significant difference in percentage reattaching at any time period when compared to controls (Fig. 5). Plating efficiency was significantly lower in cells exposed to 530 mOsm/liter at 24 and 30 hr compared to controls. Cells exposed to 570 mOsm/liter had significantly lower plating efficiency at all study periods of normoxic incubation.

Six hours of anoxia followed by 24 hr of reoxygenation resulted in a significantly lower plating efficiency for all hyperosmolar media (Fig. 6). Six hours of anoxia followed by 24 hr of reoxygenation did not significantly alter plating efficiency compared to 30 hr of normoxia in controls or cells exposed to 460 mOsm/liter. Cells exposed to 530 and 570 mOsm/liter had significantly increased plating efficiency after 6 hr of anoxia and 24 hr of reoxygenation when compared to 30 hr of normoxia (Fig. 7).

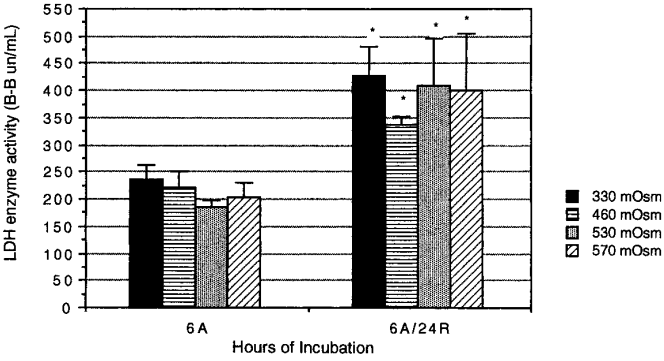

FIG. 4. LDH activity in the supernatant following exposure to hypertonic media and 6 hr of anoxia or 6 hr of anoxia and 24 hr of reoxygenation. LDH activity increased significantly in all groups with reoxygenation. $n = 9$, $^*P < 0.05$ vs 6A; 6A, 6 hr of anoxia; 6A/24R, 6 hr of anoxia followed by 24 hr of reoxygenation.

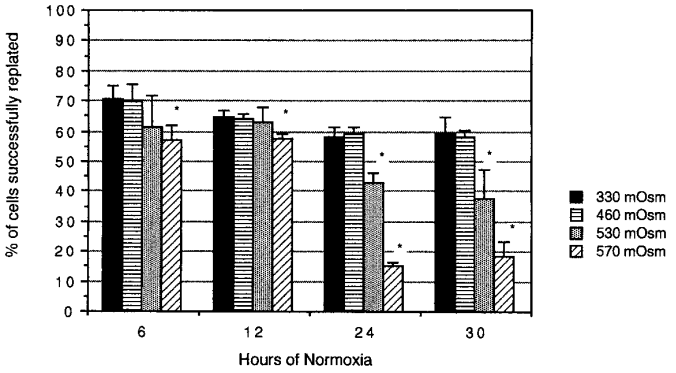

FIG. 5. Replating efficiency of cells following normoxic exposure to hypertonic media for various periods. Replating efficiency was significantly lower than controls at 24 and 30 hr in cells exposed to 530 mOsm/liter. Cells exposed to 570 mOsm/liter had significantly lower plating efficiency at all time periods compared to controls. $n = 9$, $^*P < 0.05$ vs controls.

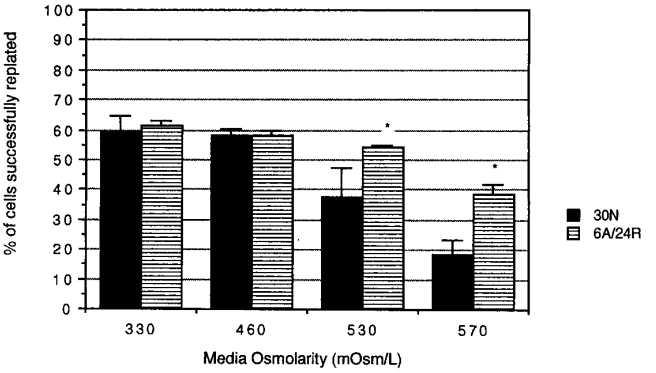

FIG. 7. Replating efficiency following a 6-hr anoxic/24-hr reoxygenation insult compared to 30 hr of normoxic incubation. Replating efficiency was unaffected in controls and in cells exposed to 460 mOsm/liter. Cells exposed to 530 and 570 mOsm/liter had significantly increased plating efficiency after exposure to anoxia/reoxygenation than after exposure to normoxic conditions of similar duration. $n = 9$, $^*P < 0.05$ vs normoxia; 30N, 30 hr of normoxia; 6A/24R, 6 hr of anoxia followed by 24 hr of reoxygenation.

DISCUSSION

Hypertonic solutions have been demonstrated to have significant advantages over isotonic fluids in the resuscitation of hypovolemic shock [2–8, 21, 22]. Hypertonic solutions expand the vascular volume by extracting water from cells down an osmolar gradient. Mazzoni *et al.* [23, 24] have demonstrated that endothelial cells are a major source of the water which replenishes the intravascular volume. Because the endothelial cell plasma membrane is impermeable to solutes but freely permeable to water, endothelial cells behave as "classic osmometers" [9]. The endothelial cell volume responds rapidly and proportionally to changes in osmolarity; the greatest and most rapid volume reductions occur at the highest osmolarity.

A potential disadvantage of resuscitation with hypertonic saline is cellular dehydration. The degree to which cells can be dehydrated and remain viable and functional is largely unknown. Such information is important because hypertonic resuscitation of all shock states has been advocated. In addition, hyperosmolar therapy is common following severe head injury. Determining the tolerance of endothelial cells to hypertonic media could provide a rational basis for limiting hypertonic resuscitation and hyperosmolar therapy of head injury [25].

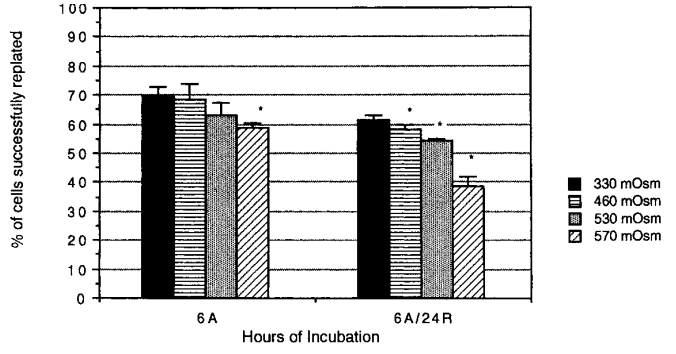

FIG. 6. Replating efficiency following exposure to hypertonic media and 6 hr of anoxia or 6 hr anoxia and 24 hr of reoxygenation. Replating efficiency was significantly lower than controls after 6 hr of anoxia in cells exposed to 570 mOsm/liter. Following 6 hr of anoxia and 24 hr of reoxygenation, replating efficiency was significantly lower than controls in all experimental groups. $n = 9$, $^*P < 0.05$ vs controls; 6A, 6 hr of anoxia; 6A/24R, 6 hr of anoxia followed by 24 hr of reoxygenation.

Most of the recent work investigating the effect of osmolarity on cell physiology has concerned the osmolar activation of the plasma membrane electroneutral sodium/hydrogen exchanger (antiport) in the control of cell volume [26, 27]. The antiport facilitates the extrusion of protons from the intracellular space by direct exchange with extracellular sodium and is essential to the regulation of intracellular pH (pH_i).

In its role in the regulation of pH_i, the antiport is thought to initiate cell swelling following anoxia or ischemic events [28, 29]. When oxygen delivery fails to meet metabolic demand anaerobic metabolism ensues with the generation of protons, resulting in a decrease in the pH_i. The antiport, which is quiescent at normal pH_i, becomes activated and exchanges a hydrogen ion for a sodium ion (Fig. 8). The extruded proton rapidly combines with bicarbonate in the extracellular space forming carbonic acid which dissociates into carbon dioxide and water. The carbon dioxide diffuses into the cell where it combines with water and the process is reversed generating a hydrogen and a bicarbonate ion. The hydrogen ion is handled by the antiport while the bicarbonate is exchanged with a chloride ion. The carbon dioxide which entered the cell is without osmotic effect, but the sodium and chloride increase the intracellular osmolarity obliging the entry of water with subsequent swelling of the cell. The exchange is not dependent on any exergenic chemical reaction, such as ATP hydrolysis, because energy is obtained from the steep sodium concentration gradient (extracellular sodium to intracellular sodium ratio >10:1). Thus, the

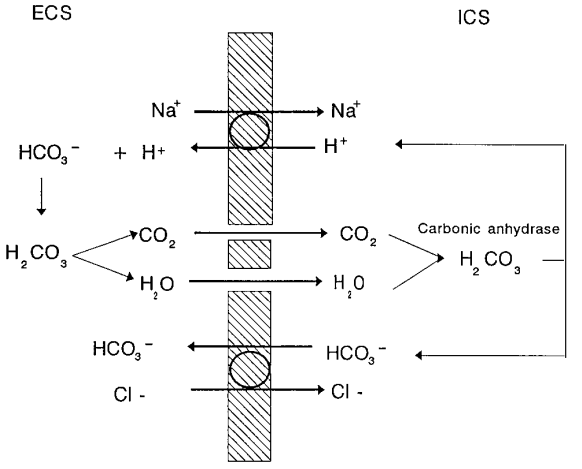

FIG. 8. Na^+/H^+ antiport. ECS, extracellular space; ICS, intracellular space; Na^+, sodium ion; H^+, hydrogen ion; HCO_3^-, bicarbonate; H_2CO_3, carbonic acid; CO_2, carbon dioxide; H_2O, water; Cl, chloride ion. See text for discussion.

maintenance of pH_i during anaerobic metabolism results in an increase in cellular volume.

The increase in volume associated with a decrease in pH_i is estimated to be 10–19% [30, 31]. Mazzoni and colleagues [32, 33] have observed that endothelial swelling results in a 20% decrease in the diameter of skeletal muscle capillaries during hemorrhagic shock. The increase in cell volume associated with the decrease in pH_i can be prevented by pretreatment with amiloride (which inhibits the antiport) [29–32] or corrected by treatment with hyperosmotic solutions [33]. In fact, it appears that function of the antiport is affected by the extracellular osmolarity and sodium concentration. Escobales and associates [27] have shown that the activity of the antiport is decreased in hypoosmolar media and increased in hyperosmolar media at both normal and lowered pH_i. Furthermore, Grinstein and colleagues [34], using hypertonic solutions, demonstrated that the function of the antiport was dependent on the extracellular sodium concentration.

In addition to regulating pH_i, the antiport also plays a major role in volume regulation after exposure to hyperosmolar media [28, 29, 34]. Previous investigation of the antiport's role in volume regulation has demonstrated that a variety of cells, including endothelium, can tolerate an osmolarity as high as 630 mOsm/liter for short periods of time [23]. Increasing the media osmolarity results in rapid intracellular alkalinization, acidification of the extracellular environment, and a reduction in cell volume [34]. Exposure to an osmolarity of 585 mOsm/liter is associated with a 35% reduction in cell volume within 10 min [34]. This suggests that protons are exchanged for osmotically active ions (perhaps sodium) which may act to prevent a further decrease in volume. There has been no investigation of osmolar tolerance during prolonged periods of normoxia nor has there been any investigation of the osmolar tolerance of cells after exposure to anoxia and anoxia followed by reoxygenation.

It would have been more clinically relevant to perform our studies in whole animals. Because of the preliminary nature of this investigation and for reasons of practicality and cost, we chose to use *in vitro* BAEC. Cultured endothelium is metabolically active and similar, in many respects, to *in vivo* vascular endothelium [10–12]. Furthermore, previous work examining the effects of osmolarity on endothelial function have utilized *in vitro* techniques [23, 35].

Cell viability was subjectively analyzed utilizing trypan blue dye exclusion because viable cells exclude trypan blue. Compared to the controls, there was no significant difference in the viability of cells incubated in hypertonic media of 460 mOsm/liter for periods of up to 30 hr. Furthermore, hypertonic medium of 460 mOsm/liter produced no significant decrease in cell viability compared to controls following 6 hr of anoxia and 6 hr of anoxia followed by 24 hr of reoxygenation. Compared to controls, 6 hr of anoxia followed by 24 hr of reoxygenation resulted in significantly decreased viability in hypertonic media of 530 and 570 mOsm/liter. However, compared to their viability after 30 hr of normoxia, cells exposed to 530 and 570 mOsm/liter had significantly increased viability after 6 hr of anoxia followed by 24 hr of reoxygenation.

The LDH enzyme release confirmed the trypan blue analysis. Lactate dehydrogenase is a cytosolic enzyme involved in the last step of glycolysis, the reduction of pyruvate by NADH to form lactate. A breakdown in the integrity of the plasma membrane would result in the release of LDH into the extracellular space. Thus, an increase in LDH activity in the supernatant should correlate with a decrease in the number of viable cells [36]. Although not statistically significant, LDH activity in the extracellular fluid of cells exposed to hypertonic media was greater than that observed in cells exposed to isotonic medium at each study interval of normoxic incubation and corresponded to the decrease in viability as assessed by trypan blue exclusion.

The cell detachment/plating efficiency assays were used to validate the cell injury assays because it has been suggested that such an assay is an index of endothelial cell dysfunction [19, 20]. Cell detachment represents the total number of cells that are lysed as well as the cells that are living but unable to maintain their adherence to the culture vessel following the stress of washing. Only those cells which have retained major metabolic and functional capacities will be capable of remaining adhered to the culture vessel following the stress of washing and will be capable of reattaching onto fresh culture plates.

Hypertonic media of 460 mOsm/liter resulted in no appreciable decline in plating efficiency compared to controls for periods of 6 to 30 hr of normoxic incubation. In addition, anoxia or anoxia and reoxygenation did not significantly decrease the plating efficiency of cells exposed to a hypertonic medium of 460 mOsm/liter. These results confirm that hypertonic medium as high as 460 mOsm/liter has no adverse affect on cell viability when compared to isotonic solutions, and that cell func-

tion is preserved for extended periods of normoxia, anoxia, and anoxia/reoxygenation. On the other hand, media of higher osmolarity (530 and 570 mOsm/liter) decreased plating efficiency during normoxia, anoxia alone, and anoxia followed by reoxygenation when compared to controls.

That BAEC exposed to moderately severe hyperosmolar media should survive and function for prolonged periods of normoxia and anaerobic stress is consistent with previous work investigating the antiport's function in pH_i and volume regulation. Although we did not measure pH_i or estimate changes in BAEC volume, we believe that the antiport prevented cellular dysfunction from dehydration by exchanging protons for osmotically active sodium ions. During anoxia, the hyperosmotic gradient favored intracellular sodium movement, thus facilitating proton extrusion, maintaining pH_i, and preventing cellular swelling. It is obvious from our work that there is a limit to the hyperosmolar tolerance of BAEC. Prolonged exposure to media with an osmolarity of greater than 460 mOsm/liter significantly reduced viability and function.

The observation that cells exposed to 530 and 570 mOsm/liter had significantly increased viability and significantly improved plating efficiency following 6 hr of anoxia and 24 hr of reoxygenation when compared to their viability and plating efficiency following 30 hr of normoxic conditions is not explained by the present data. It is interesting to speculate that the anoxic period induced stress-related proteins (hypoxia-associated proteins or oxygen-regulated proteins) [37, 38] which were associated with the antiport and improved the tolerance to the higher osmolarity resulting in increased viability. Given that the antiport is stimulated by both ischemia and increased osmolarity and that the response to osmolarity is associated with membrane protein phosphorylation by a kinase similar to protein kinase C [39], it is not unreasonable to conceive of the central role of the antiport in this stress response. Potential confirmation of the antiport's role in the stress response has been provided by the observation that the protein kinases which mediate osmosensing signal transduction also mediate responses to endotoxic lipopolysaccharides [40].

Our study has several limitations. First, our work was done in serum-free media. We did this to limit the factors which might affect BAEC function. Second, the cells were seeded directly onto plastic dishes rather than onto a prepared substrate. Again, this was done to limit those variables which might affect cell phenotype or function. Third, we observed a slight decline in the viability and function of the control cells which was not due to the effects of the hyperosmolar medium. This decline in viability and function is standard for our system and is due, we believe, to the factors previously cited; namely, growth on plastic in a serum-free environment. Fourth, the results of our study may not apply to all cellular functions because the cell plating and detachment assay examines only those functions related to cell adhesion events. Finally, the results of our work may not be applicable to the whole animal. Because we have now established a range of hyperosmolarity which can be tolerated by BAEC, an isolated *ex vivo* organ preparation or whole animal experimentation would be the next logical step. Such a study should examine the physiologic and histologic effects of hyperosmolar therapy.

Our work suggests that BAEC are capable of responding to a significant increase in osmolarity without apparent loss of viability or function. Our observation of endothelial tolerance to relatively extreme osmolarity (i.e., 460 mOsm/liter) is not without precedent. Endothelial cells lining the capillaries of the renal countercurrent system are exposed to osmolarity which can be as high as 1400 mOsm/liter [40].

Although preliminary, our work suggests that previous concerns regarding cellular dysfunction following hypertonic resuscitation may be unwarranted. Given that regulation of pH_i is facilitated by hyperosmolarity, hypertonic solutions would be ideal for the resuscitation of hemorrhagic shock. Furthermore, because hypertonic resuscitation could allow regulation of pH_i without obligatory endothelial swelling, capillary flow would be better preserved during the anoxic state.

Limiting or withholding hyperosmolar therapies when serum osmolarity exceeds 350 mOsm/liter is safe and judicious. Our data would suggest, however, that exceeding 350 mOsm/liter may not be unsafe. Furthermore, our work indicates the need for *in vivo* experimentation to determine the effect of hyperosmolarity on renal and central nervous system function. Such work could greatly advance the therapy of hypovolemic shock and severe head injury.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Mrs. Eva Champoux in the preparation of the manuscript and tables.

REFERENCES

1. Shackford, S. R., Mackersie, R. C., Holbrook, T. L., *et al.* The epidemiology of traumatic death: A population-based analysis. *Arch. Surg.* **128:** 571, 1993.
2. Shackford, S. R. Fluid resuscitation of the trauma victim. *Prob. Crit. Care* **1:** 576, 1987.
3. Peters, R. M., and Shackford, S. R. Comparison of isotonic and hypertonic fluids in resuscitation from hypovolemic shock. *Surg. Gynecol. Obstet.* **163:** 219, 1986.
4. Shackford, S. R., Sise, M. J., Fridlund, P. H., *et al.* Hypertonic sodium lactate versus lactated ringer's solution for intravenous fluid therapy in operations on the abdominal aorta. *Surgery* **94:** 41, 1983.
5. Shackford, S. R., Fortlage, D. A., Peters, R. M., *et al.* Serum osmolar and electrolyte changes associated with large infusions of hypertonic sodium lactate for intravascular volume expansion of patients undergoing aortic reconstruction. *Surg. Gynecol. Obstet.* **164:** 37, 1987.
6. Holcroft, J. W., Vassar, M. J., Turner, J. E., *et al.* 3% NaCl and 7.5% NaCl/Dextran 70 in the resuscitation of severely injured patients. *Ann. Surg.* **206:** 279, 1987.
7. Kramer, G. C., Perron, P. R., Lindsay, D. C., *et al.* Small volume

resuscitation with hypertonic saline dextran solution. *Surgery* **100:** 239, 1986.

8. Shackford, S. R., Norton, C. H., and Todd, M. M. Renal, cerebral, and pulmonary effects of hypertonic resuscitation in a porcine model of hemorrhagic shock. *Surgery* **104:** 553, 1988.
9. Pollock, A. S., and Arieff, A. I. Abnormalities of cell volume regulation and their functional consequences. *Am. J. Physiol.* **239:** F195, 1980.
10. Gimbrone, M. A., Cotran, R., and Folkman, J. Human vascular endothelial cells in culture, growth and DNA synthesis. *J. Cell Biol.* **60:** 673, 1974.
11. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. Culture of human endothelial cells derived from umbilical veins: Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52:** 2745, 1973.
12. Grinspan, J. B., Mueller, S. N., Noveral, J. P., *et al. In vitro* senescence, differentiated function, and transformation in cultured vascular endothelial cells. *Adv. Exp. Med. Biol.* **172:** 67, 1984.
13. Augustin-Voss, H. G., Voss, A. K., and Pauli, B. U. Senescence of aortic endothelial cells in culture: Effects of basic fibroblast growth factor expression on cell phenotype, migration, and proliferation. *J. Cell. Physiol.* **157:** 279, 1993.
14. Shatos, M. A., Doherty, J. A., Stump, D. C., *et al.* Oxygen radicals generated during anoxia followed by reoxygenation reduce the synthesis of tissue-type plasminogen activator and plasminogen activator inhibitor-1 in human endothelial cell culture. *J. Biol. Chem.* **265:** 20443, 1990.
15. Smith, S., and Broxmeyer, H. E. The influence of oxygen tension on the long-term growth in vitro of haematopoietic progenitor cells from human cord blood. *Br. J. Haematol.* **63:** 29, 1986.
16. Ratych, R. E., Chuknyiska, R. S., and Bulkley, B. G. The primary localization of free radical generation after anoxia/reoxygenation in isolated endothelial cells. *Surgery* **102:** 122, 1987.
17. Tennant, J. R. Evaluation of the trypan blue technique for determination of cell viability. *Transplantation* **2:** 685, 1964.
18. Cabaud, P. G., and Wroblewski, F. Colorimetric measurement of lactic dehydrogenase activity of body fluids. *Am. J. Clin. Pathol.* **30:** 234, 1958.
19. Inauen, W. D., Granger, N., Meninger, C. J., Schelling, M. E., Granger, H. J., and Kvietys, P. R. An *in vitro* model of ischemia/reperfusion-induced microvascular injury. *Am. J. Physiol.* **259**(*Gastrointest. Liver Physiol.* **22**): G134, 1990.
20. Harlan, J. M., Killen, P. D., Harker, L. A., and Striker, G. E. Neutrophil-mediated endothelial-injury *in vitro.* Michanisms of cell detachment. *J. Clin. Invest.* **68:** 1394, 1981.
21. Velasco, I. T., Pontiero, V., Rocha-e-Silva, M., and Lopes, O. U. Hyperosmotic NaCl and severe hemorrhagic shock. *Am. J. Physiol.* **239:** H664, 1980.
22. deFellipe, J., Timoner, J., Velasco, I. T., *et al.* Treatment of refractory hypovolemic shock by 7.5% sodium chloride injections. *Lancet* **2:** 1002, 1980.
23. Mazzoni, M. C., Lundgren, E., Arfors, K., and Intaglietta, M. Volume changes of an endothelial cell monolayer on exposure to anisotonic media. *J. Cell Physiol.* **14:** 272, 1989.
24. Mazzoni, M. C., Borgstrom, P., Arfors, K., and Intaglietta, M. Dynamic fluid redistribution in hyperosmotic resuscitation of hypovolemic hemorrhage. *Am. J. Physiol.* **139:** H629, 1988.
25. Shackford, S. R., Zhuang, J., and Schmoker, J. Intravenous fluid tonicity: Effect on intracranial pressure, cerebral blood flow, and cerebral oxygen delivery in focal brain injury. *J. Neurosurg.* **76:** 91, 1992.
26. Kregenow, F. M. Osmoregulatory salt transporting mechanisms: Control of cell volume in anisotonic media. *Annu. Rev. Physiol.* **43:** 493, 1981.
27. Escobales, N., Longo, E., Cragoe, E. J., Jr., Danthuluri, N. R., and Brock, T. A. Osmotic activation of Na^+/H^+ exchange in human endothelial cells. *Am. J. Physiol.* **259:** C640, 1990.
28. Cala, P. M., Anderson, S. E., and Cragoe, E. J., Jr. Na^+/H^+ exchange-dependent cell volume and pH regulation and disturbances. *Biochem. Physiol.* **90A**(4): 551, 1988.
29. Mahnensmith, R. L., and Aronson, P. S. The plasma membrane sodium–hydrogen exchanger and its role in physiological and pathophysiological processes. *Circ. Res.* **56**(6): 773, 1985.
30. Kempski, O., Staub, F., Jansen, M., Schodel, F., and Baethmann, A. Glial swelling during extracellular acidosis in vitro. *Stroke* **19:** 385, 1998.
31. Jakubovicz, D. E., and Klip, A. Lactic acid-induced swelling in C6 glial cells via Na^+/H^+ exchange. *Brain Res.* **485:** 215, 1989.
32. Mazzoni, M. C., Intaglietta, M., and Cragoe, E. J., Jr. Amiloride-sensitive Na^+ pathways in capillary endothelial cell swelling during the hemorrhagic shock. *J. Appl. Physiol.* **73**(4): 1467, 1992.
33. Mazzoni, M. C., Borgstrom, P., Intaglietta, M., and Arfors, K. E. Capillary narrowing in hemorrhagic shock is rectified by hyperosmotic saline-dextran reinfusion. *Circ. Shock* **31:** 407, 1990.
34. Grinstein, S., Rothstein, A., and Cohen, S. Mechanism of osmotic activation of Na^+/H^+ exchange in rat thymic lymphocytes. *J. Gen. Physiol.* **85:** 765, 1985.
35. Shepard, J. M., Goderie, S. K., Brzyski, N., *et al.* Effects of alterations in endothelial cell volume on transendothelial albumin permeability. *J. Cell. Physiol.* **133:** 389, 1987.
36. Chopra, J., Joist, J. H., and Webster, R. O. Loss of ^{51}chromium, lactate dehydrogenase, and ^{111}indium as indicators of endothelial cell injury. *Lab. Invest.* **57:** 578, 1987.
37. Benjamin, I. J., Kroger, B., and Williams, R. S. Activation of the heat shock transcription factor by hypoxia in mammalian cells. *Proc. Natl. Acad. Sci. USA* **87:** 6263, 1990.
38. Zimmerman, L. H., Levine, R. A., and Farber, H. W. Hypoxia induces a specific set of stress proteins in cultured endothelial cells. *J. Clin. Invest.* **87:** 908, 1991.
39. Grinstein, S., Goetz-Smith, J. D., Stewart, D., Beresford, B. J., and Mellors, A. Protein phosphorylation during activation of Na^+/H^+ exchange by phorbol esters and by osmotic shrinking. *J. Biol. Chem.* **261:** 8009, 1986.
40. Han, J., Lee, J.-D., Bibbs, L., and Ulevitch, R. J. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265:** 808, 1994.
41. Vander, A. J. *Renal physiology.* New York, McGraw-Hill, 1991. 4th ed., Pp. 99–100.