

Hypertonic Cardiopulmonary Bypass Primes and Endothelial Damage

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Abstract: The objective of this study was to investigate whether hypertonic hyperosmolar primes solution (HHPS), with an osmolarity of 2300 mOsmol/L, causes endothelial damage/loss. The bodies' normal osmolarity is ~280 mOsmol/L. Aortic endothelial cells were cultured and plated to confluence, confirmed by light microscopy, on a 96-well plate. Serial dilutions of HHPS ($n = 10$) were incubated with the cells ($n = 160$) for 1 hour. The plates were agitated to simulate flow that occurs during cardiopulmonary bypass (CPB). One half the cells ($n = 80$) were stained with crystal violet to provide a visual analogue of cell survival. The second half of the cells had the HHPS removed and replaced with culture medium and were incubated overnight before being stained with crystal violet. Optical densities were measured using an optical plate reader set at 470 nm. Analysis of the endothelium after 1 hour showed that HHPS (2300 mOsmol/L) and wa-

ter (positive control for 100% cell death) resulted in equal cell death, which was significantly higher ($p < .05$) than any of the other osmolarities tested for. There was no significant difference in the endothelial death rates for osmolarities between 260 and 400 mOsmol/L. Results of overnight incubation showed that cells in contact with a solution of osmolarity >320 mOsmol/L resulted in a significantly greater endothelial cell death rate ($p < .05$). Our results indicate that the endothelium can be irreversibly damaged by HHPS with osmolarities >320 mOsmol/L. The experimental protocol showed that this endothelial damage, which obviously occurs at the time of contact with the HHPS, may only become manifest 24 hours later. **Keywords:** hyperosmolar, prime, cardiac surgery, cardiopulmonary bypass. *JECT. 2008;40:236–240*

Hypertonic hyperosmolar primes, consisting of NaCl with an osmolarity of 2300 mOsmol/L, has emerged as a possible advance in cardiac surgery, because of the decrease in end organ edema and increased function, particularly of the heart and lungs, that is associated with its use (1–6).

Because the osmolarity of the prime is non-physiologic, endothelial damage/aberrant function may ensue. Endothelial activation, damage, and loss can result in neutrophil adhesion and complement and coagulation activation caused by direct contact of the blood components with the underlying exposed basement membrane (7). Complement and coagulation activation can result in systemic inflammatory response syndrome (SIRS), which manifests in a varied clinical picture from barely detectable to mul-

tipple organ failure. In the long term, acute endothelial damage may predispose to platelet aggregation and cholesterol influx, which can be the initiating events in the atheroma cascade, leading to accelerated graft failure (8,9).

Increasingly greater proportions of patients undergoing cardiopulmonary bypass (CPB) are elderly. Higher plasma concentrations of the measured adhesion molecules in elderly critically ill patients indicate that elderly patients are more prone than younger patients to a more pronounced activation and damage of the endothelium (10).

Under normal conditions, the body's osmolarity is ~280 mOsmol/L. On initiation of bypass, the pump prime, 2300 mOsmol/L, is in direct contact with the endothelium. After complete mixing with the blood volume, the blood's osmolarity is ~630 mOsmol/L. After whole body water distribution, the whole body osmolarity will have fallen to ~320–350 mOsmol/L. Thus, use of a hyperosmolar prime (2300 mOsmol/L) exposes vascular endothelium to a non-physiologic osmolarity for an extended period of time.

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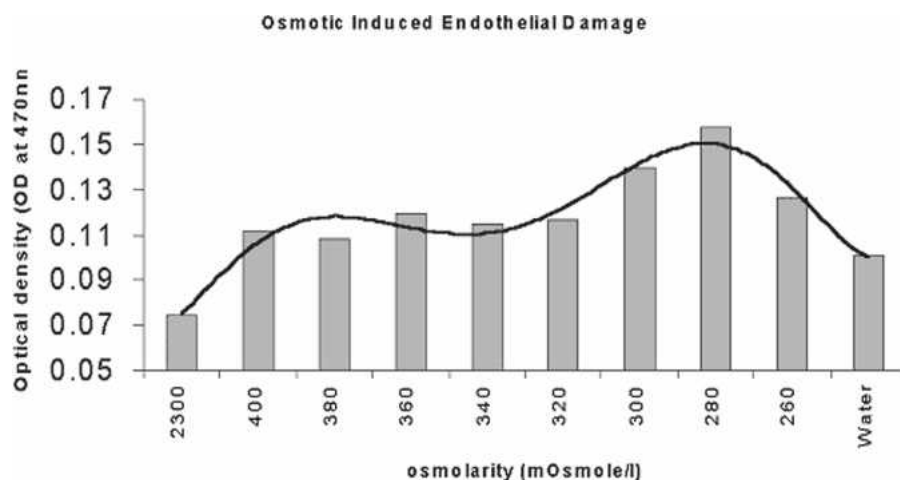


Figure 1. Osmotic-induced endothelial death. The higher the OD, the less endothelial death.

MATERIALS AND METHODS

Aortic Endothelium Harvest and Passage

This study was performed as previously described (11). Porcine thoracic aortas were obtained from a local slaughterhouse within 15 minutes of death. They were transported to the laboratory in sterile phosphate-buffered saline (PBS) containing antibiotics, gently cleaned of fat and adventitia, and opened longitudinally to expose the lumen. To harvest endothelial cells, the intima was gently scraped using a no. 22 scalpel blade. Each scrape was washed from the blades into culture flasks containing medium M199 (Gibco, Paisley, UK) containing 5% fetal bovine serum (Gibco), 2% penicillin/streptomycin, and 2% Fungizone (Gibco). Cell cultures were fed twice weekly thereafter until cultures were established. They were plated into tissue culture flasks and grown to confluency. Endothelial cells were detached from tissue culture flasks with .125% trypsin-EDTA and resuspended in growth medium. They were added to a 96-well flat bottom plate.

Osmotic Assay

Porcine aortic endothelial cells were cultured in Roswell Park Memorial Institute medium and 10% fetal calf serum and plated to confluence, confirmed by light microscopy, on a 96-well plate. Serial dilutions of prime solution (0, 260, 280, 300, 320, 340, 360, 380, 400, and 2300 mOsmol/L) were incubated with the cells (*n* = 160) for 1 hour. Water (0 mOsmol/L) was used as a positive control because this results in near 100% cell death; 260 mOsmol/L was used as a second hypotonic control, and 280 mOsmol/L (normal serum osmolarity) was used as a negative control. The plates were agitated to simulate flow that occurs during CPB. One half the cells (*n* = 80) were stained with crystal violet to provide a visual analog of cell survival (12). The second half of the cells had the serial diluted hypertonic hyperosmolar solutions removed and replaced with RPMI and 10% fetal calf serum and were

incubated overnight before being stained with crystal violet. Optical densities were measured using an optical plate reader set at 470 nm.

Cellular Death

The percentage cellular death (D%) was calculated using the equation below. It was assumed that water resulted in 100% cellular death (OD₁₀₀); the OD for each osmolarity being tested was calculated (OD_s). Percentage cell death (D%) = 100 × (OD_{sample} - OD_{zero}) / (OD₁₀₀ - OD_{zero}), where OD₁₀₀ represents 100% death (water sample) and OD_{zero} represents zero cell death (negative control; 280 mOsmol/L). Significance was determined using the paired *t* test, with a significance level of 0.05%.

Cells were photographed after 1 hour of osmotic challenge and 24 hours later after being incubated for 23 hours in normal culture conditions.

RESULTS

Analysis of the endothelium after 1 hour of incubation

Table 1. Significance levels of endothelial cell death compared with incubation with a solution with osmolarity of 280 mOsmol/L (physiologic osmolarity).

Osmolarity	Optical Density @ 450 nm	Percentage Cell Death (%)	<i>p</i>
2300	.075	100*	.003
400	.112	80	.019
380	.109	86	.008
360	.120	66	.030
340	.115	75	.017
320	.117	71	.034
300	.140	30	.360
280 (OD _{zero})	.157	0	Reference
260	.127	53	.084
Water (OD ₁₀₀)	.101	100	.018

Percentage cell death (D%) = 100 × (OD_{sample} - OD_{zero}) / (OD₁₀₀ - OD_{zero}), where OD₁₀₀ represents 100% death (water sample) and OD_{zero} represents zero cell death (negative control; 280 mOsmol/L). *Measurement of 2300 mOsmol/L leads to a false reading because of the extensive death causing a plate reading artefact.

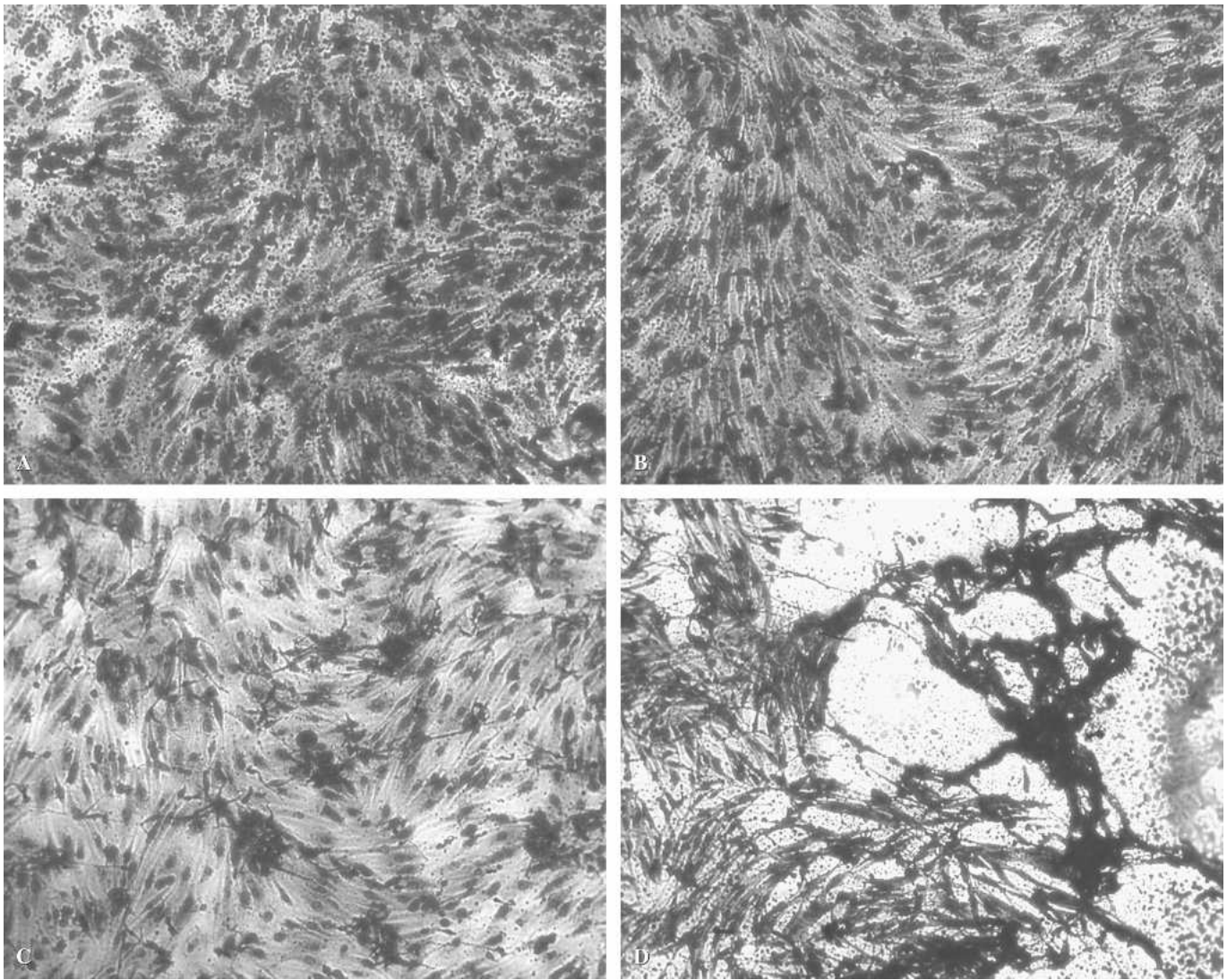


Figure 2. Typical appearance of the aortic endothelium immediately after osmotic challenge. A, Water. B, Physiologic saline (280 mOsmol/L). C, Saline (360 mOsmol/L). D, Hypertonic saline (2300 mOsmol/L).

showed that hypertonic hyperosmolar saline (2300 mOsmol/L) and water (positive control for 100% cell death) resulted in equal cell death, which was significantly higher ($p < .05$) than any of the other osmolarities tested. There was no significant difference in the endothelial death rates for any of the other osmolarities tested (range, 260–400 mOsmol/L).

Figure 1 shows the averaged results for 10 different serial dilutions carried out on cell wells ($n = 80$). These results represent 1 hour of incubation followed by overnight incubation with fresh medium. Statistical analysis showed (Table 1) that cells in contact with a solution of osmolarity >320 mOsmol/L resulted in a significantly greater endothelial cell death rate ($p < .05$).

Figure 2 shows the typical appearances of the aortic endothelium immediately after osmotic challenge. It can be seen that cell atypia and damage is only evident at

extremes of the osmolarity scale (water and hypertonic saline). The extensive dark staining indicates severely crumpled osmotically damaged clumped endothelial cells.

Figure 3 shows the cellular appearances of the endothelium after a further 23 hours of being cultured in normal cell media conditions. It can be seen that extensive endothelial damage occurs at any osmolarity that is not physiologic. This shows that cell damage/death does occur after just 1 hour of abnormal osmolarity but may only become visible after a longer period of observation (a lag period).

DISCUSSION

Traditionally, the prime for CPB has consisted of an isotonic mixture containing varying proportions of crystalloid, colloid, and blood. Recently, the introduction of hypertonic hyperosmolar primes has occurred because of the

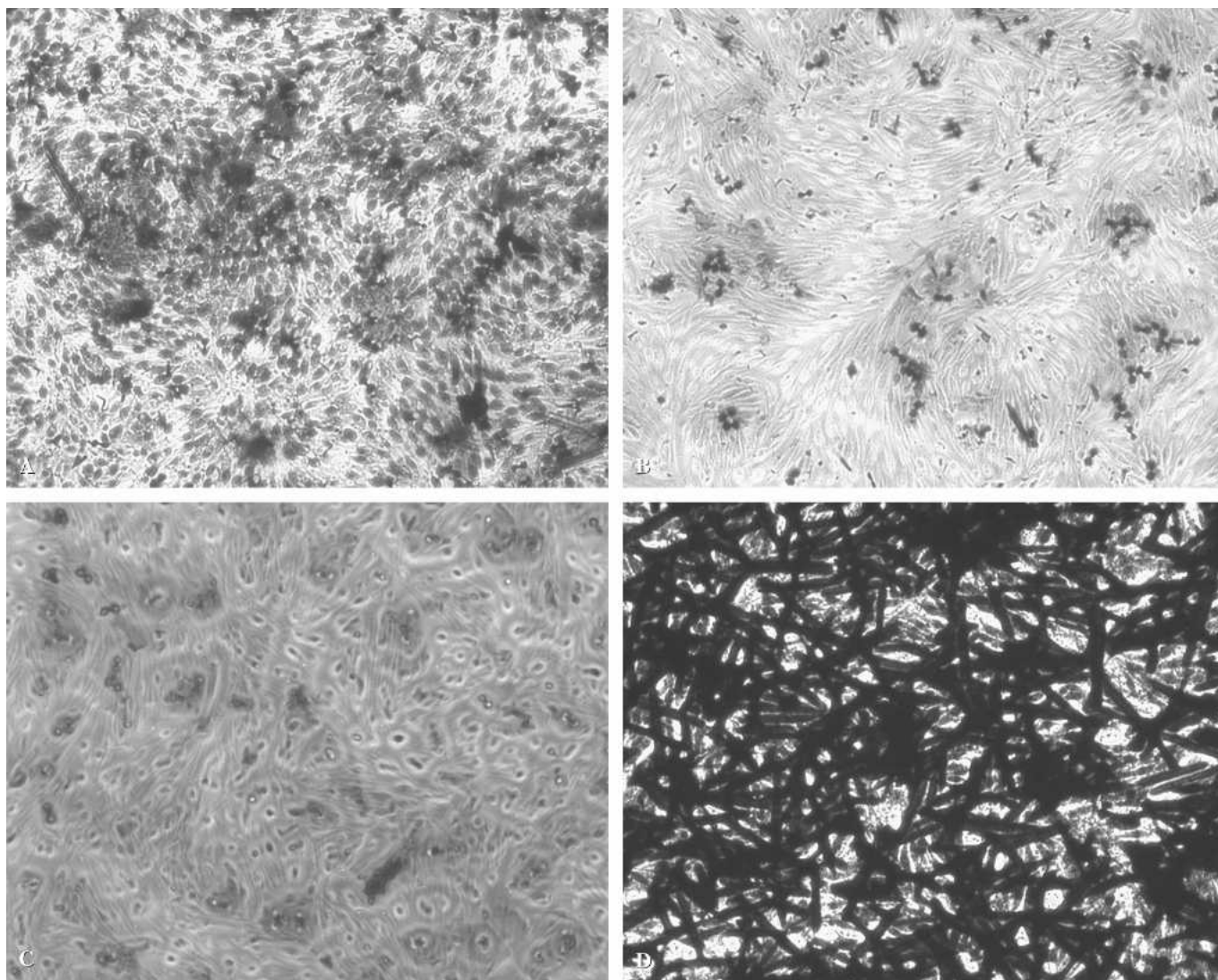


Figure 3. Cellular appearances of the endothelium after a further 23 hours of culture in normal cell media conditions. A, Water. B, Physiologic saline (280 mOsmol/L). C, Saline (360 mOsmol/L). D, Hypertonic saline (2300 mOsmol/L).

preliminary findings that these decreased end organ edema and increased end organ function. Using an in vitro setup, involving aortic endothelial cell lines, we showed that hypertonic hyperosmolar saline as used in CPB primes has a deleterious effect on the survival of aortic endothelial cells. This may have relevance to the development and subsequent manifestations of SIRS caused by CPB.

The experimental protocol we used showed that this endothelial damage, which obviously occurs at the time of contact with the hypertonic solution, is morphologically inapparent initially and may only become visible 24 hours later.

Annexin V is a 35- to 36-kd Ca^{2+} -dependent phospholipid-binding protein that binds to the plasma membrane early in apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner

to the outer leaflet of the plasma membrane, thereby exposing phospholipid phosphatidylserine to the extracellular environment. We realize that an assay based on Annexin V would be a more accurate test, but the methodology used above was found to be simple, reliable, and reproducible (adapted from a tumor necrosis factor bioassay methodology in use in our laboratory).

Hyperosmotic stress on freshly obtained human peripheral blood mononuclear cells has been shown to increase interleukin (IL)-1 α , IL-1 β , and IL-8. Reducing the osmolarity from 305 to 280 mOsmol/L results in a 50% reduction in IL-8 synthesis (13). However, bacterial lipopolysaccharide and IL-1, which are both known to increase during CPB, in combination with hyperosmolar hypertonic stress, result in synergistic synthesis of IL-1 α , IL-1 β , tumor necrosis factor- α , and IL-8 (13). The stress-induced synthesis of pro-inflammatory cytokines involves mem-

bers of the mitogen-activated protein (MAP) kinase stress pathway, particularly p38 MAP kinase and c-Jun NH2-terminal kinase.

Further study on hypertonic hyperosmolar prime should concentrate on the endothelial damage and inflammatory aspects created as opposed to the wet/dry weight and short-term clinical outcomes.

In addition, hypertonic saline has been shown to inhibit neutrophil phagocytosis and killing of bacteria. This could result in an increase incidence of sternal, valvular, leg, pulmonary, and urinary tract infections after CPB (14).

These results raise an interesting point. Once endothelial cell death has occurred, the dead cells will be unable to synthesize inflammatory mediators; hence, measurements of serum E-selectin may be low, even though significant endothelial damage has occurred. E-selectin is only synthesized in the endothelium, unlike other inflammatory markers, such as intercellular adhesion molecule, vascular cell adhesion molecule, and P-selectin. This has major implications for the inflammatory markers used in further research.

Hypertonic hyperosmolar mannitol pump primes, which incidentally are a free radical scavenger as effective as superoxide dismutase and catalase (15), are common. NaCl possesses none of these additional properties. Further work needs to be done to evaluate the effect of hypertonic primes on the endothelium.

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