Supplemental File.

Title. Shear stress-induced restoration of pulmonary endothelial barrier function following ischaemia reperfusion injury requires VEGFR2 signalling.

Authors. Don Walsh[1], Daria S. Kostyunina[1], Aoife Blake[1], John Boylan[2] and Paul McLoughlin[1]*

[1] School of Medicine and Conway Institute, University College Dublin, Dublin, Ireland,

[2] Department of Anaesthesia and Intensive Care, St Vincent's University Hospital, Dublin, Ireland.

Corresponding author. Paul McLoughlin, University College Dublin, School of Medicine, Health Sciences Centre, Belfield, Stillorgan Road, Dublin 4, Ireland, D04 C7X2.

Email. paul.mcloughlin@ucd.ie Orcid. https://orcid.org/0000-0001-6200-016X

Key words. Pulmonary oedema, endothelial permeability, shear stress

Supplement Introduction

While our studies of albumin permeability showed that perfusion with PVS reduced endothelial permeability, there remained the possibility that the reduced oedema formation observed in lungs perfused by PVS compared to those perfused by LVS may have been in part due to differences in colloid osmotic pressure (COP) exerted by these solutions. To examine this possibility, we measured the osmolality of the LVS and PVS solutions and the COP exerted by these solutions and compared them to the COP produced. In addition, we determined the COP produced by a solution of Ficoll 70kDa at a concentration of 60 g/l Fico 70 6%, which we have previously shown does not protect against oedema formation when used to perfuse isolated ventilated lungs (1).

Supplement Methods.

LVS, PVS and Ficoll 70 6% (60g/l) solutions in DMEM were made as previously described. Solutions of Bovine Serum Albumin (HyClone, USA, catalog no. SH30574.03) in DMEM at different concentrations (0 – 0.8 mmol/l) were also prepared. The COP that would be produced by each of these solutions if they behaved as ideal solvents acting across a perfect semi-permeable membrane was calculated using the van't Hoff equation.

$$\pi = RTC$$

where π is COP, R is the ideal gas constant (0.082[L.atm]/[degree.mole], T is the absolute temperature (kelvin) and C is the solute concentration (osmoles/kg of solution).

COP measurements were made using a polyarylethersulfone (PAES) based hollow fiber dialysis/ultrafiltration membrane (Prismaflex ST150, Baxter, Meyzieu, France, catalogue number. 23E0110CA) as previously described (2). Figure 1 shows a schematic diagram of the apparatus. Initially, the chamber containing the hollow fibre dialysis membrane was filled with reference solution (DMEM) to an initial fixed starting height within one arm of the manometer (tube B). Tap 1 (three-way tap) was then closed to the chamber, Tap 3 positioned so that is was closed to waste and Tap 2 positioned so that it was closed to manometer tube B to prevent any further fluid movement. The hollow fibre dialysis membrane was next filled with test solution (via Tap 4) until excess fluid drained to waste (via Tap 5). Tap 5 was then switched so that the outflow from the hollow fibre array was directed to manometer tube A. Additional test fluid was then added from the filling syringe until the height of the column of test fluid matched that of the reference solution in manometer tube B and Tap 4 was then closed so that no test fluid could subsequently drain from the

hollow fibre bundle during measurement. Finally Tap 2 was opened so that the chamber was connected to manometer tube B. Fluid could then move across the membrane, driven by the COP difference between the test and reference solutions, until the hydrostatic pressure difference between the two columns of fluid was equal (but opposite to) the COP. The difference in height between the two columns was measured and taken as the COP exerted by the macromolecules in the test solution. This was measured at 1, 2, 3, 5, 8 and 10 minutes after starting the measurement procedure and we found that the initial rapid exchange of fluid across the membrane had always been completed within two minutes of opening Tap 2. Following these pressure measurements, the solutions on either side of the dialysis membrane were then exchanged for fresh test and reference solutions and the procedure repeated. In total five separate determinations of COP were made and the mean of these five separate determinations was taken as the COP for that test solution. All measurements were made at room temperature (20C).

Osmolality of the perfusion solutions was measured by determination of solution vapour pressure (VAPRO, model 5520, Wescor, Utah).

Supplemental Results.

The COP of a series of five solutions with different concentrations of albumin (0.0 – 0.8 mmol/l) demonstrated a linear relationship between albumin concentrations and COP (Figure 2). The slope and intercept of the least squares, best fit, linear regression equation were not significantly different from the van t'Hoff predicted values (Slope = 18.3 $mmHg.mOsm^{-1}.l$ and intercept = 0 mmHg respectively).

The COP produced by LVS, PVS and Ficoll 60g/l are shown in Table 1. The osmolalities of the PVS and Ficoll 70 (60 g/l) solution were similar (Table 1) but both were increased above that of the LVS. The COP exerted by PVS was also higher than that of LVS, while the COP of the Ficoll (60 g/l) solution was higher than both the PVS and LVS (Table 1). It is interesting to note that the COP of LVS was lower than that of the albumin solution (8.7 mmHg) that had an identical molar concentration of macromolecule (0.57 mmol/l).

Supplemental Discussion

We found that PVS had a somewhat higher osmolality and higher colloidal osmotic pressure than LVS. In addition, our results showed that a solution of Ficoll 70 at a higher concentration (60g/l) than in LVS (40g/l) had a slightly higher osmolality and COP than both LVS and PVS.

Comment on COP measurements. To assess the performance of our method of measuring COP, we measured the COP produced by a set of solutions with different concentrations of bovine serum albumin for which the PAES-based membrane displays a very low sieving coefficient (SC, < 0.01), indicative of a very high reflection coefficient (σ); σ =1- SC (3). Across such a membrane a solution of bovine serum albumin should produce a pressure gradient that is linearly related to the concentration of albumin with a slope close to that predicted by the van't Hoff equation. The data obtained demonstrate a linear relationship between COP and concentration that was not significantly different from the van't Hoff predicted relationship (Figure 2), confirming that the apparatus accurately measured the COP exerted by a macromolecule to which the membrane had very low permeability.

The LVS solution produced a COP which was considerably less than the value predicted by the van't Hoff equation for a 70kDa macromolecule (approximately 55% of the expected value). There are a number of potential reasons for this. First, the Ficoll molecules are much more flexible than albumin and can pass through pores of a particular size more easily than an albumin molecule i.e. the reflection coefficient of Ficoll is lower than expected on the basis of its molecular weight (4, 5). A second contributing factor may be that the Ficoll 70 that we used was not composed of molecules of a single molecular weight but contained molecules with a range of different molecular weights whose distribution was centred on 70kDa (i.e. a polydisperse mixture of molecules). Modern high flux dialysis membranes have molecular weight cut offs up to 60kDa, while still having very high reflection coefficients for albumin. Thus, lower molecular weight Ficoll molecules within the Ficoll 70 solution could cross the membrane reducing the marcomolecular gradient and thus reduce the COP exerted by the solution. Finally, Ficoll 70 does not dissolve completely as single molecules acting independently but a fraction of the molecules form microaggregates (2-3 molecules), which by reducing the number of separate particles in solution, attenuates the COP exerted by the solution (6). Interestingly, the lower COP exerted by Ficoll 70 solutions that we observed is similar to the behaviour of Ficoll 70 solutions perfusing capillaries where it has been shown to exert a COP that is considerably lower than predicted by the van t'Hoff equation (7-9).

Our PVS solution produced a somewhat higher COP than the LVS solution (Table 1). In view of this, we were interested to compare it to a higher concentration Ficoll 70 solution (60g/l), which we had

previously shown did not exert the protective effect against oedema formation in the lung produced by a high viscosity perfusate with a relative viscosity identical to that of PVS used in the present study (Rowan et al AJP 2018). As intended, the Ficoll 70 6% solution had a higher osmolality and COP than the LVS solution (Table 1). Furthermore, its osmolality and COP were closely similar to those of the PVS solution although its relative viscosity was considerably less than that of PVS. Despite the similarities of both osmolality and COP to PVS, Ficoll 70 6% did not protect the lung against oedema formation but caused oedema after a time interval closely similar to that seen with LVS solution (1). Thus, the protective effect of PVS against oedema formation cannot be attributed to either its higher COP or osmolality compared to LVS.

REFERENCES

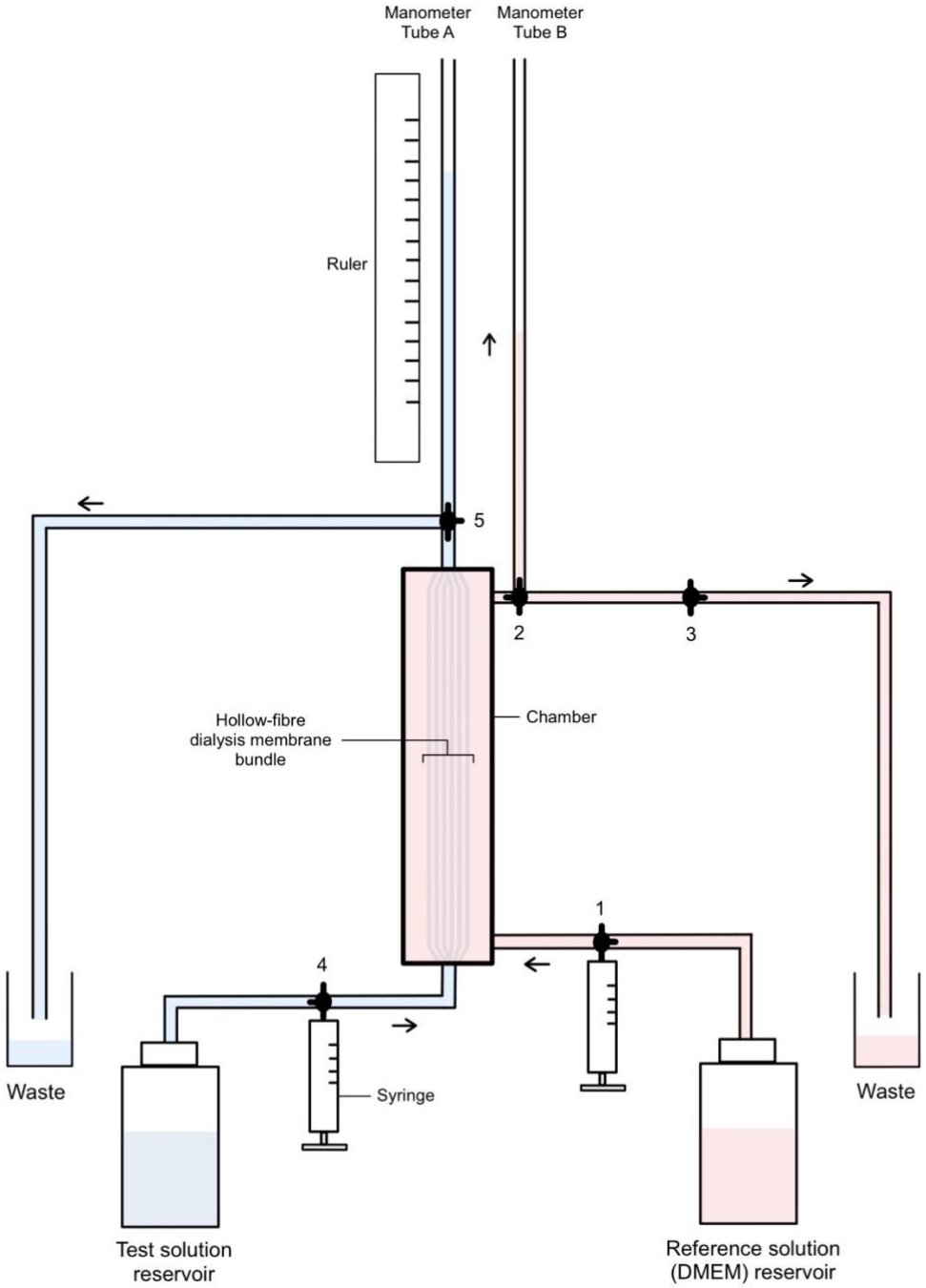
1. **Rowan SC, Rochfort KD, Piouceau L, Cummins PM, O'Rourke M, and McLoughlin P**. Pulmonary endothelial permeability and tissue fluid balance depend on the viscosity of the perfusion solution. *Am J Physiol Lung Cell Mol Physiol* 315: L476-L484, 2018.
2. **Matsumoto T, Yoshida K, Shinohara T, Miyoshi E, and Ueno T**. Development of new colloid osmotic pressure measurement method using ultrafiltration membrane during cardiopulmonary bypass. *J Extra Corpor Technol* 55: 167-174, 2023.
3. **Werynski A, Malchesky PS, Lewandowski JJ, Waniewski J, Wojcicki J, and Piatkiewicz W**. Theoretical formulation of sieving coefficient evaluation for membrane plasma separation. *Artif Organs* 9: 250-254, 1985.
4. **Fissell WH, Manley S, Dubnisheva A, Glass J, Magistrelli J, Eldridge AN, Fleischman AJ, Zydney AL, and Roy S**. Ficoll is not a rigid sphere. *Am J Physiol Renal Physiol* 293: F1209-1213, 2007.
5. **Venturoli D, and Rippe B**. Ficoll and dextran vs. globular proteins as probes for testing glomerular permselectivity: effects of molecular size, shape, charge, and deformability. *Am J Physiol Renal Physiol* 288: F605-613, 2005.
6. **Palit S, and Yethiraj A**. Dynamics and cluster formation in charged and uncharged Ficoll70 solutions. *J Chem Phys* 147: 074901, 2017.
7. **Hu X, Adamson RH, Liu B, Curry FE, and Weinbaum S**. Starling forces that oppose filtration after tissue oncotic pressure is increased. *Am J Physiol Heart Circ Physiol* 279: H1724-1736, 2000.
8. **Michel CC**. Capillary permeability and how it may change. *J Physiol* 404: 1-29, 1988.
9. **Michel CC, Phillips ME, and Turner MR**. The effects of native and modified bovine serum albumin on the permeability of frog mesenteric capillaries. *J Physiol* 360: 333-346, 1985.

Supplemental Table 1. Osmolality, colloid osmotic pressure and relative viscosity of three perfusion solutions

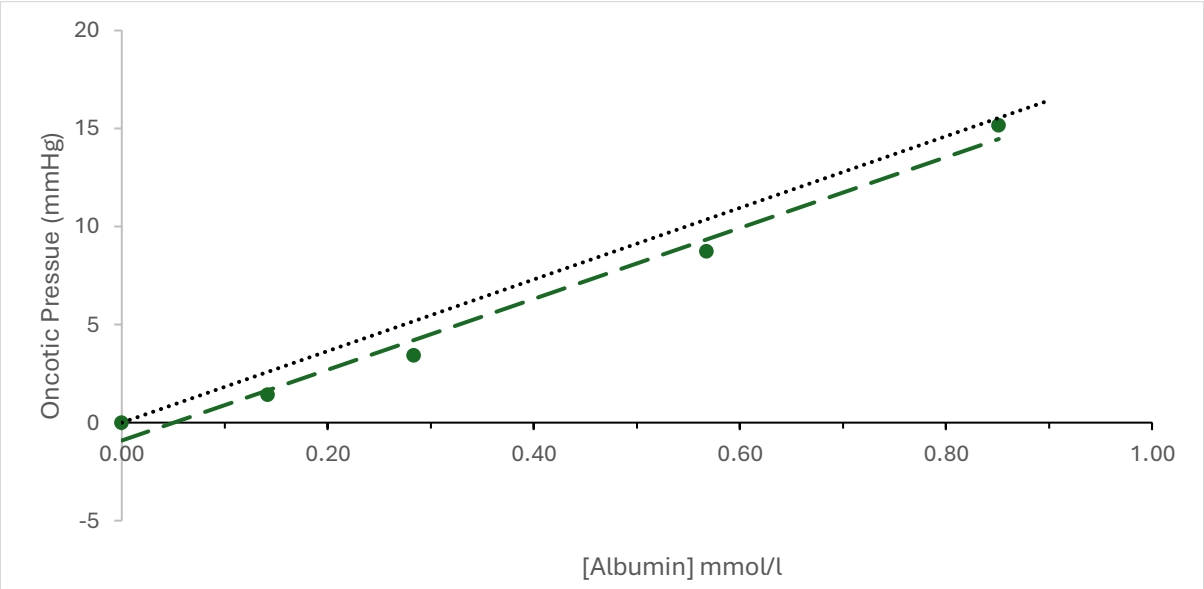
	LVS	**PVS**	**Ficoll 70 (60g/l)**
Osmolality (mOsm/kg)	325	329	331
COP (mmHg)	5.4	9.9	10.8
RV	1.5	2.5	1.8*

COP, colloid osmotic pressure. LVS, low viscosity solution. PVS, physiological viscosity solution. RV, relative viscosity.

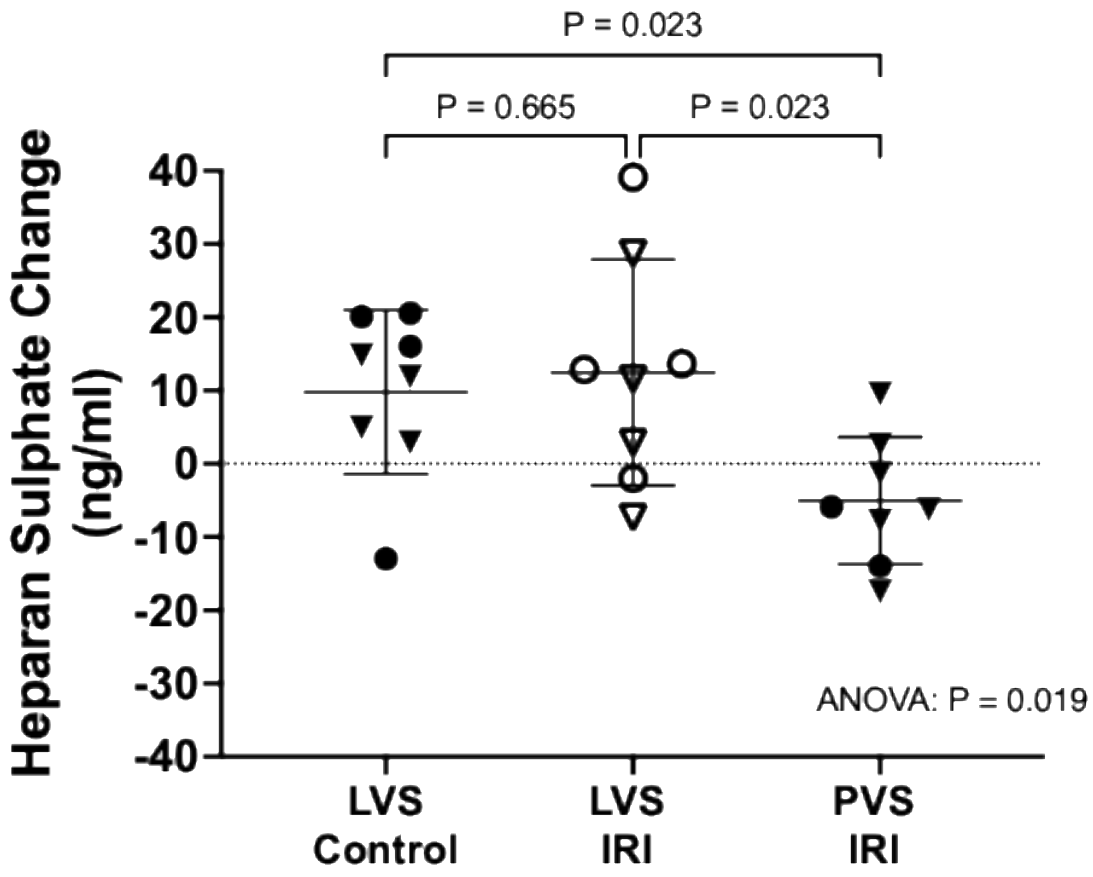
* Previously reported in Rowan et al, American Journal of Physiology 2018.



Supplemental Figure 1. Schematic diagram of apparatus for measuring COP. For details of operation see text.



Supplemental Figure 2. Plot of oncotic pressure (mmHg) versus concentration of albumin (mmol/l) in DMEM. Dotted line represents oncotic pressures predicted by the van't Hoff equation. Dashed line indicates line of best fit to experimental data: slope (SE slope) = 18.2 (1.3) mmHg/mmol, intercept (SE intercept) = -0.9 (0.6) mmHg, r^2=0.99.



Supplemental Figure 3. Effect of PVS on glycocalyx shedding.

Change in heparan sulphate concentrations in the perfusate in each of three groups, LVS Control, LVS IRI and PVS IRI, following the period of warm ischaemia injury (n=8 in each group). Open symbols represent lungs in which perfusion was terminated due to the development of edema (Pinsp > 7.5mmHg) before 180 minutes. Filled symbols represent lungs in which edema did not develop. Triangular symbols represent male lungs and circular symbols represent female lungs. Mean and SD are also shown on each panel. Exact P values were determined by ANOVA followed by Student Newman Keuls post hoc test for pairwise comparisons of specific groups.