Endothelial Homeostasis and Post-Cardiac Surgery Inflammatory Organ Injury.

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Muhammad Saqib Hayat Qureshi MBBS, MRCS.

Supervisors

Professor Gavin J. Murphy

Dr. Marcin Wozniak

Department of Cardiovascular Sciences

University of Leicester

Clinical Sciences Wing

Glenfield Hospital

LEICESTER, LE3 9QP

Abstract

Endothelial Homeostasis and Post-Cardiac Surgery Inflammatory Organ Injury.

This thesis evaluates three most important etiological factors for organ injury namely, intravenous fluid, cardiopulmonary bypass (CPB) and blood transfusion, and considers their effects on endothelial dysfunction.
This thesis evaluates 1. The safety of current fluid therapy, 2. The effects of novel versus established volume expanders on endothelial function and Acute Kidney Injury (AKI), 3. The effect of Sildenafil on renal glycocalyx in post-CPB AKI and 4. The effect of standard and modified red cell transfusions on lung and kidney injury.
Using a systematic assessment of randomised controlled trials comparing colloids and crystalloids, we found that Hydroxyethylstarches are associated with increased risk of mortality and AKI. On the contrary, there was no evidence that Gelatins, Dextrans and Crystalloids were associated with increased harm. We evaluated the supremacy of novel AQIX RS-1® for intravenous resuscitation in a swine model of general anesthesia but failed to show its superiority as an endothelial and organ protective agent compared to Hartmann's solution. We characterized that CPB mediated AKI is associated with modifications of Glycosaminoglycans and core protein components of Glycocalyx, but also that CPB induces detrimental changes in endothelial surface markers; vWF, Thrombomodulin, VE-Cadherin. These structural modifications caused direct reduction in Nitric Oxide bioavailability, renal artery vasomotor function and invoked AKI in swine. Post CPB-AKI was prevented by restoration of depleted NO bioavailability using Sildenafil Citrate without restoration of glomerular endothelial membrane (GEM) constituents. We explored the pathogenesis of Transfusion Related Acute Lung Injury (TRALI) and implicate alterations in red cell metabolomics; endothelial dysfunction, inflammation, microparticles and labile Iron and use a translational porcine model of TRALI to test red cell Rejuvenation coupled with red cell washing as a promising therapy. The work in this thesis has supported or generated ideas of novel pragmatic randomised clinical trials that seek to make direct difference in outcomes after cardiac surgery.

M.Saqib.H.Qureshi

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In the name of the One who is most gracious and merciful……..

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From here onwards there is just one person; Professor Gavin Murphy who deserves my utmost gratitude for showing the degree of patience and having endless conviction in that I will take this through till the end. In credit is in fact his ability to motivate and inspire with a balance of affection and positive critique and lastly setting only the highest standards that I here am the beneficiary of. In this journey with him though I have on one hand been humbled by the world of academic science, have developed nothing but the highest regards for scientists and have found my principal attributes mainly to persevere amongst all odds.

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AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Any views expressed in the dissertation are those of the author.

SIGNED: .....M. Saqib. H. Qureshi..... DATE:.....24 March..2016

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Abbreviations

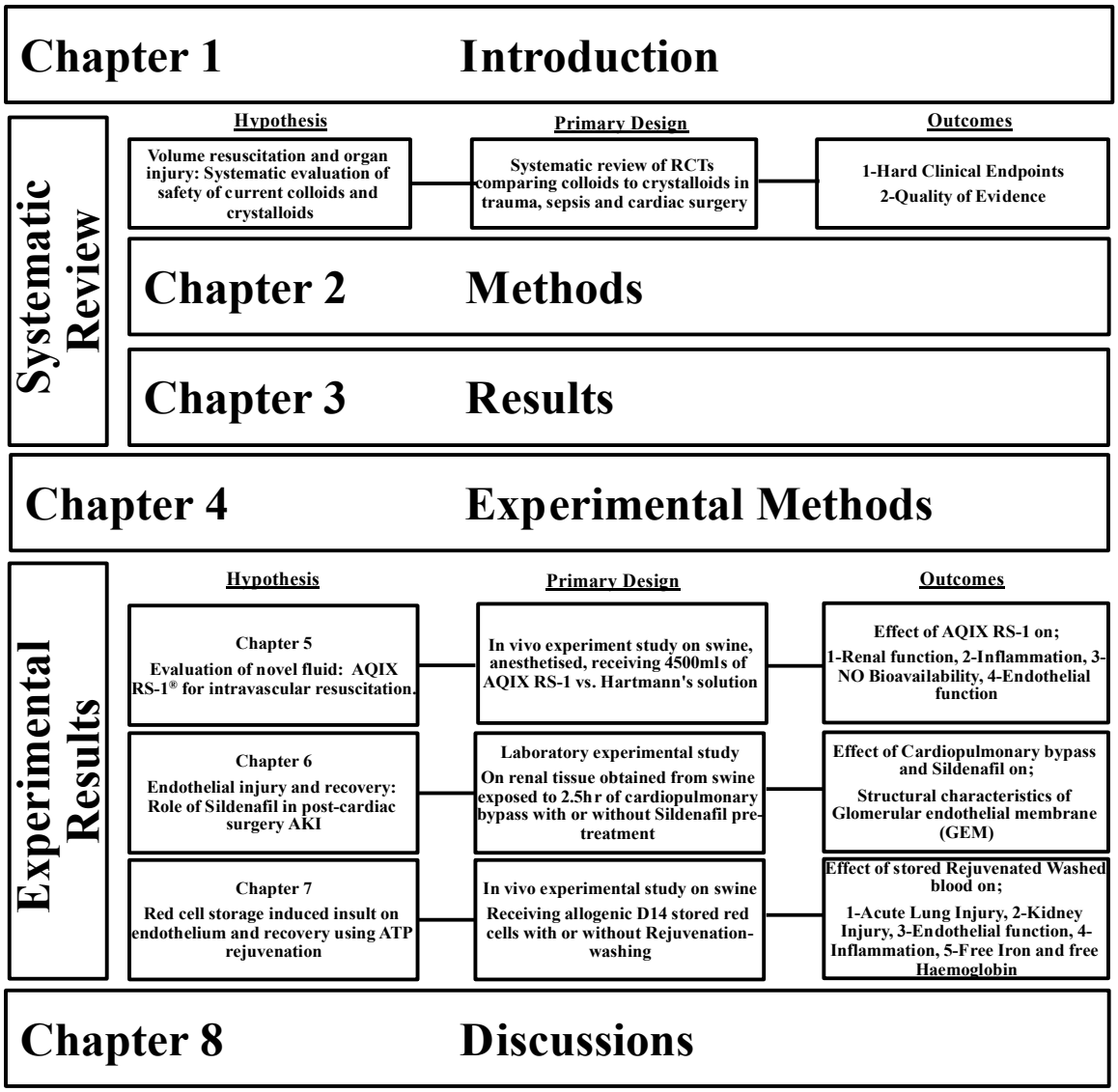
2,3-DPG	2,3-Diphosphoglycerate
95% CI	95% Confidence Interval
Ach	Acetylcholine
ADP	Adenosine Diphosphate
ADQI	Acute Dialysis Quality Initiative
AKI	Acute Kidney Injury
AKIN	Acute Kidney Injury Network
Akt	Adenylate kinase
ALI	Acute Lung Injury
ANOVA	Analysis of Variance
ANP	Atrial Natriuretic Peptide
ARF	Acute Renal Failure
AS	Additive solution
ATN	Acute Tubular Necrosis
ATP	Adenosine Triphosphate
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CaO_2	Arterial Oxygen Content
ciGEnC	Conditionally Immortalized Glomerular endothelial
cells	
Cr	Creatinine
CrCl	Creatinine Clearance
cGMP	cyclic 3'-5'-guanosine monophosphate
CI	Cardiac Index
cmH_2O	Centimeters of Water
CPB	Cardiopulmonary Bypass
CPD	Citrate-Phosphate-Dextrose

CVP	Central Venous Pressure
DAB	DiAmino Benzidine
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DBA	Dolichos Biflorus Agglutinin
dL	deciliter
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal calf serum
FE	Fractional Excretion
FiO_2	Fraction of Inspired Oxygen
GAG	Glycosaminoglycans
GFR	Glomerular Filtration Rate
GRADE	Grading of Recommendations Assessment, Development and Evaluation
Hct	Hematocrit
HES	Hydroxyethylstarch
HMVECs	Human Microvascular Endothelial Cells
HPLC	High-performance liquid chromatography
HRP	Horseradish Peroxidase
ICAM	Intercellular Adhesion Molecule
ICU	Intensive care unit
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IP_3	Inositol triphosphate
IQR	Interquartile Range
ISS	Injury severity score
kDa	kilodaltons
KDIGO	Kidney Disease: Improving Global Outcomes
kg	Kilogram
L	Liters

LPS	Lipopolysaccharide
MAP	Mean Arterial Blood Pressure
MAPKs	Mitogen-activated protein kinases
MD	Mean Difference
mg	Milligram
ml	Milliliters
mm	Millimeter
mmHg	Millimeters of Mercury
mmol	Millimoles
MP	Microparticle
MPO	Myeloperoxidase
NAG	N-acetyl- β -(D)-glucosaminidase
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric Oxide
ONCAB	On-Pump Coronary Artery Bypass
OPCAB	Off-Pump Coronary Artery Bypass
OR	Odds Ratio
PAGE	Polyacrylamide gel electrophoresis
PaO_2	Arterial oxygen tension
PBS	Phosphate Buffered Saline
PCWP	Pulmonary Capillary Wedge Pressure
PDE-5	Phosphodiesterase-5
PEEP	Positive end-expiratory pressure
pg	Picogram
PI_3K	phosphoinositide 3-kinase
PRISMA and Meta-Analyses	Preferred Reporting Items for Systematic Reviews
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
PVRI	Pulmonary Vascular Resistance Index
RBC	Red blood cell
RCT	Randomised Controlled Trial
RIFLE	Risk, Injury, Failure, Loss, End-stage kidney disease

ROB	Risk of bias
ROS	Reactive Oxygen Species
RRT	Renal Replacement Therapy
SAGM	Saline-Adenine-Glucose-Mannitol
SCr	Serum Creatinine
SD	Standard Deviation
Sildenafil	Sildenafil Citrate
SDS	Sodium dodecyl sulfate
SEM	Standard Error of the Mean
SIRS	Systemic Inflammatory Response Syndrome
SOD	Superoxide dismutase
SOFA	Sequential Organ Failure Assessment score
STS	Society of Thoracic Surgeons. USA
SVRI	Systemic Vascular Resistance Index
TBS-T	Tris Buffered Saline with Tween
TNF	Tumour Necrosis Factor
TRALI	Transfusion Related Acute Lung Injury
VCAM	Vascular cell adhesion
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand Factor

Overview of thesis



Chapter 1 Introduction

1.1 Cardiac Surgery and Acute Kidney Injury (AKI)

Current clinical definitions of acute kidney injury include measures of acute decline in glomerular filtration rate. The term ‘‘acute kidney injury/impairment’’ has been proposed to encompass the entire spectrum of the syndrome from minor changes in markers of renal function to requirement for renal replacement therapy (RRT). AKI is not ATN, nor is it renal failure.

1.1.1 Definitions

More than 35 definitions have been proposed to describe acute kidney injury (AKI) (Kellum et al., 2002). Many of these have been complex, however the more commonly used were based on urine output (UO) and/or serum creatinine (SCr) criteria. An increase in basal SCr of at least 44.2 μ mol/L (0.5 mg/dL), a decrease in Cr clearance of at least 50% or the need for renal replacement therapy (RRT) were the most frequent definitions used for AKI in clinical practice (Thadhani et al., 1996). Where UO has been used to define AKI, it is generally considered that a value less than 400–500 mL/day could be an indicator.

In May 2002, the Acute Dialysis Quality Initiative (ADQI) group proposed RIFLE classification, which was published in 2004. The RIFLE criteria (acronym for Risk of renal dysfunction; Injury to the kidney; Failure of kidney function; Loss of kidney function; and End-stage kidney disease) classify AKI into three categories of severity (Risk, Injury and Failure) and two categories of clinical outcome (Loss and End-stage kidney disease). The AKIN classification was proposed in 2007 by the Acute Kidney Injury Network. The AKIN criteria classify AKI into three stages of severity (Stages 1, 2 and 3). These criteria are detailed in table [1.1].

More recently the Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group proposed changes to the staging for AKI. This new classification was important and novel for medical practice, especially with regard to the criterion of time. KDIGO covers both the AKIN and RIFLE criteria, taking into account changes in creatinine within 48 hours or a decline in the

glomerular filtration rate (GFR) over 7 days. Moreover, patients under 18 with GFR <35mL/min and patients with serum creatinine >4.0mg/dL (absolute value) were added to AKIN stage 3.

Table [1.1]: Comparison between RIFLE, AKIN and KDIGO definitions.

	AKIN staging Serum Creatinine	Urine output (Common to AKIN and RIFLE)		RIFLE Class Serum Creatinine or GFR		KDIGO Staging 	
Stage 1	Increase in serum creatinine ≥26.2 μmol/L or increase to ≥150–199% (1.5- to 1.9-fold) from baseline	<0.5 mL/kg/h for ≥6 h	Risk	Increase in serum creatinine ≥1.5x baseline or decrease in GFR ≥25%	Stage 1	Serum creatinine 1.5-1.9 times baseline OR ≥0.3 mg/dl (≥26.5 μ mol/l) increase	Urine output <0.5 ml/kg/h for 6-12 hours
Stage 2	Increase in serum creatinine to 200–299% (>2–2.9 fold) from baseline	<0.5 mL/kg/h for ≥12 h	Injury	Increase in serum creatinine ≥2.0x baseline or decrease in GFR ≥50%	Stage 2	2.0-2.9 times baseline	<0.5 ml/kg/h for ≥12 hours
Stage 3	Increase in serum creatinine to ≥300% (≥3-fold) from baseline or serum creatinine ≥354 μmol/L with an acute rise of at least 44 μmol/L or initiation of RRT	<0.3 mL/kg/h ≥24 h or anuria ≥12 h	Failure	Increase in serum creatinine ≥3.0x baseline or decrease in GFR ≥75% or an absolute serum creatinine ≥354 μmol/L with an acute rise of at least 44 μmol/L	Stage 3	3.0 times baseline OR Increase in serum creatinine to ≥4.0 mg/dl (≥353.6 μ mol/l) OR Initiation of renal replacement therapy OR In patients <18 years, decrease in eGFR to <35 ml/min per 1.73 m2	<0.3 ml/kg/h for ≥24 hours OR Anuria for ≥12 hours

	Loss	Persistent acute renal failure=complete loss of kidney function > 4 weeks
	End-stage kidney disease	ESRD>3 months

1.1.2 Limitations of using Serum creatinine and Urine output to diagnose AKI

1.1.2.1 Serum Creatinine

Pickering *et al* (Pickering et al., 2013) have suggested that a reduction in GFR can occur in critically ill patients with unchanged creatinine levels. Importantly, the magnitude of the decrease in creatinine generation rate might correlate with illness severity (Wilson et al., 2012)thus in sicker patients, increases in creatinine concentration could be smaller and occur more slowly than in less-sick patients with the same AKI severity.

1.1.2.2 Urine Output

RIFLE, AKIN and KDIGO use the same urine output criteria to define AKI and to determine its severity. Since exact data on urine output are not always available, only a minority of retrospective studies have included urinary output criteria. Theoretically, measuring the urine output per hour in 6 or 12 h intervals as proposed by RIFLE, AKIN and KDIGO requires continuous monitoring of the diuresis which is practically limited to critically ill patients admitted in an Intensive Care Unit (ICU).

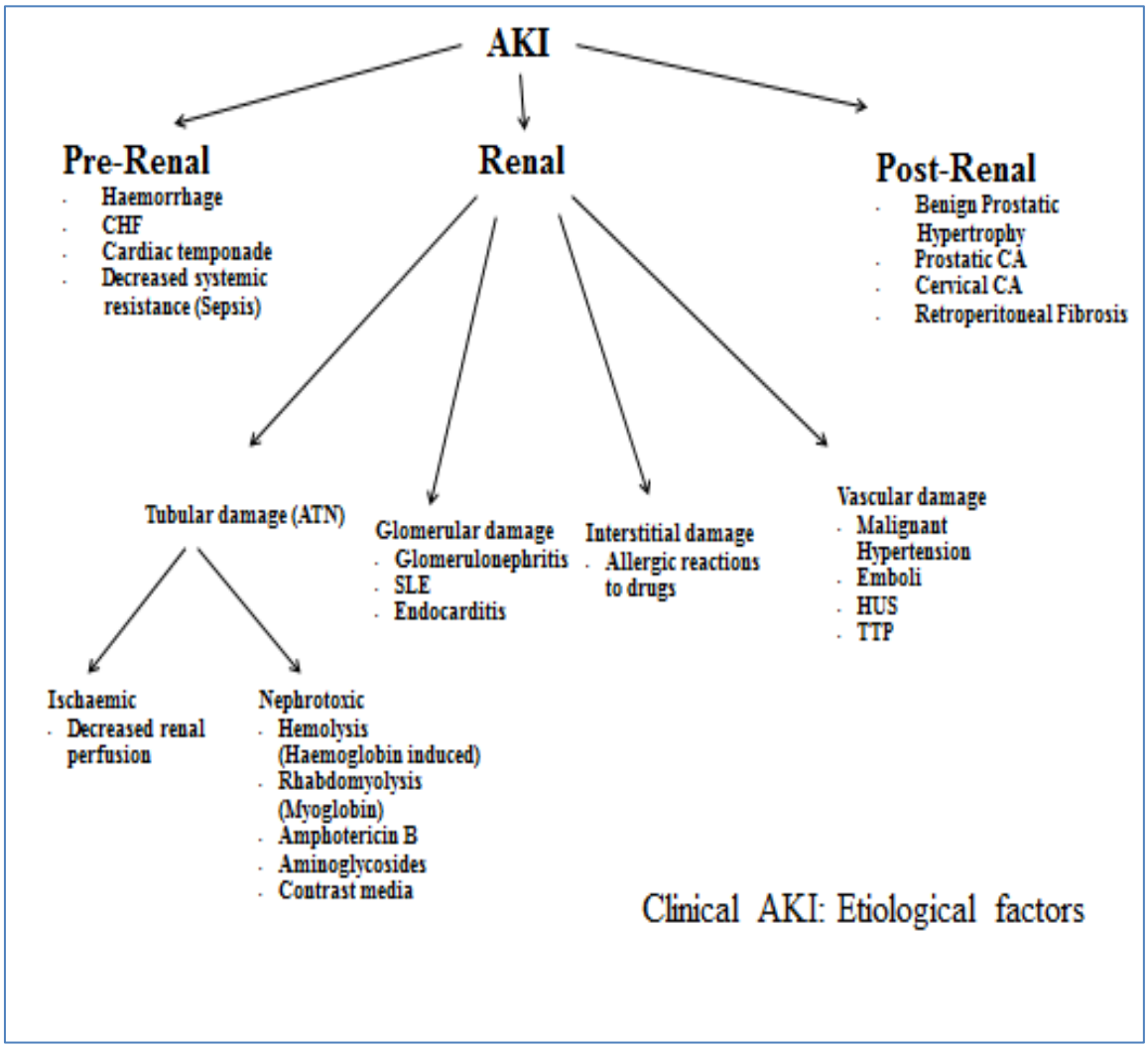
1.1.2.3 Use of Creatinine clearance as an index of GFR

Glomerular filtration rate (GFR), measured using exogenous substances such as inulin, iohexol, ^{123}I-iothalamate, diethylene triamine pentaacetic acid (DTPA) and chromium-ethylenediaminetetraacetic acid (^{51}Cr-EDTA) as filtration markers, is considered as the gold standard for assessment of renal function [(Stevens et al., 2006, Endre et al., 2011)].

Aetiology of AKI:

Broadly, etiological factors are classified as pre-renal, renal and post renal. These are highlighted in figure [1.1].

Figure [1.1]: Etiological factor of AKI. CHF: Congestive heart failure, ATN: Acute tubular necrosis, SLE: Systemic lupus erythematosus, HUS: Hemolytic uremic syndrome, TTP: Thrombotic thrombocytopenic purpura, CA: Carcinoma



1.1.3 Aetiology of AKI in cardiac surgery

The factors implicated in aetiogenesis of AKI in cardiac surgery can broadly be described as Pre-operative, Intraoperative and Post-operative factors. These are depicted in table [1.2].

Table [1.2]: Pathophysiological factors in post-cardiac surgery AKI. LV: left Ventricle, ACE: Angiotensin Converting Enzyme.

Preoperative	**Intraoperative**	**Postoperative**
Lack of renal reserve Renovascular disease Prerenal azotemia (Recent diuresis, Nil per mouth status, Impaired LV function) ACE inhibitors Nephrotoxins (IV contrasts) Endotoxemia Inflammation	Decreased renal perfusion (Hypotension, lack of pulsatile flow, vasoactive agents, anasthetic agents) Embolic events CPB induced inflammation Nephrotoxins (free hemoglobin)	Systemic inflammation Reduced LV function Vasoactive agents Hemodynamic instability Nephrotoxins Volume depletion Sepsis

1.1.4 Pathophysiology of AKI

In our current understanding, AKI is a continuum of stereotypical pathological phases; Figure [1.2], which are;

1-Initiation phase:

Early phase of vasomotor nephropathy in which there is alterations in vasoactivity and renal perfusion leading to pre-renal azotemia and eventually cellular ATP depletion and oxidative injury.

2-Extension phase:

These processes lead to activation of bone marrow derived cells, endothelial cells and renal epithelial cells, resulting in pro-inflammatory state. Inflammatory cells adherer to activated endothelium in the peri-tubular capillaries or the outer medulla, leading to medullary congestion and further hypoxic injury to S3 segment of proximal tubule.

3-Maintenance phase:

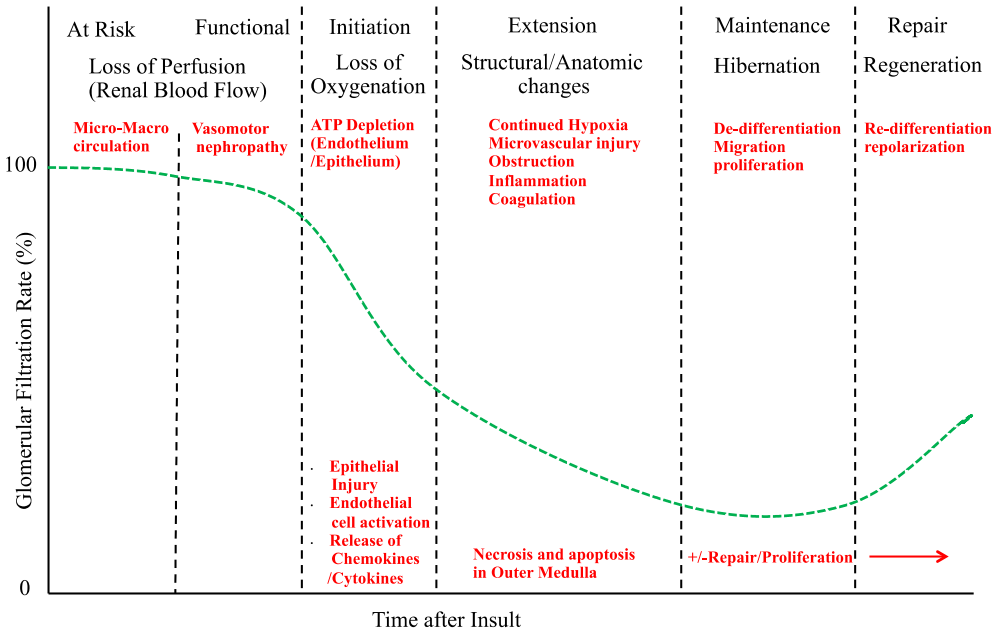
Elaboration of inflammatory mediators leads to additional cellular injury.

Tubular cells then begin process of proliferation and re-differentiation.

4-Repair phase:

Polarity and function are restored.

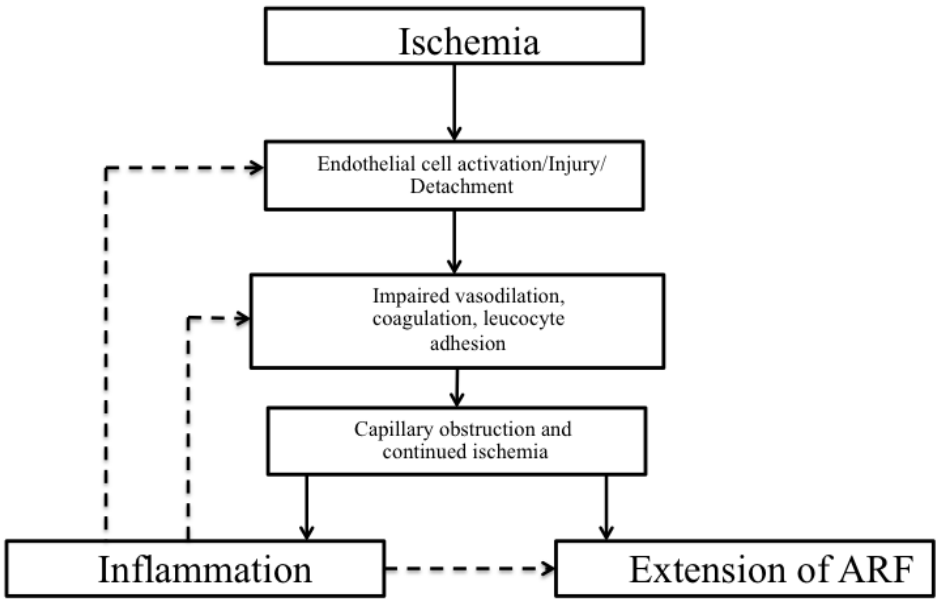
Figure [1.2]: Clinical phases of AKI; Text in red shows cellular changes responsible for each phase of AKI spectrum. Derived and modified from (Basile et al., 2012)



1.1.4.2 Endothelial cell injury in AKI

Ischemic injury leads to endothelial cell activation, which coupled with Reactive Oxygen Species (ROS) species drives dysregulated vasomotor function, EC-leucocyte adhesion, abnormal barrier function, coagulation and inflammation. This algorithm is shown in Figure [1.3].

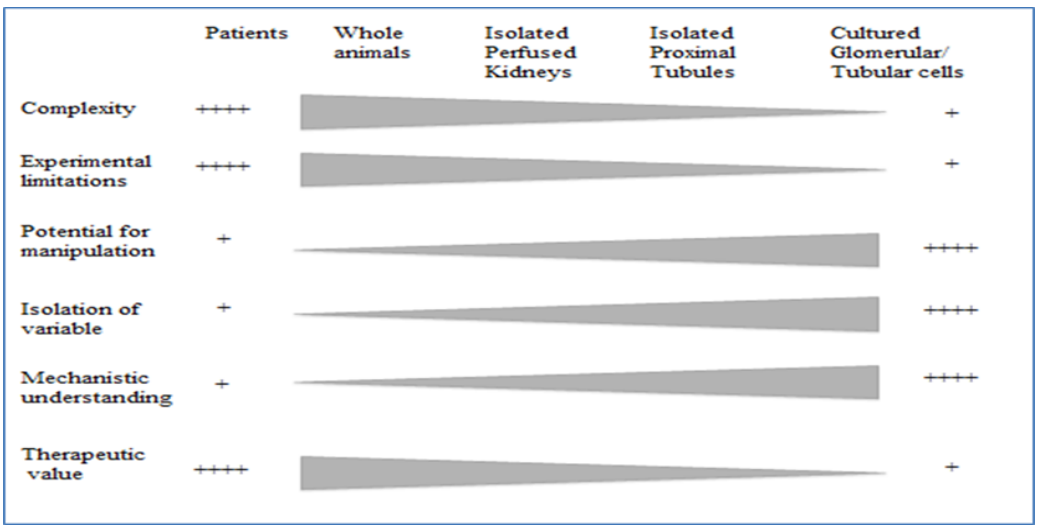
Figure [1.3]: Proposed contribution of endothelial cell activation in AKI



1.1.6 Animal models of AKI

Simple models such as (cultured cells, isolated perfused organs) provide mechanistic insight into cellular processes; however results in these models need verification in intact biological systems before they could be extrapolated to complex, evolved systems such as humans. All experimental models differ in their ability to synthesize disease conditions and generate useful data with predictive value to human disease. In addition no one experimental model affords a comprehensive insight to organ assessment while simultaneously providing mechanistic insight, figure [1.4].

Figure [1.4]: Strengths and weaknesses of available models of AKI.



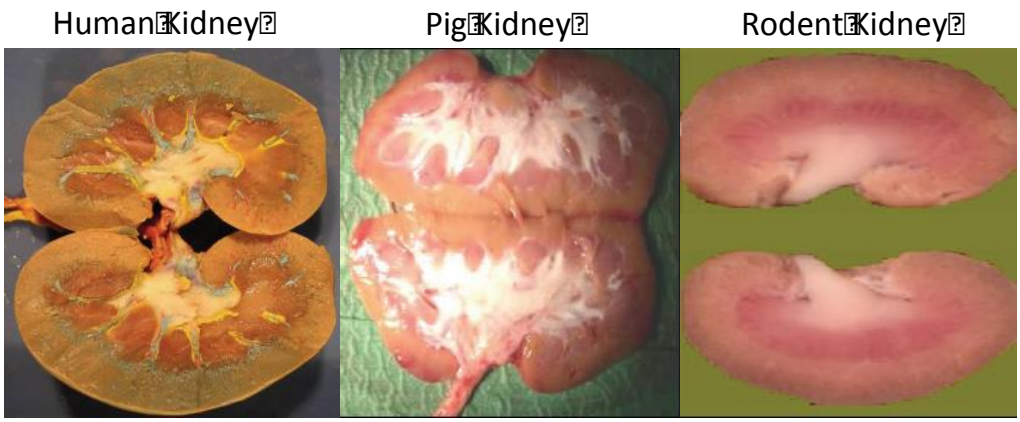
1.1.6.1 Swine as large animal model of AKI

Pigs share renal architectural characteristics; figure [1.5] and biochemical profile to humans and hence can provide translational relevance for human conditions and interventions.

1.1.6.1.1 Anatomical Similarities

Humans and pigs have multilobular and multipapillary kidneys vs. rodents that have unilobular and unipapillary kidney, Derived from (Giraud et al., 2011).

Figure [1.5]: Anatomical comparison between human, pig and rodent kidney



1.1.6.1.2 Physiological similarities (Giraud et al., 2011)

Table [1.3]: Unlike rodents, humans and swine both have morphological distinct renal architecture.

Anatomical region	**Metabolism**	**Present in humans**	**Present in swine**
Cortex	Uses O_2-dependant metabolism linked to oxidative phosphorylation.	yes	yes
Outer medulla	Uses O_2-dependant metabolism linked to oxidative phosphorylation.	yes	yes
Inner medulla	Use glucose to generate ATP via anaerobic glycolysis.	yes	yes

1.1.6.1.3 Biochemical similarities

Table [1.4]: Comparison of human and swine renal function indices. Derived from (Hannon et al., 1990)

	Swine	**Humans**
Renal weight (g)	99.1 (17.9)	147 (11)
Renal blood flow (ml/min/kg)	24 (8.0)	17.1 (7.2)
Renal blood flow (% cardiac output)	11.4 (2.1)	20 (1.0)
Glomerular filtration rate (ml/min/kg)	2.4 (0.92)	1.8 (0.2)
Filtration fraction (%)	15.3 (5.89)	19.0 (5.8)
Urine flow (ml/min/kg)	0.05 (0.05)	0.02 (0.08)
Urine osmolality (mOsm/kg H2O)	253 (127)	600 (150)
Free water clearance (ml/min/kg)	50 (2.7)	4.3 (1.5)
Fractional sodium excretion (%)	12.4 (9.6)	1.12 (0.4)
Plasma creatinine (mmol/L)	89 (19.5)	93 (20)
Plasma urea (mmol/L)	3.2 (1.15)	5.9 (1.5)
Plasma sodium (mEq/L)	138 (3.5)	140 (2.5)
Plasma potassium (mEq/L)	4.4 (0.4)	4.2 (0.4)
Plasma glucose (mmol/L)	4.6 (0.7)	5.0 (0.6)
Plasma lactate (mmol/L)	1.0 (0.3)	1.4 (0.6

1.1.6.1.4 Similarities between Renal indices in swine and humans exposed to cardiopulmonary bypass

Table [1.5]: The effect of cardiopulmonary bypass on renal function in humans and swine (Murphy et al., 2009).

	Normal ranges in human adults (16, 17, #)	**Porcine pre-CPB values median (IQR)**	**Porcine post-CPB values median (IQR)**
Urine output (ml kg_1 h_1)	>0.5	2.2 (2.0 to 4.7) ^	2.3 (1.1 to 2.9)^
Serum creatinine (mmol l_1)	71 to 124	113 (109 to 130)	119 (95 to 127)
Glomerular filtration rate (51Cr-EDTA clearance) (ml min_1)	90 to 120	114 (94 to 140)^	88 (28 to 102)^
Creatinine clearance (ml min_1)	88 to 137	97 (79 to 102) *	65 (34 to 90) *
(*) p < 0.05, (^) p < 0.1, Wilcoxon Signed Ranks test for porcine pre- versus post-values, IQR interquartile range, (#) http://www.kidney.org/professionals/KDOQI/.			

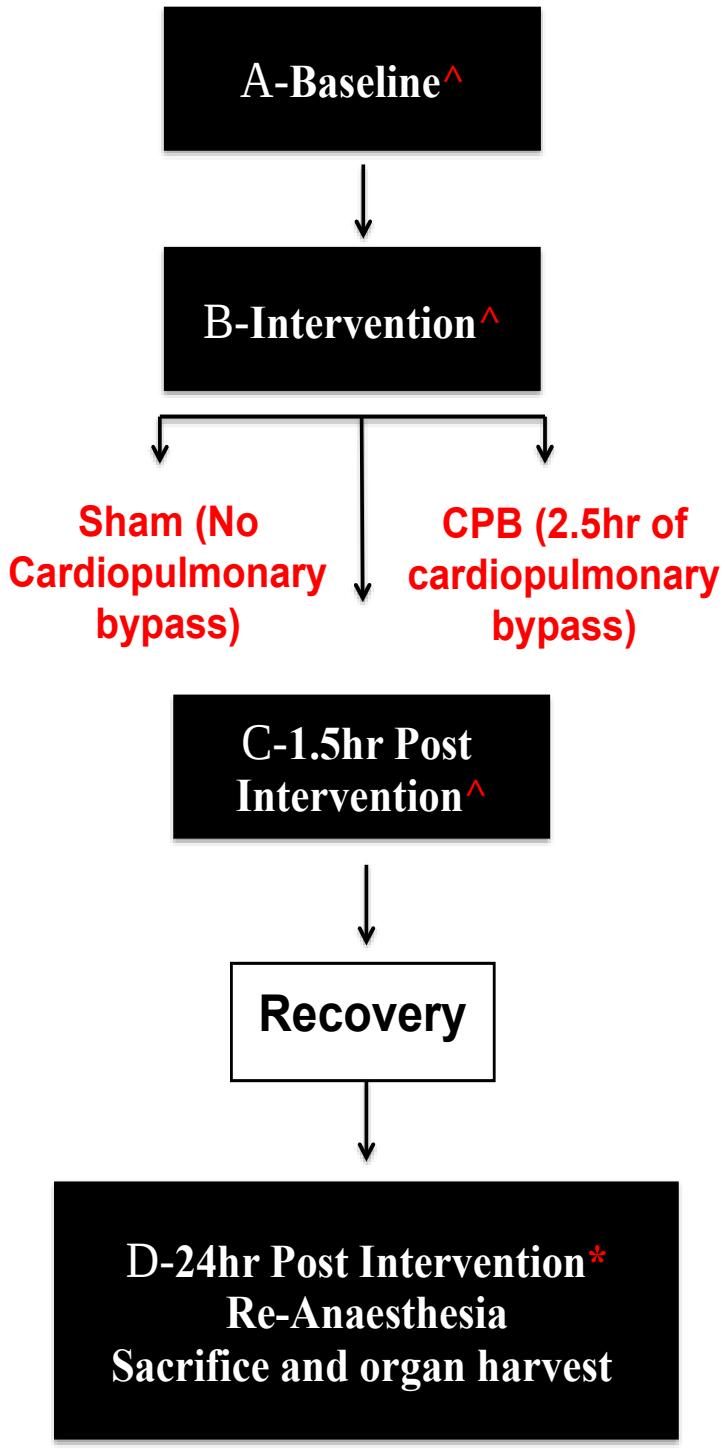
1.1.6.2 Porcine model of post CPB-AKI

Patel et al(Patel et al., 2011a) subjected healthy swine weighing 50-70kg to 2.5 hrs. of cardiopulmonary bypass utilizing minimally invasive method of central cannulation via Internal Jugular and Internal carotid vessels. The unique feature of their design was a 24hr recovery time point which allowed assessment of renal indices (Creatinine clearance and tissue assessment at 24hr post CPB) when clinically significant AKI is observed post-operatively.
In their experimental protocol swine were analysed at baseline (Pre-CPB), during Intervention (CPB), 1.5hr post intervention (Post-CPB) and 24hr post Intervention, figure [1.6]. The model incorporated assessment of renal function using Creatinine clearance as an index of GFR along with free water clearance

and fractional excretion of sodium and renal biomarkers i.e. IL-8. In additional this model incorporated detailed assessment of renal medullary and cortical hypoxia and perfusion using intra-parenchymal and surface probes, (Oxylite large area sensor and pO_2 E Series; Oxford Optronix, Oxford, UK). Nitric oxide bioavailability was measured using urinary nitrate: nitrite ratio along while renal artery flow measured in response to acetylcholine as a bench test for assessing in-vivo endothelial function using renal artery blood flow probe (T106 Transonic flow meter; Transonics Systems, Ithaca, NY). In conjunction with renal function detailed assessment of heart function (in-vivo assessment of cardiac index, SVRI, PVRI, Oxygen delivery and myocardial oxygen consumption), lung function (work of breathing, compliance, resistances) and assessment of systemic perfusion (30min interval hemodynamic and acid-base status, serum lactate, pre/peri/post and 24hr-assessment of blood indices and platelet function) were also undertaken.

Figure [1.6]: Schematic of key stages (A-D) of porcine AKI model. CPB: Cardiopulmonary Bypass, NO: Nitric Oxide, Ach: Acetylcholine, ICC: Immunocytochemistry. (A: Baseline; general anasthetic induction and intubation monitoring and analyses (90min), B: Intervention; randomisation and application of intervention (i.e. Cardiopulmonary bypass, Blood transfusion etc.) with monitoring and analyses (150min), C: Post-intervention; removal of intervention and recovery (under monitoring and analyses, 90min) followed by extubation and ambulation, D: 24hr post intervention; Re-intubation under general anasthetic and monitoring, analyses and sacrifice with organ harvesting (90min))

Porcine CPB AKI model



^Analyses (Baseline(90min), Intervention(2.5hr), 1.5hr-post-intervention(90min))

- Measures of hemodynamics.
- Measures of ventilation.
- Measures of AKI
- Measures of inflammation.
- Measures of NO bioavailability
- Platelet function

* Analyses (24hr-recovery (90min)

- Endothelial Function (Ach mediated renal artery flow assessment)
- Organ Harvest
- Histology
- ICC

1.1.6.2.1 Pathogenesis of Post-CPB AKI; insights form porcine model, Murphy et al.

The hallmark change was decline in GFR as suggested by reduction in creatinine clearance. This was associated with proteinuria and elevated levels of NAG and alpha1-microalbumin, (markers of glomerular and/or proximal tubular injury) and IL-8. These changes were associated with reduction in NO bioavailability as measured by nitrite: nitrate ratio and RBF measurement. Figures [1.7 and 1.8].

Figure [1.7]: Pathogenesis of CPB mediated AKI: (A) Changes in creatinine clearance at 1.5 hr and 24 hr post CPB. (B) Changes in nitric oxide bioavailability following CPB. (C) Changes in renal blood flow at 24 hr post CPB induced by acetylcholine. (D) Normalized densities after staining histological tissue with antibodies against macrophages; MAC 387.

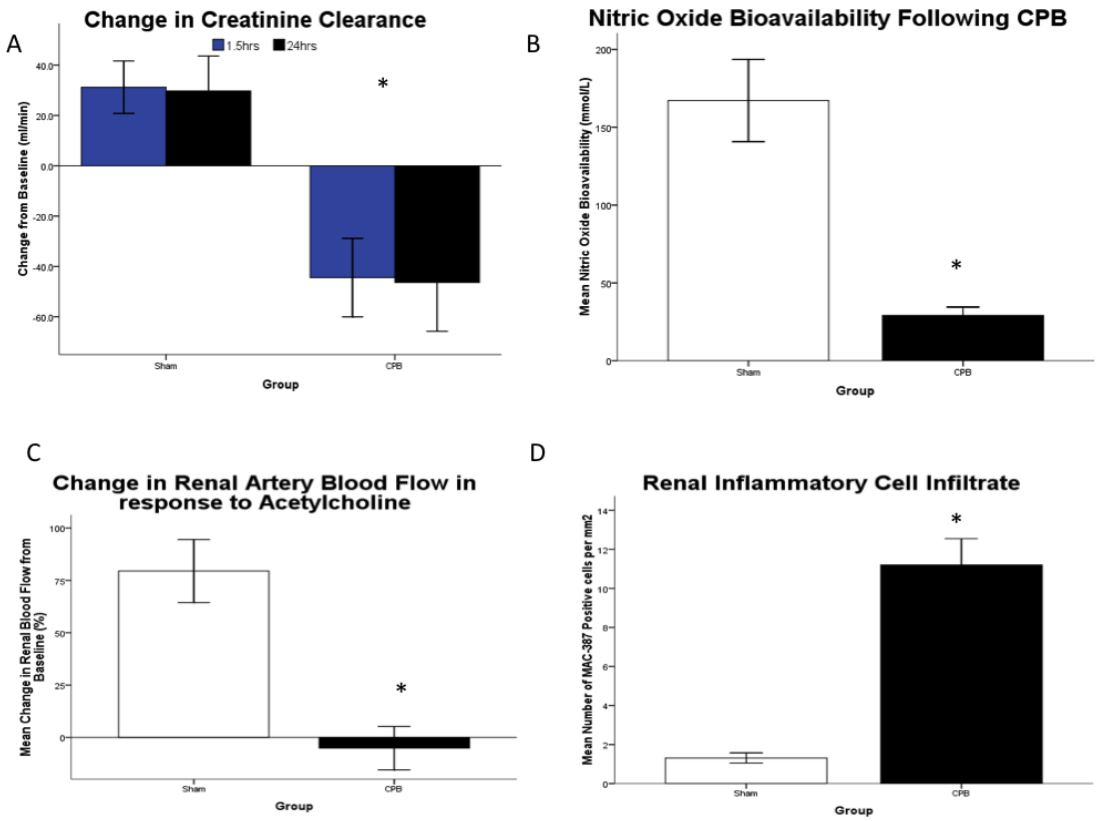
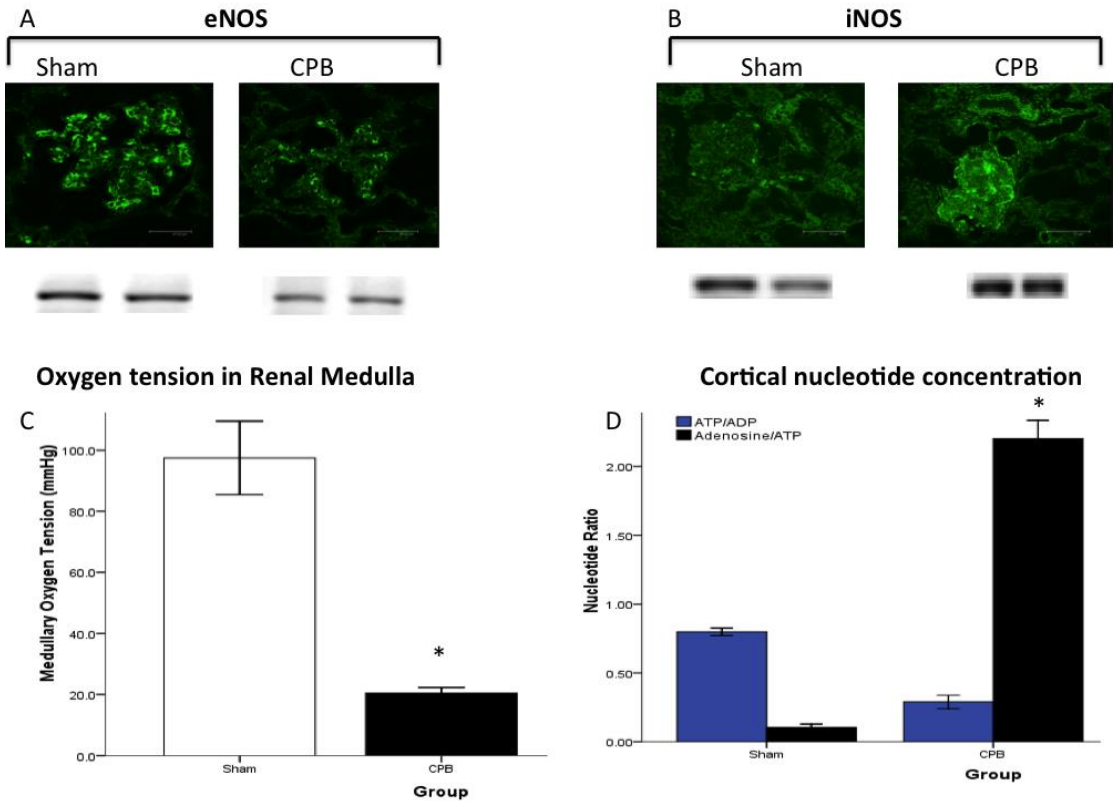


Figure [1.8]: Pathogenesis of CPB mediated AKI: (A) Immunofluorescence staining of renal frozen sections obtained at 24hr post CPB, in conjunction with protein quantification with western blotting demonstrating endothelial NOS expression. (B) Immunofluorescence staining of renal frozen sections obtained at 24hr post CPB in conjunction with western blotting demonstrating inducible NOS expression. (C) Medullary oxygen tension at 24hr post CPB (D) Cortical ATP/ADP measured using Reverse-phase high performance liquid chromatography.



The CPB-mediated endothelial dysfunction was associated with increased expression of iNOS but reduction in eNOS expression. Determination of nucleotide profile suggested depletion of cortical adenosine triphosphate (ATP) concentrations as demonstrated by reduction in ATP/ADP ratio and significant hypoxia at the level of the outer medulla at 24 hours.

1.1.7 Role of Glycocalyx in Endothelial cell homeostasis

Glycocalyx is a gatekeeper to endothelial cell and lines the luminal surfaces of all vasculature and comprises of Glycoproteins and Proteoglycans.

1.1.7.1 Structural profile

Proteoglycans have a protein core to which are attached negatively charged glycosaminoglycan (GAG) side chains. Core proteins include Syndecans, Glypicans, Perlecans, Versicans, Decorins, Biglycans and Mimecans. GAG side chains include: Heparan sulphate (50–90%), Hyaluronic acid, Chondroitin, Dermatan and Keratin sulphates. Figures [1.9] and [1.10] detail the structural characteristics of glomerular endothelial membrane (GEM) and glycocalyx respectively.

Figure [1.9]: High power electron micrograph of porcine glomerular endothelial layer using Lanthanum perfusion fixation; a: Podocyte, b: basement membrane, c: endothelial cell, d: glycocalyx, e: inter-endothelial cell cleft, f: shed glycocalyx, g: blood space, h: urinary space.

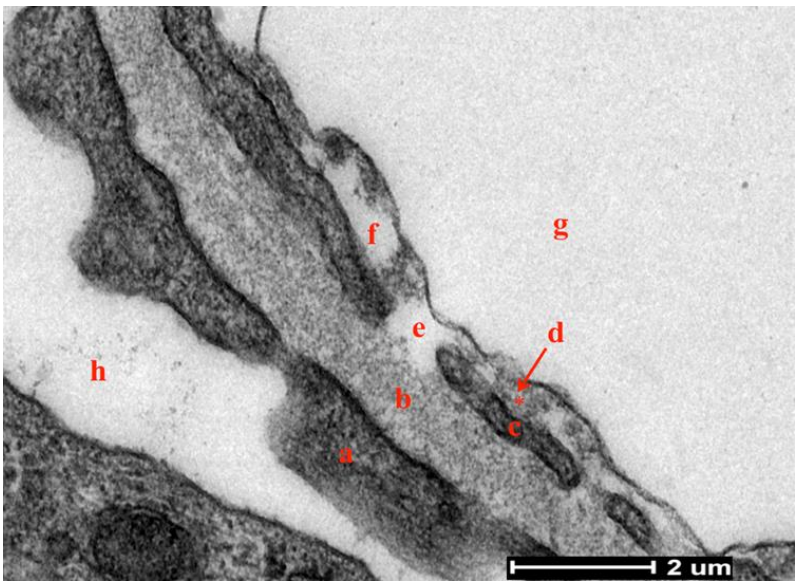
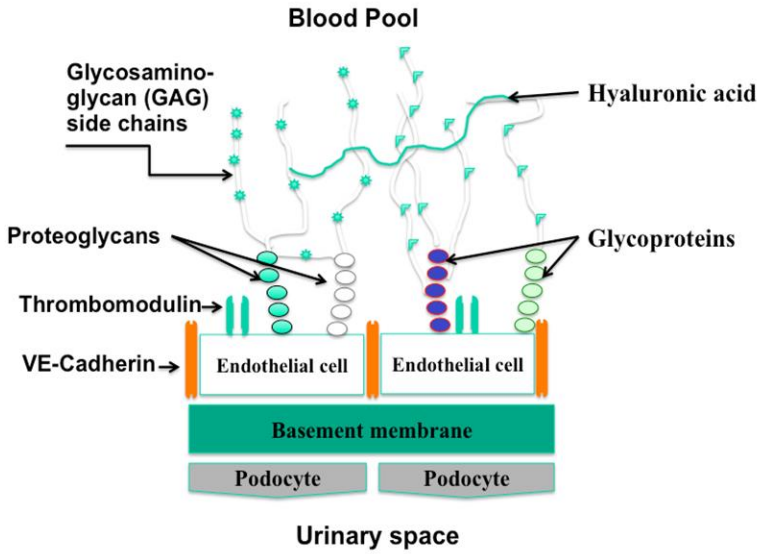


Figure [1.10]: Components of the endothelial glycocalyx. Bound to the endothelial membrane are proteoglycans, with long unbranched glycosaminoglycan side-chains (GAG-chain) and glycoproteins, with short-branched carbohydrate side-chains. Incorporated in and on top of this grid are plasma and endothelium-derived soluble components, including hyaluronic acid. EC are lined with receptors for thrombomodulin and VE-Cadherin forms tight junctions between endothelial cells.



1.1.7.2 Functional significance:

Endothelial glycocalyx has many important physiological functions, including the regulation of vascular permeability, adhesions of leucocytes, mediation of shear stress and regulation of inflammatory cascades. Destruction of glycocalyx has been implicated in many pathological states i.e. ischemic insult, redox stress, enzymatic attack, and after inflammation.

1.1.7.3 Cardiac surgery and shedding of glycocalyx

Surgical trauma has also been implicated in shedding of glycocalyx in human beings. Rehm et al showed an increase of the main components of the glycocalyx,

syndecan-1 and Heparan sulfate, in the plasma of vascular surgical patients with global or regional ischaemia (Bruegger et al., 2008). The intraoperative damage was proportional to the duration of ischaemia. Bruegger et al. described increase levels of syndecan-1 and Heparan sulfate in the arterial blood of patients undergoing coronary artery bypass surgery (Bruegger et al., 2009).

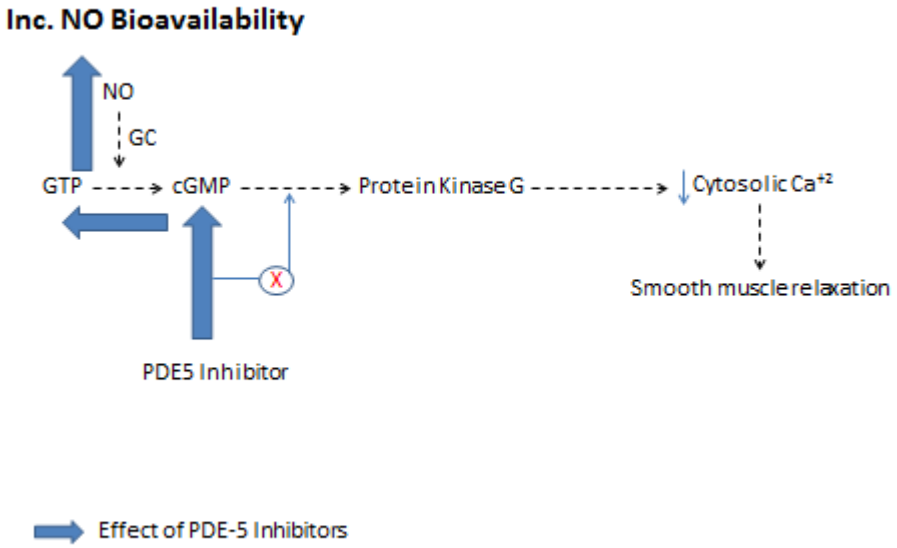
1.1.8 Renoprotection in cardiac surgery

Several strategies ranging from inhalational anasthetic agents to pharmacological agents to minimally invasive procedures have failed to translate into clinically discerning benefit against CPB mediated AKI with a greater appreciation felt now that paucity exists for such effective measures (Patel and Angelini, 2014). In a systematic review, Patel et al looked at randomised controlled trials assessing renoprotective utility of pharmacological agents in more than 4000 patients undergoing cardiac surgery and found none of the intervention to reduce mortality.

1.1.8.1 Phosphodiesterase-type-5 inhibitor as renoprotective agent.

Sildenafil, a Phosphodiesterase type 5 inhibitor is indicated for the treatment of pulmonary arterial hypertension (PAH) and erectile dysfunction. It inhibits phosphodiesterase Type 5 (PDE-5) which normally breaks down Nitric Oxide (NO). Inhibition with PDE-5 leads to increase in NO bioavailability by reducing its metabolism; Figure [1.11].

Figure [1.11]: Increased Nitric Oxide bioavailability with PDE-5 inhibitors. GC: Guanylyl cyclase. cGMP Cyclic guanosine monophosphate, GTP Guanosine-5'-triphosphate. X: Inhibition.

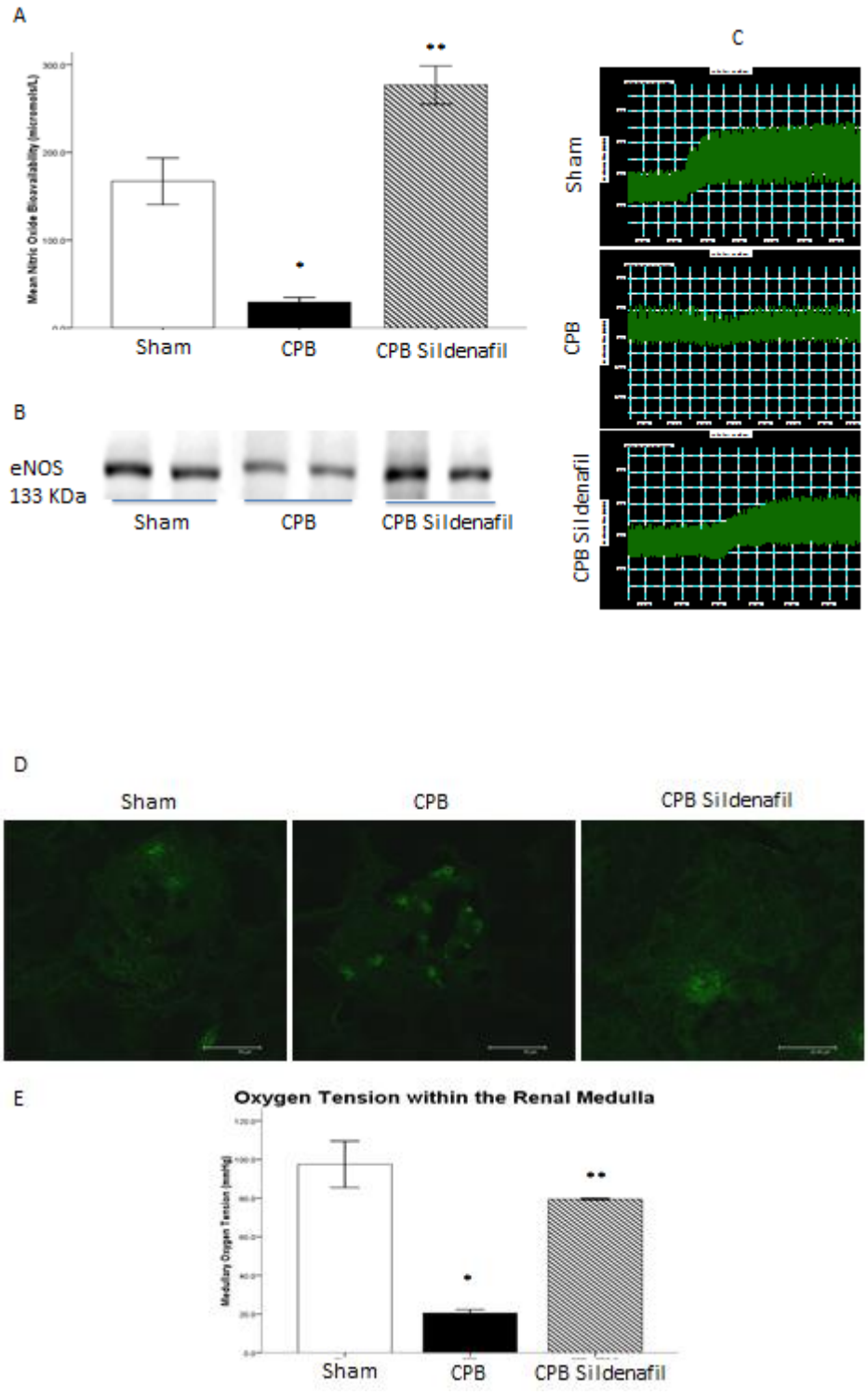


1.1.8.1.1 Effect of Sildenafil pretreatment and attenuation of post-CPB AKI in large animal model

Patel et al(Patel et al., 2011a) explored this renoprotective role in their pre-clinical porcine model of post CPB AKI. They measured Urinary nitrite and nitrate to determine NO bioavailability, which was reduced in CPB pigs but restored with Sildenafil pre-treatment. This increased NO bioavailability was associated with increased endothelial NOS activity measured in cortical lysates. This restored NO bioavailability resulted in preservation of macrovascular renal artery endothelial function measured via endothelial stimulatory response with supra-renal acetylcholine infusion. Not only did restoration of NO bioavailability resulted in improved endothelial function, but it also resulted in reduction in renal medullary hypoxia and macrophage induced renal inflammation; Figure [1.12]. (Patel et al., 2011a).

Figure [1.12]: Effect of Sildenafil pre-treatment on NO bioavailability, endothelial function, inflammation and medullary hypoxia. (A): Nitric Oxide bioavailability (μ mol/L), (B) Western blot analyses of endothelial nitric oxide synthase in kidney lysates obtained at 24hr post intervention. (C) Renal Blood Flow (Green

trace) measurement using renal artery probe in response to supra-renal acetylcholine infusion. (D) Panel representing MAC 387(Macrophage marker) positive staining (Green) in renal glomeruli. (E) Assessment of renal medullary hypoxia (mmHg) using medullary O_2 pressure sensor at 24hr post intervention. *: p <0.5 vs. Sham, ** vs. CPB.



1.2 Intravascular fluid therapy

Fluid resuscitation is required to replace and maintain intravascular volume in patients with sepsis, in intra/post-operative state or exposed to trauma. In sepsis, up to 10 liters may be easily infused in initial 24hrs (Hollenberg et al., 2004). Traditionally options have been either crystalloids or colloids. The rationale of colloid use is based on the theoretical benefits of increased intravascular volume expansion, and a subsequent reduction in overall fluid requirements.

1.2.1 Types of resuscitation fluids

1.2.1.1 Colloids

1.2.1.1.1 Human albumin solutions (HAS)

In health albumin contributes to 80% of plasma oncotic pressures (25mmHg) where as in critically ill patients there is considerable reduction (approx. 19.1mmHg) and associated poor outcomes (Nicholson et al., 2000). The Saline versus Albumin Fluid Evaluation study (SAFE trial) on 6997 patients randomised to either albumin or saline, concluded that it was not superior to saline when analysed for 28 day mortality; Relative risk, 0.99: 95%CI, 0.91-1.09, p 0.87. Since then follow on trials such as FEAST study and ALBIOS have not shown any superiority of human albumin vs. crystalloids.

1.2.1.1.2 Semisynthetic colloids

1.2.1.1.2.1 Hydroxyethyl starches

Human albumin being costly dictated the development of synthetic colloids. Currently Hydroxyethyl starches (HES) are the most commonly used synthetic colloids. HES are produced by hydroxyl substitution of amylopectin obtained

from sorghum, maize or potatoes. A high degree of substitution on glucose molecules prevents against hydrolysis by non-specific amylases in the blood, but the action increases accumulation in liver and spleen macrophages, which constitute the reticuloendothelial system (van Rijen et al., 1998) likely due to recognition as a foreign substance and promoting internalization and storage. In addition HES has shown to produce significant reduction in Endothelium Derived Relaxation in epicardial coronary arteries of the pig.
Currently used HES have a reduced concentration (6%) with a molecular weight of 130kDa and a molar substitution of 0.38-0.45. Up till recently HES was widely being used for patients undergoing anaesthesia for major surgery and in patients in ICU. Pharmacological properties of different HES preparations are provided in table [1.15].

HES preparations

Table [1.6]: Pharmacological properties of different HES preparations. Molar substitution and the C2/C6 (i.e. quotient of the numbers of glucose residues hydroxyethylated at positions 2 and 6, respectively) dictate the kinetics of degradation. A higher C2/C6 substitution protects from hydrolysis thus increasing plasma half-life and potential toxicity. Derived and modified from (Ertmer et al., 2009).

Table [1.6]: HES Preparations

Preparation	Concentration	Trade name	MMW(Da)	Specification range (kDa)	Top fraction (kDa)	Bottom fraction (kDa)	MS	C2/C6 ratio	In vitro COP (mmHg)	Initial volume effect	T1/2 Alpha (h)	T1/2 Beta (h)	Clearance (ml/min)
Hetastarch													
HES450/0.7	6%	Plasmasteril, Hespan	450000	150	2170	19	0.7	4-5	26	100	n/a	300	n/a
HES 670/0.7	6%	Hextend	670000	175	2500	20	0.75	4	n/a	100	6.3	46.4	0.98
Hexastarch													
HES 200/0.62	6%	Elohes	200000	25	900	15	0.62	9	25	110	5.08	69.7	1.23
Pentastarch													
HES 70/0.5	6%	Rheohes, Expafusin	70000	10	180	7	0.5	3	30	90	n/a	n/a	n/a
HES 200/0.5	10%	HAES-steril, Hemohes	200000	50	780	13	0.5	4-5	50-60	145	3.35	30.6	9.24
HES200/0.5	6%	HAES-steril, Hemohes	200000	50	780	13	0.5	4-5	30-35	100	n/a	n/a	n/a
HES 200/0.5	3%	HAES-steril, Hemohes	200000	50	780	13	0.5	4-5	15-18	60	n/a	n/a	n/a
Tetrastarch (waxy-maize-derived)													
HES 130/0.4	10%	Voluven	130000	20	380	15	0.4	9	70-80	200	1.54	12.8	26
HES 130/0.4	6%	Voluven,	130000	20	380	15	0.4	9	36	100	1.39	12.1	31.4

Preparation	Concentration	Trade name	MMW(Da)	Specification range (kDa)	Top fraction (kDa)	Bottom fraction (kDa)	MS	C2/C6 ratio	In vitro COP (mmHg)	Initial volume effect	T1/2 Alpha (h)	T1/2 Beta (h)	Clearance (ml/min)
		Volulyte											
Tetrastarch (Potato-derived)													
HES 130/0.42	10%	Tetraspan	130000	15	n/a	n/a	0.42	6	60	150	n/a	n/a	n/a
HES 130/0.42	6%	Venofundin, Tetraspan, VitaHES	130000	15	n/a	n/a	0.42	6	36	100	n/a	12	19

MMW: Molecular weight, Bottom fraction: less than 10% of molecules are less than the molecular weight defined by bottom fraction, top fraction: less than 10% of molecules exceed the molecular weight defined by bottom fraction, MS: Molar substitution, COP: Colloid osmotic pressure, n/a: not applicable, not available, T1/2 alpha: distribution half life, T ½ Beta: elimination half life, Data extracted from manufacturer's product information.

1.2.1.1.2.2 Gelatins

These are prepared by hydrolysis of bovine collagen. Succinylated gelatin (Gelofusion™) is produced by enzymatic alterations of the basic gelatin peptide and is presented in isotonic saline. Urea linked gelatin (Polygeline, Haemaccel™) is produced by thermal degradation of the raw material to small peptides (12000-15000 Daltons) followed by urea cross linking to produce polymers of around 35000 Daltons that are suspended in isotonic sodium chloride with 5.1mmol/L potassium and 6.25mmol/L calcium.

1.2.1.1.2.3 Dextrans

These are biosynthesized from sucrose by leuconostoc bacteria using the enzyme dextran sucrase. This enzyme catalyze the alpha-1,6-glycosidic linkage of glucose monomers. They are defined by their MW. Dextran 40 and 70 having 40,000 and 70,000 Daltons respectively. Allergic reactions have been associated with Dextrans; <0.35% (Grocott et al., 2005). The development of Dextran 1 hapten has significantly reduced the incidence; <0.0015% (Ljungstrom, 1993). This involves a 20ml injection of low molecular weight dextran-1 (1000Da MW) prior to infusion of a dextran volume expander, which leads to inactivation of anti-Dextran IgGs in the recipient.

1.2.1.1.3 Crystalloids

The most commonly used crystalloid is normal (0.9%) saline (NS)(Na conc.=154 mmol/L). This term was coined by Dutch physiologist who in 1882 suggested human blood salt to be 0.9% rather than 0.6%.
Concerns have been raised for NS to induce hyperchloremic metabolic acidosis ($NaCl + H_2O \rightarrow HCl + NaOH$), however the clinical consequences of this are unclear (Guidet et al., 2010). In addition, due to concerns regarding large volume induced

fluid overload condition, small volume resuscitation with hypertonic saline (3%, 5% or 7%) has been introduced.

1.2.2 AQIX RS-1, a novel fluid replacement intravenous solution.

Aqix RS-I (Figure [1.13]), a novel non-inorganic phosphate perfusion solution designed for use with any organ at all physiological temperature, utilizes the physiological buffering of sodium bicarbonate/carbon dioxide and the zwitterionic Good's buffer, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES). It has similar osmolality to serum (286 milli-osmol/L) with an ionic composition that maintains isosmotic characteristics (Maruyama and Chambers, 2008).

Figure [1.13]: Constituents of AQIX RS-1.

a

b

Component Aqix RS-I	Conc. (mmol/L)
NaCl	110.0
KCl	5.0
$CaCl_2$	1.25
$MgCl_2$	0.45
$MgSO_4$	--
$NaHCO_3$	25.0
KH_2PO_4	--
BES	5.0
D-Glucose	10.0
Glycerol	0.11
L-Glutamate	0.30
L-Glutamine	0.40
L-Aspartate	0.02
L-Carnitine	0.05
Choline chloride	0.01
TPP (cocarboxylase)	40.0 nmol/l
Human (recomb.) insulin	28.0 mIU

1.2.2.1 Characteristics of AQIX RS-1 beyond conventional crystalloids: potential to confer endothelial and organ preservation

Following is a description of constituents that differentiate AQIX RS-1 from other non-blood volume expanders. The inclusion of physiological buffers, metabolic

and high-energy substrates are the key elements that fill the gap between simple colloids/crystalloids and gold standard volume expander and resuscitative therapy i.e. blood. The ionic composition of AQIX RS-1 is similar to interstitial fluid.

Buffering capacity

AQIX RS-1 consists of $NaHCO_3/pCO_2$ (sodium hydrogen carbonate/dissolved CO_2) in combination with the zwitterionic Good's buffer, BES (N, N~bis [2-Hydroxyethyl]-2-amino-ethanesulphonic acid as a buffering system. This acts by maintaining an ideal pK_a over a temperature range of 10 to 37^0C, to provide a stable pH, an essential requisite for cellular preservation.

Energy substrates

Glucose and glycerol in this solution act as substrates for the organs. Apart from their ability to be metabolized, glycerol and glucose also have free radical scavenging and membrane preserving properties.

TCA intermediates

Aspartate and glutamate enhance oxidative metabolism by replenishing tricarboxylic acid (TCA) cycle intermediates, thereby maintaining high-energy phosphate levels even during ischemic insult.

Thiamine

Thiamine plays an important role in the oxidation of α -keto acids (by the action of thiamine cocarboxylase) and prevents the accumulation of pyruvate and toxic pyruvate aldehyde, thereby minimizing cellular apoptosis and accompanying necrosis of tissues and associated organs.

Carnitine

The vitaminoid carnitine improves cardiac function by improving coronary blood flow.

Insulin

Insulin acts to (1) enhance of intracellular glucokinase activity and amino- acid incorporation into protein, (2) stimulates DNA (deoxyribose nucleic acid) translation into proteins, (3) increases lipid synthesis and (4) stimulates sodium, potassium and inorganic phosphate transport across cell membranes.

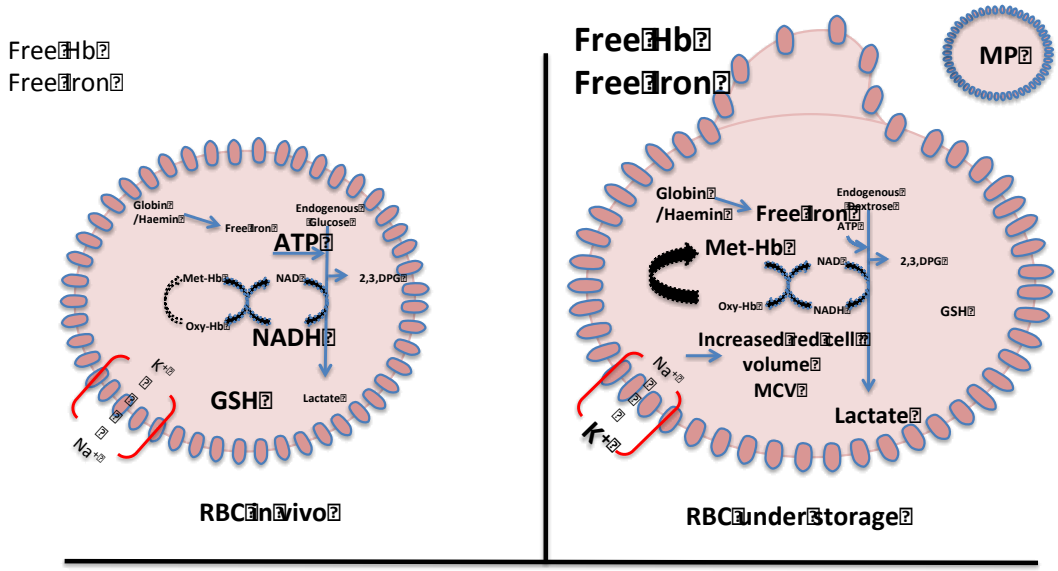
1.3 Blood transfusion

Half of patients undergoing cardiac surgery in USA and UK received blood transfusion in 2006. In UK, cardiac surgery uses 5% of all donated blood (McGill et al., 2002). Growing evidence, largely observational associates blood transfusion with increased post cardiac surgery adverse events. Transfusion with as little as 1 unit has been linked in decrease in 10- year Survival after CABG (Koch et al., 2006).

1.3.1 Red cell Storage-lesion

Red cell units undergo time dependent changes both in red cell and supernatant that includes membrane, rheological, oxidative changes resulting from increased potassium, loss of red cell ATP which leads to programmed cell death (eryptosis), reduction in 2,3-DPG, loss of S-nitrosol hemoglobin which carries nitric oxide and production of inflammatory microvesicles, collectively attributed as 'storage lesion'. Functional changes also occur such as inability to deliver O2, survival in circulation etc. Storage related RBC cellular and supernatant storage changes are highlighted in figure [1.14].

Figure [1.14]: Functional storage related changes in cellular and supernatant components of packed red cell units. With prolonged storage (Right Panel) the accumulation of lactate inhibits glycolysis. Reduction in 2,3 DPG attenuates the ability of oxygen delivery. Reduced ATP and cold storage impair Na exchange, which affects cell volume. Reduced NADH promotes auto-oxidation and formation of met-Hb, whereas reduction in NADPH decreases production of reduced glutathione (GSH). Externalization of Phosphatidylserine (PS) induces senescence. Increased production of microvesicles decrease endothelial derived NO gas. Elevated levels of free Hb and iron in the supernatant released from hemolysed cells contribute to oxidative stress. Derived and modified from(Orlov and Karkouti, 2015).



1.3.2 Red cell storage mediated organ injury; potential mechanisms

1.3.2.1 Storage induced production of free Hb and resultant loss of NO bioavailability.

Red blood cells are known to modulate local blood flow by 'hypoxic vasodilation'. These mechanisms include 1-release of ATP, 2-release of NO from its storage form i.e. S-nitrosylated hemoglobin and 3-reduction of circulating nitrite to NO during R to T allosteric transitions of Hb. An additional mechanism includes scavenging NO produced from ECs by free Hb. Ferrous oxyhemoglobin, which accumulates with storage is known to react with NO via the dioxygenation reaction to form methemoglobin and nitrate in a 1:1 stoichiometry (Donadee et al., 2011). As little as 6 μ M of plasma heme concentration has shown to cause vasomotor dysfunction in patients with sickle cell anemia (McAuslan and Reilly, 1986).

1.3.2.2 Role of RBC Fe-load and inflammatory insult, the "Iron Hypothesis".

Transfusion of 1 RBC unit into an adult human recipient would acutely deliver an iron load to the mononuclear phagocyte system that is approximately 60 times greater than it experiences during normal homeostatic conditions (i.e., approx. 60 mg of iron in the first hour post-transfusion due to clearance of 25% of the transfused RBCs present in 1 unit of 42-day-old RBCs, compared with approximately 1 mg of iron per hour due to normal clearance of senescent endogenous RBCs (Spitalnik, 2014). The delivery of this Iron to monocyte/macrophage system results in oxidative stress and inflammatory cytokine production. Some of the macrophage-ingested Iron is released back to circulation as Non-transferrin bound Iron (NTBI), where it can cause oxidative damage (Donadee et al., 2011).

1.3.2.3 MPs and Endothelial Cell adhesion

Depletion of Red cell ATP leads to dysfunction of red cell flippase/floppase/scramblase enzymes, which leads to asymmetry of cellular lipids so that Phosphatidylserine (PS) is exteriorized. PS cause MPs to bind to EC and lead to pro-thrombotic and pro-inflammatory reactions. Annexin-V suppresses RBC-EC adhesion; thereby confirming that storage induced PS externalization is responsible for the elevation of stored RBC adherence to ECs (Wautier et al., 2011). In addition red cell rejuvenation also reduces PS mediated EC adhesion (Koshkaryev et al., 2009).

1.3.3 Transfusion related acute kidney injury.

In a review 18 observational studies perioperative transfusions was found to be independent risk factor for AKI and suggested that each unit of perioperative blood transfusion is independently associated with a 10–20% increase in the risk of AKI after cardiac surgery with CPB.

1.3.4 Transfusion Related Acute Lung Injury

Popovsky et al in the 1980s coined the term transfusion-related acute lung injury (TRALI), previously called severe pulmonary hypersensitivity reaction. Incidence: 1 in 5000 units of packed RBC, 1 in 2000 plasma-containing components, and 1 in 400 units of whole-blood– derived platelet concentrates (Gazmuri and Shakeri, 2002). The incidence of ARDS among those massively transfused (>10 units) patients may be as high as 57% (Silverboard et al., 2005).

1.3.4.1 Diagnostic criteria for TRALI.

TRALI is acute lung injury temporally related to transfusion. It is not physiologically different from other forms of ALI or ARDS, and it is important to note that autopsy findings from fatal TRALI cases demonstrate that TRALI is indeed neutrophil mediated and that autopsy findings are similar to those of patients with ALI/ARDS. Thus, the diagnosis of TRALI should be considered in all cases of respiratory distress with significant hypoxemia (PaO_2/FiO_2 less than 300 mm Hg) temporally related to transfusion and should satisfy the criteria for the diagnosis of ALI. Table [1.7] displays criteria for diagnosing TRALI and possible TRALI.

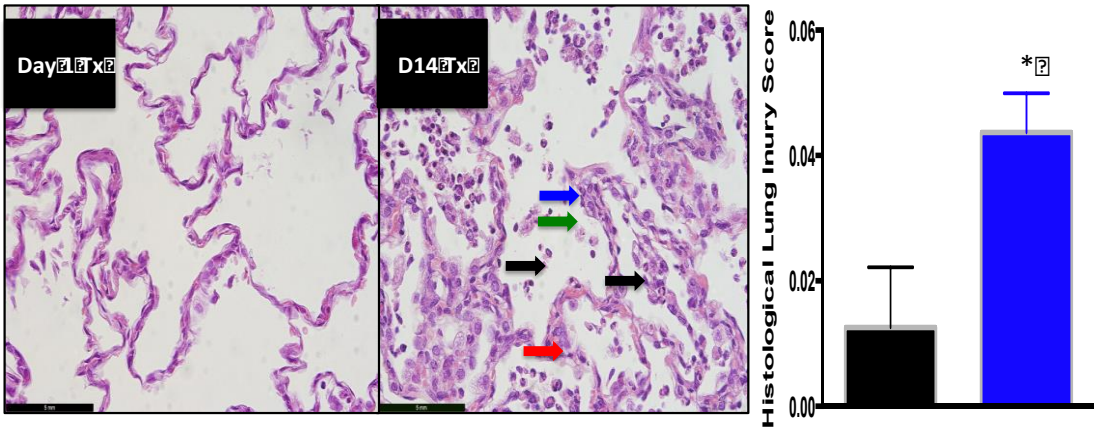
Table [1.7]: Diagnostic criteria of TRALI.

TRALI: Recommended criteria (Kleinman et al., 2004)
a. Acute Lung Injury (ALI)
i. Acute onset
ii. Hypoxemia
PaO2/FiO2 less than 300 mm Hg, or SpO2 _ 90% on room air
iii. Bilateral infiltrates on frontal chest radiograph
iv. No evidence of left atrial hypertension (i.e. circulatory overload)
b. No pre-existing ALI before transfusion during or within 6 h of transfusion
d. No temporal relationship to an alternative risk factor for ALI
Possible TRALI
a. ALI occurring during or within 6 hours of transfusion
b. No pre-existing ALI before transfusion
c. Clear temporal association for an alternative risk factor for ALI

1.3.4.2 Red cell 'storage dependent-TRALI': Evidence from porcine model of storage mediated pulmonary dysfunction

Patel et al (Patel et al., 2013) have shown homology between porcine and human erythrocytes under storage condition though, 14-day storage changes in swine corresponded to 42-day storage conditions in humans. Transfusion of 14 days stored blood in healthy swine weighing 50-70kg with allogenic matched blood, recipient swine developed histological evidence of pulmonary injury characterized by neutrophils in the interstitium and alveolar space, hyaline membrane formation, alveolar wall thickening, proteinaceous debris in the alveolar space, and significantly elevated lung injury scores compared with recipients of fresh (Day 1) blood (Figure [1.15]).

Figure [1.15]: Storage induced histological lung injury. Histological ling injury scores were calculated using characteristics of lung injury that included 1-neutrophilic infiltration (Black arrow), 2-Hyaline membranes (Blue arrow), 3-proteinaceous debris (red arrow) and 4-septal thickening (green arrow). Key: P<0.05 * versus Day 1 Tx. Graph columns (Black: Day 1 stored red cell transfusion (Tx) recipients, Blue: Day 14 red cell Tx recipients)



1.3.4.4 Elimination of storage lesion and attenuation of transfusion related organ injury

Novel methods are being tested to reduce the storage lesion and improve flow properties of red cells, these include the following;

1.3.4.4.1 Red cell washing

Red cell washing has shown to ameliorate supernatant component of storage lesion such as potassium, free Hb and MPs, without improving red cell characteristics such as ATP, 2,3-DPG or deformability (Gu et al., 2008).

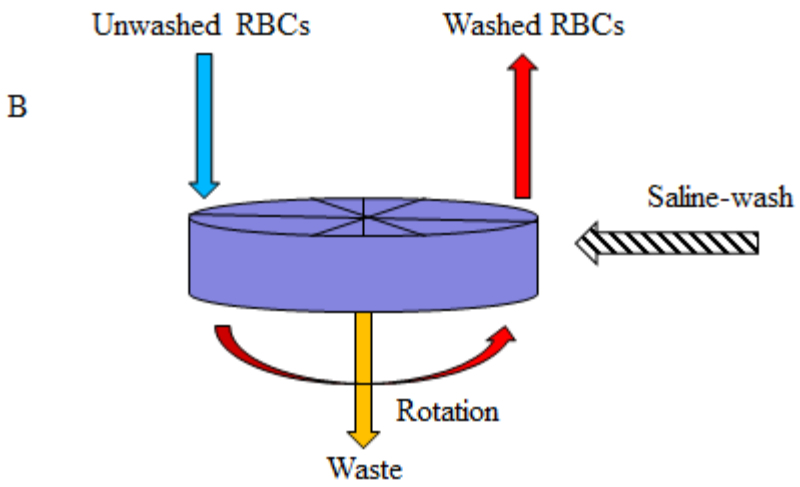
Principals of Red cell Washing (Example; C.A.T.S. Fresenius autotransfusion device (C.A.T.S))

Processing takes place in three distinct phases: fill, wash, and empty, on a unit or "batch" of shed blood. Processing and discharge of red cells are defined by the size of the bowl. C.A.T.S differs from traditional bowl systems in that all processing steps take place on a continuous basis as the individual cells flow through the washing chamber via a centrifuge.

Figure [1.16]: (a) C.A.T.S Fresenius® red cell washing device. (b) Sequential steps of a typical washing cycle.

A

B



There are three internal pumps that drive the continuous flow process. The blood-pump transports blood from the shed blood reservoir to the washing chamber, while the saline pump transports saline into the chamber. The packed red cell (PRC) pump delivers washed packed red cells from the chamber into the reinfusion bag. Due to the continuous process with three independent roller pumps, C.A.T.S facilitates high PRC and blood flow at considerably lower pump speeds compared to bowl systems, where only a single pump performs all three

tasks in succession. The C.A.T.S [1.16] system uses one set of disposables for all autotransfusion procedures. The disposable set contains a seal less, double spiral washing chamber with ports for incoming blood, waste, saline and packed red cells. The overall process is comprised of three successive stages. The blood pump drives collected blood from the reservoir into the rotating washing chamber, which is driven by a centrifuge. Blood cells introduced into the chamber separate at the edge of the spiral channel. As the chamber spins, blood cells flow by gravitational force from the inside to the outside edge of the chamber. During the first separation phase, blood cells flow along the spiral channel and the hematocrit concentrates to approximately 80%.

The red blood cells then move through a second washing phase. Here, red cells are resuspended with saline and the hematocrit drops to approximately 15%. The cells will continue along the outside edge of the chamber during a second separation phase, until they reach the outer most edge at the packed red cell (PRC) port where the red cells are reconcentrated to a hematocrit of approximately 65%. The minimum filling volume for the PRC port is 30 mL's; once this volume is attained, all excess red cells are transferred to the reinfusion bag.

1.3.4.4.2 Red cell Rejuvenation with Rejuvesol™

Red cell rejuvenation was first described by Gabrio et al (1955) and Nakao et al (1962) who showed that incubation with adenine and Inosine restored the ATP content of stored red cells and improved survival(Mollison and Robinson, 1959). The modified solution manufactured by Citra labs, USA as Rejuvesol™ has shown to provide effective reduction in in-vitro storage lesion in stored bloods by no. of investigators (table 1.8). It was our hypothesis to utilize this function and adopt it in our in vivo experiments on swine receiving allogenic 14-day stored blood to reduce transfusion related organ injury.

1.3.4.4.2.1 Rejuvesol™, a Red cell processing solution for rejuvenation

Constituents

Table [1.7]: Constituents of Rejuvesol. Derived from (Meyer et al., 2011).

Component	Concentration (mmol/L)
Sodium Pyruvate	**100**
Inosine	**99.9**
Adenine	**5**
Dibasic Na Phosphate	**70.4**
Monobasic Na Phosphate	**29.0**
pH	**6.7-7.4**

1.3.4.4.2.2 Elimination of storage lesion with Rejuvenation: Results from the in vitro studies

An up-to-date review of studies assessing effect of post storage rejuvenation on storage lesion is shown in table [1.8].

Table [1.8]: Review of in vitro studies analyzing effects of red cell rejuvenation on stored blood. OFI: Osmotic fragility index, MFI: Mechanical fragility index. RMP: Red cell derived Microparticles.

Study	Study question	Additive solutions	Rejuvenation at Day	Parameters assessed	Results
Barshtein et al 2014 (Barshtein et al., 2014)	To assess the effectiveness of Rejuvenation in repairing St-RBC mechanical properties	Non-LR, CPDA-1	35 day	Haemolysis, Osmotic fragility Index, RBC deformability, ROS, Annexin V-labeled RBC, Adhesion of RBC to EC, mechanical fragility Index	Rejuvenation effectiveness Index: OFI, % : 49.3± 26.8 MFI, % : 57.9 ±13.3 ROS, %: 55.3 ±16.1 Annexin V-labeled RBC, %a: 69.1 ± 13.4 Adhesion of RBC to EC, RBC/mm2 93.2± 6.1
Kurach et al 2014 (Kurach et al., 2014)	To evaluate the effect of cold rejuvenation treatment at multiple time points during storage on MP generation and changes in Phosphatidylserine (PS) and CD47 expression on RBCs and Red cell MPs	SAG-M	Day 28, 35, or 42	PS externalization, CD47 expression, or the rate of RMP formation	No significant differences between sham- and rejuvenation-treated RBC samples in the levels of PS externalization, CD47 expression, or the rate of RMP formation.
Tchir et al 2013	To determine the effect of a cold rejuvenation	SAG-M	Day 28, 35, or 42	ATP, Deformability, haemolysis, blood gas	On Day 28, rejuvenation was accompanied by a significant decrease in deformability 1 week

Study	Study question	Additive solutions	Rejuvenation at Day	Parameters assessed	Results
(Tchir et al., 2013)	treatment of RBCs and in particular to assess the connection between ATP levels, RBC deformability, and morphology during RBC storage			analysis.	after treatment ($p < 0.01$). Rejuvenation on Day 28, but not Day 35 or 42, was also associated with a significant change in morphology ($p < 0.01$). Of the in vitro quality variables measured, most changed during cold storage, but differences among treatment groups were not observed.
Gelderman et al 2011 (Gelderman and Vostal, 2011)	To assess the impact of rejuvenation on roller pump-induced physical stress (To model CPB) resistance of fresh and stored red blood cells	CP2D/AS3	At 42 day of human blood. This blood was then infused into SCID mice and samples obtained from mice analysed.	RBC recovery/survival, osmotic fragility, haemolysis, 2, 3-DPG	Roller pump induced haemolysis: Fresh: Non-Rej: 0.22, Rej:0.17, at day 42; Non-Rej: 0.30, Rej: 0.11

Chapter 2 Methods

Volume resuscitation and organ injury: Systematic evaluation of safety of current colloids and crystalloids

2.1 Objectives

The objective was to systematically review safety evidence from randomized control trials comparing colloids against crystalloids in sepsis, critical care, peri and post-op major surgery and trauma patients and analyzing risk of mortality, AKI, ARF, RRT, ICU, hospital length of stay.

Study Identification

Published RCTs comparing any non-blood fluid therapy (tetrastarch (balanced and unbalanced), pentastarch, hexastarch, hetastarch, dextrans, gelatins, hypertonic solutions, ringer's lactate and normal saline) in adult patients undergoing major surgery (vascular, cardiac, abdominal and orthopaedic surgery), patients who had sustained major trauma, or patients who had been admitted to critical care with sepsis or septic shock were identified.

For patients with trauma pre-hospital and post admission RCTs were included. For patients undergoing non-trauma surgery, RCT's reporting on perioperative (induction or maintenance) and postoperative use of colloid versus crystalloid solutions were included in the study. In patients undergoing cardiac surgery, RCT's reporting on use of fluid therapy as pump prime along with perioperative maintenance and postoperative resuscitation were also included.

The primary outcomes were risk of mortality, AKI and need for renal replacement therapy. The secondary outcomes were length of ICU and hospital stay.

Non-RCT studies, reviews, paediatric and transplant populations were excluded. Trials comparing albumin in single head to head analyses were also excluded as these have been recently reviewed (Patel et al., 2014).

Search Strategy

Search was undertaken using electronic databases (Medline, Embase, Cochrane central register of controlled trials and database of abstracts of reviews of effects (DARE) from inception to 2014 using pre-specified key words. A hand search of

referenced lists and bibliographical data of pertinent RCTs and systematic reviews for additional relevant articles was also undertaken.

Data collection

Data for demographics, methods, results and bias was collected by two independent reviewers. Any disagreement was resolved by discussion. Selected authors of publication were individually contacted for clarification of eligibility and analysis.

Assessment of methodological quality

Using grading of recommendations assessment, development and evaluation (GRADE) methodology, risk of bias was used to guide whether trials had limitations. Limitation' was defined as presence of poor design or its implementation, indirectness of evidence (indirect population, intervention, control, outcome), unexplained heterogeneity or inconsistency of results, imprecision of results or high probability of publication bias. GRADE Pro-software, Version 3.2 for Windows. Jan Brozek, Andrew Oxman, Holger Schünemann, 2008, was used to undertake GRADE quality assessment of outcomes.

Data extraction

Data was extracted by two reviewers using a specifically designed proforma. This incorporated assessment of trial design, demographics, and details of interventions, details of outcomes and author judgment of risk of bias. Additional data for type of analysis (per-protocol, intention to treat) and follow up was also recorded. Data was also obtained for the method of definition of AKI or acute renal failure such as risk, injury, failure, loss of kidney function, and end-stage kidney disease (RIFLE) or acute kidney injury network (AKIN). Standard errors of the means and interquartile ranges were converted to standard deviations using appropriate formulae.

Data analysis

Dichotomous outcomes (mortality, sepsis, cerebrovascular accident, myocardial infarction, need for renal replacement therapy and incidence of AKI or acute renal failure have been presented as odds ratios (OR) with 95 percent confidence intervals (c.i.) using fixed effects model. If significant heterogeneity was found, a random effect model using Mantel-Haenszel method was also used. Several trials report zero events for certain outcomes in both treatment and control groups. These trials have been included in current analyses as their exclusion could inflate the size of pooled treatment effects (Friedrich et al., 2007). Pooling of continuous outcomes (ICU stay, hospital stay) was done as weighted mean differences (MD). Heterogeneity was assessed with I^2 statistics, and I^2 value greater than or equal to 50 percent was considered to be substantial for this analysis. Quality of studies was assessed using GRADE methodology. Authors reporting per protocol analyses, interquartile range (i.q.r.) and missing data were directly contacted to seek clarification if possible. Meta-analysis was performed in line with Cochrane Collaboration and the quality of reporting of meta-analyses guidelines (Higgins JPT).

Publication bias was evaluated by inspection of funnel plots.

Subgroup and Sensitivity analyses

Sources for heterogeneity were explored by conducting subgroup analyses by type of colloid (Tetrastarch, Pentastarch, Dextrans and Gelatin) and clinical settings (trauma, sepsis, general and cardiac surgery). Sensitivity analyses were conducted by exclusion of low volume studies (fewer than 250 patients) and after exclusion of low quality studies (using GRADE assessment: studies with limitations). An additional sensitivity analysis, excluding studies by Joachim Boldt in the colloid vs. colloid comparison was also undertaken. Studies by this author were excluded (Boldt et al., 2003, Boldt et al., 2010) due to reporting of fabricated results and lack of institutional ethical approval (Wise, 2013). None of these studies were included in the colloid vs. crystalloid analyses.

All analyses were carried out using Review Manager (RevMan), Version 5.1. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011.

Chapter 3 Results

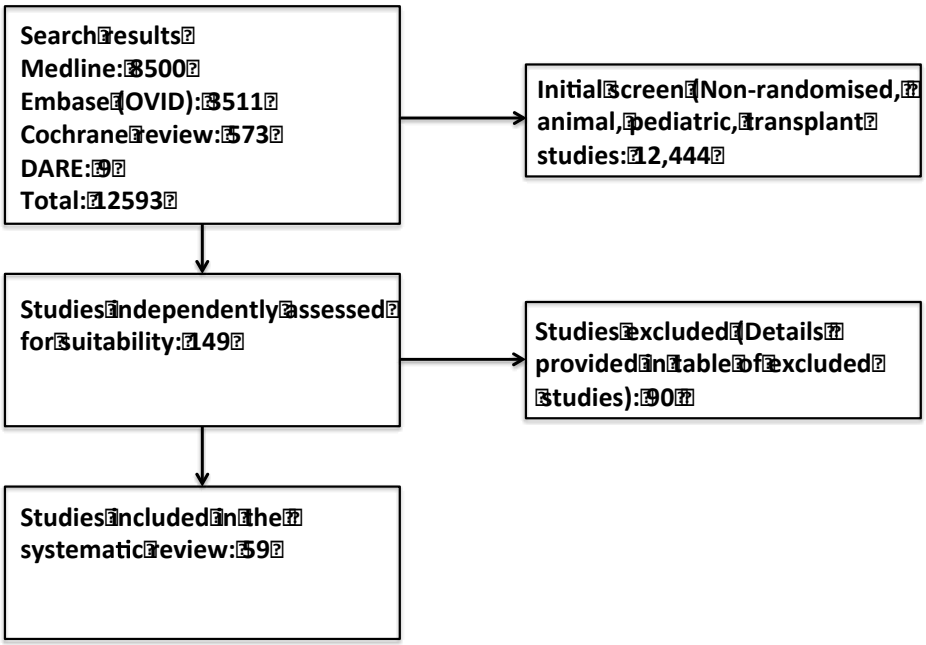
Volume resuscitation and organ injury: Systematic evaluation of safety of current colloids and crystalloids

3.1 Results

Characteristics of studies

A PRISMA flow diagram depicting the overall search strategy is provided in Figure [3.1]. A total of 59 RCTs comparing non-blood fluid therapies were included in the meta-analyses(Alavi et al., 2012, Allison et al., 1999, Alpar and Killampalli, 2004, Ando et al., 2008, Annane et al., 2013, Base et al., 2011, Beards et al., 1994, Boks et al., 2007, Boldt et al., 2003, Boldt et al., 2010, Brunkhorst et al., 2008, Bueno et al., 2004, Bulger et al., 2008, Bulger et al., 2011, Dubin et al., 2010, Fang et al., 2008, Gallandat Huet et al., 2000, Gan et al., 1999, Gombocz et al., 2007, Guidet et al., 2012, Gurbuz et al., 2013, Hamaji et al., 2013, Harten et al., 2008, Hung Ming-Hui 2012, Ickx et al., 2003, Issa et al., 2012, James et al., 2011, Kumle et al., 1999, Lee et al., 2011, Magder et al., 2010, Mahmood et al., 2007, Mattox et al., 1991, Mazhar et al., 1998, McIntyre et al., 2008, Morrison et al., 2011, Myburgh et al., 2012, Oliveira et al., 2002, Ooi et al., 2009, Parker et al., 2004, Perner et al., 2012, Rasmussen et al., 2014, Rizoli, 2003, Schortgen et al., 2001, Shahbazi SH, 2011, Sirvinskas et al., 2007, Soares et al., 2009, Tamayo et al., 2008, Tiryakioglu et al., 2008, Van der Linden et al., 2004, Van der Linden et al., 2005, Vassar et al., 1993, Veneman et al., 2004, Verheij et al., 2006, Waters et al., 2001, Wu et al., 2011, Yates et al., 2014, Younes et al., 1992, Younes and Birolini, 2002, Zarychanski et al., 2013). In the included studies, 21 (35 percent) studies were in cardiac surgery, followed by critical care or sepsis 13 (22 percent) studies, trauma 11 (18 percent), abdominal or general surgery six (10 percent), aortic surgery two (3 percent), orthopaedic surgery two (3 percent), urological surgery one (1 percent) heart failure one (1 percent), pancreatitis one (1 percent) study.

Figure: [3.1]: PRISMA diagram showing schematic of search



Of the studies reporting outcomes in cardiac surgery, all used cardiopulmonary bypass, except for two studies, which employed the off-pump technique (Lee et al., 2011, Soares et al., 2009). Of these studies, 7 (33 percent) studies evaluated the study fluid as pump prime, and the remainder 14 (67 percent) studies evaluated them as perioperative volume replacement solutions. In patients with sepsis 13 studies (22 percent), one trial analysed mechanically ventilated patients with respiratory failure (Beards et al., 1994), while another examined patients with hypalbuminaemia in ICU (Veneman et al., 2004). The remaining 11 trials considered patients with severe sepsis or septic shock.

Tetrastarch (130/0.42) was compared in 25 (42 percent) of the included studies, pentastarch (200/0.5, 250/0.45,70/0.45) in 11 (18 percent), hexastarch (200/0.62) in two (3 percent), hetastarch (450/0.7) in two (3 percent), hypertonic saline (7.5 percent)/dextran (HSD) in 13 (22 percent), dextran alone (Dextran-70) in one (1 percent), gelatin in 15 (25 percent), lactated ringers in 19 (32 percent) studies and normal saline in 22 (37 percent) studies. There was

considerable variation in the doses of pentastarch 28mls/kg to 70mls/kg and tetrastarch 27mls/kg to 50mls/kg administered.
Mortality was reported by 53 (89 percent) studies. Acute renal failure or AKI was reported in 21(35 percent) studies, need for renal replacement therapy in 13 (22 percent), myocardial infarction in nine (15 percent), sepsis in seven (11 percent), cerebrovascular accident in eight (13 percent) and length of stay in 24 (40 percent) studies. Of the RCTs reporting acute renal failure or AKI, six RCT's did not define the method of outcome assessment, four used isolated RIFLE method, six were isolated creatinine based, and remaining used combination of reduction in glomerular filtration rate (GFR), increase in serum creatinine and need for renal replacement therapy as qualifying criteria.

Risk of bias

Inter-rater agreement for both eligibility and quality of methodology was good (kappa; 0.74). Random sequence generation was reported by 31 (52 percent) RCT's, concealment of allocation by 32 (54 percent), blinding of participants by 31 (50 percent), blinding of outcome assessment by nine (15 percent), incomplete outcome reporting by 31 (52 percent) and selective reporting by six (10 percent) trials, Figure [3.2]. Six (10 percent) studies had other sources of bias that included, per-protocol analyses, violation of protocols and forged studies without institutional approval (Boldt et al, colloid vs. colloid analyses). Using the GRADE recommendations, figure [3.3] and table [3.1], 18 (30 percent) studies had no limitation, 16 (27 percent) had serious limitations and 25 (42 percent) had very severe limitations. Applying the recommendations, the evidence for the outcomes for the primary comparison (colloid vs. crystalloids) was graded to "moderate" evidence.

Figure [3.2]: Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies.

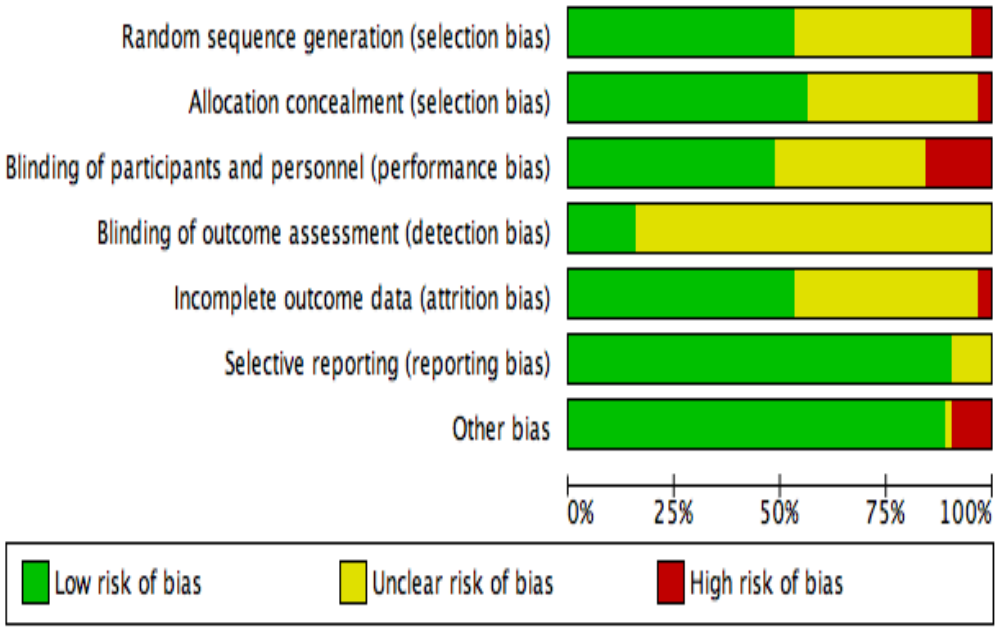


Figure [3.3]: GRADE assessment of studies with limitations

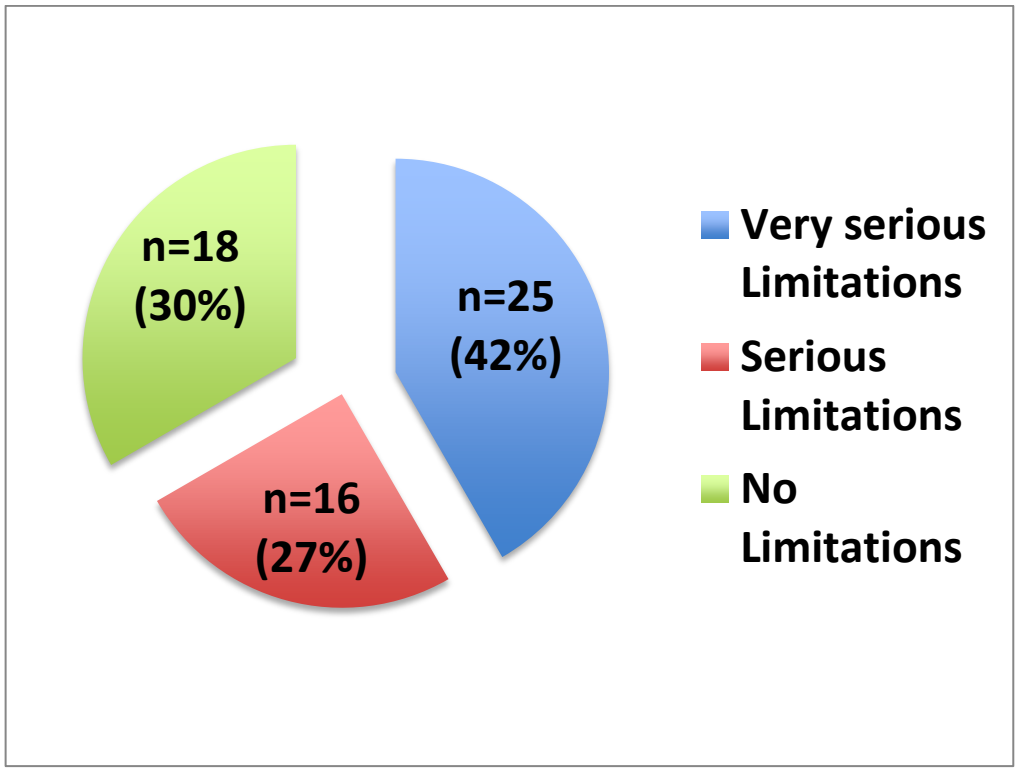


Table [3.1]: GRADE assessment of outcomes in primary comparison. PICO: Participants, Intervention, Comparison, Outcomes. NS: Not significant.

GRADE Tools Outcomes	**Studies**	**(A) Risk of Bias (Limitation)**	**(B)Indirect PICO Assessment**	**(C) Heterogeneity, p value**	**(D) Imprecision**	**(E) Publication Bias**	**GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.**
Mortality				40%, p=0.01			
1	Alavi	Very severe	No	--	Yes	No	Downgrade
2	Alpar	Severe	No	--	Yes	No	Downgrade
3	Annane	No	No	--	Yes	No	Downgrade
4	Brunkhors	No	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
	t						
5	Bueno	Very severe	No	--	Yes	No	Downgrade
6	Bulger	No	No	--	Yes	No	Downgrade
7	Dubin	Very severe	No	--	Yes	No	Downgrade
8	Guidet	No	No	--	Yes	No	Downgrade
9	Gurbuz	Very severe	No	--	Yes	No	Downgrade
10	Siegemun	No	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
	d						
11	Hamaji	Severe	No	--	Yes	No	Downgrade
12	Harten	Very severe	No	--	Yes	No	Downgrade
13	James	No	No	--	Yes	No	Downgrade
14	Magden	No	No	--	Yes	No	Downgrade
15	Mattox	No	No	--	Yes	No	Downgrade
16	McIntyre	Severe	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
17	Morrison	No	No	--	Yes	No	Downgrade
18	Myburgh	No	No	--	Yes	No	Downgrade
19	Oliveria	Severe	No	--	Yes	No	Downgrade
20	Parker	No	No	--	Yes	No	Downgrade
21	Perner	No	No	--	Yes	No	Downgrade
22	Rizoli	Very severe	No	--	Yes	No	Downgrade
23	Soares	Severe	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
24	Tamayo	Very severe	No	--	Yes	No	Downgrade
25	Vassar	Severe	No	--	Yes	No	Downgrade
26	Veneman	Severe	No	--	Yes	No	Downgrade
27	Verheij	Very severe	No	--	Yes	No	Downgrade
28	Yates	Very severe	No	--	Yes	No	Downgrade
29	Younes 02	No	No	--	Yes	No	Downgrade
30	Younes 92	No	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
MI				0%, p=0.66			
1	Bulger	No	No	--	Yes	No	Downgrade
2	Hamaji	Severe	No	--	Yes	No	Downgrade
3	Parker	No	No	--	Yes	No	Downgrade
4	Soares	Severe	No	--	Yes	No	Downgrade
5	Yates	Very severe	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
CVA				0%, p=0.44			Downgrade
1	Bulger	No	No	--	Yes	No	Downgrade
2	Gurbuz	Very severe	No	--	Yes	No	Downgrade
3	Magder	No	No	--	Yes	No	Downgrade
4	Mazhar	Very severe	No	--	Yes	No	Downgrade
5	Parker	No	No	--	Yes	No	Downgrade
6	Verheij	Very severe	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
7	Yates	Very severe	No	--	Yes	No	Downgrade
sepsis				0%, p=0.45			
1	Bulger 08	No	No	--	Yes	No	Downgrade
2	Bulger 11	Severe	No	--	Yes	No	Downgrade
3	Mattox	No	No	--	Yes	No	Downgrade
4	Mazhar	Very severe	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
5	Yates	Very severe	No	--	Yes	No	Downgrade
6	Younes	No	No	--	Yes	No	Downgrade
AKI				34%, p=0.10			
1	Brunkhorst	No	No	--	Yes	No	Downgrade
2	Bulger	No	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
3	Guidet	No	No	--	Yes	No	Downgrade
4	Gurbuz	Very severe	No	--	Yes	No	Downgrade
5	Hamaji	Severe	No	--	Yes	No	Downgrade
6	James	No	No	--	Yes	No	Downgrade
7	Magder	No	No	--	Yes	No	Downgrade
8	Mattox	No	No	--	Yes	No	Downgrade
9	Myburgh	No	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
10	Perner	No	No	--	Yes	No	Downgrade
11	Soares	Severe	No	--	Yes	No	Downgrade
12	Yates	Very severe	No	--	Yes	No	Downgrade
13	Younes	No	No	--	Yes	No	Downgrade
RRT				2%, p=0.42			
1	Annane	No	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
2	Brunkhorst	No	No	--	Yes	No	Downgrade
3	Siegemend	No	No	--	Yes	No	Downgrade
4	James	No	No	--	Yes	No	Downgrade
5	Magder	No	No	--	Yes	No	Downgrade
6	McIntyre	Severe	No	--	Yes	No	Downgrade
7	Myburgh	No	No	--	Yes	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
8	Perner	No	No	--	Yes	No	Downgrade
ICU stay				83%, p<0.00001			
1	Ando	very severe	No	--	No	No	Downgrade
2	Annane	No	No	--	No	No	Downgrade
3	Bulger	No	No	--	No	No	Downgrade
4	Guidet	No	No	--	No	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
5	Gurbuz	very severe	No	--	No	No	Downgrade
6	Harten	very severe	No	--	No	No	Downgrade
7	Magder	No	No	--	No	No	Downgrade
8	Myburgh	No	No	--	No	No	Downgrade
9	Rizoli	very severe	No	--	No	No	Downgrade
10	Shabazi	Severe	No	--	No	No	Downgrade
11	Soares	Severe	No	--	No	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
12	Tamayo	very severe	No	--	No	No	Downgrade
13	Tiryakioglu	very severe	No	--	No	No	Downgrade
Hospital stay				31%, p=0.13			
1	Ando	Very severe	No	--	No	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
2	Annane	No	No	--	No	No	Downgrade
3	Guidet	No	No	--	No	No	Downgrade
4	Gurbuz	very severe	No	--	No	No	Downgrade
5	Harten	Very severe	No	--	No	No	Downgrade
6	Magder	No	No	--	No	No	Downgrade
7	Morrison	No	No	--	No	No	Downgrade
8	Myburgh	No	No	--	No	No	Downgrade

GRADE Tools Outcomes	Studies	(A) Risk of Bias (Limitation)	(B)Indirect PICO Assessment	(C) Heterogeneity, p value	(D) Imprecision	(E) Publication Bias	GRADE recommendation in light of assessments (A-E) (Presence of single limitation leads to downgrading of level of evidence.
9	Rasmussen	Severe	No	--	No	No	Downgrade
10	Rizoli	Very severe	No	--	No	No	Downgrade
11	Shabazi	Severe	No	--	No	No	Downgrade
12	Soares	Severe	No	--	No	No	Downgrade
13	Tiryakioglu	Very severe	No	--	No	No	Downgrade

Effects of Interventions

Comparison: Colloid vs. Crystalloids

Mortality

Thirty trials with 16647 patients, comparing four colloids (tetrastarch, pentastarch, dextran, and gelatin) were included in this analysis (Figure 3.4). Sixteen of these trials were judged as GRADE 'limited', which was based on features such as serious risk of bias, lack of imprecision and significant heterogeneity, table [3.1]. There was no evidence that colloids increased mortality compared to crystalloids, OR 0.99 (95 percent c.i. 0.92 to 1.06) although there was evidence of moderate heterogeneity (I^2=40 percent). Publication bias was not observed on inspection of funnel plots (Figure [3.5]). Exclusion of low-volume studies) or low-quality studies (as judged to have GRADE limitations) did not significantly change the effect estimate (table 3.2 and table 3.3).

Figure [3.4]: Forrest plot comparing colloid vs. crystalloids for mortality

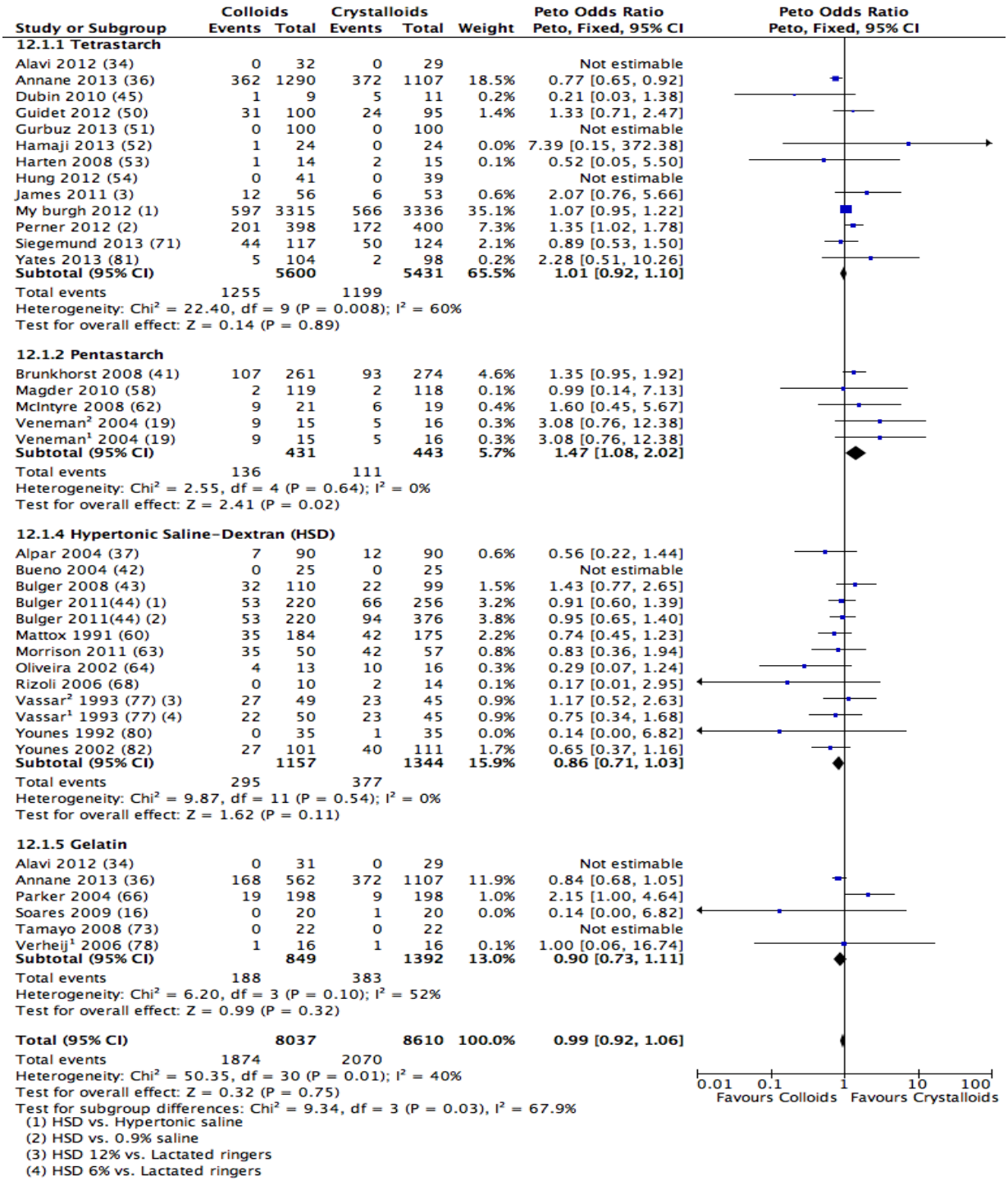
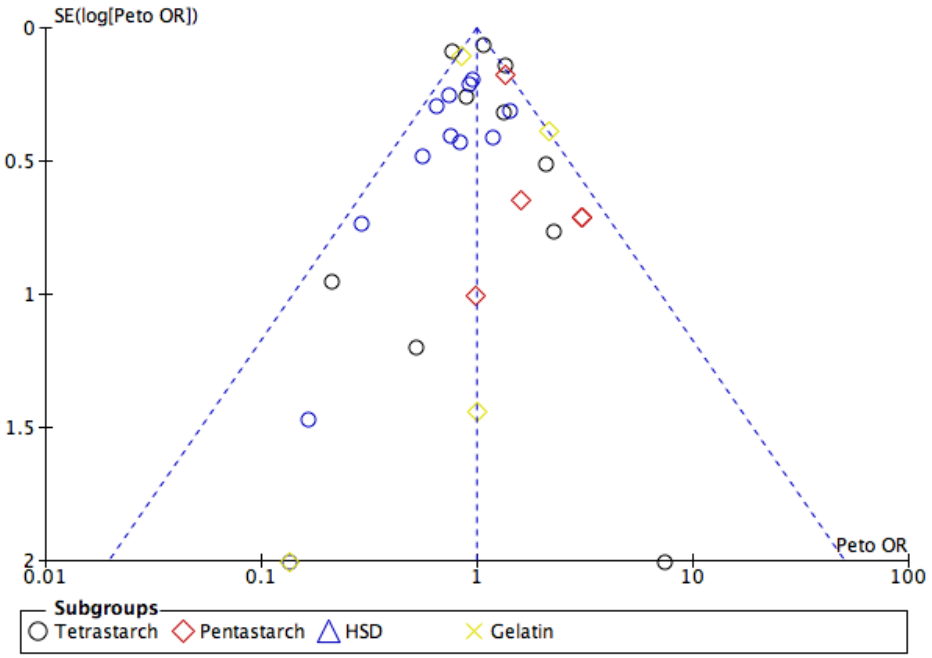


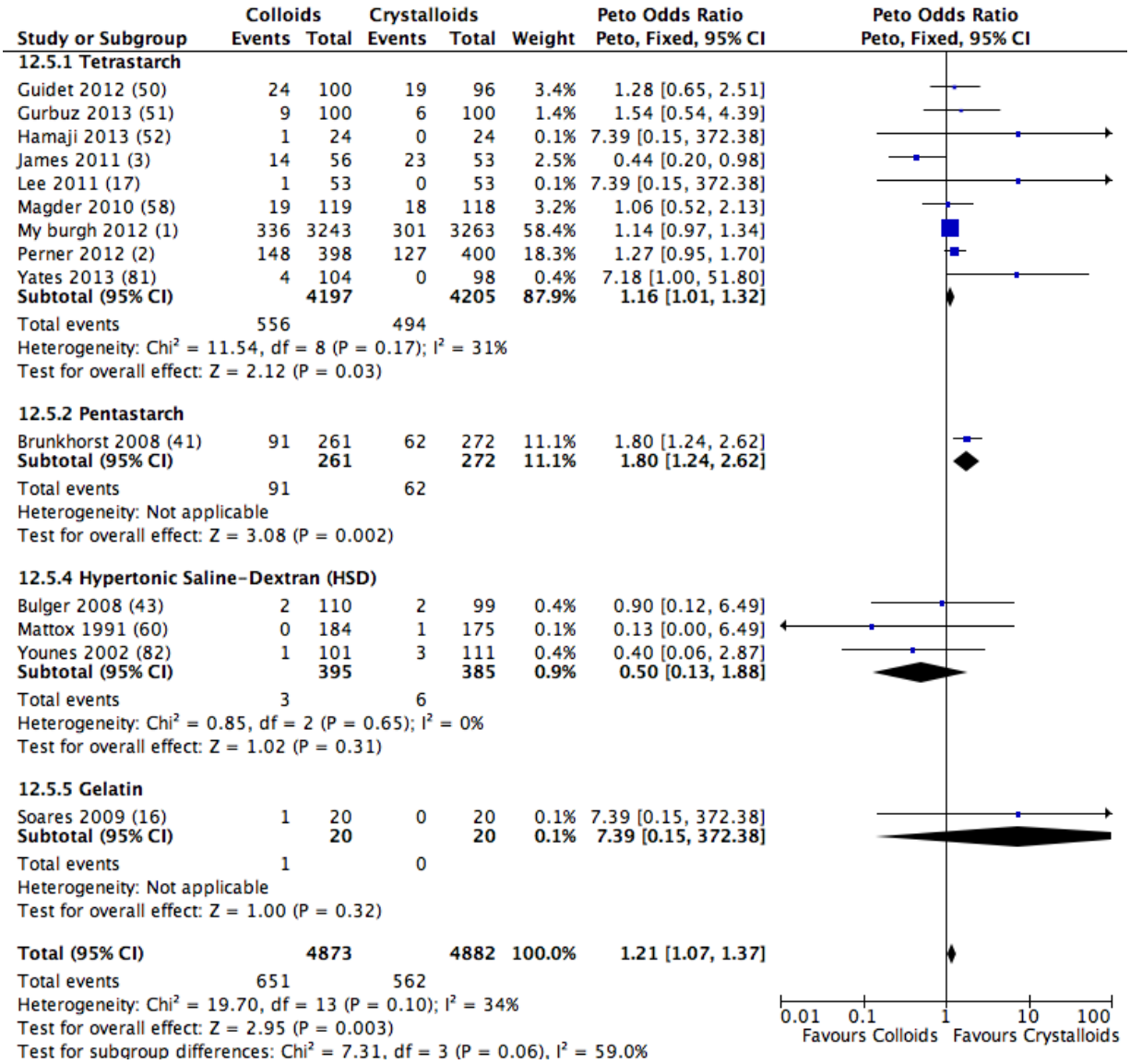
Figure [3.5]: Funnel plot of primary comparison, outcome: Mortality



Acute renal failure and acute kidney injury

Based on the results of 13 RCTs enrolling 9755 patients, colloid administration increased the risk of developing AKI or acute renal failure, OR 1.21 (1.07 to 1.37) (Figure [3.6]). No significant heterogeneity or publication bias was present. Exclusion of low volume or low quality studies did not change the effect estimate and therefore colloids increased the odds of AKI in high-volume and high-quality RCTs (Table 3.2 and table 3.3).

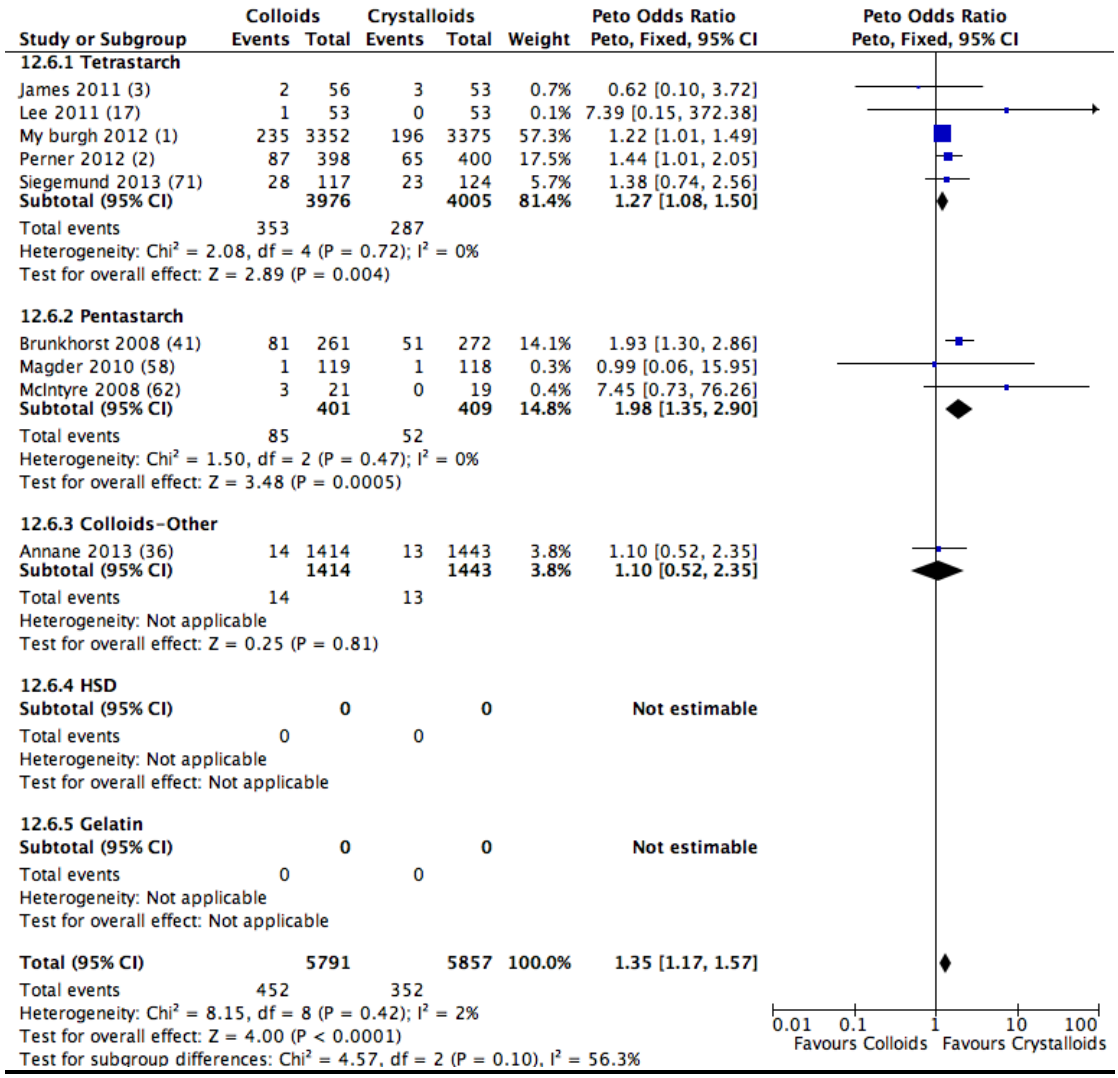
Figure [3.6]: Forrest plot comparing colloid vs. crystalloids for the risk of: acute kidney injury or acute renal failure



Renal replacement therapy

Based on the results of eight RCTs with 11648 patients, colloid administration increased the risk for renal replacement therapy, OR 1.35 (1.17 to1.57) (figure [3.7]). Heterogeneity was not significant, and no publication bias was found. The adverse effect of colloids on RRT remained valid in the sensitivity analyses (Table 3.2 and table 3.3).

Figure [3.7]: Forrest plot comparing colloid vs. crystalloids for the risk of Renal replacement therapy



Intensive care unit and Hospital Stay

Based on the results of 13 RCTs enrolling 10915 patients colloid administration increased the mean ICU stay, MD 0.40, (0.39 to 0.41) days (figure [3.8]) and hospital length of stay MD 0.20 (0.18 to 0.21 days) days (Figure [3.9]). There was significant heterogeneity between studies reporting mean ICU stay (I^2=83 percent). There was however no significant heterogeneity for hospital stay (I^2=31 percent) and no publication bias was found for either outcome. Restricting the analyses to high volume or high quality studies, colloids increased

ICU and hospital length of stay; however heterogeneity for ICU stay remained significant (tables 3.2 and 3.3).

Figure [3.8]: Forrest plot comparing colloid vs. crystalloids for length of ICU stay

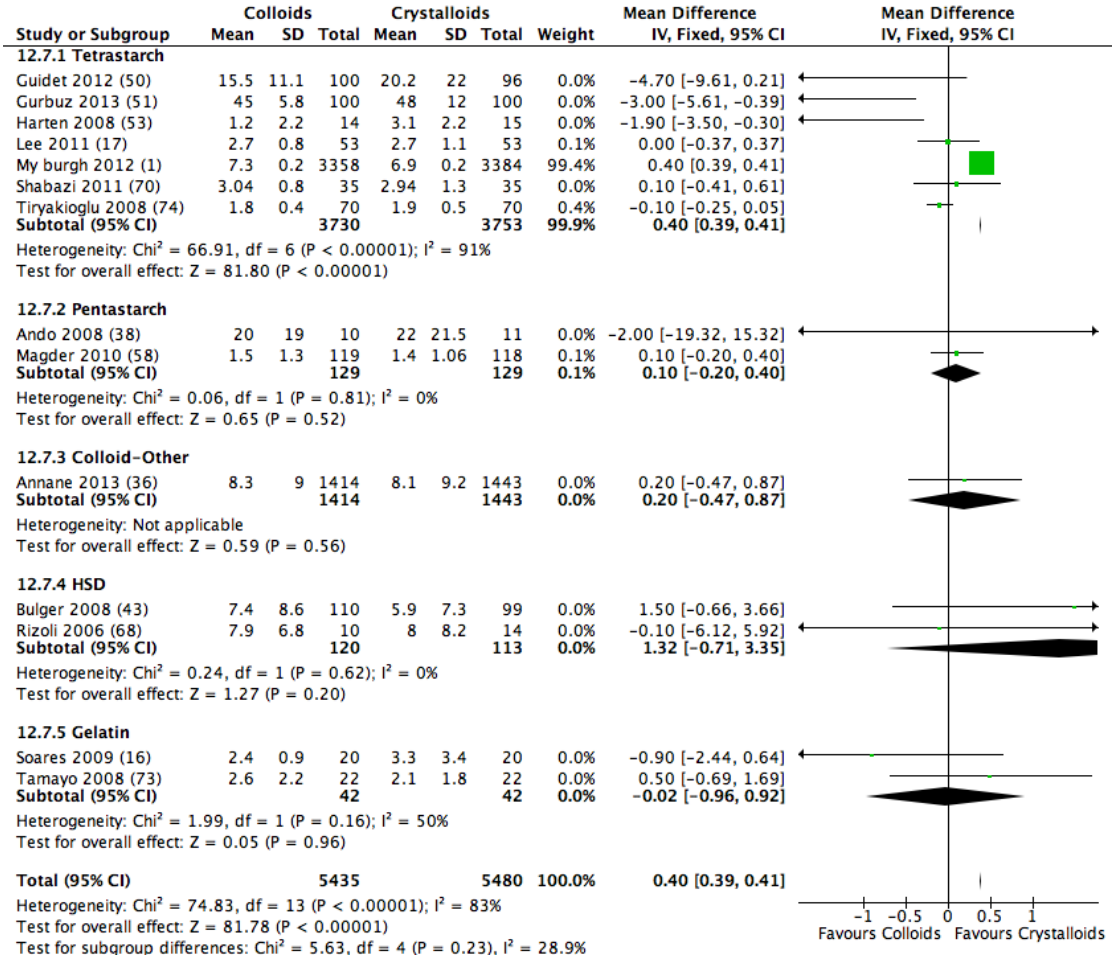
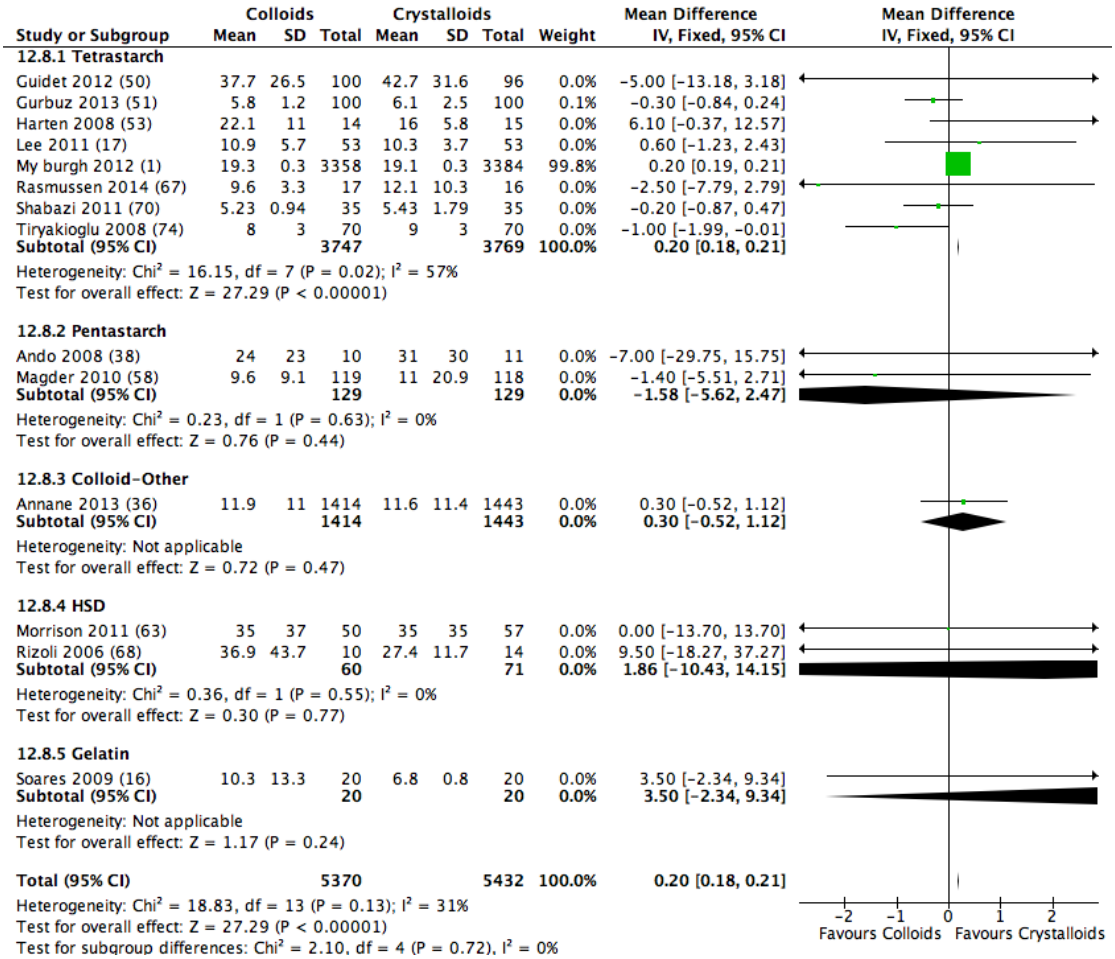


Figure [3.9]: Forrest plot comparing colloid vs. crystalloids for length of Hospital stay



Subgroup Analyses

To determine the safety and efficacy of specific colloids, analyses were stratified according to the type of colloid administered (Table 3.2). Both Tetrastarch and Pentastarch increased the incidence of adverse outcomes compared to crystalloid administration. Tetrastarch increased the odds of developing AKI (nine RCTs), OR 1.16 (1.01 to 1.32, I^2=31 percent) and the need for renal replacement therapy (five RCTs), OR 1.27 (1.08 to 1.50, I^2=0 percent). Pentastarch increased mortality (four RCTs), OR 1.47 (1.08 to 2.02, I^2=0 percent) and the need for renal replacement therapy (three RCTs), OR 1.98 (1.35 – 2.90, I^2=0 percent). Exclusion of pentastarch studies from primary analysis with or without sensitivity analyses had no resultant effect on mortality. Dextran or Gelatin administration did not increase the incidence of adverse outcomes compared to crystalloid administration.

To determine patient subgroups that may benefit from colloids or crystalloids, subgroup analyses were performed according to clinical setting (Table 3.3).
In patients undergoing cardiac surgery there was no evidence that colloids increased the risk of any adverse outcome compared to crystalloid administration. In patients undergoing general surgical operations there was no evidence that colloids increased mortality, OR 2.61 (0.59 to 11.49, I^2=0 percent), compared to crystalloid administration. However these results were derived from three suitable studies comparing HES 130/0.4 vs. control. Further comparisons in this clinical setting were not feasible due to lack of standardized subgroups, standardised outcome reporting or comparison of interest.
Amongst critically ill or septic patients, colloid administration had a borderline effect on mortality (10 RCTs): OR 1.10 (1.00 to 1.20, I^2=42 percent) but a clear impact on AKI (five RCTs), OR 1.24 (1.09 to 1.41, I^2=21 percent) and the need for renal replacement therapy (five RCTs), OR 1.37 (1.18 to 1.59, I^2=37 percent).
Amongst patients with trauma, colloids were found to reduce the risk of developing AKI (four RCTs), OR 0.46 (0.23 to 0.90, I^2=0 percent).

Table [3.2]: Subgroup analyses by choice of colloid treatment.

Outcomes															
Subgroup	**Mortality**			**Acute Kidney Injury**			**Renal Replacement Therapy**			**ICU Stay**			**Hospital Stay**		
	n/N(I:C)	Fixed Effect (Peto) Estimate	Random Effect (Mantel-Haenszel) Estimate	n/N(I:C)	Fixed Effect (Peto) Estimate	Random Effect (Mantel-Haenszel) Estimate	n/N(I:C)	Fixed Effect (Peto) Estimate	Random Effect (Mantel-Haenszel) Estimate	N(I:C)	Fixed Effect (Inverse Variance) Estimate	Random Effect (Inverse Variance) Estimate	N(I:C)	Fixed Effect (Inverse Variance) Estimate	Random Effect (Inverse Variance) Estimate
Tetrastarch	1255/5600,1199/5431	1.01(0.92,1.10),p=0.89-I^2:60%,0.008	1.06(0.48,1.33)p=0.62,I^2:58% 0.01	556/4197,494/4205	1.16(1.01,1.32)p=0.03	1.15(0.96,1.38)p=0.13	353/3976,287/4005	1.27(1.08,1.50)0.004	1.27(1.08,1.50)p=0.004	3730:3753	0.40(0.39,0.41)p<0.00001,I^2:91%,<0.00001	-0.08(-0.46,0.31)p=0.69,I^2:91%,<0.00001	3747:3769	0.20(0.18,0.21)p<0.00001,I^2:57%,0.02	-0.15(-0.63,0.32)p=0.53,I^2:57%,0.02
Pentastarch	136/431,111/443	1.47(1.08,2.02)p=0.02	1.47(1.07,2.03)p=0.02	91/261,62/272	1.80(1.24,2.62)p=0.002*	1.81(1.24,2.65)p=0.002*	85/401,52/409	1.98(1.35,2.90)0.0005	1.97(1.33,2.92)p=0.0008	129:129	0.10(-0.20,0.40)p=0.52	0.10(-0.20,0.40)p=0.52	129:129	-1.58(-5.62,2.47)p=0.44	-1.58(-5.62,2.47)p=0.44
Dextrans	295/1157,377/1344	0.86(0.71,1.03)p=0.11	0.86(0.71,1.04)p=0.12	3/395,6/385	0.50(0.13,1.88)p=0.31	0.54(0.14,2.09)p=0.37	Not estimable	Not estimable	Not estimable	120:113	1.32(-0.71,3.35)p=0.20	1.32(-0.71,3.35)p=0.20	60:71	1.86(-10.43,14.15)p=0.77	1.86(-10.43,14.15)p=0.77
Gelatin	188/849,383/1392	0.90(0.73,1.11)p=0.32	1.12(0.56,2.24)p=0.74	1/20,0/20	7.39(0.15,372.38)p=0.32*	3.15(0.12,82.16)p=0.49*	Not estimable	Not estimable	Not estimable	42:42	-0.02(-0.96,0.92)p=0.96	-0.11(-1.47,1.25)p=0.87	20:20	3.50(-2.34,9.34)p=0.24*	3.50(-2.34,9.34)p=0.24*

I: Intervention group, C: Control group, *: Effect estimate derived from single study. Dichotomous outcome (Mortality, AKI, RRT) effect estimates described as: Odds ratio (95% Confidence Interval) Significance value, Continuous outcome (ICU and Hospital stay) estimates described as Mean difference (95% Confidence Interval) Significance value. I^2: Heterogeneity.

Table [3.3]: Subgroup analyses by clinical setting

Subgroup	Outcomes														
	Mortality			Acute Kidney Injury			Renal Replacement Therapy			ICU Stay			Hospital Stay		
	n/N(I:C)	Fixed Effect (Peto) Estimate	Random Effect (Mantel-Haenszel) Estimate	n/N(I:C)	Fixed Effect (Peto) Estimate	Random Effect (Mantel-Haenszel) Estimate	n/N(I:C)	Fixed Effect (Peto) Estimate	Random Effect (Mantel-Haenszel) Estimate	N(I:C)	Fixed Effect (Inverse Variance) Estimate	Random Effect (Inverse Variance) Estimate	N(I:C)	Fixed Effect (Inverse Variance) Estimate	Random Effect (Inverse Variance) Estimate
Cardiac Surgery	3/365,4/330	0.74(0.17,3.32)p=0.70	0.79(0.18,3.39)p=0.75	30/292,24/291	1.28(0.72,2.26)p=0.40	1.26(0.71,2.22)p=0.43	2/172,1/171	1.94(0.20,18.71)p=0.57	1.60(0.20,13.19)p=0.66	419:418	-0.04(-0.16,0.08)p=0.54	0.01(-0.19,0.21)p=0.91	397:396	0.14(-0.23,0.52)p=0.46,I^2:57%,0.04	0.01(-0.74,0.76)p=0.98,I^2:57%,0.04
Sepsis/Critical Care	1227/5038,1162/5086	1.10(1.00,1.20)p=0.06	1.13(0.95,1.35)p=0.16	627/4119,532/4155	1.24(1.09,1.41)p=0.0009	1.28(1.09,1.51)p=0.003	434/4149,335/4190	1.37(1.18,1.59)<0.0001	1.43(1.15,1.77)p=0.001	3458:3480	0.40(0.39,0.41)p<0.0001,I^2:76%,<0.04	-1.53(-6.39,3.32)p=0.54,I^2:76%,0.04	3458:3480	0.20(0.19,0.21)p<0.0001	-0.72(-4.62,3.17)p=0.72
Trauma	316/1260,385/1448	0.91(0.76,1.09)p=0.31	0.91(0.76,1.10)p=0.32	17/451,29/438	0.46(0.23,0.90)p=0.02	0.46(0.23,0.92)p=0.03	2/56,3/53	0.62(0.10,3.72)p=0.60*	0.62(0.10,3.85)p=0.61*	120:113	1.32(-0.71,3.35)p=0.20	1.32(-0.71,3.35)p=0.20	60:71	1.86(-10.43,14.15)p=0.77	1.86(-10.43,14.15)p=0.77
I: Intervention group, C: Control group, *: Effect estimate derived from single study. Dichotomous outcome (Mortality, AKI, RRT) effect estimates described as: Odds ratio (95% Confidence Interval) Significance value, Continuous outcome (ICU and Hospital stay) estimates described as Mean difference (95% Confidence Interval) Significance value. I^2: Heterogeneity.															

Chapter 4 Experimental Methods

4.1 Animal procedures

Evaluation of novel therapies i.e. AQIX RS-1® fluid, Cardiopulmonary bypass (CPB), Sildenafil citrate-CPB and red cell rejuvenation with Rejuvesol®-washing) was undertaken using healthy swine of adult White Landrace species weighing 50-70 kg. The exceptions were donor animals for allogenic blood transfusion in Chapter 7, which were 80-90kg in weight.

The unifying characteristics shared in these animal procedures were that they followed a predesigned experimental design (Figure 1.6), where A- anaesthesia using a standard Halothane based regimen followed by intubation. This was followed by B-a treatment allocation period in which animals were randomised to controls or test treatment. After withdrawal of treatment the animals where C-observed and analysed for 4 hours. All animals were then recovered for the following 12 hours. The next day animals were D-re-anaesthetised and reevaluated for 90 minutes followed by organ harvesting and euthanasia. During all key stages (A-D, Figure [1.6]) animals where monitored invasively for measures of oxygenation, ventilation, cardiovascular and respiratory function. In additional measures of NO bioavailability, endothelial function, inflammation and platelet function were carried out.

In the next few pages we describe the primary porcine model followed by special consideration applicable to the model vis a vis to individual chapters.

4.1.1 Ethical considerations

Animals received care in accordance with, and under license of, the Animals (Scientific Procedures) Act 1986 and conformed to the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). All studies had received local Institutional Review Board approval. These studies adhered to the principals of 3Rs (Replacement, Refinement, Reduction).

4.1.2 Anesthesia and Monitoring

Animals were induced using Ketamine 100mg/ml and maintained using Halothane 1.5-2.0% and nitrous oxide 50% in oxygen. Venous access was gained through a neck incision and insertion of multicath VYGON® central line in the internal jugular vein. This was used to setup a CVP trace and infusion of bolus of Cefurexime 750mg, Heparin and infuse trial fluids. Arterial blood pressure was monitored via a 20G VYGON® catheter placed in the internal carotid artery. Urine output was measured via a urethral silastic 14Fr catheter. Arterial blood gas analysis to measure adequacy of ventilation and tissue perfusion was conducted every 30 min during the experiment. All data was recorded on a data sheet that was then entered in Microsoft Access database, (Microsoft Inc.) version 2010.

4.1.3 Recovery

After intervention, animals were maintained under general anasthetic for a further observation period after which they were weaned from the anasthetic machine. Animals were given intermittent analgesia and allowed to ambulate. This is a unique attribute to this model as it allows collapsed alveoli to re-expand and negate the confounding of dependent atelectasis that develops in anesthetized large animal models in research (Hedenstierna et al., 1989). At 24 hours, pigs were re-anaesthetized and underwent measurement of cardiac, pulmonary and renal indices followed by harvest of tissues and sacrifice. In a given intervention group 50% (n=4) of subjects underwent organ harvest and 50% underwent renal blood flow measurements in response to acetylcholine.

4.1.4 Organ Harvesting

Organs (Kidney and lung) were harvested post-recovery using a midline laparotomy approach and sternotomy with continued infusion of Hartmann's solution for resuscitation at 500mls/30 min.

4.1.5 Generic Experimental treatment and analyses schedule

These are described below in table [4.1]

Table [4.1]: Generic experimental treatment and analyses schedule

		Induction	Baseline		Intervention		Post intervention		24hr recovery	
			90 mins		150 mins		90 mins		90 mins	
Drugs	Ketamine 5mg/kg	X							X	
	Halothane	X	X	X	X	X	X	X	X	X
	Buprenorphine 0.05mg/kg	X			X					
	Bupivacaine						X			
	Cefuroxime (750mg)		X							
	Heparin (5000 U)		X						X	
Intervention		X	X		X	X	X		X	
	Heparin (20,000 U)				X					
	Protamine (200mg)							X		
Fluids (Hartmann's solution)	Total 4500mls over entire experiment	500 mls	500mls		2000mls		1000mls		500mls	
Blood Collection (Hematology/Biochemistry /Inflammation)			X			X		X		X
Arterial blood Gases	Every 30 mins		X	X	X	X	X	X	X	X
Multiplate platelet testing			X					X	X	
Urine Collection (for				X		X		X		X

volume, nitrate and nitrite, NGAL)										
Organ harvesting and Bronchoalveolar lavage									X	
Ketamine used as an induction agent while Halothane as general anasthetic with maintenance concentration of 2-3%. Cefurexime used as antimicrobial for surgical prophylaxis. Heparin used as a anticoagulant and revered using Protamine Sulphate. Buprenorphine is used as an intramuscular analgesic, Bupivacain used as a local anasthetic instilled around surgical wounds. Interventions: AQIX RS-1 (Chapter 5), Cardiopulmonary bypass(CPB) and CPB-Sildenafil (Chapter 6), Rejuvesol treated red cell Transfusion (Chapter 7).										

4.1.5.1 Chapter 6

Sildenafil Dosing

Sildenafil (SDF) was infused as 10 mg in 50 mL 0.9% saline infusion over 30 minutes at the commencement of CPB. This dose was identified in a preliminary study (n _ 3 pigs) as having minimal hypotensive side effects. The timing of SDF administration was chosen to reflect the likely clinical scenario were the agent to be administered in a future clinical trial.

Cardiopulmonary bypass conduct

CPB venous drainage was established via a 24Fr Smart Cannula® (Smartcanula LLC, Lausanne, Switzerland) placed in the right internal jugular vein and advanced to the right atrium. Arterial return was achieved via a 14Fr Smart Cannula® placed in the right internal carotid artery and advanced to the brachiocephalic trunk. All animals received heparin 300IU per kg. The CPB circuit was primed with 1500mls of Hartmans solution plus heparin (5000 i.u.). Normothermic (38-39°C in pigs), non-pulsatile CPB will be commenced using a Stöckert Multiflow Roller Pump (Sorin Group GmbH, Munich, Germany) to achieve a target flow rate of 80-90 ml/kg/min of blood through the hollow fibre–membrane oxygenator apparatus (Dideco D708 Compact-Flo, Sorin Biomedica, Via Crescentio, Italy). Mean arterial blood pressure (MABP) was maintained between 65 and 75 mm Hg with small incremental doses of the alpha-adrenergic agonist metaraminol, percentage inspired oxygen (FiO2) at 50% and, partial

pressure of carbon dioxide (PaCO2) between 35-45mmHG. Central venous pressure (8-12mm Hg), hydration and sodium load (500ml/h of AQIX RS-I®/Hartmann's) was be standardized. Total CPB time was 2.5 hours. This represents a prolonged CPB duration; a risk factor for post cardiac surgery AKI identified in clinical studies (Li et al., 2011) and has previously been shown to result in significant kidney injury in the porcine model (Patel et al., 2011a).

4.1.5.2 Chapter 7

Donor animals

These animals, 4 per group (A-E), where used for allogenic blood collection under general anaesthesia. The internal jugular vessels were exposed, directly cannulation passive blood collection carried out in sterile single-use, closed system processing blood bags (Pall Corporation, Cat no: WBT436CEU).

Blood collection-Rejuvenation-Washing and storage

These procedures were carried out as per NHS Blood and Transplant (NHSBT) protocol and manufacturer instructions respectively and are detailed below.

1-Porcine blood Collection

Adult female Large-White-Landrace crossbred pigs (80-100kg) were used to obtain blood. Neck vessels were exposed under sterile cover. This was followed by cannulation of Internal Jugular vein. Blood was collected in sterile single-use, closed system processing blood bags (Pall Corporation, Cat no: WBT436CEU Blood was leucodepletion, followed by centrifugation at 3600 G for 10 min to separate plasma from Red cells concentrate. Red cell concentrate was separated and Saline-Adenine-Glucose-Mannitol: SAGM) was added and mixed well. Packed red cells were stored at 4 degrees either for 1 day (Group-B) or for 14 days (Groups C-E).

2-Red cell Washing

Each bag of red cell concentrate was washed using 3-4 liters of isotonic saline (Baxter BV, Utrecht, the Netherlands), using C.A.T.S (Autotransfusion system, Fresenius AG, Bad Homburg, Germany). The device was setup and used according to the manufacturers recommended protocol. 4-5 units of red cells (Total: 1000mls) were transfused immediately following the washing procedure.

3-Red cell Rejuvenation-Washing

50mls of Rejuvesol Solution (Citra Labs. USA) was injected into red cell concentrate bag under sterile conditions.

Red cells were agitated until Rejuvesol was mixed and enclosed in heat sealed clear plastic bag. The bag was incubated in warm water bath at 37 degrees for 1 hour. Immediate post red cell rejuvenation and prior to transfusion, each bag of red cell concentrate was washed using 2 liters of Isotonic saline (Baxter BV, Utrecht, the Netherlands), using C.A.T.S (Autotransfusion system, Fresenius AG, Bad Homburg, Germany). 4-5 units of rejuvenated/washed red cells (Total: 1000mls) were immediately transfused.

4.2 Outcomes

4.2.1 Acute Kidney Injury

4.2.1.1.1 Creatinine clearance

Measurements for this key outcome were carried out at pre-specified time points; baseline, intervention, 1.5 hours and 24 hours post intervention. This was calculated as follows; urine was collected over 90 minutes at each time point. Serum for creatinine measurement was also collected at the beginning of each period. Creatinine clearance was determined by the standard formula: creatinine clearance (mL/minute) = [urine creatinine concentration (µmol/mL) × urine volume (mL/min)]/plasma creatinine concentration (µmol/mL).

4.2.1.1.2 Fractional Excretion of Sodium

FE Na is the percentage of Na+ filtered by the kidney, which is not reabsorbed and excreted in the urine. In case of decreased renal blood flow, as in hypovolemia, there is enhanced reabsorption of Na+ from renal tubules, making the fraction of the filtered Na+ that is excreted in urine less than normal(Westhuyzen et al., 2003). FE Na has been used to discriminate between pre-renal (FE Na <1%) and established AKI (FE Na >1%)(Miller et al., 1978, Espinel, 1976).

Measurement
FE Na= [(urinary sodium/plasma sodium)/(urinary creatinine/plasma creatinine)] X 100

4.2.1.1.3 Free Water Clearance

Electrolyte-free-water clearance is the amount of water present in the urine that is free of solutes, i.e., the amount of water excreted in the urine. Determination is helpful in the assessment of serum [Na] in hypernatremia and hyponatremia.

For example, hypernatremia may not improve despite volume replacement because the exact amount of free water that is reabsorbed or excreted is not known. In order to quantify how much electrolyte-free-water is being reabsorbed or excreted, the following formula can be used.

Free water= V(Ur Na + Ur K)/ (P Na) -1
where V is the total urine volume, and P Na is the plasma [Na^+].

Water clearance can be positive or negative. Positive value means that less water was reabsorbed in the nephron segments, resulting in hypernatremia. On the other hand, negative value indicates that the nephron segments reabsorbed more water with resultant hyponatremia.

4.2.1.1.4 Urine biomarkers of AKI

Neutrophil Gelatinase-associated Lipocalin (NGAL)

NGAL has been validated in several populations from cardiac surgery (ROC between 0.74 for the 3-h NGAL and 0.80 for 18-h post op) (Wagener et al., 2006) to post-transplantation AKI (AUC of 0.9)(Parikh et al., 2006) indicative of an excellent predictive biomarker.
In this study we measured urinary NGAL using ELISA (Bioporto Diagnostics A/S Denmark, Kit 044) assay on urinary samples obtained at each time point and analysed unadjusted and adjusted for urinary creatinine.

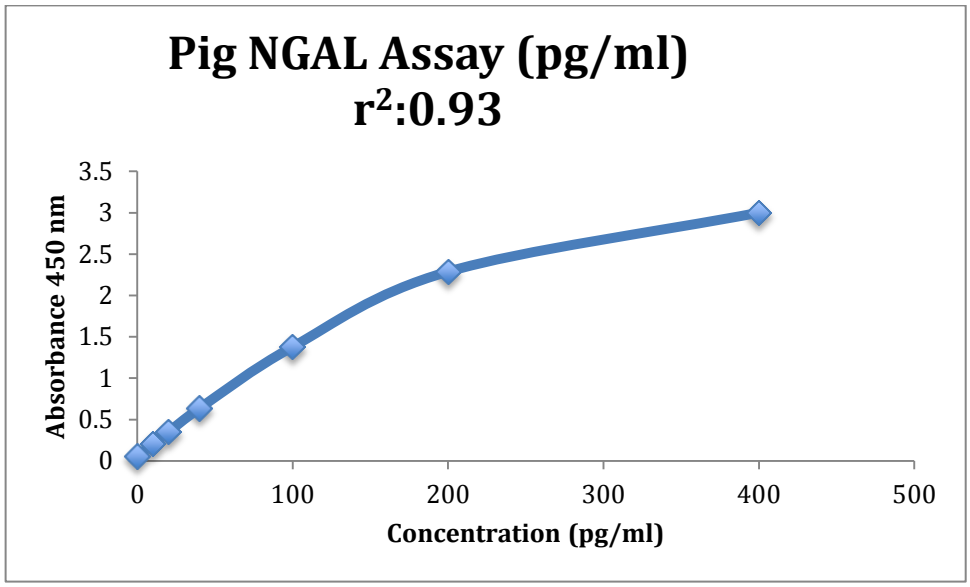
Method

ELISA assay was a four-step procedure:
Step 1. Aliquots of calibrators, diluted samples and any controls are incubated in microwells, which are pre-coated with monoclonal capture antibody. NGAL present in the solutions binds to bind to the coat. This is followed by washing to remove unbound material.
Step 2. Biotinylated monoclonal detection antibody is added to each test well and incubated. The detection antibody attaches to bound NGAL; unbound detection antibody is removed by washing.
Step 3. HRP-conjugated streptavidin is added to each test well and allowed to form a complex with the bound biotinylated antibody. Unbound conjugate is removed by washing.
Step 4. A color-forming peroxidase substrate containing tetramethylbenzidine (TMB) is added to each test well. The bound HRP-streptavidin reacts with the substrate to generate a blue color. The enzymatic reaction is stopped by adding dilute sulfuric acid (Stop Solution), which changes the color to yellow. The yellow color intensity is read at 450 nm in an ELISA reader. The color intensity (absorbance) is a function of the concentration of NGAL originally added to each well. The results for the calibrators are used to construct a calibration curve from which the concentrations of NGAL in the test samples are read (Figure [4.1]). Miss Pia Nielson and Ade Abedoye, lab scientists, University of Leicester, conducted these assays.

Validation of calibration curve

The mean absorbance value for the 400 pg/mL Pig NGAL Calibrator was taken as >1.5 as per manufacturer instructions. Minimum detection limit 4 pg/ml, within assay and between assay Coefficient of Variations (CVs) were <5 and <10% respectively.

Figure [4.1]: Standard curve NGAL assay. r^2: goodness-of-fit, linear regression.



4.2.1.1.5 In vivo renal endothelial function: Measurement of renal blood flow

At 24 hours post intervention renal blood flow was recorded using a T106 Transonic blood flow meter (Transonics Systems, Ithaca, NY) with a perivascular flowprobe (MA-5PSS, Precision perivascular flowprobe, Transonic Systems, Ithaca, NY) placed on the renal artery via a mini-laparotomy. Transonic flow meter works on the principals of Laser Doppler Flowmetry (LDF).

Laser Doppler Flowmetry for determination of RBC Flux

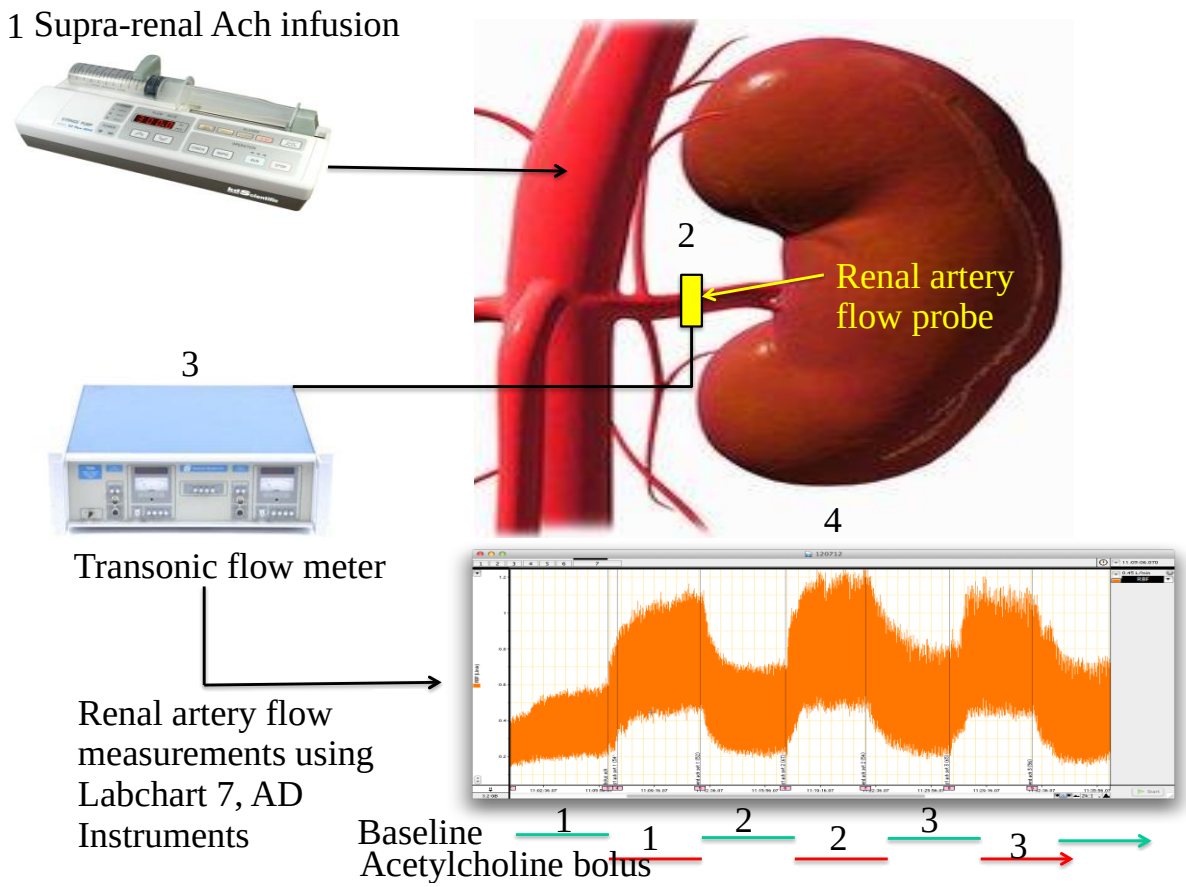
Laser Doppler flowmeters produce an output signal that is proportional to the blood cell perfusion (or flux). This represents the transport of blood cells through microvasculature and is defined as:

Microvascular Perfusion = number of blood cells × mean velocity

Microvascular perfusion, therefore, is the product of the mean cell velocity and mean blood cell concentration present in the small volume of tissue under illumination from the laser beam.

The Doppler frequency shift is detected by a photodiode detector. The frequency and magnitude of the alternating component of the photocurrent from this device is related to the mean velocity and concentration of blood cells present in the measuring volume. The signal from the photodiode is converted to digital information and then processed using a DSP (Digital Signal Processor). This device performs the spectral analysis and produces an LDF signal. This setup is shown in figure [4.2].

Figure [4.2]. Assessment of macrovascular endothelial function. (1) Infusion of supra-renal acetylcholine infusion using standard syringe driver, delivering Ach at 0.1-10µg/kg/min. (2) Renal blood flow was measured by a 5mm transonic perivascular flowprobe (MA-5PSS, Precision perivascular flowprobe, Transonic Systems, Ithaca, NY) around the left renal artery. (3) Data from Transonic-flowmeter displayed and analysed using Labchart 7, AD instruments; New Zealand.



4.2.1.1.6 Nitric Oxide bioavailability

Nitric oxide bioavailability was measured in using modified calorimetric Griess method. Miss Pia Nielson and Ade Abedoye, lab scientists, University of Leicester, conducted these assays.

Griess methodology (Assay)

Briefly 10ml urine aliquots were collected at the end of each time period, centrifuged at 3000rpm for 5 minutes to remove cell debris and frozen at -80°C until analysis in batches. Nitrate in the urine is converted to nitrite by nitrate reductase. The nitrite is then reacted with sulphanilamide and N-(1-naphthyl) ethylenediamine and detected chromogenically by measuring optical density at 540nm using spectrophotometer. Standard curves (Figures [4.3 and 4.4]) were calibrated using known sodium nitrate at concentrations from 0 to 25 μ M and the concentration of NO calculated by linear regression. Analyses of unknown samples were performed in duplicate. The assay was highly sensitive with a lower-limit cut-off of 30 nM of nitrite in the final reaction mixture. Intra-assay and interassay coefficient variations were less than 3% and 4% on average, respectively.

Figure [4.3]: Standard curve Nitrate assay. r^2: goodness-of-fit, linear regression.

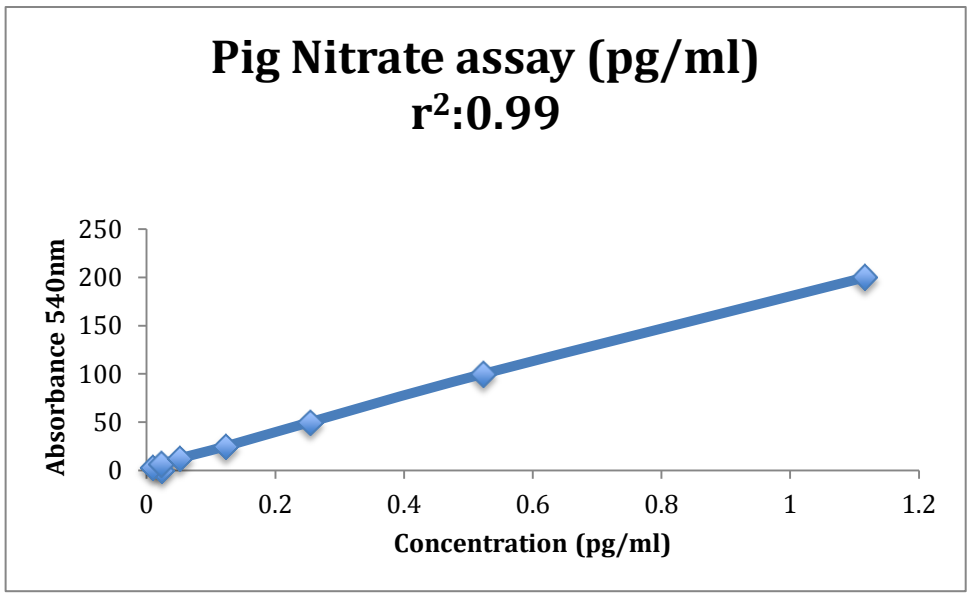
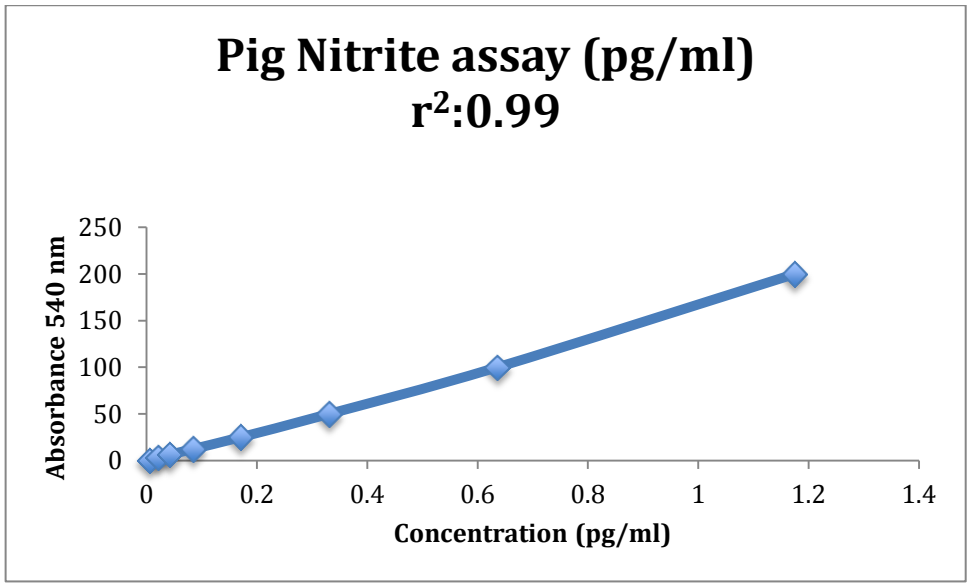


Figure [4.4]: Standard curve Nitrite assay. r^2: goodness-of-fit, linear regression.



4.2.1.1.7 Biochemical markers of Inflammation

4.2.1.1.7.1 Inflammatory cytokines

Inflammation was assessed by measuring inflammatory cytokine IL-6, IL-8 and TNF-α in serum at each time point. Miss Pia Nielson, Biotechnician at University of Leicester undertook all lab assays.

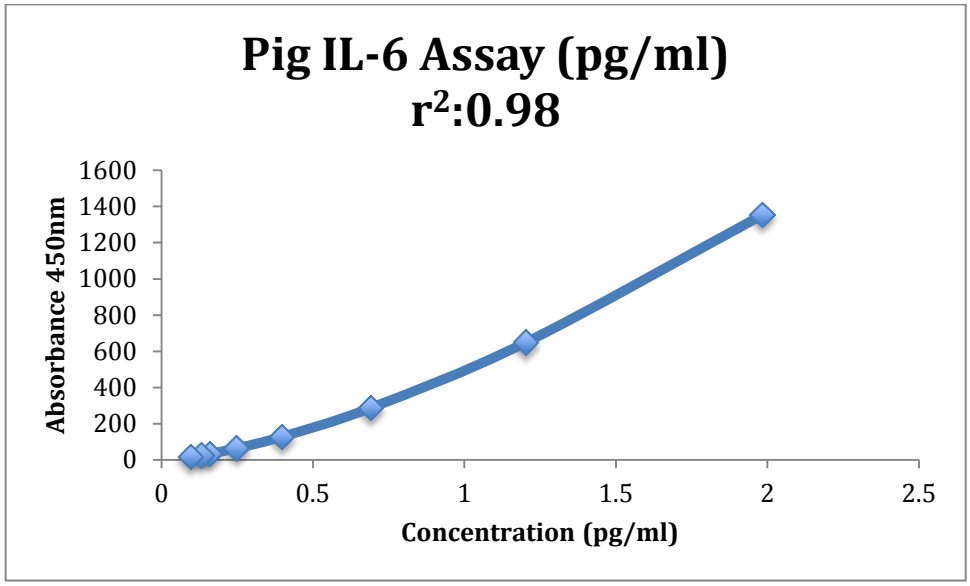
Interleukin-6

Interleukin-6 is a cytokine not only involved in inflammation and infection responses but also in the regulation of metabolic, regenerative, and neural processes.

IL-6 assay

Interlukin-6 (IL-6) was measured in serum samples using a colorimetric porcine specific assay kits (P6000, R&D Systems, Abingdon, UK) and performed according to the manufacturer's instructions. Blood samples from pigs were allowed to clot for 2 hours at room temperature prior to centrifuging for 20 mins at 1000g. The serum was then removed, aliquoted and stored at -800C until analysis. This assay employed the quantitative sandwich enzyme immunoassay technique. The optical density was read at 450 nm (correction wavelength set at 540 to 570 nm). All analyses were performed in duplicate. The intra-assay CV was < 5.1% and inter-assay CV was < 7.5%. The mean minimum detectable concentration of IL-6 for this assay is 2.03 pg/ml. Standard curve is provided in (Figure [4.5]).

Figure [4.5]: Standard curve IL-6. r^2: goodness-of-fit, linear regression.



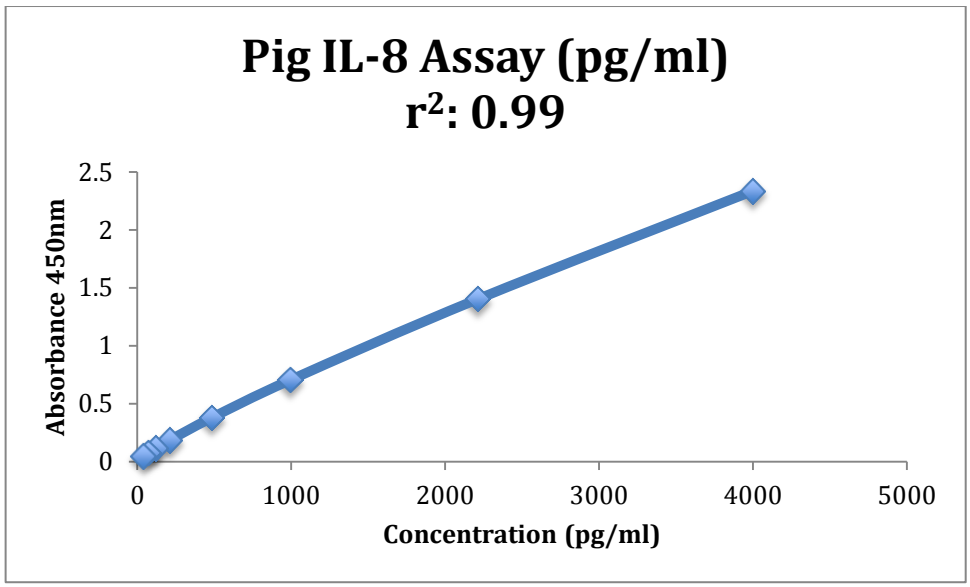
Serum IL-8

IL-8 is a proinflammatory chemokine. Expression of IL-8 is regulated by NF-Kb transcription. Expression of IL-8 is affected by several stimuli i.e. TNF-alpha or hypoxia. Biological effects of IL-8 include angiogenic responses in endothelial cells, proliferation and survival of endothelial and phagocytes, and migration of cancer cells, endothelial cells, and infiltrating neutrophils at the tumor site (Waugh and Wilson, 2008). IL-8 also primes neutrophils by recruiting NADPH oxidase, which drives the respiratory burst (Guichard et al., 2005).

Assay Principle

This assay employs the quantitative sandwich enzyme immunoassay technique similar to IL-6. The standard curve is provided in Figure [4.6].

Figure [4.6]: Standard curve, porcine IL-8 assay. r^2: goodness-of-fit, linear regression. Minimum limit of detection for the assay is 1 pg/ml. Inter-assay and intra-assay CV's were less than 10%.



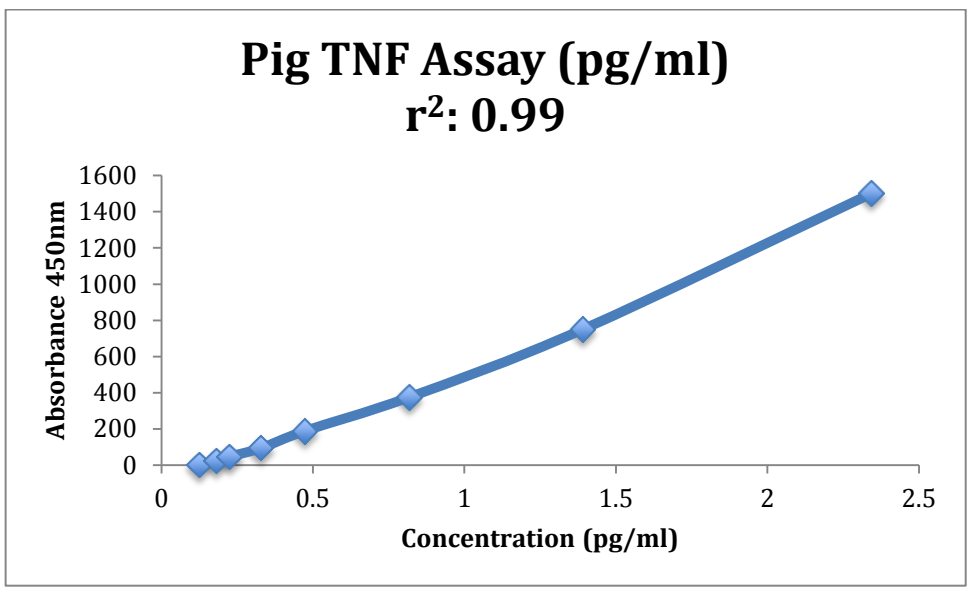
Tumor Necrosis Factor- α

TNF is produced predominantly by activated macrophages and T lymphocytes as a 26 kDa protein, pro-TNF, which is expressed on the plasma membrane, where it can be cleaved in the extracellular domain by the matrix metalloproteinase, which result in the release a soluble 17 kDa soluble form.

TNF Assay

The principle of the assay is as described above for IL-6; however the microplates were pre coated with a monoclonal antibody specific for porcine TNF α. The optical density was read at 450 nm (correction wavelength set at 540 to 570 nm). The sample values were then read off the standard curve (Figure [4.7]) for TNF α. All analyses were performed in duplicate. Intra-assay CVs were < 7.0% and inter-assay CVs were less than 10%. This assay was highly sensitive and had a minimum detectable concentration of TNF α of 5 pg/ml. All ELISA measurements were performed using the DS2 dynex automated ELISA platform (Dynex Technologies, Worthing, England).

Figure [4.7]: Standard curve TNF α. r^2: goodness-of-fit, linear regression.



4.2.1.1.8 Tissue analysis of kidney injury and inflammation

4.2.1.1.8.1 *Immunofluorescence: Effect of AQIX RS-1 on glycocalyx and endothelial integrity.*

Dual-stained Immunofluorescence: 5mm cortical frozen sections were fixed, blocked and stained with DBA lectin, (Biotinylated Kit, Vector Labs) a marker of endothelial glycocalyx and VE-Cadherin, (Santa Cruz Biochemicals, Santa Cruz, CA), an endothelial marker. 6 gloms per group were imaged using Leitz DMRB fluorescence microscope (Leica, Solms, Germany). Image J software (National Institute of Health) was used to undertake quantification of densities. Qualitative differences in IF-staining were confirmed using western blotting.

Tissue samples

Renal tissue was harvested at 24hr post intervention and cut into 2-5 grams. Representative 5um cryosections from renal cortex were then cut and stored at -80 degrees until staining.

Method

We used the indirect method of immunofluorescence where our primary antibodies where unlabeled and a second anti-immunoglobulin antibody directed towards the constant portion of the first antibody (i.e. secondary antibody) was tagged with Fluorescein Isothiocyanate (FITC). The advantage of this method over direct method (where antibody against the molecule of interest is chemically conjugated to a fluorescent dye) is that there is amplification of the signal in this technique as more than one secondary antibody can bind to primary Ab.

Imaging

Using Leitz DMRB fluorescence microscope (Leica, Solms, Germany), 4 slides with 2 sections per animal/condition (n=4) were screened for best staining regions and followed by selection of 6 gloms per animal (24 per condition, 96 in total).

4.2.1.1.8.2 Immunofluorescence: Effects of CPB and Sildenafil on constituents of renal glycocalyx (GAGs/Core proteins).

Lectins selected for IF experiments

We selected DBA, WGA, SBA and UEA as test lectins for our hypotheses testing. Properties of these four lectins are shown in table [4.2].

Table [4.2]: Characteristics of selected lectins.

Common Abbreviation	Lectin	Source	Ligand motif	Optimization results	
				Specificity	Co-localization
DBA	Dolichos biflorus	(Horse Gram) seeds	GalNAc	Yes	Yes
WGA	Wheat Germ Agglutinin, WGA	Triticum vulgaris	GlcNAc β 1-4GlcNAc β 1-4GlcNAc, Neu5Ac (sialic acid)	Yes	Yes
SBA	Soybean	Glycine max (soybean) seeds	>GalNAc	Yes	Yes
UEA	Ulex europaeus agglutinin	Ulex europaeus	Fuc α 1-2Gal-R	Yes	Yes
PNA	Arachis hypogaea (peanut) agglutinin	Arachis hypogaea peanuts	Galactose	No	No
CON A	Concanavalin A	Canavalia ensiformis (Jack beans)	Mannose	No	No
Abbreviations: Gal D-Galactose, GalNAc N-Acetylgalactosamine, Glc D-Glucose, GlcNAc N-Acetylglucosamine, Fuc L-Fucose					

Antibodies against core proteins

Further staining with antibodies against glycocalyx core proteins i.e. Syndecan-1, Syndecan-4, (all Zymed Laboratories, San Francisco, CA) and endothelial marker VE-Cadherin (Santa Cruz Biochemicals, Santa Cruz, CA, USA) were tested. Syndecan-4 antibody was selected for its specificity to glomeruli in pigs.
Both lectins and antibodies were tested on all treatment groups, and visualized for co-localization at 100X. Visual evidence of specificity and co-localization were considered parameters for antibody selection. (Syndecan-1 and Syndecan-4 both were tested, however only syndecan-4 showed specificity and co-localization, data not shown). The final choice of lectins and antibodies used with respective dilutions are provided in the following table [4.3].

Table [4.3]: Lectins, primary and secondary antibodies for Immunofluorescence experiments.

Antibody	Dilutions tested	Final dilution used
Biotinylated Lectins		
Dolichos biflorus agglutinin (DBA)	1:50, 1:100, 1:200	1:50
Wheat germ agglutinin (WGA)	1:50, 1:100, 1:200	1:50
Ulex europaeus agglutinin (UEA)	1:50, 1:100, 1:200	1:50
Soybean agglutinin (SBA)	1:50, 1:100, 1:200	1:50
Primary Antibody		
1-Endothelial marker		
VE-Cadherin	1:50, 1:100, 1:20	1:100
2-Core protein marker		
Syndecan-4	1:50, 1:100, 1:200	1:100
FITC-conjugated Secondary Antibody		
-Alexa Fluor® 488 Anti-Mouse IgG	1:100, 1:200	1:100
- Alexa Fluor® 546 Anti-Mouse IgG	1:100, 1:200	1:100

Method

This is previously detailed in section [4.2.1.1.8.1].

Imaging

Using Leitz DMRB fluorescence microscope (Leica, Solms, Germany), 4 slides with 2 sections per animal/condition (n=4) were screened for best staining regions and followed by selection of 6 gloms per animal (24 per condition, 72 in total).

4.2.1.1.8.4 Immunofluorescence: Effect of storage lesion on $CD14^+$ $CD16^+$ monocytes in kidney

$CD14^+$ and $CD16^+$ are well-recognized markers of monocytes and neutrophil activation that are expressed during immunological reactions of innate nature in human diseased conditions (Belge et al., 2002). Our hypothesis was that systemic stress due to stored blood transfusion drives neutrophils and monocytes to transmigrate through vascular endothelium and invade lungs and kidneys leading to tissue injury, hypoxia and organ dysfunction.

Methodology

Immunofluorescence on kidney tissue was performed on 5μm sections and imaged with an inverted Zeiss Axio Observer Z1 microscope, equipped with 20x, N/A 0.4 objective and Hamamatsu Flash 4.0 camera. Four different animals per treatment with at least 2 slices per animal were analysed; at least 30 images per slice were acquired. Antibody against CD14 (clone MIL2) was a generous gift from Prof. Mick Bailey (Haverson et al., 1994). Antibody against CD16 (clone G7) was purchased from BD Biosciences, respectively. Fluorophore-conjugated, secondary antibodies against mouse IgM (Alexa Fluor 546), IgG1 (Alexa Fluor 546) and IgG2b (Alexa Fluor 488) were purchased from Life Technologies. Images were analysed using ImageJ (NIH USA).

Controls

Adequate controls incubated with only secondary antibodies where simultaneously stained and imaged under similar exposure conditions as of test slides. The final analysis achieved involved adjusting every test slide to their corresponding control.

Image analysis

We used Image J to analyse test images for respective integrated densities that were adjusted for controls and DAPI. The results were averaged for all subjects within treatment groups and analysed with ANOVA plus Bonferroni adjustment.

4.2.1.1.9 Chemical histological staining for determination of Redox active Iron (Fe^{2+}) in kidney

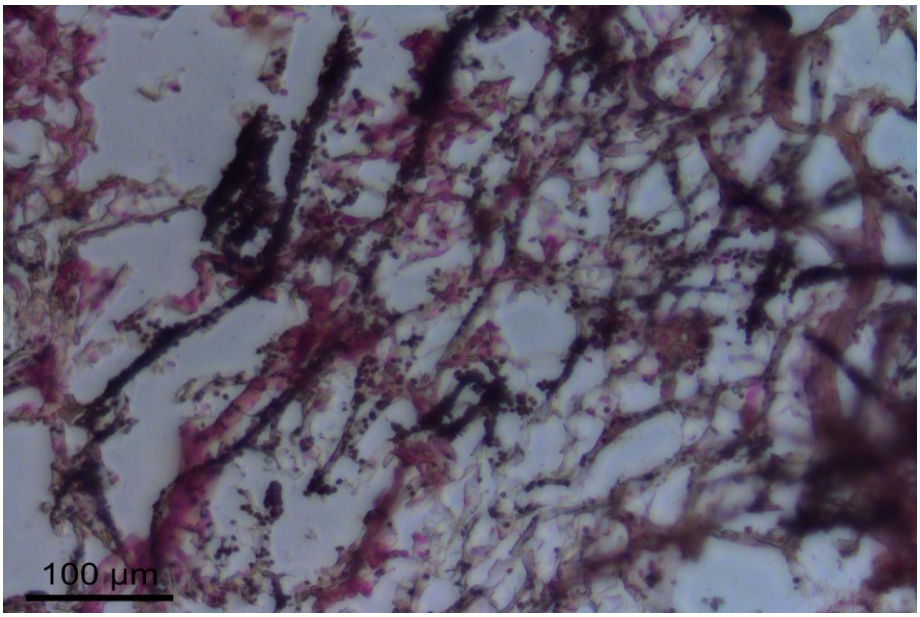
Redox active Iron (Fe^{2+}) was detected in porcine frozen lung tissue obtained at 24hrs using a modified Nguyen-Legros method (Nilsson et al., 2002)).

Principle

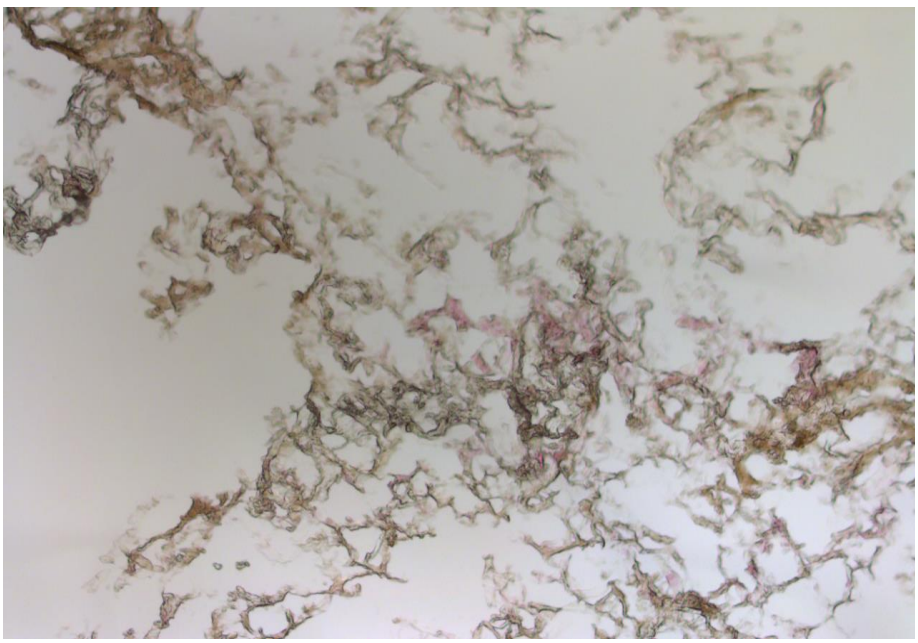
This method starts by inhibition of endogenous red cell peroxidases using a blocking solution comprising 30% H_2O_2 in 300mls of methanol with 0.15 grams of NaN_3, mix well, pH: 7.5, Figure [4.8]. This step is followed by classical pearls stain reaction that involves binding of iron (II) cyanide (ferrocyanide) to iron (III) in tissue to give a brilliant blue mixed-valence iron (II+III) complex. In the second stage this complex is enhanced to a secondary complex (dark brown pigmentation) by a peroxidase-like H_2O_2-dependent oxidation of 3,3'-diaminobenzidine (DAB). The optical densities of this secondary complex are measured using bright-field microscopy and adjusted for control tissue sections that lack the DAB step.

Figure [4.8]: Blocking of endogenous red cell peroxidases. (A) Without blocking solution; red cell endogenous peroxidases oxidise DAB and show up as black dots. (B) With blocking solution red cells are not highlighted. Image taken at 40X of frozen 10-micron porcine lung section.

A-Blocking solution $^{-}$/DAB^{+}



B-Blocking solution $^{+}$/DAB^{+}



Imaging

Imaging was carried out using bright field mode of Leica DM2500 upright microscope using Leica Application Suit version 4.0.0, Leica Microsystems (Switzerland) Limited. Ten Pearls-positive (Fe+) and 10 Pearls-negative (Fe-) images were taken per animal (N=4 per each treatment group) using at 40X and analysed for integrated density using Inage J (NIH) USA.

4.2.1.1.10 Western blotting: Quantification of expression of glycocalyx constituents (Core-proteins: Syndecan-1, Syndecan-4, Glypican, Perlecan) and endothelium (Thrombomodulin, vWF, VE-Cadherin) and podocin in response to CPB.

Tissue samples

Renal cortex was harvested at 24hr post intervention and immediately cut into 2-5 grams cubes and stored at -80 degrees.

Protein extraction-Cortex Lysates

In order for proteins of interest to run through gels, physical and enzymatic solubilization was carried out using a homogeniser and RIPA buffer. The concentrations were measured using Bradford assay.

SDS-PAGE Electrophoresis Preparation

The electrophoresis was undertaken with standard methodology using 6 and 10% gels, using PVDF membranes for transfer. And blocked with 3-5% BSA in TBS. Detection was carried out using chemiluminescent detection using CCD; MultiDoc-It imaging System (UVP, Upland, CA). Images where analysed using Image J and protein bands quantified using densitometry. All target protein bands were adjusted to tubulin or beta actin as internal control.

4.2.1.1.11 Cell culture

Hypothesis

To provide a mechanistic insight into the effects of post-bypass serum with or without Sildenafil treatment on expression of Glycocalyx of conditionally immortalized cultured glomerular endothelial cells (ciGEnC's).

4.2.1.1.12 Conditionally immortalized cultured human glomerular endothelial cells (ciGEnC's)

These cells have been developed after transduction with a temperature-sensitive mutant of simian virus 40 large tumour antigen SV40 large T antigen and the catalytic subunit of human telomerase. At the permissive temperature of 33°C the tsSV40LT transgene is activated, causing cell proliferation, while at 37°C the transgene is inactivated, causing cells to become non-proliferative. Conditionally immortalized GEnC were used for experiments after they were maintained at the non-permissive temperature for 7 days. GEnC were cultured in endothelial growth medium-2 microvascular (EGM2-MV, Cambrex, Wokingham, UK) containing fetal calf serum (5%) and growth factors as supplied with the exception of VEGF.

Splitting and propagation of endothelial cells

When cell splitting was required, it was done after removing old media, washing with PBS followed by addition of trypsin and incubating for 10 min at 33 degrees. After this the cells were collected in 10ml falcon tube and centrifuged at 1300rpm for 5 min. The pallet was resuspended in media and then onto 6 or 96 well plate and left at 33 degrees until 70-80% confluent.

Experimental design

Experiment 1&2

A 96 well plate impregnated with 70-80% confluent *ciGEnC's* was used. Cells were incubated with 5% (in Endothelial cell Basal Medium-2, Lonza Group Ltd), Pre-CPB and Post-CPB sera for 2.5, 12 and 24hrs. Serum from 4 different CPB animals (N1-4) was used in triplicate. At 24hrs, cells were washed with PBS/TBS-T, incubated with 3% BSA/5%FCS for 1hr and then left for overnight incubation with biotinylated WGA lectin (1:50). Next morning, they were washed, treated with DAPI and imaged.

Experiment 3&4

A 96 well plate impregnated with 70-80% confluent *ciGEnC's* was used. Cells were incubated with 5% Pre-CPB or Post-CPB sera ± Sildenafil for 24hrs. Sera from 4 different CPB animals (N1-4) were used in triplicate. Culture medium (EBM-2) or sera were treated with Sildenafil at a final conc. of 0.0024mgs/ml (Corresponding to 10mg per 4.5L circulating blood volume of adult swine). At 24hrs, cells were washed, incubated with 3% BSA/5%FCS for 1hr and then left for overnight incubation with biotinylated WGA lectin (1:50). After 24hr interval they were washed, treated with DAPI and measured for optical density.

Fluorometric determination of lectin uptake by ciGEnC's

We used fluorometric module (detection limit < 2 fmol/well, 10pM) of Wallac 1420 VICTOR2™ multi-label counter/plate reader. The following wavelengths were used; WGA lectin: 485nm/535nm, DAPI: 355nm/460nm.

Analysis

Each sample in the 96-well plate was analysed as triplicate. Using Image J, WGA-lectin values were adjusted for their respective DAPI signals and averaged values were compared using ANOVA with Bonferroni adjustment (significance cut-off: <0.05). For experiments 1,2,3 and 4 culture medium treated cells were used as negative controls whereas in 3 and 4 Sildenafil treated in cells in culture medium were considered positive controls. Calibration of the unit was not required as each experiment had a negative control that was considered as a blank.

4.2.2 Acute lung injury

4.2.2.1.1 Assessment of storage lesion mediated TRALI

TRALI was determined according to functional, histological and biochemical parameters (Table [4.4]) of acute lung injury (ALI) as recommended by the American Thoracic Society (Matute-Bello et al., 2011).

Table [4.4]: Main features of Acute Lung Injury in experimental animal models; Very relevant and some-what relevant measures. A-a: Alveolar-arterial difference, MPO: Myeloperoxidase, Kf: lung vascular filtration coefficient. Parameters highlighted in red were chosen for our assessment of TRALI. Derived from (Matute-Bello et al., 2011).

Main features of experimental ALI	Very relevant	Some what relevant
Physiological dysfunction	Hypoxemia	PaO2/FIO2 < 200
	Increased A-a oxygen difference	Increase in spontaneous minute ventilation
		Increase in spontaneous respiratory rate
Histological evidence of tissue injury	Accumulation of neutrophils in the alveolar/interstitial space	Evidence of haemorrhage
	Formation of hyaline membranes	Areas of atelectasis
	Proteinaceous debris in alveolar space	Gross macroscopic changes such as discoloration of the lungs
	Thickening of the alveolar wall	
	Injury by a standardised histology score	
Inflammation	BAL total neutrophil counts Lung	Increases in procoagulatory activity
	MPO activity	Increased expression of adhesion molecules
	Concentrations of cytokines	Conversion of the neutrophilic

Main features of experimental ALI	Very relevant	Some what relevant
		alveolitis into a mono- nuclear alveolitis with time Increase in levels of complement factors and matrix metalloproteinases
Alteration of the alveolar capillary barrier	Increased extravascular lung water content	Increase in lung wet/dry weight ratio Translocation of a protein from the airspaces into plasma Increased lung lymph flow High lymph protein concentration
	Accumulation of protein/tracer in airspaces/extravascular space	
	Total BAL protein concentration	
	BAL concentration of high molecular weight proteins	
	(Micro-) Vascular filtration coefficient (Kf)	

4.2.2.1.1.1 Physiological dysfunction

Lung compliance, PaO_2/FiO_2 ratio, airways resistance and work of breathing were measured in-vivo at baseline, 1.5hrs and 24hrs post-intervention using the SERVO-i Universal Ventilator (Maquet Gmbh, Germany) using volume controlled ventilation with a tidal volume of 10ml/kg, FiO_2 of 0.5, respiratory rate of 12 breaths per minute and PEEP of 5 cm/H_2O.

4.2.2.1.1.2 Alteration of the alveolar capillary barrier

This is described in section; Biochemical markers of lung inflammation [4.2.2.1.2.1].

4.2.2.1.1.3 Histological evidence of tissue injury

This is described in section; Tissue analysis of lung injury [4.2.2.1.3.1].

4.2.2.1.1.4 Pulmonary Inflammation

This is described in section: Tissue analysis of lung injury [4.2.2.1.3.2].

4.2.2.1.2 Biochemical markers of lung inflammation

4.2.2.1.2.1 BAL total protein

Total protein in bronchoalveolar lavage (BAL) samples was measured using a Bradford Protein Assay (Quick Start Bradford Protein Assay, Hercules, USA) at 24hrs post intervention by an investigator blinded to intervention allocation.

4.2.2.1.2.2 Inflammatory cytokines

IL-6 and IL-8 were measured in serum at each key time point and these are described in section [4.2.1.1.7]. TNF-α was measured in lung lysates.

4.2.2.1.2.3 Endothelial and Platelet derived Microparticles

Hypothesis

MP estimation in circulation of stored blood recipients and their reduction with homeostatic interventions such as washing and red cell rejuvenation could highlight their important role in these conditions.

Flow cytometry analysis of Microparticles

Preparation of Microparticle (MP) samples

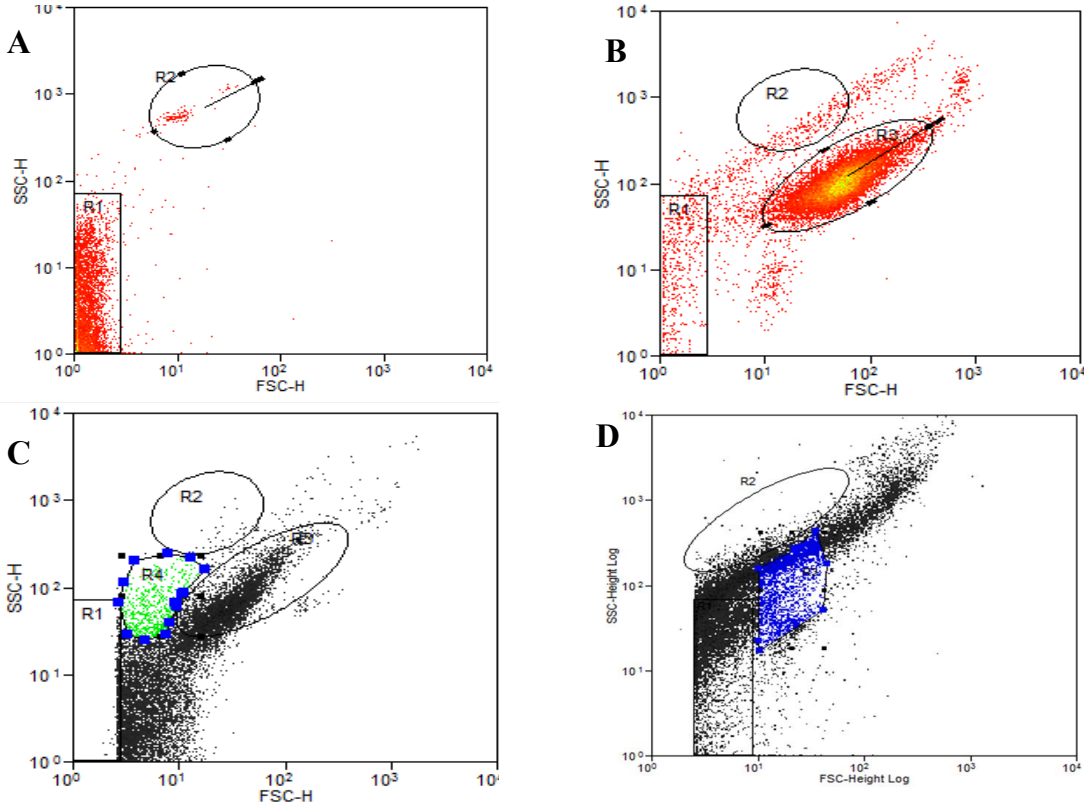
Five mls of blood anticoagulated with sodium citrate was obtained from recipient animals and centrifuged at 1550 × g for 20 mins followed by a second centrifugation at 13,000 × g for 2 mins. Aliquots of platelet poor plasma (PPP) were taken from each sample and immediately frozen at -80^{0}C until analysis.

Establishing a MP gate

To establish a suitable MP gate on a flow cytometry plot of forward scatter (FSc) vs. side scatter (SSc), it was necessary to distinguish between MPs and small platelets. Platelet-rich-plasma (PRP) or storage supernatant was prepared by centrifugation of citrated porcine whole blood at 200 × g for 10 min. The PRP

was analysed on a FACSort flow cytometer using (Summit V4.3.02 Build 2451, Beckman Coulter, Inc.) to establish a platelet gate. This technique was described by Nieuwland and Sturk (Nieuwland and Sturk, 2007). 60 μ l of PRP was then incubated with 20 μ l of 2 μ M calcium ionophore A23187 (Sigma I0634) for 5 hours. Calcium ionophore is known to stimulate platelets to produce MPs(Nieuwland and Sturk, 2007, van der Zee et al., 2006). The stimulated PRP was used to establish the upper limit of the MP gate and was confirmed in each experiment with 1 μ m beads (L2778, Sigma-Aldrich, Gillingham, UK). 0.1 μ m beads (L9904, Sigma-Aldrich, Gillingham, UK) were used to determine the lowest detectable limit, and therefore used to set the threshold. The threshold was set on SSc so to exclude background noise, which is determined by running 0.1 μ m filtered PBS-calcium. A fluorescence threshold was avoided in this study to allow analysis of both the Annexin-V positive and negative MPs. In order to separate true events from background noise and unspecific binding of antibodies to debris, we defined microparticles as particles that were less than 1.0 μm in diameter, had positive staining for Annexin V. Figure [4.9].

Figure [4.9]: Establishing a MP gate: Scatter plot representation showing side and forward scatter for (A) localization of 0.1 μ m (R1) and 1.0 μ m (R2) distribution derived from beads. (B) Localization of 0.1 μ m (R1), 1.0 μ m (R2) distribution and (R3) Unactivated, Annexin V/CD41 positive platelets. (C) Localization of 0.1 μ m (R1), 1.0 μ m (R2) distribution and (R3) Unactivated, Annexin V/CD41 positive platelets and activated, Annexin-V/ CD41 positive platelet derived MPs population (R4 in green) and (D) Annexin-V/E-selectin positive Endothelial derived MPs (R3 in blue).



Labelling of MPs

Annexin-V-FITC (556420, BD Pharmingen, Oxford, UK) was used as a general marker for MPs. Ann-V FITC was used to label phosphatidylserine-positive MPs in Annexin-V binding buffer (556454, BD Pharmingen). All antibodies were titrated, using either control or porcine plasma, to determine optimal concentrations. Five μ l AnnV- FITC was added to PBS-calcium (2.5 mmol/L) to make up a total volume of 50 μ l followed by incubation with 10 μ l of sample for 30 min at room temperature, protected from light. After incubation, 900 μl of PBS-calcium (2.5 mmol/L) was added.

Endothelial derived microparticles were measured in whole blood using FACS analysis according to the protocol described below using an anti-CD62 antibody (Anti E-selectin, clone 1.2B6, FITC conjugated, Merck Millipore, Darmstadt, Germany). Platelet derived microparticles were similarly measured using anti-CD41, AbSerotec, Kidlington, United Kingdom), and activated platelets using (antiplatelet-activating complex-1; BD Bioscience, Oxford, United Kingdom). All laboratory analyses were carried out by Miss Ade Abedoye, Technician, Cardiovascular sciences, University of Leicester.

Controls and calibration

Annexin-V-FITC in the absence of calcium (PBS-Citrate (0.32%) buffer) was used as a negative control for MP marking in general, while IgG1 was used as a negative control for CD41 PE and CD62 PE. Calibrations were done every day before running the flow cytometer, while controls were run with every test samples.

4.2.2.1.3 Tissue analysis of lung injury

4.2.2.1.3.1 Histological lung injury

The lower lobe of the left lung was harvested 24hrs post intervention, immediately fixed in 10% formalin and 6 sections taken sequentially across the resected lung were stained with haematoxylin & eosin. Investigator blinded to intervention allocation used a histological scoring system for lung injury assessment.

Lung Injury scoring system (Matute-Bello et al., 2011)
This is described in the table [4.5]. To generate a score, sum of each of the five parameter is weighted according to the relevance ascribed to each feature and then normalized to the number of fields evaluated. The resulting score is a continuous value between zero and one.
These scores were obtained by blinded assessment of formalin fixed H&E stained sections undertaken by Dr. John Le Quesne, Pathologist, MRC Toxicology Unit, Hodgkin Building, University of Leicester. The formalin fixed H&E slides were prepared by Centre for core biotechnology, University of Leicester.

Table [4.5]: Lung injury scoring system, Derived from (Matute-Bello et al., 2011).

Score per field				
Parameter		0	1	2
A	Neutrophils in alveolar space	none	1-5	>5
B	Neutrophils in the interstitial space	none	1-5	>5
C	Hyaline membrane	none	1	>1
D	Proteinaceous debris filling the airspaces	none	1	>1
E	Alveolar septal thickening	<2X	2X-4X	>4X
Score=[(20 X A)+(14 X B)+(7 X C)+(7 X D)+(2 X E)]/(number of fields X 100)				

Data Analysis

The calculated lung injury scores where averaged among animals under control and intervention conditions and analysed using ANOVA with Bonferroni adjustment. Data is presented as Mean ± SD. Significant level was set at $p < 0.05$.

4.2.2.1.3.2 Immunofluorescence: $CD14^+$ $CD16^+$ monocytes in lung

The SOP is similar to previous section [4.2.1.1.8.4].

4.2.2.1.3.3 Chemical histological staining for determination of Redox active Iron in lung

This is similar to section [4.2.1.1.9].

4.2.3 Platelet dysfunction

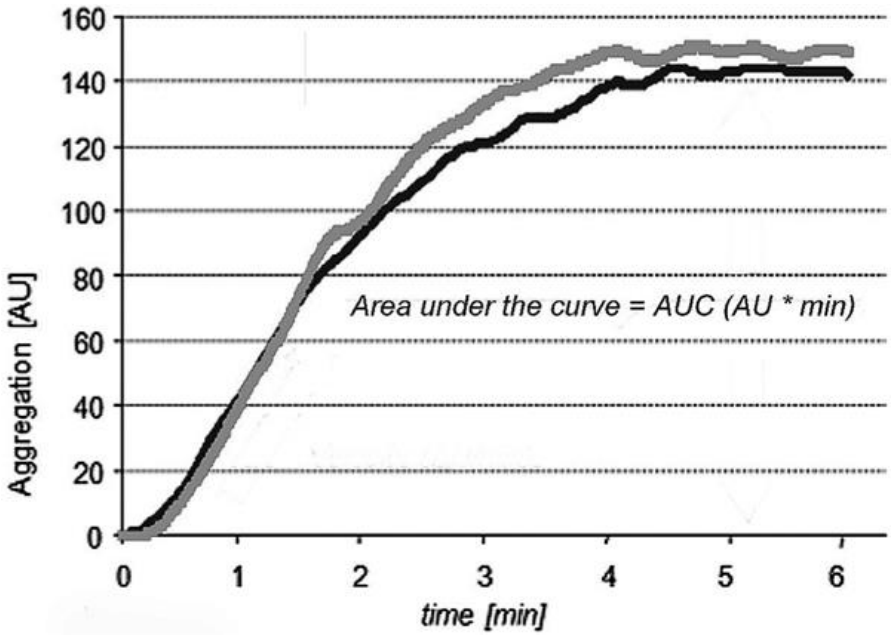
Platelet dysfunction assessment using Multiplate™ aggregometer.

At each pre-specified (A-D, Figure [1.10]) time point platelet aggregation (using ASP and ADP test reagents) was measured in hirudin blood using the Multiplate analyzer (Dynabyte GmbH, Munich, Germany). Platelet aggregation performance was assessed using the Multiplate analyzer (Dynabyte GmbH, Munich, Germany), a recently developed, point-of-care, multiple-electrode platelet aggregation monitoring system that is based on whole-blood impedance measurements.

Multiplate test output

Multiplate continuously records platelet aggregation. The increase of impedance by the attachment of Platelets onto the Multiplate sensors is transformed to arbitrary aggregation units (AU) and plotted against time (Figure [4.10]). Three parameters are calculated: The most important parameter is the area under the aggregation curve (AUC). It is affected by the total height of the aggregation curve as well as by its slope and is best suited to express the overall platelet activity. Two more parameters are calculated for research use: The aggregation is the height of the curve. The velocity is the maximum slope of the curve.

Figure [4.10]: Multiplate test output: The unit of the AUC is AU * min (as the y-axis is the aggregation, expressed in Aggregation units (AU) and the x-axis is the time, expressed in minutes). The aggregation and velocity are calculated for research use. The correlation coefficient (cc) between the values of the 2 individual curves is determined. The analysis is accepted when the cc is at least 0.98. The difference from the mean curve (DIF) is calculated based on the AUC values of the 2 individual measured curves. The analysis is accepted when the difference is lower than 20% (vs. the mean value of the 2 curves). Derived from (Hanke et al., 2010).



Data Analysis

The respective test outputs for all above (clinical and research) measures where averaged among animals under control and intervention conditions and analysed using ANOVA with Bonferroni adjustment. Data is presented as Mean ± SD. Significant level was set at $p < 0.05$.

Limitations

The results obtained with Multiplate™ Analyzer are influenced by platelet function as well as platelet count thus displaying the overall platelet aggregability within the blood sample rather than platelet function alone (Hanke et al., 2010). In addition platelet aggregation results are influenced by time between sampling and analysing (Johnston et al., 2013).

Multiplate assays

We used ADP and ASP assays for platelet function assessment.

- ADP: binds to a specific platelet membrane receptor and causes platelet activation and release of dense granule stored ADP, Figure [4.20]. Shows biphasic aggregation.
- Aspirin induced activation (ASP): Aspirin inhibits PAR (Protease Activated Receptor)-induced platelet aggregation, which prevents activation of thromboxane production, which is a powerful platelet agonist.

4.3 Additional methods

4.3.1.1 ***In vitro assessment of storage lesion***

Twenty ml aliquots were removed from representative red cell bags for evaluation of storage related changes at Day 0, 7 and 14 of storage and following washing or Rejuvesol-Washing.

A-Storage lesion Erythrocyte Factors

1. **-RBC count (x10^{12}/L)**

Hematology analyzer (Advia 2120, Siemens, Frimley, UK)

2. **-Mean corpuscular volume (f/L)**

Hematology analyzer (Advia 2120, Siemens, Frimley, UK)

3. **-Supernatant Hemoglobin conc. (g/dL)**

Supernatant Haemoglobin by Spectrophotometric Harboe Method (Cookson et al., 2004).

4. **-Hematocrit (%)**

Hematology analyzer (Advia 2120, Siemens, Frimley, UK)

5. **-Erythrocyte 2,3 DPG Conc. (µmol/g Hb)**

Commercially available kit from Roche Diagnostics, Welwyn Garden City, UK, using a UV spectrophotometer (Thermospectronic UV-540, Thermo Electron Corporation, Hemel Hempstead, UK).

Summary of steps:

The initial step is deprotenization of blood sample using perchloric acid followed by its neutralization. Simultaneous working solutions of glycolytic metabolites are prepared according to manufacturer guidelines. The sample and working solutions are co-treated according to the recommended protocol and absorbance's of the supernatant are read before and after the completion of reactions at 340nm using a spectrophotometer.

6. **-Erythrocyte ATP Conc. (μ mol/g Hb)**

This was undertaken using coupled enzyme method as described by Lagerberg and colleagues (Lagerberg et al., 2007) using a microplate reader (Synergy HT, BioTek, Winooski, VT) and controlled against methodology drift(i.e. greater than 10% shift in control assays), using a frozen control prepared from a commercial ATP (Roche Diagnostics). The principles of this method involve conversion of phosphoenolpyruvate to pyruvate kinase coupled to the conversion of pyruvate to lactate by lactate dehydrogenase. The latter step requires NADH which is oxidized to NAD+. NADH absorbs strongly at 340nm but NAD+ does not, enabling utilization of NADH to be followed by monitoring absorbance's at 340nm. The decrease on absorbance can be converted into ATPase activity where 1 molecule of NADH oxidized to NAD+ corresponds to the production of 1 molecule of ADP by ATPase. For these assays, RBC samples were deproteinized with perchloric acid and stored below -70°C before testing (Wilsher et al., 2008).

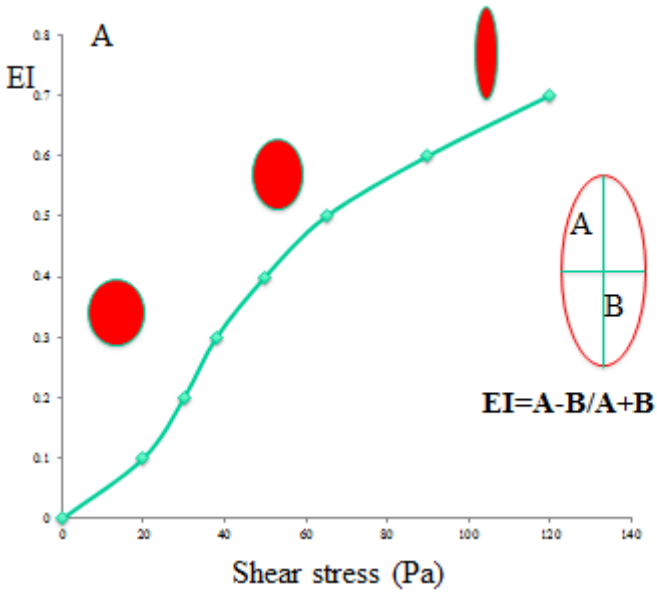
7. **-Elongation Index: Red cell deformability by Laser-assisted Optical Rotational Cell Analyzer (LORCA)**

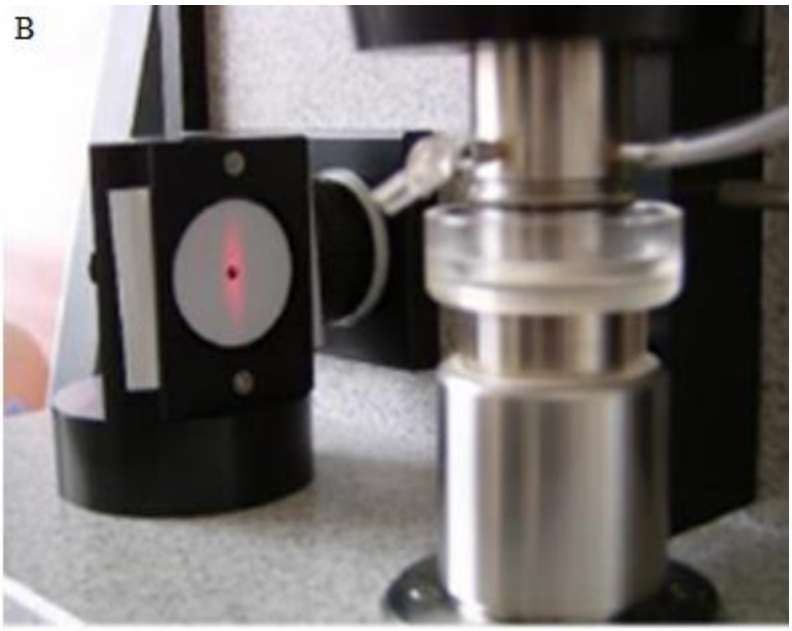
Reticulocyte elongation index (EI), the unit of deformability was measured over a range of shear stresses (0.3-30 Pa) by ektacytometry using a laser- assisted optical rotational cell analyser (LORCA, Mechatronics). The elongation index was defined as the ratio of the difference between the axes of the ellipsoid diffraction pattern and the sum of these two axes. This is discussed below in detail.

In this instrument a dilute suspension of erythrocytes (~ 2 x 107 cells/ml) in a viscous medium is placed in the gap of a Couette shearing system, created by an outer rotating cylinder and an inner static cylinder, having a laser-diode projected through the gap. The presence of red blood cells in the gap diffracts the laser light that creates a diffraction pattern on a diaphragm changing from circular to elliptical as cells deform and elongate. The pattern is captured and analysed by a video camera and a computer system that calculates an elongation

index (EI) as the (length - width) / (length + width) of the pattern for each shear stress (SS). Results are shown on an elongation index-shear stress (EI-SS) diagram, Figure [4.11].

Figure [4.11]: Red cell deformability. (A) Change in diffraction pattern during RBC elongation and measurement of Elongation index (EI), (B) Two cylinders of LORCA with the laser beam creating an ellipsoidal diffraction pattern on a diaphragm.





B-Storage lesion Supernatant Factors

We measured partial pressure of Oxygen PO_2 (mmHg) in the red cell supernatant using ABL-800 Flex blood gas analyser (Radiometer, Copenhagen, Denmark). The remaining supernatant factors such as, Potassium (mmol/L), Sodium (mmol/L), Glucose (mmol/L) and Lactate (mmol/L) were measured using biochemistry analyzer (Vitros DT60 II, Ortho-Clinical Diagnostics, HighWycombe, UK). The Hemolysis Index (%) was calculated using the following equation; (Plasma hemoglobin × Hematocrit)/Donor Unit hemoglobin.

4.4 Statistical analyses

4.4.1 Power calculation for animal procedures

These studies were powered to detect significant intergroup differences in creatinine clearance as the primary outcome. On the basis that there will be one baseline and two post intervention measurements it was estimated that a study with 8 recipients per group will have 90% power to detect a large effect size of 0.7 standard deviations (equivalent to a difference of 16 ml/hr in creatinine clearance between groups assuming a within group SD of 23.5).

4.4.1.1 Special consideration

Analysis of acute lung injury in chapter 6 is based on experiments powered to detect differences in creatinine clearance as a primary endpoint (Murphy et al., 2009, Patel et al., 2011a). Analysis of pulmonary dysfunction was a prespecified secondary outcome in these experiments, however, no power calculation was performed a priori, and this analysis was considered exploratory.

4.4.2 Data analysis for functional measurements

Differences between groups or time-dependent changes within groups were calculated using ANOVA with Bonferroni adjustment. For normally distributed data, values are expressed throughout as mean (_ SE), and treatment differences are reported as mean difference (95% confidence intervals [CI]). Non-normally distributed data are expressed as geometric means, with treatment differences expressed as ratios of geometric means (95% CI). Interaction for time and treatment effects were also calculated using mixed model design. All p values less than 0.05 were considered to be statistically significant. All analyses were carried out using SPSS 14.0 (SPSS, Chicago, IL).

4.4.3 Data analyses for histological organ injury, labile iron staining, cell culture and western blotting

Using SPSS 14.0 (SPSS, Chicago, IL), signal densities were analysed using ANOVA with Bonferroni correction, with significance cut off at <0.05.

Chapter 5

Experimental Results

Evaluation of novel AQIX RS-1® for intravenous fluid replacement in a swine model of general anaesthesia, recovery and end organ assessment.

5.1 Plan of Investigation

5.1.1 Rationale

Current fluid therapies may promote organ injury and there is need to identify and assess novel fluids for IV resuscitation. I have explored a novel organ preservative, AQIX-RS-1® for IV resuscitation. This safety is assessed in a large animal model. This is an in-depth in vivo investigation of an organ preservative while simultaneously analyzing critical parameters of organ stress and function.

5.1.2 Aims

Our primary aim was to assess end organ function and measures of organ stress in a head to head comparison of AQIX RS-1® vs. Hartmann's solution when infused in a large recovery model of organ assessment. If superiority of AQIX® was established in this model, further evaluation will be undertaken in a large animal recovery model of post CPB AKI.

5.1.3 Hypotheses

1. End organ assessment for AQIX RS-1 administered swine will be superior to swine receiving Hartman's solution.
2. Swine receiving AQIX –RS-1 will have better glomerular endothelial structural and functional preservation.
3. Swine receiving AQIX RS-1 will have less inflammatory stress and platelet activation.
4. Swine receiving AQIX RS-1 will have better Nitric Oxide bioavailability.

5.1.4 Design

This was an experimental parallel design, pretest-posttest repeated measures design in pigs randomised to;

Group 1: (n=8), General anaesthesia, neck dissection with volume replacement (500mls/ 30 minutes) of Hartmann's Fluid (Baxter Compound Sodium Lactate 1000 mL FKE2324), Total volume to be infused: 4500mls.
Group 2: (n=9), General anaesthesia, neck dissection with volume replacement (500mls/ 30 minutes) of AQIX RS-1 (AQIX® Ltd.), Total volume to be infused: 4500mls.

5.1.5 Outcomes

1. Biochemical Markers of Renal Injury such as creatinine clearance, free water clearance, and fractional sodium excretion were measured.
2. Endothelial Function measured as change in renal blood flow in response to acetylcholine.
3. Renal Tissue was obtained post intervention and analysed for morphological changes in glomerular endothelial membrane (GEM).

5.2 **Results**

5.2.1 Conduct of experiment

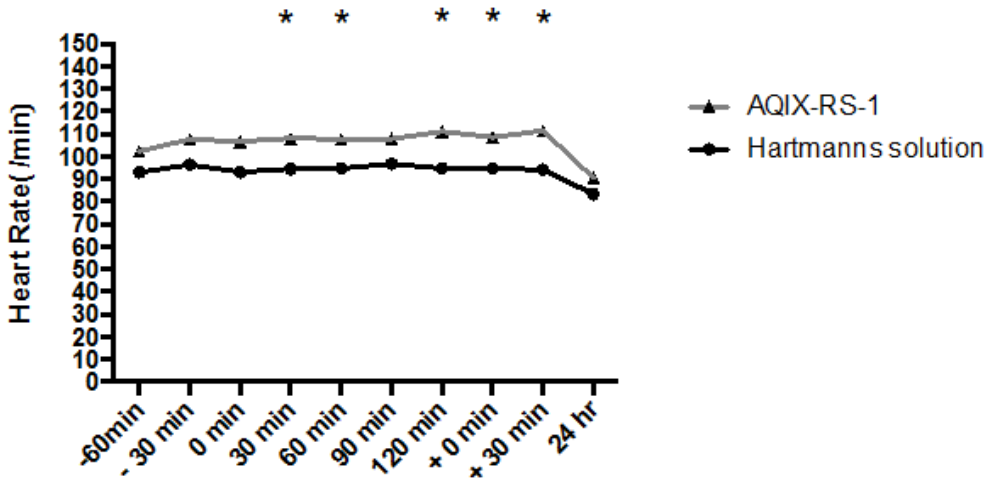
All pigs were recovered and re-anaesthetised for organ retrieval. There were no significant differences between baseline characteristics (Table [5.1]). Mean volumes infused were 4500mls for both AQIX and Hartmann's group. AQIX pigs demonstrated intermittent tachycardia as compared to Hartmann's group, however this was not significant at 24hrs (Figure 5.1-A). Similarly AQIX pigs demonstrated intermittent hypotension compared to controls (Figure 5.1-B). Despite these trends there was no significant evidence of systemic malperfusion as measured by serum lactate (Figure 5.1-C). There were no differences between groups for measures of oxygenation and ventilation (Figure 5.2 A-B).

Table [5.1]: Baseline Characteristics.

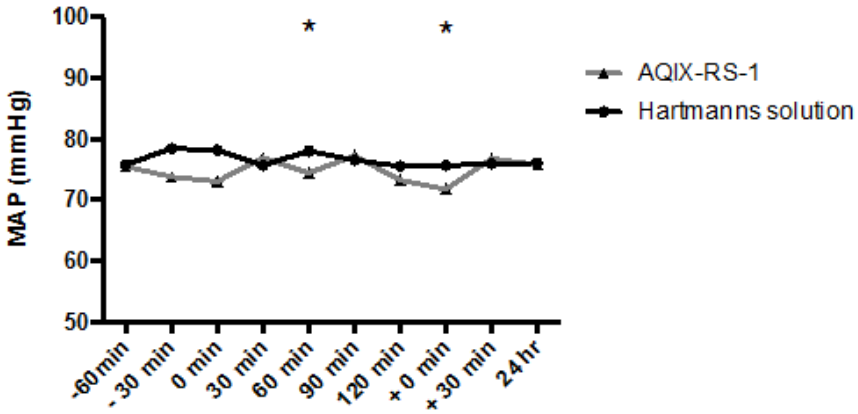
Factor	Mean±SD		95% Confidence Interval		P value
	Hartmann's Solution	AQIX RS-1	Hartmann's Solution	AQIX RS-1	
Weight (kg)	60±5.5	59.7±3.9	55.5,64.8	56.6,62.7	0.613
Baseline Serum Creatinine (mmol/L)	129.6±14.6	120.7±18	117.3,141.9	106.4,135.1	0.248
Baseline Urine volumes (mls/1.5hrs)	185±88.6	203±170	111,259	73.5,334.3	0.101*
Baseline Fractional Excretion of Sodium (%)	0.45±0.53	1.09±1.63	-0.4, 1.3	-0.27,2.4	0.671*
Baseline Free water clearance (mls/min)	2.7±4.9	4.8±11.7	-5.1,10.4	-4.2,13.7	0.758*
Creatinine Clearance (mls/min)	127.5±31.6	112±46.2	88.2,168	76.4,147.4	0.101*
Mortality	0	0	--	--	--
Data expressed as mean± standard deviation. P Value derived from t-test for normally distributed data, and Kruskall-Wallis test for non-normally distributed data*.					

Figure [5.1]: Hemodynamics and tissue perfusion: (A) Heart rate (beats/min), (B) Mean Arterial Blood Pressure (mmHg), (C) Serum Lactate (mmol/L). * $p<0.05$ for intergroup differences. Repeated measures ANOVA not significant for time interaction. Data displayed as Mean (SD).

A



B



C

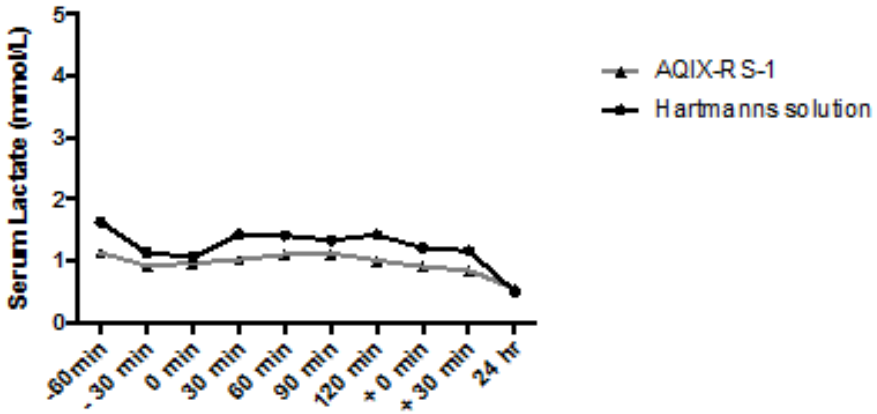
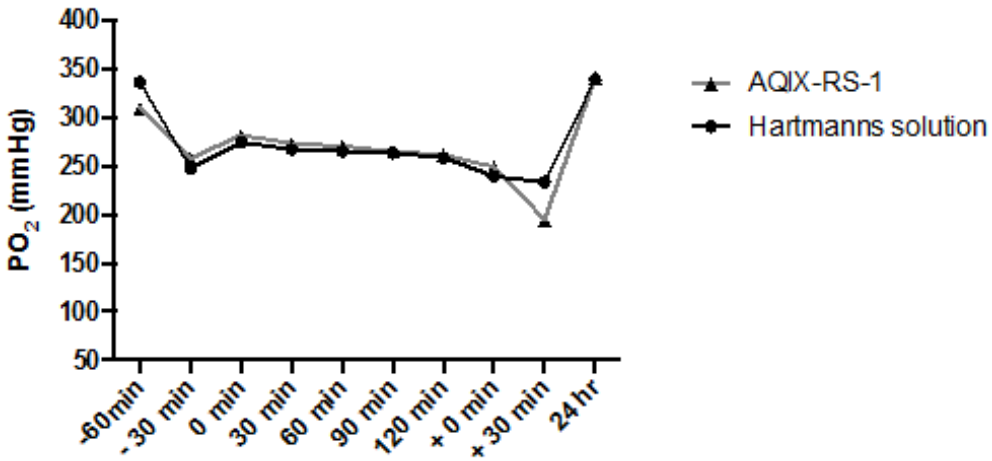
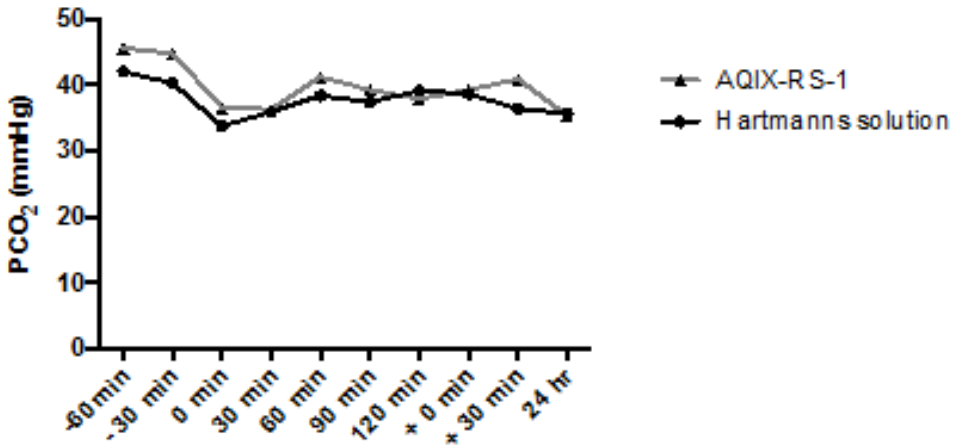


Figure [5.2]: Oxygenation and adequacy of ventilation: (A) Partial pressure of Oxygen (PO_2) (mmHg), (B) partial pressure of CO_2 (PCO_2) (mmHg). Repeated measures ANOVA was not significant. Data displayed as Mean (SD).

A



B

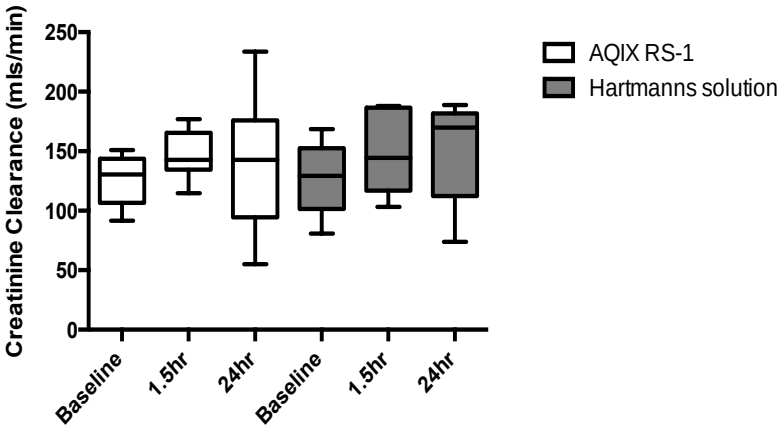

50
40
30
20
10
0
PCO2 (mmHg)
-60 min
-30 min
0 min
30 min
60 min
90 min
120 min
+ 0 min
+ 30 min
24 hr
AQIX-RS-1
Hartmanns solution

5.2.2 Assessment of Renal function

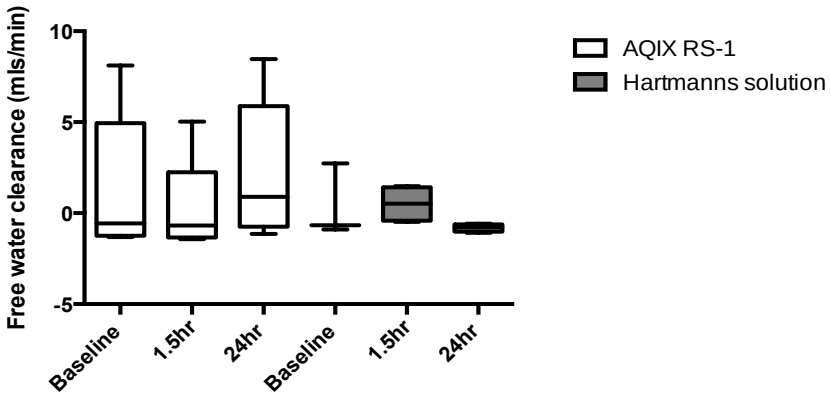
Functional measures of renal function such as creatinine clearance, free water clearance and fractional excretion of sodium were not different among groups (Figure [5.3] A-C), In additional there was no difference in release of novel renal biomarker; NGAL (Figure [5.3] D-E).

Figure [5.3]: Renal Function; (A) Creatinine clearance (mls/min), (B) Free Water Clearance (mls/min), (C) Fractional excretion of Sodium (%), (D) Urinary NGAL (ng/ml), (E) Urinary NGAL: Creatinine ratio. Significant inter-group differences ($p < 0.05$); (D) The interaction effect of treatment and time was not statistically significant ($p=0.134$) on the mean NGAL values. The mean NGAL values of AQIX and Hartmans treatment groups were not significantly different. However, the mean NGAL at 24h, irrespective of treatment groups, increased significantly ($p<0.001$) compared with the mean NGAL at 1.5h (*), (E) The interaction effect of treatment and time was not statistically significant ($p=0.125$) on the mean NGAL/Cr ratio. The mean NGAL/Cr ratio values of AQIX and Hartmans treatment groups were not significantly different ($p=0.421$). However, the mean NGAL/Cr ratio at 24h, irrespective of treatment groups, increased significantly ($p<0.001$) compared with the mean NGAL/Cr ratio at 1.5h (*) . Data presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data.

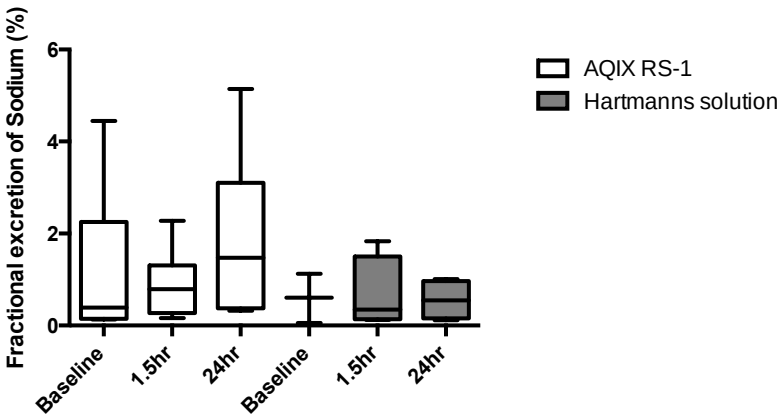
A

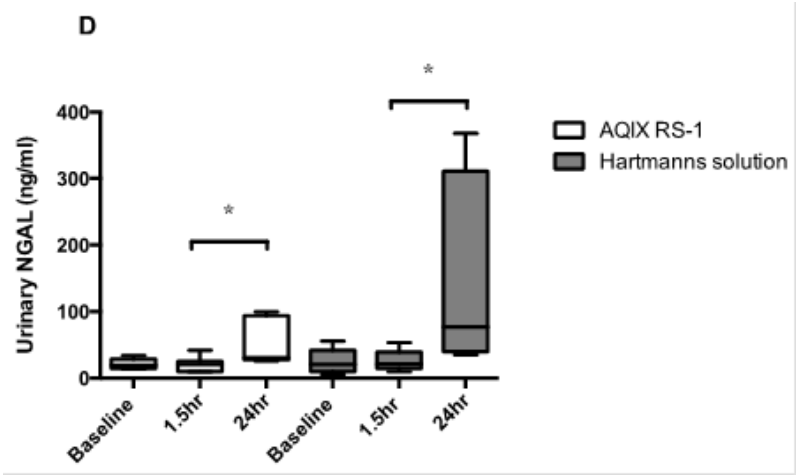

Creatinine Clearance (mls/min)
250
200
150
100
50
0
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

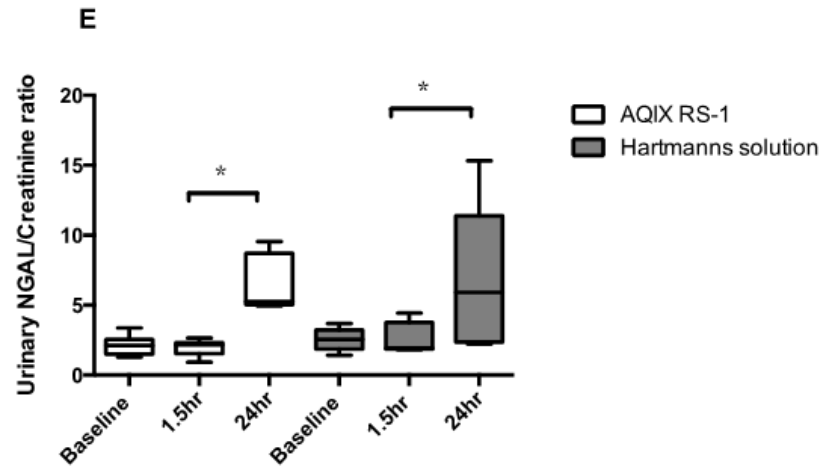
B


Free water clearance (mls/min)
10
5
0
-5
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

C


Fractional excretion of Sodium (%)
6
4
2
0
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

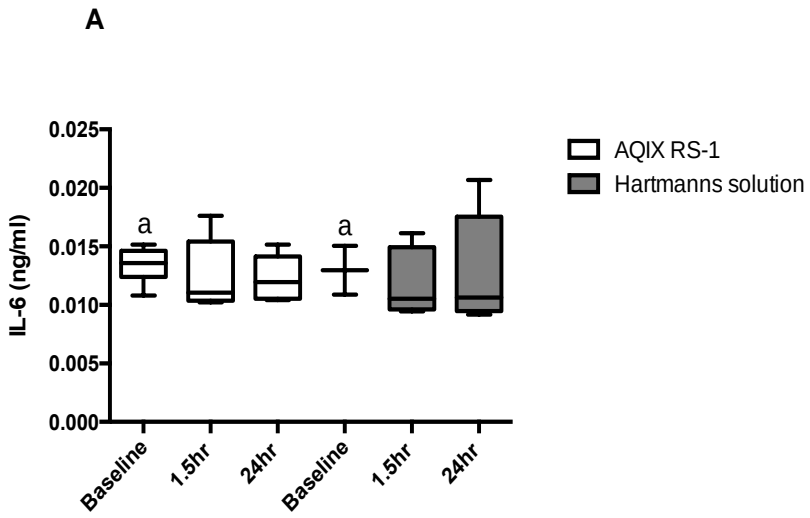

D
Urinary NGAL (ng/ml)
400
300
200
100
0
*
*
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution


E
Urinary NGAL/Creatinine ratio
20
15
10
5
0
*
*
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

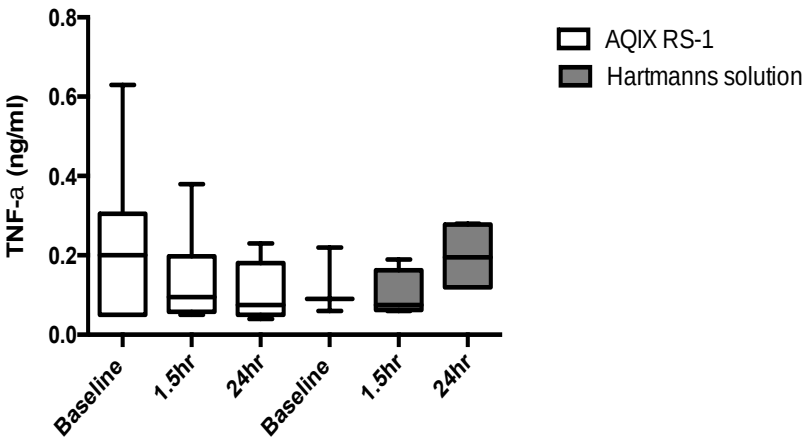
5.2.3 Assessment of systemic inflammation

We assessed systemic inflammation using cytokines; IL-6 and TNF- α . There were significant differences in baseline for IL-6, however no group differences were observed for both markers at any time point (Figure [5.4] A-B).

Figure [5.4]: Assessment of systemic inflammation; (A) Serum IL-6 (ng/ml), (B) TNF-Alpha (ng/ml). [a] : Significant baseline differences between groups. Repeated measures ANOVA was not significant for time or treatment effects or their interaction. Data presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data.



B

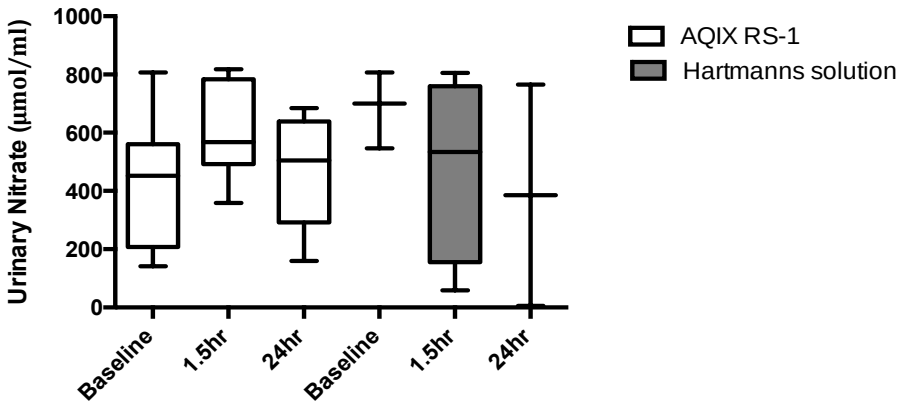

TNF-α (ng/ml)
0.8
0.6
0.4
0.2
0.0
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

5.2.4 Assessment of NO bioavailability and In vivo Endothelial function

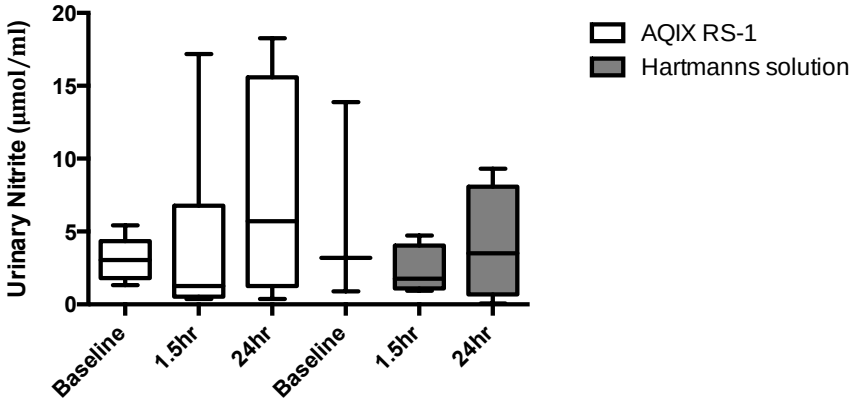
When assessed for measures of NO bioavailability using urinary nitrate and nitrite, we found no significant differences (Figures [5.5] A-B) between groups, in addition there was no difference in there combined ratio i.e. NO bioavailability (Figure [5.5] C). There was no difference in vasomotor renal endothelial function as assessed by supra-renal acetylcholine infused changes in RBF (Figure [5.5] D).

Figure [5.5]: Assessment of nitric oxide bioavailability. (A) Urinary Nitrate (μ mol/L), (B) Urinary Nitrite (μ mol/L), (C) Nitric Oxide (μ mol/L), (D) Acetylcholine induced changes on renal artery flow (mls/kg/min). Three consecutive 5-minute baseline periods are shown with successive three 5-minute experimental periods using supra-renal acetylcholine infusion. Significant intergroup difference ($p<0.05$): (C): The interaction effect of treatment and time was not statistically significant ($p=0.163$) on the mean NO values. The mean NO values of AQIX and Hartmans treatment groups were not significantly different ($p=0.927$). Data presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data. Data for renal blood flow presented as Mean with error bars representing SD.

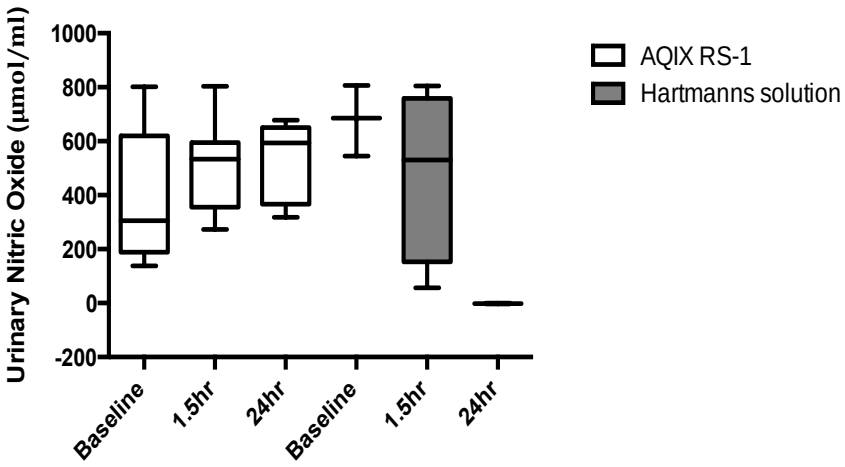
A


Urinary Nitrate (µmol/ml)
1000
800
600
400
200
0
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

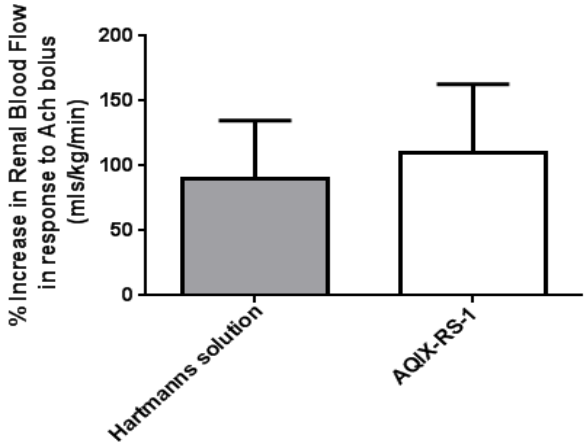
B


Urinary Nitrite (µmol/ml)
20
15
10
5
0
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

C


Urinary Nitric Oxide (µmol/ml)
1000
800
600
400
200
0
-200
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

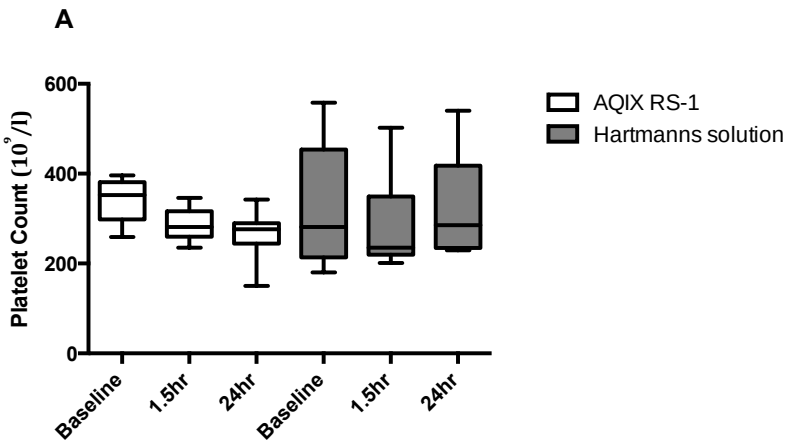
D


% Increase in Renal Blood Flow in response to Ach bolus (mls/kg/min)
200
150
100
50
0
Hartmanns solution
AQIX-RS-1

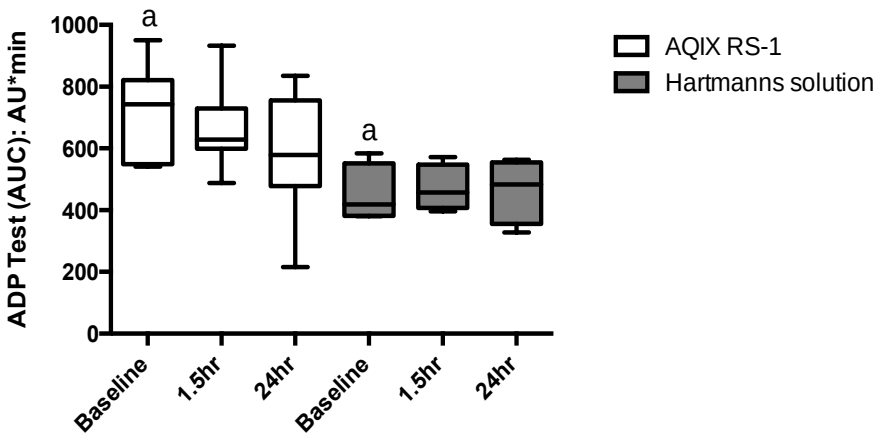
5.2.5 Assessment of Coagulation

There was no difference on platelet counts at either time points (Figure [5.6] A). Significant baseline differences were observed for ADP test between groups, however no inter-group differences were observed for platelet aggregation (Figure [5.6] B-C).

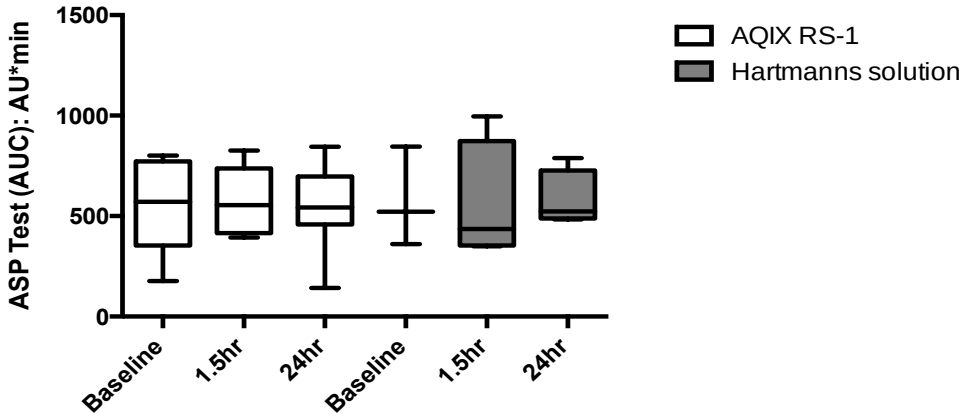
Figure [5.6]: Assessment of coagulation. (A) Platelet count (10^9/l), (B) ADP test (AU* min), (C) ASP test (AU*min), Significant intergroup difference ($p<0.05$): (A): (B): [a]: Significant baseline differences between groups. Repeated measures ANOVA was not significant for time or treatment effects or their interaction. Data presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data.



B


ADP Test (AUC): AU*min
1000
800
600
400
200
0
a
a
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

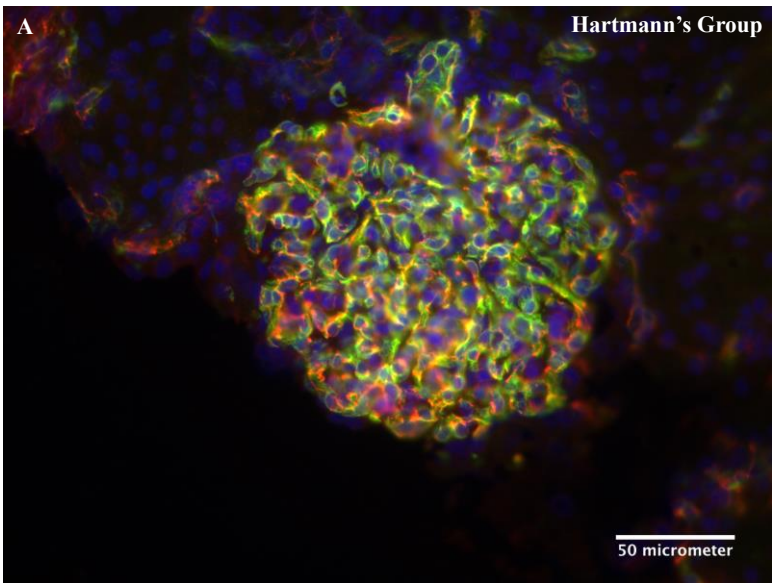
C

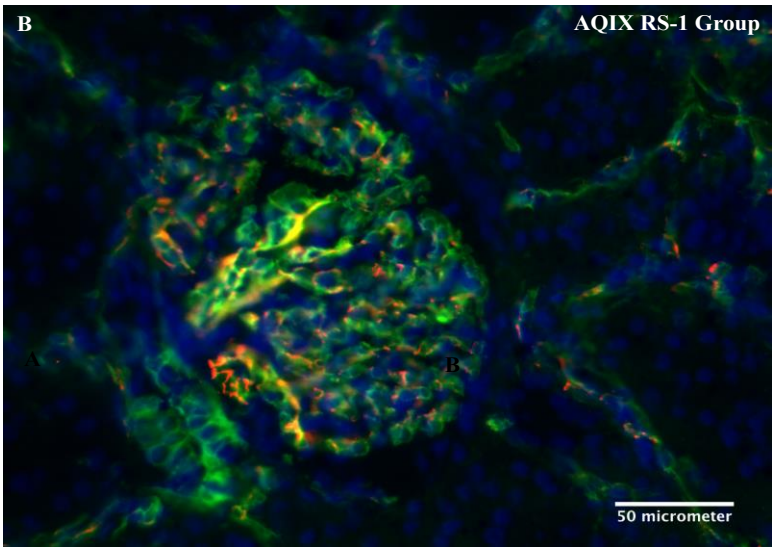

ASP Test (AUC): AU*min
1500
1000
500
0
Baseline
1.5hr
24hr
Baseline
1.5hr
24hr
AQIX RS-1
Hartmanns solution

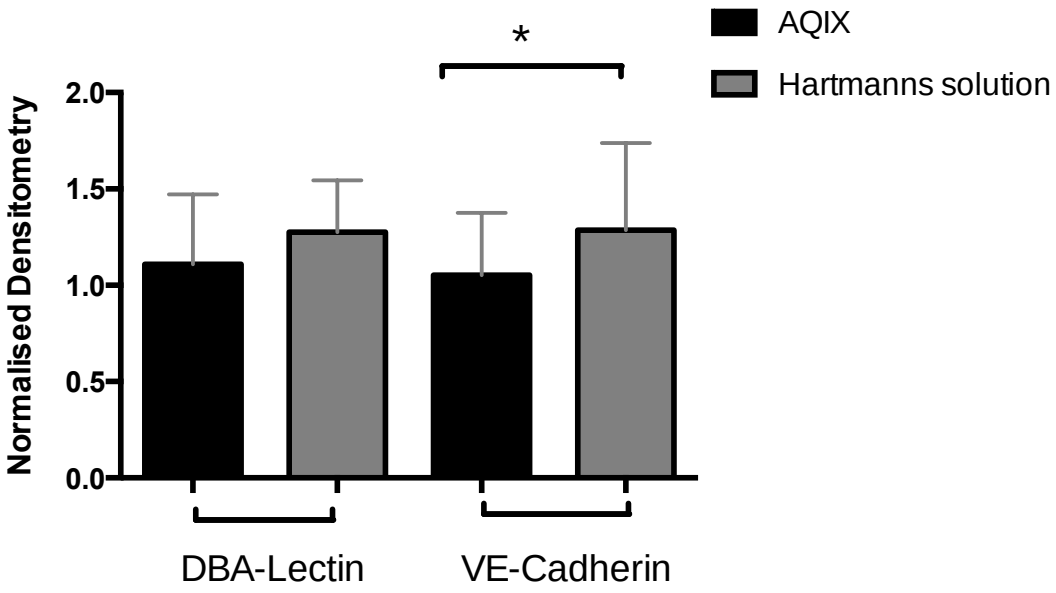
5.2.6 Histological characterization of components of glomerular endothelial membrane.

We assessed for group differences for potential changes in glycocalyx GAG and endothelial cell-cell junctional proteins using DBA lectin and VE-Cadherin respectively. There was a significant reduction in staining for VE-Cadherin in AQIX group but no changes were observed for DBA lectin staining (Figure [5.7] A-C).

Figure [5.7]: Effect of AQIX RS-1 vs. Hartman treatment on components of glomerular endothelial membrane. (A) Representative single glomerulus from image samples analysed in Hartmann's group, (B) Representative image of a single glomerulus from image samples analysed in AQIX group, (C) Immunofluorescence densitometry. *: $p<0.05$. 48 glomeruli were simultaneously analysed for AQIX group (n=24, 4 animals) and Hartmann's group (n=24, 4 animals) using co- staining for GAG components of glycocalyx (DBA lectin: green), Endothelial cell-cell junctional proteins (VE-Cadherin: Red) and adjusted for DAPI: blue, Imaged at 40 X. Data presented as Mean (SD).




B
AQIX RS-1 Group
A
B
50 micrometer


AQIX
Hartmanns solution
*
Normalised Densitometry
2.0
1.5
1.0
0.5
0.0
DBA-Lectin
VE-Cadherin

Chapter 6

Experimental results

Endothelial injury and recovery: Role of CPB and sildenafil on morphological expressions of glomerular endothelial membrane in post-cardiac surgery AKI.

6.1 Plan of Investigation

6.1.1 Rationale

Using large animal model of post CPB AKI it has been shown that Sildenafil pretreatment negates post CPB Acute Kidney Injury (Patel et al., 2011a). The effects could be either due to preservation of structural integrity (constituents of glycocalyx ± surface antigens) or functional changes i.e. ischemic preconditioning with Sildenafil or macro/microvascular flow changes. This insight will further characterize the effects of CPB on renal glomerular endothelial membrane.

6.1.2 Aims

To evaluate the effects of Sildenafil Citrate, a PDE-5 inhibitor that promotes endothelial homeostasis, on post cardiopulmonary bypass (CPB) acute kidney injury and to determine whether the effects were exerted by preservation of endothelial and glycocaleceal integrity.

6.1.3 Hypotheses

1. CPB leads to damage of Endothelial cell-cell junctions and surface proteins.
2. CPB causes loss of Glycosaminoglycan (GAG) and Core-protein components of glycocalyx.
3. CPB will lead to damage to other components of GEM i.e. podocytes and basement membrane.
4. The extent of GEM damage is proportional to exposure of pro-inflammatory CPB serum.
5. Pre-treatment with sildenafil will protect against CPB mediated damage to GEM.

6.1.4 Design

An experimental single factor, pretest-posttest repeated measures design was used; Pigs (n=8 per group) were randomised to;
Group 1: Sham Operation: negative control. Pigs underwent a neck dissection under general anaesthesia and received 4500ml of Hartmann's solution.
Group 2: Cardiopulmonary bypass, Positive control. Pigs underwent 2.5hrs of CPB and received 4500mls of Hartmann's solution including as pump prime.
Group 3: Cardiopulmonary Bypass plus Sildenafil (Revatio®) pretreatment.

6.1.5 Outcomes

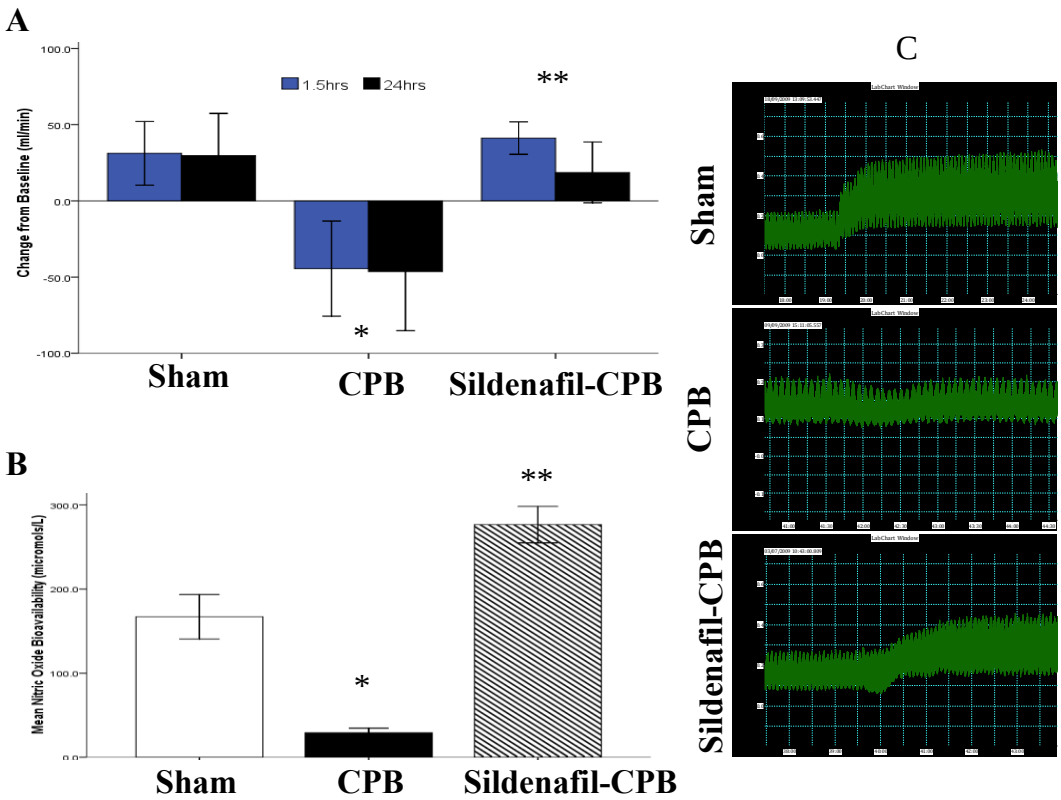
1. Characterization and evaluation of morphological changes in expression of GAG and Core protein constituents of glycocalyx including endothelial surface antigens in sham and CPB +/- sildenafil using immunofluorescence.
2. Quantitative measurements of changes in expression of glycocalyx/endothelial components with gel electrophoresis.
3. Mechanistic investigation of effect of CPB with or without sildenafil treated sera on expression of glycocalyx expressed on conditionally immortalized glomerular endothelial cells (ciGEnC's) using cell culture.

6.2 Results

6.2.1 Effect of Cardiopulmonary bypass and Sildenafil on in vivo renal function and Nitric Oxide bioavailability

CPB exposure is an independent risk factor for post-CPB AKI. In order to investigate the characteristics of this lesion, Mr. Nishith Patel (Patel et al., 2011a) and our research team investigated the role of Sildenafil in post CPB AKI in a porcine model. Swine in this model were exposed to 2.5hr of CPB with or without sildenafil pretreatment and assessed for primary outcome; Acute Kidney Injury defined as a > 25% reduction in Creatinine Clearance and simultaneous measures of in vivo endothelial function (using acetylcholine induced changes in renal blood flow) and NO bioavailability (using urinary nitrate and nitrite) were undertaken. Results demonstrated clear reduction in creatinine clearance with CPB that was associated with loss of NO bioavailability and renal artery endothelial dysfunction. Not only did Sildenafil pretreatment prevented measures of NO bioavailability but preserved endothelial function that translated in preservation of renal function (Figure 6.1]).

Figure [6.1]:Effect of Sildenafil pre-treatment on in vivo renal function measured using creatinine clearance, NO bioavailability and endothelial function, (A) Creatinine clearance (mls/min)(B): Nitric Oxide bioavailability (μ mol/L), (C) Renal Blood Flow(Green trace) measurement using renal artery probe in response to supra-renal acetylcholine infusion. *: p <0.5 vs. Sham, ** vs. CPB.



6.2.2 Effect of CPB and Sildenafil pre-treatment on the expression of glycosaminoglycans (GAG) side chains and Core proteins of glycocalyx.

The results of immunostaining are shown in figure [6.2] with representative optical density analyses shown in figure [6.3]. Western blot quantifications of expression of core protein in renal cortex lysates are shown in figure [6.4] along with optical densities.

Cardiopulmonary bypass caused a significant loss of GAG components of glycocalyx as demonstrated with immunofluorescence targeting DBA and WGA specific carbohydrate ligands. Interestingly, SBA specific ligands were significantly altered in glomeruli from Sildenafil treated swine. UEA specific ligands remained unmodified. Co-staining syndecan-4 (core-protein marker) either with a lectin probe; DBA or EC cell-cell junctional marker; VE-Cadherin, demonstrated significant loss of this core protein with CPB with no effect of Sildenafil pre-treatment. Expression of Syndecan-4 in cortex lysates was not significantly different among groups however Syndecan-1 core protein was overexpressed in CPB group (Figure [6.4]).

Figure [6.2]: Immunofluorescence panel-Effect of CPB and Sildenafil on co-expression of GAG component of glycocalyx and core proteins: (A) Effect of CPB and Sildenafil on co-expression of GAG component of glycocalyx using DBA-lectin and EC cell-cell junctional marker; VE-Cadherin. Green: DBA-lectin, Red: VE-Cadherin, Blue: DAPI (B) WGA-lectin and EC cell-cell junctional marker; VE-Cadherin. Green: WGA-lectin, Red: VE-Cadherin, Blue: DAPI. (C) Effect of CPB and Sildenafil on co-expression of GAG component of glycocalyx using SBA-lectin and EC cell-cell junctional marker; VE-Cadherin. Green: SBA-lectin, Red: VE-Cadherin, Blue: DAPI. (D) Effect of CPB and Sildenafil on co-expression of GAG component of glycocalyx using UEA-lectin and EC cell-cell junctional marker; VE-Cadherin. Green: UEA-lectin, Red: VE-Cadherin, Blue: DAPI. (E) Effect of CPB and Sildenafil on co-expression of Syndecan-4 core protein and Glycosaminoglycans using co-staining with anti sydecan-4 antibody and DBA lectin respectively. Green: DBA lectin, Red Syndecan-4, Blue: DAPI. (F) Effect of CPB and Sildenafil on co-

expression of Syndecan-4 core protein and EC cell-cell junctional protein; VE-Cadherin using co-staining with anti sydecan-4 and VE-Cadherin antibodies respectively. Green: Syndecan-4, Red: VE-Cadherin, Blue: DAPI. All Immunofluorescence carried out on 5um frozen sections of renal cortex. Imaged at 40X primary objective. 24 glomeruli where analysed in each condition (4 animals per group). Scale bar: 50 μ m.

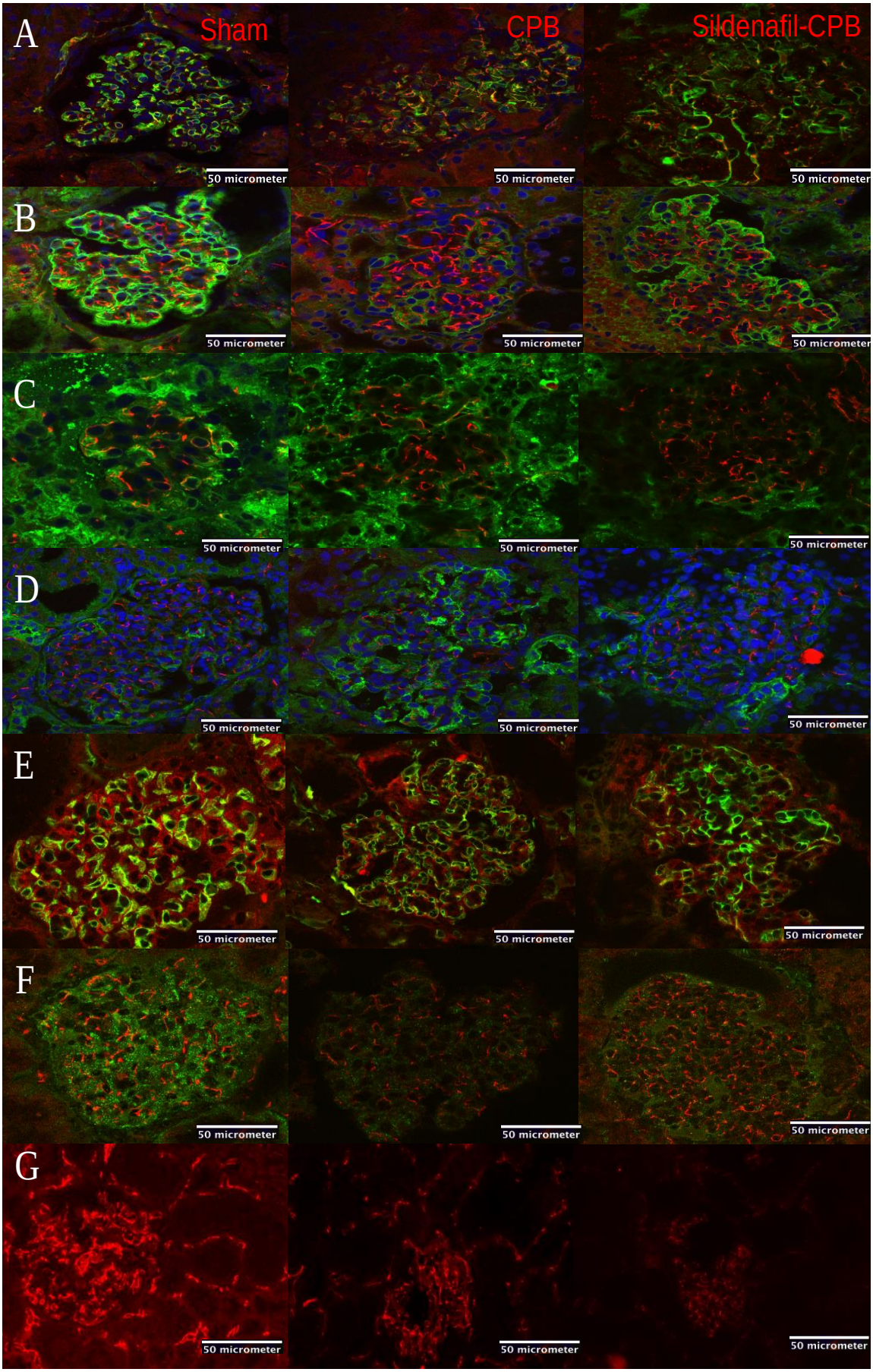
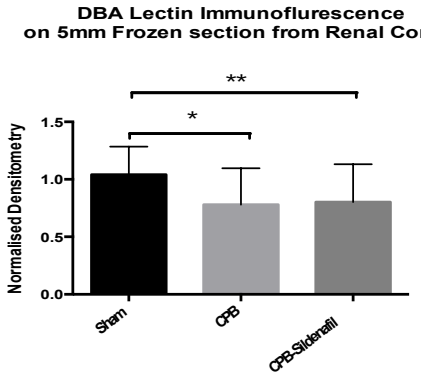
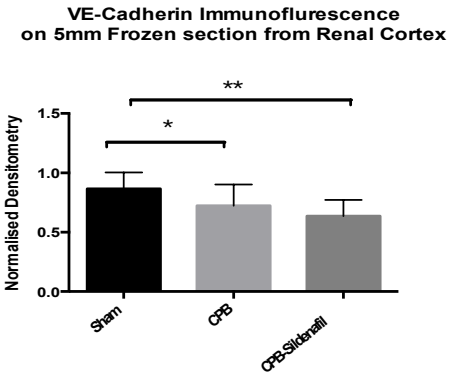

A
Sham
CPB
Sildenafil-CPB
B
C
D
E
F
G
50 micrometer

Figure [6.3]: Immunofluorescence densitometry-Effect of CPB and Sildenafil on co-expression of GAG component of glycocalyx and core proteins: Effect of CPB and Sildenafil on co-expression of GAG component of glycocalyx using DBA-lectin (A-1) and EC cell-cell junctional marker; VE-Cadherin (A-2). (B-1): WGA-lectin and EC cell-cell junctional marker; VE-Cadherin (B-2). Effect of CPB and Sildenafil on co-expression of GAG component of glycocalyx using SBA-lectin (C-1) and EC cell-cell junctional marker; VE-Cadherin (C-2). Effect of CPB and Sildenafil on co-expression of GAG component of glycocalyx using UEA-lectin (D-1) and EC cell-cell junctional marker; VE-Cadherin (D-2). Effect of CPB and Sildenafil on co-expression of Syndecan-4 core protein (E-1) and Glycosaminoglycans using co-staining with DBA lectin (E-2) respectively. Effect of CPB and Sildenafil on co-expression of Syndecan-4 core protein (F-1) and EC cell-cell junctional protein; VE-Cadherin (F-2). * $p<0.05$ sham vs. CPB, **$p<0.05$ CPB vs. Sildenafil-CPB, NS: not significant.

A-1



A-2



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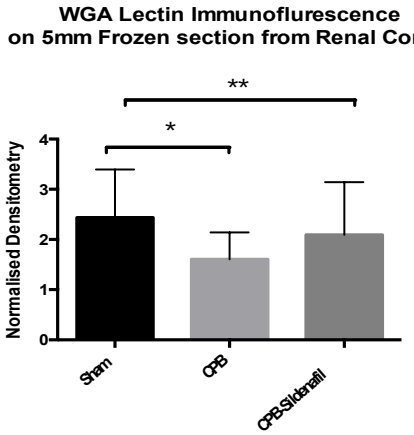
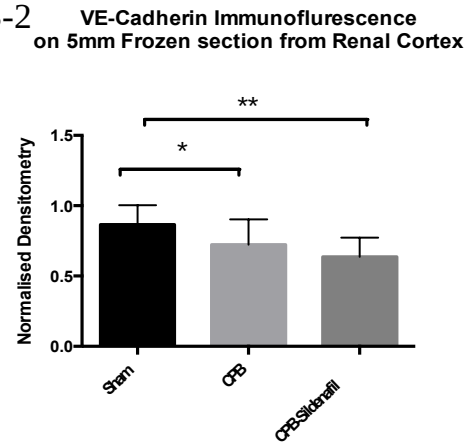
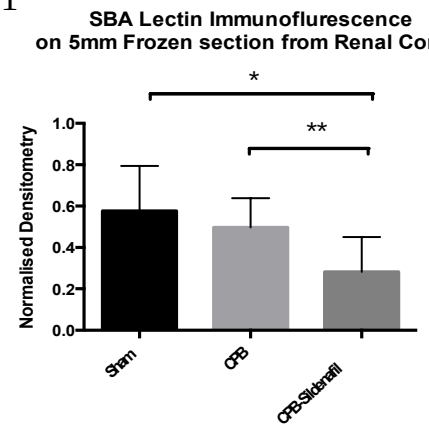


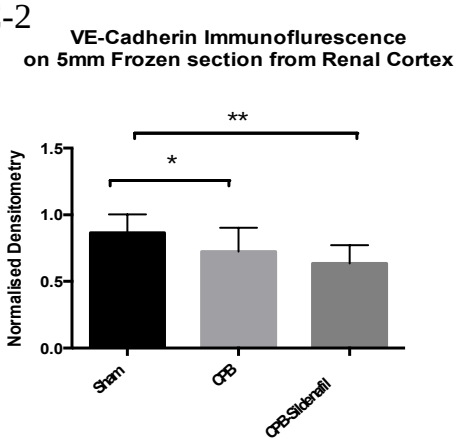
B-2



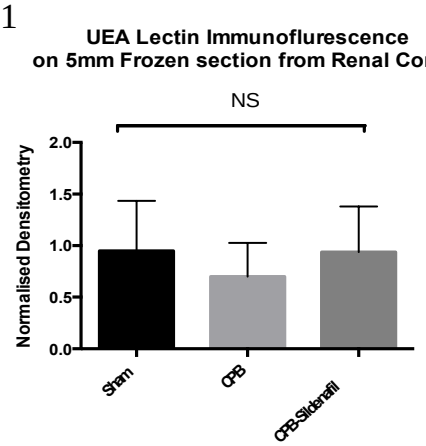
C-1



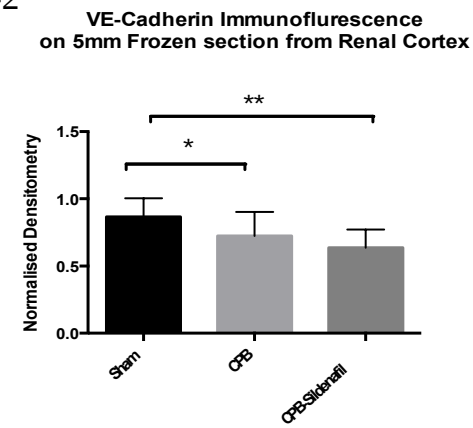
C-2



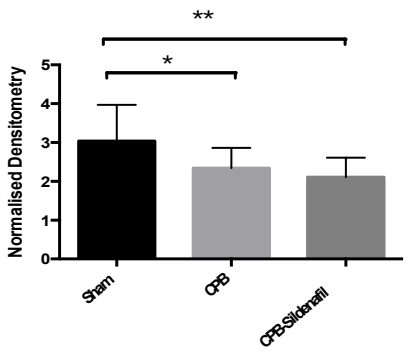
D-1



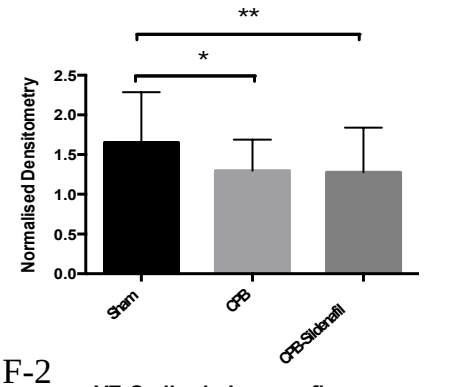
D-2



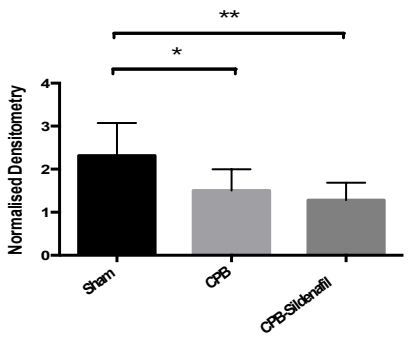
E-1



E-2



F-1



F-2

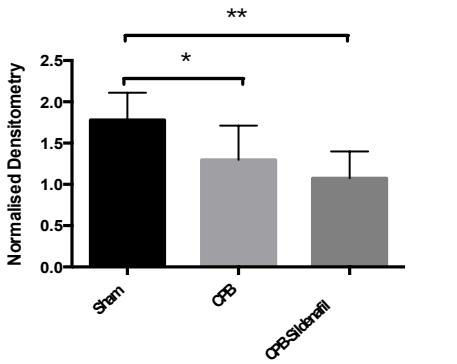
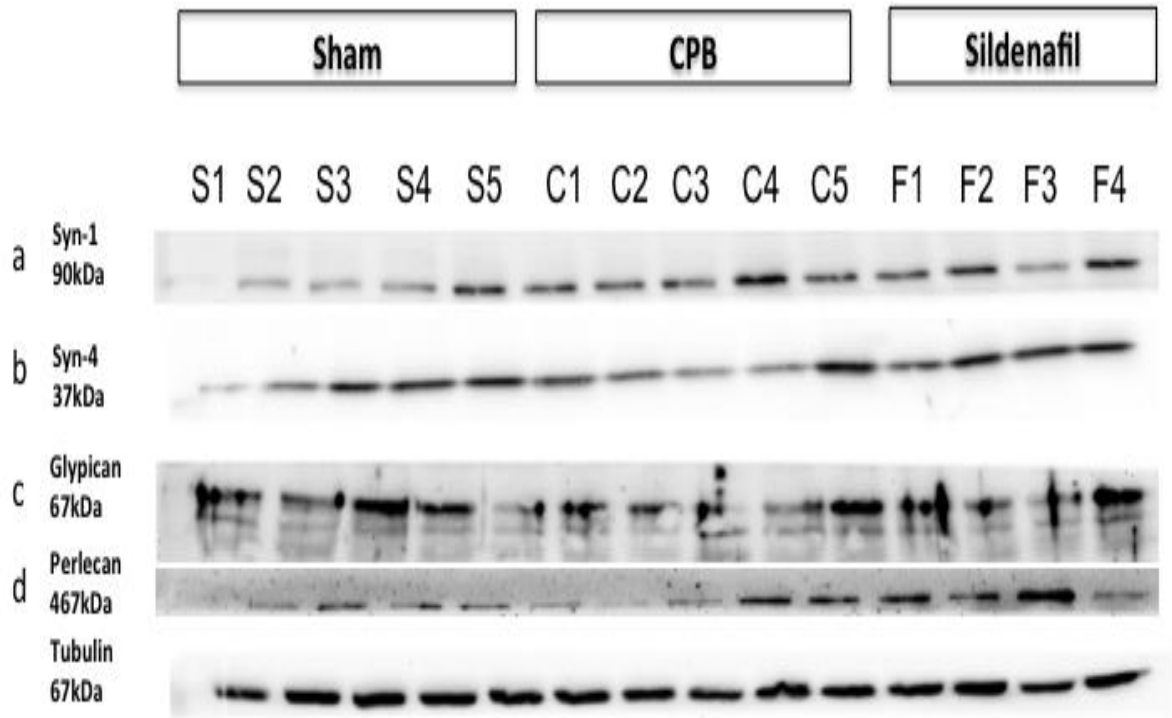
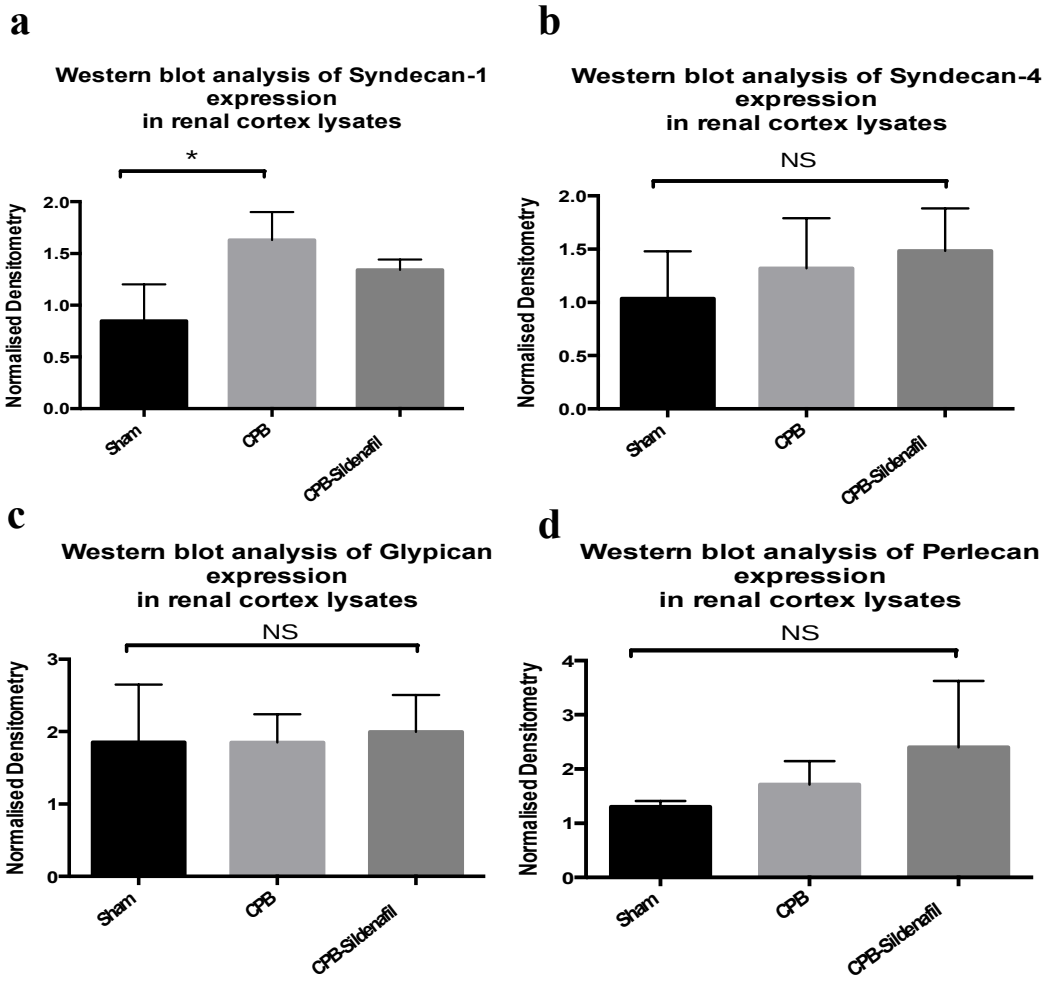


Figure [6.4]: Western blot analyses with densitometry-Expressions of glycocalyx core proteins; (a) Syndecan-1(Syn-1) (b) Syndecan-4 (Syn-4) (c) Glypican and (d) Perlecan. Sham animals (S1-S5), CPB animals (C1-C5), Sildenafil-CPB animals (F1-F4). * $p<0.05$ sham vs. CPB. Tubulin was used as internal control. NS: not significant.



Western Blot Densitometry



6.2.3 Effect of CPB and Sildenafil pre-treatment on the expression of endothelial surface markers

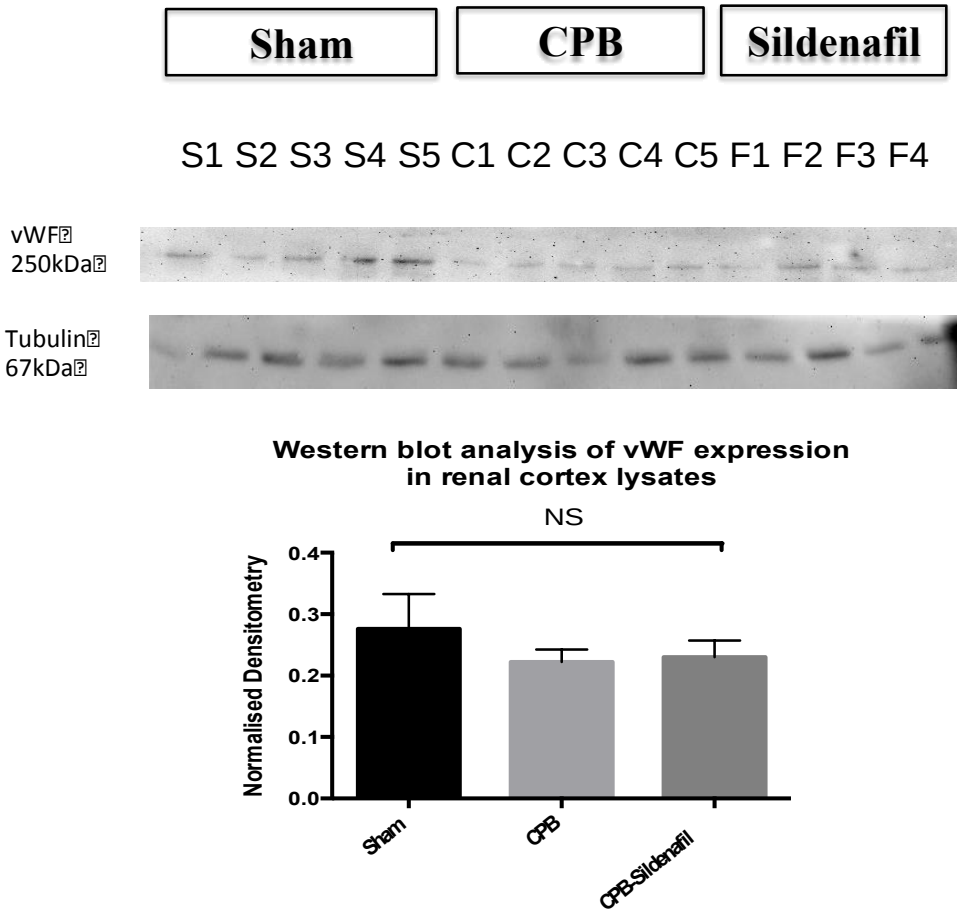
6.2.3.1 Endothelial cell-cell junctional protein; VE-Cadherin

VE-Cadherin, a key endothelial cell-cell junctional protein regulating permeability was significantly lost in CPB exposed glomeruli, while Sildenafil pre-treatment had no effect on its expression (Immunofluorescence panel, Figure [6.2-G])

6.2.3.2 Endothelial surface vWF

Von Willebrand factor (vWF) is an endothelial cell surface glycoprotein involved in critical interactions between endothelium, factor 8, platelets and collagen during coagulation. Using western blotting, we demonstrated that CPB or sildenafil pre-treatment had no significant effect on vWF expression (Figure [6.5]).

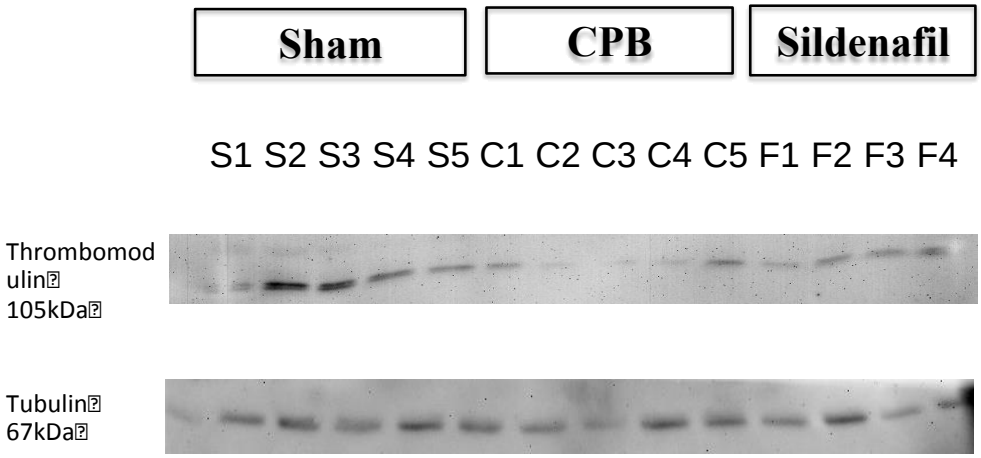
Figure [6.5]: Western blot analysis of vWF expressions using cortex lysates. Sham animals (S1-S5), CPB animals (C1-C5), Sildenafil-CPB treated animals (F1-F4).


Sham
CPB
Sildenafil
S1 S2 S3 S4 S5 C1 C2 C3 C4 C5 F1 F2 F3 F4
vWF
250kDa
Tubulin
67kDa
Western blot analysis of vWF expression in renal cortex lysates
NS
Normalised Densitometry
0.4
0.3
0.2
0.1
0.0
Sham
CPB
CPB-Sildenafil

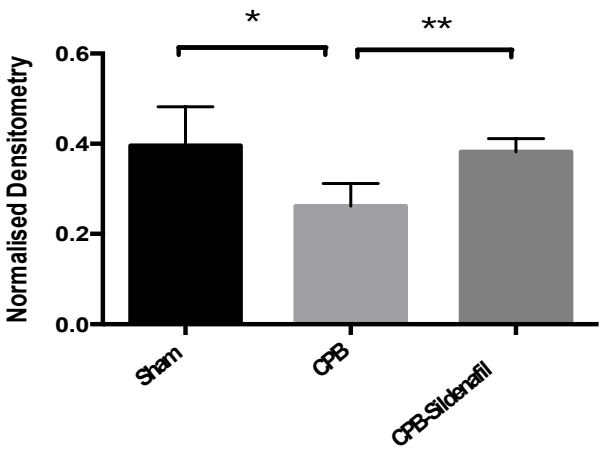
6.2.3.3 Endothelial surface Thrombomodulin

Thrombomodulin is an EC surface protein, which acts as a cofactor for thrombin and regulates C3b inactivation by factor I. Thrombomodulin-Thrombin complex activates protein C and initiates an anticoagulant cascade. Our western blot analysis demonstrated that CPB causes loss of this protein, while Sildenafil pre-treatment prevents its degradation; Figure: [6.6].

Figure [6.6]: Western blot analysis of thrombomodulin expressions using cortex lysates. Sham animals (S1-S5), CPB animals (C1-C5), Sildenafil-CPB treated animals (F1-F4). Densitometry: * $p<0.05$ Sham vs. CPB, **: CPB-Sildenafil vs. CPB.



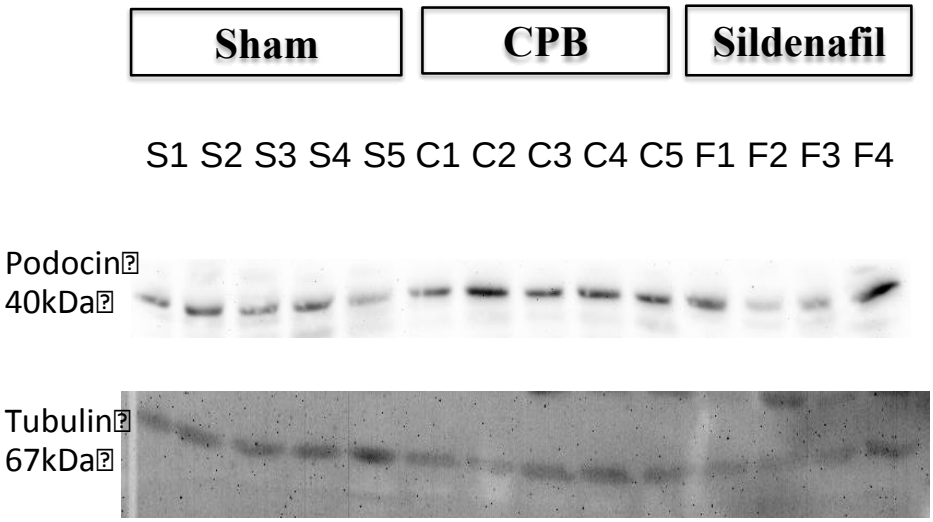
Western blot analysis of Thrombomodulin expression in renal cortex lysates

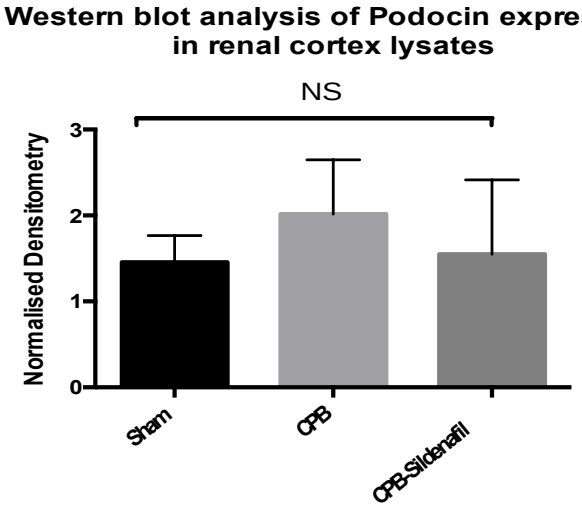


6.2.4 Effect of CPB ± Sildenafil pre-treatment on Podocytes

Morphologic abnormalities of podocyte foot processes and slit diaphragms caused by CPB are well known. Podocin an integral component of slit diaphragm and interact with basement membrane to prevent proteinuria. Our analysis did not demonstrate a significant change in its expression (Figure [6.7])

Figure [6.7]: Western blot analysis of podocin expressions using cortex lysates. Sham animals (S1-S5), CPB animals (C1-C5), Sildenafil-CPB exposed animals (F1-F4). Densitometry: not significant.


Sham
CPB
Sildenafil
S1 S2 S3 S4 S5 C1 C2 C3 C4 C5 F1 F2 F3 F4
Podocin
40kDa
Tubulin
67kDa

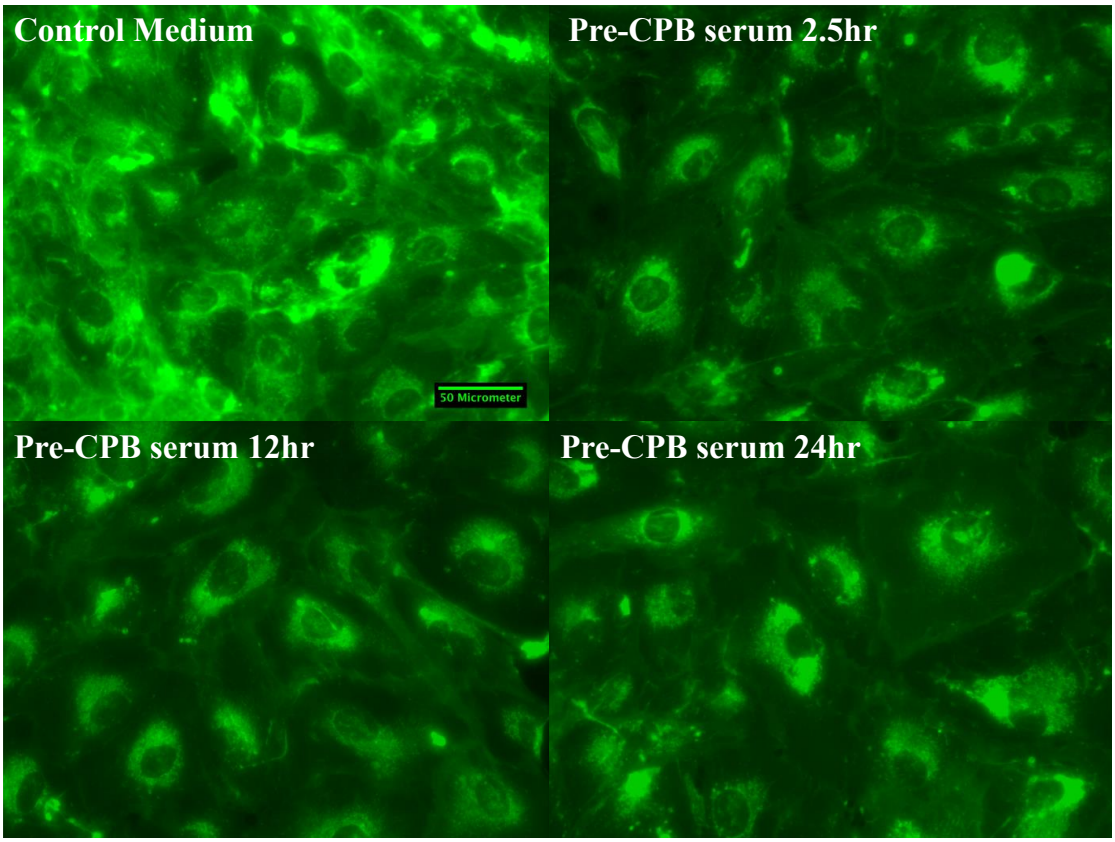

Western blot analysis of Podocin expression in renal cortex lysates
NS
Normalised Densitometry
0
1
2
3
Sham
CPB
CPB-Sildenafil

6.2.6 In vitro assessment of effect of differential exposure of CPB sera on ciGnEC's expression of GAG side chains of glycocalyx.

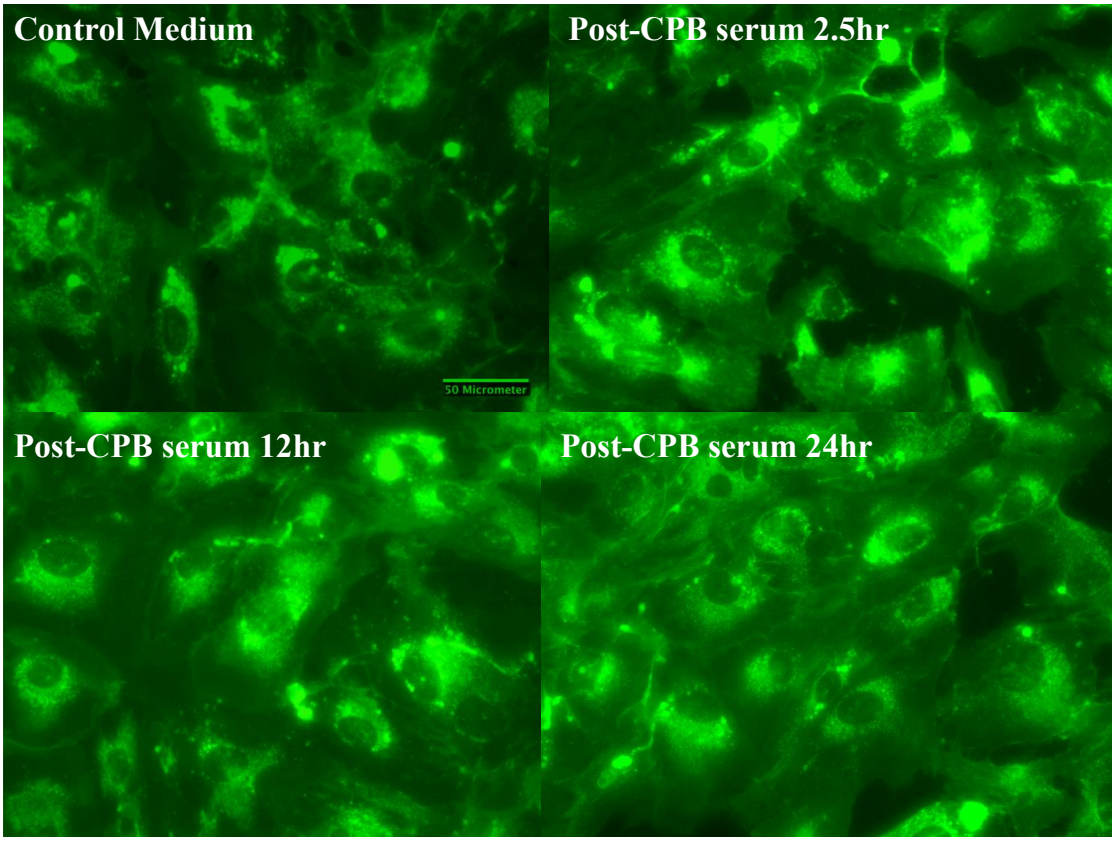
To further explore the relationship between CPB and Sildenafil pretreatment on endothelial expression of glycocalyx we explored the effect of pre-CPB and post-CPB sera on WGA lectin specific ligands of endothelial glycocalyx. We investigated the effects of differential exposures of pre CPB (Experiment 1; figure [6.8] Panel A) and post CPB sera (Experiment 2; figure [6.8] Panel B) using 2.5, 12 and 24hr incubation periods. The control ciGnEC's were incubated in EGM-2 without any sera. Incubation with pre CPB sera (No intervention) demonstrated reduction in WGA lectin-GAG expression vs. control, however incubation with post CPB serum caused overexpression of WGA specific GAG ligands in a exposure-time-response manner on ciGnEC's.

Figure [6.8]: Effect of differential CPB serum exposures on WGA lectin-GAG expression of ciGnEC's. (Panel A: Experiment 1): Effect of differential exposure analysis of Pre CPB sera on WGA lectin-GAG expression, (Panel B: Experiment 2): Effect of differential exposure analysis of Post CPB sera on WGA lectin expressions. Green: WGA lectin, Blue: DAPI, Imaged at 40 X. Bar graph: Normalized Densitometry: a: Experiment 1, * p< 0.05 Sham vs. Pre CPB serum 2.5hr-24hrs. B: Experiment 2, **p< 0.05 Post CPB serum 24hr vs. Complete medium. Scale bar: 50 μ m.

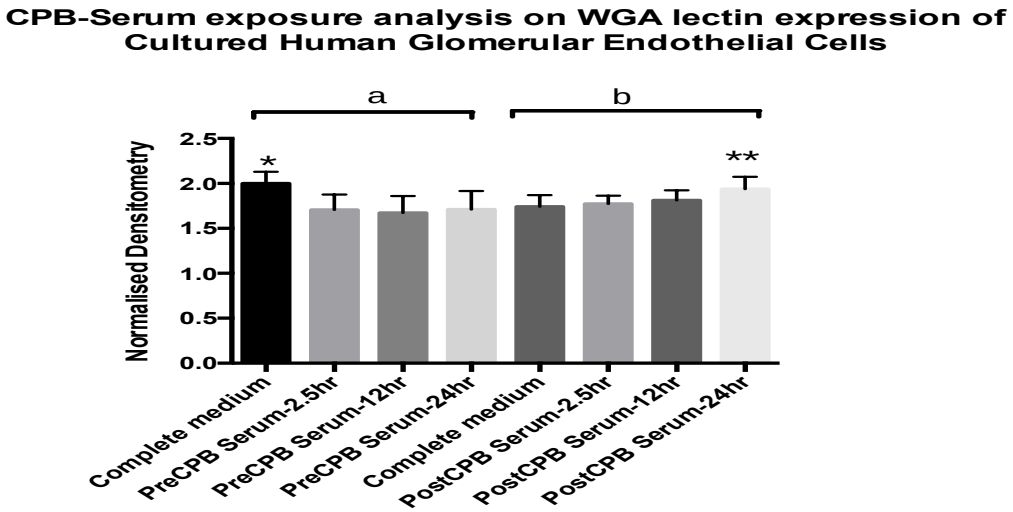
Panel A



Panel B



Immunofluorescence densitometry

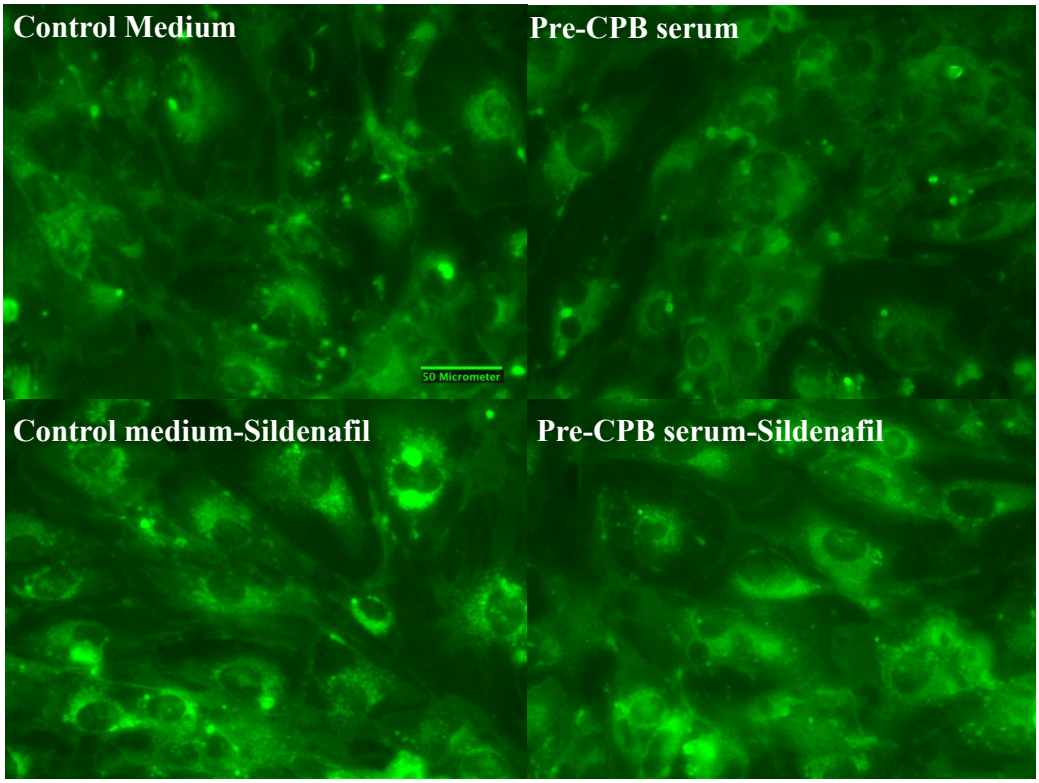


6.2.7 In vitro assessment of effect of sildenafil pre-treated CPB sera on ciGnEC's GAG side chains of glycocalyx.

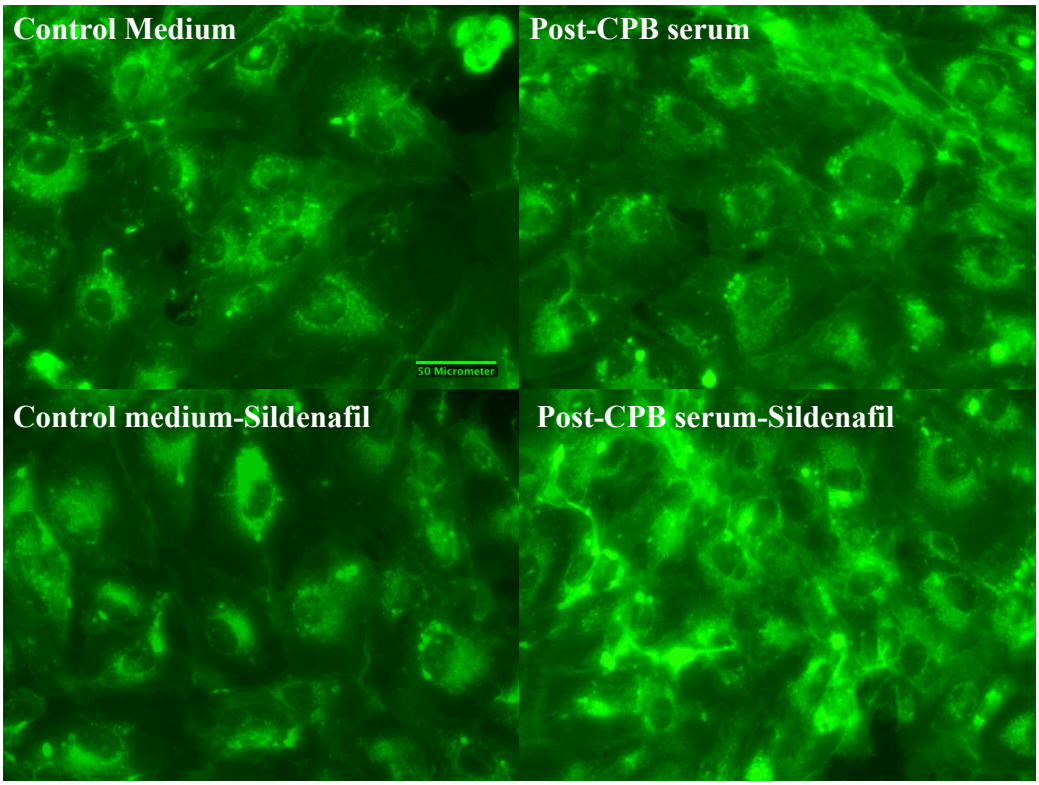
After ascertaining the effect of CPB sera on WGA lectin-GAG specific ligands in experiment 1 and 2, we proceeded to investigate the effect of Sildenafil pre-treatment on GAG expression with 24hr incubation using Complete medium as control and pre CPB and post CPB sera as interventions. 24hr incubation with pre CPB (No intervention) had no effect on WGA lectin-GAG expression, however 24 hr incubation with either CPB or sildenafil pre-treated CPB sera induced overexpression of these ligands. Sildenafil pre-treatment did not alter the changes induced by CPB sera (Figure 6.9, Panel A and B).

Figure [6.9]: Effect of differential CPB serum exposures on WGA expression of cultured Human Glomerular Endothelial cells. (Experiment 3, Panel A): Effect of Pre CPB serum ± Sildenafil pre-treatment on WGA lectin-GAG expression, (Experiment 4, Panel B): Effect of Post CPB serum ± Sildenafil pre-treatment on WGA lectin-GAG expressions. Green: WGA lectin, Blue: DAPI, Imaged at 40 X. Bar graph: Normalized Densitometry: Experiment 3, no significant results B: Experiment 4, *p< 0.05 Post CPB serum vs. Complete medium, ** Post CPB serum with Sildenafil pre-treatment vs. Complete medium. Scale bar: 50 μ m.

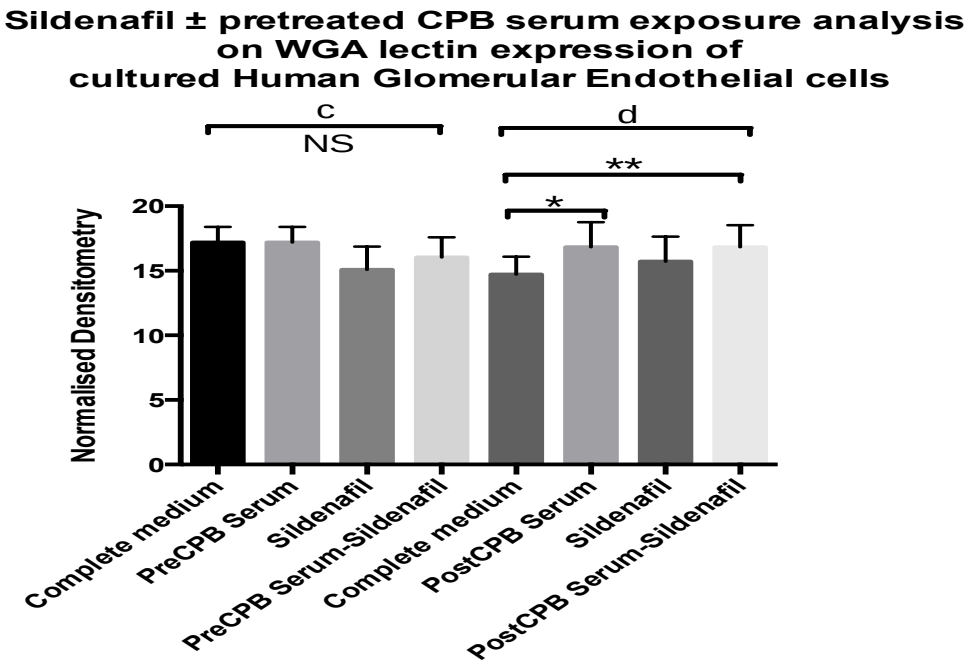
Panel A



Panel B



Immunofluorescence densitometry



Chapter 7

Experimental results

Effect of Red cell Rejuvenation-washing on post-storage characteristics of red cell units and In vivo assessment of organ injury in a porcine model of allogenic transfusion.

7.1 Synopsis

The experimental work in this chapter has three distinct primary hypotheses;
1-Effect of storage, washing and red cell rejuvenation on in Vitro assessment of storage lesion
This study was conducted on 7 red cell units obtained from two large (80-100kg) animals and processed according to NHSBT protocol and stored for 14 days. These units were serially analysed over the following 14-day period on 0, 1, 7 and 14 days. On the 14th day, these units were rejuvenated and washed by myself and analysed for measures of storage lesion. This study is shown in Figure 7.1-A.

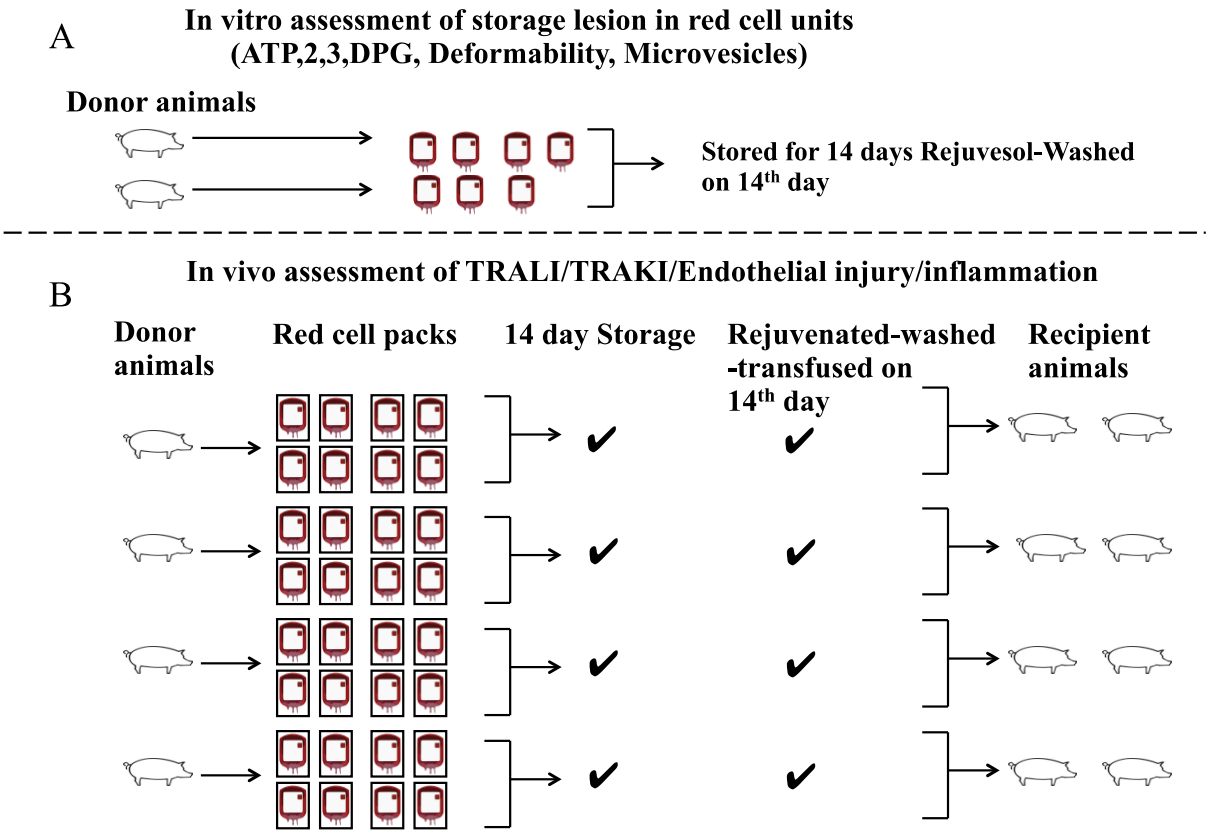
2- Effect of storage, washing and red cell rejuvenation on in vivo assessment of Transfusion Related Acute Lung Injury (TRALI), endothelial injury, inflammation and organ injury

This study was commenced with allogenic blood retrieval from donor animals similar to in vitro study, stored for 14 days, followed by Rejuvenation-washing on 14th day and transfusion in recipient pigs weighing 50-60kg on the same day. Single donor animal allowed collection of 8 red cell units, which were transfused, to two recipient animals (4 units per animal). These sequential steps are shown in Figure 7.1-B. Recipient experiments involving Groups 1-4 (See 7.2.4) were carried out by Mr. Nishith Patel.

3- Effect of storage, washing and red cell rejuvenation on in vivo assessment of Transfusion Related Acute Kidney Injury (TRAKI), renal endothelial injury and renal inflammation.

This is a parallel analysis in TRALI assessment using porcine model (Figure 7.1-B). Recipient experiments involving Groups 1-4 (See 7.2.4) were carried out by Mr. Nishith Patel.

Figure [7.1]: Sequential steps involved in Rejuvenation-washing transfusion study. (A): In vitro analyses of storage lesion in post rejuvenated-washed red cell units. (B): In vivo assessment of TRALI/TRAKI, endothelial injury, inflammation and organ injury.



7.2 Plan of Investigation

7.2.1 Rationale

Considerable knowledge gap exists in our understanding of effects of storage on red blood cells and effect of stored blood on in vivo organ function in recipient. To bridge this gap, I have extended the experimental work of allogenic transfusion mediated TRALI of our research group by investigating the effect of red cell rejuvenation-washing to eliminate storage lesion in vitro and organ dysfunction in vivo.

7.2.2 Aims

To determine whether removal of supernatant and cellular components of storage lesion using Rejuvenation-washing preserves quality of stored RBCs and prevents organ injury in swine.

7.2.3 Hypotheses

1. Red cell rejuvenation combined with cell washing will restore the qualitative degradation of RBC cellular and supernatant components of stored units.
2. Transfusion of rejuvenated red cells will prevent acute lung injury in a porcine model of transfusion related inflammatory organ injury compared to un-rejuvenated washed or unwashed cell transfusion.
3. Transfusion of rejuvenated red cells will prevent acute kidney injury in a porcine model of transfusion related inflammatory organ injury compared to un-rejuvenated washed or unwashed cell transfusion.

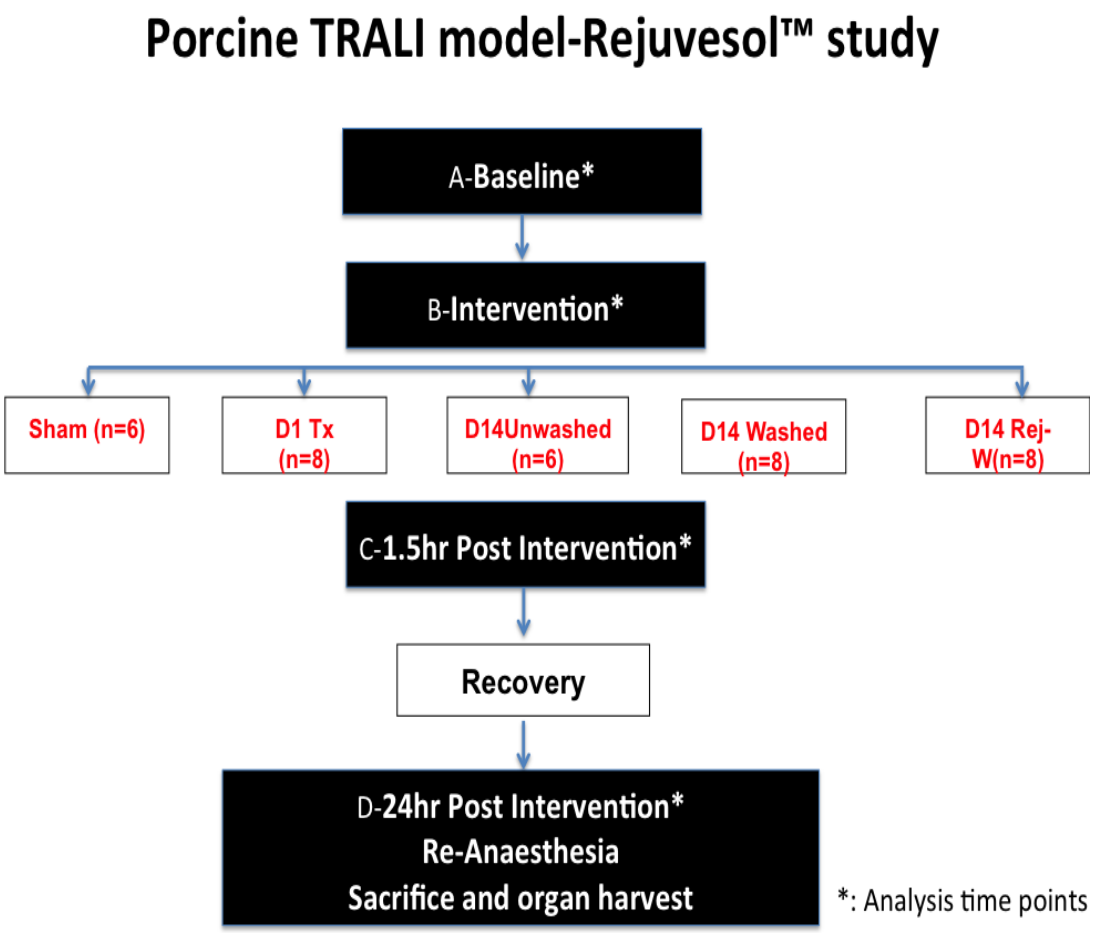
7.2.4 Design

An experimental full factorial, pretest-posttest repeated measures design was used, Treatment allocation is shown in Figure [7.2]. Pigs (n=8 per group) were randomised to;
Group 1: Sham transfusion (0.9% normalized saline, n=6).
Group 2: Transfusion of 1-day old allogenic RBC (D1, n=8)
Group 3: Transfusion of 14-day old stored allogenic RBC (D14, n=6)
Group 4: Transfusion of washed, 14-day old stored allogenic RBC (D14 Washed, n=8)
Group 5: Transfusion of washed, 14-day stored allogenic RBC+ treated with Rejuvesol™ (Citra Labs. Warsaw, Indiana, USA) (Rejuvesol-Washed, n=8).
Transfused pigs received 1000mls of allogenic RBCs.

7.2.5 Outcomes

1-Assessment of storage lesion in packed red cell units.
2-Assessment of Transfusion Related Acute lung Injury in a porcine model of allogenic transfusion.
3-Assessment of Transfusion Related Acute Kidney Injury using the same model.
4-Assessment of endothelial dysfunction, measures of inflammatory stress, redox active iron and free hemoglobin and coagulation in the same model.

Figure [7.2]: Porcine model of TRALI.



7.3 **Results**

7.3.1 In vitro assessment of Storage lesion

7.3.1.1 Effect of storage

Storage of packed cell units for 14 days resulted in reductions in red cell ATP and 2,3-DPG levels, as well as cell deformability and increased the haemolysis index; Table [7.1]. Storage also resulted in the accumulation of phosphatidylserine positive microparticles in the supernatant, along with lactate and potassium, and an increase in PaO_2 reflecting increased affinity of Hb for oxygen.

7.3.1.2 Effect of washing

Red cell washing reversed the storage changes in the supernatant. Washing resulted in a modest reversal of red cell ATP and 2,3 DPG depletion but did not restore the reduction in deformability attributable to storage; table [7.1]. Washing increased the haemolysis index, although levels remained within quality control standards for human RBC units.

7.3.1.3 Effect of Rejuvenation washing

Rejuvesol-washing increased ATP and 2,3 DPG levels beyond those observed with washing alone. Rejuvesol-washing also restored deformability, and reduced the haemolysis index to levels similar to those observed in day 1 units; Table [7.1].

Table [7.1]: Effects of storage, washing and rejuvenation on red cell units in-vitro. Data is presented as mean (SD). Post hoc intergroup comparisons $p<0.05$: Key: *: D14 Rejuvesol-Washed vs. Day 0, †: D 14 Rejuvesol –Washed vs. D14 Unwashed, ‡: D14 Rejuvesol-Washed vs. D14 Washed, §: D14 Washed vs. Unwashed, ||: D14 Washed vs. Day 0, #: D14 Unwashed vs. Day 0ᵹ: D14 Rejuvesol Washed vs. Day 7).**: Experiment not repeated for Rejuvesol-washing group.

Variable	Day 1	Day 14 Unwashed	Day 14 Washed	Day 14 Rejuvesol-Washed	ANOVA
Erythrocyte Factors					
RBC ($x10^{12}$/L)	7.7±1.3	7.5±1.2	7.2±0.92	6.2±2.15	0.227
MCV (f/L)	63.6±0.95	64.6±1.0	58.1±9.4	67±0.34	0.081
Hb (g/dL)	145±24.5	142±23	128±13	124±46	0.368
Hct (%)	49.4±8.4	48.8±7.9	49.7±6.5	42±14	0.377
Erythrocyte ATP Conc. (µmol/g Hb)	1.96±0.58	0.80±0.34#	1.3±0.09§	1.76±0.43†	0.001
Erythrocyte 2,3 DPG Conc. (umol/g Hb)	14.4±4	6.5±1.6#	13.8±0.06§	18.3±7.5†	0.010
Elongation Index (30Pa)	0.47±0.03	0.43±.005	0.43±0.001	0.52±0.02*,†,‡	0.000
Red Cell Survival (% of maximum)	94%	72%	71%	68%	--
Supernatant Factors					
PO_2 (mmHg)	86±7.6	176±21.8 #	137±5.6||	293±18*,†,‡	0.000
Potassium (mmol/L)	1.1±0.4	19±7.38#	3.2±1.5§	2.2±0.71†	0.002
Sodium (mmol/L)	140±3.2	124.8±8.7#	146±2.4§	144±2†	0.002
Glucose (mmol/L)	34.5±3.0	33.3±2.2#	3.6±1.3§,||	0.41±0.3*,†,‡	0.000
Lactate (mmol/L)	0.85±0.2	2.3±0.25#	1.0±0.43§	5.4±1.8*,‡	0.002
Haemolysis Index (%)	0.07±0.08	0.11±0.04	1.02±0.017§,||	0.05±0.03‡	0.000
Annexin-V^+ Microparticles (FACS: Events/µl)	1113.8±363.34	2589.3±184.84#	1113.8±363.34§	**	0.000

7.3.2 In vivo assessment of Transfusion Related Organ Injury

7.3.2.1 Baseline characteristics of recipient animals

All baseline measures of cardio-pulmonary function were similar between the groups (Table 7.2). Four pigs did not survive the experiments. Two experiments in the D14 group were terminated early for refractory hypoxia. Two pigs in the Rejuvesol-washed group were terminated early for cardiovascular instability.

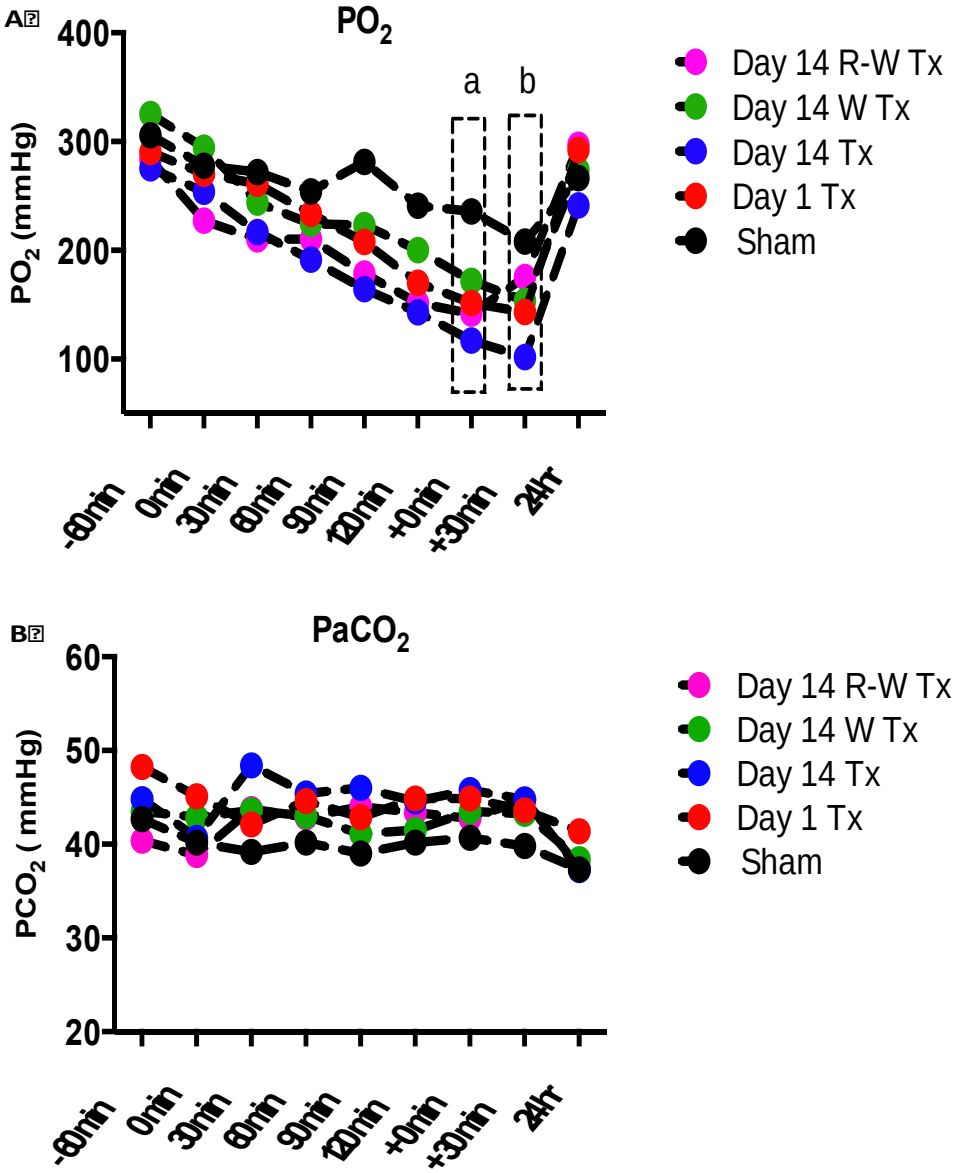
Table [7.2]: Baseline characteristics: Data expressed as mean (SD). MAP: Mean arterial pressure, WBC: White blood cell, CRP: C-reactive protein, CI: Cardiac Index, SVRI: Systemic Vascular Resistance Index, PVRI: Pulmonary Vascular Resistance Index, WOB: Work of Breathing.

	Sham	**D1-Tx**	**D14-Tx**	**D14-Washed Tx**	**D14 Rejuvesol Washed Tx**	**ANOVA**
	(n=6)	(n=8)	(n=6)	(n=8)	(n=8)	
Weight*,(Kg)	56(52,59)	61.3(54,68.5)	60.5(52.8,68)	59.8(55,64)	53(50,56)	0.68
Baseline MAP	74.33±1.86	77.25±5.20	74±2.44	75±3.62	71.88±3.8	0.09
Baseline CVP (mmHg)	7.67±3.7	6.5±4.4	6.83±3.5	9.88±2.0	5.13±1.9	0.079
Baseline PO_2 (mmHg)	306±63	290.38±79	275.5±52	325.63±104	283.75±86	0.772
BaselinePCO_2 (mmHg)	42.67±8.7	48.25±5.1	44.83±5.9	43.5±4.2	40.38±14	0.50
Baseline SvO_2(mmHg)	74.35±13	55.825±15	64.8±9	61.686±16	66.438±12	0.190
Baseline serum Lactate (mmol/l)	1.1±0.65	1.3±0.21	1.267±0.4	1.243±0.28	1.125±0.55	0.890
Baseline Hematocrit	0.3±0.01	0.2888±0.02	0.304±0.02	0.3±0.01	0.3225±0.02	0.071
Baseline WBC ($x10^9$/l)	17.14±4.09	18.075±5.83	14.517±4.86	18.325±6.82	18.825±4.11	0.627
Baseline Platelets ($x10^9$/L)	328.8±71	308.25±104	257.17±59	309.75±67	320.88±87	0.592
Baseline CI(L/min/m^2)	3.90±1.06	3.94±0.91	4.28±1.22	3.94±0.68	4.49±0.59	0.689
Baseline SVRI (dynes/sec/cm^5/m^2)	1248.83±396	1366±96	1395.8±300	1346.17±153.5	1294.5±157	0.866
Baseline PVRI (dynes/sec/cm^5/m^2)	178±53.11	174.5±94.76	146.2±64.84	173.8±54.55	117.75±28.57	0.297
Baseline Tidal volume (ml/kg)	9.94±0.05	9.9±0.05	9.96±0.06	9.94±0.05	9.9±0.11	0.489
Baseline WOB, (J)	1.048±0.11	1.0863±0.21	1.0767±0.21	1.0788±0.08	1.06±0.17	0.997
Baseline Lung Injury Score*	1.14(0.5,1.78)	0.91(0.7,1.1)	0.94(0.60,1.2)	0.95(0.58,1.33)	0.96(0.75,1.17)	0.860
Mortality	0	0	2	0	2	

7.3.2.2 Effects of storage, washing and Rejuvesol-washing on pulmonary gas exchange

We assessed adequacy of gas exchange by measuring indices of oxygenation, ventilation and PaO_2: FiO_2 ratio, which is an index of ALI, used clinically. These measures were obtained by serial half-hourly blood gas analysis. During the course of experiments PCO_2 remained stable, however a trend of serial decline in PaO_2 was seen, which was significantly reduced in all transfused groups at post-intervention time point but restored to normal levels at 24hour (Figure [7.3]).

Figure [7.3]: Assessment of gas exchange: (A) Arterial tension of oxygen; PO_2 (mmHg): All transfused groups had lower PO_2 post intervention (a,b) compared to shams. (B) Arterial tension of CO_2; PCO_2 (mmHg). (-60: baseline, 0-120: intervention, +0-30: post intervention, 24hr: 24hr recovery assessment)

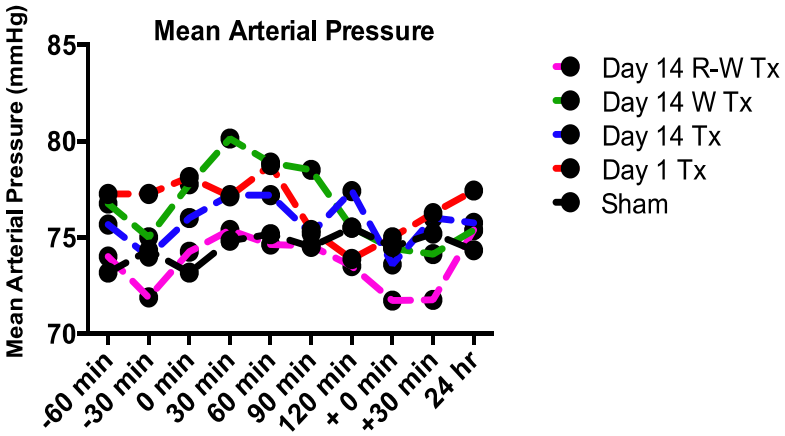


7.3.2.4 Effects of storage, washing and Rejuvesol-washing on invasive hemodynamics

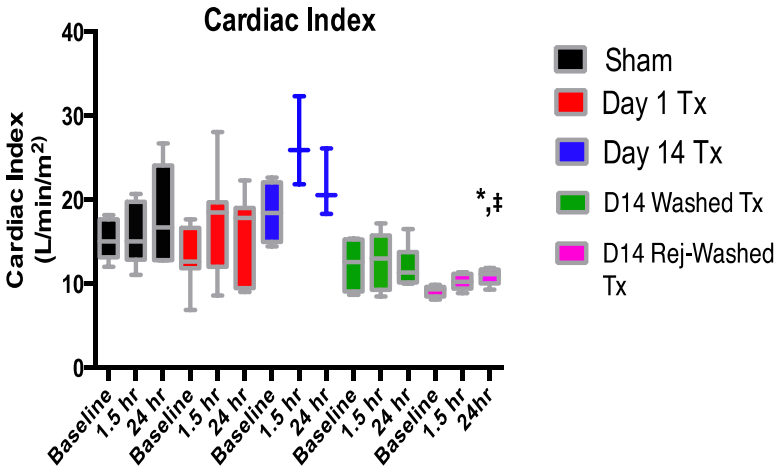
We undertook serial measurements of mean arterial blood pressure (MAP) using an indwelling carotid arterial pressure line. No significant alterations were observed during the course of the experiment (Figure [7.4] A). Cardiac index (CI) and systemic vascular resistance index (SVRI) are measurements obtained via centrally placed Pulmonary Artery floatation catheter (swans Ganz). Rejuvesol-treated washed blood transfusion group had a lower CI at 24-hour compared to un-transfused and unwashed group (Figure [7.4] B). In addition transfused animals of washed and rejuvenated-washed blood had higher SVRI during intervention (Figure [7.4] C).

Figure [7.4]: Assessment of invasive hemodynamics: (A) Mean Arterial Pressure (B) cardiac Index (C) Systemic Vascular Resistance Index. Key: $P<0.05$ * versus Sham, † versus D14 Unwashed, ‡ versus D14 Washed, § versus D14 Rejuvesol-washed. Data for Cardiac Index and Systemic Vascular Resistance Index presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data. (-60 and -30: baseline, 0-120: intervention, +0-30: post intervention, 24hr: 24hr recovery assessment)

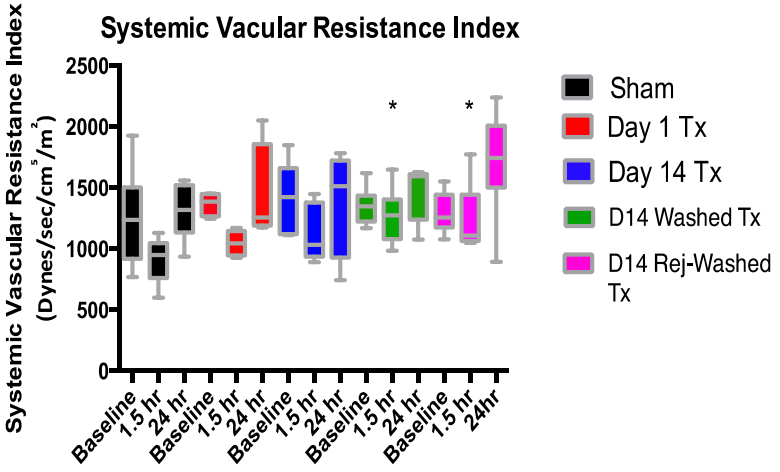
A


Mean Arterial Pressure
Mean Arterial Pressure (mmHg)
85
80
75
70
-60 min
-30 min
0 min
30 min
60 min
90 min
120 min
+ 0 min
+30 min
24 hr
Day 14 R-W Tx
Day 14 W Tx
Day 14 Tx
Day 1 Tx
Sham

B


Cardiac Index
Cardiac Index (L/min/m2)
40
30
20
10
0
*,‡
Baseline
1.5 hr
24 hr
Sham
Day 1 Tx
Day 14 Tx
D14 Washed Tx
D14 Rej-Washed Tx

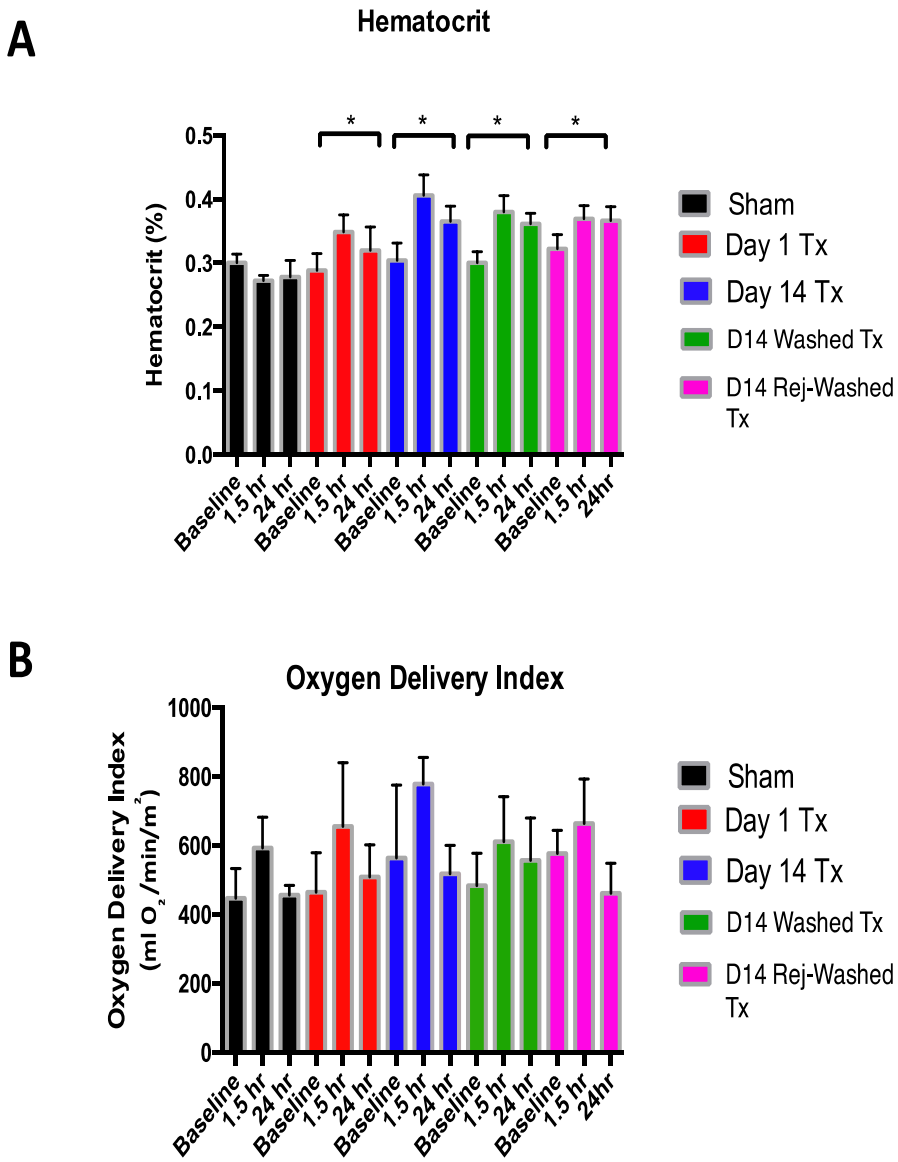
C


Systemic Vacular Resistance Index
Systemic Vascular Resistance Index (Dynes/sec/cm5/m2)
2500
2000
1500
1000
500
0
*
*
Baseline
1.5 hr
24 hr
Sham
Day 1 Tx
Day 14 Tx
D14 Washed Tx
D14 Rej-Washed Tx

7.3.2.5 Effects of storage, washing and Rejuvesol-washing on oxygen delivery

All groups that received allogenic RBC had significantly higher Hb concentrations relative to Sham pigs (Figure [7.5] A). Trends in oxygen delivery (product of cardiac index and arterial oxygen content (CaO_2), where arterial oxygen content= [(1.34 (1.34 is carrying capacity of O_2 a gram of Hb) X Hb X Mixed Venous Saturations)+(PaO_2 X 0.0031(0.0031 is solubility coefficient of oxygen in blood (aO_2). It is equal to 0.0031 mL / mmHg of oxygen / dL of blood)] were similar among groups. (Figure [7.5] B)

Figure [7.5]: Oxygen delivery; (A) changes in hematocrit, (B) Oxygen delivery: No differences among treatment group. Key: * p <0.05 vs. sham. Data presented as Mean (SD)



7.3.2.6 Effects of storage, washing and Rejuvesol-washing on TRALI in vivo

TRALI in vivo was assessed using ATS criteria recommended for establishing ALI in experimental animal models, (1) PaO_2: FiO2 ratio was used as a physiological parameter (2) BAL protein conc. was considered as a marker for alveolar membrane dysfunction and (3) formalin fixed lung section H&E analysis was undertaken to obtain a histological lung injury score.

7.3.2.6.1 Effects of storage on TRALI in vivo

Pigs receiving D14 unwashed blood developed significant reduction in PaO_2:FiO_2 ratio at 1.5hr, compared to Sham pigs. Transfusion of older cells resulted in an increased capillary leak of protein into the bronchoalveolar lavage fluid. In addition transfusion of older (D14) red cells increased histological markers of lung injury (Figure [7.6] A-C).

7.3.2.6.2 Effects of washing on TRALI in vivo

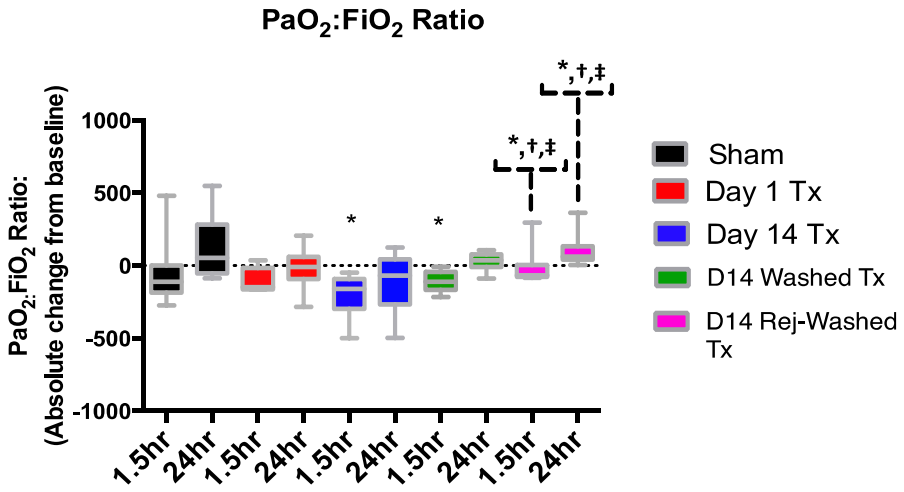
Recipients of D14 washed RBC developed significant reduction in PaO_2:FiO_2 ratio at 1.5hr, compared to Sham pigs. Washing reduced both capillary protein leak and histological markers of lung injury (Figure [7.6] A-C).

7.3.2.6.3 Effects of Rejuvesol-washing on TRALI in vivo

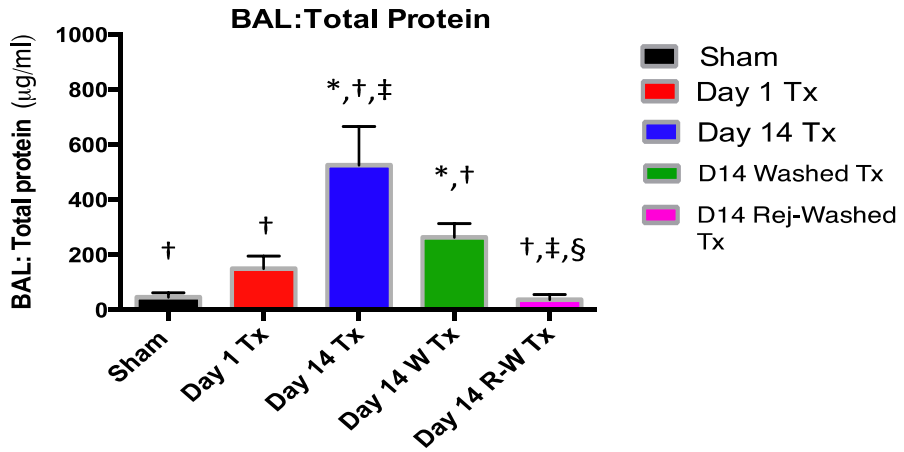
Hypoxia at 1.5 hours was prevented by Rejuvesol-washing. PaO_2:FiO_2 was also higher in the Rejuvesol-washed group at 24 hours post transfusion. Rejuvesol-washing significantly reduced BAL protein beyond that observed with washing alone. Rejuvesol-washing significantly attenuated histological marker of lung injury (Figure [7.6] A-C)

Figure [7.6]: Assessment of TRALI (A) PaO2: FiO2 ratio: Absolute change from baseline of 1.5 and 24 hr post intervention data points. (B) Protein levels from bronchoalveolar lavage (BAL) fluid at 24 hours post intervention. (C) Densitometry and photomicrographs showing H&E stained sections of lung tissue obtained at 24hr (x 400). Alveolar neutrophils (Black arrows), septal thickening (Green arrows), proteinaceous debris (Red arrows) and hyaline membranes (Blue arrows). Data for PaO_2: FiO_2 ratio (change from baseline) presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data. Data for BAL protein and histological injury scores presented as Mean (SD). Key: $P<0.05$ * versus Sham, † versus D14 Unwashed, ‡ versus D14 Washed, § versus D14 Rejuvesol-washed.

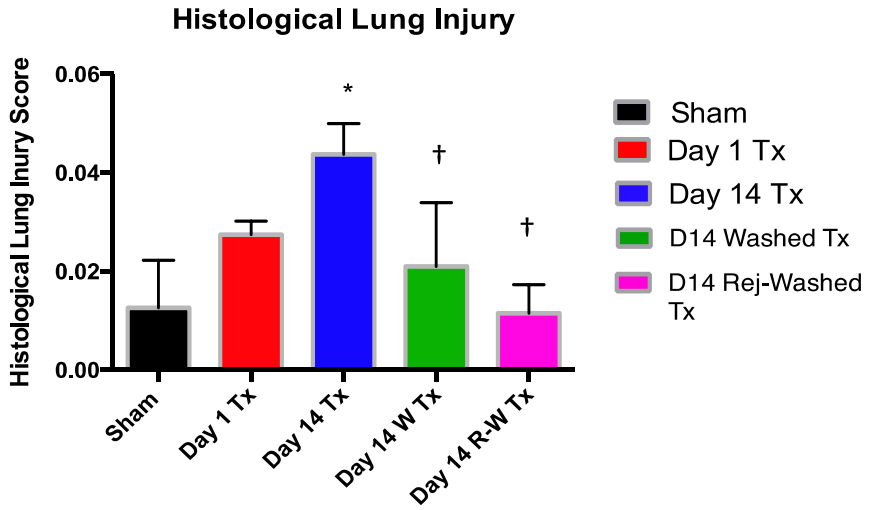
A


PaO2:FiO2 Ratio
PaO2:FiO2 Ratio: (Absolute change from baseline)
1000
500
0
-500
-1000
*
*
*,†,‡
*,†,‡
1.5hr
24hr
1.5hr
24hr
1.5hr
24hr
1.5hr
24hr
1.5hr
24hr
Sham
Day 1 Tx
Day 14 Tx
D14 Washed Tx
D14 Rej-Washed Tx

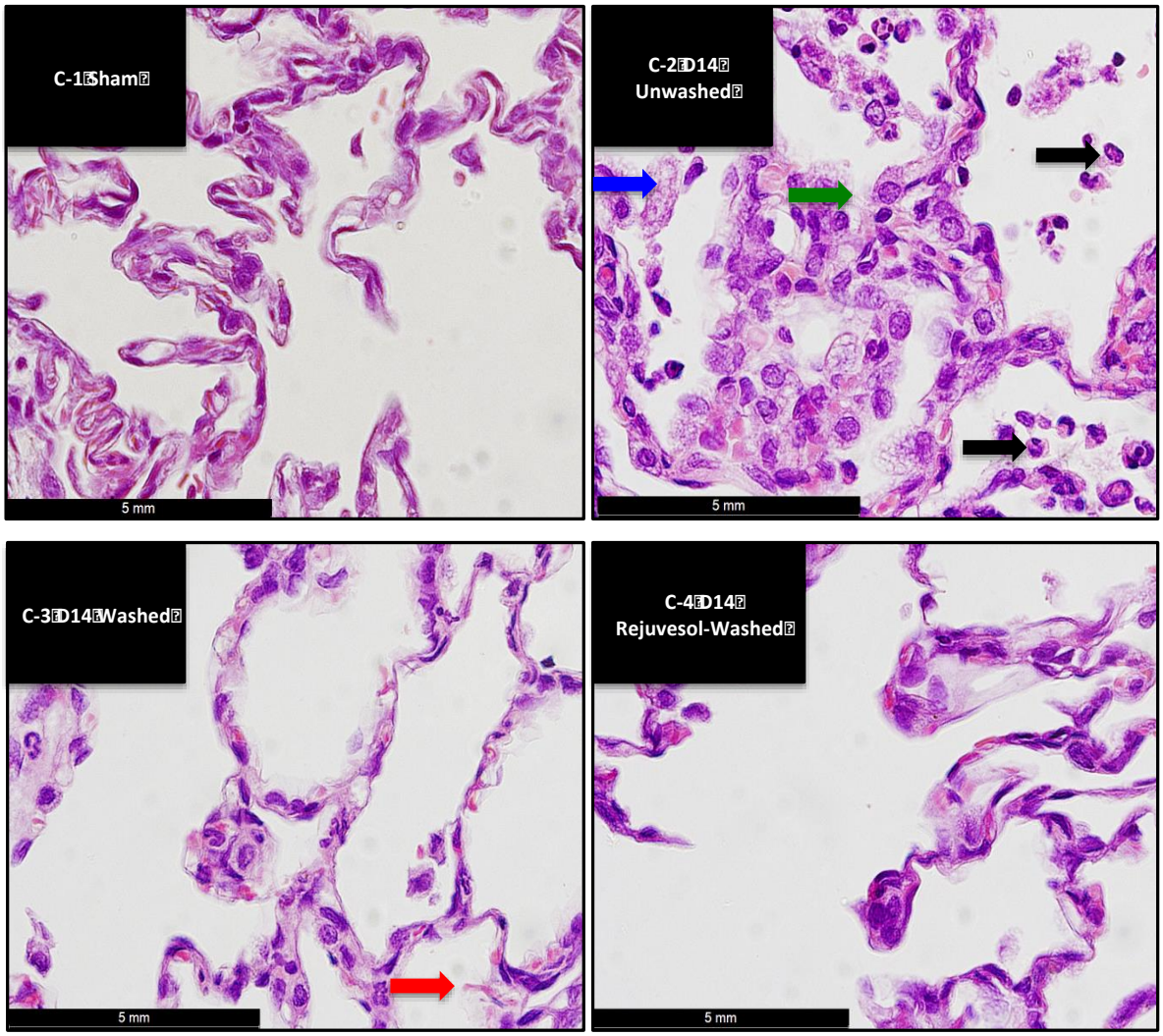
B


BAL:Total Protein
BAL: Total protein (μg/ml)
1000
800
600
400
200
0
†
†
*,†,‡
*,†
†,‡,§
Sham
Day 1 Tx
Day 14 Tx
Day 14 W Tx
Day 14 R-W Tx
Sham
Day 1 Tx
Day 14 Tx
D14 Washed Tx
D14 Rej-Washed Tx

C


Histological Lung Injury
Histological Lung Inury Score
0.06
0.04
0.02
0.00
*
†
†
Sham
Day 1 Tx
Day 14 Tx
Day 14 W Tx
Day 14 R-W Tx
Sham
Day 1 Tx
Day 14 Tx
D14 Washed Tx
D14 Rej-Washed Tx

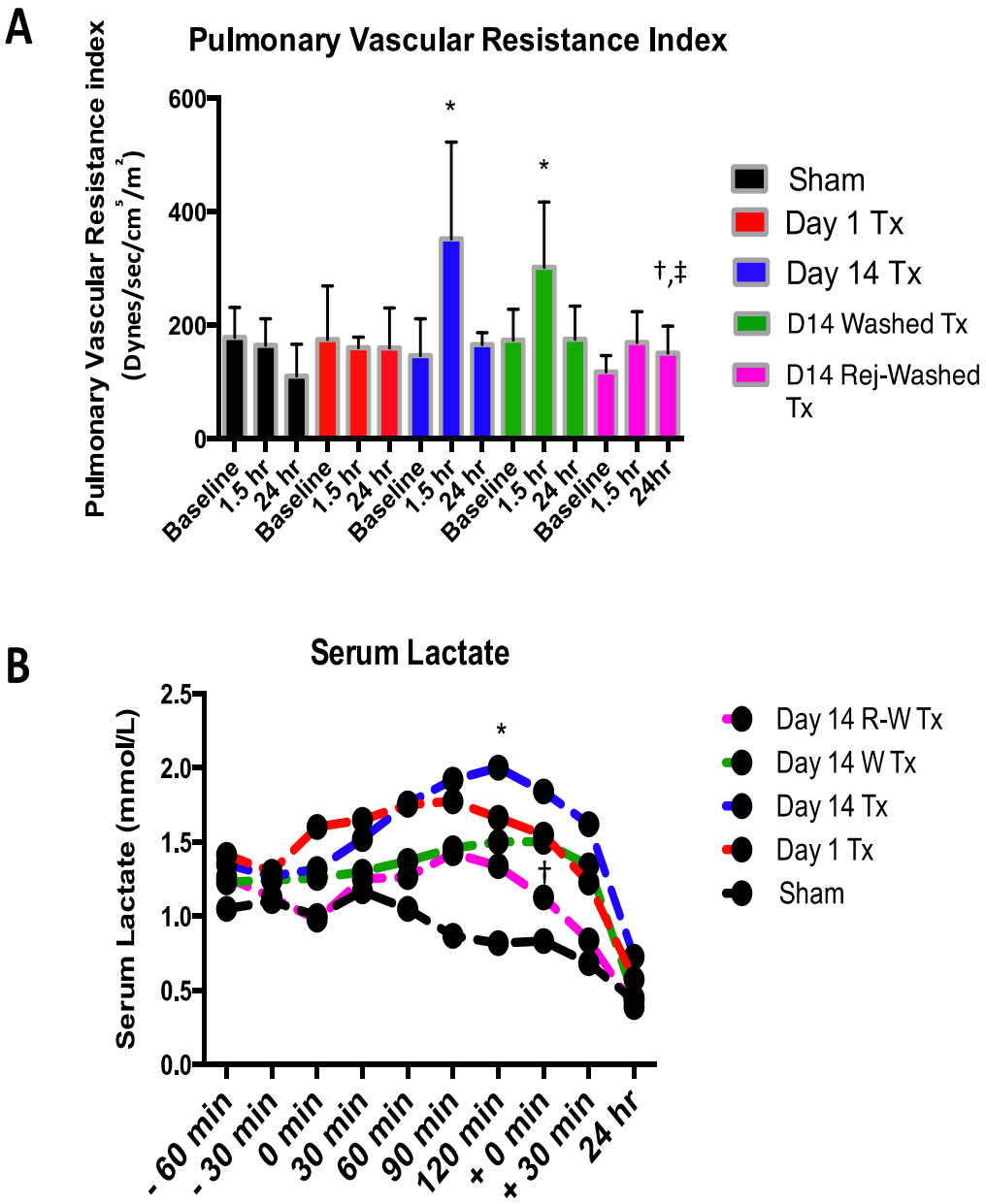
C


C-1 Sham
5 mm
C-2 D14 Unwashed
5 mm
C-3 D14 Washed
5 mm
C-4 D14 Rejuvesol-Washed
5 mm

7.3.2.7 Effects of storage, washing and Rejuvesol-washing on macrovascular pulmonary endothelial function

Transfusion of D14 RBC resulted in pulmonary vasoconstriction (endothelial dysfunction) as demonstrated by higher Pulmonary Vascular Resistance Index (Figure [7.7] A). Pulmonary vascular dysfunction was also observed with washed group, however Rejuvesol-washing reversed pulmonary vasoconstriction. Serum lactate levels are known to gauge adequacy of systemic perfusion, which is an end effect of endothelial dysfunction. We observed D14 recipients to have higher serum lactate levels, unaffected by washing but significantly reduced by Rejuvesol-washing, figure [7.7] B.

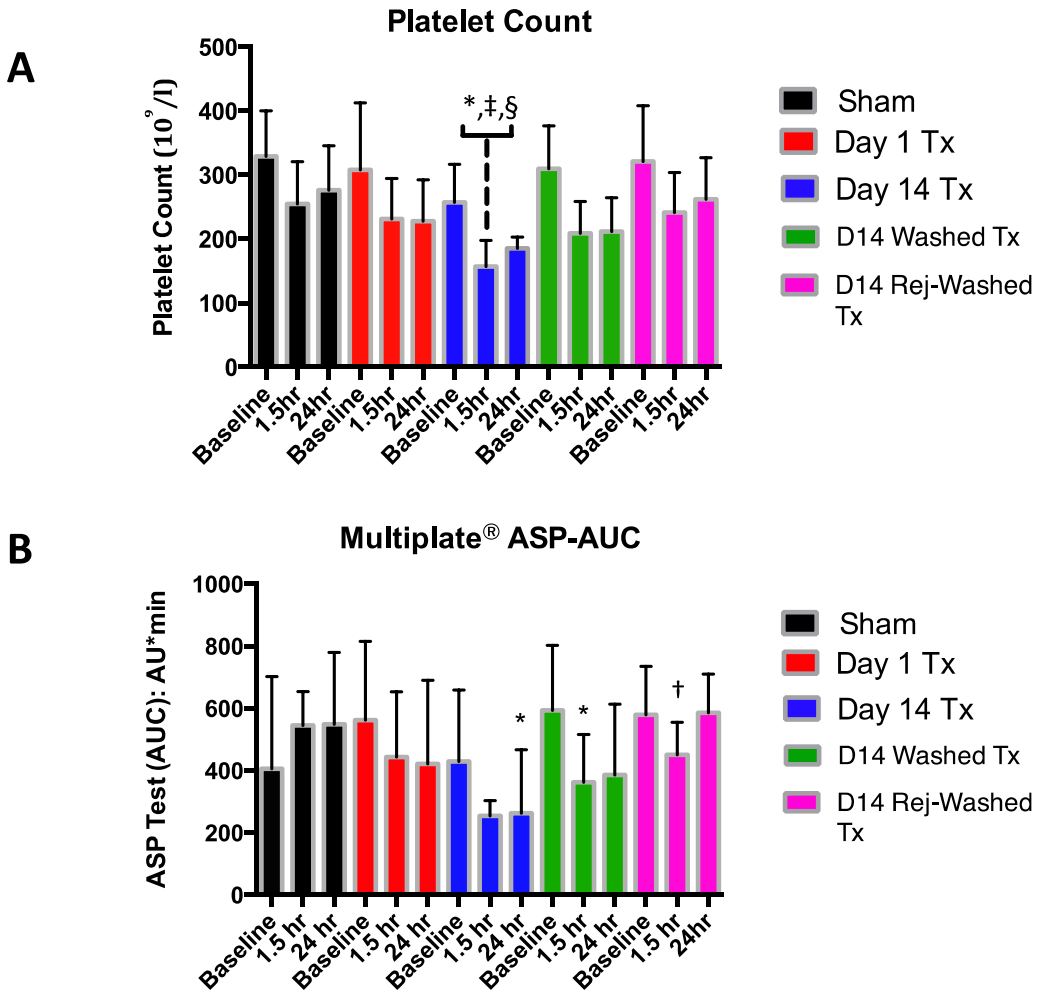
Figure [7.7]: Assessment of endothelial dysfunction; (A) Measurement of PVRI at specified time points via PA floatation catheter (B) Serial measurement of serum lactate from arterial blood gas analyses. Key: P<0.05 * versus Sham, † versus D14 Unwashed, ‡ versus D14 Washed, § versus D14 Rejuvesol-washed, || vs. D1. Data for Pulmonary Vascular Resistance Index presented as Mean (SD). Serum lactate presented as mean of all data points. (-60 and -30: baseline, 0-120: intervention, +0-30: post intervention, 24hr: 24hr recovery assessment)



7.3.2.8 Effects of storage, washing and Rejuvesol-washing on platelets

Transfusion of older (D14) red cell units led to reduction in platelet count (Figure [7.8] A) and aggregation (Figure [7.8] B) at 1.5 hours post transfusion. Rejuvesol-washing prevented functional defect is ASP test led platelet aggregation as assessed using Multiplate™ technology.

Figure [7.8]: Assessment of coagulation: (A) Platelet count, (B): ASP test for platelet aggregation. Key: P<0.05 * versus Sham, † versus D14 Unwashed, ‡ versus D14 Washed, § versus D14 Rejuvesol-washed, || vs. D1. Data presented as Mean (SD).



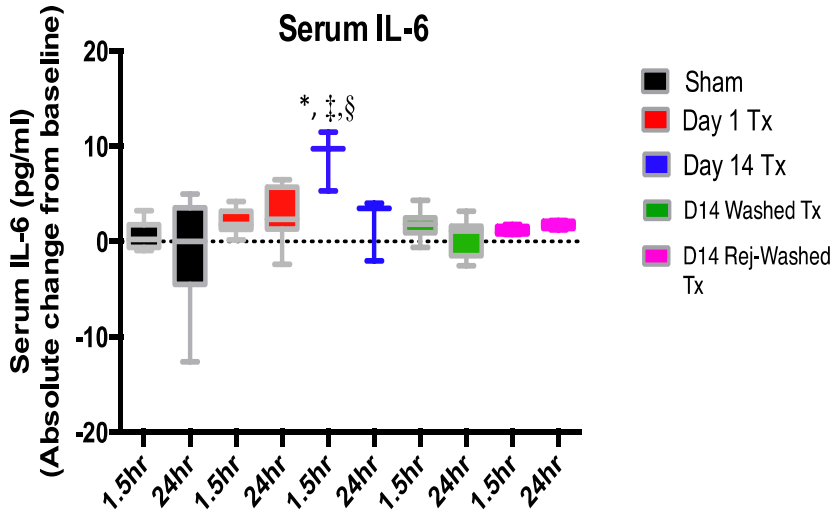
7.3.2.9 Effects of storage, washing and Rejuvesol-washing on inflammation

7.3.2.9.1 Inflammatory cytokines

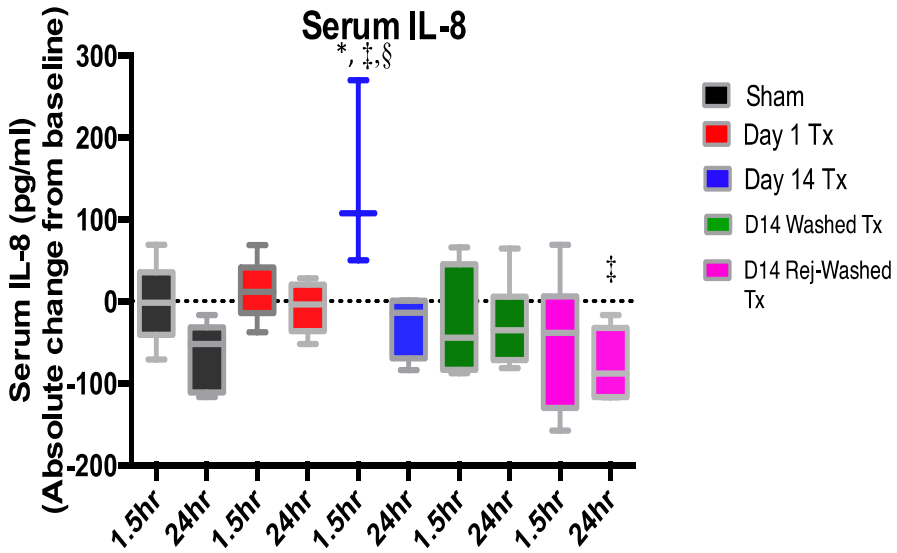
We analysed serum levels of IL-6 and IL-8 at key time points and interpreted these using change from baseline plots due to baseline heterogeneity. We observed levels of inflammatory cytokines to rise immediately post intervention in recipients of D14 untreated blood (Figures [7.9]). Rejuvesol-washing but not washing alone prevented inflammatory surge at 24hr compared to D14 RBC recipients.

Figure [7.9]: (A) Change from baseline in serum IL-6, (B) change from baseline in serum IL-8. Key: P<0.05 * versus Sham, † versus D14 Unwashed, ‡ versus D14 Washed, § versus D14 Rejuvesol-washed, || vs. D1. Data presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data.

A



B

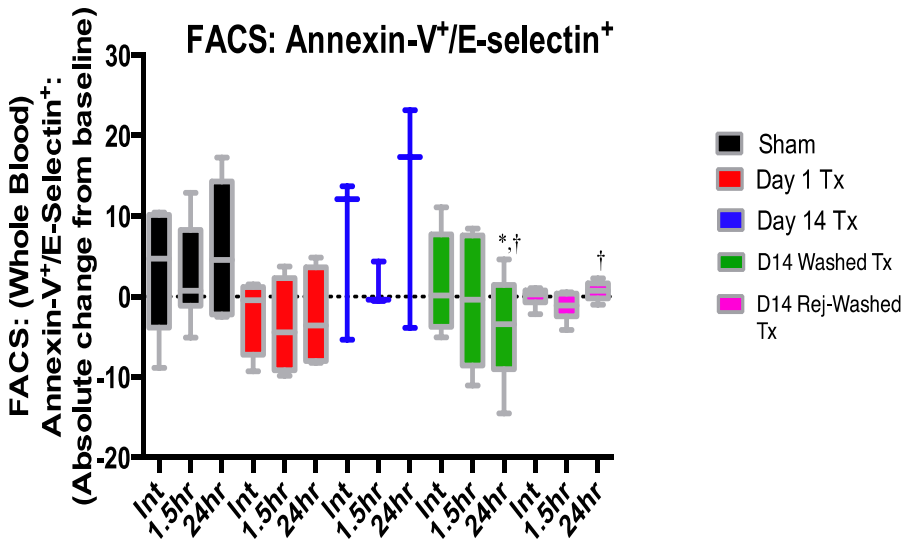


7.3.2.9.2 Inflammatory microparticles

7.3.2.9.2.1 Endothelial derived MPs

Annexin-V⁺/E-selectin⁺ MPs were sampled at all key time points, quantified using FACS and analysed as change from baseline. Levels of the MPs were significantly reduced with washing and Rejuvesol washing at 24hr (Figure [7.10]).

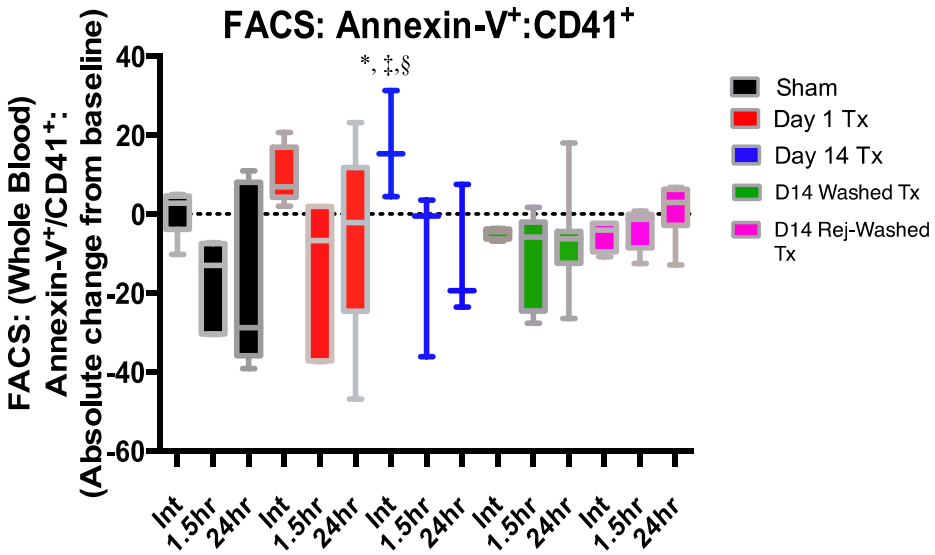
Figure [7.10]: Changes in Annexin-V⁺/E-selectin⁺ MPs. Key: P<0.05 * versus Sham, † versus D14 Unwashed, ‡ versus D14 Washed, § versus D14 Rejuvesol-washed, || vs. D1. Absolute change from baseline data presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data.



7.3.2.9.2.2 Platelet derived MPs

Similarly, platelet derived MPs were also analysed using FACS. A post-intervention surge was readily visible in D14 Tx group, which was prevented by washing and Rejuvesol-washing (Figure [7.11]).

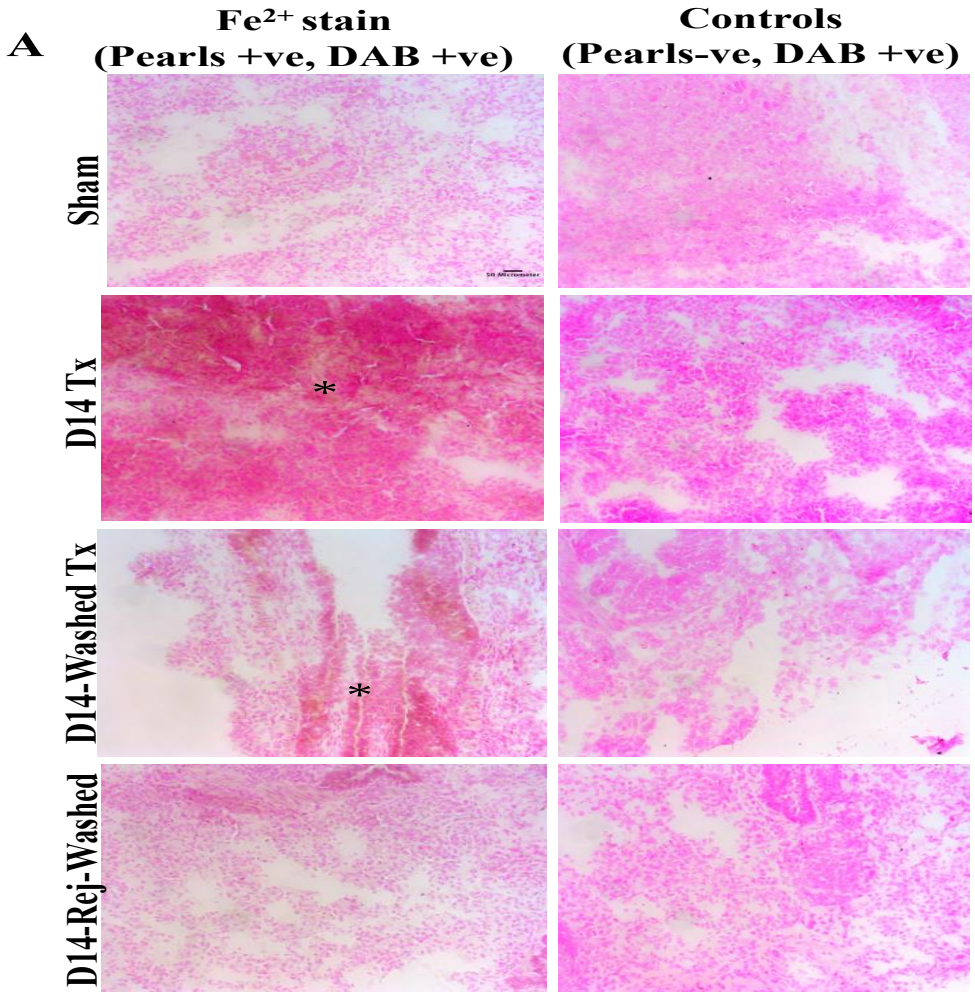
Figure [7.11]: Changes in Annexin-V⁺/CD-41⁺ MPs. Key: P<0.05 * versus Sham, † versus D14 Unwashed, ‡ versus D14 Washed, § versus D14 Rejuvesol-washed, || vs. D1. Absolute change from baseline data presented as box and whiskers plot where bottom and top of the box represent first and third quartiles, the band inside the box is second quartile (the median) and the ends of the whiskers represent minimum and maximum of all the data.

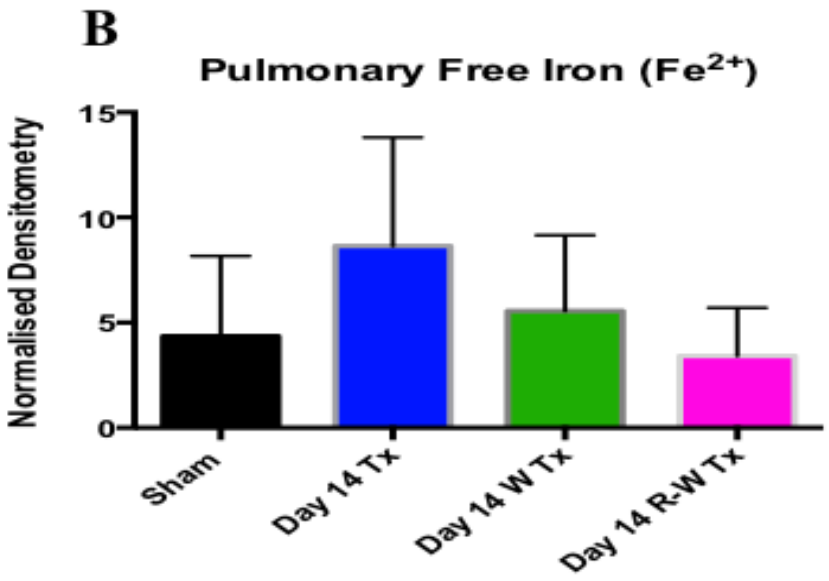


7.3.2.9.3 Redox active Iron

We measured pulmonary precipitation of free Iron on 10 μ m frozen section using Pearls/DAB technique. The results displayed a non-significant trend of increased free iron in D14 Tx group and lesser levels in washed and Rejuvesol-washed groups (Figure [7.12]).

Figure [7.12]: (A) Panel: Iron staining on 10 μ m frozen sections. Pearls –ve, DAB +ve control lacks substrate provided by pearls stain-Fe^{2+} complex that is oxidation by DAB leading to lack of brown pigmentation. Key: * Iron deposition. (B) Optical densitometry. Scale bar: 50 μ m, Data displayed as Mean (SD).




B
Pulmonary Free Iron (Fe^{2+})
Normalised Densitometry
15
10
5
0
Sham
Day 14 Tx
Day 14 W Tx
Day 14 R-W Tx

7.3.2.10 Effects of storage, washing and Rejuvesol-washing on Acute Kidney Injury; Assessment of renal function, free iron and inflammation.

7.3.2.10.1 Renal function

In D14-Washing group we observed a non-significant rise in NGAL/creatinine ratio (Figure [7.13] A1-A2) associated with a significant failing in endothelial function (Figure [7.13] A3), as measured with acetylcholine induction of renal blood flow.

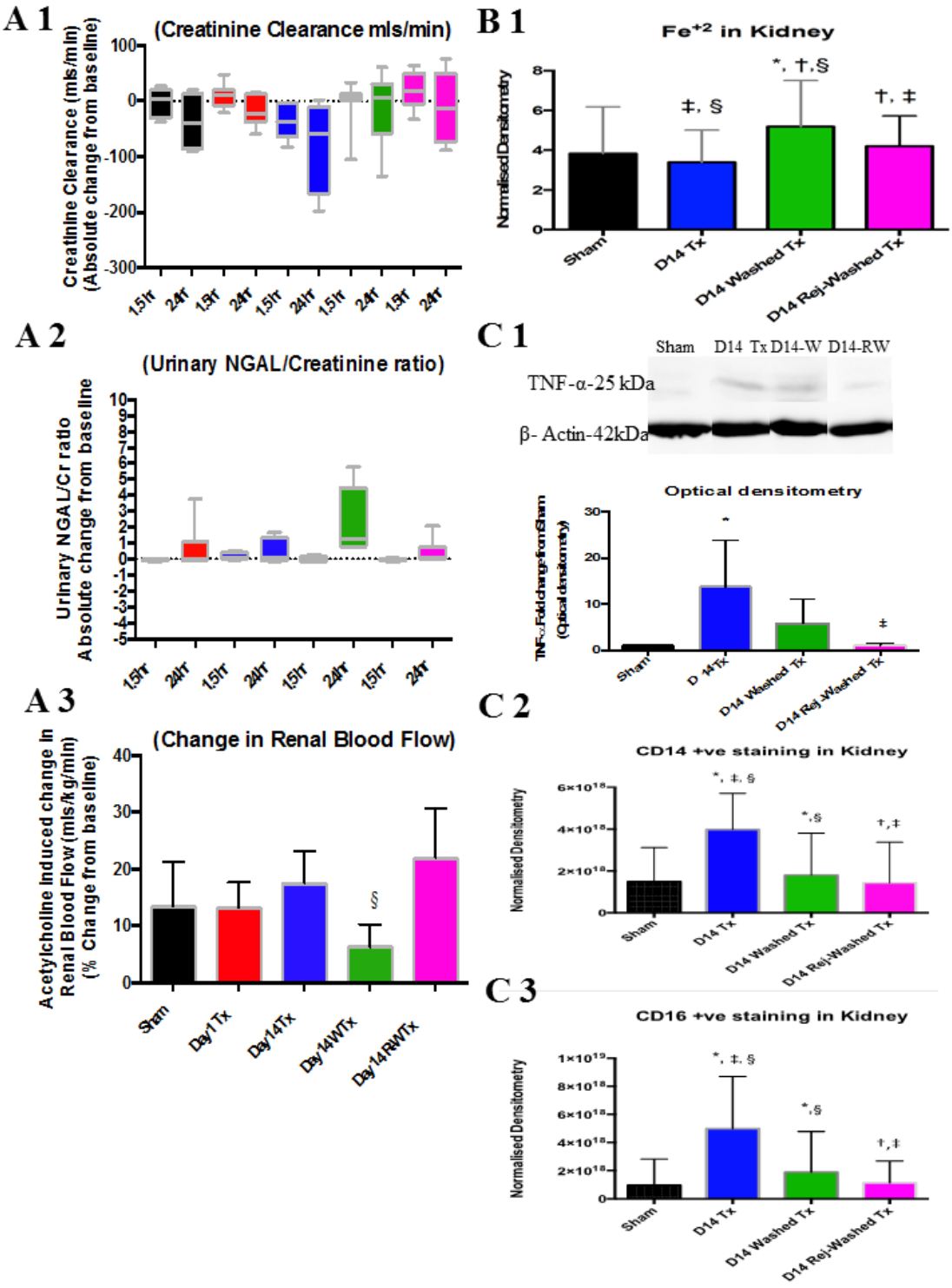
7.3.2.10.2 Fe^{2+} staining in kidney

Recipient animals of washed stored red cell units had significantly higher levels of free iron in kidney compared with unwashed group, however transfusion of rejuvenated red cell units prevented this despite being coupled with washing (Figure [7.13] B1-B2).

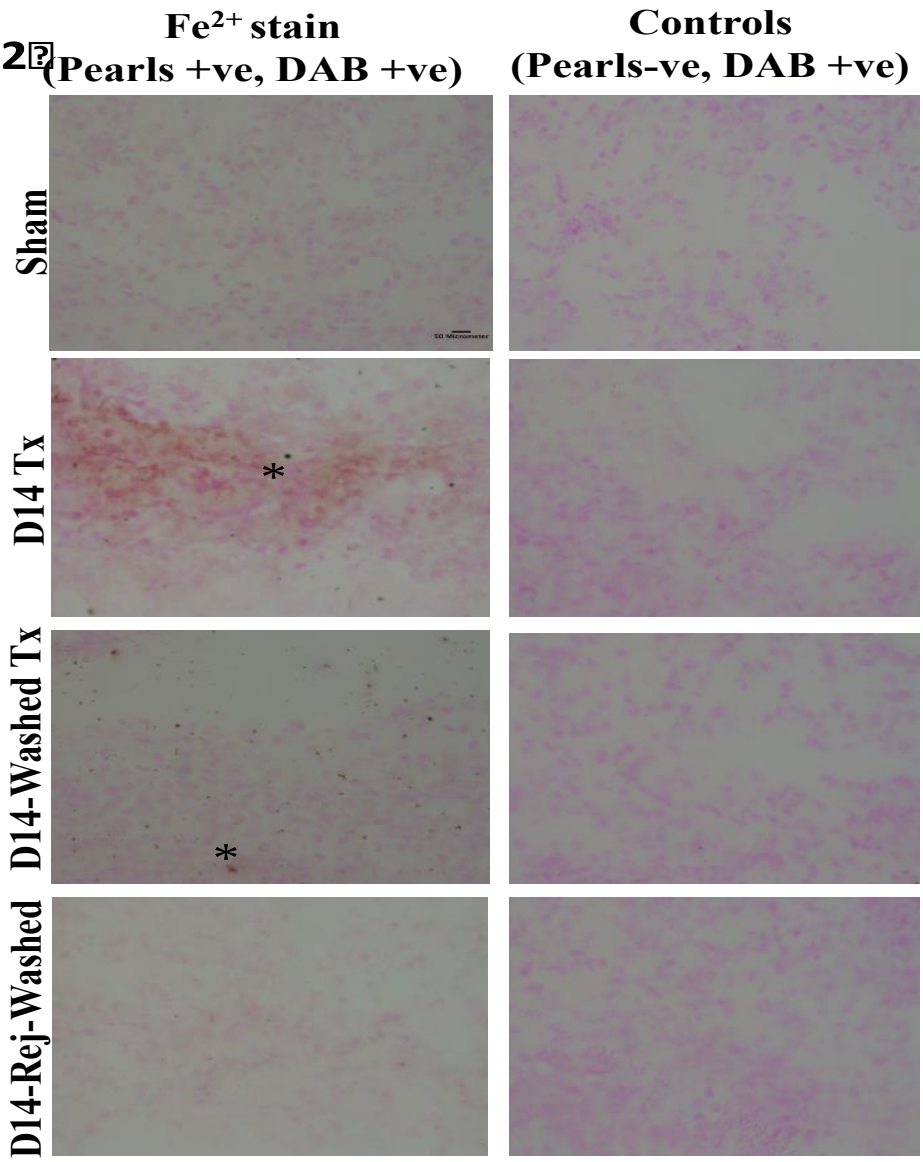
7.3.2.10.3 Renal inflammation

Recipients of unwashed blood had higher levels of TNF-α (Figure [7.13] C1) in lung lysates and higher levels of CD14 and CD16 (figures [7.13] C2-C3) positive monocytes on cryostaining. These measures were significantly reduced by washing and even further reduced with Rejuvenation-washing.

Figure [7.13]: Effects of storage, washing and Rejuvesol-washing on Acute Kidney Injury; Assessment of renal function, free iron and inflammation. Assessment of renal injury; (A-1): Changes in creatinine clearance presented as change from baseline, (A-2) changes in NGAL/creatinine ratio displayed as change from baseline, (A-3): Endothelial functional assessment with acetylcholine bolus and measurement of RBF, presented as change from baseline. (B-1): Panel of representative photomicrographs showing staining of labile iron (Fe^{2+}) on 5 μ m kidney frozen sections. Pearls –ve, DAB +ve control lacks substrate provided by pearls stain-Fe^{2+} complex that is oxidation by DAB leading to lack of brown pigmentation. Key: * Iron deposition. Scale bar: 50 μ m. (B-2), Optical densitometry for iron staining, Data presented as Mean (SD). (C-1): Western blot analysis of TNF- α with optical densitometry, Data presented as Mean (SD). β -Actin used as internal control. 4-renal cortical lysates per treatment condition were blotted on 10% Gel and repeated thrice (C-2) Optical densitometry for renal inflammation measured using CD-14 +ve staining on 5 μ m renal cortex tissue, (C-3) Optical densitometry for renal inflammation measured using CD-16 +ve staining on 5 μ m renal cortex tissue, Key: $P<0.05$ * versus Sham, † versus D14 Unwashed, ‡ versus D14 Washed, § versus D14 Rejuvesol-washed. Data presented as Mean (SD).


A 1
(Creatinine Clearance mls/min)
Creatinine Clearance (mls/min)
(Absolute change from baseline)
100
0
-100
-200
-300
1.5hr
24hr
B 1
Fe+2 in Kidney
Normalised Densitometry
8
6
4
2
0
*, †,§
‡, §
†, ‡
Sham
D14 Tx
D14 Washed Tx
D14 Rej-Washed Tx
A 2
(Urinary NGAL/Creatinine ratio)
Urinary NGAL/Cr ratio
Absolute change from baseline
C 1
Sham
D14 Tx
D14-W
D14-RW
TNF-α-25 kDa
β- Actin-42kDa
Optical densitometry
30
20
10
0
*
‡
Sham
D 14Tx
D14 Washed Tx
D14 Rej-Washed Tx
A 3
(Change in Renal Blood Flow)
Acetylcholine Induced change in
Renal Blood Flow (mls/kg/min)
(% Change from baseline)
40
30
20
10
0
§
Sham
Day1Tx
Day14Tx
Day14WTx
Day14RWTx
C 2
CD14 +ve staining in Kidney
Normalised Densitometry
6×10¹⁸
4×10¹⁸
2×10¹⁸
0
*, ‡, §
*,§
†,‡
Sham
D14 Tx
D14 Washed Tx
D14 Rej-Washed Tx
C 3
CD16 +ve staining in Kidney
Normalised Densitometry
1×10¹⁹
8×10¹⁸
6×10¹⁸
4×10¹⁸
2×10¹⁸
0
*, ‡, §
*,§
†,‡
Sham
D14 Tx
D14 Washed Tx
D14 Rej-Washed Tx

B-2


Fe2+ stain
(Pearls +ve, DAB +ve)
Controls
(Pearls-ve, DAB +ve)
Sham
D14 Tx
D14-Washed Tx
D14-Rej-Washed
*
*

Chapter 8

Discussions

Volume resuscitation and organ injury: Systematic evaluation of safety of current colloids and crystalloids

Principal findings

The principal findings of this systematic review are that colloid administration does not increase mortality but does increase the risk of developing AKI when compared to the use of crystalloid for volume replacement. Subgroup analyses demonstrated an increased risk of mortality and AKI attributable to pentastarch and an increased risk of AKI attributable to tetrastarch. The adverse effects attributable to these interventions were observed in septic critically ill patients but not in patients with trauma and patients undergoing non-trauma surgery. Dextrans and gelatin were not investigated by high quality RCTs and it was not possible to resolve uncertainty as to the clinical risks and benefits of these colloids from the available evidence.
The review used comprehensive search strategies, contemporary risk of bias assessments (GRADE), and assessed a wide range of outcomes in critically ill patients, patient with trauma and patients undergoing non-trauma surgery. It identified important limitations of existing published data; 41 (69 percent) of the RCTs identified had serious limitations in terms of methodological quality. This finding, along with important sub-group interactions such as clinical setting, was considered as contributors to the heterogeneity observed in the results. After adjustment for these factors in an a priori sensitivity analyses, and with the exception of patient with sepsis, colloids in general resulted in no specific harm in the studied patient groups.

Comparison with contemporary evidence

The results of the current study are at odds with the conclusions of another recent meta-analyses (Serpa Neto et al., 2014) that included data from the recent 6S and CHEST trials that suggested increased mortality with HES. Neto et al (Serpa Neto et al., 2014) failed to find an increase ICU stay and 28-day mortality with HES but showed increased 90-day mortality in this subgroup. However, the

analyses of 90 day mortality was essentially based on four studies, CHEST and 6S being the only high quality trials (i.e. free from GRADE limitations), and it lacked data from the recent CRISTAL and BaSES trials that are included in the current meta-analysis. Similarly, a meta-analysis by Zarychanski et al (Zarychanski et al., 2013), concluded a pooled mortality estimate in favour of crystalloid but lacked sensitivity analyses.

Limitations

There are some important limitations of the present investigation. The study relied on the reported information on confounding variables that were controlled for; consistent analyses of all studies can only be done when data on individual patients are combined. The study was unable to determine whether attributes of interventions in some trials influenced the results, such as for example the use of six percent vs. 10 percent HES, molar substitution 140/0.3 vs. 140/0.32, or dosage.

Evaluation of novel AQIX RS-1® for intravenous fluid replacement in a swine model of general anaesthesia, recovery and end organ assessment.

Principal findings

The key finding of this study is that AQIX-RS-1® infused in healthy swine does not afford superior endothelial or organ protection compared with traditional hartmans solution. Our findings show that AQIX infusion causes loss of endothelial cell-cell junctional protein VE-Cadherin in renal glomeruli but as such this was not sufficient to increase NGAL release from kidneys.
AQIX RS-1infusion has been associated with increased Hyaluronic acid loss (marker of glycocalyx) from isolated perfused porcine livers (Vekemans et al., 2009) but no evidence for effect of AQIX RS-1 on renal glycocalyx exists in published literature. Our observation did not reveal alteration in DBA specific glycosylation patterns but did demonstrate loss of endothelial marker VE-Cadherin compared to Hartmann's group. Kay et al have demonstrated glomerular shrinkage with AQIX infusion in isolated porcine kidneys without affecting creatinine clearance when compared to other perfusates (Kay et al., 2011) which likely characterizes renal injury with AQIX to be real but not enough to cause decline in renal function. Measurement of creatinine clearance however is not a sensitive marker of AKI where as NGAL/creatinine ratio is and hence we have typically shown no evidence of renal injury in otherwise healthy animals with similar baseline demographics receiving optimal peri-operative fluid resuscitation with minimal surgical stress.

Limitations

We have measured urinary NGAL, which becomes positive in filtrate only in the presence of proximal tubular injury however we have no histological evidence for this. Urinary NGAL could have been from extra renal sources (likely in this case due to systemic infusion of AQIX RS-1) and a simultaneous assay of plasma NGAL could have increased the confidence in our results.

Endothelial injury and recovery: Role of CPB and sildenafil on morphological expressions of glomerular endothelial membrane in post-cardiac surgery AKI.

Principal findings

This study demonstrates the direct effect of cardiopulmonary bypass on all constituent layers of renal capillary endothelium i.e. Glycocalyx (both GAG glycosylation patterns and core protein expressions), endothelial surface markers that interact with important pathways such as coagulation, fibrinolysis and complement and basement membrane podocytes which regulate the glycocalyx constituents (Foster et al., 2013). Very importantly CPB leads to loss of endothelial cell-cell junctional proteins regulating functional attributes such as permeability.

The effect of CPB on glycocalyx GAG motifs is more intriguing, as certain GAG components are easily lost such as DBA, WGA and SBA while others are unaltered such as UEA specific ligands. Core proteins display similar pattern. The functional significance of these heterogeneous alterations in the context of cardiac surgery has been demonstrated by Bruegger et al who measured syndecan-1 in infants after cardiac surgery and demonstrated syndecan-1(core protein) loss in urine to rise proportionately with the ischemic burden (cardiac ischaemia using aortic cross clamping < cardiopulmonary and systemic ischaemia-reperfusion using deep hypothermic circulatory arrest) (Bruegger et al., 2015). In contrast Hyaluronan (GAG) increased significantly in the course of the operation in all groups, irrespective of the burden of ischaemia.

Limitations

One limitation of our investigation is that we did not measure generation of reactive oxygen species (ROS), heparanase's TNF-Alpha, ANP and other sources of direct contributors of glycocalyx shedding. However these factors are known to increase in serum of patients in cardiac surgery or other models of ischaemia-reperfusion (Tossios et al., 2003).

Translational Relevance

The porcine model affords a controlled, reproducible insult, which is akin to post-cardiac surgical AKI. In addition the results of preclinical evaluation of Sildenafil Citrate in this model have paved way for first ever pragmatic trial in cardiac surgery; (REVAKI: The effect of Sildenafil (REVATIO) on post-cardiac surgery Acute Kidney Injury, A pharmacokinetic-dose finding study ISRCTN06134609 and a randomised controlled trial planned to included 220 cardiac surgical patients at increased risk of AKI allocated to Sildenafil vs. Placebo with primary outcome being differences in AKI.

Effect of Red cell Rejuvenation-washing on post-storage characteristics of red cell units and In vivo assessment of organ injury in a porcine model of allogenic transfusion.

Principal findings

The key findings of this study are that stored red cells develop toxic metabolites, which induce organ injury in large animal model of allogenic transfusion. Washing of stored blood negates some inflammatory metabolites but it is Rejuvenation-washing that fully eliminates in vitro storage lesion and in vivo organ injury. Rejuvesol, an additive solution imparts this benefit by preserving endothelial responses, reductions in labile Iron, monocyte/macrophages invasion in lungs and kidneys and systemic cytokines of transfused swine.

The current model effectively replicates human storage lesion both in vitro and in vivo. In our study swine did not develop severe ALI, akin to clinical syndrome TRALI that manifests as acute respiratory failure <6 hours (Matute-Bello et al., 2011). However, animal distress due to severe ALI is unacceptable in a recovery model and likely to result in confounding. Our model may therefore be more akin to more common, but less severe forms of ALI attributable to transfusion, including Transfusion Associated Circulatory Overload and Transfusion Associated Dyspnea(Muller et al., 2012, Tuinman et al., 2011, Vlaar, 2012, Vlaar et al., 2011). The recovery component is a key attribute of the model. We have documented previously that pigs develop hypoxia due to atelectasis during prolonged ventilation (Patel et al., 2013, Patel et al., 2011b). In this model animals are recovered and are ambulatory for up to 24 hours before re-assessment, reducing the likelihood that atelectasis may confound assessment of ALI and lung function.

Limitations

Mediators of TRALI in healthy swine may be different to those in critically ill or post- operative patients receiving transfusion and that is why while this study

highlights the primary lesion and its implications on organ injury yet cannot be considered an exhaustive assessment of TRALI mechanism.
A further evaluation of the pro-inflammatory role of reactive oxygen species (Chaudhary and Katharia, 2012) or plasma free-Hb induced NO scavenging by old red cells (Donadee et al., 2011, Liu et al., 2013, Kanias and Gladwin, 2012), protein carboxylation from lipid peroxidations from ROS and Iron Hypothesis that considers not just organ precipitation but also assesses Fe pathways in circulation and liver, should be considered for future work.

Translational relevance

This is the first preclinical study that evaluates the effect of storage lesion on organ injury in a translational swine model of TRALI. The measures of organ injury used in this preclinical study namely PaO_2: FiO_2 ratio for ALI and creatinine clearance for AKI are well established indices used in critical care units around the world that are used for both establishing diagnosis and monitoring recovery.
Commercially available cell washing systems such as C.A.T.S Fresenius used in present study are widely used in cardiac surgical units to hemoconcentrate field losses during cardiac surgery. Our results highlight the double-edge sword nature of this intervention. On one hand they remove the supernatant of stored blood containing potassium, lactate and inflammatory microparticles, which translate into reduction in inflammatory cell invasion into tissue but increase iatrogenic hemolysis due to shear stress. This excess free hemoglobin produced causes endothelial dysfunction that is evident in our flow studies of pulmonary and renal vasculature. The relevance of these findings are currently being tested in a first ever definitive trial; Randomised Controlled Trial of Red Cell Washing (REDWASH®) for the Attenuation of Transfusion Associated Organ Injury in Cardiac Surgery ISRCTN 27076315.

8.1 Summary

Post cardiac surgery inflammatory organ injury is penultimate outcome of pathological sequelae that originate from dysregulation of homeostatic mechanisms of intact endothelium. From an exhaustive list of inciting factors I have focused on conventional fluid therapy, cardiopulmonary bypass and stored blood transfusion because 1-they are liberally used, 2-have mechanisms of inculcating organ inflammation and injury that are poorly understood and lastly 3-lead to significant morbidity and mortality without any curative treatment strategy. In this thesis I have investigated the centrality of endothelial dysfunction in two of the most lethal and challenging complications i.e. acute kidney injury and acute lung injury. This investigation has focused on the critical importance of nitric oxide bioavailability, structural modifications of endothelial glycocalyx or surface proteins and inflammation as pivotal to organ homeostasis. In addition I have shown a translational link between dysregulation of these parameters and functional decline in clinical indices of organ function such as creatinine clearance and PaO_2/FiO_2 ratio for AKI and ALI respectively. This translational relevance was proven using swine as a bench model of replicating the hemodynamic, biochemical and inflammatory stresses exactly how these factors drive organ injury in humans. I have also explored novel therapies that challenge current conventions; such as AQIX RS-1® for intravenous resuscitation, use of Sildenafil Citrate to replenish nitric oxide bioavailability lost during CPB and reinvigoration of red cell ATP to curb the loss in functionality and viability of stored blood and with it the ensuing organ injury.

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