CONFIDENTIAL REPORT

Phase II – *Blood Volume Replacement Study*

Assessment of a Novel Non-Phosphate pH Buffered Solution

**AQIX® RS-I solution**

as a blood volume expander in a porcine model of hemorrhagic shock in comparison to Whole Autologous Blood and Lactated Ringer solution

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Preamble

This study was undertaken to evaluate the efficacy of AQIX® RS-I solution as a hypovolemic therapy when administered as an intravenous, infusion agent into a large animal (pig) model in comparison to current therapies adopting autologous whole blood or Lactated Ringer solution as blood volume replacement fluids. A prior study using a similar pig model indicated that there were no ‘safety’ issues involved (ref. Phase I - Pilot ‘Safety’ Study Report, November, 2007).

Resuscitation can exacerbate cellular injury caused by hemorrhagic shock, and the type of fluid used for resuscitation may play an important role in this injury. Traditional resuscitation has involved administration of large volumes of isotonic crystalloid or colloid solutions followed by blood products as necessary. Experimental studies have demonstrated that the use of these fluids has been associated with neutrophil activation and tissue reperfusion injury.

AQIX® RS-I is a non-phosphate, pH-buffered physiological solution that has been designed and developed to simulate human serum and interstitial fluid in terms of the extracellular ionic composition, anion gap, osmolality, conductivity, pH stability and contains essential amino acids, intermediary metabolites and recombinant human insulin (expressed in S.cerviceria).

All ingredients comprising the formulation of AQIX® RS-I solution are at concentrations observed from detailed analyses of human serum (e.g. Insulin; 28.0 mIU/L) with each ingredient chosen to facilitate acknowledged biochemical, biophysical and osmoregulatory mechanisms. For example, thiamine pyrophosphate (TPP) is an essential co-factor for the pyruvate and α-ketoglutarate dehydrogenase catalyzed reactions as well as the transketolase catalyzed reactions of the pentose phosphate pathway.

AQIX® RS-I solution, in accordance with the American and British Pharmacopoeia contains no pyrogenic or toxic, harmful substances as previously verified from some 4000 in vitro animal and human cadaver tissue and organ studies and recent in vivo studies involving mouse, rat, dog, pig and human cells, tissues and organs (see, Appendix ‘A’).

The properties of the AQIX® RS-I solution may minimize tissue hypoperfusion induced injury as well as minimize the activation of the inflammatory cascade leading to an improved outcome in situations of hemorrhagic shock.

Background and Significance:

Hypotension is predictive of very high mortality in trauma patients and an absence of palpable pulse is associated with almost certain death unless the source of hemorrhage can be controlled within minutes. The time available for surgical interventions is severely limited by the warm ischemia time that can be tolerated by the brain (5 minutes), and the heart (about 20 minutes). Thus a significant number of patients with potentially reparable traumatic injuries die due to irreversible cerebral or myocardial damage.

Resuscitation can often exacerbate the injury sustained during hemorrhagic shock. This “resuscitation injury” is thought to be multifactorial in etiology, with over-activated neutrophils playing a key role in causing the cellular damage. In recent years there has been increasing
evidence that choice of resuscitation strategy is an important variable and different fluids can have widely divergent impact on the immune response, neutrophil activation and tissue injury.  

Traditional resuscitation has involved administration of large volumes of isotonic crystalloid or colloid solutions followed by blood products as necessary. Using blood obtained from healthy human volunteers, Alam et. al. have also shown that LR and artificial colloids (Dextran and Hetastarch) cause neutrophil activation in a dose dependent fashion. The same group has also previously demonstrated in a swine model, that resuscitation with lactated Ringer’s (LR) solution causes increased neutrophil “oxidative burst” activity. In that study, no significant neutrophil activation was noted following fresh whole blood or hypertonic saline (HTS) resuscitation. Hydroxyethyl starch-based solutions have well-established uses as plasma volume expanders. They provide plasma volume expansion greater than one and one half times the volume administered, and they have been investigated for use in the treatment of hypovolemia secondary to hemorrhage. Their use is limited because of the known risks of induction of disseminated intravascular coagulopathy.

In light of the above, it appears that there remains a need for an effective plasma expander with specific properties to reduce reperfusion injury. A plasma expander with such properties given early after traumatic injury with severe hemorrhage could reduce the life threatening complications and improve survival and outcomes in hemorrhagic shock patients. AQIX® RS-I is a potential plasma expander with such properties.

AQIX® RS-I is a ‘universally’ applicable, in vitro perfusion solution for mammalian cells, tissue and organ preparations that based under standardized conditions, facilitates reproducible physiological, pharmacological and toxicological observations from isolated preparations. AQIX® RS-I solution uniquely uses organic buffers in tandem with the ‘natural’ bicarbonate/pCO₂ buffering system used by all mammalian species. While phosphate ions are a requisite for the production of high-energy phosphate molecules (ATP) inside all mammalian cells, the presence of extracellular inorganic phosphate ions has been shown to cause deleterious and irreversible alterations in cell structure and numerous biochemical processes (e.g. glycolysis) within a few hours.

**Aims**

To investigate the efficacy of AQIX® RS-I as a blood volume replacement and resuscitation solution following hemorrhagic trauma in the pig in comparison to conventional clinical therapies utilising autologous blood or a non-physiological electrolyte solution, Lactated Ringer’s.

**Objectives:**

1. To ascertain the unit volume of AQIX® RS-I required to replace 1 unit volume of pig blood to normalize hemodynamics.
2. To compare the efficacy of this volume of AQIX® RS-I with that of autologous ‘shed’ blood in maintaining hemodynamic parameters during the resuscitation period.
3. To compare this volume of AQIX® RS-I with an equally effective volume of Lactated Ringer (LR) Solution.
4. To determine from Objectives (2) and (3) the degree of reperfusion injury, apoptosis and necrosis in the brain, liver, kidney and lung.
5. To statistically analyze data based on four experimental trials comprising
FOUR groups of animal, namely

**Group 1** - 8 of `dry’ (SHAM) `non-replacement’ studies

**Group 2** - 6 of `shed’ autologous blood (BLOOD) replacement procedures

**Group 3** - 6 of AQIX® RS-I (AQIX) replacement procedures

**Group 4** - 6 of Lactated Ringers (LR) replacement procedures

**Research Design**

**Vertebrate Animal Selection:** For years, farm and miniature pigs have been widely used in laboratory medicine. Since the introduction of surgical laparoscopy, the pig model has been increasingly used as a reliable model of hypovolemic shock.

The selection of the pig as a model stems from a number of factors:

1. The pig is a readily available, cheap laboratory animal which has been successfully used in such studies before and the hemorrhagic shock model is well established.
2. The pig anatomy and hemodynamic profiles shares many similarities with the human patient.
3. It allows the use of readily available surgical instruments which are also used on human patients.

The anatomy and morphology of the porcine organs as well as the circulating blood volume in pigs closely resembles that of humans and therefore adopting such a model to examine the effects of AQIX® RS-I and Lactated Ringer solutions in blood replacement studies would allow a closer appreciation of the clinical situation.

**Experimental Protocol**

**Hemorrhagic Shock Model:** All animals used in this study were be handled according to the *Guide for the Care and Use of Laboratory Animals* after approval of the institutional animal care committee of the American University of Beirut Medical Center.

A total of 29 farm swine weighing between 30 and 50 kg were used in this study. An additional number of animals (maximum 8) were available if required in the event of accidental deaths during the surgical manipulation part of the procedures. The animals were sedated with an intramuscular injection of ketamine (15–20 mg/kg) and atropine (0.04–0.4 mg/kg) followed by induction of anesthesia with 2.5% sodium thiopental (6.6%, 25 mg/kg) and endotracheal intubation.

Anesthesia was maintained with Isoflurane to achieve absence of response to surgical stimulation without depression of heart rate or mean arterial pressure (MAP). Animals were paralyzed with intravenous Pancuronium (0.05–0.07 mg/kg) and maintained under neuromuscular blockade with repeated doses as needed to prevent spontaneous ventilation. Mechanical ventilation was maintained within the following parameters: respiratory rate, 10 breaths/min; tidal volume, 7 to 10 ml/kg; FIO2, 0.50; and positive end-expiratory pressure, 3 mm Hg. Tidal volume was adjusted to achieve normocarbia before baseline measurements and maintained at that volume for the remainder of each experiment.
**Surgical procedures:** A 8.5-French introducer sheath (Baxter Healthcare, Irvine, CA) was placed via cutdown into the right external jugular vein through which a VIP thermodilution pulmonary artery catheter (Baxter) was inserted. The catheters were attached to a hemodynamic monitoring platform for continuous monitoring of blood pressure, mixed venous oxygen saturation, and pulmonary artery catheter parameters (measured and derived). Electrocardiogram electrodes were used for continuous cardiac electrical activity monitoring throughout the procedure.

Bilateral lower extremity cutdowns allowed exposure to the femoral vessels, and bilateral femoral arteries and a single femoral vein were cannulated. Systemic arterial pressure was continuously monitored through one arterial line, whereas hemorrhage was performed via the second arterial line. Resuscitation fluids were administered into the femoral vein. Finally, a suprapubic bladder catheter was placed using an open technique for monitoring urine output. All incisions were closed with skin staples and all tubing and catheters secured in place. Electrocardiogram electrodes were used for continuous cardiac electrical activity monitoring throughout the procedure.

After completion of the instrumentation phase, animals were allowed to equilibrate for 15 minutes. Baseline measurements of heart rate, systemic and pulmonary arterial pressures, core temperature, and cardiac output were taken and repeated at intervals throughout the experimental protocol.

**Experimental procedures:** Using a well established and previously characterized model of hemorrhagic shock and resuscitation in the swine, after baseline measurements, pigs were rapidly hemorrhaged from the femoral artery until the MAP reached 30 mm Hg. Hemorrhage was continued as needed to maintain the MAP at 30 +/- 2 mm Hg for 45 minutes. No intervention was made if the MAP decreased below 30 mm Hg during the shock period. Shed blood was collected in ACD treated bags and the net weight used to estimate volume of hemorrhage. The same volume of shed blood was then re-infused into the autologous blood group of pigs (AB-group) via the same vein after the predetermined shock period of 45 minutes. Alternatively, at the conclusion of the 45-minute shock period, animals received either no resuscitation (‘SHAM’ group) or were infused with a volume of AQIX® RS-I (AQ-group) or Lactated Ringer (LR-group) solutions sufficient to restore the MAP to 60±2 mmHg within the 120 minute resuscitation period. An additional control group of six pigs underwent anesthesia only as a negative control or baseline.

The following three resuscitation fluids were used: ‘shed’ autologous, whole blood, Lactated Ringer (LR) and AQIX® RS-I solutions as detailed in Table 1 and summarized below:

**Control groups**

1) Anesthesia only, instrumentation, No hemorrhage (SH) (n=6).
2) Hemorrhage with No resuscitation (SR) (n=6).

**Blood resuscitation group**

Resuscitation with fresh, autologous whole blood over 2 hours (n=5). Resuscitation volume was approximately equal to ‘shed’ autologous blood.

**Isotonic crystalloid LR resuscitation groups**

Resuscitation with LR solution over 2 hours (n=6). Resuscitation volume was 258.1 ± 43.99 % of ‘shed’ blood volume.
**AQIX® RS-I resuscitation group**

Resuscitation with AQIX® RS-I solution over 2 hours (n=6). Resuscitation volume was 290.5 ± 35.82 % of 'shed' blood volume.

The resuscitation fluids were either administered by gravity or pump assisted infusion into the femoral vein until the MAP increased to 60 mm Hg. Fluid administration was given in a dynamic manner whereby it continued over a 2-hour period to re-establish and then maintain the MAP at 60 +/− 2 mm Hg. No intervention was made if the MAP exceeded 60 mm Hg.

Blood was sampled serially during the experiment at the following time points: at baseline; at initiation of the 45- minute shock period (commenced at initial decrease of MAP to 30 mm Hg); at 30 minutes of shock; at 45 minutes (completion) of shock; at 30, 60, 90, 120 minutes of resuscitation and on post-operative days 1, 2 and 7 (POD7). Samples were drawn from the femoral artery and pulmonary artery for blood gas analysis and lactate measurement. Femoral arterial samples were also collected and placed into standard blood tubes for complete blood counts, serum chemistry determination: electrolytes, glucose, lactate, osmolarity, serum enzymes (aspartate aminotransferase, alanine aminotransferase, total creatine kinase [CK] + LDH, coagulation times and fibrinogen levels. All the above blood tests were measured using standardized calibrated systems that are used for patient care purposes.

Pulmonary arterial pressures and core body temperature were measured continuously throughout the experiment. Pulmonary artery wedge pressure and cardiac output were measured at the following time points: at baseline; at initiation of the 45-minute shock period (commenced at initial decrease of MAP to 30 mm Hg); at 30 minutes of shock; at 45 minutes (completion of shock); and at 30, 60, 90 and 120 minutes of resuscitation. Cardiac output and pulmonary artery wedge pressure were measured at end-expiration. Cardiac output was measured using the thermodilution method and calculated as the mean of three serial injections. Analysis and calculations were made using an HP M1092A monitor with a 54S transducer module (Hewlet Packard). Oxygen consumption was calculated from the Swan Ganz Data.

After resuscitation, the animals had their cannulae removed leaving the neck venous catheter for daily blood withdrawals. This catheter was secured in place. All incisions were then sutured and the animals allowed to recover.

During the ensuing 7 post-operative days the animals were observed for any behavioral changes and underwent blood withdrawals as described in the post operative protocol. The animals were subsequently humanely sacrificed on post-operative Day 7 (POD7) to investigate any evidence of reperfusion injury in the brain, kidney, liver and lung.

All procedures were performed under strict aseptic conditions and under general anesthesia. All animals were given appropriate analgesics post operatively (Tramadol 50-100 mg and Ketoprofen 50-100 mg administered intramuscularly in the immediate post-operative periods).
Post-Operative procedures: In the immediate post-operative periods, the animals were kept in boxes warmed by blankets and monitored. Long acting analgesics were administered by intramuscular or intravenous route, at the discretion of the anesthesiologist and at the completion of the procedure before the animal is awakened. When awake, the animals were allowed to drink milk. Beginning on post-operative Day 1, all pigs were allowed free access to normal food and water. Post-operative care was routinely carried out thereafter.

On post-operative days (PODs) 1, 2 and 7, surviving animals had blood samples drawn to measure electrolytes, glucose, lactate, osmolarity, Hb, hematocrit, serum enzymes (AST, ALT, total Creatine Kinase [CK] + LDH) and additional samples stored at -80 °C for further analysis of TNF-alpha, IL-6 and levels of neutrophil activation.

On POD 7, all surviving animals were humanely sacrificed by the intravenous administration of 80 mEq of KCl and their organs harvested for histologic examination. Gross and microscopic examination was performed on the following organs: brain (hippocampus), kidney, lungs and liver.

Follow up surveillance: The pigs were monitored closely for any signs of ill health and euthanized if any undue suffering was detected (e.g., weight loss, poor appetite, fatigue).

Outcomes: The primary outcome measured was a 7-day survival. Secondary outcome parameters examined were as follows: hemodynamic measurements (Day 0) (group comparisons); global perfusion/oxygenation indices (group comparisons); blood profiles (Day 1, 2 and 7); cell counts, blood gas analyses, electrolytes, glucose, cellular Hb, hematocrit, and serum enzymes (group comparisons); and histology (group comparisons of animals surviving to POD 7).

Statistical Analysis: Data were reported as mean +/- SD for each group. Statistical analysis was performed using repeated measures. Significance was tested using Student’s t-test of the means and defined as p < 0.05.

Results

The collated data (excl. TNF-α; IL-6) for this study is shown in Appendix ‘B’ and the range of values published (Appendix ‘D’) for constituent blood profiles and hemodynamic values of swine (pig) species worldwide. The acknowledged specie variance in several of the blood profiles relates to age, sex and husbandry of the swine species documented.1-6 However, non-parametric statistical analysis of the results presented in this study showed consistency among and within the various blood profile categories of the farm swine (pigs) investigated in this study.

Out of the 29 pigs studied, one LR-pig died, one AQ-pig died after dislodging the neck venous catheter; both pigs were replaced by additional pigs in each group during the trial. All pigs in the trial survived the 7-day experimental period, with one AQ-pig developing pneumonia and pulmonary congestion in a LR-pig, but all pigs showed behavioral and feeding habits that were within normal limits.

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## Table 1: Blood & Fluid Replacement Volumes used in Trial Groups

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<th>Blood: Fluid Ratio</th>
<th>Infusion rate over 't' mins [mL/min/Kg]</th>
<th>Infusion rate over 60 mins [mL/min/Kg]</th>
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**Data Analyses:**

**Section 1 - Blood Profile Categories**

### 1a: Blood components

A very significant (P< 0.01) hemodilution of RBC count was observed in both AQ and LR-group pigs in comparison to the AB-group pigs during the experimental period (Fig. 1a,b) but all groups returned to normal values (27±3%) by post-operative day 7 (POD7). No significant change in either WBC or lymphocyte count (Fig. 2a) was observed except for a significant (P< 0.04) elevation in WBC count in LR-groups on POD1. Lymphocyte count returned to baseline levels only in the AQ-group pigs over the seven days investigated with a 7% and 17% decrease observed in the AB and LR-groups respectively (Fig. 2b).
Platelet count in all group pigs remained depressed during the 120 minute resuscitation period with only the AB-group platelet count remaining decreased (37.6%) in comparison to a 28.9% increase in the AQ-group (Fig.3a,b). There was no significant difference between groups by POD7, all platelet counts being within normal levels (Appendix ‘D’).

Fibrinogen levels were significantly elevated (P< 0.02) in the AQ and LR-group pigs on POD1,2 in comparison to the AB-group (Fig. 4a,b) with only the AQ-group showing levels significantly elevated (P< 0.04) in comparison to AB-group pigs on POD7 (Fig.4b). All levels remained within the published range (2.06-6.13 g/L) for porcine species.11

In terms of the blood clotting parameters investigated, aPTT remained essentially unchanged in the AB-group during the experimental period (Fig. 5a). In comparison, there was an observed significant 6% increase in the AQ-group (P< 0.03) compared to that recorded in the AB-group pigs (Fig.5b) on POD7. Only the LR-group showed a decrease (18%) at the end of the experimental period. Prothrombin clotting indices (INR) remained within normal values (0.8 – 1.2) throughout the trial period in all groups studied.

**Ib: Electrolyte and Biophysical Parameters**

No significant differences were observed in serum electrolytes in all groups studies except for a highly significant elevation (P< 0.003) in the sodium ion levels in the LR-group pigs on POD1 which returned to within the normal range for porcine species (135-150 mmole/L) by POD7.

There was no difference in osmolality amongst or between the AB and AQ-group pigs over the 7-day experimental period in comparison to baseline values whereas in the LR-group pigs there was a significant increase in comparison to the AQ-group pigs on POD 1 and 2 (P< 0.03) and the AB-group pigs (P< 0.03) on POD7 (see, Appendix ‘D’).

The anion gap (GAP) was slightly elevated in the AB-group pigs (13-20 mmole/L) in comparison to the AQ-group (9-16 mmole/L) and LR-group (13-17 mmole/L) pigs but returned to normal (<16.5 mmole/L) by POD7 (Fig.6a,b; Appendix ‘D’). Strong Ion Difference (SID) levels remained basically constant during the seven day experimental period in all groups studied.

The pH, pO2 and pCO2 levels were not of statistical difference among or between the three trial groups of pigs.

**Ic: Serum Metabolites**

Serum glucose and creatinine (Fig. 8a,b) baseline levels were characteristically elevated in all the groups of pigs on Day 1 following the trauma of surgical manipulative procedures but were restored to normal by POD7.

Lactic acid levels remained significantly elevated (P< 0.006) in all groups during the 120 minute resuscitation period (Fig.7a,b) with the LR-group pigs exhibiting the greatest elevation (324%).
By POD7, only the AQ-group showed a decline (45%) in lactic acid levels in comparison to a sustained elevation in both the AB (>39.7%) and LR (>38.7%) group pigs (Fig. 7b). Blood Urea Nitrogen (BUN) levels remained relatively stable throughout the post-operative period in the AB-group pigs in contrast to that observed in the two other groups of pigs (Fig. 8a). At the conclusion of the experimental period (POD7), BUN levels in the LR-group were significantly (P< 0.02) elevated 72.7% above baseline levels (Fig. 8b) being double that observed in the AQ-group pigs (35.2%) but not statistically different.

Id: Serum Enzymes:

Changes in the serum levels of Lactate Dehydrogenase (LDH), Aspartate Aminotransferase (AST), alanine aminotransferase (ALT) and Creatine Phosphokinase (CPK) showed no significant changes to baseline values in the three groups of pigs studied. On POD 1 and 2 there was elevation of all serum enzymes examined (e.g., Figs. 10a,b; 11a,b). All showed a decline by POD7 to acceptable serum values for porcine species (see, Appendix ‘D’).
Fig. 1a  Hematocrit % Levels during Experimental Period

[Range value: 15 - 37 %]

-60 min -30 min -15 min 0 min 30 min 60 min 90 min 120 min po day 1 po day 2 po day 7

Fig. 1b  Percentage Change from baseline Hematocrit levels after 30 and 120 minutes resuscitation and on post-operative Day 7

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Fig. 2a  Lymphocyte % Levels during Experimental Period  
[Range: 40 - 60 %]

Fig. 2b  Percentage Change from baseline Lymphocyte count after 30 and 120 minutes resuscitation and on post-operative Day 7
Fig. 3a  Platelet levels during Experimental Period
[Range: 120,000 - 720,000]

Fig. 3b  Percentage Change from baseline Platelet count after 30 and 120 minutes resuscitation and on post-operative Day 7
Fig. 4a  Fibrinogen levels during Experimental Period
[Range value: 2.06 - 6.13 g/L]

Fig. 4b  Percentage Change from baseline Fibrinogen levels after 30 and 120 minutes resuscitation and on post-operative Day 7
Fig. 5a  Activated Partial Thromboplastin Time (aPTT) during Experimental Period
[Range value: 17.8 - 23.2 sec]

Fig. 5b  Percentage Change from baseline activated Partial Thromboplastin Time after 30 and 120 minutes resuscitation and on post-operative Day 7
**Fig. 6a** Anion Gap levels during Experimental Period

[Range: 12.5 - 16.5 mmole/L]

**Fig. 6b** Percentage Change from baseline Anion Gap levels after 30 and 120 minutes resuscitation and on post-operative Day 7
Fig. 7a  Lactate levels during Experimental Period
[Range value: 1.91 - 2.47 mmoles/L]

Fig. 7b  Percentage Change from baseline Lactate levels after 30 and 120 minutes resuscitation and on post-operative Day 7
Fig. 8a Creatinine levels during Experimental Period
[Range: 0.8 - 3.6 mg/dL]

Fig. 8b Percentage Change from baseline Creatinine levels after 30 and 120 minutes resuscitation and on post-operative Day 7
**Fig. 9a** Blood Urea Nitrogen levels during Experimental Period

[Range: 6 - 30 mg/dL]

![Graph showing Blood Urea Nitrogen levels during experimental period with different treatments.](image)

**Fig. 9b** Percentage Change from baseline Blood Urea Nitrogen levels after 30 and 120 minutes resuscitation and on post-operative Day 7

![Bar graph showing percentage change from baseline for different treatments.](image)
**Fig. 10a**  Aspartate Aminotransferase levels during Experimental Period  
[Range: 29 - 1,140 IU/L]

**Fig. 10b**  Percentage Change from baseline Aspartate Aminotransferase levels after 30 and 120 minutes resuscitation and on post-operative Day 7
Fig. 11a  Creatine Phosphokinase levels during Experimental Period

[Range: 311 - 16,700 IU/L]

Fig. 11b  Percentage Change from baseline Creatine Phosphokinase levels after 30 and 120 minutes resuscitation and on post-operative Day 7
Section 2 – Hemodynamic Profiles

2a:  Mean Arterial Pressure (MAP)

Only the AB and AQ-group pigs quickly restored the MAP to +60 mmHg within the critical, first 30 minute resuscitation period which further increased throughout the remaining 90 minutes finally achieving 7% and 22% of their initial baseline values (Fig. 12a,b). There was no significant difference in the rate of restoration of MAP in either the AB or AQ groups (Table 2). In contrast, the LR-group pigs showed the slowest rate of resuscitation in comparison the AQ-group (P<0.03) in the first 30 minutes and barely achieved 60 mmHg over the entire 120 minute resuscitation period.

2b:  Heart Rate (HR)

AQ and LR-group pigs, in comparison to the AB-group pigs, showed characteristic tachycardia during the hemorrhagic procedure (Fig. 13a) which decreased during the first 30 minutes of fluid replacement but persisted as such in the AQ-group pigs during the resuscitation period being significantly different (P<0.03) to that observed in the AB-group. Both the AQ and LR groups remained tachycardic at the end of the 120 minute resuscitation period (Fig. 13b).

2c:  Body temperature (BT)

The AQ and LR group pigs had similar body temperatures at the commencement of the hemorrhagic procedure but were not significantly higher than that observed in the AB-group (Fig. 14a). All groups showed lower body temperatures when compared to baseline values at the end of the resuscitation period (Fig. 14b).

2d:  Cardiac Output (CO)

The rate of recovery of cardiac output was significantly faster and greater in magnitude in the AQ-group pigs (P<0.003) compared to both the AB-group and LR-group pigs (Fig.15a) during the first 30 to 60 minutes and remained as such at the end of the resuscitation period (Fig. 15b).

2e:  Central Venous Pressure (CVP)

As was the case with the restoration of MAP, the AB and AQ group pigs restored the CVP quicker and maintained the increased pressures in comparison to the LR-group (Fig. 16a), which finally achieved baseline values by the end of the 120 minute resuscitation period (Fig. 16b).

2f:  Pulmonary Arterial Occlusion Pressure (PAOP)

Only the AB-group pigs restored the PAOP to baseline levels within the first 30 minute resuscitation period with the AQ-group taking a further 30 minutes (Fig. 17a). The LR-group never achieved baseline levels at the end of the 120 minute resuscitation period having decreased by 7.1 mmHg (91%) from baseline values in comparison to 0.2 mm Hg (9%) and 2.1 mmHg (29%) in the AB and AQ groups respectively (Fig. 17b).
2g: Central Venous Pressure:Pulmonary Arterial Occlusion Pressure (CVP:PAOP)

Indicative of the compliance of the vasculature following fluid replacement was the observation that baseline ratios were achieved in the AB and AQ group pigs within the first 30 minutes of resuscitation which remained steady over the final 90 minutes (Fig. 18a). In contrast, the LR-group pigs showed a dramatic increase in the CVP:PAOP ratio (400%) during the final 90 minutes of resuscitation (Fig. 18b).

2h: Systemic Vascular Resistance (SVR) and Pulmonary Vascular Resistance (PVR)

In terms of changes in the total peripheral resistance (SVR) (Fig. 19a) and resistance to fluid flow in the pulmonary circulation (PVR) (Fig. 20a) during the 120 minute resuscitation period, there was a significant difference in the vasodilatory status of the systemic circulation (SVR) of the AQ-group pigs compared to either the AB (P<0.002) or LR-group pigs (P<0.01), which remained as such during the entire resuscitation period (Figs. 19b). The pulmonary vascular resistance (PVR) was not significantly different in AQ-group pigs in comparison to that observed in the AB-group at the end of the resuscitation period (Fig. 20b), both having increased to around 12% from baseline levels by the end of the resuscitation period. However, the LR-group pigs showed a 65.6% increase in pulmonary vascular resistance within the pulmonary vasculature at the end of the resuscitation period but this was not statistically significant.

2i: Capillary Hydrostatic pressure (pCAP)

Both the rate and magnitude of recovery of the capillary hydrostatic pressure in the microvasculature (pCAP) were significantly greater in the AQ-group in comparison to the LR-group pigs (P<0.04) as shown in Fig. 21a. At the end of the resuscitation period the AB-group pigs showed a return to baseline values whereas that of the AQ and LR-group pigs had decreased by 18% and 41% respectively (Fig. 21b) but which were not significantly different to that observed for the AB-group pigs.
Fig. 12a  Variation in Mean Arterial Pressure (MAP) within Trial Groups over Experimental Period

Fig. 12b  Absolute Change in Mean Arterial Pressure (MAP) within Trial Groups during Resuscitation Period
Fig. 13a  Variation in Heart Rate (HR) within Trial Groups over Experimental Period

Fig. 13b  Absolute Recovery of Heart Rates (HR) within Trial Groups during Resuscitation Period
Fig. 14a  Variation in Body Temperature (BT) within Trial Groups over Experimental Time

Fig. 14b  Absolute Recovery of Body Temperature (BT) within Trial Groups during Resuscitation Period
Fig. 15a  Variation in Cardiac Output (CO) within Trial Groups over Experimental Period

0.00  1.00  2.00  3.00  4.00  5.00  6.00  7.00
-60 min -30 min -15 min 0 min 30 min 60 min 90 min 120 min

CO mL/beat

Experimental Period

AB  AQIX  LR

Fig. 15b  Absolute Recovery of Cardiac Output (CO) within Trial Groups during Resuscitation Period

Absolute change over 30 mins from resuscitation  Absolute change over 120 mins from resuscitation  Absolute change @ 120 mins from baseline value
Fig. 16 a  Variation in Central Venous Pressure (CVP) within Trial Groups over Experimental Period

Fig. 16 b  Absolute Change in Central Venous Pressure (CVP) within Trial Groups over Experimental Period

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Fig. 17a  Variation in Pulmonary Arterial Occlusion Pressure (PAOP) within Trial Groups over Experimental Period

Fig. 17b  Absolute Change in Pulmonary Arterial Occlusion Pressure (PAOP) within Trial Groups over Experimental Period
Fig. 18a  Variation in the ratio between the Central Venous Pressure and Pulmonary Occlusion Arterial Pressure within Trial Groups over Experimental Period

Fig. 18b  Absolute Change in the ratio between Central Venous Pressure and Pulmonary Occlusion Arterial Pressure within Trial Groups over Experimental Period
Fig. 19a  Variation in Systemic Vascular Resistance (SVR) within Trial Groups over Experimental Period

![Graph showing variation in SVR over time among different trial groups.]

Fig. 19b  Absolute Change in Systemic Vascular Resistance (SVR) within Trial Groups over Experimental Period

![Bar chart showing absolute change in SVR over 30 mins, 120 mins, and baseline value.]
Fig. 20a  Variation in Pulmonary Vascular Resistance (PVR) within Trial Groups over Experimental Period

Fig. 20b  Absolute Change in Pulmonary Vascular resistance (PVR) within Trial Groups during Resuscitation Period
Fig. 21 a  Variation in Capillary Hydrostatic Pressure (pCAP) within Trial Groups over Experimental Period

Fig. 21 b  Absolute Change in Capillary Hydrostatic Pressure (pCAP) within Trial Groups over Experimental Period
Section 3 – Pathological Changes

3a: Kidney Morphology

In comparison to the normal morphology of the pig kidney (Fig. A1), 5 out of 6 AQ-group kidneys showed no abnormality in the glomeruli, tubule arrays or peritubular capillaries (Fig. B2) at the end of the experimental period (POD7). However, in one kidney in the AQ-group there was minimal damage in the tubular elements in the form of cell debris, pyknotic and enucleation changes in the nuclei (Figs. B3).

Out of all the trial groups the most severe damage occurred in the LR-group kidneys whereby loss of nuclei (Fig.C1), karyolitic and mitotic changes in the tubular elements (Fig.C2) with cellular debris and general vacuolization (Fig. C3) a common feature observed in this group of pigs.

3b: Liver and Lung Morphology

Examination of the lung morphology indicated no significant pathological changes in the either the AB or AQ-group pigs in comparison to the normal morphology (e.g., lung; Fig. D1). However, widespread oedema was evident in the lungs of the LR-group pigs (Fig. D2). While the liver morphology was normal in the AQ-group pigs, that of the AB and LR-group pigs showed evidence of sinusoidal damage in both these groups (Table 3).

3c: Brain (Hypocampus) Morphology

In direct contrast to the AB or AQ-group pigs, the LR-group pigs showed a general necrosis of the nuclei within sections of the hypocampus (Fig. E1).

3d: Reperfusion Injury Indices

A tabulated, morphometric assessment of the damage resulting from reperfusion of either autologous blood (AB-group) or the electrolyte-type fluids, AQIX RS-1 (AQ-group) or Lactated Ringer’s (LR-group), based upon a modified, Bamff99 tissue scoring system, is presented below in Table 3.

Morphological examination of the liver showed that while no evidence of damage had occurred in the AQ-group pigs, sinusoidal elements of both the AB and LR-group pigs had become damaged. Equally, while no RI damage was evident in the lungs of AB-group pigs, two of the LR-group pigs showed focal edema and one pig from the AQ-group had developed lobular pneumoniae.

In terms of RI damage to the kidneys in the trial groups, the least (score) damage was observed in the AB-group. In contrast, tubular debris was significantly more prominent (P< 0.04) in the AQ-group pigs when compared to AB-group pigs but not significantly different in the other forms of tubular damage.

Tubular dilation was most prominent in the LR-group pigs in comparison to either the AB (P< 0.02) or AQ-group pigs (P< 0.05) with no significant difference observed between AQ and AB-
group pigs (Table 3). Equally, cytoplasmic vacuolization was a significant, prominent feature observed in LR-group pigs when compared to the AB-group (P< 0.02) but not a significant feature in the AQ-group kidneys.

From a statistical analysis of the overall results presented in Table 3 it can be seen that the AB-group pigs scored significantly better than the LR (P< 0.03) or AQ-group pigs (P< 0.04) in preserving the integrity of the kidney when blood was administered as the volume replacement therapy. No significant difference overall was found when comparing AQ and LR-group pigs.

The causative factors involved in creating the damage observed in both AQ and LR-group pigs awaits further confirmation following analysis of TNF-α, IL-6 and neutrophil activity levels.

Table 3  Organ Reperfusion Injury (RI) - Morphometric Graded Assessment

<table>
<thead>
<tr>
<th>Pig [N]</th>
<th>Trial Group</th>
<th>Liver</th>
<th>Lung</th>
<th>Brain [Hypocampus]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Blood</td>
<td>Some WBC in sinusoids and mild vacuolization of cytoplasm [N=1]</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>LR-Saline</td>
<td>Apoptotic nuclear debris in sinusoids [N=1]</td>
<td>Focal lung edema [N=2]</td>
<td>Necrosis of Neurons [N=1]</td>
</tr>
<tr>
<td>6</td>
<td>AQIX RS-I</td>
<td>Normal</td>
<td>Acute lobar pneumonia [N=1]</td>
<td>Normal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pig [N]</th>
<th>Trial Group</th>
<th>Kidney: Tubular dilation</th>
<th>Kidney: Cytoplasmic vacuolization</th>
<th>Kidney: Tubular debris Scale 0-1-2</th>
<th>Kidney: Nuclear Absence or shrinkage</th>
<th>Kidney [composite score]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Blood</td>
<td>0.50</td>
<td>0.50</td>
<td>0.33</td>
<td>0.50</td>
<td>1.83</td>
</tr>
<tr>
<td>6</td>
<td>LR-Saline</td>
<td>1.33 ttest: LR vs AB = P &lt; 0.0219 ttest: LR vs AQ = P &lt; 0.0493</td>
<td>1.33 ttest: LR vs AB = P &lt; 0.0219 ttest: LR vs AQ = P &lt; 0.2131</td>
<td>0.83 ttest: LR vs AB = P &lt; 0.2131 ttest: LR vs AQ = P &lt; 0.2961</td>
<td>1.00 ttest: LR vs AB = P &lt; 0.2720 ttest: LR vs AQ = P &lt; 0.6870</td>
<td>4.50 ttest: LR vs AB = P &lt; 0.0280 ttest: LR vs AQ = P &lt; 0.6000</td>
</tr>
<tr>
<td>6</td>
<td>AQIX RS-I</td>
<td>0.67 ttest: AQ vs AB = P &lt; 0.5995</td>
<td>0.83 ttest: AQ vs AB = P &lt; 0.4029</td>
<td>1.33 ttest: AQ vs AB = P &lt; 0.0335</td>
<td>1.17 ttest: AQ vs AB = P &lt; 0.1778</td>
<td>4.00 ttest: AQ vs AB = P &lt; 0.0465</td>
</tr>
</tbody>
</table>

[Refer to accompanying file : / RI-Figs. A1 – E1/.. for further detail of morphological changes]
Concluding Remarks

It is to be acknowledged that numerous investigative studies involving swine species have noted great diversity in blood serum profile and hemodynamic values, which differ in many respects to that of humans.\(^1\)\(^-\)\(^6\) However, in the present study, these values were consistent within the swine species examined there being good statistical correlation.

The experimental design and protocols adopted in this investigation involving the intravenous administration of volume replacement fluids in a hemorrhagic pig (swine) model were based on the critique that following near fatal blood loss the accompanying changes in hemodynamics and blood chemistry should be restored to normality within the critical 30-60 minutes following hemorrhagic trauma in order to minimize the reported incidence of reperfusion injury that develops over the next 48-72 hours in essential organ systems.

To achieve this outcome, the study was particularly focused on monitoring specific hemodynamic and blood chemistry parameters, namely, how quickly and maintained effectively during the resuscitation period could these parameters be restored to baseline values as stated below;

1. the mean arterial pressure (MAP) to within 65\% of baseline
2. the cardiac output (CO) and stroke volume (SV)
3. the central venous (CVP) and pulmonary arterial occlusion (PAOP) pressures
4. the ratio of central to pulmonary pressures (CVP:PAOP)
5. the capillary hydrostatic pressure (pCAP)
6. the systemic (SVR) and pulmonary (PVR) vascular resistances

Overall, from the data presented in this study, all of the above parameters recovered quicker and were better maintained in the AQ-group pigs over the experimental periods than that observed in either the AB or LR-group pigs.

While it is acknowledged that the rapid and sustained restoration of the hemodynamic parameters, MAP, CVP and PAOP do not categorically indicate an absolute recovery from hemorrhagic shock, these parameters along with that indicating a restoration of intravascular flow (CO, SV) does suggest that in a clinical setting, AQIX RS-I has an equivalence to that of autologous blood replacement. In addition, the restoration of normalcy of the vascular compliance indices (i.e., CVP:PAOP), peripheral (SVR) and pulmonary (PVR) and microvasculature (pCAP) would all contribute in optimizing intravenous fluid flow during the ‘golden’ hour to the conclusion of the resuscitation period.

Understandably, no hemodilution occurred in the AB-group pigs when infused with approximately the same volume of shed blood during the experimental period. In contrast, the significantly, greater volumes of AQIX RS-I (3.8±0.8L) and LR-saline (3.2±0.7L) infused over the 2 hour resuscitation period and, in the case of 3 pigs in the AQ-group, 4.0L infused within 60 minutes, compared to that of autologous blood (1.0±0.3L) infused within the first 60 minutes, naturally caused a state of over perfusion resulting, predictably, in a state of hemodilution which remained as such until the end of the trial period when the hematocrit levels returned to within normal levels (Fig. 1a; Appendix ‘D’).
Characteristically, volume replacement therapies are susceptible to the incidence of inflammatory, anaphylactic, hypercoagulability episodes and seemingly related to the antigenicity and/or toxicity of the various excipients used in the formulation of the blood replacement fluids administered. Such does not appear to be the case in the intravenous administration of AQIX® RS-I solution from the analysis of the blood cell profile data obtained (Appendix ‘D’) but awaits ratification of the TNF-α and IL-6 analysis yet to be completed for this study.

Of importance in this study was the observation that the reperfusion of intravenous solutions, while elevated initially (CPK, SGOT, SGPT) associated with damaged organs (e.g., heart, lung, liver, kidney) during reperfusion injury, was still slightly elevated in the AB and LR-group pigs (Fig. 9a, b) but both groups still showed elevated levels of 39.7% and 38.7% respectively to baseline levels on POD7 in contrast to a 45% decrease in the AQ-group pigs. Interestingly, levels of LDH, an enzyme intimately linked with lactate/pyruvate metabolism and characteristically released from tissues and organs during reperfusion injury, was still slightly elevated in the AB and LR-group pigs but below baseline levels in AQ-group pigs (Appendix ‘D’).

Perhaps indicative of the RI damage observed in the LR-group pigs on POD7 was a significant 70% elevation (P < 0.02) in serum blood urea nitrogen levels from initial baseline levels in comparison to AB-group pigs (Fig. 9a, b) whereas the 37.5% increase of this enzyme in the AQ-group pigs was not statistically significant. However, the levels recorded during the experimental period were well within published levels (6 – 30 mg/dL) for porcine species of pigs.

Of particular interest in this study was the observation that the release of those enzymes (e.g., CPK, SGOT, SGPT) associated with damaged organs (e.g., heart, lung, liver, kidney) during reperfusion of intravenous solutions, while elevated initially in all trial groups on POD1 and 2, gradually declined to within normal published levels (see, Fig. 10b; 11b; Appendix ‘D’).

From the aforementioned comments, it is not surprising to find evidence of reperfusion injury predominately within the LR-group pigs (Table 2; Figs. A - E) and, in line with the better performance overall of the AQ-group pigs, that there was no resultant damage evident within the
lungs, livers or brains (hypocampus) of the AQ-group pigs when compared to either the AB or LR-groups and only minimal tubular damage observed in their kidneys.

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Aqix Ltd, UK
September 15th 2008
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Appendix ‘A’ – AQIX® Solution Technology Applications

AQIX® Solution Technology Publications

AQIX® Solution Technology Publications

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**Bulletin No.3** Historical Repercussions : Donor Organ Transplantation (1994)

**Bulletin No.4** Toxicity of Ingredients in Commercial Preservation Solutions (2008)[web]
**Bulletin No.5**  AQIX® RS-C - A Cardioplegic solution for use in Cardiothoracic Surgical Procedures under Hypothermic or Normothermic conditions via single, multiple bolus or continuous perfusion conditions *(2008)* [web site]

**Bulletin No.6**  The *Quest* - *A universal* mammalian organ and tissue perfusate and preservation solution *(2008)* [web site]

**Bulletin No.7**  Unique Design Features: AQIX RS-I Solution *(2008)* [web site]
### Appendix 'D' - Reference Data Comparisons

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Mean Values at end of Experimental Period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood &amp; Cell Profile</strong></td>
<td>AB-group</td>
</tr>
<tr>
<td>RBC's 5 – 8 mil/mm$^3$</td>
<td>4.92</td>
</tr>
<tr>
<td>Hg 6.9 - 12.7 g/dL</td>
<td>9.25</td>
</tr>
<tr>
<td>Hct 15 – 37 %</td>
<td>27.17</td>
</tr>
<tr>
<td>WBC's 7 – 20 mil/mm$^3$</td>
<td>18,433</td>
</tr>
<tr>
<td>Lymphocytes 40 – 60 %</td>
<td>46.67</td>
</tr>
<tr>
<td>Platelets 120,000 – 720,000 mm$^3$</td>
<td>389,500</td>
</tr>
<tr>
<td>aPTT 17.8 - 23.3 sec</td>
<td>17.50</td>
</tr>
<tr>
<td>Pt [INR] 0.8 - 1.2</td>
<td>0.88</td>
</tr>
<tr>
<td>Fibrinogen 2.06 - 6.13 g/L</td>
<td>2.03</td>
</tr>
<tr>
<td><strong>Electrolytes</strong></td>
<td></td>
</tr>
<tr>
<td>Sodium 135 – 150 mmole/L</td>
<td>138.50</td>
</tr>
<tr>
<td>Potassium 4.1 – 6.9 mmole/L</td>
<td>4.03</td>
</tr>
<tr>
<td>Calcium 8.3 - 13.3 mg/L</td>
<td>9.88</td>
</tr>
<tr>
<td>Magnesium 1.7 - 2.44 mg/L</td>
<td>2.55</td>
</tr>
<tr>
<td>Hydrogen Carbonate 22 – 46 mmole/L</td>
<td>29.17</td>
</tr>
<tr>
<td>Chloride 280 - 306 mOsm/kg</td>
<td>97.33</td>
</tr>
<tr>
<td>Osmolality</td>
<td>290.0</td>
</tr>
<tr>
<td>Anion Gap 10.6 – 16.5 mmole/L</td>
<td>16.03 ± 5.07</td>
</tr>
<tr>
<td><strong>Metabolites</strong></td>
<td></td>
</tr>
<tr>
<td>Glucose 65 – 150 mg/dL</td>
<td>82.00</td>
</tr>
<tr>
<td>Lactic acid 1.91 - 2.47 mmole/L</td>
<td>3.49</td>
</tr>
<tr>
<td>BUN 6 – 30 mg/dL</td>
<td>14.67</td>
</tr>
<tr>
<td>Creatinine 0.8 – 3.6 mg/dL</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>LDH 286 -12,000 IU/L</td>
<td>684.33</td>
</tr>
<tr>
<td>SGOT (AST) 29 – 1140 IU/L</td>
<td>59.83</td>
</tr>
<tr>
<td>SGPT (ALT) 7 - 161 IU/L</td>
<td>47.00</td>
</tr>
<tr>
<td>CPK 311 - 16,700 IU/L</td>
<td>775.50</td>
</tr>
</tbody>
</table>
Fig. A - Autologous Blood - Pig No. 4 - Normal Kidney profiles of collecting tubules

Fig. B - Aqix RS I - Pig No 22 - Typical Kidney profile on post-operative day 7

- Nuclei
- Mitosis
- Peritubular capillaries
- Glomerulus
Fig. C1 - Lactated Ringers Pig No. 17 – Loss of Nuclei from tubular components in kidney

Cellular Debris

Loss of nuclei

Fig. C2 - Lactated Ringers Pig No. 17 – Debris and Mitotic events in glomeruli

Debris

Mitosis

Karyolysis
Fig. C3 - Lactated Ringers Pig No. 17 – Widespread Vacuolization of tubules

Fig. C4 - Aqix Pig No. 25 Kidney showing enucleation, pyknosis and cellular debris as part of acute tubular necrosis process
Fig. D1 - Lactated Ringers Pig No. 13 – Sample section of normal lung structure.

Fig. D2 - Lactated Ringers Pig No. 13 – Sample section with evidence of edema.
Fig E - Lactated Ringers Pig No. 13 – Hypocampus – Necrotic Neurons

Necrotic neurons