Optimising the composition of irrigation fluid to reduce the potency of S. aureus -toxin: potential role in the treatment of septic arthritis

Optimising the composition of irrigation fluid to reduce the potency of *S. aureus* α-toxin: potential role in the treatment of septic arthritis.

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Running Head: Optimising cartilage irrigation fluid

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Abstract

Objective: Septic arthritis is commonly caused by *Staphylococcus aureus* and is a medical emergency requiring antibiotics and joint irrigation. The bacteria produce α-toxin causing rapid cartilage cell (*chondrocyte*) death. Saline (0.9%NaCl) lavage is normally used to remove bacteria and toxins, however, its composition might be sub-optimal to suppress the lethal effects of α-toxin. We utilised rabbit erythrocyte haemolysis as a sensitive, biologically-relevant assay of α-toxin levels to determine if changes to osmolarity, temperature, pH, and divalent cation (Mg^{2+}, Ca^{2+}) concentration were protective.

Design: Erythrocytes were incubated in the various conditions and then exposed to α-toxin ('chronic' challenge) or incubated with α-toxin and then exposed to experimental conditions ('acute' challenge).

Results: Raising osmolarity from 300mOsm (0.9%NaCl) to 400, 600 or 900mOsm (sucrose addition) when applied chronically, significantly reduced haemolysis linearly. As an acute challenge, osmotic protection was significant and similar over 400-900mOsm. Reducing temperature chronically from 37°C to 25°C and 4°C significantly reduced haemolysis, however, when applied as an acute challenge although significant, was less marked. Divalent cations (Mg^{2+}, Ca^{2+} at 5mM) reduced haemolysis. Varying pH (6.5, 7.2, 8.0) applied chronically marginally reduced haemolysis. The optimised saline (0.9%NaCl;900mOsm with sucrose), 5mM $MgCl_2$, (37°C) rapidly and significantly reduced haemolysis compared to saline and Hank's buffered saline solution (HBSS) applied either chronically or acutely.

Conclusions: These results on the effect of *S. aureus* α-toxin on erythrocytes showed that optimising saline could markedly reduce the potency of *S. aureus* α-toxin. Such modifications to saline could be of benefit during joint irrigation for septic arthritis.

Introduction

Septic arthritis, a destructive joint disease leading to permanent cartilage damage and disability, affects all ages with an incidence in Western Europe of 4-10 cases/100,000 persons/yr[1,2]. *Staphylococcus aureus* (*S. aureus*) accounts for 40-70% of all cases of septic arthritis[1,3,4] and the incidence is rising due to factors including an ageing population, increased use of immunosuppressive agents, musculoskeletal prosthetics and surgical procedures[5]. Management of septic arthritis is to rapidly eliminate the bacteria and associated toxins through intravenous antibiotics and copious joint irrigation[5]. Despite eradication of the bacteria, the damage caused to articular cartilage persists in >50% of cases and may lead to osteoarthritis (OA)[1,2,6].

S. aureus produces an array of toxins including exotoxins (including alpha (α), beta (β), gamma (γ) and delta (δ) haemolysin)[7] which are potent virulence factors[8-11]. Recent work using *in vitro* bovine cartilage and an *in vivo* murine model of septic arthritis with isogenic mutants of *S. aureus*, has identified α-toxin as the primary agent causing the rapid death of cartilage cells (chondrocytes)[12-14]. Chondrocytes are the only cell type capable of maintaining the tissue's resilience through the turnover of extracellular matrix proteins and their loss, through the action of α-toxin, will result in cartilage degradation. *S. aureus* α-toxin also has longer-term damaging effects on chondrocytes, for example, it may increase expression of catabolic factors including matrix metalloproteinases (MMPs) and inducible NO synthase (iNOS) leading to deleterious changes to cartilage metabolism[15-18]. While there has been considerable attention given to the development of antibacterials for treating *S. aureus* infection, the protection of chondrocytes against the deleterious effects of α-toxin has not been as intensively investigated.

S. aureus α-toxin binds to the A Disintegrin And Metalloproteinase 10 (ADAM10) receptor present on animal and human articular chondrocytes and rabbit erythrocytes[19-21]. This leads to the formation of a heptameric pore and rapid influx of Na^+ and water, causing cell swelling and lysis, leading to the release of intracellular components resulting in inflammation[22]. Rabbit erythrocytes show only low sensitivity to other haemolysins[23] (in contrast to human erythrocytes[24]) and are therefore an extremely flexible and sensitive model system for studying the interaction between this α-toxin and cell lysis[24]. Additionally, the release of haemoglobin can easily be measured spectrophotometrically, allowing the dynamic effects of biologically-relevant activity of α-toxin on cell viability to be assessed[24].

The fluid used for joint irrigation is normally isotonic saline (0.9%NaCl;300mOsm) which is hypo-osmotic compared to normal synovial fluid (400mOsm)[25]. Previous work has shown that the sensitivity of chondrocytes to other forms of injury may be markedly reduced when the osmolarity of isotonic saline or culture medium (typically 300mOsm) is increased[26,27]. This raised the possibility that the saline currently used for irrigation might be sub-optimal and that altering some of its properties might reduce the injurious effects of α-toxin and thus be chondroprotective against α-toxin. Accordingly, we have tested the effects of osmolarity (300, 400, 600, 900mOsm),

temperature (4°C, 25°C, 37°C), divalent cations (Ca^{2+} and Mg^{2+}) and pH (6.5, 7.2, 8.0) on the potency of *S. aureus* α-toxin using the sensitive rabbit erythrocyte haemolysis assay. The aim of this study therefore was to determine if these relatively simple alterations to the properties of standard saline could reduce the damaging effect of *S. aureus* α-toxin.

Materials and Methods.

(a) Biological materials, tissue culture, reagents. Saline (0.9%;300mOsm) used clinically for irrigation was obtained from Baxter Healthcare Ltd., Norfolk, UK. Hank's buffered saline solution (HBSS;300mOsm) was purchased from Invitrogen Ltd., Paisley, UK. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer was obtained from Sigma-Aldrich Chemical Co., Gillingham, UK. TSA (tryptone soya agar), TSB (tryptone soya broth) and skimmed milk were obtained from Oxoid Ltd., Basingstoke, UK.

(b) Rabbit red blood cells. The rabbit red blood cell (RBC) haemolysis assay was used to semi-quantitatively determine biologically relevant α-toxin activity[24]. Fresh, heparinised RBCs from Orygen Ltd., Penicuik, UK, were prepared at ~5% haematocrit in saline (0.9% NaCl) and kept at 5°C until required. Fresh blood was obtained weekly and was suitable for up to 5 days of experimentation after receipt.

(c) Preparation of bacterial pellets and supernatant samples. S. aureus strain 8325-4 was kindly provided by Prof. T.J. Foster and stored at -80°C in 10%v/w skimmed milk. This strain is a well-characterised prophage-cured derivative of strain NCTC8325 that produces large amounts of α-toxin[28] and has comparable potency to clinical strains of *S. aureus* in terms of chondrocyte-damaging potential[12]. When required, bacteria were thawed and streaked onto TSA plates and cultured (24hrs;37°C). TSB plates containing 2μg/ml tetracycline were then prepared and inoculated with several single bacterial colonies from the TSA plate and incubated (24hr;37°C) with shaking. From this culture, serial dilutions were performed in saline to 10^{-6}, spread on TSA plates and incubated (24hr;37°C). The number of Colony Forming Units/ml (CFU/ml) in TSB was typically ~$1x10^9$ CFU/ml. α-toxin-containing supernatants were obtained by centrifugation (800xg;10min) of the TSB cultures which were then filter-sterilised and stored (4°C) until required which was within one week. To establish an appropriate time-course at the beginning of a week's experiments, the sensitivity of rabbit RBCs to α-toxin were assessed by adding a small volume of toxin to a 5% RBC suspension and incubating at 37°C for 60mins. Samples were taken every 10mins, centrifuged (10,000xg;10secs) and haemolysis determined by the absorbance of haemoglobin at 540nm (Abs_{540}) on a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, USA). Maximum (100%) haemolysis was determined by freeze/thawing a sample of the RBC suspension. Percent haemolysis (%H) was then calculated ((Abs_{540} of sample – Abs_{540} of negative control)/(Abs_{540} of 100% haemolysis – Abs_{540} of negative control) x100%) to give a measure of α-toxin activity. The sensitivity of erythrocyte samples to α-toxin and the amount and potency of α-toxin produced from each *S. aureus* culture, were variable. Rabbit erythrocytes which were relatively insensitive to toxin requiring >1hr of incubation before haemolysis was detectable, were not studied further. A rabbit blood sample which produced ~50% haemolysis after about 15mins was considered acceptable for experimentation.

(d) Chronic or acute exposure of α-toxin-treated erythrocytes to various solutions and temperatures. Chronic exposure: Suspensions of rabbit erythrocytes were initially exposed to the

experimental conditions of osmolarity, temperature, pH or divalent cations for 10mins before an aliquot of α-toxin was added, the cell suspension mixed quickly, and the time course of % haemolysis (%H) measurements commenced. *Acute exposure:* The α-toxin treated erythrocyte suspensions were initially incubated under control conditions and haemoglobin release measured until this reached 20-30% haemolysis. The cell suspension was then challenged with the various experimental conditions, and the extent of haemolysis determined until the end of the time course. The rate of change in % haemolysis/10mins for the chronic challenge was measured over 10-20mins and for the acute challenge, the time course was measured over 20-30mins after the start of the experiment. Data were shown as the change in % haemolysis (%H)/10mins. For the control condition in the 'acute exposure' experiments for osmolarity and divalent cations, an identical volume of saline was added at the same time point to correct for α-toxin dilution. For the pH experiments, HEPES (10mM) was present and pH adjusted using HCl or NaOH. For some experiments, erythrocytes were suspended in Hank's balanced salt solution (HBSS;pH 7.2).

(e) Data analysis and statistics. Data are shown as means ± standard error of the mean (s.e.m.) for *N* independent experiments and *n* replicates for each experiment (*N*(*n*)), and analysed using GraphPad Prism Ver. 7.0b (GraphPad, San Diego, USA). Non-parametric t-tests and ANOVAs (Analysis of Variance) were performed as indicated and significant differences accepted when $P<0.05$.

Results

(a) Suppression of α-toxin damage by raising saline osmolarity. To assess the effects of raising saline osmolarity (300mOsm) above that of synovial fluid (i.e. ≥400mOsm) on the damaging effect of α-toxin on rabbit erythrocytes, two types of experiments were performed: *(i) chronic challenge* – where erythrocytes were exposed to the various osmotic conditions including α-toxin throughout (Fig. 1A), or *(ii) acute challenge* – where erythrocytes were exposed to α-toxin until approx. 20-30% haemolysis had occurred, before the hyper-osmotic challenge was delivered (Fig. 1B). For the chronic challenge, by the end of the time-course, control percent haemolysis (%H) (300mOsm; ~93%) was significantly greater than 400mOsm (72%;P=0.0008), 600mOsm (47%;P<0.0001) and 900mOsm (21%;P<0.0001) (Fig. 1A(i)). An inverse linear relationship was evident between osmolarity and %H induced by α-toxin (R^2=0.9626;P=0.0189; (Fig. 1A(ii)). The change in % haemolysis/10mins was calculated after toxin exposure and was 3.9% for the control and although reduced at 400mOsm, was not significant (2.2%;P=0.506). However a reduction was observed at 600mOsm (0.7%;P<0.0001) and 900mOsm (0.1%;P<0.0001; (Fig. 1A(iii)).

When osmolarity was raised >300mOsm, approximately 10mins after α-toxin addition ('acute challenge') there was rapid and almost complete protection of erythrocytes (Fig. 1B(i)). There was a significant decrease in haemolysis between the control (300mOsm;82%) and hyperosmotic solutions (44%, 41%, 41% for 400, 600, 900mOsm respectively) by the end of the experiment (P<0.0001 for all osmolarities compared to 300mOsm). Interestingly, in contrast to the chronic challenge, the effects of 400mOsm and greater were not significantly different when compared to each other (P=0.327;Fig. 1B(ii)). The rates of change in %H were significantly decreased for osmolarities of ≥400mOsm (Fig. 1B(iii)). Raising osmolarity using sucrose was thus strongly protective particularly after α-toxin damage to erythrocytes had been initiated.

The osmotic protection conferred by sucrose was compared with that of a different osmolyte (NaCl) to the same osmotic pressure. At 40mins, NaCl (600mOsm) reduced %H from the control (300mOsm) value of 66±6% to 56±8%, whereas with sucrose this was decreased further to 20±3%. In additional experiments at the same time point, when the osmolarity was raised to 900mOsm, the %H for the control was 68±8%, and reduced to 21±7% in the presence of NaCl but was only 4±2% with sucrose (data are means ± s.d., N=2). Although NaCl protected erythrocytes against α-toxin, it appeared less effective when present at the same osmolarity as sucrose.

(b) Reducing temperature of saline conferred protection against α-toxin-induced haemolysis. RBC suspensions were incubated for 10mins at the various temperatures, treated with α-toxin and then haemolysis determined. The chronic exposure to reduced temperature protected erythrocytes treated with α-toxin (Fig. 2A(i)). After 60mins at 25°C, the %H was 48% and significantly less compared to 37°C (75%;P=0.0056). However, when the α-toxin treated red blood cells were incubated at 4°C, haemolysis was virtually abolished over the time course studied (P<0.0001;Fig.

2A(i)). An inverse linear regression was observed between decreasing temperature and haemolysis (R^2=1.00;*P*=0.002) (Fig. 2A(ii)). The rate of change in haemolysis (%H/10mins) for 4ºC, 25ºC, and 37ºC was 0.02%, 2.0%, and 2.6% respectively (4ºC *vs.* 37ºC, *P*=0.0115;4ºC *vs.* 25ºC, *P*=0.0451) (Fig. 2A(iii)). When temperature was changed quickly during the time course ('temperature switch protocol'), no significant difference in haemolysis was observed between 37ºC and 25ºC until the 50min time-point (71% *vs.* 66%; *P*=0.0232; Fig. 2B(i)). A significant difference was also found between 37ºC and 4ºC after 40mins (65% *vs*. 59%;*P*=0.001). Although a linear relationship was evident, there was no significant deviation from a gradient of zero (R^2=0.979;*P*=0.0923;Fig. 2B(iii)). Therefore, reducing temperature was more protective before α-toxin addition. However, after its addition, reducing the temperature from 37ºC to 4ºC reduced haemolysis by ~10% (Fig. 2B(i)).

(c) Protective effect of divalent cations against α-toxin induced erythrocyte haemolysis. For the chronic exposure experiments, divalent cations (Ca^{2+} or Mg^{2+} at 5mM) produced a modest decrease in haemolysis with significant protection observed as early as 10mins with $CaCl_2$ (*P*=0.0149) and 20mins with $MgCl_2$ (*P*<0.0001;Fig. 3A(i)). This was maintained throughout the exposure protocol. By the end of the experiment, lower haemolysis levels resulted from cell suspensions containing Ca^{2+} (58.8%, *P*=0.0063) or Mg^{2+} (60.7%, *P*=0.0231) compared to the control (71.7%;Fig. 3A(i)). The protective effects of the divalent cations were indistinguishable (*P*>0.99;Fig. 3A(i, ii)). The addition of Mg^{2+} after α-toxin exposure ('acute exposure') gave significant protection (*P*=0.0002) against haemolysis, however, no difference was recorded with Ca^{2+} (*P*=0.12) at 60mins (Fig. 3B(i)). Furthermore, the rate of change showed no differences between the control and divalent ions (*P*>0.05;Fig. 3B(ii)). Thus, divalent ions provided only minor protection against haemolysis induced by α-toxin.

(d) Influence of pH on α-toxin induced erythrocyte haemolysis. Altering saline pH might provide some protection against the damaging effects of α-toxin. A significant decrease in %H occurred between pH 6.5 and 8.0 (two-way ANOVA; *P*=0.0015) at 60mins (Fig. 4(i). However, there were no significant differences between normal pH (pH 7.2) and pH 6.5 (*P*=0.1995) or pH 8.0 (*P*=0.0892). The data points fitted a linear regression but the slope was not significantly different from zero (R^2=0.9685, *P*=0.1136;Fig. 4(ii)). The rate of change of %H between 10-20mins of toxin exposure showed little variation across the pH values (*P*>0.99) (Fig. 4(ii)) suggesting that increasing saline pH may only offer marginal protection.

(e) Effect of optimised saline on α-toxin-induced erythrocyte haemolysis. On the basis of the previous results, an optimised saline solution (0.9%NaCl, 900mOsm, 5mM $MgCl_2$, 37ºC) was prepared. This was compared to normal saline (0.9%NaCl) and HBSS (which contains Ca^{2+}) in its ability to protect erythrocytes against α-toxin (Fig. 5A(i)). When the red blood cells were pre-incubated in these solutions for 10mins before addition of α-toxin, the optimised saline abolished subsequent haemolysis in contrast to both normal saline and HBSS (both *P*<0.0001), where levels of haemolysis were 78% (normal saline), 63% (HBSS), 4% (optimised saline) at 60mins (Fig.

5A(i)). The rate of change of haemolysis was greatest for normal saline (3.3%) followed by HBSS (1.7%) then optimised saline (0.05%;Fig. 5A(ii)). The addition of optimised saline after toxin exposure also significantly suppressed haemolysis compared to the other solutions. With optimised saline, haemolysis showed a small increase from 36% to 46% by the end of the experiment, whereas these levels rose considerably more with the use of normal saline (75%) and HBSS (72%;Fig. 5B(i)). Likewise, the rate of change was much reduced with optimised saline (0.2%H/10mins) compared to normal saline (1.3%H/10mins, P=0.0374) and HBSS (1.4%H/10mins, P=0.03;Fig. 5B(ii)).

Discussion

During treatment for *S. aureus* septic arthritis, it is important that the synovial fluid and infected tissues of the joint are rinsed quickly with a benign solution to remove bacteria and associated toxins. Traditionally, saline (0.9% NaCl) is used, however this might be sub-optimal for suppressing α-toxin activity and there may be opportunities for its composition and other properties to be modified to protect chondrocytes. To assess the protective effects of these modified solutions, the release of haemoglobin from rabbit erythrocytes was used as a sensitive and biologically-relevant measure of *S. aureus* α-toxin activity. The results suggested that increased osmolarity, reduced temperature, divalent cations, and to a lesser extent alkaline pH, could significantly reduce the damaging effect of α-toxin suggesting that relatively simple modifications to saline could be of benefit during joint irrigation for septic arthritis.

It could be considered that the rabbit haemolysis assay for determining methods for protecting cells against the damaging effects of α-toxin would not be an appropriate model. However, rabbit erythrocytes contain the *S. aureus* α-toxin receptor ADAM10 which is also present on chondrocytes of animals and normal and degenerate human cartilage[19-21]. The measurement of haemoglobin release following the interaction between α-toxin and erythrocytes provided a dynamic and sensitive assay for the lethal effects of α-toxin, and was highly flexible experimentally and reproducible. While it would be possible test these conditions on cartilage explants, interpreting the results obtained under these various conditions might not as straightforward, as for example access to the receptor in cartilage zones could be delayed and/or restricted. While studies on isolated chondrocytes could be of benefit, the receptor may be damaged or its sensitivity altered as a result of the enzymic treatment of cartilage which is required for release of chondrocytes. Our previous work has demonstrated that *S. aureus* α-toxin is the key damaging agent to chondrocytes in a cartilage model of septic arthritis[12,14]. Thus, although the rabbit erythrocyte model could be considered a limitation in this study, it nevertheless yielded valuable information about whether protection against α-toxin was possible and identified alterations to the irrigation fluid which could potentially be extended to detailed *in vivo* and clinical studies on *S. aureus* septic arthritis.

Two protocols were used which would broadly correspond to different stages of α-toxin action on the cell membrane which is time-dependent with the binding and pore-formation occurring within 2-3 mins[24]. For the chronic challenge, erythrocytes were equilibrated with the various conditions (osmolarity, temperature, pH, divalent cations), before toxin was added and the haemolysis time-course commenced. For the acute challenge, the time-course was started by α-toxin addition to the RBC suspension, and when there was 20-30% haemolysis, the erythrocytes were exposed to the experimental conditions. Thus, the chronic exposure would mainly represent the effect of experimental conditions on early steps of toxin action but for the acute exposure, pore formation would be complete and the pathological changes (i.e. increased ion permeability, cell swelling) would be underway with haemolysis following. The acute exposure would be closer to the clinical situation where the majority of cells in the joint would already have been exposed to

prevailing levels of α-toxin, and cell injury/death would be proceeding. While the overall effects of chronic and acute challenges of osmolarity, reduced temperature and divalent cation concentration (Figs. 1-4) were similar and gave significant protection, the time courses appeared different.

Raising osmolarity prior to toxin addition (chronic osmotic challenge) reduced the rate of haemolysis in a dose-dependent manner (Fig. 1A(i)-(iii)). This suggested that erythrocyte shrinkage could have interfered with early events of toxin action i.e. monomeric α-toxin binding to the cell membrane and pore formation. However studies by Cooper *et al*.[24] where an osmolyte (polyethylene glycol) was added after α-toxin addition, suggested that binding and pore formation were unaffected and that pore permeability instead was more sensitive to osmolarity. An acute hyper-osmotic challenge to erythrocytes in which α-toxin pores would already have formed, was rapidly (within 10mins) effective over the range studied (400-900mOsm). However, there was no difference between the osmolarities (Fig. 1B(i)-(iii)). This protective effect may be different compared to the chronic challenge, with the acute hyper-osmotic medium causing rapid erythrocyte shrinkage thereby conferring protection against the cell swelling induced by α-toxin. The raised osmolarity might simply shrink the cells rapidly meaning that it would take longer for the cells to swell to a critical volume. It was noted that NaCl was less effective at protecting erythrocytes compared to sucrose to the same osmolarity. This may be because Na^+ can enter *via* Na^+ channels and $Na^+/K^+/2Cl^-$ cotransporter[29] effectively reducing the osmolarity compared to sucrose. Thus, sucrose would be the preferred osmolyte for the optimised irrigation fluid as it is impermeable and metabolically inert (see[26]).

Pre-incubating erythrocytes at 25ºC delayed the damaging action following α-toxin addition. However once established, the rate of increase in haemolysis was the same as for cells equilibrated at a physiological temperature (Fig. 2A(iii)). This suggested that the early steps of pore formation were sensitive to reduced temperature. However, pre-equilibration at 4ºC completely protected erythrocytes against α-toxin (Fig. 2A(i)). This may accord with Reichwein *et al.,*[30] who demonstrated a temperature-dependent transition from toxin monomers to a functional heptameric pore. They pre-incubated rabbit erythrocytes with α-toxin (0ºC;30mins), and then washed and maintained the erythrocytes at either 0ºC or 37ºC. Enzyme-linked immunosorbent assays (ELISA) showed that there were no α-toxin oligomers on the cell membrane nor any haemolysis at the lower temperature. In contrast, the cells that were incubated at 37ºC experienced haemolysis, suggesting that α-toxin binding/pore formation was suppressed at low temperature. This is supported by Freer[31] who showed that α-toxin binding did not necessarily lead to erythrocyte destruction and that lysis (i.e. functional pore formation) did not occur until temperatures were >12ºC. Notably, the effects of reduced temperature in the acute challenge experiments (Fig. 2B(i)) were less marked than for the chronic challenge as there was a delay before the inhibition occurred (Fig. 2(A,B)). This could be because the pores had formed, and reduced temperature had little effect on the cation flow and subsequent erythrocyte swelling. Clinically, the acute challenge methodology would represent the situation where irrigation fluid was introduced into the infected

joint during treatment. The toxin would already be present, bound to the cell membranes and acting on chondrocytes and other cells in the joint to cause its damaging effects.

There was a mild but significant protection of erythrocytes when pH was increased from 6.5 to 8.0 (Fig. 4(i)). The pH of 0.9% NaCl should be ~7.0 but the true value often oscillates around pH=5.5 due to varying levels of dissolved CO_2[32]). Work by others[33] suggested that acidity converted α-toxin from an amphipathic form into a more hydrophobic molecule, thus accelerating pore formation. It has also been proposed that acidic pH enhanced H^+ binding to histidine residues on the toxin molecule vital for polymerisation, accelerating pore formation[34]. There may therefore be benefit to introduce a benign pH buffer to stabilise irrigation fluid pH, perhaps at a slightly alkaline level, to provide some protection against α-toxin and also dampen any elevated pCO_2 levels present in the clinical environment.

Ca^{2+} and Mg^{2+} produced small but significant protection against α-toxin (Fig. 3A,B) with no differences between these ions. Previous studies on Ca^{2+} suggested that it reduced the lateral movement of monomeric α-toxin in the plane of the membrane thereby reducing the rate of pore formation[35]. Depletion of Ca^{2+} was sufficient to remove the protection suggesting a reversible effect. However, it was unclear if this was Ca^{2+}-specific or whether due to the osmolarity of the $CaCl_2$ which would contribute ~90mOsm[35]. Apart from the protective effects of divalents on the action of α-toxin on cells, antibacterial roles for Ca^{2+} and Mg^{2+} have been reported[36]. Raised divalent concentrations disrupted *S. aureus* membranes possibly by forming complexes with cardiolipin which introduced membrane bending and destabilised its integrity. Stationary-phase bacteria, which are resilient against environmental pressures, were subjected to either divalent ion for 40mins and ~60% of the bacterial culture did not survive. A threshold of 10mM Ca^{2+} and 20mM Mg^{2+} to destroy *S. aureus* was established[36]. This study used concentrations greater than the present work, and therefore it may be interesting to further investigate increasing Ca^{2+} and Mg^{2+} concentrations on *S. aureus* survivability and α-toxin potency.

On the basis of these experiments, we tested an optimised saline applied as either a chronic or an acute challenge and observed substantial protection of erythrocytes against α-toxin (Fig. 5A,B). That there was little difference between normal saline and HBSS suggested that the majority of the protection was due to the raised osmolarity. With the acute challenge (Fig. 5B), the protection was very rapid indicating the quick suppression of the damaging effect of α-toxin which could be considered potentially clinically relevant for joint irrigation. There was still, however, a small increase in %H (Fig. 5B) possibly because further optimisation may be required, and/or there are other toxic elements produced by *S. aureus* which could have a relatively minor damaging effect on rabbit erythrocytes. A modified irrigation solution may also have benefits beyond those of protecting cells against α-toxin. For example, cooled irrigation fluid could offer pain relief and anti-inflammatory effects. A study involving patients who underwent total knee arthroplasty found that saline administered at 4°C alleviated pain, localised swelling, and decreased analgesia intake, as well as improving the quality of post-operative recovery[37]. Furthermore, a hyper-osmolar irrigation

saline, in addition to rapid protection against α-toxin, could be beneficial as Chan and Foster[38] found that addition of 20mM sucrose in growth media suppressed α-toxin gene (*hla*) expression by ~98% of the control.

While the present results were obtained using the rabbit red blood cell model, some caution should be exercised when extrapolating these results to the protection of chondrocytes within the cartilage matrix. Previous studies using a bovine osteochondral explant model have shown that *S. aureus* α-toxin can rapidly penetrate the matrix and cause chondrocyte death[12]. We have also shown that chondrocyte volume changes very quickly (within mins) following alterations to extracellular osmolarity[39]. Thus there is the expectation that by raising osmolarity, protection of *in situ* chondrocytes against α-toxin should be achieved in the same way this has been demonstrated with rabbit erythrocytes. With these observations in mind and taking the results from the present study together with previous observations, the beneficial effects of modifying the irrigation saline used during joint lavage should be considered in further *in vivo* animal and/or clinical research. The use of a relatively benign, inexpensive, drug-free and rapidly-effective modified saline as part of the normal lavage process is potentially an attractive novel method for limiting the damaging action of *S. aureus* α-toxin during septic arthritis.

S. aureus infections have been treated with β-lactams (e.g. penicillin) for decades, but the appearance and rapid spread of methicillin-resistance *S. aureus* (MRSA) have all but eliminated these antibacterials for treatment[40]. Non-antibiotic treatment is therefore an area of important research interest since suppressing activity of bacterial toxins either by influencing toxin production, or blocking their action would not only make the bacteria less pathogenic, but may also increase their susceptibility to host immune defence[41]. For example, inhibition of *S. aureus* pathogenesis by interfering with the signal transduction pathways for virulence using the RNA III inhibiting peptide has been described[42]. This peptide reduced the pathology and delayed the onset of disease symptoms in models of *S. aureus* infection including septic arthritis[42]. Other methods include an α-toxin antibody[43], cyclodextrin-lipid complexes to suppress the damaging effect of *S. aureus* α-toxin[44] and nanoparticle-based α-toxin entrapment to deliver the non-disrupted pore-forming toxin for immune processing[45]. These methods could be particularly important for cells with high levels of the ADAM10 receptor[21] which would render them particularly sensitive to *S. aureus* α-toxin. In summary, the development of the optimised irrigation saline described here potentially offers a cheap, very rapid (within minutes) and relatively benign method to suppress the damaging effects of α-toxin and may be of benefit during joint irrigation for septic arthritis caused by *S. aureus*.

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Figures

Figure 1. **Raised osmolarity reduced rabbit erythrocyte haemolysis induced by *S. aureus* α-toxin.** The % haemolysis (%H) was measured over 60mins under either (A) chronic or (B) acute challenge with various osmolarities as follows; 0.9% saline (control, 300mOsm (●)) and saline osmolarity raised by sucrose addition to 400mOsm (□), 600mOsm (▲) or 900mOsm (inverted triangle) either before toxin addition (chronic) or at approx. 20-30% haemolysis (indicated by the bar and arrow – 'osmolarity change'). The panels labelled (ii) show the % haemolysis data at t=60mins plotted as a function of osmolarity, and the panels labelled (iii) show the rate of change of % haemolysis/10mins plotted as a function of osmolarity. In this and subsequent figures, significant differences are indicated as follows: * P<0.05; ** P<0.01; *** P<0.001). Results are means ± s.e.m. from (5(2)).

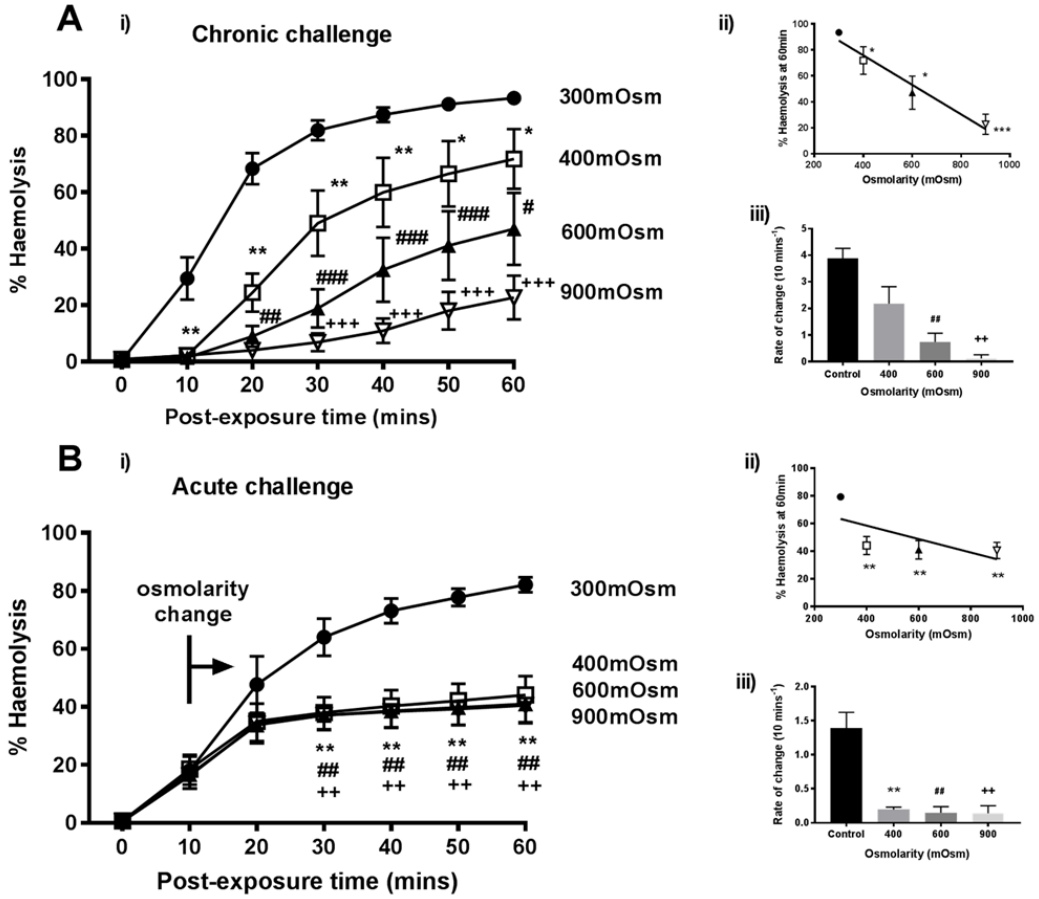
Figure 2. **Decreasing temperature suppressed rabbit erythrocyte haemolysis induced by *S. aureus* α-toxin.** The % haemolysis (%H) was measured over 60mins under either (A) chronic or (B) acute challenge at different temperatures as follows; 37ºC (control) (●), 25ºC (□), 4ºC (▲) either before toxin addition (chronic) or at about 20% haemolysis (indicated by the bar and arrow – 'temperature change').The panels labelled (ii) show the % haemolysis data at t=60mins plotted as a function of temperature, and the panels labelled (iii) show the rate of change of % haemolysis/10mins plotted as a function of temperature. Results are means ± s.e.m. from (4(2)).

Figure 3. **Divalent cations inhibited rabbit erythrocyte haemolysis induced by *S. aureus* α-toxin.** The % haemolysis (%H) was measured over 60mins under either (A) chronic or (B) acute challenge in the presence of Ca^{2+} or Mg^{2+} normal saline (NS; 0.9% NaCl control) (●), Ca^{2+} (5mM) or Mg^{2+} (5mM) (▲) either before toxin addition (chronic) or at about 20% haemolysis (indicated by the bar and arrow – divalent challenge).The panels labelled (ii) show the % haemolysis data at t=60mins plotted as a function of temperature, and the panels labelled (iii) show the rate of change of % haemolysis/10mins plotted as a function of temperature. Results are means ± s.e.m from (4(2)).

Figure. 4. **Effect of varying pH on rabbit erythrocyte haemolysis induced by *S. aureus* α-toxin.** The % haemolysis (%H) was measured over 60mins under chronic challenge at pH values of 7.2 (control) (□), 6.5 (●), and 8.0 (▲) in HBSS containing the buffer HEPES (10mM) with pH altered using HCl or NaOH (all at 300mOsm). The panel labelled (ii) shows the % haemolysis data at t=60mins plotted as a function of temperature, and the panel labelled (iii) shows the rate of change of % haemolysis/10mins plotted as a function of temperature. Results are means ± s.e.m. from (3(2)).

Figure 5. **The protective effect of optimised saline on rabbit erythrocyte haemolysis induced by *S. aureus* α-toxin.** The % haemolysis (%H) was measured over 60mins under either (A) chronic or (B) acute challenge in the presence normal saline (NS; 0.9% NaCl control) (●), HBSS (□), or optimised saline (OS; 0.9% NaCl, 900mOsm, 5mM $MgCl_2$, 37°C) (▲) either before toxin addition (chronic) or at about 20% haemolysis (indicated by the bar and arrow – challenge). The panels labelled (ii) show the rate of change of % haemolysis/10mins for chronic and acute challenges respectively in the various solutions. Results are means ± s.e.m. from (5(2)).

Liu & Hall, 2018 Figure 1.



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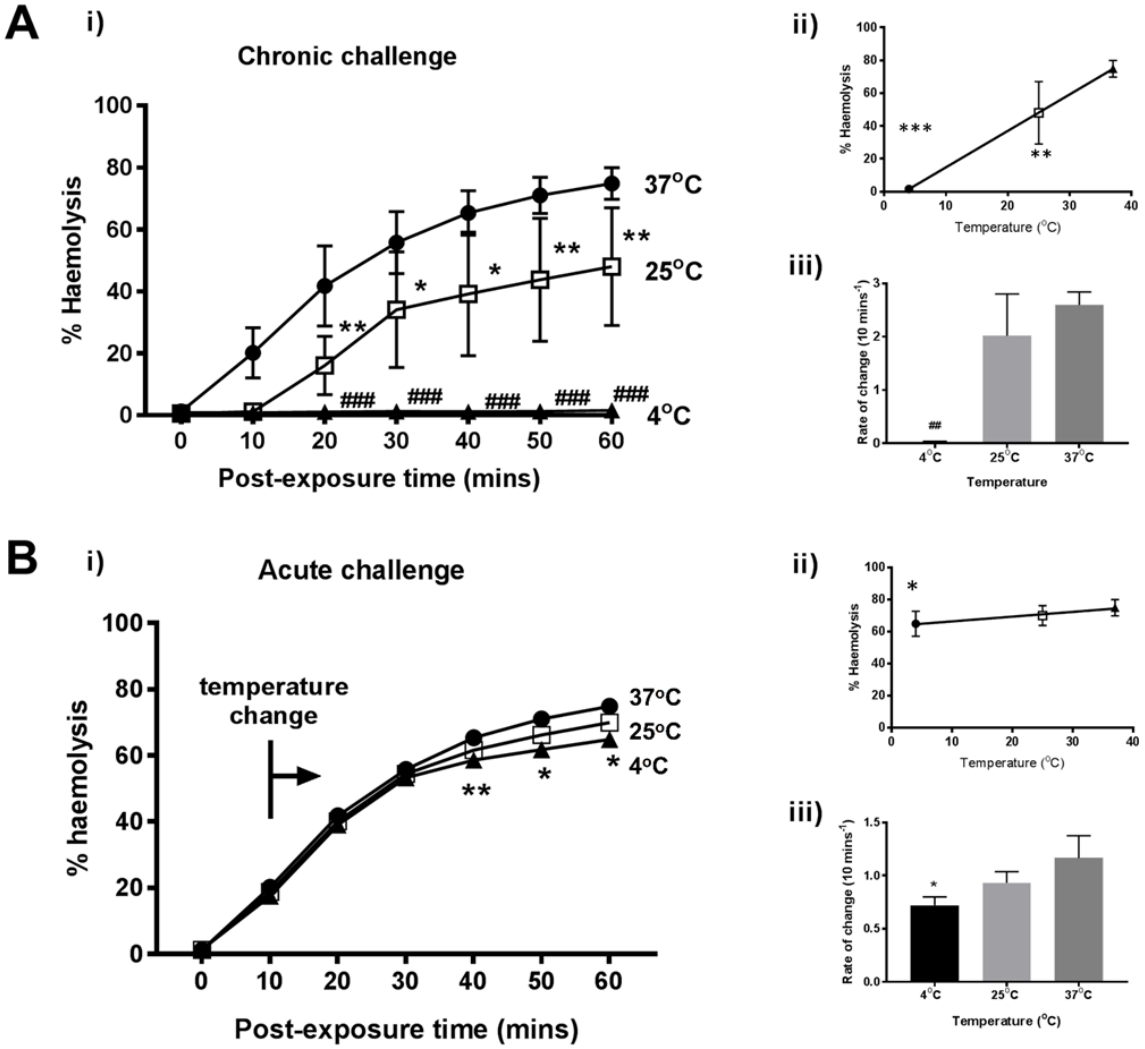
Liu & Hall, 2018 Figure 2.



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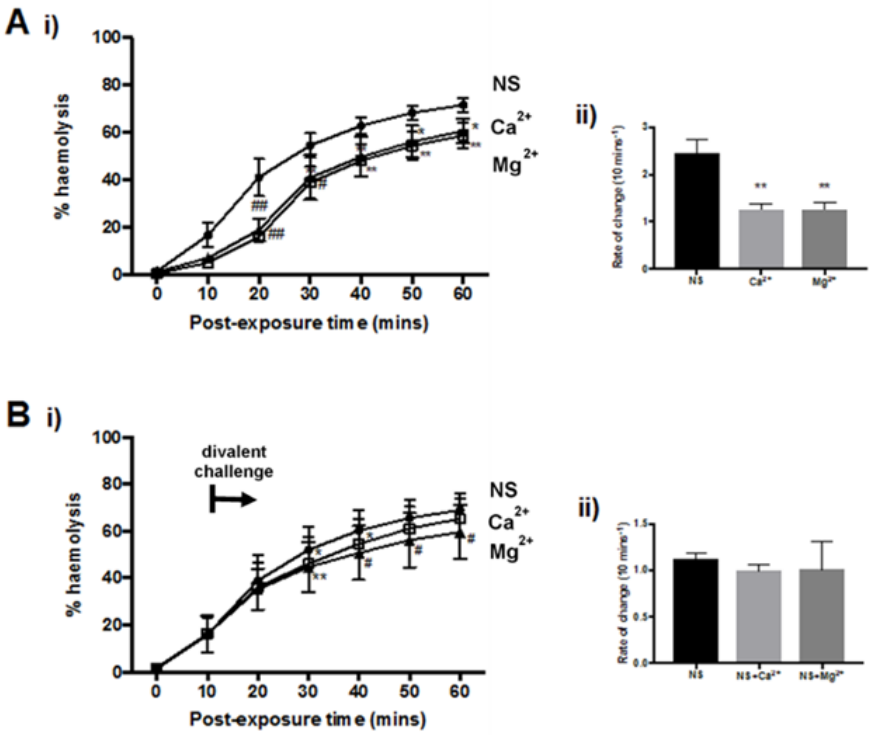
Liu & Hall, 2018 Figure 3.



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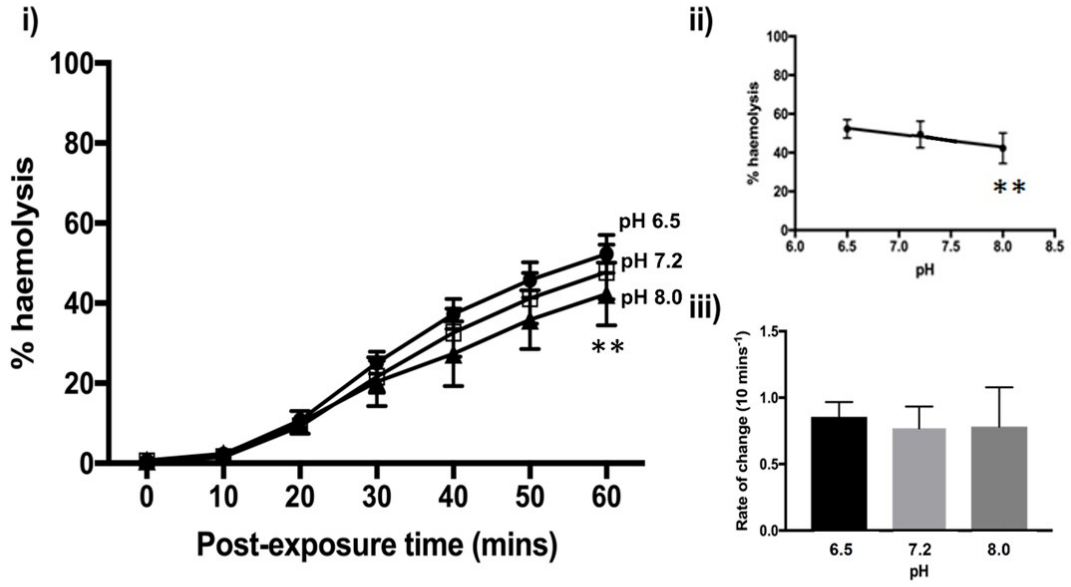
i)
% haemolysis
Post-exposure time (mins)
pH 6.5
pH 7.2
pH 8.0
**
ii)
% haemolysis
pH
**
iii)
Rate of change (10 mins-1)
pH
6.5
7.2
8.0

610

Liu & Hall, 2018 Figure 5.