Effects of antibiotics on -toxin levels during Staphylococcus aureus culture: implications for the protection of chondrocytes in a model of septic arthritis.

Effects of antibiotics on α-toxin levels during *Staphylococcus aureus* culture: implications for the protection of chondrocytes in a model of septic arthritis.

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Running title: Antibiotics on *S. aureus* α-toxin levels

Abstract

Objective: Septic arthritis results from joint infection by *Staphylococcus aureus* which produces potent alpha (α)-toxin causing cell death, potentially leading to permanent cartilage damage. Treatment is by joint irrigation and antibiotics, although it is unclear if, following treatment with antibiotics which cause bacterial lysis, there is release of additional stored (α)-toxin.

Design: A rabbit erythrocyte haemolysis assay was optimised to assess biologically-active α-toxin from cultured *S. aureus* (α)-toxin strain DU5946. Haemoglobin release was measured spectrophotometrically following addition of a bacteriostatic/bactericidal antibiotic (linezolid) or a bacteriolytic antibiotic (penicillin). A bovine cartilage model of septic arthritis was utilised to test the protective effects of antibiotics against *S. aureus* infection.

Results: During *S. aureus* culture, α-toxin levels increased rapidly but the rate of rise was quickly (within 20mins) suppressed by linezolid (25μg/ml). Penicillin also reduced the increase in α-toxin levels, however the time course was relatively slow compared to linezolid even at high concentrations (50,000U/ml). The efficacy of penicillin (250,000U/ml) at reducing the rise in α-toxin was approximately 8% less than that of linezolid ($P<0.05$) suggesting the presence of additional toxin. This could be due to a delayed action of penicillin, and/or release of a small pool of stored α-toxin from dying bacteria. In a bovine cartilage model however, there was no difference between the protection of *in situ* chondrocytes against *S. aureus* by penicillin or linezolid ($P>0.05$).

Conclusion: The results suggested that equally effective protection of chondrocytes against *S. aureus* septic arthritis may be obtained by the bacteriostatic/bactericidal or bacteriolytic antibiotics tested.

Key words: *Staphylococcus aureus*; bacteriostatic; bactericidal; antibiotic; α-toxin; septic arthritis.

Introduction

Septic arthritis, resulting from joint inflammation secondary to infection[1], can be highly destructive leading to cartilage damage and joint failure[2-4]. It affects all ages with an incidence in Western Europe of 4-10 cases/100,000 persons/yr[5]. The incidence of septic arthritis is rising globally due to various factors including an ageing population, increased use of immunosuppressive agents, musculoskeletal prosthesis and surgical procedures[5-7]. Although numerous bacterial species may induce septic arthritis, *Staphylococcus aureus* accounts for 40-60% of cases[2,3,8] with invasive healthcare-associated methicillin-resistant *S. aureus* (MRSA) infections accounting for 18 per 100,000 persons/yr[9]. The bacteria enter the synovial joint mainly by haematogenous spread[5], and the infection results in chondrocyte death, leading to cartilage degeneration and tissue erosion[2,3]. Chondrocyte death is particularly important as these are the only cell type capable of maintaining cartilage resilience through their regulation of extracellular matrix metabolism[10]. Septic arthritis can lead to osteoarthritis (OA[11]) which contributes to the high morbidity associated with the disease, and may also lead to fatal septicaemia[7].

S. aureus produces an array of potential virulence factors (e.g. toxins, adhesins), which contribute to colonisation and subsequent tissue destruction. The toxins may damage host cells directly or facilitate evasion of the host immune response[12]. Depending on the strain, *S. aureus* may release toxins including α, β, and γ-toxins in addition to damaging agents including Panton-Valentine leucocidin (PVL) and super-antigens (e.g. toxic shock syndrome toxin)[13]. A bovine cartilage explant model of *S. aureus*-induced septic arthritis, however reported that the 'pore forming' alpha (α)-toxin is the key damaging agent causing rapid chondrocyte death whereas the β and γ-toxins were relatively ineffectual[14,15]. Alpha-toxin is active against a variety of mammalian cells, but has marked potency against rabbit red blood cells (RBCs) *via* the ADAM-10 receptor which is also present on human chondrocytes[16-18]. Rabbit RBCs haemolysis can be quantified spectrophotometrically and thus is a very useful and sensitive biological assay for *S. aureus* α-toxin[19,20].

Treatment for septic arthritis includes intravenous antibiotics with joint lavage[1], however retrospective studies indicate that some permanent cartilage damage develops in ~50% of cases[2-4], thus its rapid and effective treatment is essential. When patients present, the joint is aspirated to obtain a microbiological profile of the synovial fluid. As identification of the causative micro-organism may take ~48hrs, patients are traditionally empirically commenced on intravenous antibiotics with joint lavage. Beta (β) lactam type antibiotics such as benzyl penicillin (penicillin-G) and flucloxacillin are classed as bacteriolytic as they kill the bacteria through damage to the cell wall[21,22]. However, this may release cellular contents including *S. aureus* α-toxin potentially causing additional chondrocyte death above that occurring when the bacteria were alive. In contrast, antibiotics such as linezolid and erythromycin are bacteriostatic at low concentrations by inhibiting bacterial growth and replication, which may then be followed by their removal by the immune system[23,24], whereas at higher levels they are bactericidal[24,25]. Thus these agents can be described as bacteriostatic/bactericidal. The choice of antibiotic for *S. aureus* septic arthritis treatment is important as it could influence the amount of α-toxin in the synovial space, chondrocyte death and subsequent cartilage damage.

Here, we have utilised two isogenic mutants (DU5946, DU5938) from the well-characterised prophage-cured derivative of NCTC8325 *S. aureus* 8325-4[26]. NCTC8325 was originally isolated from a patient with joint sepsis and its lineage remains a valuable resource for *S. aureus* research[27]. These mutants produce the range of toxins[26,28,29] and the mutations only affect the synthesis of alpha-haemolysin (α)-toxin) (Hla::Em^r), beta (β)-haemolysin (*Hlb*::φ42E) and gamma (γ)-haemolysin (Δ*hlg*::Tc^r). Thus, while both strains produce damaging agents, the mutant strain DU5946 only produces α-toxin and not β or γ-haemolysin, whereas the DU5938 mutant does not produce α, β, or γ-toxins. Levels of biologically active α-toxin during *S. aureus* culture, were assessed semi-quantitatively using the sensitive rabbit haemolysis assay[19,20]. Using this technique, we tested the hypothesis that α-toxin levels in the culture medium would be higher after the addition of high concentrations of a bacteriolytic antibiotic (penicillin G) compared to a bacteriostatic/bactericidal antibiotic (linezolid). The results demonstrated that there was only a small (<10%) further increase in α-toxin levels from *S. aureus* cultures following penicillin treatment compared to linezolid. We then tested these two antibiotics (in addition to two other antibiotics, flucloxacillin (bacteriolytic) and erythromycin (bacteriostatic/bactericidal)) for their ability to protect articular chondrocytes in a bovine cartilage model of septic arthritis. The results suggested that both penicillin and linezolid (as well as flucloxacillin and erythromycin) protected chondrocytes with equal efficacy suggesting that there was a negligible intracellular α-toxin released from *S. aureus* following antibiotic treatment.

Materials and Methods

(a): Bacterial strains and reagents. Two isogenic mutants of *S. aureus* 8325-4 (DU5946, DU5938) containing mutations affecting the synthesis of alpha-haemolysin (α)-toxin (Hla::Em^r), beta-haemolysin (*Hlb*::φ42E) and gamma haemolysin (Δ*hlg*::Tc^r;Table 1) were used. The DU5946 strain produced α-toxin whereas this was not produced by the DU5938 strain, otherwise the strains were identical. Antibiotics were from Sigma-Aldrich, Irvine, UK. Fresh, filter-sterilised stock solutions of linezolid (PZ0014), Na-flucloxacillin (F0150000) and penicillin G (P3032;1477U/mg) were prepared in dH_2O whereas erythromycin (E6376) was dissolved in ethanol-water (6mg/ml).

(b): Preparation of bacterial pellets, supernatant samples and antibiotics. Bacteria were stored at -80°C in 10%v/w skimmed milk (Oxoid, Basingstoke, UK). When required, they were thawed and streaked onto tryptone soya agar (TSA;Oxoid) plates containing 2μg/ml tetracycline (Cat. 87128; Sigma-Aldrich). Following incubation (24hrs;37°C), tryptone soya broth (TSB;Oxoid), containing 2μg/ml tetracycline, was inoculated with several single bacterial colonies from the TSA plate and incubated (24hr;37°C) with shaking. From this TSB culture, serial dilutions were performed in saline to 10^{-6}, in order to calculate the number of colony forming units (CFU). A CFU is defined as a unit used to estimate the number of viable bacteria capable of reproducing to form a colony of the same bacterial species – the number of CFUs is therefore a measure of the number of active bacteria in 1ml of TSB cultured for 24hr. Thereafter, 100μl of 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread evenly onto TSA plates and incubated (37°C;24hr). Colonies were then counted using a colony counter (Stuart®, Bibby Scientific, Stone, UK). Bacterial counts were performed on several cultures for each strain and a count of ~$1x10^9$ CFU/ml (range 0.8-$1.25x10^9$ CFU/ml) was routinely obtained. Cultures were then centrifuged (2,000xg;15min) and the pellets resuspended by vortexing, washing in HBSS and centrifugation (x3). The supernatant was then removed and the pellets air-dried before being stored (-20°C) and used within one week. Based on the colony counts, a bacterial pellet contained ~$1x10^5$ CFU and the bacteria were used in the experiments described here at ~$0.2x10^5$ CFU in HBSS. TSA plates were prepared to assess effective doses of antibiotics against *S. aureus* whose viability was assessed by counting colonies plated on TSA in the presence/absence of antibiotics following incubation overnight at 37°C. For penicillin and linezolid, doses of 1000U/ml and 25μg/ml respectively were sufficient. For flucloxacillin and erythromycin, doses of 2mg/ml and 10μg/ml respectively were adequate and no viable bacteria were detected. For some experiments where α-toxin levels were assessed by the haemolysis method, higher doses of penicillin (to 250,000U/ml) were used. Table 2 summarises the antibiotics used with doses, together with sites of action and references. All doses used were substantially greater than the MIC (minimum inhibitory concentration) in the literature (Table 2).

(c): Rabbit erythrocyte haemolysis assay for α-toxin. The rabbit red blood cell (RBC) haemolysis assay was used to determine biologically-relevant levels of α-toxin released during *S. aureus* culture[19,20]. Fresh, heparinised RBCs (Orygen, Penicuik, UK), were prepared at ~5% haematocrit in Hank's balanced salt solution (HBSS;pH 7.4;Invitrogen, Paisley, UK). At time zero and at specific points throughout the culture of *S. aureus* (see Figures), aliquots were taken and centrifuged (8,000xg;30secs) and the supernatant aliquotted into microcentrifuge tubes, taking care not to disturb the pellet. The supernatants which contained α-toxin, were

then added to microcentrifuge tubes containing 5% RBC suspensions, mixed gently and incubated (37°C;60mins). The samples were then centrifuged (8,000xg;10secs) and the supernatant assessed for haemoglobin at 540nm (Abs_{540}) on a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, USA). 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%). The sensitivity of RBC samples to α-toxin and the amount of α-toxin produced from each culture were variable. It was important to minimise this at the beginning of an experimental week, by initially testing erythrocytes from several rabbits on a freshly-prepared batch of α-toxin. Erythrocytes which were relatively insensitive to α-toxin requiring >1hr of incubation before haemolysis was detectable, were not studied further and this reduced the variation in results obtained.

(d): Assessment of in situ chondrocyte viability by confocal scanning laser microscopy (CLSM). Fresh, healthy (non-degenerate) metacarpophalangeal joints of 3-yr old cows from a local abattoir, were washed, skinned, de-hoofed and opened under aseptic conditions. Cartilage explants were cultured in Dulbecco's Modified Eagle's medium (DMEM;pH 7.4;37°C; Invitrogen) in the presence or absence of *S. aureus* and after 15hrs the bacteria had caused measurable (~20%) chondrocyte death. Explants were then exposed to antibiotics (or control medium) and the incubation continued for up to 23hrs. At the time points indicated, explants were removed, rinsed in DMEM and chondrocyte viability assessed as described[30]. Explants were incubated with 5-chloromethylfluorescein diacetate (CMFDA) and propidium iodide (PI;both at 10μM from Invitrogen, Paisley, UK) in DMEM (1hr;21°C). These probes labelled living or dead cells green or red, respectively. Explants were then fixed (4% formaldehyde;4hrs;Fisher Scientific, Loughborough, UK), rinsed and stored in PBS, until imaged within 24hrs. Imaging of fluorescently-labelled *in situ* chondrocytes was performed as described[14,30]. An upright Zeiss LSM510 Axioskop (Carl Zeiss, Welwyn Garden City, UK) CLSM with a x10 (0.3NA) dry objective was used to acquire axial images. The percentage cell death ((number of dead cells/total number of living and dead cells) x100%) was calculated within a standard region of interest (ROI) using Volocity™ 4 software (Improvision, Coventry, UK). Within each ROI, individual cells, in both green (living) and red (dead) channels, were quantified based on percentage voxel (volumetric pixel) intensity[14].

(e): Data analysis and statistics. Data are shown as means ± standard error of the mean (S.E.M) from at least 3 independent experiments, and were analysed using GraphPad Prism Ver.6 (GraphPad, San Diego, CA, USA). Non-parametric t-tests (Kruskal-Wallis) and ANOVAs were performed and significant differences accepted when $P<0.05$.

Results

(a) S. aureus α-toxin mediated rabbit RBC haemolysis. The strain of *S. aureus* (DU5946) that harboured the α-toxin (*hla*) gene was haemolytic on rabbit blood agar whereas the strain deficient in this gene (DU5938) was non-haemolytic (Fig. 1(a)). Supernatants were prepared from cultures of these strains and added to suspensions of rabbit erythrocytes in HBSS. There was a rapid increase in %H for the α-toxin-positive strain, however the α-toxin-deficient strain had no detectable effect (Fig. 1(b)). Thus, by using rabbit erythrocytes in suspension as a model and determining haemoglobin release spectrophotometrically, biologically active α-toxin could be quickly assessed semi-quantitatively.

(b) Time course of linezolid action. Single doses of linezolid (to concentrations of 5-50μg/ml) were added to the α-positive *S. aureus* cultures (DU5946) at a point corresponding to ~50% haemolysis (after 80mins) and samples analysed for α-toxin-induced haemolysis (Fig. 2(a)). For all concentrations, there was a rapid reduction in the rate of rise of α-toxin-induced haemolysis, and at 25 and 50μg/ml, there was no significant change in %H (Fig. 2(b)). Addition of linezolid to 50μg/ml at 80, 100, 130mins time points, rapidly suppressed any further increase in α-toxin-induced haemolysis (Fig. 2(c)). Linezolid (50μg/ml) addition 60mins after the commencement of the *S. aureus* culture, abolished subsequent α-toxin-induced haemolysis compared to the untreated (infected) control (Fig. 3(a)). The same concentration of linezolid added after 100mins when there was ~40% haemolysis, also completely inhibited subsequent α-toxin-induced haemolysis to the same level after 40 and 60mins as that present at the start of the experiment ($P<0.001$; Fig. 3(b)). These results indicated that the linezolid addition to *S. aureus* cultures rapidly (within ~20 mins) inhibited α-toxin-induced haemolysis and that 25 and 50μg/ml prevented any further rise.

(c) Time course of penicillin action. Single doses of penicillin G (to 5000, 10,000, 25,000 or 50,000U/ml) were added to the α-toxin positive *S. aureus* cultures at a time point corresponding to ~50% haemolysis (after 60mins) and samples analysed for α-toxin-induced haemolysis (Fig. 4(a)). While at penicillin concentrations of 50,000U/ml and 250,000U/ml at 140 mins there was a significant reduction in the rate of rise of α-toxin-induced haemolysis, the effect of lower concentrations was not significant (Fig. 4(b)). Addition of penicillin to 50,000U/ml at different time points, suppressed the increase in α-toxin-induced haemolysis (Fig. 4(c)), however the time course of action was slower compared to linezolid (Fig. 2(c)). Penicillin addition 20mins after the start of the culture, significantly inhibited haemolysis compared to the untreated (infected) control after 40 and 60mins (Fig. 5(a)). While in the presence of penicillin, there were significant increases in haemolysis at 40 and 60mins compared to the values at the time of drug addition ($P<0.05$), these were not different ($P=0.12$), indicating a plateau in α-toxin levels. When the same concentration of penicillin was added after 60mins, there was ~60% haemolysis at time zero and this significantly ($P<0.001$) inhibited subsequent α-toxin-induced haemolysis after 40 and 60mins (Fig. 5(b)). The extent of α-toxin-induced haemolysis at 60mins was significantly ($P=0.05$) higher than the time zero point but not significantly different compared to the 40min time point (Fig. 5(b)). These results suggested that penicillin addition to *S. aureus* cultures progressively reduced the rate of increase of α-toxin-induced haemolysis. However, the time course appeared

relatively slow compared to linezolid, and at the highest concentrations studied (50,000U/ml) there was still a slight rise in α-toxin-induced haemolysis until the inhibition appeared complete.

To directly compare the inhibitory effects of linezolid and penicillin using the same blood samples and *S. aureus* cultures, DU5946 cultures were incubated for 60mins, and then untreated (control) or dosed with penicillin (250,000U/ml) or linezolid (25μg/ml), and the medium analysed for α-toxin for up to 80mins (Fig. 6). After 20mins, both antibiotics significantly ($P<0.05$) inhibited haemolysis, however at 40, 60 and 80mins after antibiotic addition, the inhibition was significantly (by ~8%) greater ($P<0.05$) in the presence of linezolid (Fig. 6). The inhibition by penicillin did not change significantly compared to linezolid over the 40-80mins period after antibiotic addition, suggesting that the inhibitory effect of penicillin was maximal.

(d) Chondrocyte protection in a bovine cartilage septic arthritis model by antibiotics. S. aureus DU5946 was cultured with cartilage explants and in control (no antibiotics) samples, there was almost complete (>90%) chondrocyte death after ~21hrs (Fig. 7(a,b)). In contrast, when infected cartilage was treated with penicillin or linezolid after 15hrs, there was no subsequent chondrocyte death and no difference between the potency of the two drugs (Fig. 7(a,b)). Similar experiments were performed with flucloxacillin and erythromycin (data not shown). In the untreated infected samples, there was complete (~100%) chondrocyte death after ~24hrs. Treatment of infected cartilage samples with flucloxacillin (2mg/ml) or erythromycin (10μg/ml) after 18hrs, prevented any subsequent chondrocyte death and there was no significant difference ($P>0.05$) between the two drugs at any time point studied up to 26hrs after antibiotic addition. Levels of chondrocyte death in the control (i.e. uninfected and untreated) cartilage samples were <2%. These results suggested that both bacteriolytic (penicillin, flucloxacillin) and bacteriostatic/bactericidal (linezolid, erythromycin) antibiotics were equally effective at protecting chondrocytes in the bovine cartilage model of *S. aureus* septic arthritis.

Discussion

The rabbit erythrocyte haemolysis assay determined the time course of biologically-relevant α-toxin produced during culture of *S. aureus* in the presence or absence of bacteriostatic/bactericidal and bacteriolytic antibiotics. The antibiotics tested could limit or prevent a rise in α-toxin levels, however there were differences in their time courses of action. The bacteriostatic/bactericidal agent, linezolid, appeared more rapid and potent than the bacteriolytic antibiotic, penicillin. There was, however, no evidence of a detectable 'pulse' of α-toxin–induced haemolysis following addition of any of the antibiotics studied, suggesting there was no substantial intracellular 'pool' of stored α-toxin in the *S. aureus* strain DU5946. In the bovine cartilage model of septic arthritis, *S. aureus* cultures rapidly caused chondrocyte death. However chondrocytes were completely protected against *S. aureus* by the antibiotics tested (linezolid, flucloxacillin, penicillin, erythromycin). These results were important for elucidating whether or not there was a significant intracellular store of α-toxin as this may be relevant for antibiotic selection during *S. aureus*-induced septic arthritis.

The rabbit RBC haemolysis bio-assay was used to determine the time course of α-toxin release during *S. aureus* culture. The measurement of haemoglobin released 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, allowing several conditions (e.g. drug concentrations) to be tested at the same time points. The other toxins/damaging agents produced by *S. aureus* had a negligible effect on rabbit erythrocytes emphasising the specific nature of this assay for α-toxin (Fig. 1). It was noted that some blood samples were very sensitive to α-toxin whereas others were relatively unresponsive and so it was necessary to initially screen each blood sample for sensitivity against a *S. aureus* culture. Once a blood sample was deemed acceptable, the time course of haemolysis to α-toxin was highly reproducible. In addition, the ability of *S. aureus* cultures to produce sufficient quantities of potent α-toxin was variable and probably depended on prevailing culture conditions. The difference in potency of batches of α-toxin was observed, as for example, in one series of experiments, 60mins elapsed before the *S. aureus* culture started to produce measurable α-toxin levels (Fig. 2(a)). In another set of experiments performed with a different α-toxin preparation, after only 40mins haemolysis had already increased to 20% (Fig. 4(a)). The rabbit erythrocyte model for determining the effects of antibacterials on α-toxin levels might be considered a limitation. However, rabbit erythrocytes contain the *S. aureus* α-toxin receptor ADAM-10 also present on chondrocytes of animals and normal and degenerate human cartilage[16-18]. Our previous work has demonstrated that in a cartilage model of septic arthritis, α-toxin is the key damaging agent to chondrocytes and thus the rabbit erythrocyte hemolysis assay is appropriate to determine the effects of antibacterials on α-toxin release from *S. aureus* cultures[14,15].

Linezolid at 25 or 50μg/ml rapidly abolished the rise in α-toxin–induced haemolysis (Fig. 2(c)) probably because of its potent action on bacterial protein synthesis and replication[31]. However, linezolid may have both bacteriostatic and/or bactericidal effects on *S. aureus* depending on concentration and experimental conditions[22]. Therefore the reduced rate of haemolysis in linezolid-treated cultures may be the result of bacteriostatic, bactericidal or combined bacteriostatic/bactericidal action. Whatever the mechanism, at 50μg/ml linezolid (Fig. 3(a,b)), there was no progressive change in haemolysis ((Fig. 2(c); Fig. 3(b)) indicating no additional release of α-toxin from dying/dead bacteria. However, future work would be required to

investigate the effect of varying concentrations of linezolid on the bacteriostatic and/or bactericidal modes of actions on *S. aureus* α-toxin. It should be noted that to test our hypothesis of whether there was a detectable intracellular store of α-toxin, we used concentrations considerably above the MIC (minimum inhibitory concentrations;Table 2) and have not atttempted to study the effects of clinically-relevant doses.

The action of penicillin was slower compared to linezolid as evidenced by the relatively tardy inhibition of α-toxin-induced haemolysis even at a high concentration (50,000U/ml;Fig. 4(c);Fig. 5(a,b)). There are two possible explanation for this finding. The most likely interpretation of these results is because of the slower time course of the antibiotic inhibiting bacterial cell wall synthesis[21,22]. The level of haemolysis after penicillin addition was higher than that with linezolid, and did not change with time (Fig. 6). This suggested that the inhibition by both drugs was maximal with no further bacterial death or inhibition of division. However it is also possible that the slightly elevated level of haemolysis (~8% of the total) with a high dose of penicillin (Fig. 6) could have been because of the release of the small additional amount of intracellular α-toxin from the dying/dead bacteria.

The presence of a small amount of intracellular *S. aureus* α-toxin is implied by others using different methods to cause bacterial damage. For example Duncan and Cho[32] suggested that mechanical disruption of *S. aureus* released 1-2% of total toxin present in cultures. However this might have been influenced by the simultaneous release of degradative enzymes leading to an underestimation in the amount of active toxin. Monecke et al[33] using alkali treatment, noted that there was only a 'small' (but unquantified) level of stored α-toxin following bacterial lysis. For *S. aureus* therefore, there might only be a small intracellular store with the α-toxin released rapidly after synthesis. On the other hand, pneumolysin, the toxin produced by *Streptococcus pneumoniae*, is almost exclusively stored intra-cellularly and only released through bacterial autolysis[34,35]. In a *S. pneumoniae* rabbit meningitis model, addition of the bacteriolytic agent ceftriaxone, released substantial additional quantities of pneumolysin compared to the non-bacteriolytic agent rifampin, with the additional toxin causing further cellular damage and inflammation[36].

In the *S. aureus* septic arthritis cartilage model, linezolid and penicillin (Fig. 7a,b) and flucloxacillin and erythromycin (see Results) were equally effective This might appear to conflict with the suggestion above that there was a small intracellular 'pool' of α-toxin. It is possible that the methods for determining chondrocyte death were not sufficiently sensitive to detect the small increase from the release of stored α-toxin by penicillin. However there was no significant difference in % chondrocyte viability between linezolid and penicillin-treated explants so this does not seem likely. Alternatively, α-toxin levels in the medium and thus the cartilage matrix may already be high and any small increase following penicillin treatment could be without further detectable effect. The α-toxin released from *S. aureus* will have a rapid effect on the rabbit erythrocytes as pore formation and haemolysis proceeds promptly[19]. However in cartilage, toxin permeability may be hindered by the cartilage extracellular matrix[37]. It is also possible that the dynamics of toxin binding and pore formation to bovine chondrocytes proceeds with a different time course compared to rabbit erythrocytes. ADAM-10 (A Disintegrin And Metalloproteinase domain-containing protein-10) has been identified as an important receptor for α-toxin binding[38] mediating changes to intracellular Ca^{2+} signalling[39] and is present on human chondrocytes

and up-regulated in osteoarthritis[16-18]. The binding site density on chondrocytes may also be different compared to rabbit erythrocytes which could further influence the time course of the α-toxin effect.

The rapid inhibition by linezolid on the rise in *S. aureus* α-toxin levels compared to penicillin is likely due to a bacteriostatic action on *S. aureus* replication, and *in vivo* this would be followed by bacteria removal by the immune system. Septic arthritis may be regarded as a medical emergency[3] and thus clinically there would be benefit in administering this antibacterial agent or others in this class for the most rapid action possible to protect joint tissues from further damage. It is possible that bacteriolytic antibiotics (e.g. penicillin, flucloxacillin) may lead to the release of additional inflammatory components (e.g. teichoic acids, peptidoglycans) from *S. aureus* potentially causing further chondrocyte damage. However all the drugs tested (penicillin, linezolid (Fig. 7); flucloxacillin, erythromycin (see Results)), were equally effective at protecting chondrocytes against *S. aureus*. This suggests that these agents, if released from dead/dying bacteria, do not have a significant effect on chondrocyte viability and support the view that the α-toxin alone was the primary cause of chondrocyte death[15]. It is also worth noting that bacteriostatic/bactericidal agents e.g. linezolid, reduce the expression of *S. aureus* α-toxin[29,31]. This is in marked contrast to sub-inhibitory doses of β-lactams which increase *S. aureus* hla (α-toxin) mRNA expression[40,41] and therefore might cause concern in the treatment of osteoarticular infection by these bacteria. Furthermore, linezolid reduces expression of other staphylococcal toxins such as PVL in PVL-associated staphylococcal pneumonia[42]. Since PVL-producing strains of *S. aureus* may also be present and can cause complications in septic arthritis[43], linezolid may offer a further advantage over bacteriolytic antibiotics. Although our results demonstrated that these antibacterials are protective against chondrocytes *in vitro*, the response *in vivo* is likely to be far more complex as our model of *S. aureus*-induced septic arthritis does not include the host's immune response.

While it can be particularly challenging to extrapolate the results from the *in vitro* situation to the clinical setting, there are three main findings from this study which are relevant. (a) The time course of the bacteriostatic/bactericidal antibiotic linezolid on limiting the rise α-toxin was clearly more rapid than for the bacteriolytic antibiotic penicillin (Fig. 2 *vs* Fig. 4). (b) There was a negligible amount of stored α-toxin released following antibacterial treatment (Fig. 6). (c) There was no significant difference in the chondroprotective effect between exemplars of the two classes of antimicrobials in the septic arthritis model (Fig. 7). It is also relevant to note that Monecke et al.[33] have presented evidence to indicate that secretion of *S. aureus* α-toxin does not appear to correlate with the progression of septic arthritis. There is nevertheless a strong link between levels of α-toxin and chondrocyte death[14,15] and it is known that cartilage which does not possess viable chondrocytes will degenerate[44]. Thus, while there is probably a relationship between levels of α-toxin in *S. aureus* – induced septic arthritis and *in vivo* cartilage viability, a direct correlation is yet to be established. Despite this, the dominant importance of α-toxin produced by *S. aureus* in septic arthritis as opposed to other damaging agents released by this bacterium, has been underscored by recent preliminary *in vivo* studies[45]. In these experiments, the intra-articular injection of *S. aureus* 8325-4 which produces all toxins including α-toxin and other damaging agents, was compared to the injection of an *S. aureus* mutant (DU1090) which produces the full range of toxins/enzymes, *except* α-toxin[15]. Mice injected with either *S. aureus* strains developed septic arthritis with evidence of weight loss, limb swelling and gait changes whereas these were absent in the control

(PBS – injected) group. Notably, there was significantly ($P<0.05$) more chondrocyte death in the group infected with *S. aureus* 8325-4 (approx. 93% chondrocyte death) when compared to DU1090 (approx. 26% chondrocyte death) and PBS-injected (i.e. control) mice (5% chondrocyte death). The results suggested that α-toxin was the major chondrocyte damaging agent, but also any adverse effect of the immune system during this time course with this animal model of septic arthritis was negligible in comparison. While antimicrobial treatment of *S. aureus* to limit further production of α-toxin is clearly an essential clinical approach, strategies aimed at quickly suppressing the action of α-toxin already present in the infected tissue could be of additional benefit. For example, the damaging effect of α-toxin is markedly suppressed by raising the osmolarity of the culture medium[20].

This present study utilised a rabbit RBC assay to determine α-toxin levels produced by *S. aureus* in culture following treatment with antibiotics. Linezolid rapidly limited any further increase in α-toxin levels by *S. aureus* whilst penicillin had a slower time course of action. There was a small additional release of α-toxin following penicillin addition suggesting a delayed action and/or a small intracellular store of α-toxin. However, both drugs were equally effective at protecting chondrocytes in our *S. aureus* septic arthritis model.

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539 **Tables**

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542 **Table 1. Miller et al., (2018)**

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***S. aureus* strain**	**Genotype**	**Phenotype**	**Haemolysins produced**	**Given name**
DU5946	*Hlb*::ϕ42E Δ*hlg*::Tc^r	Hla$^+$ Hlb$^-$ Hlg$^-$	Hla	α-toxin positive α+ β- γ-
DU5938	*Hlb*::ϕ42E *hla*::Em^r Δ*hlg*::Tc^r	Hla$^-$ Hlb$^-$ Hlg$^-$	None	α-toxin deficient α- β- γ-

545

546 **Table 1. The bacterial strains used in study.** The strain of *S. aureus* is shown with its genotype,

547 phenotype and the toxins produced. Tc ^{r} = Tetracycline resistance. Em ^{r} = Erythromycin resistance.

548

549 **Table 2. Miller et al., (2018)**

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			Antibiotic Dose		
Antibiotic	**Class (action)**	**Mode of action (on bacteria)**	**Dose used**	**MIC**	**Reference**
Linezolid	Oxazolidinone (bacteriostatic/ bactericidal)	Ribosomal 50S inhibitor – interrupts protein synthesis	50μg/ml for haemolysis experiments; 25μg/ml for cartilage experiments.	0.12-8μg/ml	46
Erythromycin	Macrolide (bacteriostatic/ bactericidal)	Ribosomal 50S inhibitor – interrupts protein synthesis	10μg/ml for cartilage experiments.	0.06-128μg/ml	46
Penicillin	β-lactam (bacteriolytic)	Blocks cross-linking of peptidoglycan - inhibits cell wall formation	50,000U/ml (34mg/ml) for haemolysis experiments; 1000U/ml (0.70μg/ml) for cartilage experiments.	0.015-128μg/ml	46
Flucloxacillin	β-lactam (bacteriolytic)	Blocks cross-linking of peptidoglycan - inhibits cell wall formation	2mg/ml for cartilage experiments.	0.4μg/ml (fully effective)	21

551

552 **Table 2. Antibiotics and concentrations used in study.** The class, mode of action and concentration of
553 antibiotics used are shown. The MIC (minimum inhibitory concentration) for linezolid against *S. aureus* ranges
554 over 0.12-8μg/ml[46,47]; and the MIC90 (minimum inhibitory concentration required to block 90% bacterial
555 growth) ranges over 2-4μg/ml[48]. Doses used in the present work ranged from 5-50μg/ml and were effective
556 (Fig. 2(c), Fig. 7a)). The MIC for erythromycin on Staphlococci ranges over 0.06-128μg/ml[46] and the dose
557 used for cartilage experiments was 10μg/ml. The MIC for penicillin action on Staphlococci ranges over 0.015-
558 128μg/ml[46]. The present study used 50,000U/ml (34mg/ml) for haemolysis experiments and 1000U/ml
559 (0.70μg/ml) for cartilage experiments which were effective (Figs. 4(c) and 7); see text). Flucloxacillin has
560 been shown to be completely effective against *S. aureus* when present at 0.4μg/ml[21]. In the present study a
561 concentration of 2mg/ml was used (see text).

Figure Legends

Figure 1. The haemolysis of rabbit erythrocytes following incubation with α-toxin positive or α-toxin negative strains of *S. aureus*. (a) Cultures of *S. aureus* (i) α-toxin positive, β and γ-toxin negative strain or (ii) α-toxin negative, β and γ-toxin negative strain (DU5938; Table 1) were incubated on tryptone soya agar (TSA) containing rabbit erythrocytes. Clear areas surrounding colonies of α-toxin secreting *S. aureus* represent the haemolysis of rabbit erythrocytes. No haemolysis was observed around colonies of the α-toxin negative strain. (b) The time course of haemolysis in suspensions of rabbit erythrocytes during culture with α-toxin positive or α-toxin negative strain of *S. aureus*. For this and subsequent Figures, when error bars were not shown they were smaller than the symbols. Data shown are means ± s.e.m. for 3 independent experiments for each condition.

Figure 2. Effects of linezolid on α-toxin-induced haemolysis of rabbit erythrocytes. Panel (a) shows the effect of linezolid (0 (HBSS control), 5, 10, 25 or 50μg/ml) addition at 80mins to *S. aureus* α-toxin positive strain (DU5946) cultures on α-toxin-induced haemolysis (see Materials and Methods for details). Panel (b) presents the rate of change of haemolysis over 100-140mins ((expressed as the % change/min) x10) extent following addition of linezolid with increasing concentration. Asterisks (* or **) represent significant differences at the $P<0.05$ or $P<0.001$ levels respectively. Panel (c) demonstrates the effects of linezolid (50μg/ml) addition to *S. aureus* cultures at 60, 80, 100 and 130mins corresponding to approximately 0, 10, 40 or 80% α-toxin-induced haemolysis. Data shown are means ± s.e.m. for at least 3 independent experiments.

Figure 3. Inhibition of α-toxin-induced haemolysis of rabbit erythrocytes at different time points by prior treatment with linezolid. Linezolid (50μg/ml) was added (a) 60mins or (b) 100mins after the initiation of *S. aureus* α-toxin positive strain (DU5946) cultures which corresponded to approximately 0 or 40% α-toxin-induced haemolysis respectively at the start of the experiment. Measurements of %H were then taken at 0, 40 and 60mins as shown. At 40 and 60mins, the prior treatment with linezolid significantly ($P<0.001$) reduced α-toxin-induced haemolysis to levels that were not significantly different from the control (i.e. levels of %H at the start of the time course). Data shown are means ± s.e.m. for at least 3 independent experiments.

Figure 4. Effects of penicillin on α-toxin-induced haemolysis of rabbit erythrocytes. Panel (a) shows the effect of penicillin (0, 5,000, 10,000, 25,000 or 50,000U/ml) addition at 60mins to α-toxin positive strain (DU5946) *S. aureus* cultures on α-toxin-induced haemolysis (see Materials and Methods for details). Panel (b) presents the extent of α-toxin-induced haemolysis at 140mins following addition of penicillin. The inhibition by penicillin was only significantly different from the HBSS control at 50,000 and 250,000U/ml ($P<0.001$) whereas there was no significant difference between these two antibiotic concentrations. Panel (c) demonstrates the effects of penicillin (50,000 U/ml) addition to *S. aureus* cultures at 20, 40, 60 and 100 mins

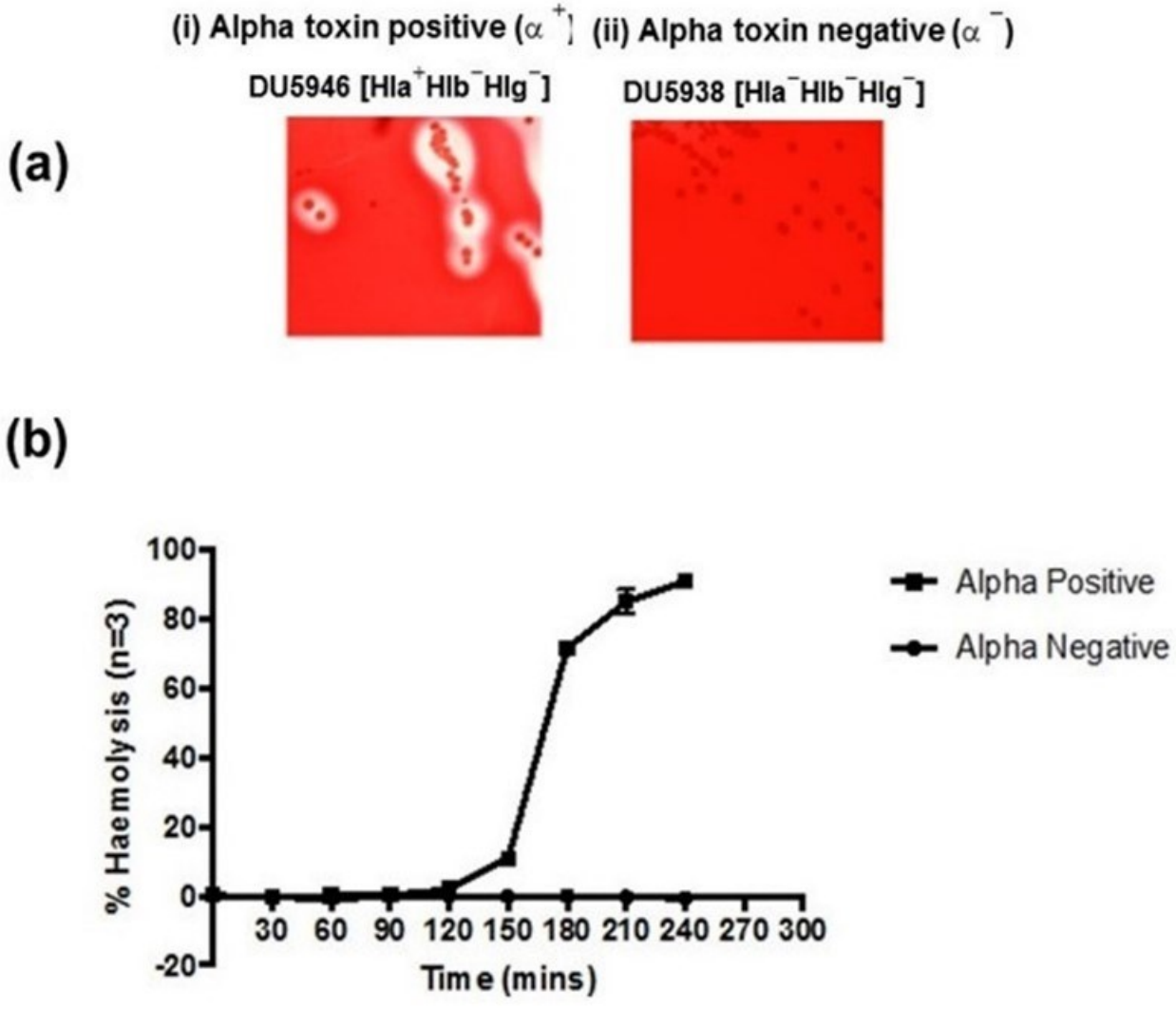
corresponding to approximately 0, 10, 40 or 80% α-toxin-induced haemolysis. Data shown are means ± s.e.m. for at least 3 independent experiments.

Figure 5. Inhibition of α-toxin-induced haemolysis of rabbit erythrocytes at different time points by prior treatment with penicillin. Penicillin (50,000U/ml) was added (a) 20mins or (b) 60mins after the initiation of α-toxin positive strain (DU5946) *S. aureus* cultures which corresponded to approximately 0 or 60% α-toxin-induced haemolysis at the start of the experiment. Measurements of %H were then taken at 0, 40 and 60mins as shown. At 40 and 60mins, the prior treatment with penicillin significantly ($P<0.001$) reduced α-toxin-induced haemolysis. For the penicillin-treated cultures, there were small but significant ($P<0.05$) increases in %H for (a) at 40 and 60mins, and for (b) at 60mins compared to the %H values at 0mins. Data shown are means ± s.e.m. for at least 3 independent experiments.

Figure 6. Comparison between the inhibitory effects of penicillin and linezolid on the extent of α-toxin-induced haemolysis. A single dose of penicillin (250,000U/ml) or linezolid (25μg/ml; final concentrations) was added to α-toxin positive strain (DU5946) *S. aureus* cultures after 60mins (corresponding to approximately 55% haemolysis) and α-toxin-induced haemolysis determined. There was a significant reduction in levels of α-toxin-induced haemolysis for both drugs at 20, 40, 60 and 80mins compared to the untreated control at the same time points. However the inhibition was significantly greater for the linezolid-treated cultures compared to those following penicillin addition at 40, 60 and 80mins ($P<0.01$). Data shown are means ± s.e.m. for 3 at least independent experiments.

Figure 7. Protection of *in situ* chondrocytes by penicillin or linezolid against *S. aureus* infection in a bovine model of septic arthritis. Bovine cartilage was incubated with α-toxin positive strain (DU5946) *S. aureus* cultures for 15hrs, which produced approximately 20% chondrocyte death (infected), or in the absence of bacteria (uninfected). Cultures were then continued untreated, or following the addition (at the arrow) of penicillin (1,000U/ml) or linezolid (25μg/ml). Data shown are means ± s.e.m. for 6 independent experiments. Examples of confocal images of chondrocytes in bovine cartilage taken after 23hrs are shown for the following conditions (a) uninfected (<2% cell death), (b) infected penicillin – treated (about 20% cell death), (c) infected linezolid – treated (~21% cell death) and (d) infected untreated (~100% cell death). The scale bar is 100μm for all panels.

Miller et al., (2018) Fig. 1.



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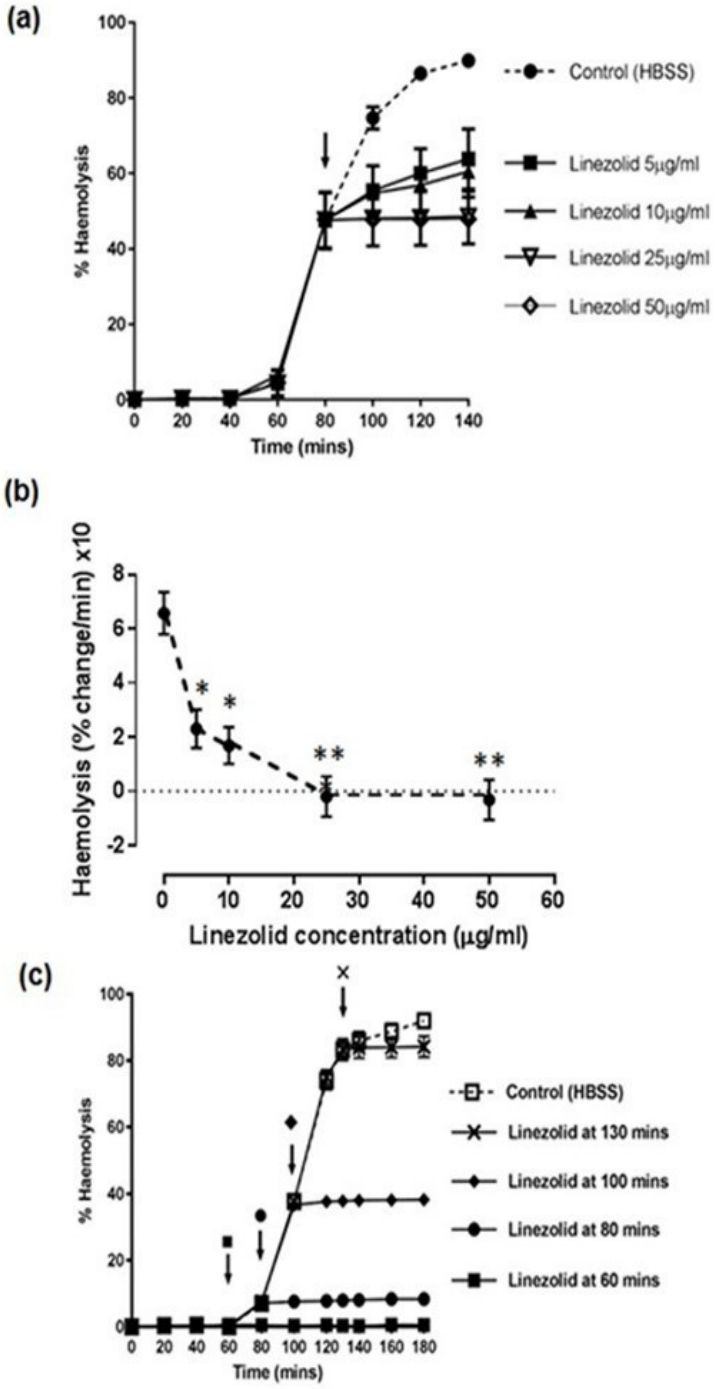

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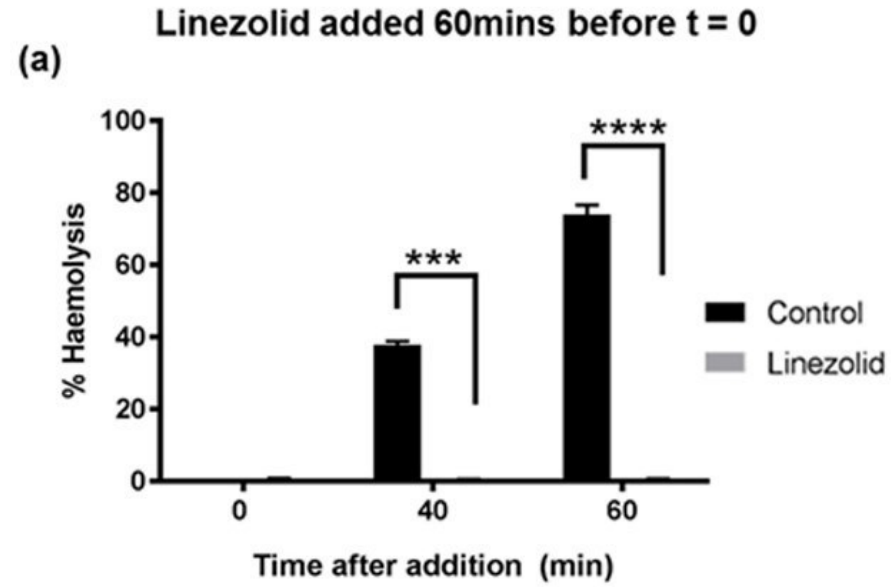

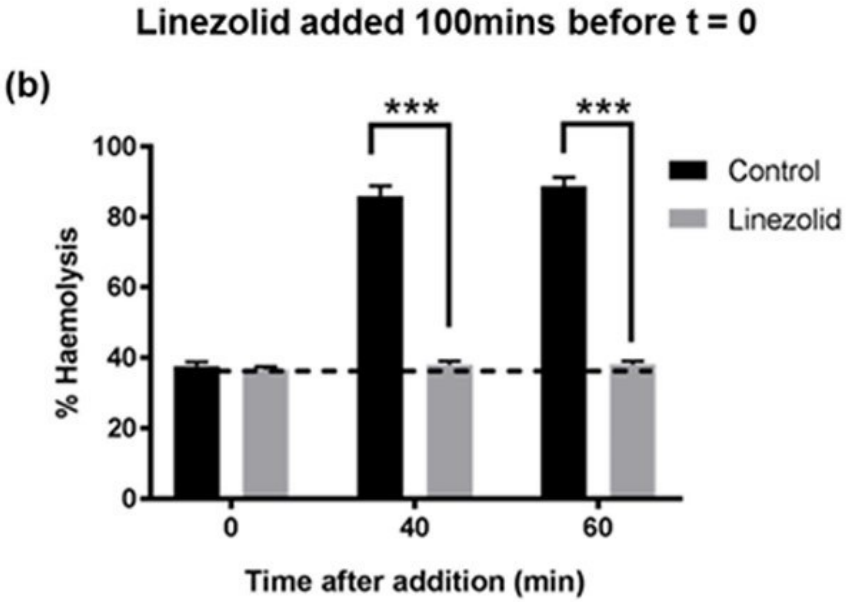

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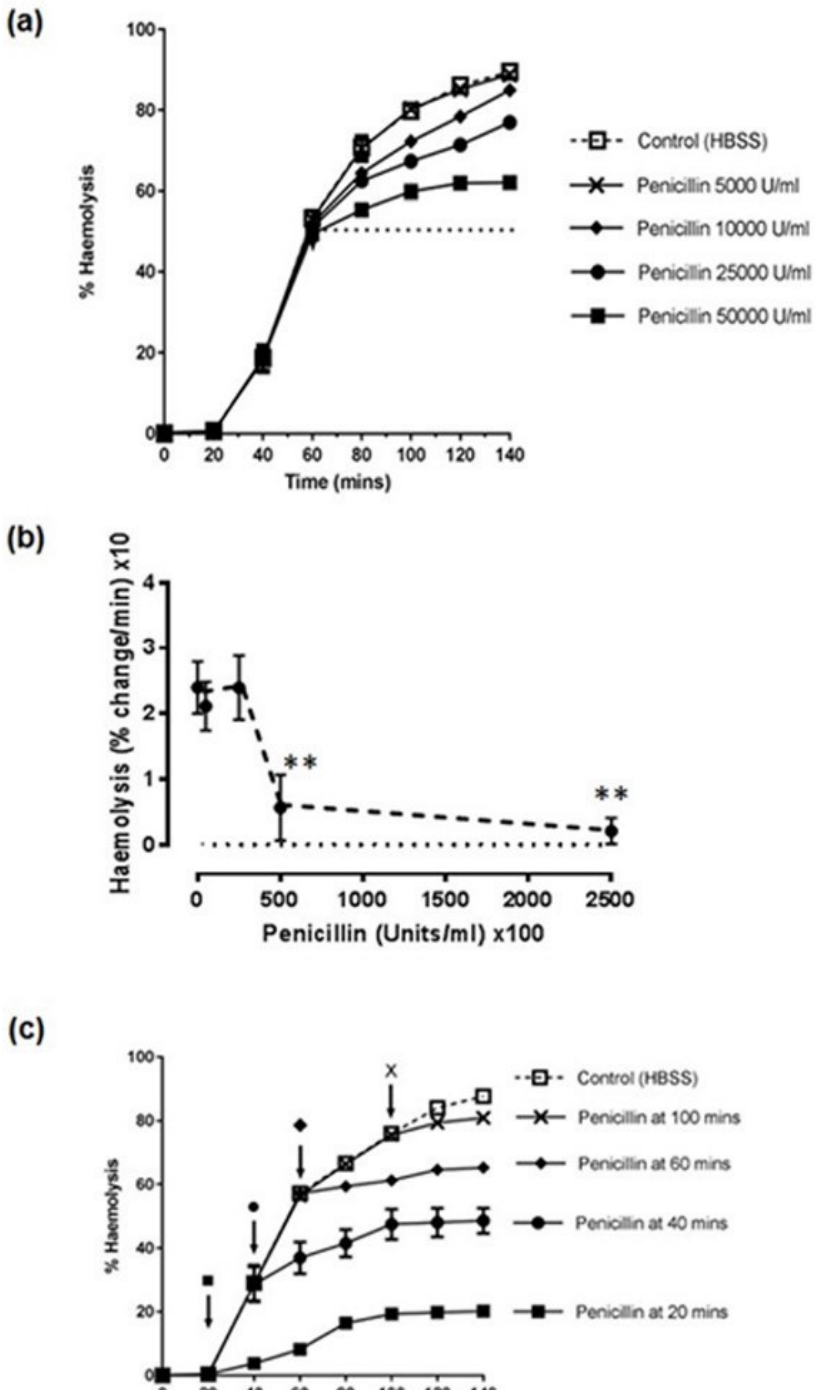
Miller et al., (2018) Fig. 4.



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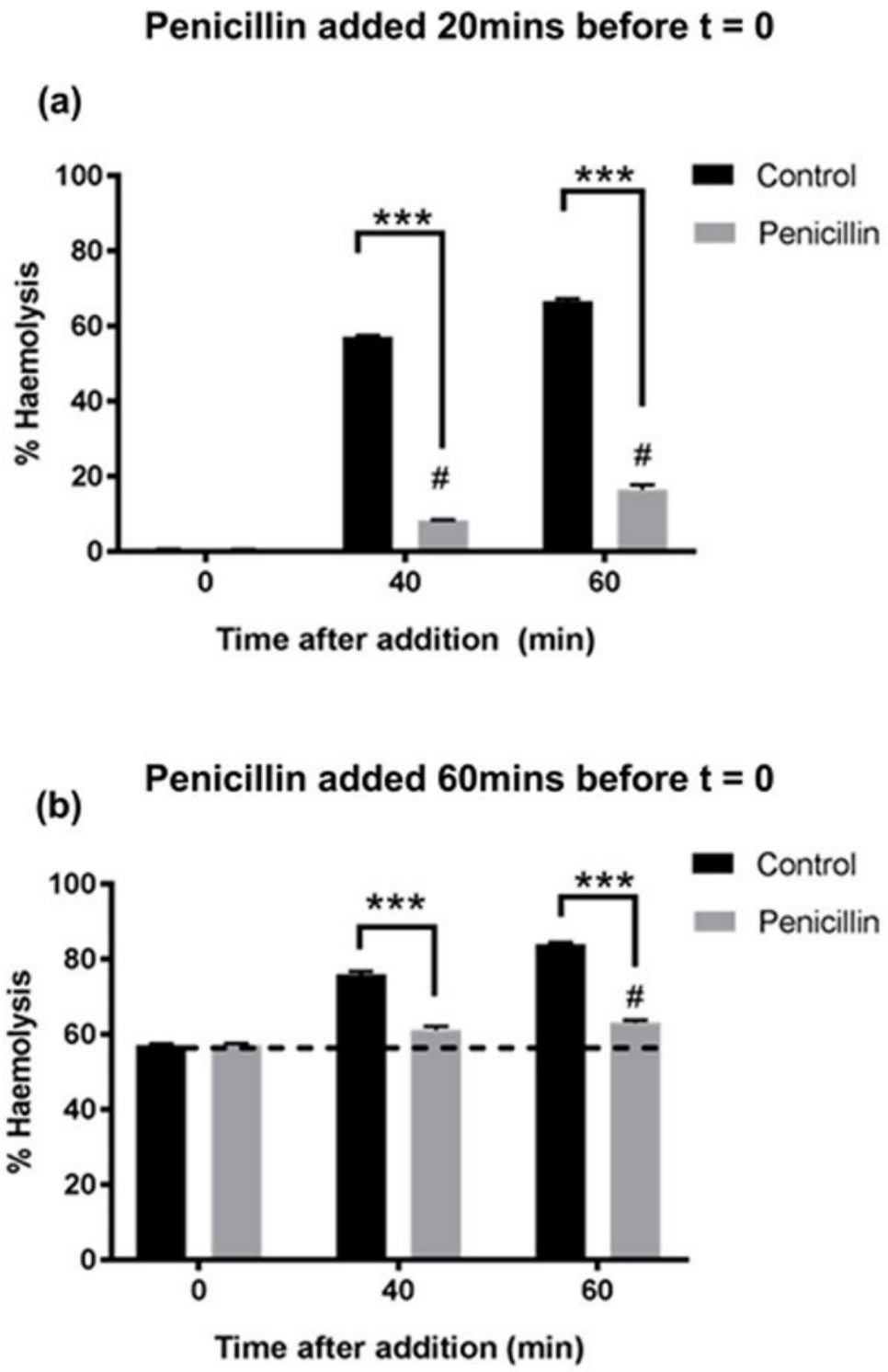
Miller et al., (2018) Fig. 5.



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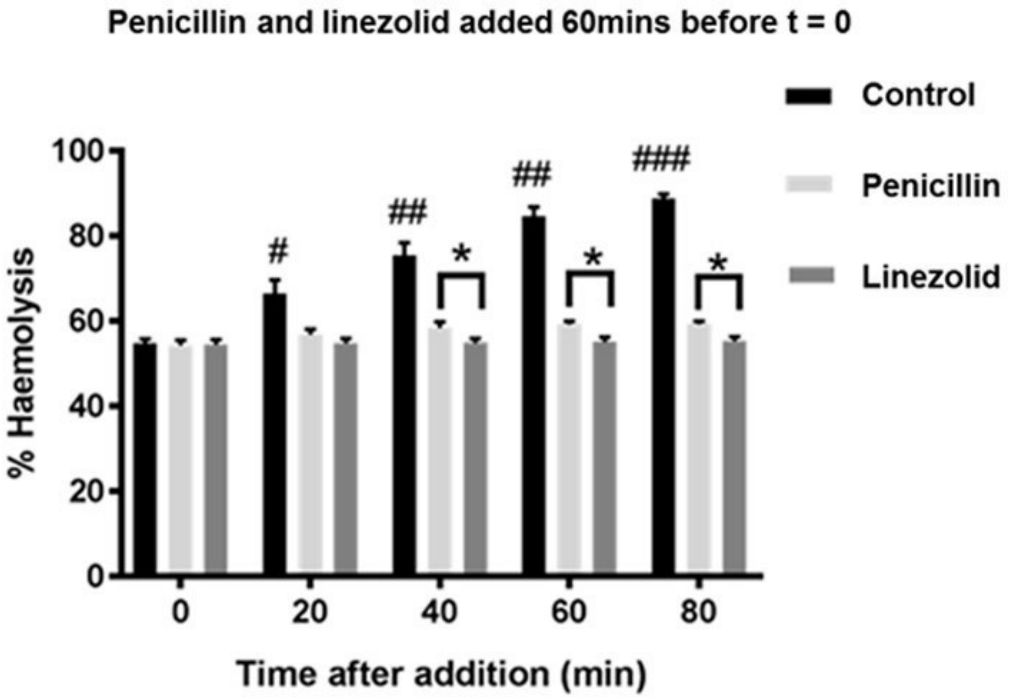

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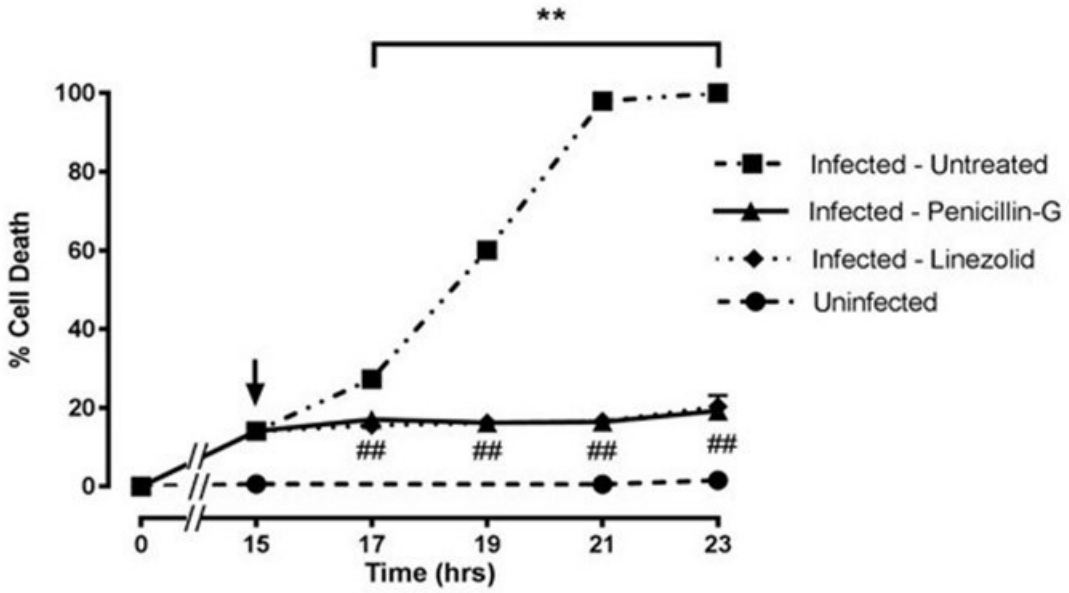
(a)



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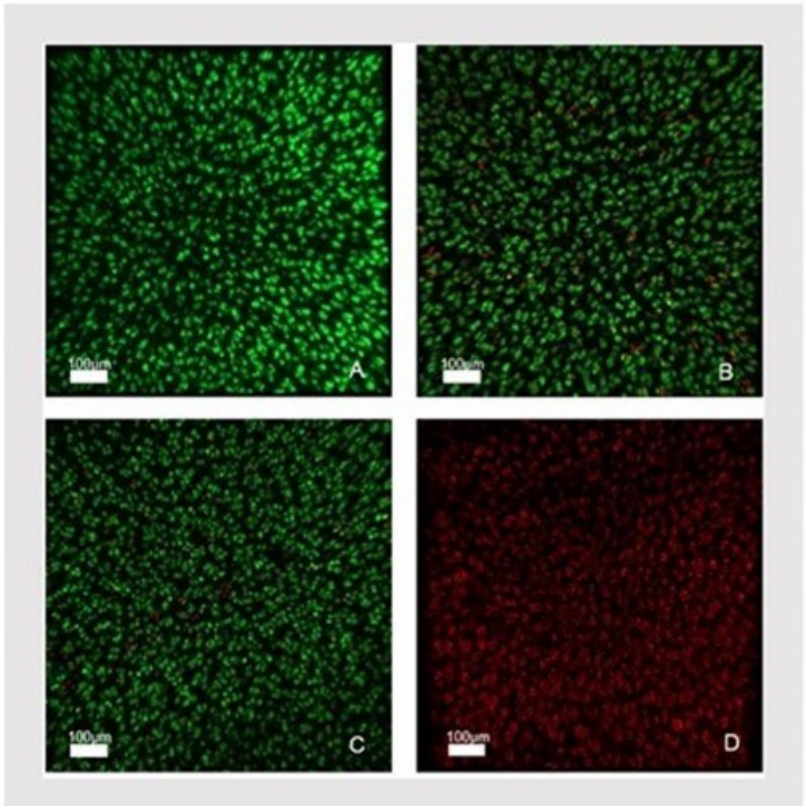

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