Effects of *Pseudomonas aeruginosa* elastase and Exotoxin A on subcutaneous tissue following dermal trauma.

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen of the skin, especially following severe trauma such as burning. It produces a large array of virulence factors, including Exotoxin A and elastase that can kill susceptible cells and digest collagenous tissue. To observe and measure their effects, these purified virulence factors were applied directly to subcutaneous tissue using a transdermal chamber implanted in mice. Elastase disrupted collagen formation in tissue, induced haemorrhage, and increased exudation into the chamber which was quickly resolved by the host. Exotoxin A reduced the number of fibroblasts and leukocytes in tissue, decreased collagen formation, and reduced exudation and the concentration of monocytes in the chamber reservoir; these effects being observed four days post treatment. The ability of these two virulence factors to alter collagen formation and production, and influence local populations of leukocytes, provides further insight into the aetiology of bacteraemia following *P.aeruginosa* infection of skin.

Key Words

Exotoxin A, Elastase, *Pseudomonas aeruginosa*, Dermis

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1. Introduction

P.aeruginosa is a renowned pathogen of burns patients that can produce a large number of soluble proteins, enzymes and toxins that collectively degrade host structures and kill host cells in order to establish infection and penetrate host tissue.[1] One of these toxins, Exotoxin A (ETA), enters the host's cells via the Alpha-2 macroglobulin receptor (α_2MR) and this is followed by ribosylation of elongation factor-2, culminating in cell death.[2] As many as 80% of clinical isolates produce ETA which is a significant virulence factor (VF) due to its extreme toxicity [3], and because the α_2MR is common to a wide range of cells in various systems in humans, including resident cells in sub-epidermal layers of the skin, comprising of fibroblasts and some leukocytes.[4]

Another protease in its arsenal of VFs is *P.aeruginosa* elastase (PAE), and several studies have highlighted its potency and potential to digest a range of collagen and elastin substrates. It can digest both collagenous and non-collagenous bovine basement membranes of glomerular, alveoli of the lung, anterior-lens proteins *in-vitro* [5], and human interstitial and basement membrane collagens.[6] PAE injected into muscle of mice results in damage to muscle fibres and the basement membrane of blood vessels.[7] In the lung, Azghani et al. found PAE increased the permeability between cell-to-cell junctions (transcellular transport) in the epithelial lining to allow the passage of large, 69KDa molecules.[8] Subsequent *in-vitro* analysis using PAE and ETA suggested that specific tight junction (TJ) proteins were being either digested or redistributed by PAE in epithelial cell monolayers, and that ETA blocked protein synthesis and prevented cells from restoring the damaged TJs [9], thereby highlighting the synergistic mechanism whereby PAE and ETA both destroy structural integrity of lung tissue and prevent reconstruction and repair. The reported toxicity of ETA and digestive capabilities of PAE in other tissue caused us to hypothesise that these VFs may digest the extracellular matrix of subcutaneous tissue (ST) and kill fibroblasts that function to maintain the integrity of ST during *P.aeruginosa* infection following dermal injury.

Numerous studies have collectively provided valuable insight into *P.aeruginosa* infections but very few experimental techniques allow for studies involving the direct application of purified bacterial products onto ST. This study used a transdermal chamber [10] to isolate ST in a mouse model of dermal injury permitting the direct application of purified ETA and PAE onto ST. This innovative approach has significant advantages over *in-vitro* analyses and to our knowledge this is the first time the application of these VFs directly on ST has been investigated to examine the effect on fibroblasts, inflammatory cells, and the structural integrity of tissue in a mouse model of dermal injury.

2. Materials and Methods

2.1 Mice

All animal experiments were conducted with permission from the Animal Ethics Committee of Murdoch University, Western Australia. Ten to twelve week old C57Bl/J6 mice approximately 22 to 30 grams in weight were obtained from the Animal Resource Centre, Murdoch Western Australia. Mice were housed five per cage and subjected to 12 hour light cycle with food and water *ad libitum*, in the small animal holding facility at Murdoch University, Western Australia.

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2.2 Transdermal chamber implantation and sampling

The transdermal chamber is depicted in Figure 1. Transdermal chambers were implanted as described.[10] To collect fluid samples from the implanted transdermal chamber, mice were anesthetized by controlled delivery of Halothane (Merial, Australia) to a perspex box through a Fluotec Mark 2 Vaporiser (Cyprane Ltd, England). Once under deep sedation, a sterile micropipette was used to transfer sample exudate from the chamber.

2.3 Purification of P.aeruginosa elastase

A PAE^+ clinical strain of *P.aeruginosa* from a burns patient was kindly donated by the Burns Unit at Royal Perth Hospital, Perth Western Australia. PAE production followed the protocol of Komori et al.[7], with some modifications. *P.aeruginosa* was cultured in TSB at 37°C with shaking, for 24 hours before being centrifuged at 5250 x *g* for five minutes. The cells were resuspended in 3L of Dulbecco's Modified Eagle's Medium (DMEM) with 2% FCS and incubated with shaking for 48 hours then filtered through 0.22µm vacuum filters (Millipore, USA) before being transferred and sealed in a 6-8KDa MWCO Spectra/Por dialysis membrane (Spectrum laboratories Inc, USA). The bag was dialysed against solid 45KDa polyethylene glycol (Fluka, Switzerland) at 4°C and the reduced supernatant was dialysed extensively against Tris HCl (pH 7.2) at 4°C then stored at -20°C. This sample was loaded onto a DEAE sepharose (GE Healthcare, Sweden) column (15 x 200mm) which had been equilibrated with 5mM Tris HCl (pH 7.2). Proteins were eluted with increasing NaCl concentration to 1M and fractions were assayed for elastase activity using the Elastin Congo Red assay as described [11], and pooled. PBS (pH 7.4) buffer exchange was performed using Amicon Ultra (Millipore, USA) centrifuge filters with 10KDa MWCO at 4000 *x g* followed by protein separation on Sephacryl S-200 (GE Healthcare, USA). Elastase rich fractions were identified using the Elastin DQ Assay Kit (Invitrogen, USA) according to the manufacturer's standards and instructions, and fluorescence was recorded using a Beckman Coulter DTX-880 Multimode Detector with EX485nm/EM530nm. Purity was confirmed by a single band of approximately 33KDa by SDS-PAGE (Figure 2).

2.4 Exotoxin A purity & toxicity

Lyophilised ETA was purchased from List Biological Laboratories Inc, USA and had an LD_{50} of approximately 3µg/kg IP. Purity was confirmed by SDS-PAGE revealing a single band of approximately 72KDa. Western blot using rabbit anti-ETA sera (Sigma, USA) confirmed the band as ETA. MDCK cells known to be susceptible to ETA toxicity 12 were grown to confluency and cytotoxicity was confirmed using In-Vitro Toxicology Assay Kit (Sigma, USA) for spectrophotometric measurement of cell viability by mitochondrial dehydrogenase, according to the manufacturer's instructions.

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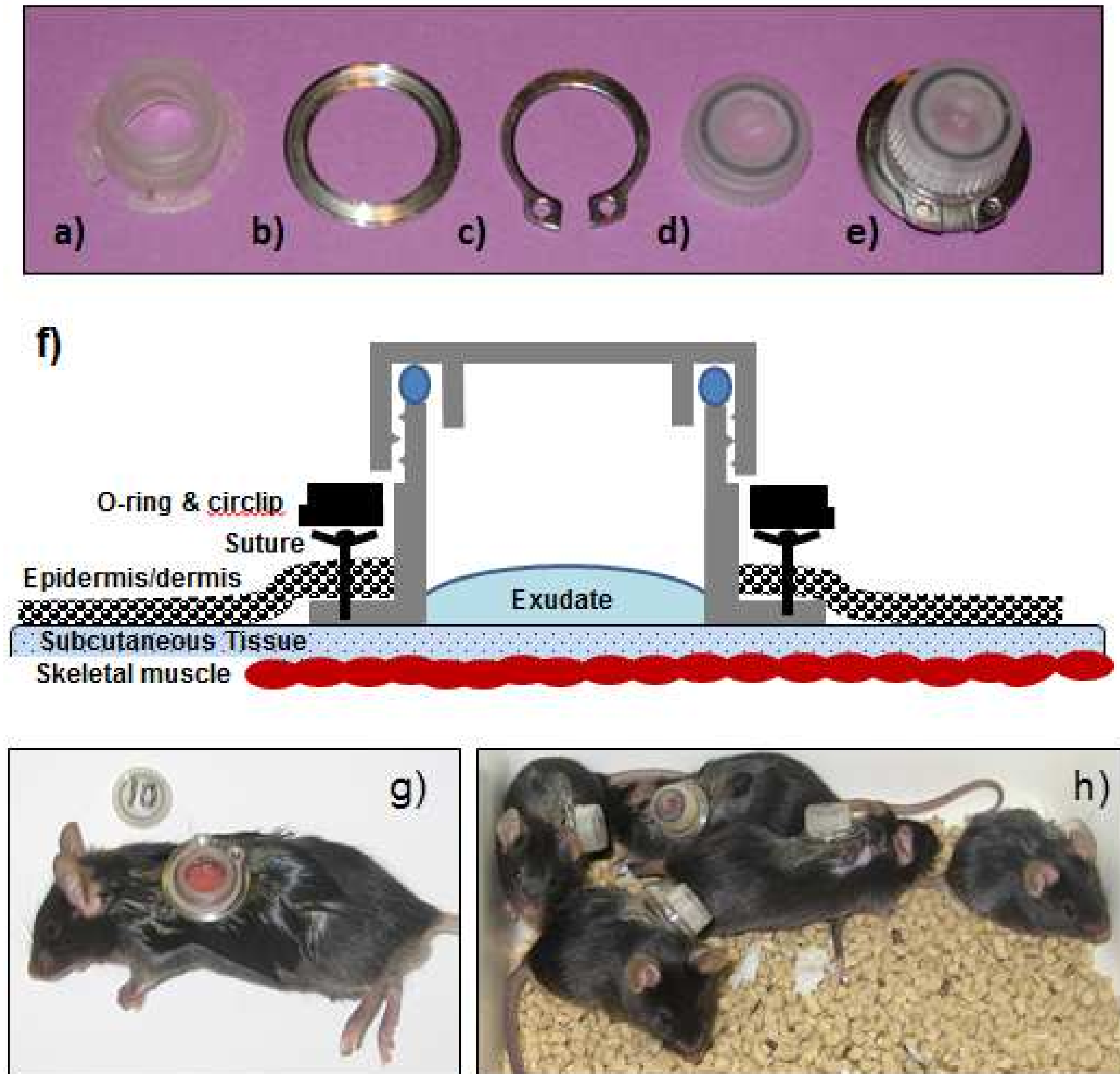

Figure 1. The transdermal chamber. a) The transdermal chamber components comprising a) the body b) o-ring, c) circlip, d) lid, and e) assembled chamber. f) diagram of the chamber in-situ. The dermis and epidermis are separated from ST and sutured over the chamber flange, which is seated on the ST. g) The transdermal chamber implanted in an anaesthetised C57BL/J6 mouse. The screw cap has been removed to show that the chamber is seated on the fascia overlying the muscle. h) group of mice with chambers implanted.

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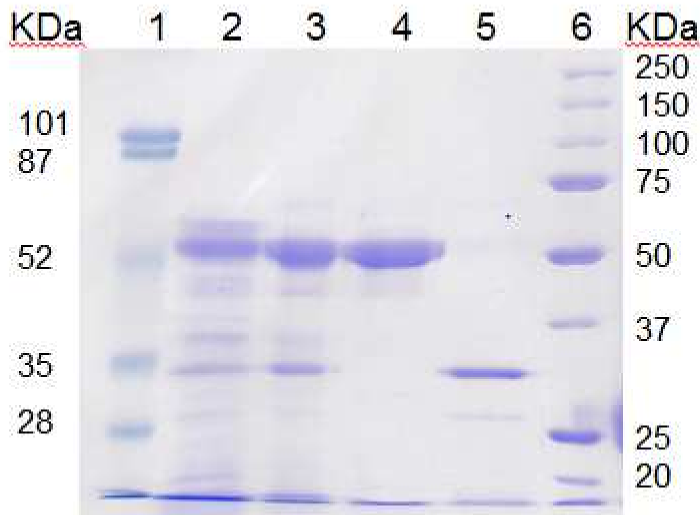

Figure 2. SDS-PAGE of PAE purification. 1- Molecular weight standard. 2- Concentrated culture supernatant. 3- PAE rich fraction from DEAE chromatography. 4- flow through fraction from Sephacryl 200 chromatography. 5- purified PAE. 6- High molecular weight standard.

2.5 Removal of pyrogens from PAE & ETA preparations

Purified PAE was passed through Detoxi-Gel^{TM} Endotoxin Removing Gel (Thermo Scientific, USA) in accordance with the manufacturer's instructions, to remove pyrogens from the solution prior to use in the transdermal chamber.

2.6 Virulence factor application in-vivo

Transdermal chambers were implanted in mice; designated as day '0'. Prior to administering a VF to the chamber, the entire volume of exudate was collected from each chamber.

2.7 Treatment with elastase

To examine the effect of PAE on exudate volume and host cellular response purified PAE was administered to implanted transdermal chambers on day 1 and day 8 (not day 5) and left for 18 hours after each application. On Day 2, 6 and 9 the exudate in the chambers was collected for measurement and analysis.

2.8 Treatment with Exotoxin A

On day 2 mice were treated with either, 50 or 100ng of ETA, or PBS for control mice. Without further intervention, entire chamber exudates were collected for analysis again on day 3 and day 6. Doses of 50ng and 100ng were selected as they were non-lethal but sufficient to produce pathological changes in tissue, based on earlier observations.

2.9 Estimation of concentration & identification of cells in the chamber exudate

Chamber exudate was aspirated from the chamber and the volume measured using a microtiter pipette, diluted and counted in a Neubauer Counting Chamber. Differential cell identification was performed based on cell staining and morphology after being loaded onto glass slides using a Shandon Cytospin 2 and stained with Wright-Geimsa stain.

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2.10 Histology
The chamber and surrounding tissue was excised and fixed in 10% buffered formalin, pH 7.4, then embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (H & E) or Martius, Scarlet and Blue (MSB) by the Histology Laboratory, School of Veterinary and Life Sciences, Molecular and Biomedical Sciences at Murdoch University, Western Australia.

2.11 Data analysis
Statistical evaluations were performed using the Student's *t* test. Differences were considered significant if the *p*-value was less than 0.05.

3. Results

3.1 P.aeruginosa elastase applied to subcutaneous tissue
PAE treated mice had a significantly higher exudate volume compared to control mice on day 2 and day 9 (18 hours after PAE treatment), but not day 6 (when mice had not been pretreated with PAE) (Figure 3a). The chamber exudate of PAE treated mice also had a significantly higher concentration of leukocytes (Figure 3b), however there was no significant difference in the proportion of neutrophils and monocytes between mice treated with PAE and control mice (Figure 3c). The chamber fluid of PAE treated mice also showed the presence of red blood cells (RBC) on day 9 (Figure 4).

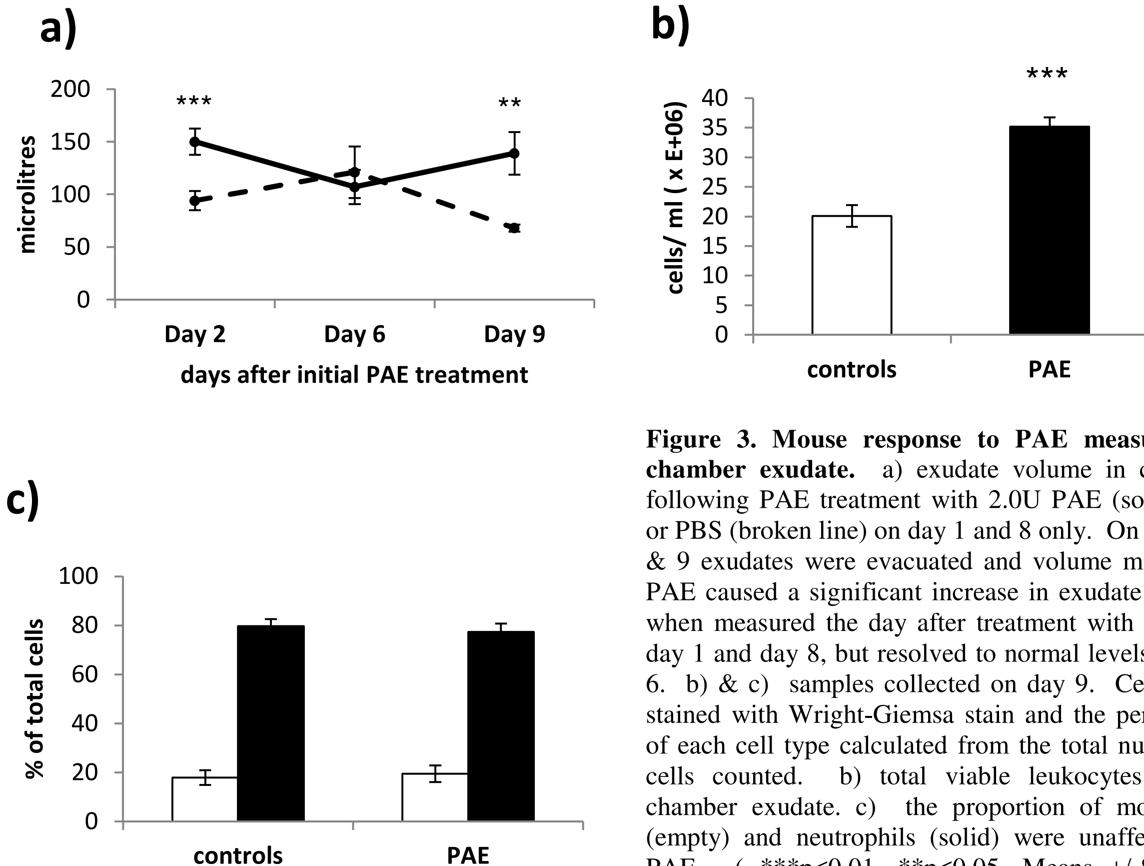

Figure 3. Mouse response to PAE measured in chamber exudate. a) exudate volume in chamber following PAE treatment with 2.0U PAE (solid line) or PBS (broken line) on day 1 and 8 only. On day 2, 6 & 9 exudates were evacuated and volume measured. PAE caused a significant increase in exudate volume when measured the day after treatment with PAE on day 1 and day 8, but resolved to normal levels on day 6. b) & c) samples collected on day 9. Cells were stained with Wright-Giemsa stain and the percentage of each cell type calculated from the total number of cells counted. b) total viable leukocytes in the chamber exudate. c) the proportion of monocytes (empty) and neutrophils (solid) were unaffected by PAE. (***p<0.01, **p<0.05. Means +/-SEM, 5 mice/group).

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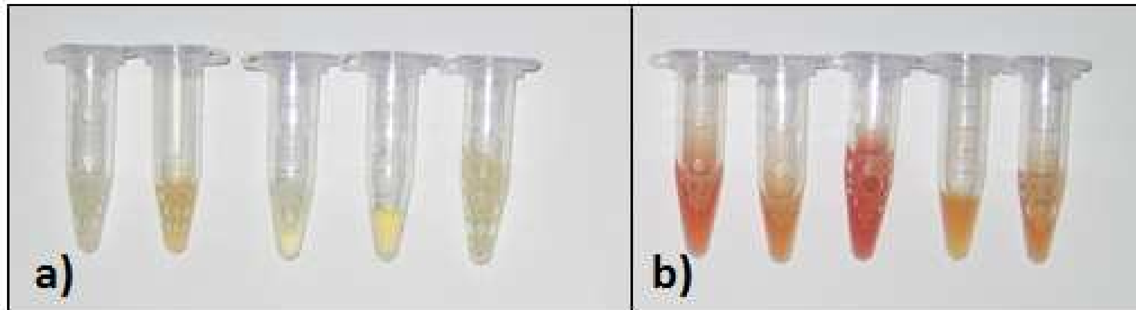

Figure 4: Exudate recovered from the chamber of mice on day 9 contained RBC. a) negative control mice were pretreated with PBS on day 1 and day 8. b) mice were treated with 2.0U of PAE on day 1 and day 3. On day 9 total exudate was recovered. PAE treated mice showed the presence of RBC in the exudate.

Thin tissue sections from beneath the chamber were stained with MSB trichrome stain which highlights connective tissue (blue) and fibrin (red). Microscopic examination of these sections from PAE treated and PBS control mice showed several differences, and a typical section from each group is shown in Figure 5. The collagen in the ST was less dense and there was less superficial granulation tissue formation in PAE treated mice; which also appeared fragmented at the surface. PAE treated mice also had more leukocytes in the hypodermis and there was an absence of adipocytes. The myocytes beneath the fascia of PAE treated mice appeared smaller in diameter and less dense with more leukocytes within the muscle compared to control mice. They also frequently displayed central nuclei compared to myocytes in control mice which had peripheral nuclei, indicative of cellular distress.

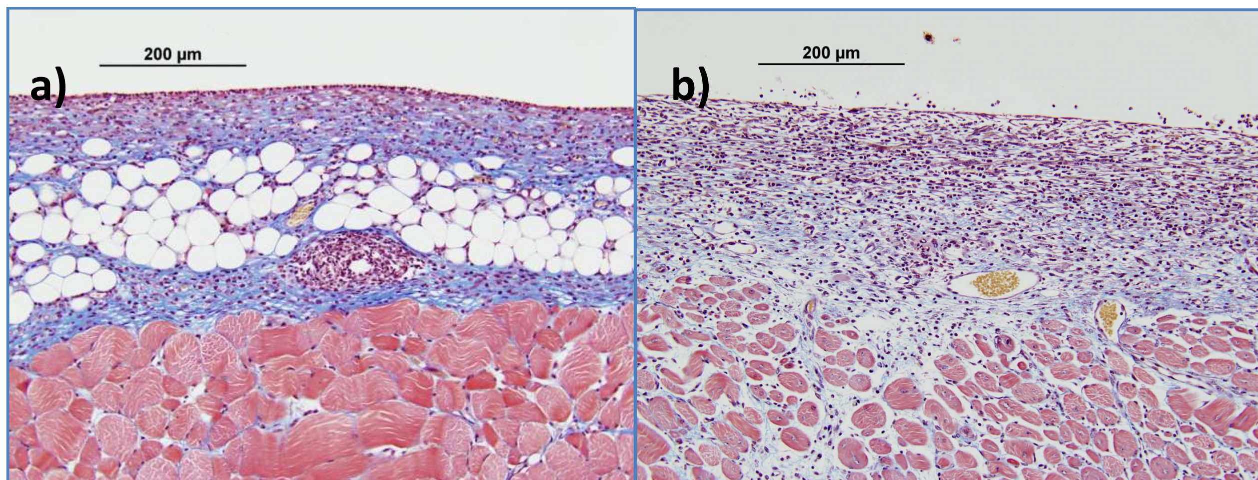

Figure 5. Histology of fascia and muscle following PAE treatment. MSB stained tissue sections of ST beneath the transdermal chamber. Tissue was harvested on day 9 following a) PBS or b) 2.0U PAE treatment on day 1 and 8. In PAE treated mice, collagen (stained blue) was less dense and was infiltrated by more leukocytes compared to control mice. Adipocytes are absent in PAE treated mice, and the underlying myocytes are smaller, less dense and many have central nuclei.

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3.2 Exotoxin A applied to subcutaneous tissue

On day 2 (pre ETA treatment) and day 3 (18 hours post ETA treatment) there was no significant difference in the exudate volume collected between ETA treated and PBS treated control mice. There was an increase in exudate volume from day 2 to day 6 for the control mice and the mice that received 50ng of ETA. However on day 6 (four days after ETA treatment), mice that received 100ng ETA had a significantly lower volume ($p<0.01$) of exudate in the chamber compared to control mice, indicating that higher doses of ETA decreased the exudate volume (Figure 6a). There was no significant difference in the concentration of leukocytes in the chamber fluid of ETA treated mice compared to PBS controls. Predictably, neutrophils accounted for the greatest proportion of leukocytes in the exudates in control mice (85%), and there was a small but significant increase ($p<0.05$) in the proportion of neutrophils when 100ng (increase to 98% neutrophils) of ETA was added to the chamber compared to PBS controls (Figure 6b). However, differential cell counts of chamber exudates from ETA treated mice on day 3 showed that monocytes were reduced in response to ETA treatment. About 15% of the leukocyte population in control mice exudates were monocytes, however this proportion was significantly reduced to 2.5% ($p<0.05$) when mice were treated with 100ng ETA (Figure 6b).

Tissue was harvested directly under the chamber body, sectioned and stained with MSB on day 6; four days after ETA treatment. Typically, control mice had a dense layer of collagenous tissue containing fibroblasts. Beneath this, a thicker layer of collagen extended to the skeletal muscle, which also had resident fibroblasts and some leukocytes. Blood vessels, and nerves were apparent, and there was an organised dense layer of collagen rich in fibroblasts adjacent to healthy muscle (Figure 7a). In contrast, the ETA treated mice showed an absence of fibroblasts throughout the ST and a qualitative reduction in the number of leukocytes present. In ETA treated mice, older collagen (less intense blue) was present. Adipocytes appeared less plump and round in mice that received 100ng of ETA. Overall, higher doses of ETA resulted in a greater degree of disorganisation to tissue (Figure 7b and 7c).

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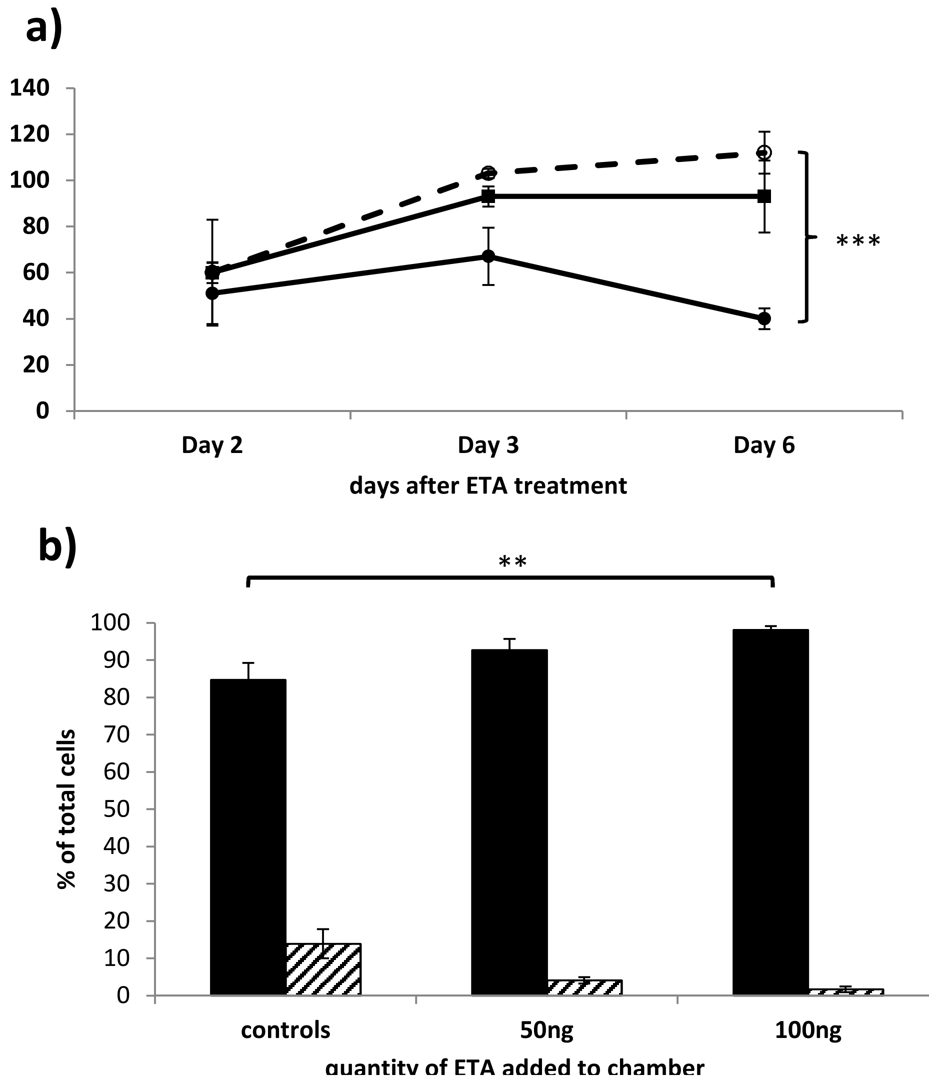

Figure 6. Total and differential leukocytes and exudate volume following of ETA treatment. Chambers were evacuated on Day 2 after chamber implantation and treated with ETA. a) mice were treated with PBS (broken line), 50ng (square), or 100ng (solid circle). On Day 3 and Day 6 chambers were evacuated and exudate volume measured. High doses of ETA significantly reduced the exudate volume in the chamber on Day 6. b) A dose of 100ng significantly increased the proportion of neutrophils (solid) measured on Day 6, and significantly reduced the proportion of monocytes (striped) in the chamber fluid. (***p<0.01, **p < 0.05. Means +/- SEM, 5 mice/group).

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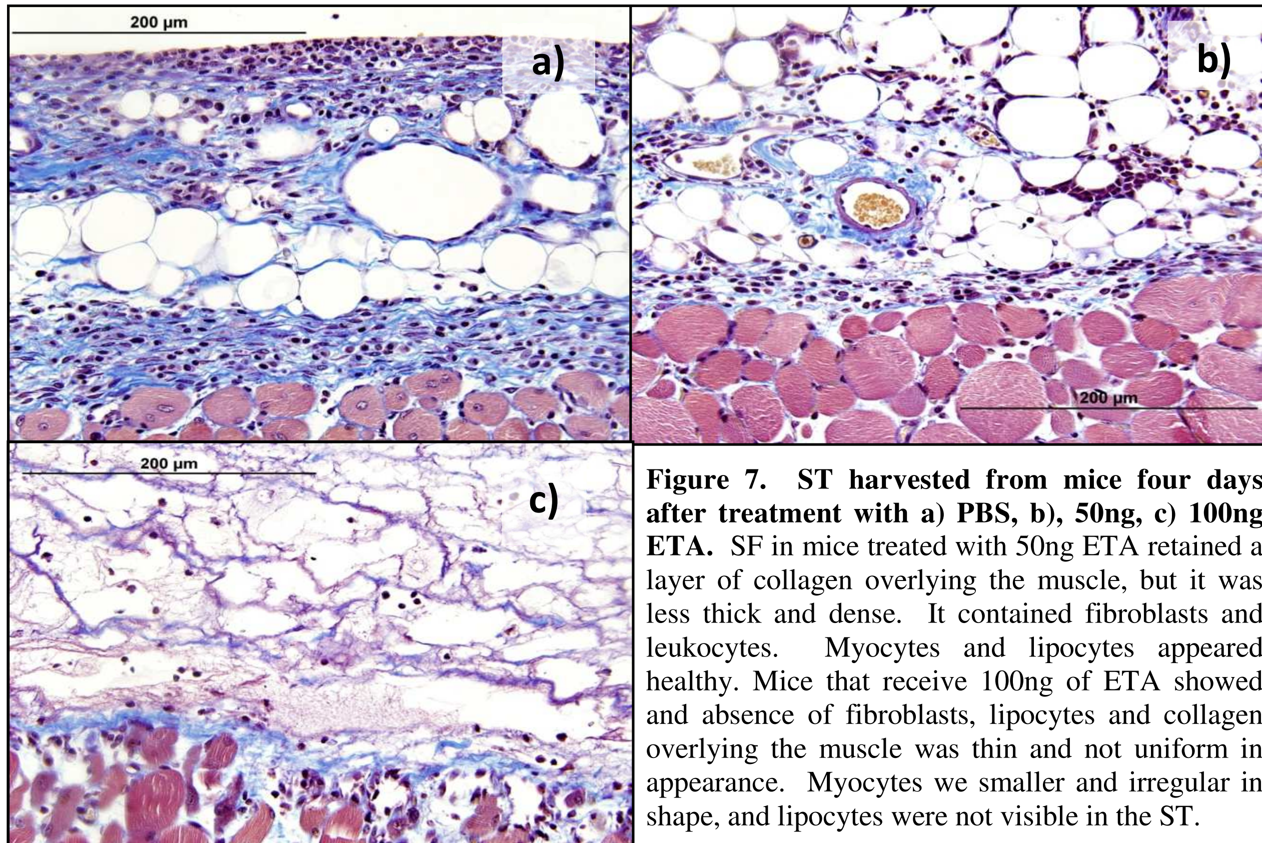

Figure 7. ST harvested from mice four days after treatment with a) PBS, b), 50ng, c) 100ng ETA. SF in mice treated with 50ng ETA retained a layer of collagen overlying the muscle, but it was less thick and dense. It contained fibroblasts and leukocytes. Myocytes and lipocytes appeared healthy. Mice that receive 100ng of ETA showed and absence of fibroblasts, lipocytes and collagen overlying the muscle was thin and not uniform in appearance. Myocytes we smaller and irregular in shape, and lipocytes were not visible in the ST.

4. Discussion

This study used a transdermal chamber to examine the effect of purified ETA and PAE from *P.aeruginosa* on fibroblasts, inflammatory cells and the structural integrity of ST in a mouse model of dermal injury. This model simulates trauma or surgery resulting in the dermis and epidermis being excised to expose subcutaneous tissue to environmental pathogens (similar to severe burning). The distinct advantage of this model is that the ST is protected by a removable protective cap allowing direct application of reagents to ST whilst protecting the tissue from contamination. Although these two prominent virulence factors target different cells and molecules in ST, the evidence presented shows the influence each has on tissue regeneration, and their inhibition of host cellular immune function through the localised reduction of leukocytes. To our knowledge this is the first time that these two *P.aeruginosa* VFs have been applied directly to ST using a transdermal device, and these data clearly show how ETA and PAE may facilitate tissue penetration by *P.aeruginosa* and cause disease following trauma.

As expected, *P.aeruginosa* elastase applied to the fascia was pro-inflammatory. Neutrophils were the most abundant cell in the exudate, which is normal during acute inflammation [13], and although the specific mechanism for the initiation of inflammation following PAE administration has not been elucidated, Kon et al. reported that heat inactivated PAE did not result in the same level of inflammation compared to the active enzyme.[14] Therefore, inflammation is likely to have been initiated by enzymatic digestion of substrates in ST including

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collagen [6] and elastin[15], and possibly the digestion of fibrinogen; the soluble plasma protein involved in the clotting cascade. [7] Although damaging to components of ST, our study showed the effects to be acute and were rapidly resolved by the host.

Increased concentration of RBCs in the chamber exudate suggests that PAE had damaged blood vessels within the fascia thereby increasing leakage of serum components into the extracellular space. Aside from its elastolytic activity, PAE has previously been shown to hydrolyse components of the endothelium and cause severe haemorrhage in muscle.[7] In addition, PAE is fibrinogenolytic [7] so can digest the principal component involved for the formation of a blood clot [16], perhaps causing any blood clots beneath the chamber floor to be more susceptible to destruction. The presence of RBC in the exudate of mice treated with PAE nine days after chamber implantation may also be due to ST being more vascularised. By this time, angiogenesis and healing had progressed and blood vessel density increased in accordance with normal healing [17] so the tissue would also have had increased blood supply compared to previous days. Azghani et al. reported that PAE digests TJ proteins in epithelial tissue [8], so it is possible that PAE digested endothelial TJ proteins thereby liberating RBCs into the interstitial space, but this was not confirmed by our study. On the other hand, it is clear that PAE caused physical disruption to ST and underlying muscle. The damage caused to collagen is likely to be the result of both the direct hydrolysis of the collagen by PAE, and neutrophilic elastase production to facilitate migration by leukocytes in response to inflammatory chemokines and cytokines [13], and was accompanied by increased exudation.

The muscle damage observed has been previously reported [7] and found to result in elevated creatine phosphokinase (CPK) in serum; evidence of deep tissue injury. [18] Therefore, the observed infiltration of leukocytes into the muscle could have been in response to heat shock proteins liberated following myocyte death[19], but remains unconfirmed from this study. The histology indicated that PAE had altered the structure of collagen in ST and damaged myocytes thereby facilitating penetration of deeper tissue during *P.aeruginosa* infection.

To the contrary, ETA treatment reduced the volume of exudate in the chamber which may have been induced fluid reabsorption within ST. Although this study has not established the reason for this, at least two reports have suggested that ETA may be able to stimulate fluid reabsorption from local tissue. These studies focussed on *P.aeruginosa* infection in the epithelium of the lung, and suggested that the fluid movement across the epithelium was in response to sodium uptake initiated by ETA. [20, 21] It has been suggested that other bacterial products including LPS may act in a similar way, but at this stage the precise cause remains unclear. [22] The prolonged response (4 days) between ETA treatment and exudate volume reduction also suggests that this is may be a secondary effect or symptom of localised ETA toxicity.

ETA also reduced the concentration of leukocytes in the exudate and ST. A direct cytotoxic effect on murine fibroblasts and macrophages is very likely given that in rats these cells express the α_2-MR [23], as in humans [24], making these cells directly susceptible to ETA cytotoxicity. However, α_2-MR has not been isolated from neutrophils to date, despite a report suggesting direct cytotoxicity of ETA on polymorphonuclear cells (PMN). [25]

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Miyazaki et al. administered 100ng ETA IV and reported a significant reduction to circulating PMNs 0.5-2.0 hours afterwards, however the concentration of PMNs returned to normal levels after 24 hours. In-vitro incubation of PMNs and *P.aeruginosa* with 200ng [2µg/ml] ETA reportedly decreased the PMN population by 68% with affected PMNs displaying what is described only as swollen nuclei. [25] It remains inconclusive whether α_2-MR is expressed by PMNs, although it is unlikely that cell death results from receptor-mediated cytotoxicity. Alternatively, we speculate that ETA could have inserted itself into the lipid bilayer of the cell membrane to form ionic channels resulting in cellular death as previously demonstrated [26], however this warrants further research.

4.1 Limitations of the study

The purpose of this study was to demonstrate the effect of purified *P.aeruginosa* VFs on tissue and cells in ST, however there are limitations to this study. Due to practical considerations, only a limited range of doses and timing of dose in relation to trauma were trialled, and their combined effects were not investigated. Additionally, although the decision to use an inbred mouse strain, the choice to use a single strain of mouse may have introduced a source of bias, as C57BL mice favour a TH1 response over a TH2. [27]

4.2 Conclusion

This brief study provides further insight on the effect *P.aeruginosa* PAE and ETA have on tissue of the ST in mice following dermal injury. Although this study involved a relatively small number of animals and used a limited range of VF doses, the results provided initial data of the gross effects of these VFs on local ST. During an infection by *P.aeruginosa* it is likely that PAE results in acute destruction of tissue that would normally be neutralised quite quickly, but ETA ensures that collagen within ST is not restored by resident fibroblasts. ETA also causes a reduction in exudation and local leukocyte populations. The combination of these VFs may provide *P.aeruginosa* with the opportunity to penetrate deeper tissue and cause bacteraemia. Future studies could focus on determining how ETA in local tissue results in the reduction of leukocytes in local tissue, including its influence on Mast cells and the expression of cytokines in subcutaneous tissue.

5. Acknowledgements

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