The Role of Mitochondrial DNA in the Post-Injury Inflammatory Response Following Major Trauma

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List of Publications in the Order Included in This Thesis:

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5. Tuboly E, McIlroy D, Briggs G, Lott N, Balogh ZJ, **Clinical implications and pathological associations of circulating mitochondrial DNA.** *Front Biosci (Landmark Ed).* 2017; 22:1011-1022 **(*Background: Appendix 1*)**

6. Balogh ZJ, McIlroy DJ, Smith DW, Hansbro PM, **The origin and the role of mitochondrial DNA in postinjury inflammation.** *J Crit Care,* 2013 ;28(6):1099-100 ***(Hypothesis: Appendix 2)***

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List of Abbreviations:

AIF – Apoptosis Inducing Factor
ARDS – Acute Respiratory Distress Syndrome
AST – Aspartate Transferase
ATLS® - Advanced Trauma Life Support
ADP – Adenosine Di-Phosphate
ATP - Adenosine Tri-Phosphate
ACTH - Adrenocorticotropic hormone
bDNA – Bacterial DNA
C - Complement
CARS - Compensatory Anti-inflammatory Response Syndrome
CD18 – Integra beta-2 protein
Cf-DNA – Cell-free DNA
CK – Creatine Kinase
CNS – Central Nervous System
Cyp-D - Cyclophilin D
Cyt c – Cytochrome C
DAMP – Damage Associated Molecular Pattern
DNA – Deoxyribonucleic Acid
DNase – Deoxyribonuclease
Fas – Fas ligand / receptor
FP – Formyl Peptides
GM-CSF - Granulocyte-macrophage colony-stimulating factor
H_2O_2 – Hydrogen Peroxide
HIF – Hypoxia Inducible Factor
ICAM – Intracellular Adhesion Molecule
ICU – Intensive Care Unit
IKK - inhibitory kappa B Kinase
IL – Interleukin
IL1-ra - IL-1 receptor antagonist
IR – Ischaemia Reperfusion
ISS – Injury Severity Score
LDH – Lactate Dehydrogenase
LOS – Length of Stay
LPS – Bacterial Lipopolysaccharide
MOD – Multiple Organ Dysfunction
MODS – Multiple Organ Dysfunction Syndrome
MOF – Multiple Organ Failure
mtDNA – Mitochondrial DNA
nDNA – Nuclear DNA
NET – Neutrophil Extracellular Trap
NFκB – Nuclear Factor Kappa B
P - Protein

PAMP – Pathogen Associated Molecular Pattern
PaO_2 – Partial Oxygen Pressure
PARP – Poly-ADP-Ribose Polymerase
PCO_2 – Partial Carbon Dioxide Pressure
PICS - Persistent Inflammatory, immunosuppressed Catabolic Syndrome
PKC – Protein Kinase C
PMA - Phorbol Myrisate Acetate
PRR – Pathogen recognition receptor
RIPK - Receptor Interacting Protein Kinases
RNA - Ribonucleic acid
rRNA – Reporter Ribonucleic Acid
tRNA – Transfer Ribonucleic Acid
ROI – Reactive Oxygen Intermediate
SIRS – Systemic Inflammatory Response Syndrome
TLR – Toll-Like Receptor
TNFα – Tumour Necrosis Factor Alpha
VCAM – Vascular Cellular Adhesion Molecule

Abstract:

Introduction:

Trauma is the leading cause of death in the developed world in those aged under 45 years. The main potentially modifiable cause of late death after injury is post-injury multiple organ failure (MOF). Early MOF is characterised by a lethal combination of systemic inflammatory response syndrome (SIRS) which underpinned by neutrophil proliferation and "priming" as a result of the initial injury and haemorrhagic shock. If primed neutrophils are then exposed to "second hit" then dysregulated neutrophil driven inflammation can occur, resulting in end organ sequestration, parenchymal damage, MOF and ultimately death. Interest has increased in endogenous drivers of the innate immune system that exert a potent pro-inflammatory effect by activating pathogen recognition receptors (PRR's), which are designed to respond to pathogen associated molecular patterns (PAMPs) found in bacteria. Endogenous factors that can trigger this response in the absence of sepsis have been termed "alarmins" or damage associated molecular patterns (DAMPs). Mitochondrial DNA (mtDNA) is a potently pro-inflammatory DAMP, which has been found to be highly elevated in the post-injury state. Mitochondrial DAMPs have also been associated with neutrophil mediated end organ injury.

Primary aims:

The primary aim of this thesis was to characterise the effect of post-injury non-life saving orthopaedic surgery on circulating mtDNA levels. No study had looked at the effect of surgical intervention on levels of mtDNA after initial injury and possible sources of mtDNA release. Initially a pilot study of 35 trauma patients who subsequently underwent orthopaedic surgery was performed primarily measuring cell-free mtDNA and nuclear DNA (nDNA) with sequential plasma measurements over a 5-day perioperative period with comparison to 20 healthy control subjects. MtDNA levels continued to rise over the 5-day observation period following surgery and had no correlation to markers of cell-necrosis either in the form of direct musculoskeletal injury, or secondary inflammatory end organ injury. Whilst nDNA levels were elevated when compared to healthy controls no increase was observed in the 5-day observation period. Elevated mtDNA perioperative levels were directly correlated with the magnitude and early timing of surgical intervention. MtDNA levels were inversely proportional to the volume of crystalloid infused indicating a possible role for adequate resuscitation in modulating circulating mtDNA levels. A positive trend between mtDNA levels and incidence of post-

injury SIRS and MOF was observed but this failed to reach statistical significance. This lead to the genesis of the hypothesis that the persistently elevated mtDNA levels may have a primary inflammatory source.

Secondary aims:

The secondary aims of this thesis were threefold. Firstly, to determine whether there was a primary inflammatory source of mtDNA, namely focussing on possible neutrophil extracellular trap (NET) formation or "NETosis". Secondly, to determine what factors may propagate and influence mtDNA release. Finally, to investigate mechanisms for modulating circulating mtDNA levels following injury and subsequent surgery by looking at DNase activity. NETosis is characterised by the release of chromatin in conformational net-like structures in response to sepsis, however some authors had shown that under certain conditions NETs could be composed of mtDNA (mtDNA-NETs). The next study performed focussed on demonstrating whether NETs were formed after injury and subsequent surgery and what type of DNA they were composed of. The presence of NETs had been postulated after traumatic injury by one group based on observed high concentrations of cell-free DNA but they failed to define any microscopic evidence of NET formation. In our next paper we definitively demonstrated that NETs were formed after injury and subsequent surgery and also in response to elective orthopaedic hip replacement surgery. This was achieved microscopically using fluorescent DNA avid dyes to demonstrate the presence of conformational DNA-NET structures. Molecular genetic analysis of the NETs formed in response to injury and subsequent surgery or in response to elective surgery alone revealed that the NETs were mtDNA-NETs. Due to molecular similarities between mtDNA and bacterial DNA (bDNA) we hypothesised that mtDNA might trigger NETosis through a PRR mediated pathway. In the next paper we studied the effect of exposing healthy neutrophils and post-injury perioperative neutrophils to physiological concentrations of mtDNA we had measured in our initial pilot over the 5-day observation period. We then conducted a series of positive control experiments using phorbol myrisate acetate (PMA), a known potent stimulator of NETosis. NETs were triggered after trauma and healthy neutrophils were exposed to mtDNA. Notably the NETs formed in response to mtDNA were mtDNA-NETs in both trauma and healthy neutrophils, however trauma neutrophils were less responsive compared to healthy control neutrophils. This observation was thought to be possibly due to the exposure of trauma neutrophils to high levels of mtDNA after injury and surgery causing prior mtDNA-NET production. NETs formed in response to PMA

exposure were composed almost exclusively of nDNA (nDNA-NETs). Finally, we studied the plasma activity of DNase alongside mtDNA and nDNA concentrations in a much larger cohort of trauma patients (n=103) compared to our initial pilot (n=35). Circulating DNase isotypes are responsible for the digestion of extracellular DNA whether mtDNA or nDNA and also digest NETs. DNase activity was significantly reduced compared to that measured in healthy controls. This greater powered study did reveal a statistically significant positive correlation between perioperative mtDNA levels and SIRS but not MOF, despite a strong trend.

Conclusion / Clinical Relevance:

Our data suggests that after traumatic injury, the timing/magnitude of surgery and adequacy of resuscitation influence the levels of circulating mtDNA. Neutrophils contribute a significant amount of mtDNA through mtDNA-NET formation in the post-injury and perioperative period. MtDNA can essentially drive its own release through a positive feedback loop. This occurs through circulating mtDNA triggering further mtDNA-NET release, resulting in a vicious cycle of dysregulated inflammation and associated SIRS with a likely link to post-injury MOF. Most excitingly the finding of reduced DNase levels in the face of high mtDNA levels. This may offer up a novel therapeutic target for modulation of aggressive post-injury SIRS, through the potential administration of exogenous DNase in the post-injury and peri-operative recovery period.

Chapter 1: Literature Review

1.1 Trauma Significance:

Trauma represents the leading cause of death in those under the age of 45 years in the developed world (ABS 2008, Mackenzie 2000, Trunkey et al. 1983). Major trauma can be defined as those who have an injury severity score (ISS)>15 (Baker et al. 1974).

An initial prospective observational study by Baker et al. over a 12-month period in San Francisco in 1977, led to traumatic deaths being defined as having a trimodal distribution (Baker et al. 1980). The first peak represents those individuals who die immediately or on scene, having suffered a non-survivable central nervous system injury (CNS), organ injury or rapid exsanguination. In a later study these were found to represent 52-73% of trauma related deaths (Trunkey et al.1983). The second peak, within 24hrs of injury, represents those individuals with severe head injuries, hypovolaemia and hypoxia (Acosta et al. 1998, Baker et al. 1977, Sauaia et al. 1995).

These first two peaks, both immediate and early, are difficult to influence as they largely reflect non-survivable injuries. When reviewing the epidemiology of traumatic deaths nearly 18 years later, many cases that died in the pre-hospital setting in Baker's initial 1977 study now represent early hospital deaths within the first 24hrs (Sauaia et al. 1995). This is largely attributed to advances in pre-hospital care and the use of preventative safety measures such as the mandatory use of seatbelts in motor vehicles, resulting in more people making it to hospital alive.

The third peak represents early survivors, who then develop multiple organ failure (MOF) described as causing 75% of late deaths (Baker et al. 1980). Whilst the incidence of MOF has fallen in more recent population based studies, attributed to advances in care of the critically injured (Evans et al. 2010), it still affects up to 25% of patients who suffer major trauma (Ciesla et al. 2005). It is still the leading cause of late in-hospital mortality (Dewar et al. 2009, Evans et al. 2010), accounting for between 51% and 61% of deaths (Dewar et al. 2009), with total mortality between 27% and 100%, dependent on the number of organs affected (Dewar et al. 2009). Those who survive MOF are four times more likely to need assistance with basic activities of daily living (Ulvik et al. 2007). They also have mortality 6 times higher than other

trauma survivors in the seven years following recovery (Ulvik et al. 2007). The socioeconomic burden of the condition is substantial.

The greatest interventional opportunity today for reducing post-injury mortality, involves focussing on those early survivors who go on to develop MOF. MOF is a complex pathophysiological process that results, at least in part, from deleterious post-injury immune activation, and it can be subdivided into early and late phases. In the early phase, MOF is propagated by the aggressive systemic inflammatory response syndrome (SIRS) that likely represents the stage with greatest potential for intervention.

1.2 Post-Injury Inflammatory Response:

Inflammation is a normal physiological response to tissue injury. Through the localised release of pro-inflammatory mediators and endothelial cellular adaptation, neutrophils can be recruited into damaged tissue as the primary effector cells in this early acute inflammatory response. This process facilitates immune protection in the event of subsequent bacterial invasion, if an epithelial surface has been breached. The acute phase response also triggers a cascade of cellular activation and recruitment that aids healing and repair of damaged tissues. In trauma, extensive tissue damage is often sustained and inflammation can manifest itself systemically in SIRS. This can have negative implications if tissues of vital organs are affected, leading to multiple organ dysfunction (MOD), and MOF. It is therefore necessary to understand the mechanisms underpinning the post-injury inflammatory response.

1.3 Acute Inflammatory Response:

The acute phase inflammatory response manifests itself early after injury is sustained. It is mediated by interactions between a variety of cell types including macrophages, neutrophils, fibroblasts and vascular endothelial cells. The cellular events that characterise the response are regulated and controlled by a wide range of cytokines. The most active cytokines involved are interleukin (IL)-1, IL-2, IL-6 and tumour necrosis factor-alpha (TNF-α). More recently IL-8 and IL-10 have also been shown to play distinct roles in the response. IL-1 and TNF-α released from activated macrophages in response to cellular injury initiate the acute phase response. Il-

1 and TNF-α then stimulate the release of other pro-inflammatory cytokines such as IL-6 (Winterborn et al. 2003).

The action of pro-inflammatory cytokines is modulated by anti-inflammatory cytokines such as IL-4, IL-10 and IL-13. These inhibit the production of the pro-inflammatory mediators by leukocytes. There are also pro-inflammatory cytokine antagonists that help to dampen the response by competing for cytokine receptor sites. An example of this is IL-1 receptor antagonist (IL-1ra). It is a soluble receptor antagonist which competitively inhibits the action of IL-1 (Paterson et al. 2000).

Characteristic features of the acute phase response:

Malaise
Headache
Musculoskeletal pain
} Encourage energy conservation
Fever
Activation of the immune system
Release of ACTH and glucocorticoids (Activation of the Hypothalamic-Pituitary-Adrenal Axis)
Activation of the clotting cascades (promoting the formation of blood clots)
Increased vascular permeability
Production of acute phase proteins by the liver

(Adapted from Winterborn et al. 2003)

The role of complement in the acute phase inflammatory response:

Complement plays a key role in initiating the acute inflammatory response. The alternative pathway appears to be predominantly involved following traumatic injury. The anaphylotoxins C3a, C5a and C5-C9 membrane attack complex are generated and activate mast cells, which degranulate releasing a host of other cytochemical mediators. These mast cell cytokines, together with complement, serve to trigger the activation of other immune cells involved in the response, such as neutrophils and macrophages. Platelets are also stimulated and together with mast cells and basophils produce histamine and serotonin, which induce vasodilation and increased vascular permeability. Nitric oxide is a powerful vasodilator being released from a number of cells including macrophages, neutrophils and endothelial cells. The action of nitric oxide and other vasodilators is opposed by endothelins, which are potent vasoconstrictors. The

relaxation of the arteriolar walls induced by this raft of cytokines allows increased blood flow to injured tissue. Complement components also act as powerful chemotaxins such as C3a and C4a, drawing phagocytic cells up the concentration gradient (Winterborn et al. 2003).

Vascular endothelial cells and neutrophil interaction in the acute phase response:

In order for the effector cells of the immune system to respond to tissue injury they must migrate from the blood into surrounding tissue. In order for this to occur the vascular endothelium must adapt to facilitate the extravasation of cells and proteins by increasing vascular permeability. A host of cytokines including IL-1, IL-6 and TNF-α are responsible for initiating conformational structural changes in the endothelial cells. Endothelial cells contract (marginate), allowing the inflammatory exudate, primarily comprised of neutrophils and plasma proteins, to escape from the bloodstream into the surrounding tissue.

Increased capillary permeability can also be induced by a host of noxious stimuli that cause damage to endothelial cells:

- Bacteria
- Endotoxins
- Hydrogen peroxide
- Reactive oxygen intermediates (ROI's)

Neutrophils adhere to the vascular endothelium after stimulation from chemical mediators including C5a, TNF-α, and leukotriene (LTB4) (Mattox et al. 2003). They do this through the interaction of cellular adhesion molecules expressed on both endothelial cells and neutrophils. The expression of these molecules is increased during acute inflammation.

Adhesion molecules include: P-selectin, E-selectin, L-selectin, Intracellular adhesion molecule-1 (ICAM-1), Vascular cell adhesion molecule-1 (VCAM-1), CD11 and CD18 (Mattox et al. 2003)

The increased expression of these adhesion molecules facilitates increased neutrophil binding to the vascular endothelium

Neutrophils then migrate through the vascular endothelium into the interstitial space. Neutrophil oxidative burst activity is also stimulated in response to injury. The ROI's released when neutrophils are bound to the vascular endothelium can damage endothelial cells leading to excessive vascular permeability and oedema formation that causes tissue damage. This process can result in end organ damage and subsequent MOD/MOF.

1.4 Innate Immune System Activation Pathways: PAMPs / DAMPs

Neutrophils are polymorphonuclear granulocytes and are phagocytic cells of the innate immune system. Cells of the innate immune system are programmed to respond to 'danger signals' in order to initiate an appropriate immune response. These molecular danger signals that signify potentially life-threatening events through our evolution have resulted in development of patter recognition receptors (PRRs) being displayed on the surface of cells in the innate immune system. Cells types that possess PRRs include neutrophils, and monocytes/macrophages. They have been developed to respond to highly conserved molecular motifs displayed by pathogenic products such as cell bacterial cell wall constituents like lipopolysaccharide (LPS), bacterial DNA (bDNA), toxins and viral RNA. These molecules are termed collectively as pathogen associated molecular patterns or PAMPs. A well characterised family of PRRs are the toll-like receptors TLRs, which have various subclasses, each corresponding to a specific set of PAMPs (Harris et al. 2006). PRR activation leads to intracellular signal transduction which activates the immune cell. This leads to upregulated pro-inflammatory gene expression leading to increased cytokine production. In circulating phagocytic cells such as neutrophils and monocytes, cytoskeletal changes enabling phagocytosis can be triggered, together with increased oxidative burst activity and adhesion molecule expression.

More recently focus has shifted to how endogenous cellular products may also provoke inflammation. Endogenous factors, known as "alarmins", released from damaged host cells also activate the innate immune system. Alarmins have been defined as a diverse group of intracellular proteins released through necrosis, but not apoptosis (Oppenheim et al. 2005, Harris et al. 2006). They have the ability to induce innate immunity through inflammatory pathways (Oppenheim et al. 2005, Harris et al. 2006) at physiological concentrations (Kono et al. 2008). Alarmins activate the innate immune system through the action of endogenous "damage" associated molecular patterns (DAMPs) binding to PRRs. There is believed to be

molecular commonality between DAMPs and PAMPs, which means through a conserved evolutionary molecular arrangement they can cross react at PRRs (Seong et al. 2004). Mitochondrial DNA has been identified as a potent "alarmin" (Harris et al. 2006). Mitochondrial DNA has also been shown to be released in large amounts following injury (Zhang et al. 2010a), and exerts a potent pro-inflammatory effect on neutrophils through a TLR9 mediated pathway (Zhang et al. 2010b). MtDNA and its role as a DAMP in the post-injury inflammatory response will be discussed in depth later.

1.5 Systemic Inflammatory Response Syndrome (SIRS):

When massive tissue injury is sustained following major injury, activation of the immune system can be overwhelming and systemic. When certain physiological parameters are met, a clinical diagnosis of SIRS can be made.

At least 2 of the following must be present:

- Heart Rate >90/min
- Respiratory Rate >20/min or PCO2 less than 32mmHg
- Temperature >38C or <36C
- White Cell Count >12,000/ml or <4,000/ml or >10% immature forms

(Bone et al. 1992, Nathans et al. 1996)

Sepsis can be defined as SIRS with confirmed or high suspicion of bacterial infection (Bone et al. 1992). The key implication when considering SIRS and sepsis is that SIRS is a sterile inflammatory process that is related to the degree of tissue injury sustained. Clinically in early stages, the physiological response is initially the same for both sepsis and SIRS. The mechanisms through which sterile tissue injury initiates and self-propagates inflammation are still poorly understood. The study of alarmins is exposing possible pathways through which endogenous factors released from direct cellular damage and lysis may drive post-injury SIRS (Oppenheimer et al. 2005, Harris et al. 2006, Kono et al. 2008).

1.6 Traumatic Haemorrhagic Shock:

In the post-injured state, haemorrhagic shock often causes systemic hypoperfusion and ischaemia in combination with massive tissue injury. This causes systemic tissue hypoxia and oxidative stress as the tissues cannot use oxygen effectively. This relative period of ischaemia is often reversed by resuscitation. However, resuscitation fluids further complicate matters by having their own immunostimulatory effects.

The combination of extensive tissue damage and shock (followed by resuscitation) leads to defence systems being activated such as inflammatory cascades, complement system, innate and adaptive immune responses and the coagulation cascade (Dewar et al. 2009).

Endothelial cell adhesion molecule expression increases in shock. This allows neutrophil adhesion with oxidative burst damage to the endothelial cell layer and underlying parenchymal tissue. This results in increased capillary permeability and oedema formation, a so-called ischaemia-reperfusion (IR) injury.

In the lungs this can have catastrophic implications where oedema formation and neutrophil action can cause acute respiratory distress syndrome (ARDS) (Hickling et al. 1998). The inflammatory response causes damage to pulmonary tissue resulting in impaired oxygen delivery, respiratory failure and in the most severe cases, death.

1.7 Multiple Organ Failure:

Multiple organ failure (MOF) is a complex post-injury inflammatory complication leading to the failure of multiple organ systems. MOF can be defined as failure of 2 or more organ systems greater than 48hrs after serious injury (Dewar et al. 2009). It is important to distinguish it from early multiple organ dysfunction syndrome (MODS) which can occur in the first 48hrs post-injury, and which is usually correctable through complete resuscitation (Ciesla et al. 2004). MOF presentation is bimodal and can be subdivided into early and late MOF which are both separate in their aetiology. Early MOF can be attributed to the manifestation of uncontrolled, aggressive SIRS. Simultaneously with the manifestation of SIRS subsequent activation of anti-inflammatory pathways in an attempt to resolve the dysregulated inflammation has been termed compensatory anti-inflammatory response syndrome (CARS) (Dewar et al. 2009). CARS has

been attributed to the late development of septic complications which can result in late MOF occurring after day 3 post-injury.

Early MOF

Already present at day 3 post-injury as a result of uncontrolled SIRS.

Late MOF

New onset multiple organ failure presenting after day 3 post-injury often as a result of late sepsis due to CARS.

Risk Factors: Post-Injury MOF

Patient Factors:	**Injury Factors:**	**Treatment Factors: (Potential "second hits")**
Age >55yrs	Injury severity	Severity and duration of haemorrhage
Obesity	Blunt trauma > Penetrating	Units of packed red cells transfused
Gender (male > female)	Wound contamination	Volume of crystalloid infusion
Medical Co-morbidities	Lengthened pre-hospital time	Long bone fixation (damage control Vs. definitive early fixation)
Genetic predisposition	Organ ischaemia/reperfusion	Fat embolism
		Abdominal compartment Syndrome
		Sepsis
MOF ↑		

(Table modified from Dewar et al. 2009)

Of the risk factors outlined in the table above the most modifiable risk factors are related to treatment. One of the most readily modifiable of these treatment factors is the timing and nature of non-life saving surgery (Balogh et al. 2012).

There have been numerous MOF scoring systems developed however the Denver Score has been extensively evaluated and has been shown to both sensitive and specific for predicting outcome measures associated with MOF (Sauia et al. 2009).

Denver MOF Score:

Dysfunction	Grade 0	Grade 1	Grade 2	Grade 3
Pulmonary PaO2/FiO2 ratio	> 208	208 - 165	165 - 83	< 83
Renal Creatinine (umol/L)	<159	160 - 210	211 - 420	> 420
Hepatic Total Bilirubin (umol/L)	< 34	34 – 68	69 - 137	> 137
Cardiac Inotropes	No inotropes	Only one inotrope at a small dose	Any inotrope at moderate dose or >1 agent, all at small doses	Any inotrope at large dose or > 2 agents at moderate doses

(Modified from Ciesla et al. 2005)

A period of haemorrhagic shock leading to IR injury has been implicated heavily in the pathophysiology of MOF (Dewar et al. 2009). The "two event" hypothesis has gained credence following major trauma where injury acts as the first hit, priming the immune system, and a subsequent untimely second hit (such as long bone fixation, blood transfusion and abdominal compartment syndrome) leads to uncontrolled SIRS and subsequent end organ damage and failure (Moore et al. 2005).

Haemorrhagic shock leads to systemic hypoperfusion which is then corrected through subsequent reperfusion of ischaemic tissues. As the ischaemic gut is reperfused, pro-inflammatory mediators such as cytokines, lipids and protein are released into the splanchic circulation (Dewar et al. 2009, Moore et al. 2005). These pro-inflammatory mediators then

serve to prime neutrophils which then mobilise into the circulation causing a transient neutrophilia at 3hrs post injury (Moore et al. 2005). If an untimely "second hit" occurs in this period, primed neutrophils can become activated and then sequestered in end organ tissues (Botha et al. 1995). Parenchymal tissue damage and subsequent multiple organ failure occurs through the release of ROI's and lysozymes from activated neutrophils. In multiple organ failure the initial neutrophilia is followed by neutropaenia between 6 and 12 hrs post injury supporting the concept of end organ sequestration (Botha et al. 1995). Non-multiple organ failure patients have no such early neutropaenia and the initial neutrophilia resolves over the following 36hrs without end organ damage (Moore et al. 2005).

Endothelial adhesion molecules such as CD18 and L-selectin are elevated immediately after traumatic injury (Botha et al. 1995, Ciesla et al. 2005). This increased expression of adhesion molecules can persist for up to 120hrs post-injury in patients with MOF (Ciesla et al. 2005). The combination of increased adhesion molecule expression with high numbers of circulating neutrophils can facilitate the migration of large numbers of neutrophils from the circulation into end organ parenchyma long after the initial injury.

There is an extensive body of evidence supporting the idea that there is a prolonged period of relative immune vulnerability following injury where a "second hit" could propagate MOF (Botha et al. 1995, Moore et al. 2005, Ciesla et al. 2005). Therefore, the timing and nature of urgent non-life saving surgical intervention requires critical consideration. Surgical interventions that are of most concern with respect to optimal timing are long bone fixation, pelvic fixation and spinal stabilisation (Balogh et al. 2012).

The relationship between the timing of surgery and post-injury complications became apparent in a study performed by Bone and colleagues (Bone et al. 1989). The study included 178 femoral shaft fractures in multiply injured patients randomised to early fixation (within 24hrs post-injury) versus delayed fixation (after 24hrs). The investigators found that the early group suffered a lower incidence of pulmonary complications including ARDS, fat embolism and pneumonia compared to the delayed fixation group. It also suggested that those in the early fixation group had shorter intensive care unit (ICU) and overall hospital stay. This was the first reasonably powered randomised controlled trial of early fixation versus late fixation of femoral fractures. It further substantiated findings in earlier retrospective studies, that early definitive fixation was associated with fewer complications in multiply injured patient (Goris et al. 1982, Johnson et al. 1985)

In the 1990s severely injured patients were often resuscitated with large volumes of crystalloid and colloid in line with Advanced Trauma Life Support (ATLS®) teaching at that time. Early definitive fracture fixation in those multiply injured patients who were physiologically compromised and resuscitated in this fashion, combined with further surgical trauma was associated with higher incidence of inflammatory complications such as ARDS and multiple organ failure (Regel et al. 1995). This led to the adoption of "damage control surgery" in the multiply injured patient. In orthopaedic surgery this involved early external fixation with definitive fracture fixation being delayed until the patient was physiologically stable. A study performed by Pape and colleagues aimed to guide surgical decision making by categorising patients as physiologically stable, borderline, unstable or in extremis (Pape et al. 2009). The study recommended early definitive fixation of fractures in those patients who were stable, with damage control for those who were unstable or in extremis. A further randomised control trial involving those borderline patients by the same research group concluded that they suffered a higher incidence of transient inflammatory lung injury, but no increase in incidence of serious adverse inflammatory complications such as ARDS and MOF (Pape et al. 2007). More recently there have been two meta-analyses comparing early definitive fixation of femoral fractures versus damage control surgery in the multiply injured patient (Tuttle et al. 2009, Nahm et al. 2012). Both concluded that there was no significant difference in the incidence of seriously adverse inflammatory complications such as ARDS or MOF between the early definitive fixation group and the damage control group. There was also no difference in ICU length. However, interestingly, Tuttle and colleagues noted that the operative time was reduced in the damage control group to a mean of 22 minutes versus 125 minutes in the definitive surgery group. Blood loss was also significantly less in the damage control group (Tuttle et al. 2009).

The evidence suggests fracture stabilisation is paramount (whether damage control or definitive fixation) to reduce pain, bleeding and other complications such as fat embolism. Whilst definitive fixation may be safe for the majority of patients with stable and even borderline physiology, those with unstable physiology should be managed conservatively in the first instant with damage control measures. This reduces the length of surgery associated physiological stress through increased blood loss and length of time ventilated at a time of significant immune vulnerability. Avoiding such a potentially significant "second hit" in this unstable patient group may reduce the risk of them going on to develop fulminate MOF. The optimal timing of definitive surgery in this unstable group will remain paramount to reduce

associated morbidity through long periods of immobility such as joint stiffness, muscle wasting and deep vein thromboses.

1.8 Mitochondria in the Eukaryotic Cell:

Evolution

Mitochondria derive their evolutionary origins from bacteria (Sagan 1967), namely α-proteobacteria (Gray et al. 1999, Andersson et al. 2003). They are believed to have been incorporated into the eukaryotic cell as energy producing endosymbionts (Andersson et al. 2003). The exact event that led to the incorporation of the mitochondrial precursor into the primordial proto-eukaryote (likely Archaebacteria) is still a contentious area of debate amongst evolutionary biologists.

Biology

Mitochondria are double-membrane bound intracellular organelles that produce energy in the form of adenosine tri-phosphate (ATP) through oxidative phosphorylation (Ballard et al. 2004). They can move, fuse and divide within a cell independently of the cell cycle (Bereiter-Hann et al. 2004). They possess their own genome which varies in size in mammalian species (between 15,000 and 17,000 base pairs). In humans it is 16,569 base pairs versus some 3 billion base pairs in the nuclear genome. Despite their seemingly independent existence within the cell transcription and replication are co-dependent on nuclear encoded factors which are transported into mitochondria (Taanman 1999). Mitochondria possess several copies of their genome (between 2 and 10) which resembles bDNA containing unmethylated CpG repeats and formyl-peptides (FP's) (Hochauser 2000). Thirty-seven genes are encoded on in the mitochondrial genome 24 of these represent translational genes (22 tRNA's and 2 rRNA's) that enable translation of mitochondrial genome-derived transcripts (Ballard et al. 2004). A further 13 genes encode protein subunits essential to the electron transport chain and oxidative phosphorylation, and therefore mitochondrial ATP production (Ballard et al. 2004).

Through the oxidation of one molecule of glucose through oxidative phosphorylation, 30 ATP molecules are produced in comparison to 2 ATP produced by the nuclear controlled glycolytic

cycle. As a result, mitochondria (under control of mtDNA) are responsible for the production of 90% of our energy requirements (Ballard et al. 2004).

Mitochondria also produce toxic ROIs as a by-product of oxidative phosphorylation that damage cellular DNA, proteins and lipids. This increases with age and led to one of the many developments of the conceptual role of mitochondria in the ageing process (Harman et al. 1992). Mitochondrial ROI production increases in disease states, which induces "oxidative stress" (Turren 2003). Mitochondrial ROI production increases in moderately hypoxic conditions and also in supra-normal oxygenation states (Turren 2003). This could have implications in trauma as the two states can exist simultaneously with supra-normal oxygen delivery in the lungs (certainly in the intubated-ventilated patient), but with systemically compromised oxygen delivery to tissues while in a state of haemorrhagic shock-induced hypoperfusion.

1.9 Release of Mitochondrial DAMPs: Apoptosis, Necrosis, Necroptosis or NETosis?

The mode of cell injury and subsequent death is key to the potential release of intracellular contents including mtDNA and mitochondrial DAMPs (mtDAMPs) into the extra-cellular environment. There have been significant advances in the last decade in the understanding of different modalities of cell death (Vanden Berghe et al. 2010) with subtle and significant elaboration on the rather crude definitions of "apoptosis" and "necrosis" that previously existed.

Apoptosis:

Apoptosis is "programmed cell death" and can be triggered through two pathways: extrinsic and intrinsic. The extrinsic pathway is triggered when cell surface receptors bind to pro-apoptotic ligands such as TNFα which trigger intracellular signalling cascades leading to the initiation of apoptosis (Nagata 1997). The intrinsic pathway is controlled through the release of pro-apoptotic factors from mitochondria such as cytochrome c (cyt c) and apoptosis inducing factor (AIF) (Fridman et al. 2003). These pathways are often activated simultaneously to ensure the co-ordinated and timely death of the cell. Ultimately both extrinsic and intrinsic pathways lead to the activation of pro-apoptotic genes such as p53 (Fridman et al. 2003) triggering a cascade of events, which result in the controlled breakdown of the intracellular contents (autophagy) without loss of cell membrane continuity. Morphologically apoptosis is

characterised through nuclear shrinkage and condensation, loss of cell volume, membrane blebbing, formation of apoptotic bodies, and caspase activation (Degterev et al. 2008). Caspases are intracellular proteases responsible for the execution of apoptosis (Hengartner 2000). The fact that cell membrane continuity is not lost means that the intracellular contents do not leak into the intra-vascular or extracellular space. The apoptotic cell can then be recognised by the immune system for phagocytosis. There is no evidence to suggest that mtDNA or mitochondrial DAMPs are released from an apoptotic cell that is appropriately phagocytosed.

Secondary Necrosis:

Once a cell has died through an apoptotic pathway if phagocytosis fails then "secondary necrosis" can occur. This culminates in lysis and the release of the intracellular contents (Silva et al. 2008). Morphologically this process is characterised by swelling of the cell and apoptotic bodies due to activation of self-hydrolyzing enzymes leading to eventual cell lysis (Silva et al. 2008).

Phagocytosis can fail for a multitude of reasons:

1. Failure to express appropriate cell surface markers to trigger phagocytosis
2. Defective phagocytic mechanisms in the scavenger cell population (e.g neutrophils)
3. Overwhelming the immune systems scavenging capacity through the sheer numbers of cells undergoing apoptosis

Necrosis and "Necroptosis":

In contrast, if a cell is directly injured through a physical insult or physiologically stressed so severely it may become necrotic. Necrosis is characterised morphologically by cell rounding, swelling (oncosis), dilation of organelles and the absence of nuclear chromatin condensation (Vanden Berghe et al. 2010). This process culminates in cell lysis. Previously this process has been regarded as uncontrolled, without any specific regulatory mechanisms. There is an increasing body of evidence, however, which suggests there are complex signal transduction mechanisms that control necrosis (Vanden Berghe et al. 2010). Necrosis can be triggered by exogenous molecules such as TNFα and Fas ligand binding to cell surface receptors (Vanlangenakker et al. 2011). The activation of such receptors can lead to a tightly regulated

and controlled form of necrosis being initiated. This process is mediated through caspase-8 (anti-cell death enzyme) and receptor interacting protein kinases (RIPK family), and has led to the term "necroptosis" (Kaczmarek et al. 2013). The role of DAMPs in triggering necroptosis has also been explored through their activation of PRRs which then trigger intracellular signalling cascades through RIPK1 and RIPK3 (Kaczmarek et al. 2013). RIPK1 and RIPK3 can play a role, but are not essential in necrosis following IR injury (Vanlangenakker et al. 2011). This is characterised by exposure to high levels of hydrogen peroxide (H_2O_2) and is dependent on the activity of a different enzyme, poly (ADP-ribose) polymerase (PARP) (Vanlangenakker et al. 2011). Free intracellular iron redox reactions with H_2O_2 appear to play a pivotal role in this modality of cell death by inducing lysosomal permeability (Vanden Berghe et al. 2010). Intracellular chelation of free iron was demonstrated to be cell-protective in such conditions (Vanden Berghe et al. 2010). Mitochondrial components also promote necrosis following IR injury. Cells that lack cyclophilin D (CypD), which is a component of the mitochondrial transition pore, are relatively resistant to necrosis triggered by H_2O_2 (Baines et al. 2005).

Neutrophil Extracellular Trap (NET) Formation – "NETosis"

Neutrophil homeostasis requires the generation and controlled death of an exceptionally large population of neutrophils. These numbers dramatically increase following pro-inflammatory insults such as major injury, surgery, and infection. Neutrophils were previously believed to have a lifespan of approximately 1 day in the circulation. A recent in vivo study that studied the lifespan of circulating neutrophils using $^2H_2O_2$ labelling noted that the average lifespan was actually 5.4 days (Pillay et al. 2010). This persistence in the circulation and delayed apoptosis in the extravascular space may increase the risk of suffering a secondary inflammatory injury, days after the initial priming injury, if a "second hit" occurs within this timeframe.

Apoptosis is the default mechanism of neutrophil cell death and is mediated via stimulation from a variety of extracellular signalling molecules including TNFα and Fas ligand (Maianski et al. 2004).

Neutrophil extracellular trap (NET) formation was first described by Brinkmann and colleagues in 2004 (Brinkmann et al. 2004). It is characterised by smooth extracellular filaments 17nm in diameter which are composed of stacked and probably modified

nucleosomes (Brinkmann et al. 2012). This filamentous chromatin backbone is then adorned with globular domains approximately 50nm diameter, containing neutrophilic granular proteins. The principal function of the NET is believed to be to entrap circulating pathogens and indeed bacteria and other micro-organisms have been demonstrated to be ensnared and killed by NETs (Brinkmann et al. 2012).

There are a number of factors that have been demonstrated to induce NET formation (von Köckritz-Blickwede et al. 2009).

Those that are of particular interest following major trauma include:

- IL-8 (von Köckritz-Blickwede et al. 2009)
- LPS (von Köckritz-Blickwede et al. 2009)
- GM-CSF + C5a (von Köckritz-Blickwede et al. 2009)
- Hypoxia Inducible Factor (HIF) (McInturff et al. 2012)
- Platelet TLR4 activation (Clark et al. 2007)
- H_2O_2 (Fuchs et al. 2007)

It is a unique form of cell death which is distinct from necrosis, apoptosis and necroptosis (Remijsen et al 2011). It is not regulated through the RIPK pathway as with necroptosis or controlled by caspases, inhibition of both of these pathways did not inhibit NET formation.

Regardless of the initiating stimulus, loss of membrane continuity and lysis leads to the extravasation of the intracellular contents (including DAMPs) into the extra-cellular environment.

1.10 Relationship Between Injury and the Release of Mitochondrial DAMPs:

Recently, evidence has emerged that increased titres of mtDNA are released following major injury. For example, increased levels were present in a rat model of traumatic haemorrhagic shock (laparotomy and subsequent haemorrhage) (Zhang et al. 2010). What is unclear is whether the release was as a result of direct tissue injury sustained through the laparotomy, or necrosis as a result of the induced shock state. It has been reported that tissue damage as a result

of surgical trauma (femoral fracture reaming and fixation) can liberate mtDNA into the circulation (Hauser et al. 2010).

There was a sub-group identified in a study performed in a polytrauma ICU patient population where cell free DNA (cf-DNA) was measured over 10 consecutive days following admission (minimum ICU admission of 3 days) (Margraf et al. 2008). In this patient sub-group numbering 8 patients (within the 37-patient study cohort), cf-DNA was noted to increase from day 3 post injury, peaking at day 7 (Margraf et al. 2008). The authors did not delineate the type of DNA measured (mtDNA vs. nuclear DNA) but measured total cf-DNA. These findings are also echoed by an earlier study that confirmed persistence of increased nuclear DNA (nDNA) titres in the plasma of major trauma patients up to 28 days following injury (Lam et al. 2004). An earlier study by the same group noted increased mtDNA identified in the plasma of major trauma patients <4hrs post-injury (Lam et al. 2003). This increased mtDNA titre was positively correlated with ISS (Lam et al. 2004). These studies (Lam et al. 2003, Lam et al. 2004 and Margraf et al. 2008) were limited by the fact that the surgical interventions and potential sepsis were not accounted for. Another potential limitation is in possible failure to accurately quantify plasma free DNA. The centrifugation methodology employed in processing the plasma (Lam et al. 2003, Lam et al. 2004 and Margraf et al. 2008) may have failed to eliminate the cellular fraction in the plasma samples by not including a second high-speed centrifugation step (12,000rpm ~ 10mins) prior to DNA extraction as outlined by Chiu and colleagues (Chiu et al. 2003).

Another group have recently shown that initial release of mtDNA following trauma correlated with creatinine kinase (CK) concentrations on day 1 indicating that direct tissue injury with subsequent muscle necrosis may be a significant contributory source (Yamanouchi et al. 2013). This study also positively correlated elevated mtDNA levels with injury severity. More robust methodology for mtDNA measurement in plasma (Chiu et al. 2003) was employed in this study.

1.11 Pro-Inflammatory Role of mtDNA in the Post-Injury Response:

MtDNA has been demonstrated to induce neutrophil activation through activation of TLR 4 (Hauser et al. 2010), TLR 9 (Zhang et al. 2010b) through p38 (Zhang et al. 2010b) and p44/42 (Hauser et al. 2010). MAP-Kinase pathways are of significant interest in the post-injury setting. Also, mtDAMPs have been shown to potentiate inflammatory lung injury when introduced into

healthy rats in the landmark paper by Zhang and colleagues (Zhang et al. 2010a). It remains to be elucidated whether titres of mtDNA liberated following traumatic injury are sufficient to independently potentiate remote end organ damage.

The pro-inflammatory action of mtDNA has resulted in the "Trojan Horse" analogy being used to describe the action of products released from these vital intracellular organelles (Manfredi et al. 2010).

1.12 The Role of DNase in Degradation of Extracellular DNA:

Deoxyribonuclease I is the free plasma enzyme responsible for the digestion of 90% of circulating extracellular DNA (Tamkovich et al. 2006). Other enzymes also contribute to DNA degradation in the circulation including de-oxyribonuclease II, phosphodiesterase I and DNA hydrolysing autoantibodies (Tamkovich et al. 2006). Given that this is a multi-enzymatic process, for the purposes of further discussion these enzymes will be collectively referred to as "DNase". The potential capacity for processing pro-inflammatory circulating extracellular mtDNA has never been studied in trauma.

1.13 Study Rationale:

The release of mtDNA from necrotic cells as a result of direct tissue injury, whether through surgical or traumatic injury, and subsequent elevated titres in the circulation is well documented (Hauser et al. 2010, Lam et al. 2003, Lam et al. 2004, Yamanouchi et al. 2013, Zhang et al. 2010a, Zhang et al. 2010b).

The possible role of mtDNA in inflammatory injury to remote organs is a very real and clinically relevant prospect when considering the pathogenesis of secondary remote organ injury and subsequent MODS/MOF following major trauma.

Tissue injury at the time of the trauma is an important but non-modifiable factor for post-injury inflammation associated complications. Surgical interventions on trauma patients cause secondary tissue injury and can act as a deleterious second hit negatively influencing the clinical outcome. More importantly, the timing and the magnitude of these procedures are

potentially modifiable predictors of multiple organ failure (MOF). The natural history of mtDNA plasma concentrations of trauma patients following injury and subsequent surgery is unknown.

1.14 Research Proposal:

Hypothesis:

1. Plasma mtDNA levels increase in a dose-dependent fashion with the degree of tissue damage sustained in surgery. Elevated mtDNA levels would then decrease rapidly over time in an uncomplicated surgical recovery period.

Primary Aims:

To further investigate the potential role of mtDNA in the post-injury response following major traumatic injury in humans and the impact of subsequent surgical intervention on these levels. (Chapter 2)

1. Assess the relationship between type of surgery, magnitude of tissue injury, level of inflammation, clinical outcome and patient plasma mtDNA levels. (Chapter 2)

2. To assess potential sources of mtDNA release following major injury and subsequent surgery. Measurement of markers of cell necrosis including CK (Yamanouchi et al. 2008), aspartate aminotransferase (AST) (Sursal et al. 2012) and lactate dehydrogenase (LDH) (Sursal et al. 2012).

Secondary Aims:

1. The modality of neutrophil cell death will be assessed as a possible contributor to free plasma DNA including study of potential NETosis following trauma and subsequent surgery. As this has yet to be definitively demonstrated following trauma due to the

limitations of previous studies measuring cf-DNA as a measure of NET formation in trauma (Margraf et al. 2008) (Chapter 3)

2. NETosis is triggered in response to bacterial sepsis (Brinkmann et al. 2004). Due to similarities between mtDNA and bDNA (Hochauser 2000) it is possible that mtDNA could trigger NETosis through interaction with PRR's. We will explore the possibility of mtDNA being a potential trigger for NET formation in the post-injury and peri-operative setting. (Chapter 4)

3. To study the mechanism for clearance of mtDNA from the circulation after injury and subsequent surgery. The plasma activity of DNase post-injury and subsequent surgery will be measured and correlated with circulating mtDNA levels. (Chapter 5)

1.15 Ethics

Ethical approval for the study was obtained from Hunter New England Human Research Ethics Committee. All blood samples were obtained with informed consent for sampling, archiving and further analysis in accordance with the aims of the scientific studies performed in this thesis. *(Approval Number 10/11/17/4.05)*

1.16 Thesis Overview:

The main body of this thesis is comprised of a comprehensive literature review (Chapter 1) and of 4 original basic science papers (Chapters 2-5). These are followed by discussion and conclusion (Chapter 6).

These primary original research papers focus on 4 main key areas:

1. The initial pilot study consisted of 35 trauma patients who subsequently underwent subsequent orthopaedic surgical intervention. Levels of mtDNA and nDNA over a 5-day perioperative period were correlated with other parameters including markers of tissue injury/cell necrosis. This was to better understand the possible source of mtDNA after injury and surgery. (Chapter 2)

2. Investigated the possibility of NETosis occurring after injury and subsequent surgery, and the potential impact on circulating mtDNA levels. (Chapter 3)

3. Evaluation of whether mtDNA could trigger NET formation in healthy neutrophils and also in trauma neutrophils post-injury and following subsequent surgery. (Chapter 4)

4. Assessed the activity of circulating DNase against levels of cell-free DNA both mtDNA and nDNA. Correlation between circulating DNase activity, mtDNA levels and deleterious inflammatory sequelae such as SIRS and MOF would then be made. (Chapter 5)

Chapter 2:

Mitochondrial DNA after Trauma and Subsequent Surgery (Pilot Study)

McIlroy DJ, Bigland M, White AE, Hardy BM, Lott N, Smith DW, Balogh ZJ**. Cell necrosis–independent sustained mitochondrial and nuclear DNA release following trauma surgery.** *J Trauma and Acute Care Surgery*, 2015;78(2):282

Introduction

The inception for this study came from the seminal work performed by Zhang and colleagues who were the first group to demonstrate massively elevated mtDNA following traumatic injury in 2010 (Zhang et al. 2010).

This pilot study of 35 trauma patients allowed me to assimilate many of the basic laboratory skills that would carry me through the other studies that followed. I started work on samples in mid-November 2012. In order to accurately quantify mtDNA in plasma I had to learn to robustly produce cell free plasma and perform quantitative polymerase chain reaction (qPCR) targeting mtDNA, nDNA and screening samples for bDNA to exclude bacteraemia. I designed the mtDNA, nDNA and bDNA primers used with expert supervision from Dr Doug Smith, who helped me gain insight into the science behind our proposed study.

The measurement of markers of cell necrosis CK, LDH and AST were all measured externally through Hunter Area Pathology Service (HAPS).

Other parameters such as the amount of crystalloid used in the initial resuscitation, time from injury to surgery and incidence of SIRS/MOF were all obtained from the trauma database.

The laboratory work was completed by late April/May 2013. I then collated the data and analysed the findings. I presented the preliminary data at the Australian Society for Surgical Research meeting in Adelaide, November 2013. The study was then accepted for a podium presentation at the annual Western Trauma Association Meeting in Steamboat Springs, Colorado, USA in March 2014. This meant that an embargo was placed on publication until after this and due to delays with revisions and publishers, only went to press in 2015.

The lack of correlation that we observed between mtDNA and markers of tissue necrosis really triggered my drive to really pursue NETs as a possible source of mtDNA in the post injury and perioperative setting. This was after reading the work of Yousefi and colleagues who were the first group to suggest that NETs could be formed from almost pure mtDNA (Yousefi et al. 2009).

OPEN

Cell necrosis–independent sustained mitochondrial and nuclear DNA release following trauma surgery

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BACKGROUND: Mitochondrial DNA (mtDNA), a potent proinflammatory damage-associated molecular pattern, is released in large titers following trauma. The effect of trauma surgery on mtDNA concentration is unknown. We hypothesized that mtDNA and nuclear DNA (nDNA) levels would increase proportionately with the magnitude of surgery and both would then decrease rapidly.

METHODS: In this prospective pilot, plasma was sampled from 35 trauma patients requiring orthopedic surgical intervention at six perioperative time points. Healthy control subjects (n = 20) were sampled. DNA was extracted, and the mtDNA and nDNA were assessed using quantitative polymerase chain reaction. Markers of cell necrosis were also assayed (creatine kinase, lactate dehydrogenase, and aspartate aminotransferase).

RESULTS: The free plasma mtDNA and nDNA levels (ng/mL) were increased in trauma patients compared with healthy controls at all time points (mtDNA: preoperative period, 108 [46–284]; postoperative period, 96 [29–200]; 7 hours postoperatively, 88 [43–178]; 24 hours, 79 [36–172]; 3 days, 136 [65–263]; 5 days, 166 [101–434] [healthy controls, 11 (5–19)]) (nDNA: preoperative period, 52 [25–130]; postoperative period, 100 [35–208]; 7 hours postoperatively, 75 [36–139]; 24 hours postoperatively, 85 [47–133]; 3 days, 79 [48–117]; 5 days, 99 [41–154] [healthy controls, 29 (16–54)]). Elevated DNA levels did not correlate with markers of cellular necrosis. mtDNA was significantly elevated compared with nDNA at preoperative period ($p = 0.003$), 3 days ($p = 0.003$), and 5 days ($p = 0.0014$). Preoperative mtDNA levels were greater with shorter time from injury to surgery ($p = 0.0085$). Postoperative mtDNA level negatively correlated with intraoperative crystalloid infusion ($p = 0.0017$). Major pelvic surgery (vs. minor) was associated with greater mtDNA release 5 days postoperatively ($p < 0.05$).

CONCLUSION: This pilot of heterogeneous orthopedic trauma patients showed that the release of mtDNA and nDNA is sustained for 5 days following orthopedic trauma surgery. Postoperative, circulating DNA is not associated with markers of tissue necrosis but is associated with surgical invasiveness and is inversely related to intraoperative fluid administration. Sustained elevation of mtDNA levels could be of inflammatory origin and may contribute to postinjury dysfunctional inflammation. (*J Trauma Acute Care Surg*. 2015;78: 282–288.

LEVEL OF EVIDENCE: Prospective study, level III.

KEY WORDS: DAMPs; SIRS; mtDNA; trauma.

The release of mitochondrial DNA (mtDNA) following trauma has been documented in both humans and animal models.[1,2] High titers of mtDNA have also been found in reaming material collected during the intramedullary nailing of fractured femora with subsequent rises in patient plasma levels.[3] mtDNA[4] released during cell necrosis stimulates the innate immune system and is associated with postinjury sterile systemic inflammatory response syndrome (SIRS)[1,2] and acute lung injury.[1]

The origin of the eukaryotic cell is a debated topic, but one of the widely accepted theories is that during evolution, archaebacteria internalized α-proteobacteria,[5,6] which became the energy producing endosymbiont (mitochondrion) of the eukaryotic cell.[6] In contrast to nuclear DNA (nDNA), bacterial DNA (bDNA) is circular and contains unmethylated CpG repeats.[7] Mitochondria possess several copies of their genome (between 2 and 10 per mitochondrion), which resembles bDNA containing unmethylated CpG repeats.[8]

These mtDNA and other cellular derivatives have been termed *damage-associated molecular patterns* (DAMPs) or *alarmins*.[4] Notably, alarmins can induce an inflammatory response at physiologic concentration.[4] Human neutrophils have been shown to be activated by exposure to mtDNA through activation of TLR4,[3] TLR9,[2] and FPR1.[3] Acute inflammatory lung injury was induced by injecting mitochondrial DAMPs in a healthy rat model.[3]

The tissue injury at the time of trauma is an important but nonmodifiable factor for postinjury inflammation-associated complications. Surgical interventions on trauma patients cause secondary tissue injury, releasing DAMPs, and can potentially act as a deleterious second hit to negatively influence the clinical

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This study was presented at the 44th Annual Meeting of the Western Trauma Association, March 2–7, 2014, in Steamboat Springs, Colorado.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.jtrauma.com).

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outcome.[9] More importantly, the timing and the magnitude of these procedures are potentially modifiable predictors of multiple-organ failure (MOF). The perioperative profile of mtDNA plasma concentrations of trauma patients is unknown. It has been suggested to that the initial high titers rapidly decrease within hours after injury.[10] mtDNA plasma concentrations are recently reported in isolation without addressing the simultaneous release of nDNA.[10] nDNA has been correlated with injury severity.[11]

We aimed to describe the natural history of mtDNA and nDNA release after surgery and the potential association between DNA release and resuscitation and the invasiveness of the surgical intervention.

We hypothesized that the mtDNA levels (and similarly nDNA levels) would increase in a dose-dependent fashion directly related to the degree of tissue damage sustained in surgery. Levels are then anticipated to rapidly decrease after the surgical intervention.

PATIENTS AND METHODS

Research Compliance

Ethical approval for the study was obtained from Hunter New England Human Research Ethics Committee (HNEHREC). All plasma samples were obtained with informed consent.

Patients and Blood Samples

Blood was sampled from 35 trauma patients with informed consent at six time points in the perioperative period (immediate preoperative and postoperative periods and then 7 hours, 24 hours, 3 days, and 5 days postoperatively). The rationale for the sampling time points was based on previous publications examining immune responses to orthopedic trauma interventions.[12,13] Consecutive trauma patients older than 17 years with skeletal injuries, requiring standardized orthopedic trauma surgical fixation including pelvic (symphyseal plating, iliosacral screw fixation, or open reduction and internal fixation of the sacrum or sacroiliac joint), acetabulum (open reduction and internal fixation from ilioinguinal and/or Kocher-Langenbeck approaches), femoral (intramedullary nailing of the femur), or tibia/fibula fractures (intramedullary nailing of the tibia), were included between January 2013 and June 2013. Patients operated on between Friday and Sunday have not been included because of the potential logistic problems with sample collection and procession during the weekends. There were no refusals or withdrawals during the 6-month recruitment period. Pelvic and acetabulum surgery cases were further categorized as major open surgery or percutaneous/minimally invasive surgery. Patients were excluded if they had underlying autoimmune or any chronic inflammatory condition. SIRS was defined based on consensus; for the definition of MOF, the Denver score was used.[14,15] Healthy control subjects who were age and sex matched with the study cohort (n = 20) were recruited through the Hunter Medical Research Institute registry. The registry tends to have more senior and more female volunteers than the usual trauma populations. The primary goal was better matching for age, and the secondary goal was to match sex. Plasma was separated from 5 mL of whole blood and frozen at −80°C before analysis.

mtDNA and nDNA Extraction From Plasma

Plasma samples were thawed on ice, pulse vortexed for 15 seconds, and then centrifuged at 12,000 G for 10 minutes to pellet cell debris. Cell free plasma sample (200 μL) was aspirated and used for DNA extraction[16] using a blood DNAeasy extraction kit (Qiagen Chadstone, Victoria, Australia) according to the manufacturer's instructions and using a 200-μL elution volume.

Real-Time quantitative polymerase chain reaction (qPCR) Protocols

DNA eluates were diluted 10-fold with nuclease free water, and 5 μL of the diluent was then used for each qPCR reaction. The 5 μL of diluted DNA was added to 7 μL of SYBR green master mix (SensiFast, Biolines, Alexandria, New South Wales, Australia). The real-time qPCR analysis was performed using Applied Biosystems Real-Time 7500 analyser (Applied Biosystems, Life Technologies, Foster City, CA). mtDNA primers were designed and synthesized for COX3 and ND3; and the nDNA primer, for GAPDH (Geneworks Hindmarsh, South Australia, Australia) (Supplementary Digital Content 1, http://links.lww.com/TA/A504). Standard curves were constructed using highly purified mtDNA/nDNA to enable calculation mtDNA and nDNA sample concentrations in nanogram per milliliter. All samples were screened for bDNA, the presence of which could indicate sepsis.

Data Analysis

Data are presented as mean (SD) for parametric variables and as median (interquartile range [IQR]) for nonparametric variables. Data were visually examined for skew. Hypothesis testing of changes in DNA concentrations between the time periods were performed using the Friedman test, a nonparametric equivalent of repeated-measures analysis of variance (ANOVA). Differences between mtDNA and nDNA were compared at each time point using the Wilcoxon signed-rank test. DNA levels in the trauma cohort at different time points and healthy controls were compared using the Kruskal-Wallis test. Multiple comparisons were performed by using the Holm-Sidak approach.[17] Differences between operative interventions were tested using two-way repeated-measures ANOVA. Correlation between continuous variables was calculated using Spearman's correlation coefficient.

RESULTS

Study Population Demographics

Blood samples were obtained from 35 trauma patients (25 males, 10 females), with a median age of 38 years (IQR, 29–48) and a median Injury Severity Score (ISS) of 14 (IQR, 9–22). The 20 healthy controls' (12 males, 8 females, $p = 0.57$ compared with trauma patients) median age was 38 years (IQR, 28–50) ($p = 0.87$ compared with trauma patients). All patients had experienced high-energy blunt trauma resulting in fractures that required surgical stabilization. Seventeen patients experienced polytrauma, and 18 experienced monotrauma. Median initial base deficit was −1 mEq/L (IQR, −3 to 0.9). The following interventions were performed: major pelvic surgery (n = 10), minor pelvic surgery (n = 11), femoral nailing (n = 7), tibial nailing (n = 7), and combined femoral and tibial nailing (n = 2). No patients had clinical signs of sepsis or microbiologically proven bacteremia during the perioperative period. Median time to surgery was 48 hours (IQR, 18–96) from injury. Thirteen patients received a blood product transfusion

before surgery, and an additional nine received a transfusion in the postoperative period. Thirteen patients were admitted to the intensive care unit (median stay, 6 days; IQR, 3–11 days) (mean [SD] ventilator days, 3 [3]). Twelve patients developed clinical SIRS. Three patients developed MOF. All patients survived, and the median length of hospital stay was 18 days (IQR, 8–33).

Perioperative Changes in DNA Concentration

The median (IQR) plasma mtDNA concentration (ng/mL) (preoperative period, 108 [46–284]; immediate postoperative period, 96 [29–200]; 7 hours postoperatively, 88 [43–178]; 24 hours, 79 [36–172]; 3 days, 136 [65–263]; 5 days, 166 [101–434]) was elevated compared with that of the healthy controls (11 [5–19]) at all six perioperative time points (Kruskal-Wallis test, $p < 0.0001$) (Fig. 1*A*).

The median (IQR) plasma nDNA concentration (ng/mL) (preoperative period, 52 [25–130]; immediate postoperative period, 100 [35–208]; 7 hours, 75 [36–139]; 24 hours, 85 [47–133]; 3 days, 79 [48–117]; 5 days, 99 [41–154]) was elevated compared with that of the healthy controls at all postoperative time points (29 [16–54]). (Kruskal-Wallis test, $p = 0.0069$) (Fig. 1*B*).

Plasma concentration of mtDNA was also found to be significantly elevated compared with nDNA levels in the study population at preoperative period (Wilcoxon test, $p = 0.003$), 3 days postoperatively ($p = 0.003$), and 5 days postoperatively ($p = 0.0014$) (Fig. 2).

Those patient who had low preoperative serum mtDNA concentration had less pronounced postoperative mtDNA concentrations than those who had high concentrations ($p = 0.0138$, Wilcoxon matched-pairs rank-sum test). No correlation was found between the preoperative and postoperative nDNA concentrations.

There was no detectable bDNA in any patient included in the study cohort at any time point or in the healthy controls (data not presented).

Magnitude and Timing of Surgery

Major pelvic surgery was associated significantly higher mtDNA levels when compared with percutaneous, minimally invasive pelvic surgery, at day 5 postoperatively (two-way ANOVA and then Holm-Sidak test, $p < 0.05$) (Fig. 3).

There was a statistically significant correlation between the preoperative plasma mtDNA concentration and the time elapsed from injury. No significant correlation was found between time to surgery and preoperative nDNA levels (Fig. 4*A* and *B*).

Tissue Necrosis, Fluid Resuscitation, and SIRS/MOF Association With mtDNA

No correlation was found between mtDNA levels and markers of cell necrosis, namely, creatine kinase (CK), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST) (Fig. 5*A–C*). There was no correlation between CK and nDNA serum concentrations, but there was a significant correlation between AST and nDNA (72 hours postoperatively) and also between LDH and nDNA concentration (preoperative period, 24 hours postoperatively, and 72 hours postoperatively) (data not presented here). Immediate postoperative mtDNA levels were

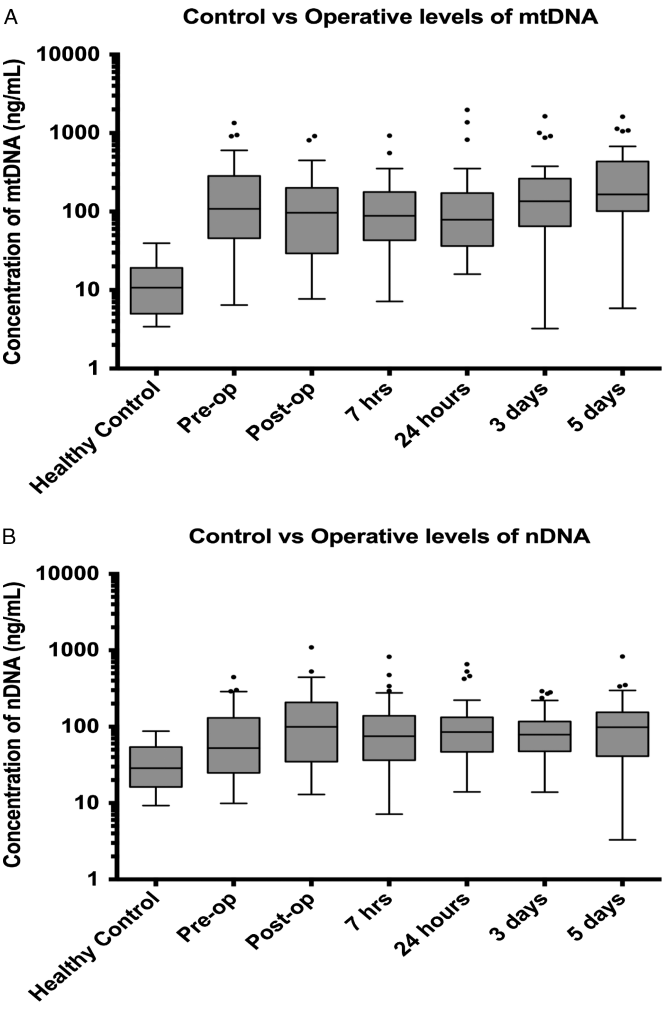

Figure 1. *A*, Free plasma mtDNA concentration of the trauma cohort was significantly higher than that of the healthy control subjects (Kruskal-Wallis test, $p = <0.0001$; Dunn post hoc test, $p = <0.05$) at all perioperative time points. There was no significant change in mtDNA concentration measured between any time points in trauma patients' plasma. Spearman's test was applied for nonparametric data comparing changes in mtDNA (omnibus $p = 0.054$). *B*, Free plasma nDNA concentration of the trauma cohort was significantly higher than that of healthy control subjects (Kruskal-Wallis test, $p = 0.0069$; Dunn post hoc test, $p = <0.05$) at all postoperative time points (preoperative comparison with healthy controls: Kruskal-Wallis test was nonsignificant). There was no statistically significant change in nDNA concentration measured between any time points in trauma patients' plasma. Spearman's test was applied for nonparametric data comparing changes in nDNA (omnibus $p = 0.075$).

negatively correlated with intraoperative crystalloid fluid administration ($p = 0.0017$) (Fig. 6). No significant difference in plasma free mtDNA/nDNA was observed with SIRS (n = 12) versus no SIRS (n = 23) or MOF (n = 3) versus no MOF (n = 32).

DISCUSSION

We demonstrated that mtDNA release and subsequent elevated plasma titers are sustained for at least 5 days following

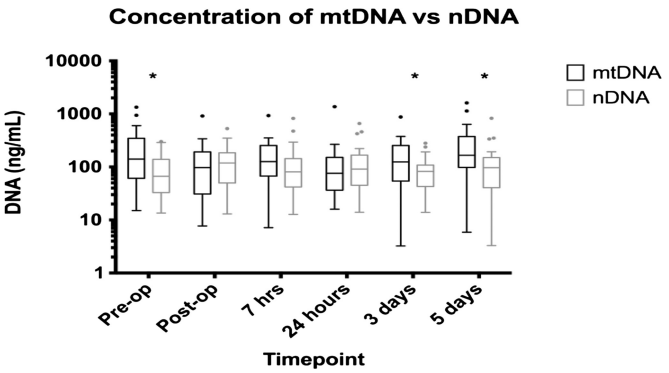

Figure 2. Statistically significant difference between free plasma mtDNA and nDNA levels at preoperative period ($p = 0.003$), 3 days postoperatively ($p = 0.003$), and 5 days postoperatively ($p = 0.0014$). Statistical comparison was made using the Wilcoxon matched-pair signed-rank test.

major orthopedic interventions on trauma patients. Trauma patients have elevated mtDNA plasma levels preoperatively days after the initial traumatic tissue injury. The detected mtDNA concentration in the plasma is independent of tissue necrosis markers but negatively correlated with the magnitude of intraoperative fluid administration. Major pelvic surgery was associated with a higher elevation in plasma mtDNA titers compared with minor pelvic surgery toward the end of the observation period. Our cohort was not powered to demonstrate association between mtDNA or nDNA titers and outcomes. These findings are novel because while it has been demonstrated that mtDNA is released by injury,[1] it has not previously been demonstrated in plasma days after surgery and the late increases in mtDNA levels rising significantly above nDNA levels were unknown so far.

The correlation between the preoperative mtDNA and time from injury to surgery indicates that mtDNA could be a valuable marker for optimal surgical timing. Independently from the elapsed time between injury and surgery, the preoperative mtDNA titers were significantly higher than those

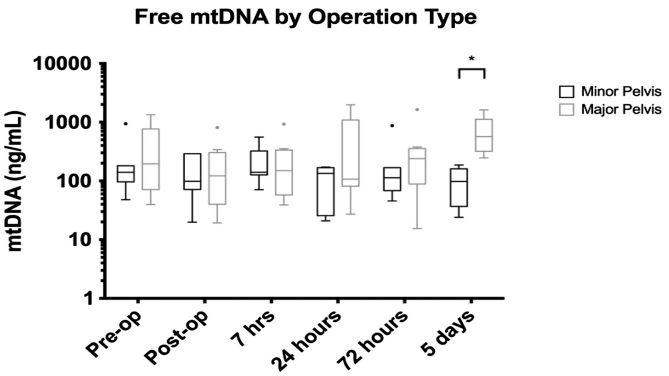

Figure 3. Statistically significant difference between free plasma mtDNA levels at 5 days postoperatively when comparing major pelvic versus minor pelvic surgery ($p < 0.05$). Statistical comparison was made using two-way ANOVA test.

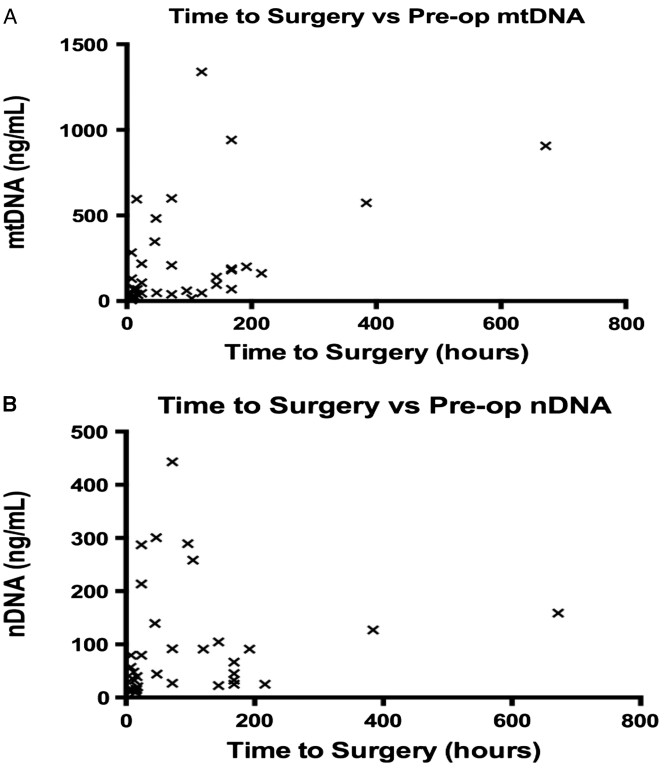

Figure 4. Statistically significant correlation between reduced time from injury to surgery and elevated preoperative levels of mtDNA (Spearman's test, $p = 0.0085$). No significant correlation was found with preoperative nDNA levels (Spearman's test, $p = 0.055$).

of the healthy controls. Most patients had comparable levels of plasma mtDNA between 3 days and 10 days after trauma with the previously published initial (within hours of injury) mtDNA concentrations.[1] The magnitude of preoperative mtDNA concentrations was correlated with the postoperative increase of mtDNA concentrations (but not the nDNA), which could make mtDNA a valuable marker for optimal timing of major secondary surgical interventions.

Our data suggest that rather than increasing rapidly following surgery, absolute plasma levels of mtDNA actually declined in the immediate postoperative period (up to 7 hours postoperatively), when compared with preoperative titers. This could be explained by intraoperative hemodilution. Increased intraoperative fluid administration was correlated with decreased postoperative titers of mtDNA. This finding highlights the need to include fluid resuscitation among the reported variables in mtDNA research on trauma patients.

mtDNA concentration increased sequentially from 24 hours and 3 days postoperatively before peaking at Day 5. There was a strong trend ($p = 0.054$) in mtDNA concentration increase from 24 hours versus 3 days and 5 days. mtDNA was significantly elevated compared with nDNA concentration at 5 days postoperatively when comparing major and minor pelvic surgery. This indicates the potential link between the magnitude of surgery and postoperative DNA release. This could be caused by the fact that more invasive surgery can result in more severe inflammatory response. Unlike the initial trauma-related increase in mtDNA plasma concentration that occurs

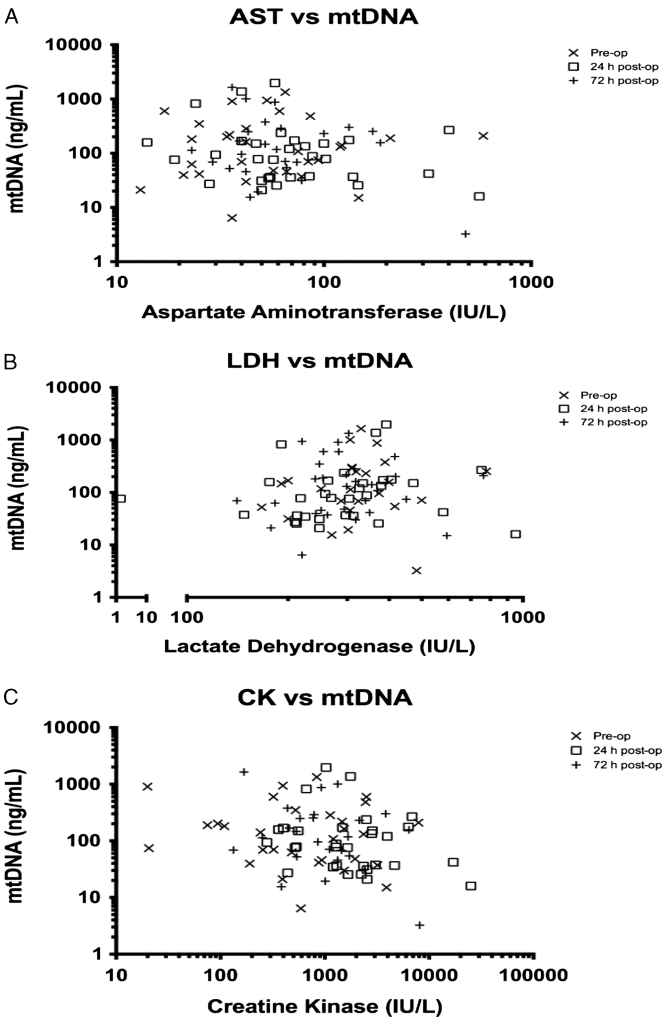

Figure 5. No statistically significant correlation to mtDNA levels (Spearman's test) between AST (p = 0.47–0.84) (*A*), LDH (p = 0.13–0.62) (*B*), and CK (p = 0.39–0.48) (*C*).

within hours of injury and correlates with CK concentrations,[10] this sustained elevation of plasma mtDNA is independent of tissue necrosis markers, while nDNA shows correlation with AST and LDH concentrations. The mechanism of this mtDNA release is very likely to be other than direct tissue injury and subsequent cell necrosis.

The current literature suggests rapid increases followed by rapid decline in mtDNA titers following major trauma in humans[1] and a rat model of traumatic hemorrhagic shock and subsequent surgery.[2] There was a subgroup (eight patients) in a study performed in a polytrauma intensive care unit patient population where cell free DNA was measured over 10 days following admission where cell free DNA was noted to increase from Day 3 after injury, peaking at Day 7.[18] The authors did not delineate the type of DNA measured (mtDNA vs. nDNA). The persistence of increased nDNA was confirmed in plasma of major trauma patients up to 28 days following injury.[19] The same group noted increased mtDNA identified in the plasma of major trauma patients less than 4 hours after injury.[20] This increased mtDNA titer was positively correlated with ISS, but the surgical interventions and potential sepsis were not accounted for.

Some studies[18–20] did not specify their methodology to eliminate the cellular fraction in the plasma samples by including a second high-speed centrifugation step (12,000 rpm in approximately 10 minutes) before DNA extraction.[17] Without this step, the white cell fraction may have been lysed during DNA extraction and could have contributed to the "free circulating DNA" measured.

There was also no correlation between AST or LDH and mtDNA. In sepsis, positive correlations had been shown between liver enzymes and plasma mtDNA levels, suggesting inflammatory end-organ damage and subsequent DNA release.[21]

With a lack of correlation between markers of tissue necrosis, that is, CK (skeletal muscle), AST, and LDH (liver) failing to correlate with mtDNA levels, alternative possible sources need to be considered. The default mode of cell death for neutrophils remains apoptosis, where little intracellular DNA, whether nDNA or mtDNA, escapes into the extracellular environment. Small amounts of DNA from apoptotic cells do escape degradation by scavenging macrophages[22] and are released into the circulation. Under normal physiologic conditions, some 1 g to 10 g of DNA from nucleated leucocytes is degraded and cycled daily.[23] Circulating DNA from dying cells is rapidly degraded by DNAse.[24] The large titers of plasma DNA in trauma patients suggests deviation from normal physiologic process.

The possible source of free circulating DNA without proven cell necrosis could be that under certain conditions, neutrophils can extrude their DNA into the extracellular environment to form neutrophil extracellular traps (NETs).[25] Our current data raise suspicion that an inflammatory mechanism (in contrast to necrosis) could be behind the sustained elevated plasma mtDNA titers in the postoperative period.

The significance of elevated mtDNA compared with nDNA concentration at several perioperative time points

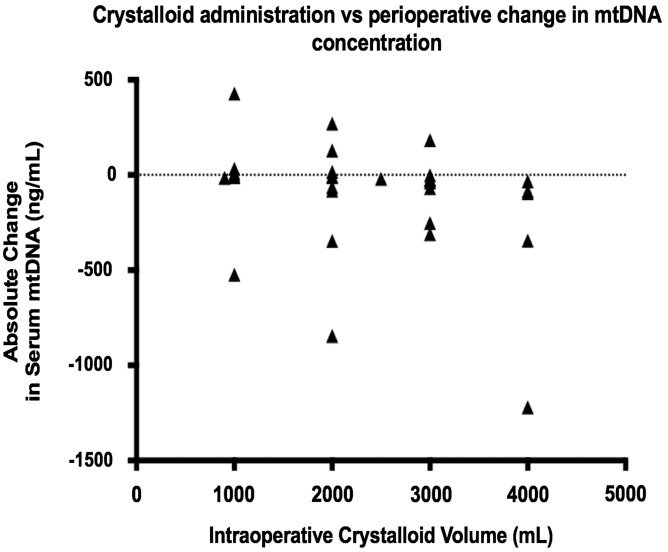

Figure 6. There was a statistically significant correlation (Spearman's test) between volume of intraoperative crystalloid infused and postoperative mtDNA level (p = 0.0017).

(preoperative period, 3 days postoperatively, and 5 days postoperatively) can be better understood by considering the size of the respective genomes. Human mitochondrial genome is 16,569 base pairs, and the human nuclear genome is 3.3 billion base pairs.[8] This makes the mtDNA molecule 200 thousand times smaller than nDNA. There are variable numbers of mitochondria in different cell types and between 2 and 10 copies of the mitochondrial genome per mitochondria.[8] Those cells with the greatest numbers of mitochondria such as striated muscle and liver would still have approximately six to seven times more nDNA if concentration was expressed as nanogram per milliliter as in this study. The potential release of mtDNA from viable nucleated cells without nDNA release has already been alluded to by studies on NET formation or NETosis.[26,27]

Most authors accept that the process of NETosis is a form of controlled cell death[28,29] and nuclear chromatin is a major constituent of NETs.[30,31] It has been postulated that some neutrophils remain viable while form NETs exclusively from mtDNA[26] and eosinophil leukocytes have a mechanism for the "catapult-like release" of mitochondria.[27] If such mechanisms for active mitochondrial product expulsion exist as a component of the innate immune response following major injury and subsequent surgery, perhaps, this may help explain why such high titers of mtDNA were measured in the plasma of patients in this study. Recently, we have demonstrated that trauma surgical interventions generate NETs and they primarily contain mtDNA in contrast to NETs produced in response to bacteria, which primarily consists of nDNA.[30]

That no significant bDNA was measurable in any patient at any time point confirms that the cellular processes occurred under essentially sterile conditions. This eliminates the possibility of sepsis-related inflammatory tissue damage driving the mtDNA or nDNA release.

The present study is limited by the lack of power to demonstrate statistical differences between groups such as SIRS versus no SIRS, which has been described before.[31,32] Our study focused on the standard orthopedic trauma surgical interventions and mtDNA/nDNA release days after those; consequently, our study did not include a very high-risk, severely shocked, and major tissue injury cohort. Although the relatively standard surgical interventions were selected for inclusion, some heterogeneity with respect to the nature of surgery, surgical technique, and the period between injury and surgery are also potential confounding elements in this study.

In conclusion, our pilot study highlights the sustained presence of primarily mtDNA in trauma patients' plasma following surgical interventions. That the timing and the magnitude of surgery correlates with mtDNA concentration makes it potentially attractive as a future marker for second hits and the development of postinjury complications.[32,33] Our results and conclusions should be interpreted in the context of the heterogeneity of our population and the pilot nature of our study. Nevertheless, in future research related to postinjury DAMPs, we confidently recommend addressing routinely the timing and effect of surgical interventions and also fluid resuscitation to distinguish between trauma and treatment-associated responses.

AUTHORSHIP

A.E.W. and N.L. collected blood samples and patient demographics. D.J.M., M.B., and D.W.S. designed the experimental protocol. D.J.M. conducted the laboratory experiments. Initial data analysis was performed by D.J.M. Further statistical analysis was performed by B.M.H. with the generation of the figures used in the manuscript. The manuscript was written by D.J.M and Z.J.B. Critical appraisal of the manuscript was performed by D.W.S.

DISCLOSURE

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Chapter 3:

NETs formed after Trauma and Subsequent Surgery are Composed of mtDNA

McIlroy, DJ, Jarnicki AG, Au GG, Lott N, Smith DW, Hansbro PM, Balogh ZJ, **Mitochondrial DNA neutrophil extracellular traps are formed after trauma and subsequent surgery.** *Crit Care*, 2014; 29:1333e1 – 1333e5

Introduction

The trigger for this line of investigation came from our pilot study finding that elevated mtDNA levels did not correlate with markers of cellular necrosis. In order to highlight the importance of our hypothesis that NETs could play a pivotal role in mtDNA release we published an editorial in the *Journal of Critical Care* in July 2013 (Balogh et al. 2013).

The investigation of potential NETosis following injury and subsequent surgery commenced in October 2013. This demanded the assimilation of a whole new laboratory skill set. Dr Andrew Jarnicki was pivotal in teaching me the necessary techniques for careful neutrophil isolation. Dr Gough Au helped me perform the initial fluorescence microscopy, using specific DNA fluorescent dyes staining extracellular DNA (NETs) and intracellular DNA (viable neutrophils). The excitement was palpable when we looked down the microscope for the first time and captured the bright filamentous green structures, confirming that NETs were present after injury and subsequent surgery.

We then took our study a step further than other authors at that time by actually analysing NETs both quantitatively and qualitatively to determine whether they were composed of nDNA or mtDNA. We revealed that neutrophils were producing mtDNA-NETs following our qPCR analysis, and realised that we had made a significant discovery.

I presented this work at the Royal Australasian College of Surgeons Annual Scientific Congress in Singapore, Malaysia, in May 2014 and won the Trauma Research Paper Prize. As part of the prize, I was sent to present the work at the ATLS® Region XVI Meeting in Taipei, Taiwan in November 2014.

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Mitochondrial DNA neutrophil extracellular traps are formed after trauma and subsequent surgery☆

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ABSTRACT

Introduction: Neutrophil extracellular traps (NETs) have not been demonstrated after trauma and subsequent surgery. Neutrophil extracellular traps are formed from pure mitochondrial DNA (mtDNA) under certain conditions, which is potently proinflammatory. We hypothesized that injury and orthopedic trauma surgery would induce NET production with mtDNA as a structural component.

Methods: Neutrophils were isolated 8 trauma patients requiring orthopedic surgery postinjury and up to 5 days postoperatively. Four healthy volunteers provided positive and negative controls. Total hip replacement patients acted as an uninjured surgical control group. Neutrophil extracellular traps were visualized with DNA (Hoechst 33342TM/Sytox Green/MitoSox/MitoTracker) stains using live cell fluorescence microscopy with downstream quantitative polymerase chain reaction analysis of DNA composition.

Results: Neutrophil extracellular traps were present after injury in all 8 trauma patients. They persisted for 5 days postoperatively. Delayed surgery resulted in NET resolution, but they reformed postoperatively. Total hip replacement patients developed NETs postoperatively, which resolved by day 5. Quantitative polymerase chain reaction analysis of NET-DNA composition revealed that NETs formed after injury and surgery were made of mtDNA with no detectable nuclear DNA component.

Conclusions: Neutrophil extracellular traps formed after major trauma and subsequent surgery contain mtDNA and represent a novel marker of heightened innate immune activation. They could be considered when timing surgery after trauma to prevent systemic NET-induced inflammatory complications.

1. Background

The formation of neutrophil extracellular traps (NETs) is a recently described cellular phenomenon of the innate immune system. Neutrophil extracellular traps were first characterized in 2004 and comprise a diffuse extracellular filamentous chromatin-histone scaffold adorned with neutrophil granular proteins such as neutrophil elastase, myeloperoxidase, lactoferrin, and gelatinase [1]. They were demonstrated to mediate neutrophil extracellular bacterial killing in sepsis [1]. It was thought that neutrophils had to die to form NETs [2], and this novel form of neutrophil cell death is often referred to as "NETosis" [3]. More recently, Yousefi et al [4] showed that, under certain conditions, NETs could be formed from pure mitochondrial DNA (mtDNA) and despite NET formation, neutrophils remained viable.

Neutrophil extracellular traps have now also been implicated in the pathogenesis of sterile chronic inflammatory conditions such as systemic lupus erythematous [5] and small vessel vasculitis [6]. More recently, their presence was demonstrated in a mixed intensive care unit (ICU) population with systemic inflammatory response syndrome [7]. Sequestration of activated neutrophils into end-organ parenchyma is a pivotal step in the pathophysiology of postinjury multiple-organ failure (MOF) [8]. Neutrophil extracellular traps have also been implicated in the pathogenesis of acute lung injury and in sterile transfusion-related acute lung injury [9], which are often antecedents of MOF. Neutrophil extracellular traps have been recently described in muscle fiber damage in a mouse hind limb ischemia-reperfusion injury model [10]. Although not reflective of a traumatic injury or shock model, this further substantiates the ability of NETs to cause direct tissue injury. More recently, interest in the potential role of NETs in the posttraumatic injury setting and their possible role in the subsequent inflammatory response has gained significant attention [11]. These data presented in this review are predominantly

☆ Conflicts of interest: None declared.

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mechanistic based on animal studies. There are no clinical studies to date that evaluate NETs after trauma or the impact of subsequent surgery on their formation. The formation of NETs after major trauma has never been definitively demonstrated.

Limited research has shown the release of greatly increased titers of extracellular DNA after major injury [12,13]. One study presumed that cell-free DNA is an indirect measure of NET formation after injury without visualizing their presence microscopically [13]. The formation of NETs after major trauma has never been definitively demonstrated. Mitochondrial DNA has also been shown to be released in large titers after injury and is potently proinflammatory [14]. Indeed, mtDNA was demonstrated to cause inflammatory lung injury when injected into uninjured rats at physiological concentrations [14]. Recently, our group measured mtDNA after orthopedic trauma and subsequent surgery. We found large increases in plasma mtDNA concentrations up to day 5 postoperatively, which did not correlate with conventional markers of cellular necrosis. Given that under certain conditions, neutrophils form mtDNA NETs [4], it is feasible that the circulating mtDNA observed may have been released through NETosis.

Suboptimal timing of surgical interventions can act as a deleterious proinflammatory "second hit" after major injury associated with the development of complications such as acute respiratory distress syndrome (ARDS) and MOF in some cases. [15,16]. Neutrophil extracellular traps are part of the innate immune response, and their release after trauma could represent a component of the postinjury inflammatory response. Furthermore, NET formation triggered by a second hit in the form of nonlifesaving orthopedic surgery could have inflammatory consequences leading to organ failure.

We hypothesized that injury and orthopedic trauma surgery would induce NET production with mtDNA as a structural component.

2. Methods

2.1. Research compliance

Ethical approval for the study was obtained from Hunter New England Human Research Ethics Committee. All blood samples were obtained with informed consent for sampling, archiving, and further analysis in accordance with the aims of this study.

2.2. Patients and blood samples

Blood was sampled from 8 trauma patients with informed consent postinjury (on admission to the emergency department) and at 6 consecutive time points in the perioperative period: immediately preoperatively and then 0 hours, 7 hours, 24 hours, 3 days, and 5 days postoperatively. This was based on previous studies examining immune responses to orthopedic trauma interventions [15,16]. Inclusion criteria were those patients older than 17 years and able to give informed consent. Patients with skeletal injuries, requiring standardized orthopedic trauma surgical stabilization including pelvic fractures (n = 2, symphyseal plating, iliosacral screw fixation, open reduction, and internal fixation of the sacrum or sacroiliac joint), acetabulum fractures (n = 2, open reduction and internal fixation from ilioinguinal and/or Kocher-Langenbeck approaches), femoral shaft fractures (n = 2, intramedullary nailing of the femur), and tibia/fibula fractures (n = 2, intramedullary nailing of the tibia).

The total hip replacement (THR) group (n = 8) had severe osteoarthritis of the hip joint requiring elective total hip joint replacement. None had any primary inflammatory comorbidity or ongoing infections. Blood samples were taken at the specified perioperative time points.

Control samples were taken from healthy volunteers (n = 8) recruited from the Hunter Medical Research Institute registry.

2.3. Neutrophil isolation

Neutrophils were isolated from 2 mL of whole blood layered onto Ficoll Paque Premium (Sigma-Aldrich Pty, Ltd, Sydney, NSW, Australia) using gradient density separation. The granulocyte/erythrocyte layer was treated with ammonium chloride erythrocyte lysis solution (on ice for 10 minutes) and then centrifuged at 900*g* for 5 minutes. The cell pellet was resuspended and washed for 5 minutes in chilled phosphate-buffered saline. The granulocyte pellet was then resuspended in RPMI + 1% Fetal Calf Serum (FCS) culture medium. Neutrophils were then enriched from the mixed granulocyte population using an EasySep human neutrophil enrichment kit (STEMCELL Technologies, Tullamarine, VIC, Australia) yielding an approximately 98% pure neutrophil population; 2×10^5 neutrophils were then seeded into Corning Costar 24-well cell culture plates (Sigma-Aldrich Pty, Ltd) for staining and microscopy. Cell-free supernatant was frozen at −80°C before downstream quantitative polymerase chain reaction (qPCR) analysis to assess NET-DNA composition.

2.4. Real-time qPCR protocols

2.4.1. Neutrophil extracellular trap–DNA analysis

Cell-free supernatant from NET experiments was used to assess the composition of NET-DNA. Five microliters of cell-free supernatant was added to 7 μL of SYBR green master mix (SensiFast; Biolines, Alexandria, NSW, Australia). The real-time qPCR analysis was performed using Applied Biosystems Real-Time 7500 analyzer (Applied Biosystems; Life Technologies, Foster City, CA). Mitochondrial DNA primers were designed and synthesized for Cytochrome Oxygenase III (COX_3) and NADH dehydrogenase, subunit 3 (ND_3), and the nuclear DNA (nDNA) primer targeted GAPDH (Geneworks, Hindmarsh, SA, Australia) (Table 1).

2.4.2. Screening for bacteremia

Patient- and time point–matched blood samples were taken postinjury and at all perioperative time points used for neutrophil isolation and NET analysis. Plasma was separated from 5 mL of whole blood sampled and frozen at −80°C before analysis. Plasma was mixed by pulse vortex for 15 seconds. DNA was extracted from 200 μL of plasma using a blood DNAeasy extraction kit (Qiagen, Chadstone, VIC, Australia) according to the manufacturer's instructions.

The 5 μL of DNA eluate was added to 7 μL SYBR green master mix (SensiFast) before performing qPCR using an Applied Biosystems Real-Time 7500 analyzer. Primers targeting the bacterial 16S ribosomal subunit were used (Geneworks) (Table 1).

2.4.3. Systemic inflammatory response syndrome criteria

Systemic inflammatory response syndrome was defined by the presence of 2 or more criteria outlined consensually by the American College of Chest Physicians and Society of Critical Care Medicine in 1992 [17].

Table 1
Primers used for real-time qPCR

Target gene	Sequence
Human COX3 (mtDNA)	5′-AGGCATCACCCCGCTAAATC-3′ (Forward) 5′-CCGTAGATGCCGTCGGAAAT-3′ (Reverse)
Human ND3 (mtDNA)	5′-ACTACCACAACTCAACGGCT-3′ (Forward) 5′-GCGGGGGATATAGGGTCGAA-3′(Reverse)
Human GAPDH (nDNA)	5′-AGGGCCCTGACAACTCTTTT-3′ (Forward) 5′-TTACTCCTTGGAGGCCATGT-3′ (Reverse)
Bacterial 16S (bacterial DNA)	5′- CGTCAGCTCGTGTTGTGAAA-3′ (Forward) 5′-GGCAGTCTCCTTGAGTTCC-3′ (Reverse)

Temperature greater than 38°C or less than 36°C
White cell count greater than 12×10^6/mL or less than 4×10^6/mL
Heart rate greater than 90 beats per minute
Respiratory rate greater than 20 breaths per minute

2.4.4. Neutrophil extracellular trap induction

Positive controls were generated by stimulating neutrophils from healthy volunteers to form NETs with 600 nmol/L phorbol myristate acetate (PMA) [18] and incubating in 5% CO_2 at 37°C for 45 minutes.

2.4.5. Staining of NETs

Five micromoles per liter Hoechst 33342 (Invitrogen; Life Technologies) was applied to each well to stain viable neutrophil nuclear DNA, as it is membrane permeable. Five micromoles per liter Sytox Green (Invitrogen) was used to stain NETs as a result of its membrane impermeable properties. Mitochondrial specific stains were added to identify mitochondria in live neutrophils (MitoTracker, Invitrogen; Life Technologies). Previously, mtDNA NETs had been visualized with a dye that relies upon mitochondrial superoxide [19] and has a high affinity for mtDNA in the extracellular environment [4]. We used this same counterstain technique with the aim of staining potential NET mtDNA (MitoSox, Invitrogen; Life Technologies). Cells were incubated in 5% CO_2 at 37°C before microscopy.

2.4.6. Visualization of NETs

Laser excitation of each well was performed at 504 nm (Sytox Green—emission 523 nm) and at 350 nm (Hoechst 33342—emission 461 nm) before microscopy. Fluorescent microscopy was performed using an Olympus IX70 inverted microscope (Olympus, NSW, Macquarie Park (Sydney), Australia) with appropriate filters for the detection of Sytox Green (green) and MitoTracker/MitoSox (red) Hoechst 33342 (blue). Images were captured with a DP72 digital camera (Olympus) at magnification ×100 and composited using ImageJ software (http://rsbweb.nih.gov/ij/).

3. Results

The 8 trauma patients included in the study had a mean age of 44 ± 17 years with a mean injury severity score of 13 ± 7. All patients were male and had high-energy blunt trauma resulting in fractures requiring surgical fixation. Mean initial base deficit was −2.5 ± 2.8 mEq/L. Four patients had major pelvic fractures (including 2 acetabular fractures), 2 had femoral shaft fractures, and 2 had isolated tibia/fibula fractures. Patients underwent surgical fixation of their fractures (major pelvic surgery, n = 4; femoral nailing, n = 2; and tibial nailing, n = 2). The mean operative time was 184 ± 103 minutes. Two patients were admitted to ICU postoperatively with a mean length of stay (LOS) of 2 days ± 1 day. Five patients developed systemic inflammatory response syndrome during the study period. Two patients required a blood product transfusion perioperatively. Total length of stay in hospital was a mean of 13 ± 7 days.

Eight healthy control subjects (4 females and 4 males) were enrolled with a mean age of 35 ± 14 years. None of the healthy controls had any NETs present after neutrophil isolation and staining (Fig. 1A). All positive controls (from the same healthy volunteer samples stimulated with 600 nmol/L PMA for 45 minutes) generated NETs (Fig. 1B). Neutrophil extracellular traps were detected after injury in the blood of all 8 trauma patients included in the study (Fig. 2A). Of the 8 patients, 6 (2 unstable pelvic fractures, 2 femoral fractures, and 2 tibia/fibula fractures) who had surgery in the first 24 hours had NETs at all perioperative time points.

The 2 major acetabular fracture cases had delayed surgery (7 and 10 days postinjury, respectively). In these patients, NETs were detected after injury but were absent preoperatively on days 7 and 10 (Fig. 2B). Postoperatively, NETs were visualized immediately after the operation (Fig. 2C) and at all postoperative time points up to day 5 (Fig. 2D).

All 4 THR patients enrolled had no NETs preoperatively (Fig. 3A), but all had NETs postoperatively (Fig. 3B) and at all subsequent postoperative time points up to day 3 (Fig. 3C). Mean operative time in this group was 132 ± 34 minutes. The only THR case to remain in hospital for the full 5-day observation period had no NETs at this later time point (Fig. 3D).

No bacterial DNA was detectable in any of the matched patient plasma samples.

Mitochondrial DNA is the major structural component of NETs demonstrated after traumatic injury, trauma surgery, and elective THR surgery. Mitochondrial DNA was detected in abundance in the supernatant demonstrated by qPCR amplification of mitochondrial gene targets (COX3/ND3) from the trauma cohort and THR cohort. The nDNA gene target (GAPDH) was undetectable in these cases. This suggests that the NETs formed after trauma and subsequent surgery or after THR surgery were composed of mtDNA. Extracellular DNA in pure neutrophil culture is directly proportional to NET formation [19]. We further substantiated this by counterstaining NETs with MitoSox, which requires the presence of mitochondrial superoxide to bind the NET-mtDNA [4] generating red fluorescence (Fig. 4A) [20]. It can be seen that extracellular NET-DNA (nonspecifically stained with Sytox Green; Fig. 4B) powerfully colocalizes MitoSox. This is demonstrated

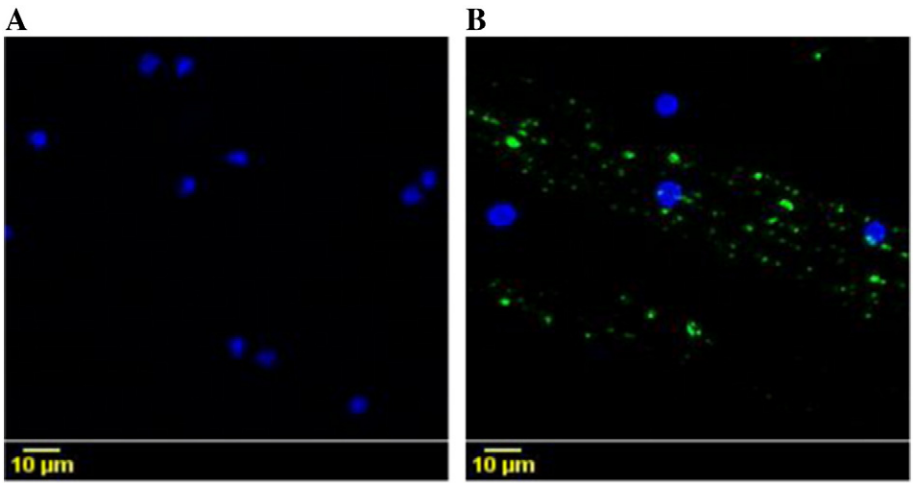

Fig. 1. Viable neutrophil nuclear DNA was stained with membrane permeable Hoechst 33342 (blue), and NETs were visualized by membrane impermeable Sytox Green (green) staining extracellular DNA. Negative control, healthy volunteer without stimulation (A) and positive control, healthy volunteer stimulated with 600 nmol/L PMA for 45 minutes (B).

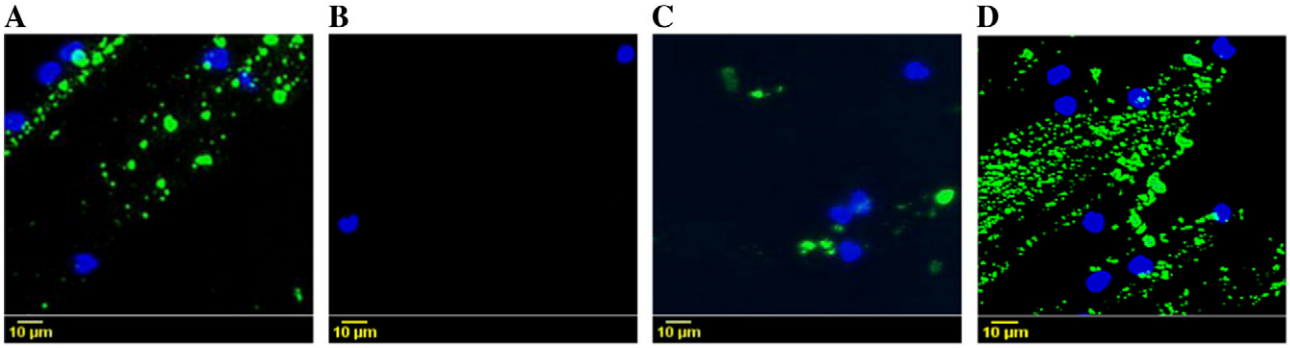

Fig. 2. Viable neutrophil nuclear DNA was stained with membrane permeable Hoechst 33342 (blue), and NETs were visualized by membrane impermeable Sytox Green (green) staining extracellular DNA. Acetabular fracture patient, immediately postinjury (A); 7 days postinjury, immediately preoperative (B); immediately postoperative (C); and 5 days postoperative (D).

as yellow when the images of NETs stained with MitoSox (red) and Sytox (green) are overlaid. Nuclear chromatin in viable neutrophils was stained blue using Hoechst stain (Fig. 4C).

4. Discussion

Given that no negative controls formed NETs and all positive controls stimulated with PMA indicates that our isolation technique was sound and did not generate NETs through experimental cellular handling. It also indicates that when stimulated, neutrophils produced NETs, which could be successfully visualized [17].

Neutrophil extracellular traps were absent preoperatively in the 2 major acetabular fracture cases that had delayed surgery, indicating that if sufficient time elapses after initial injury (7 and 10 days, respectively, in these cases), NETs resolve; however, their formation is then propagated again by major surgery. By demonstrating that NETs are no longer formed after 7 days after initial injury may indicate a reduction in the degree of innate immune activation and a less volatile window to perform nonlifesaving surgery.

This is the first time that NETs have been definitively demonstrated after major trauma. Other authors have alluded to their presence after major injury by detecting cell-free DNA released after injury and equating this proportionately to NET formation. However, no other study has demonstrated NETs experimentally [13]. Given that NETs are rapidly degraded by DNAse in the circulation [5], it is likely that NETs are actively produced throughout the 5-day observation period after injury and surgery without new traumatic, surgical, or septic insult.

We showed further evidence that surgery can stimulate NET formation independent of prior injury by demonstrating NET formation after elective THR.

No patient (injured or elective THR) had evidence of sepsis during the study period. This eliminates the possibility that bacterial sepsis may have been an initiating factor causing NET formation in these patients. Neutrophil extracellular trap formation in these cases can be viewed as part of the sterile inflammatory response of the innate immune system to traumatic injury, major surgery on trauma patients, or major elective surgery without previous injury.

Neutrophil extracellular traps produced in response to bacterial invasion contain nuclear DNA as their principle structural component [3]. Our data show NETs observed after injury and subsequent surgery and after elective THR surgery can be composed of mitochondrial DNA as other authors have found under certain conditions [4]. Interestingly, this group found that reactive oxygen species were essential for the release of mtDNA-NETs [4] and also for the red fluorescence signal of MitoSox dye bound to mtDNA [4]. The exact molecular mechanism of mtDNA-NET release is unclear; however, when a reactive oxygen species production inhibitor (diphenyleneiodonium) was used, mtDNA-NET formation was also blocked and no DNA was released [4]. Mitochondrial DNA is a potent driver of postinjury systemic inflammatory response, and it is associated with the development of ARDS and MOF [21,22]. It has gained recognition as an "alarmin" [23] meaning that it powerfully stimulates the innate immune system at physiological concentrations. Free mitochondrial DNA from tissue and necrosis after injury or from mtDNA-NETs could potentially reactivate the neutrophils through the toll-like receptors [24] and generate further NET production. Toll-like receptors have been shown to mediate NET formation stimulated through activation of toll-like receptor 4 by another alarmin, namely, high-motility group box protein 1 (HMGB1) [25]. It is, therefore, feasible that mtDNA release after tissue injury and subsequent mtDNA-NET release could perpetuate a vicious cycle of immune activation. Such a phenomenon could contribute to pathophysiological processes underpinning persistent inflammation and catabolism syndrome recently described on trauma ICUs [26].

The timing and invasiveness of major, but nonlifesaving, surgical interventions after trauma are modifiable independent risk factors

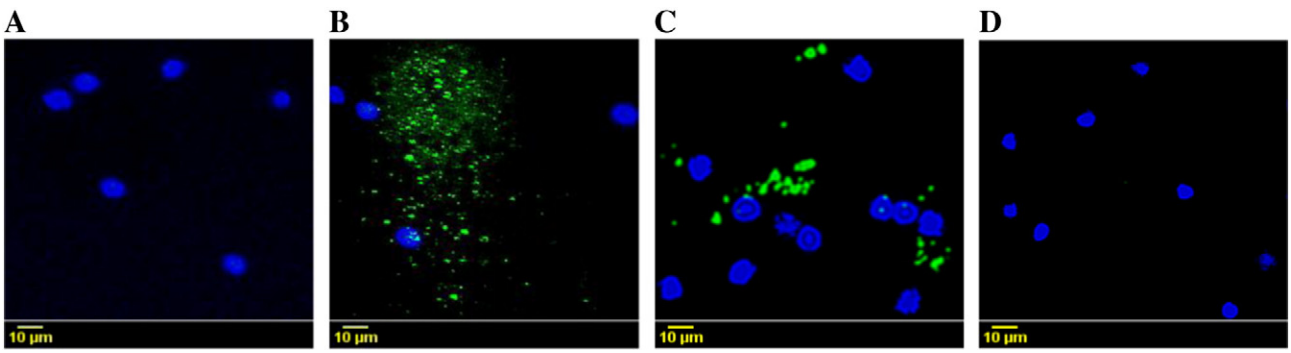

Fig. 3. Viable neutrophil nDNA was stained with membrane permeable Hoechst 33342 (blue), and NETs were visualized by membrane impermeable Sytox Green (green) staining extracellular DNA. Total hip replacement patient immediately preoperative (A), immediately postoperative (B), 3 days (C), and 5 days postoperative (D).

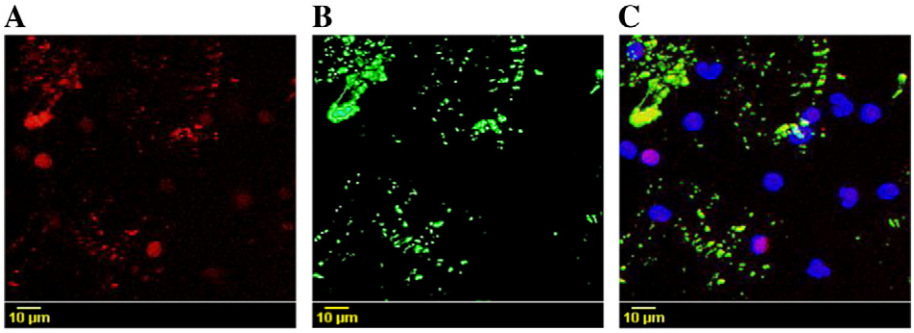

Fig. 4. Neutrophil extracellular traps isolated from a pelvic fracture patient 5 days postoperative. Viable neutrophil nDNA was stained with membrane permeable Hoechst 33342 (blue), and NETs were visualized by membrane impermeable Sytox Green (green) staining extracellular DNA. Extracellullar mtDNA was stained with MitoSox [4] (red). Intracellular mitochondria are labeled with MitoTracker (red) (scale bar, 10 μm). Neutrophil extracellular trap stained with MitoSox (A), NET stained with Sytox Green (B). C, Overlaid image: viable neutrophils (blue, Hoechst), colocalized mtDNA-NET (yellow, Sytox Green + MitoSox Red), and intracellular mitochondria (red/pink, Mitotracker).

(second hits) implicated in the development of serious postinjury complications such as ARDS and MOF [15,16]. Neutrophils play a pivotal role in the development of these complications, which are likely to be contributed to by NETs causing secondary inflammatory tissue injury [9]. Neutrophil extracellular traps are a potent source of proinflammatory compounds including cell-free DNA and other danger-associated molecular patterns [1]. The potential for NETs to be made of mtDNA after injury and surgery may have implications for the postinjury inflammatory response due to a potent immunostimulatory profile [12] and association with inflammatory lung injury [12], ARDS, and MOF [19]. The presence of NETs indicates a state of heightened innate immune reactivity. Accurate quantification of NET formation and greater understanding of their role in the postinjury inflammatory paradigm could enable more optimal timing for nonlifesaving major surgical interventions after trauma to prevent the development of second hit–related MOF.

Author contribution statement

DJM: Conducted all experimental work and wrote the manuscript.

AGJ: Supervised and advised on experimental techniques and methodology.

GA: Supervised and advised on microscopic techniques.

NL: Collected the patient and volunteer blood samples.

DS: Reviewed and critically appraised the manuscript.

PMH: Reviewed and critically appraised the manuscript.

ZJB: Designed and funded the study, mentored the first author, coordinated the collaboration, and reviewed and critically appraised and edited the manuscript and the revision.

Acknowledgment

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Chapter 4:

Mitochondrial DNA Induces Mitochondrial DNA-NET Formation After Trauma and Subsequent Surgery

McIlroy DJ, Jarnicki AG, Au GG, Lott N, Briggs G, Smith DW, Hansbro PM, Balogh ZJ, **Mitochondrial DNA Induces Mitochondrial DNA Neutrophil Extracellular Trap Formation After Trauma and Subsequent Surgery.** *Frontiers in Immunology* (under review).

Introduction

We concluded our initial work on NETs demonstrating that they were composed of mtDNA following trauma and subsequent surgery. We then progressed with our hypotheses and began investigating the possibility that mtDNA may actually stimulate NET formation. In order to conduct this study, I first had to identify a method for generating purified human mtDNA. I identified a kit produced by Qiagen^{TM} (Repli-g, detailed methodology described in the paper), that used a DNA polymerase and specific long mtDNA primers than enabled whole mtDNA genome amplification from a mixed DNA isolate obtained from buccal cheek cells. From this highly purified mtDNA we generated standards of clinically relevant mtDNA concentrations to expose both naïve neutrophils from healthy volunteers and trauma neutrophils isolated at the previously determined peri-operative timepoints after injury and subsequent surgery.

This work yielded some very interesting results. We demonstrated that mtDNA triggered mtDNA-NET formation in both healthy and trauma neutrophils in a dose and time dependent fashion after a 45min and 90min incubation period at clinically relevant concentrations. We used concentrations of mtDNA that we had measured under clinical conditions in the plasma of patients in the initial mtDNA pilot study.

I then used phorbol myrisate acetate (PMA) as a known stimulator of NETosis (Brinkman et al. 2010). PMA was used as a positive control to run on isolated neutrophils from the same blood samples as those incubated with mtDNA. The NETs produced in response to PMA were composed of almost pure nDNA indicating a likely different mechanism for NETs generated when compared to mtDNA triggered mtDNA-NETs. This finding was further substantiated by another group who found that mtDNA stimulated NETs through a TLR9 mediated pathway

(Itagaki et al. 2015). The authors found that when a TLR9 antagonist was used mtDNA stimulated NETs were inhibited but PMA stimulation of NET production was unaffected. They did not analyse the DNA composition of the NETs observed in response to mtDNA or PMA stimulation.

I presented this work at the Royal Australasian College of Surgeons Annual Scientific Meeting in Brisbane, May 2016 and won the Damian McMahon Trauma Research Prize. As a result, I was invited to present at the ATLS® Region XVI Meeting in Hong Kong in November 2016.

The paper is currently under review for publication in Frontiers in Immunology.

MITOCHONDRIAL DNA INDUCES MITOCHONDRIAL DNA NEUTROPHIL EXTRACELLULAR TRAP FORMATION FOLLOWING INJURY AND SUBSEQUENT SURGERY

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Funding:

Conflicts Of Interest:

None declared

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ABSTRACT:

BACKGROUND:

Neutrophil extracellular traps (NETs) have are formed after injury and subsequent surgery. These were found to be composed mitochondrial DNA (mtDNA). We hypothesised that mtDNA would induce NET formation in both healthy control neutrophils and trauma neutrophils and that the response would be dose dependent.

METHODS:

Neutrophils were isolated from eight healthy control subjects and eight trauma patients who required surgical intervention for orthopaedic trauma. Neutrophils were subsequently incubated with mtDNA at 30ng/ml/300ng/ml/600ng/ml or PMA 300nM/600nM for 45mins and 90mins respectively. NET formation was assessed visually with DNA (Hoechst 33342/Sytox Green) stains using live cell fluorescence microscopy with downstream quantitative polymerase chain reaction analysis of DNA composition using primers targeting both nuclear DNA (nDNA) and mtDNA.

RESULTS:

NETs were produced in a dose and time dependent response to both mtDNA and PMA stimuli. NETs produced in response to PMA stimulus were composed of nDNA. NETs produced in response to mtDNA were composed of mtDNA. Trauma neutrophils produced less NETs when exposed to mtDNA when compared to healthy neutrophils.

CONCLUSION:

MtDNA induces NETs composed of mtDNA (mtDNA-NETs) in contrast PMA induces NETs composed of nDNA (nDNA-NETs). The observed NET response is both dose and time dependent. This is the first time the DNA composition of NETs produced in response to both PMA and mtDNA has ever been assessed in the post injury setting.

Keywords:

Mitochondrial DNA, Neutrophil extracellular traps, NETosis, trauma, injury.

BACKGROUND:

Mitochondrial substances (like their DNA) evolved without exposure to the human innate immune system, they are considered as "alarmins" or danger associated molecular patterns (DAMPs), activating host inflammatory responses (1). Recently the high concentration of serum mitochondrial DNA (mtDNA) following injury (2) and orthopaedic trauma surgery (3) has been documented. MtDNA stimulates neutrophils through toll-like receptor (TLR) pathways (3, 4) and has been demonstrated to induce inflammatory lung injury when administered at clinically relevant titres to healthy rats (2). Clinical correlation was shown in humans between elevated levels of mtDNA and post-injury multiple organ failure (MOF) (5). MtDNA is a potentially promising marker of postinjury inflammation. Beyond that, its crucial role as an instigator of postinjury inflammation makes it an attractive target for therapy to prevent and/or treat postinjury organ dysfunction, organ failure and MOF.

Neutrophil extracellular traps (NETs) were first characterised in 2004 and structurally are diffuse extracellular filamentous chromatin-histone scaffolds adorned with neutrophil granular proteins such as neutrophil elastase (NE), myeloperoxidase (MPO), lactoferrin and gelatinase (6). NETs have been shown to contain nuclear and/or mtDNA, and uncertainty remains about the conditions or stimuli that produce nuclear DNA (nDNA) versus mtDNA NETs. The primary function of NETs was thought to be extracellular bacterial killing in sepsis (6) and the process of NET generation from nuclear DNA (nDNA) was originally believed to result in neutrophil death (NETosis) (7,8). More recently, NET generation was shown in viable neutrophils following stimulation with GM-CSF and complement fragment C5a. NETs formed in response to GM-CSF/C5a stimulus were demonstrated to be formed from mtDNA (9).

Our group has recently proposed the potential convergence of these two (free mtDNA release and NET formation) as fundamental proinflammatory mechanisms in trauma patients (10). Consequently, we demonstrated cell necrosis-independent high circulating levels of mtDNA following major orthopaedic trauma and subsequent surgery and we hypothesised a potential inflammatory source from living cells (NETs) (11). More recently we visualised that NETs are formed after trauma and subsequent surgery without bacterial presence and are primarily composed of mtDNA (12).

Considering the previously shown proinflammatory effect of mtDNA on neutrophils, we hypothesised that mtDNA itself would stimulate NET formation in naive human neutrophils and would increase NET formation in neutrophils isolated peri-operatively from major trauma patients.

METHODS:

Research Compliance:

Ethical approval for the study was obtained from Hunter New England Human Research Ethics Committee. All blood samples were obtained with informed consent for sampling, archiving and further analysis in accordance with the aims of this study.

Patients and Blood Samples:

After gaining informed consent, blood samples were obtained from eight healthy volunteers identified through the Hunter Medical Research Institute volunteer registry. Blood was sampled from eight trauma patients post-injury (on admission to the emergency department) and at six consecutive time points in the peri-operative period; immediately pre-op and then 0 hrs, 7hrs, 24hrs, 3 days and 5 days post-operatively. The timeframes were based on previous studies examining immune responses to orthopaedic trauma interventions (13,14); we also routinely use these timepoints for sample collection in our clinical immune monitoring and human inflammatory response to trauma surgery projects. Inclusion criteria were those patients over 16 years of age, and sustained major musculo-skeletal injuries requiring standardised orthopaedic trauma surgical interventions (femur, pelvis and acetabulum fractures). This study's trauma patients underwent the following surgical interventions to address their orthopaedic injuries: symphyseal plating plus iliosacral screw fixation (n=4), open reduction and internal fixation of acetabulum fracture (n=2), intramedullary nailing of femoral

shaft fractures (n=2). Our previous pilots confirmed that these procedures results in comparable mtDNA release and similar time pattern of NET formation (12).

SIRS Criteria:

SIRS was defined by the presence of two or more criteria outlined consensually by the American College of Chest Physicians and Society of Critical Care Medicine in 1992 (15).

Neutrophil Isolation:

Neutrophils were isolated from 5mls of whole blood layered onto double gradient was formed by layering an equal volume of histopaque-1077 over histopaque-1119 (Sigma-Aldrich, Germany) before spinning at 2500rpm, 20^0C for 30mins gradient density separation. The granulocyte layer was separated and treated with ammonium chloride erythrocyte lysis solution (NH4Cl, NaHCO3 and EDTA, diluted with molecular grade water) on ice for 10mins the reaction was terminated with the addition of 10mls RPMI + 10% FCS and then centrifuged at 2800rpm for 5mins. The granulocyte pellet was then resuspended in RPMI + 1% FCS culture medium. Neutrophils were then enriched from the mixed granulocyte population using an EasySep™ human neutrophil enrichment kit according to manufacturer's instructions (STEMCELL™ Technologies Tullamarine, VIC, Australia) yielding a ~98% pure neutrophil population.

2 x 10^5 neutrophils were then seeded into Corning® Costar® 24 well cell culture plates (Sigma-Aldrich Pty. Ltd, NSW, Australia) with 500uls of RPMI + 1% FCS culture medium for stimulation, staining and microscopy. All incubation steps were performed in a humidified 5% CO_2 environment at 37^0C.

Screening Blood Samples For Bacteraemia:

The presence of bacteria or bacterial components in trauma patient blood samples was undesirable in this study as it would confound any effect of mtDNA on NET formation. In order to screen samples for bacteraemia, quantitative polymerase chain reaction (qPCR) was performed using primers targeting the bacterial 16S ribosomal subunit. Plasma was obtained from healthy controls and from the trauma cohort (at all time points) and was separated from 5mls of whole blood by centrifuging at 1400rpm for 10 mins at 4^0C. Plasma was then centrifuged at 12000rpm for a further 10 mins to generate cell free plasma (16). The top 1ml of cell-free plasma was extracted and frozen at -80˚C prior to analysis. Plasma was thawed and mixed by pulse vortex for 15 seconds. DNA was extracted from 200µls of plasma using a blood DNAeasy extraction kit (Qiagen™ Chadstone, VIC, Australia) according to the manufacturer's instructions.

NET Experiments:

All NET evaluation experiments were standardised by seeding 2x10^5 neutrophils per well and performed in duplicate with each sample being incubated for 45mins and 90mins respectively prior to staining and live cell microscopy.

Negative Controls:

Neutrophils from healthy volunteers were incubated in RPMI+1% FCS.

PMA Stimulation:

Positive controls were generated by stimulating neutrophils from healthy volunteers with 300nM or 600nM PMA (phorbol myrisate acetate) (12) to induce NET formation.

mtDNA Stimulation:

Neutrophils from the eight healthy volunteers were incubated with different concentrations of purified human mtDNA (see method below or generation of mtDNA). The concentrations (600ng/ml, 300ng/ml or 30ng/ml) were selected based on previous reports and our previous measurements of mtDNA content in major trauma patients' plasma (11).

Generation of nDNA / mtDNA Standards:

Nuclear DNA:

DNA was isolated and purified from a buccal cheek swab taken from a healthy volunteer before suspending the cells in 200µls of molecular grade H_2O before extracting and purifying the DNA using a blood DNAeasy extraction kit (Qiagen™ Chadstone, VIC, Australia) according to the manufacturer's instructions. The final concentration of extracted DNA was checked using spectrophotometry at A260nm. DNA extracted was 99.9% nDNA after running both ND3 (mtDNA primers) and GAPDH (nDNA primers) against the DNA extraction stock solution. Serial dilutions were then performed to generate standards of known concentration using spectrophotometry UV absorbance at 260nm, with qPCR performed to generate a standard curve using GAPDH primer set.

Mitochondrial DNA:

Purified DNA from a cell-free trauma plasma sample was then amplified using the Human Mitochondrial REPLI-g kit (Qiagen™ Chadstone, VIC, Australia) to generate purified human mtDNA. Briefly, DNA was denatured at 75^0C for 5mins. REPLI-g human mtDNA polymerase was added and an isothermal multiple displacement amplification proceeded overnight at 33°C for 8hrs. Since spectrophotometry using UV at 260nm was unreliable with the REPLI-g amplified mtDNA product due to nucleic acid components in the buffering solution used, DNA was quantitated using PicoGreen assay (Invitrogen) which uses an ultra-sensitive fluorescent stain that specifically binds double stranded DNA (17). Highly concentrated mtDNA stock solution (5µM) was produced which was then used to generate mtDNA standards and to construct a standard curve using qPCR and ND3 (mtDNA primers). The purity of mtDNA product was confirmed by performing qPCR with GAPDH (nDNA primers) and ND3 (mtDNA primers). Nuclear DNA was undetectable confirming 100% pure mtDNA product. The purified human mtDNA stock solution was used to stimulate neutrophils in subsequent experiments.

Visualisation of the Isolated Neutrophils/NETs:

5 µM of cell membrane and nuclear membrane permeable Hoechst 33342 (Invitrogen, Life Technologies™, Foster City, CA, USA) was applied to each well to stain viable neutrophil nuclear DNA. 5 µM per well of membrane impermeable extracellular DNA stain (Sytox Green®, Invitrogen, Life Technologies™, Foster City, CA, USA) was used to stain the extracellular DNA component of NETs. Fluorescent microscopy was performed using an Olympus IX70 inverted microscope (Olympus, NSW, Australia) with relevant filters for the detection of Sytox® green (green), and Hoechst 33342™ (blue). Images were captured with a DP72 digital camera (Olympus, NSW, Australia) at 100x magnification and composited using ImageJ software (http://rsbweb.nih.gov/ij/). NETs were visually identified by the presence of a conformational extracellular DNA based structure (as confirmed by positive Sytox® green staining).

Quantification of NETs:

Quantification of extracellular DNA in pure neutrophil culture can be used as a direct measure of NET formation as long as other forms of cell death are excluded based on the experimental design (8). NETs were

quantitated by comparing extracellular levels of mtDNA/nDNA in each sample to intracellular mtDNA and nDNA from 2 x 10^5 neutrophils.

DNA was extracted from $2x10^5$ neutrophils from the eight healthy volunteers using a blood DNAeasy extraction kit (Qiagen™ Chadstone, VIC, Australia) according to the manufacturer's instructions and using a 200µl elution volume. DNA fractions were quantitated using qPCR with primers designed for GAPDH (nDNA), ND3 (mtDNA) and standard curves for (as described above). We determined that $2x10^5$ healthy neutrophils contain: 1.04 ± 0.1µg nDNA and 120 ± 34ng mtDNA.

Supernatants were generated from neutrophil isolates used in the NET experiments (after microscopy had been performed) by treating with proteinase K to liberate NET-DNA in solution. The sample was then spun at 12000rpm for 10mins at 4^0C to generate a pellet, with the top 200µls of cell-free culture medium drawn off and frozen for qPCR analysis and NET quantification.

NET formation was quantified using the supernatant from neutrophil isolates used in the NET experiments (as described above) and measuring cell-free nDNA and mtDNA in the culture medium. DNA was extracted from 200µls supernatant using a DNAeasy extraction kit (Qiagen™ Chadstone, VIC, Australia) according to the manufacturer's instructions. Composition of the DNA within the supernatant from the NET experiments was assessed using qPCR targeting mtDNA (ND3) and nDNA (GAPDH) using standard curves for both mtDNA and nDNA generated (described above). The percentage of DNA released as NET's was calculated by dividing supernatant concentrations of mtDNA/nDNA by intracellular DNA from the equivalent amount of $2x10^5$ intact healthy control neutrophils to represent cell-free NET DNA (see above). Corrections were made when interpreting titres of extracellular mtDNA/nDNA present in supernatants by subtracting the concentration of mtDNA added to solution to stimulate neutrophils experimentally and also mean values for cell-free mtDNA/nDNA present in unstimulated healthy control supernatants.

Real-Time qPCR Protocols:

5uls of template DNA was added to 7µls of SYBR green master mix (SensiFast™, Biolines®, Alexandria, NSW, Australia). The real-time qPCR analysis was performed using Applied Biosystems Real-Time 7500 analyser (Applied Biosystems™, Life Technologies™, Foster City, CA, USA). Mitochondrial DNA (mtDNA) primers were designed and synthesised for ND3, nuclear DNA (nDNA) primers for GAPDH and bacterial DNA primer for 16S ribosomal subunit (Geneworks™ Hindmarsh, SA, Australia) (Table 1). Standard curves were constructed using purified mtDNA/nDNA (as described above) to enable calculation of mtDNA and nDNA sample concentrations.

Table 1. Primers utilised for real-time qualitative polymerase chain reaction:

Target Gene	Sequence
Human ND3 (mtDNA)	5'-ACTACCACAACTCAACGGCT-3' (Forward) 5'-GCGGGGGATATAGGGTCGAA-3'(Reverse)
Human GAPDH (nDNA)	5'-AGGGCCCTGACAACTCTTTT-3' (Forward) 5'-TTACTCCTTGGAGGCCATGT-3' (Reverse)
Bacterial 16S (bDNA)	5'- CGTCAGCTCGTGTTGTGAAA-3′ (Forward) 5′-GGCAGTCTCCTTGAGTTCC-3′ (Reverse)

Statistical Analysis:

Data are presented as mean ± standard deviation for parametric variables and as median (IQR) for non-parametric variables. Data were visually examined for skew.

Testing of possible statistically significant differences among means was performed using ANOVA with Tukey's multiple comparisons test. To measure significant differences between healthy controls and trauma groups, two-tailed unpaired t-test with Welch's correction was applied (GraphPad PRISM version 7.0).

Results:

The eight trauma patients included in the study had a mean age of 38 ± 15 years with a median injury severity score (ISS) of 21 (IQR 8 - 45). There was one female in the group and all had suffered high energy blunt trauma resulting in pelvic, acetabular or femur shaft fractures requiring surgical fixation. Mean initial base deficit (BD) was -2.9 ± 4.5 mEq/l. Three patients were admitted to ICU post-operatively with a median length of stay (LOS) of 4 days (2 – 7). Six patients developed systemic inflammatory response syndrome (SIRS) during the study period (15). Three patients required a blood product transfusion peri-operatively. Total median LOS in hospital was 8 days (IQR 5 - 20).

Eight healthy control subjects (four females and four males) were enrolled with a mean age of 35 ± 14 years. Healthy control neutrophils were isolated and stimulated for 45 or 90 mins with respective concentrations of mtDNA 30ng/ml, 300ng/ml and 600ng/ml. Stimulation induced a statistically significant increase in released NET mtDNA compared to unstimulated controls (ANOVA $p<0.0001$) as shown in Figure 1 with representative images shown in Figure 2. NET formation was also demonstrated to be dose dependent with significantly more mtDNA release at higher concentrations of mtDNA stimulation 30ng/ml vs. 300ng/ml and 300ng/ml vs. 600ng/ml (ANOVA, Tukey $p<0.0001$). Within groups there was also a time dependent increase in NET formation when assessing NETs at 45mins vs. 90mins at the different mtDNA concentrations (ANOVA, Tukey $p<0.0001$). No difference was observed in the unstimulated controls between 45mins and 90mins incubation. MtDNA stimulation produced no significant increase in nuclear DNA (nuclear DNA was detectable, however it only represented 1.1% of total DNA released), indicating that mtDNA-induced NETS consist almost exclusively of mtDNA (Figure 1).

Figure 1:

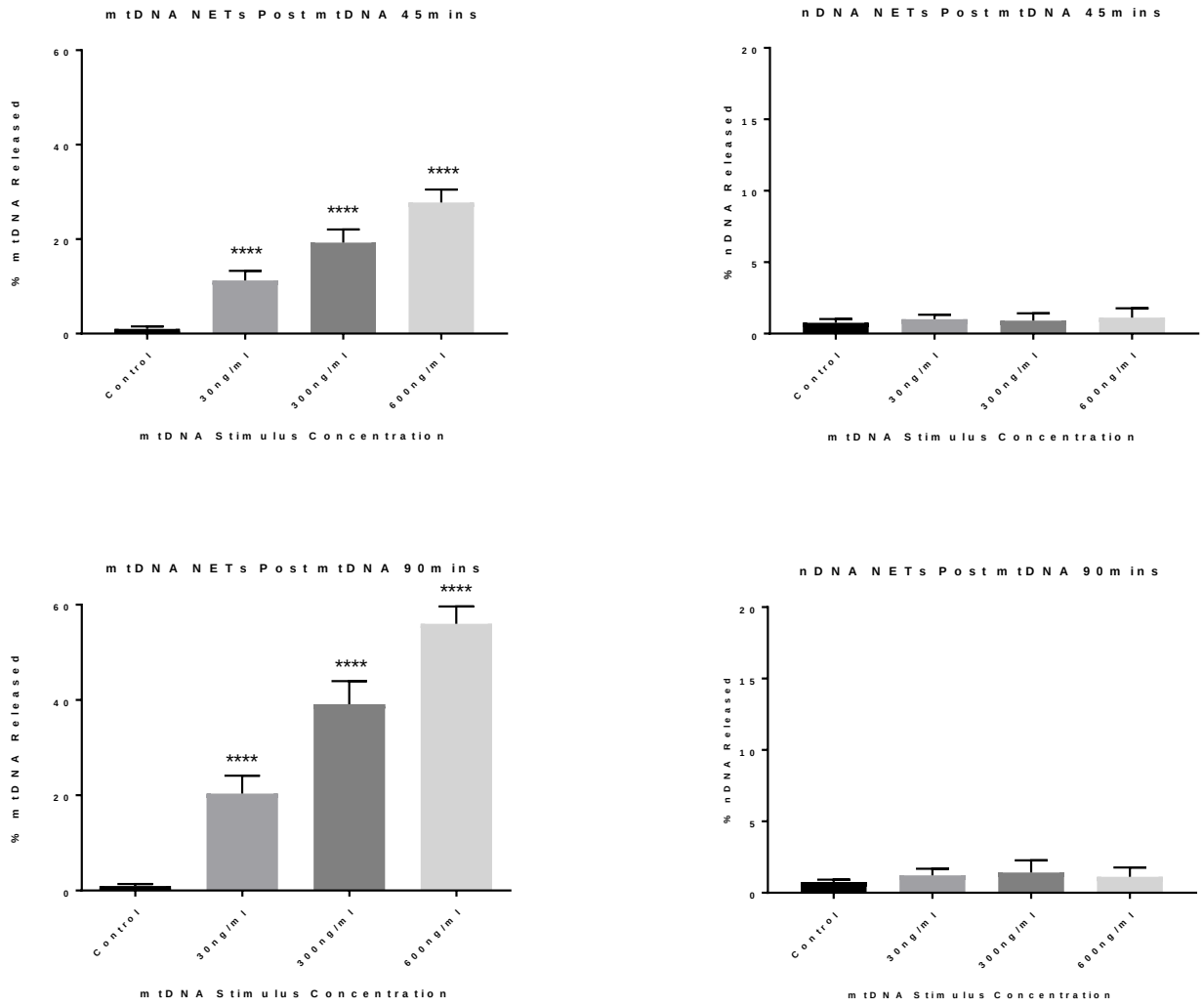
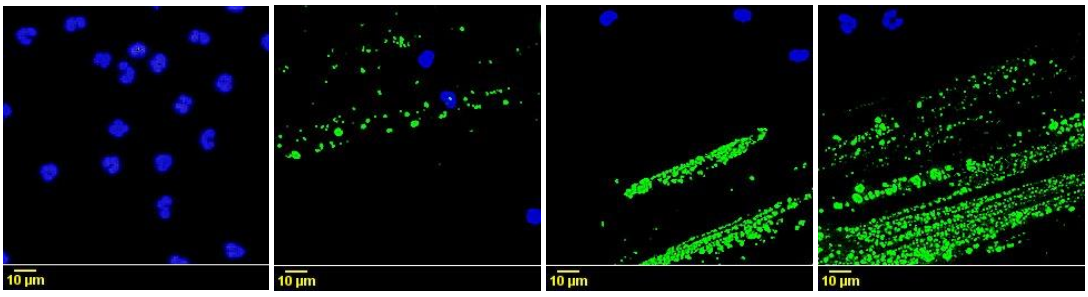


Figure 2: (Images mtDNA Induced NETs)

Control 45mins **30ng 45mins** **300ng 45mins** **600ng 45mins**



Control 90mins **30ng 90mins** **300ng 90mins** **600ng 90mins**

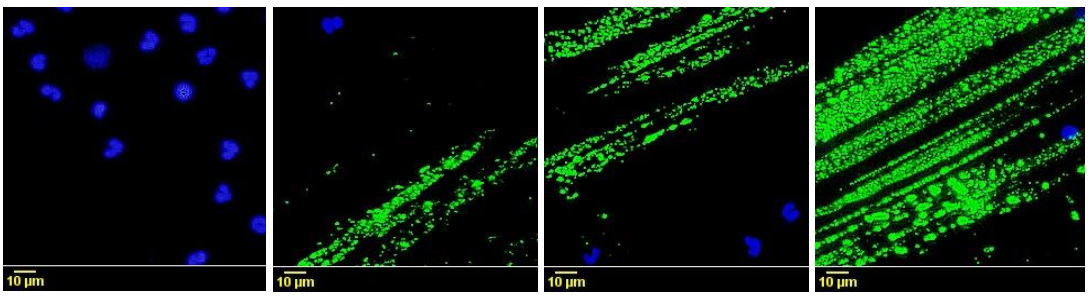


Figure 2 Legend:
This figure demonstrates the mtDNA-NETs formed in response to mtDNA stimulus after incubation with either 30ng/ml, 300ng/ml or 600ng/ml for 45mins and 90mins respectively. The extracellular NET DNA can clearly be seen stained green with Sytox Green® and viable neutrophils are stained blue with Hoechst 33342.

PMA stimulation produced no significant increase in released mtDNA (mtDNA was detectable however it only represented <1.0% of total DNA released), indicating that PMA-induced NETs consist almost exclusively of nDNA (Figure 3) with representative images (Figure 4). PMA induced a dose dependent increase in nDNA-NETs at the respective 300nM and 600nM concentrations when compared to controls after 45 minutes (ANOVA p<0.0001, Tukey p=0.02 and p=0.0006 respectively). Within groups, 90-minute incubation with PMA produced a significantly increased the amount of nDNA-NETs released than 45-minute incubation for both 300nM and 600nM concentrations (ANOVA and Tukey p<0.0001 for both).

Figure 3:

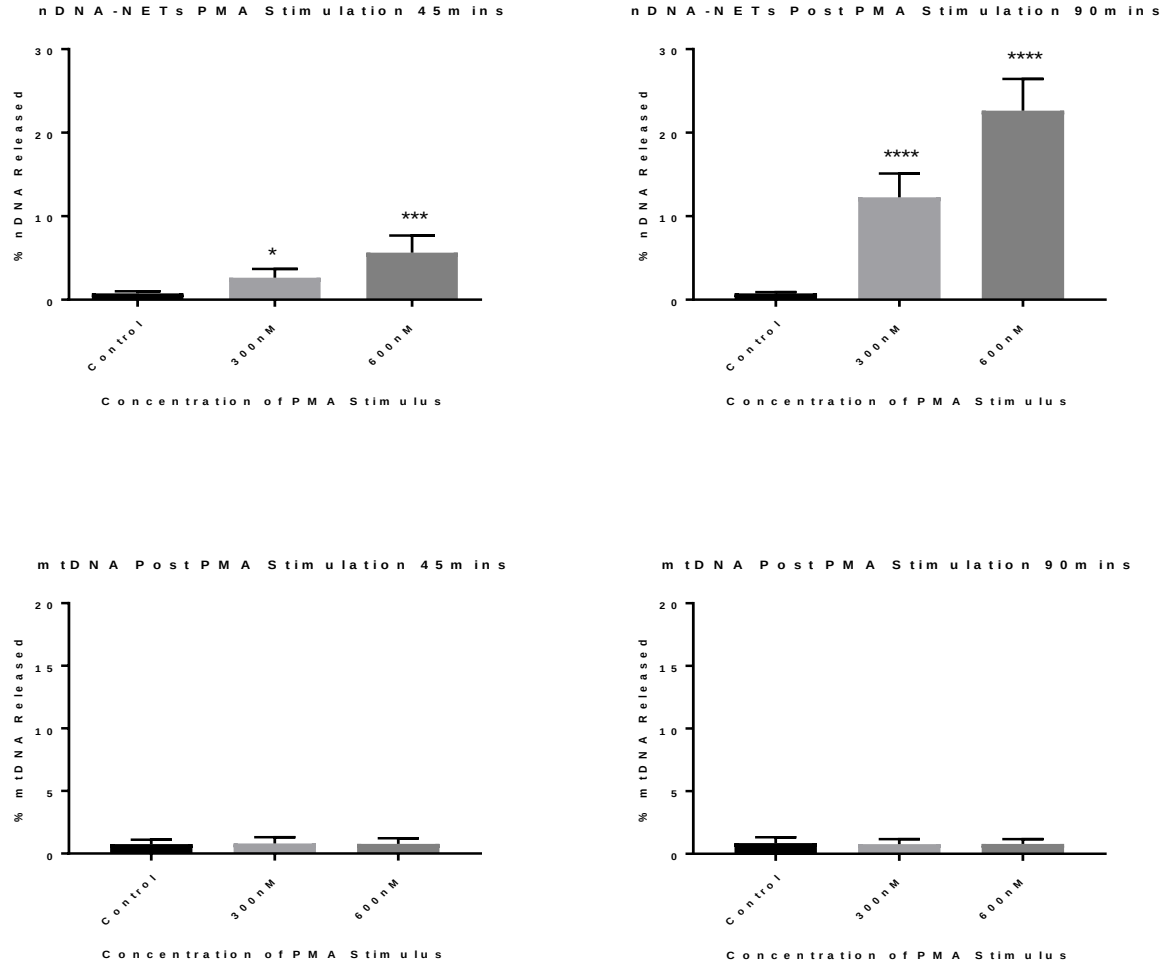
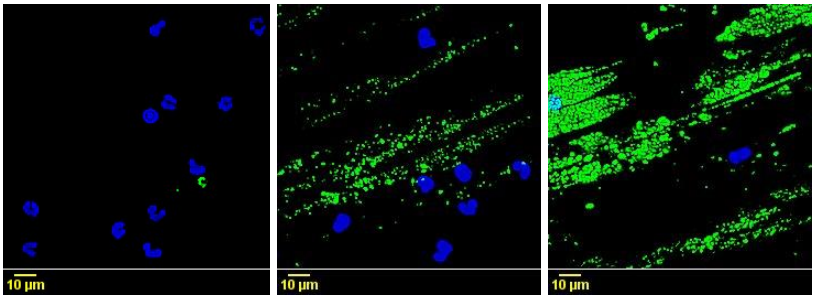


Figure 4: (Images for PMA NETs)

Control 45mins **300nM 45mins** **600nM 45mins**



Control 90mins **300nM 90mins** **600nM 90mins**

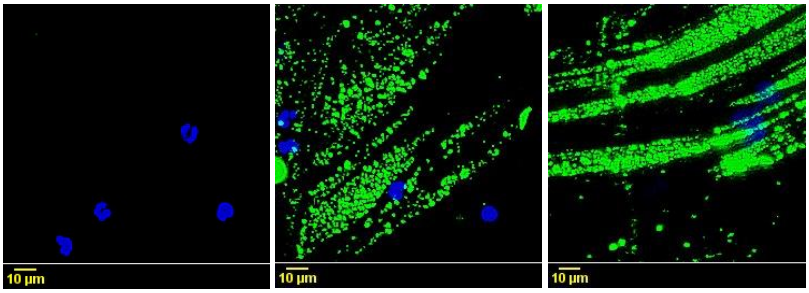
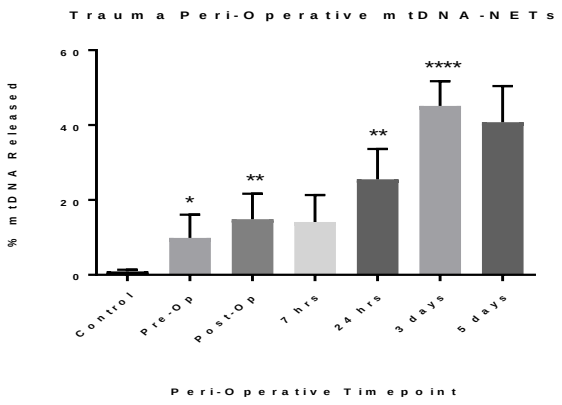


Figure 4 Legend:
This figure demonstrates the nDNA-NETs formed in response to PMA stimulus after incubation with either 300nM or 600nM concentration for 45mins and 90mins respectively. The extracellular NET DNA can again be seen stained green with Sytox Green® and viable neutrophils are stained blue with Hoechst 33342.

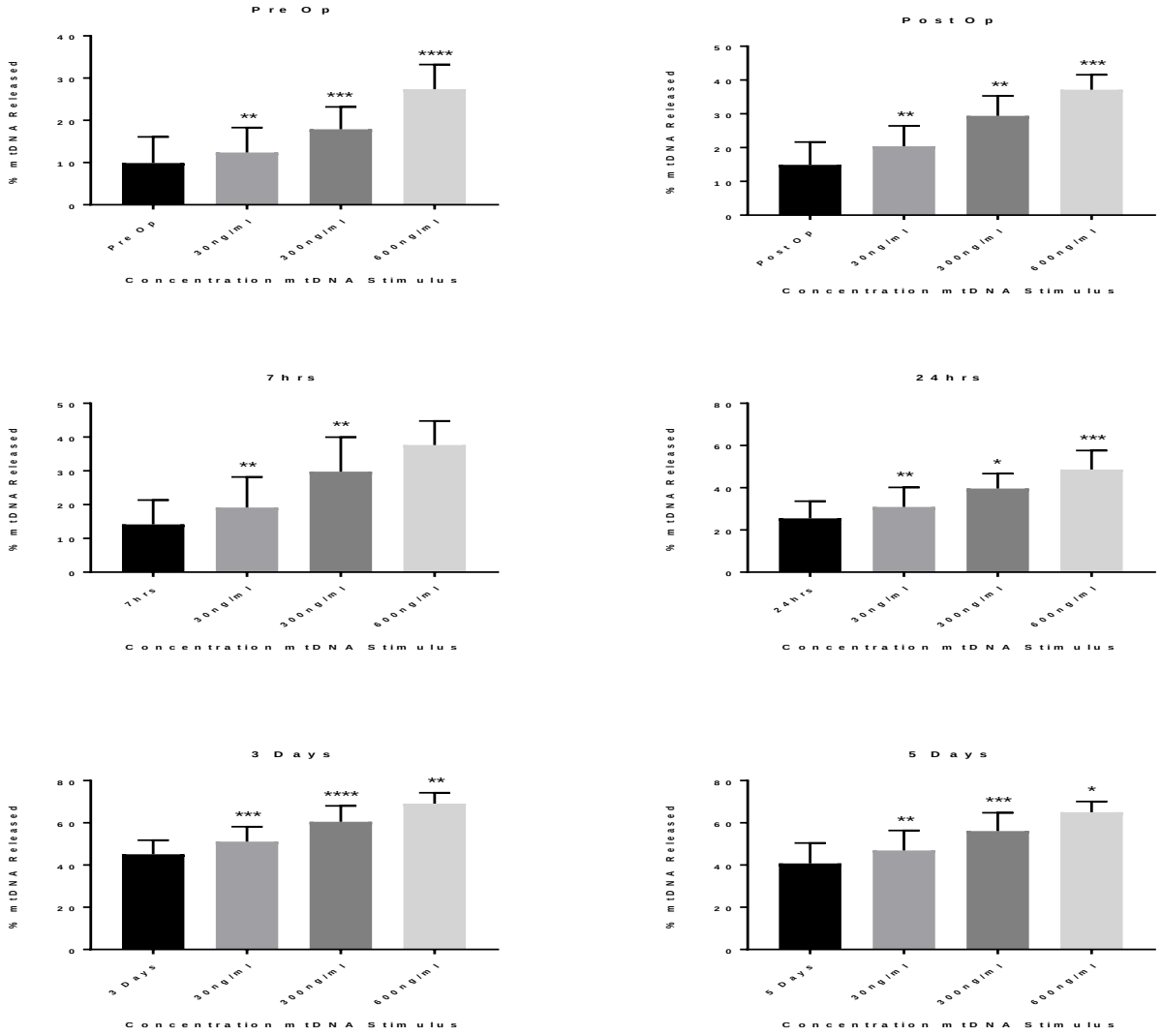
In the trauma group, significantly more NETs were formed at all peri-operative timepoints when compared to healthy unstimulated controls (ANOVA p<0.0001, Tukey's Test; Pre-Op p=0.046, Post-Op p=0.007, 7hrs p=0.014, 24hrs p=0.001, 3days p=<0.0001, 5days p=<0.0001). NETs in the trauma group were again almost exclusively composed of mtDNA (nDNA<1.0%, data not shown). Significant differences existed in mtDNA-NET formation between time points using Tukey's Test; pre-op vs. post-op p=0.0013, 7hrs vs. 24hrs p=0.003 and 24hrs vs. 3 days p<0.0001 (See Figure 5). The formation of mtDNA-NETs peaked at 3 days post-operatively.

Figure 5:



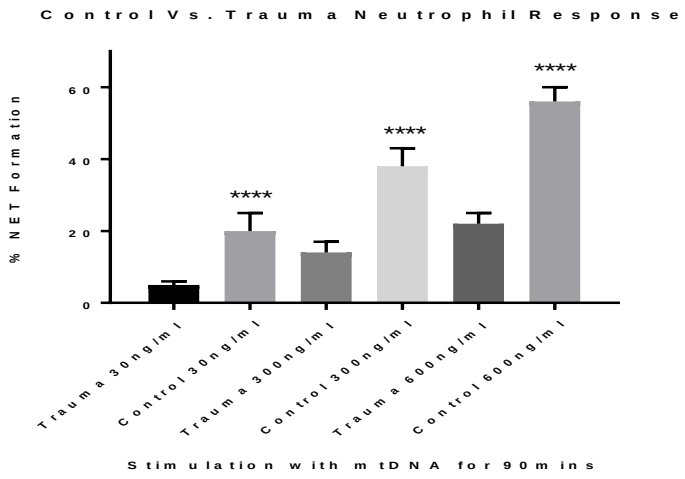
Subsequent stimulation of trauma neutrophils with mtDNA at 30ng/ml, 300ng/ml and 600ng/ml isolated at the 6 peri-operative timepoints resulted in significantly increased mtDNA-NET formation in a dose dependent fashion (ANOVA $p<0.0001$, Tukey $p<0.05$)

Figure 6:



The mtDNA-NET formation response to stimulation with mtDNA in neutrophils isolated from the trauma cohort pre-op and at all subsequent perioperative timepoints was significantly reduced, when compared to increases observed to the same stimuli in healthy neutrophils when incubated for 90 minutes. Unpaired t-test with Welch's correction Control 30ng/ml vs: Trauma 30ng/ml $p<0.0001$, Control 300ng/ml vs. Trauma 300ng/ml $p<0.0001$ and Control 600ng/ml vs. Trauma 600ng/ml $p<0.0001$ (See Figure 7).

Figure 7:



Discussion:

Our experiments showed that mtDNA induces NET formation in a dose and time dependent fashion (Figure 1.) when healthy volunteers' neutrophils are exposed to clinically relevant titres of mtDNA. Furthermore, the NETs formed in response to mtDNA stimulation were composed of mtDNA (mtDNA-NETs) as confirmed by our qPCR analysis of the NET-DNA composition.

MtDNA-NETs have been demonstrated with different stimuli, after incubation with GM-CSF and either complement fragment C5a or LPS (9). Interestingly the authors of this work demonstrated that cell viability was not compromised in response to the observed mtDNA-NET formation. In our recent study we demonstrated that mtDNA-NETs were formed after major orthopaedic trauma and subsequent surgery (12). This raises the question that mtDNA-NET formation after injury could be a self-propagating process, with mtDNA released through NETosis consequently activating quiescent neutrophils, stimulating them to also produce mtDNA-NETs.

This data suggests that mtDNA is indeed a highly potent initiator of NET formation even compared to NET induction using PMA as the primary stimulus. In our current study PMA induced comparable NETosis response in neutrophils but these NETs primarily consist of nDNA. It is accepted that PMA induced NETosis is an NADPH oxidase dependent process (18). It appears that mtDNA triggers an alternative intracellular signalling cascade in neutrophils resulting in mtDNA-NETs compared to PMA activation which produces nDNA-NETs. Perhaps this could in part be explained by the fact while PMA (19, 20) and mtDNA (20) activate Nuclear Factor kappa B (NF-κB) they do so in distinctly different ways. PMA activates protein kinase C (PKC) (19), triggering a cascade resulting in subsequent activation of (NF-κB) through an inhibitory kappa B Kinase epsilon (IKKε) dependent pathway (20). MtDNA can result also in NF-κB activation via TLR9 and subsequent reduction in IKKα expression (21). The exact intracellular signalling mechanisms involved in NET formation (whether from nDNA or mtDNA) are far from fully understood (22). Other authors have also demonstrated that mtDNA has the ability to stimulate NET formation following trauma (23). The authors found that NETs produced in response to mtDNA stimulus was mediated through a TLR9 dependent pathway. This is the first study that has demonstrated that NETs are formed in a dose and time dependent fashion in response to mtDNA stimulus and also, most notably that the NETs formed are composed of mtDNA.

One element of the observed mtDNA-NET response is particularly striking and that is the apparent reduced number of mtDNA-NETS produced by trauma neutrophils when compared to those isolated from healthy volunteers. A possible explanation for this is that trauma neutrophils have less mtDNA available for release, if they have already undergone mtDNA NETosis and less likely to be stimulated further.

Our results could provide a possible explanation for the sustained presence of mtDNA in circulation after major trauma and trauma surgical interventions. It is reasonable to consider the injury (direct trauma and hypoperfusion related cell death) associated mtDNA presence as primary source and the NETosis associated circulatory mtDNA presence as secondary source. While the primary source (injury) is difficult to modify, the secondary source of proinflammatory mtDNA could be modified through better understanding of postinjury NETosis induced by mtDNA.

The main limitation of this study is the ex vivo nature of our experiments and the fact that our blood samples were not from the microcirculation where the actual organ injury occurs. Clinically relevant models and potential bed-side visualisation of microcirculatory changes including cell-cell interactions and NETosis could provide further relevance and practical proof to these findings.

In conclusion, our ex vivo investigations showed the dose and time dependent NETosis of isolated trauma patient and healthy PMN in response to previously measured clinically relevant concentrations of circulating mtDNA. In response to trauma and mtDNA neutrophils produce mtDNA-NETs rather than nDNA-NETs commonly seen after artificial pharmacological stimuli such as PMA. The capacity of post-injury neutrophils to mount a further response to a subsequent mtDNA stimulus is reduced compared to healthy control neutrophils.

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Chapter 5:

Reduced DNase Activity In response to High Post-Injury Concentrations of Mitochondrial DNA

McIlroy DJ, Minahan K, Keely S, Lott N, Smith DW, Hansbro PM, Balogh ZJ, **Reduced DNase Enzyme Activity In Response to High Post-Injury Concentrations of Mitochondrial DNA Provides a Therapeutic Target for SIRS.** *J Trauma and Acute Care Surgery* (under review).

Introduction

This final paper looked to assess DNase activity in the plasma using an assay that quantified DNase activity across the range of isotypes found in plasma (Tamkovitch et al. 2006). The same peri-operative timepoints in trauma patients undergoing surgery as had been used previously. The activity of trauma DNase was then compared to that in healthy volunteers.

I also used qPCR on the same cell-free plasma samples to measure the nDNA and mtDNA concentrations present. We changed the methodology for performing qPCR after finding substantiating evidence that qPCR could be reliably performed directly on cell-free plasma, without the need for prior DNA isolation and purification (Breitbach et al. 2014). By removing the need for DNA isolation and purification, this greatly increased my efficiency for downstream qPCR analysis of mtDNA and nDNA concentrations. This is particularly useful when considering bench-to-bedside application of research, where results are required in a clinically relevant timeframe, reducing processing time from 4hrs to 2hrs.

The primary finding was that DNase activity was greatly reduced in trauma subjects when compared to healthy controls. This was despite highly elevated concentrations of pro-inflammatory mtDNA. I also found a positive correlation on this occasion with mtDNA levels and post-injury SIRS, which failed to reach significance in the pilot. We feel this is most likely due to the much larger of trauma subjects enrolled in this study n=103 vs. n=35 in the initial pilot. This presented a potentially exciting future treatment target for post-injury and post-operative inflammation. Exogenous DNase administration could be used as a treatment for SIRS aimed at digesting free mtDNA and mtDNA-NETs formed in response to injury and subsequent surgery.

This paper was accepted for podium presentation at the 75th American Association for the Surgery of Trauma (AAST) Annual Meeting in Waikoloa, Hawaii in September 2016.

The manuscript is currently under review for publication in the Journal of Trauma and Acute Care Surgery (revision of the manuscript based on the reviewers suggestions).

REDUCED DNASE ENZYME ACTIVITY IN RESPONSE TO HIGH POST-INJURY MITOCHONDRIAL DNA CONCENTRATION PROVIDES A THERAPEUTIC TARGET FOR SIRS

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Conflicts Of Interest: None declared

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ABSTRACT:

BACKGROUND: Cell free mitochondrial DNA (mtDNA) is pro-inflammatory and has been detected in high concentrations in trauma patients' plasma. Deoxyribonuclease (DNase) is the free plasma enzyme responsible for the digestion of extracellular DNA. The relationship between mtDNA and DNase after major trauma is unknown. We hypothesized that DNase activity would be elevated after injury and trauma surgery, and would be associated with high concentrations of extracellular DNA.

METHODS: 2-year prospective study was performed on 103 consecutive trauma patients (Male 81%, Age: 38 IQR 30-59 years; ISS: 18 IQR 12-26) who suffered high energy blunt force trauma with pelvic and femoral fractures and subsequently underwent open reduction - internal fixation or intramedullary nailing. Blood was collected at 5 peri-operative time points (pre-op, post-op, 7hrs, 24hrs and 3 days post-operatively). Healthy control subjects (n=20) were also sampled. Cell free mtDNA and nuclear DNA(nDNA) was measured using quantitative polymerase chain reaction. DNase was also assayed in the same plasma samples.

RESULTS: Increased levels of mtDNA (from Pre-op 163±86ng/ml to 3 days 282±201ng/ml $p<0.0001$) and nDNA (from pre-op 28±20ng/ml to 3 days 37±27ng/ml $p<0.037$) were present in trauma patients at all peri-operative time points compared to healthy controls (mtDNA: 4±2ng/ml; nDNA: 10±5ng/ml). DNase activity was lower in the trauma cohort (from Pre-op 0.059±0.033U/ml to 3 days 0.082±0.044U/ml $p<0.0001$) compared to healthy controls (DNase: 0.174±0.031U/ml). There was no correlation between DNase and peri-operative DNA concentrations. Elevated mtDNA (but not nDNA) correlated with the development of SIRS ($p=0.026$) but not MOF.

CONCLUSIONS: The significant perioperative elevation in plasma free MtDNA concentration is associated with the development of SIRS. The fact that increased cell free DNA concentrations present with significantly lower than healthy control DNase activity

suggests a potential therapeutic opportunity with DNase administration to modulate post-injury severe SIRS

LEVEL OF EVIDENCE:

Level II: Prospective comparative study.

KEYWORDS – SIRS, MOF, mtDNA, NETs, Trauma

BACKGROUND:

Mitochondrial DNA (mtDNA) has been demonstrated to be a potent endogenous stimulator of the innate immune system and it has been termed an "alarmin" (1). Alarmins are endogenous factors that are released from host cells that can generate a potent innate inflammatory response at physiological concentrations (1). MtDNA is known to be released in high titres following initial injury (2) and also after orthopaedic trauma surgery (3). More recently our group looked at the progressive, sustained nature of mtDNA release in trauma patients who underwent major orthopaedic surgery in the immediate 5 days following surgery (4).

Deoxyribonuclease I is the free plasma enzyme responsible for the digestion of 90% of circulating extracellular DNA (5). Other enzymes also contribute to DNA degradation in the circulation including de-oxyribonuclease II, phosphodiesterase I and DNA hydrolysing autoantibodies (5). Given that the degradation of DNA is a multi-enzymatic process, the enzymes involved will be collectively referred to as "DNase". The relationship between circulating DNase activity and cell-free DNA has never been demonstrated in trauma patients. If DNase activity in the circulation is augmented in the post-injury and peri-operative trauma surgical setting it could play a protective role by digesting pro-inflammatory extracellular mtDNA preventing excessive innate immune activation and reducing

development of systemic inflammatory response syndrome (SIRS) and subsequent multiple organ failure (MOF).

We hypothesised that DNAse activity would be elevated in response to high circulating levels of cell-free DNA released following injury and subsequent surgery.

METHODS:

Research Compliance:

Ethical approval for the study was obtained from Hunter New England Human Research Ethics Committee. All blood samples were obtained with informed consent for sampling, archiving and further analysis in accordance with the aims of this study.

Patients and Blood Samples:

After gaining informed consent, blood samples were obtained from 20 healthy volunteers identified through the Hunter Medical Research Institute volunteer registry. Blood was sampled from 103 trauma patients and at 5 consecutive time points in the peri-operative period; immediately pre-op and then 0 hrs, 7hrs, 24hrs and 3 days post-operatively. The timeframes were based on previous studies examining immune responses to orthopaedic trauma interventions (6, 7); we also routinely use these time points for sample collection in our clinical immune monitoring and human inflammatory response to trauma surgery projects. Inclusion criteria were those patients over 16 years of age, and sustained major musculoskeletal injuries requiring standardised orthopaedic trauma surgical interventions (intramedullary femoral and tibial nailing, pelvic and acetabular fractures requiring open reduction and internal fixation).

Plasma was separated from 5mls of whole blood by spinning at 1400rpm for 10 mins at 4^0C , then at 12000rpm for a further 10 mins to generate cell free plasma (8). Plasma was obtained from healthy controls and from the trauma cohort (at all time points) and frozen at -80˚C prior to analysis. 200µls of cell-free plasma sample was aspirated and used as cell-free plasma template for quantitative polymerase chain reaction (qPCR) and DNase assay testing.

Generation of nDNA / mtDNA Standards:

Nuclear DNA:

DNA was isolated and purified from a buccal cheek swab taken from a healthy volunteer before suspending the cells in 200µls of molecular grade H_2O before extracting and purifying the DNA using a blood DNAeasy extraction kit (Qiagen™ Chadstone, VIC, Australia) according to the manufacturer's instructions. The final concentration of extracted DNA was checked using spectrophotometry at A260nm. DNA extracted was 99.9% nDNA after running both ND3 (mtDNA primers) and GAPDH (nDNA primers) against the DNA extraction stock solution. Serial dilutions were then performed to generate standards of known concentration using spectrophotometry UV absorbance at 260nm, with qPCR performed to generate a standard curve using GAPDH primer set.

Mitochondrial DNA:

Purified DNA from a cell-free trauma plasma sample was then amplified using the Human Mitochondrial REPLI-g kit (Qiagen™ Chadstone, VIC, Australia) to generate purified human mtDNA. Briefly, DNA was denatured at 75^0C for 5mins. REPLI-g human mtDNA polymerase was added and an isothermal multiple displacement amplification proceeded

overnight at 33°C for 8hrs. DNA was then quantitated using PicoGreen assay (Invitrogen) which uses an ultra-sensitive fluorescent stain that specifically binds double stranded DNA (9). Spectrophotometry using UV at 260nm was unreliable with the REPLI-g amplified mtDNA product due to nucleic acid components in the buffering solution used. Highly concentrated mtDNA stock solution (5µM) was produced which was then used to generate mtDNA standards and to construct a standard curve using qPCR and ND3 (mtDNA primers). The purity of mtDNA product was confirmed by performing qPCR with GAPDH (nDNA primers) and ND3 (mtDNA primers). Nuclear DNA was undetectable confirming 100% pure mtDNA product.

Direct Quantitative PCR Protocols Using Cell-Free Plasma:

Dilute plasma as a direct template for qPCR was used, instead of DNA extraction and purification which reduces actual DNA yield (10). Plasma was diluted 40-fold with molecular grade nuclease free water prior to use in the qPCR assay. 5 µls of the diluent was then used for each qPCR reaction. The 5uls of diluted DNA was added to 7µls of SYBR green master mix (SensiFast™, Biolines®, Alexandria, NSW, Australia). The real-time qPCR analysis was performed using Applied Biosystems Real-Time ViiA 7 analyser (Applied Biosystems™, Life Technologies™, Foster City, CA, USA). Mitochondrial DNA primers were designed and synthesised for ND3, nuclear DNA primers for GAPDH (Geneworks™ Hindmarsh, SA, Australia) Samples were screened for bDNA using primers targeting the bacterial 16S ribosomal subunit, the presence of which could indicate sepsis. All primers were previously optimised during our earlier pilot study (4).

DNase Assay:

DNase activity was assayed 10µls plasma (1:40 dilution with nuclease free water as described above) using DNase detection kit (Jena Bioscience GmbH, Jena, Germany) in line with the manufacturer's instructions. (11) The kit allowed the assessment of all DNAse activity in the sample through conversion of substrate to fluorescent tagged product.

Positive controls to assess DNase activity and ability to degrade DNA were also performed on standard solutions of mtDNA and nDNA. After incubation with DNase both nDNA and mtDNA levels were undetectable on subsequent qPCR analysis. Subsequently aliquots of plasma dilutions were also incubated with DNase and again subsequent qPCR yielded undetectable nDNA and mtDNA levels.

RESULTS:

Study population demographics:

The one hundred and three trauma patients included in the study had a median age of 38 years (IQR 30 - 59 years) with a median injury severity score (ISS) of 18 (IQR 12 - 26). Eighty three of the patients (81%) were male and all had suffered high energy blunt trauma resulting in pelvic, acetabular fractures requiring open reduction – internal fixation or femoral shaft fractures requiring intramedullary nailing. Mean initial base deficit (BD) was -3.3 ± 4.1 mEq/l. Twelve patients had an admission SBP<90mm/Hg. Thirty four patients were admitted to ICU post-operatively with a median length of stay (LOS) of 4 days (IQR 2 – 7 days). Twenty two developed SIRS during the study period. Six patients developed MOF. Thirteen patients required a blood product transfusion peri-operatively. Total median LOS in hospital was 7 days (IQR 6 - 18).

Twenty healthy controls were enrolled with a median age 32 years (IQR 28 – 52 years). Sixteen were male (80%).

Data Analysis:

Data are presented as mean ± standard deviation for parametric variables and as median / interquartile range (IQR) for non-parametric variables. Data were visually examined for skew. Hypothesis testing of changes in DNA concentrations between the time periods were performed using the Friedman Test, a non-parametric equivalent of repeated measures ANOVA with Dunn's Post-hoc multiple comparison test. Differences between outcomes (e.g SIRS Vs. No SIRS and MOF Vs. No MOF) were compared using the Wilcoxon-Signed Rank Test. DNA levels in the trauma cohort at different time points and healthy controls were compared using the Kruskal-Wallis test, with Dunn's Post-hoc.

Changes in Peri-Operative DNA Concentration:

The plasma mtDNA concentration was significantly elevated compared to pre-op at all post-operative time points peaking at 3 days (Friedman Test: Pre-op 163±86ng/ml, immediately post-op 232±118ng/ml $p<0.05$, 7hrs 253±154ng/ml $p<0.05$, 24hrs 269±200ng/ml $p<0.01$, 3 days 282±201ng/ml $p<0.0001$). The trauma cohort's plasma mtDNA concentration was significantly higher compared to healthy controls (4±2ng/ml) at all 5 peri-operative time points. (Kruskall-Wallis Test $p<0.0001$) (Figure A).

The plasma nDNA concentration did not change over the peri-operative period (pre-op 28±20ng/ml, immediately post-op 43±32ng/ml, 7hrs 23±11ng/ml, 24hrs 31±25ng/ml, 3 days 37±27ng/ml) but was higher than the healthy controls' (10±5ng/ml) at all post-operative time points. (Kruskall-Wallis Test $p<0.037$) (Figure B).

There was no detectable bDNA in any patient included in the trauma cohort at any time point or healthy controls (data not presented).

Changes in Peri-Operative DNase Activity:

DNase activity was significantly lower in the trauma cohort at all time points (Pre-Op 0.059±0.033U/ml, Immediately Post-Op 0.063±0.037U/ml, 7hrs 0.063±0.036U/ml, 24hrs 0.071±0.036, 3 days 0.082±0.044U/ml) than in healthy controls (0.174±0.031U/ml) $p<0.0001$ (Kruskall-Wallis Test). (Figure C)

MtDNA Levels in SIRS:

MtDNA concentrations were found to be elevated in SIRS (but not nDNA) at the 3 day time point $p<0.026$ (Wilcoxon Matched Pair Signed Rank Test). (Figure D).

DISCUSSION:

The most striking and novel finding in this study is the greatly reduced activity of circulating DNase in the trauma cohort compared to those present in healthy controls. We observed this pre-operatively and it remained persistently low throughout the perioperative period despite consistently elevated concentrations of mtDNA and nDNA. This finding refuted our hypothesis. This study demonstrates repeatedly (4) but on a larger dataset the prolonged elevation of mtDNA following injury and subsequent surgery, peaking at 3 days post operatively. In contrast to mtDNA, there was no significant increase in peri-operative levels of nDNA following surgery in the trauma group. The profound difference between increased

amounts cell free mtDNA compared to nDNA after surgery is particularly surprising in the context of the mitochondrial genome being 200,000 times smaller than the nuclear genome (12).

The potently pro-inflammatory effects of mtDNA are well characterised with its activation neutrophils through TLR-4 (13) and TLR-9 (3), however this is the first time that mtDNA has been positively associated with the manifestation of post-surgical SIRS after major trauma, indicating a potentially clinically significant role in the development of deleterious inflammatory sequelae. MtDNA has already been linked with the development of post-injury MOF (14).

When considering the possible source of mtDNA in our previous study, no correlation was found between markers of tissue injury (4). This led us to consider the possibility of mtDNA release as part of the post-injury, post-operative inflammatory response (14).

We proposed that neutrophils could be a major source of high circulating levels of mtDNA following trauma and subsequent surgery by producing neutrophil extracellular traps (NETs) (15). Neutrophil extracellular traps (NETs) were first characterised in 2004 and structurally are diffuse extracellular filamentous chromatin scaffold (16). The primary function of NETs was thought to be extracellular bacterial killing in sepsis and they were believed to be composed of predominantly nuclear DNA (nDNA) (16). Under certain conditions NETs have been shown to be exclusively composed of mtDNA (17). Our group then studied NETosis following major trauma and subsequent surgery and demonstrated that NETs were produced and were composed of mtDNA (18). DNase is the principle enzyme responsible for degrading NETs in the circulation (16).

DNase treatment was recently shown to reduce the severity of MOF and reduced mortality in a mouse model of sepsis (19). There have been other clinical conditions where DNase treatment has been shown to be of benefit in humans. It has been given as an inhaled

preparation to help reduce viscosity of sputum in cystic fibrosis (20). It has also been used to good effect via endotracheal tube in ventilated neonates to treat atelectasis, with improvement in clinical and radiological findings (21). DNase has also been used through daily intravenous administration at a substantive dose in a randomized human Phase Ib placebo controlled trial for the treatment of systemic lupus erythematous (SLE) (22). No adverse effects were observed in the DNase treatment groups in the 40 day study period (22). Evidence of safe intravenous administration without adverse effect in SLE (whilst a numerically small study) raises the real possibility of potential therapeutic use in the trauma population. The strengths of this study are that it compares a substantial number of prospective consecutive blunt force trauma patients who underwent standardised orthopaedic trauma surgery for pelvic and femoral fractures to a healthy control group. By comparing both mtDNA and nDNA against fluctuations in DNase activity it allowed the comparison of these variables in a post-injury, perioperative setting for the first time. This allows greater insight into the dynamics at play with respect to extracellular DNA release and clearance in a trauma setting, and how this may influence post-injury inflammation.

Limitations include that the control group were slightly younger than the trauma cohort. The study also could have included larger numbers to demonstrate any potential relationship between mtDNA and MOF as we believe it was still underpowered in this regard.

By finding DNase activity to be low indicates the ability to clear pro-inflammatory NETs and circulating mtDNA is impaired following trauma and for days after major orthopaedic trauma surgery. This potentially increases susceptibility to inflammatory sequelae such as SIRS and MOF.

Exogenous DNase treatment may represent a potential new and innovative way for treating the deleterious inflammatory effects of high circulating mtDNA after trauma and subsequent surgery, potentially reducing SIRS and subsequent MOF.

Author Contribution Statement:

DJM contributed to study design, performed all experimental work and wrote the manuscript. KM contributed to study design, and provided DJM with principle laboratory support. SK contributed to study design, and critically appraised the manuscript. NL collected patient blood samples and maintained a database detailing patient events and outcomes. PH and DW critically appraised the manuscript. ZJB contributed to study design, provided funding and was the chief investigator in the study.

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Tables and Figure Legend:

Table 1): Primers utilised for quantitative polymerase chain reaction

Figure A): Peri-operative change in mtDNA concentration

Figure B): Peri-operative change in nDNA concentration

Figure C): Peri-operative DNase activity vs. healthy controls

Figure D): Peri-Operative mtDNA concentration and relationship to SIRS

Table 1. Primers utilised for real-time qualitative polymerase chain reaction:

Target Gene	Sequence
Human ND3 (mtDNA)	5'-ACTACCACAACTCAACGGCT-3' (Forward) 5'-GCGGGGGATATAGGGTCGAA-3'(Reverse)
Human GAPDH (nDNA)	5'-AGGGCCCTGACAACTCTTTT-3' (Forward) 5'-TTACTCCTTGGAGGCCATGT-3' (Reverse)
Bacterial 16S (bDNA)	5'- CGTCAGCTCGTGTTGTGAAA-3′ (Forward) 5′-GGCAGTCTCCTTGAGTTCC-3′ (Reverse)

Figure A:

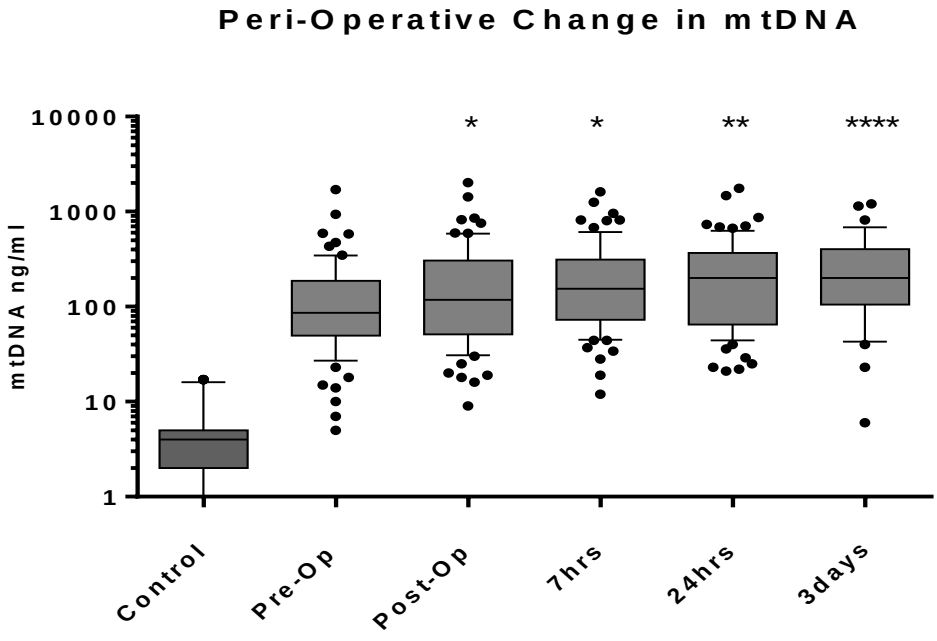

Figure A:

Free plasma mtDNA concentration of the trauma cohort (n=103) was significantly higher than healthy control subjects (n=20) (Kruskal-Wallis test p=<0.0001, Dunn Post-hoc test p=<0.05) at all peri-operative time points. There was a significant increase in mtDNA concentration measured between pre-op vs. all subsequent post-operative timepoints using Friedman's test (with Dunn's Post-hoc multiple comparison test) for non-parametric data (Post-op p<0.05, 7hrs p=<0.05, 24hrs p<0.01, 3 days p<0.0001)

Figure B:

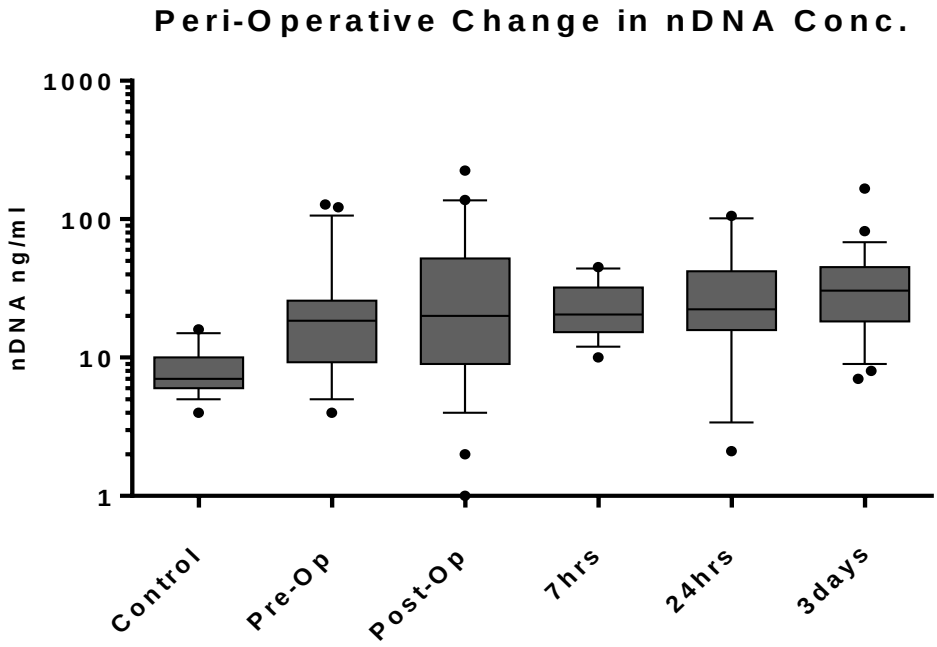

Figure B:

Free plasma nDNA concentration of the trauma cohort (n=103) was significantly higher than healthy control subjects (n=20) (Kruskal-Wallis p=0.0037, Dunn Post-hoc test p=<0.05) at all peri-operative time points. There was no statistically significant change in nDNA concentration measured between any time points in trauma patients' plasma. Friedman's test was applied for non-parametric data comparing changes in nDNA (omnibus p-value =0.09).

Figure C:

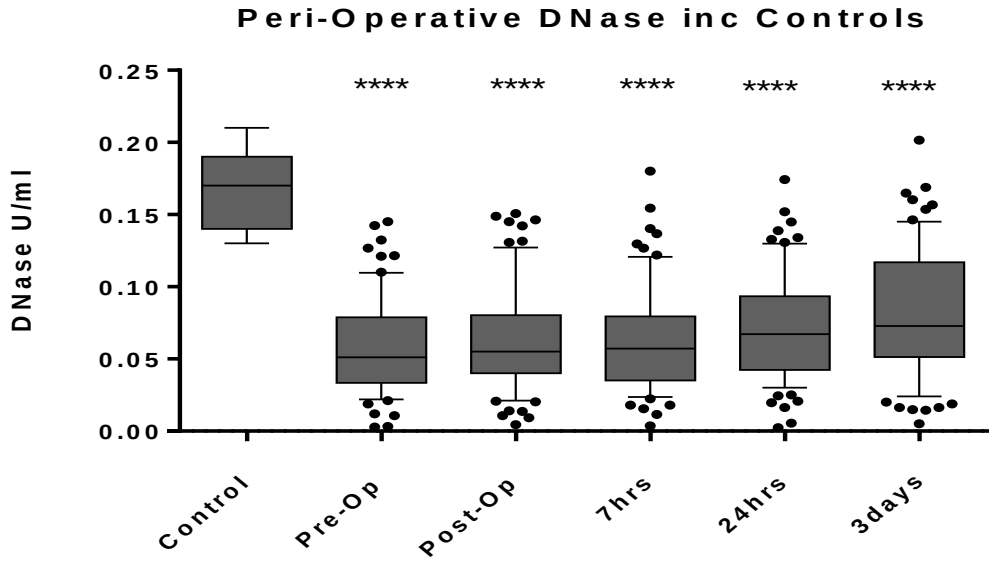

Figure C:

DNAse activity was significantly lower in the trauma cohort (n=103) compared to controls (n=20) at all peri-operative timepoints ($p<0.0001$ Kruskall-Wallis Test, $p<0.05$ Dunn's Post Hoc Test).

Figure D:

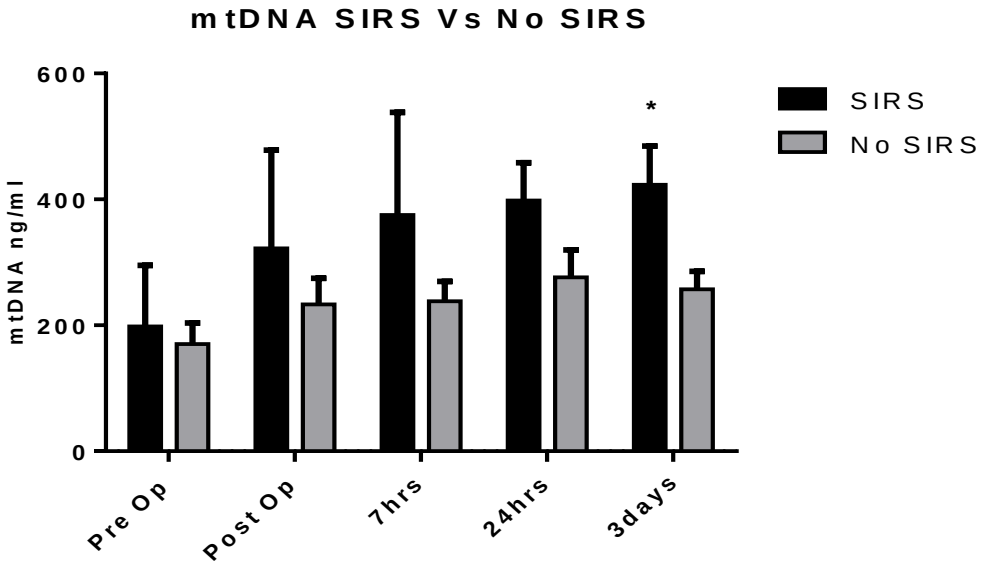

Figure D:

Free plasma mtDNA was significantly higher in SIRS Vs. No SIRS at 3 days post op $p<0.026$ (Wilcoxan Matched Pair Signed Rank Test.)

Chapter 6:

Discussion and Conclusion

Mitochondrial DNA has previously been demonstrated to be released in large quantities following injury (Zhang et. al 2010) and subsequent fracture fixation surgery (Hauser et al. 2009). In the present work we have extended these findings and demonstrated, in contrast to other groups (Yamanouchi et al. 2013, Sursal et al. 2012), that mtDNA released following trauma and subsequent surgery has no positive correlation with other markers of tissue injury. For example, there was no correlation with either CK, an indicator of musculoskeletal damage, or the liver enzymes LDH and AST, which are indicators for inflammatory liver injury (McIlroy et al. 2015). This means that the cell free mtDNA was unlikely to be solely related to cell necrosis. We also demonstrated the elevated mtDNA levels were sustained for the entirety of the 5-day post-operative observation period. While our initial hypothesis posited mtDNA levels would increase and then drop rapidly after surgery, our findings did not support this assumption, with the increased mtDNA levels not only sustained following surgery, but actually increasing to 5 days post-operatively. Interestingly the amount of mtDNA measured in plasma after surgery was directly proportional to the magnitude of surgery undertaken. A positive correlation between the amount of mtDNA released and the incidence of SIRS was identified in the study population. There was a trend towards higher concentrations in patients who developed MOF, but the correlation failed to reach statistical significance, most likely due to the relatively low incidence of MOF in our cohort. We consider these initial findings as being indicative of a potent inflammatory response that constitutes the primary source of the sustained mtDNA release we observed. Previously, other groups have demonstrated mtDNA concentrations were indeed elevated for up to 28 days after the initial injury (Lam et al. 2004). This concept of pro-inflammatory mtDNA provoking cells of the innate immune system over a prolonged time course, primarily neutrophils through interaction with TLR 9 (Hauser et al.2010), has added to the evolving concept of post-injury persistent inflammatory, immunosuppressed catabolic syndrome (PICS) (Rosenthal et al. 2015). PICS is characterised by a loss of muscle mass through protein catabolism, which is refractory to nutritional supplementation and an abundance of immature granulocytes which are dysfunctional, predisposing major trauma survivors to increased risk of late bacterial sepsis and death from MOF months after initial injury and surgery.

We hypothesised that different forms of cell death could potentially lead to the high concentrations of mtDNA observed after injury and surgery. Although others have demonstrated that under certain conditions NETs could be composed of pure mtDNA (Yousefi et al. 2009). This contradicts work suggesting NETs, in the context of sepsis, are composed of nDNA (Fuchs et al. 2007). In contrast to the popularly held belief that NETosis is a form of cell suicide (Brinkmann et al. 2007), Yousefi and colleagues also demonstrated neutrophils could remain viable after forming mtDNA-NETs. This was the genesis of our hypothesis that neutrophils undergoing NETosis after injury and subsequent surgery could potentially be driving the high concentrations of mtDNA we initially observed (Balogh et al. 2013). No group had definitively demonstrated that NETs were produced after injury and subsequent surgery. The possible existence of post-injury NETs had been postulated by one group, that equated the high concentrations of cell-free DNA to the existence of NETs even though the conformational NET structure was not demonstrated or substantiated with microscopy (Margraf et al. 2008). We have now demonstrated NETs form initially post injury and then at peri-operative time points (McIlroy et al. 2015), up to 5 days post-op (McIlroy et al. 2014). We found that the NETs that are formed after trauma, whether as a result of initial injury or subsequent surgery, were indeed composed of mtDNA (mtDNA-NETs). We confirmed this with qPCR using primers specifically targeting nDNA and mtDNA genes on isolated NETs from trauma patients. MtDNA-NETs persisted in the circulation of trauma patients who underwent surgery for the full 5-day perioperative observation period. The possibility of transient bacteraemia or sepsis as a driver of NET formation after trauma or elective surgery was excluded by screening patient plasma using qPCR targeting bDNA. No amplification product was found for any patient, indicating bacteria were not the source of NET DNA and further supporting our mtDNA hypothesis.

One group has shown that mtDNA released by injury could indeed trigger NETosis via a TLR9 mediated pathway (Itagaki et al. 2015). To confirm and further extend this work we conducted a series of experiments where we exposed both healthy volunteer neutrophils and trauma neutrophils to clinically relevant concentrations of purified human mtDNA as determined in our previous work (McIlroy et al. 2015). PMA, a known potent stimulator of NETosis, was used as a positive control (Brinkmann et al. 2007, Fuchs et al. 2007). The NETs formed in response to mtDNA stimulation were formed from mtDNA. Interestingly, trauma neutrophils generated less mtDNA-NETs compared to healthy control neutrophils. We postulated that this may be due to prior exposure of neutrophils to mtDNA in the post-injury circulation where

NETosis may already have been triggered. In contrast, NETs formed in response to PMA stimulation were composed of nDNA and not mtDNA. When considering the reason for this it is critical to note that PMA and mtDNA act through very different receptors, triggering signalling cascades that, while sharing some similarities, ultimately end in a different output signal. PMA activates protein kinase C (PKC) (Wolfson et al.1985), triggering a cascade resulting in subsequent activation of Nuclear Factor kappa B (NF-κB) through an inhibitory kappa B Kinase epsilon (IKKε) dependent pathway (Peters et al. 2000). MtDNA can also result in NF-κB activation via TLR9 and subsequent reduction in IKKα expression (Zhang et al. 2014). Itagaki and colleagues had also demonstrated that by blocking TLR9 they prevented mtDNA from triggering NETosis in trauma and healthy neutrophils. (Itagaki et al. 2015). Our work has now shown mtDNA is a mtDNA-NET trigger in both healthy and trauma neutrophils, as demonstrated by our downstream molecular analyses of the NETs using specific nDNA and mtDNA gene targets. Another group has demonstrated mtDNA-NETs can be formed under sterile inflammatory conditions in SLE (Wang et al. 2015) adding further weight to our experimental findings and the earlier initial discovery of mtDNA-NETs (Yousefi et al. 2009).

We have demonstrated a positive correlation with mtDNA levels and deleterious inflammation in SIRS (McIlroy et al. 2015), the prevalence of mtDNA-NETs after injury and surgery (McIlroy et al. 2014), and the ability of mtDNA to stimulate the release of further mtDNA-NETs. We therefore began to consider possible therapeutic options to arrest this vicious cycle of mtDNA driven inflammation. In order to address the natural clearance of mtDNA from the circulation post-injury and following surgery, we looked at the relationship between circulating deoxyribonuclease (DNase) activity and mtDNA levels. DNase is the principle enzyme for the clearance of extracellular DNA in the circulation and also for the degradation of NETs (Brinkmann et al. 2004). Using a DNase assay that quantified the collective activity of all DNase isotypes found in plasma, we show DNase activity was significantly less following injury and surgery when compared to healthy volunteers. We also confirmed the positive correlation between mtDNA and SIRS with the larger number of trauma subjects included in this study (n=103) compared to our initial (n=35) work (McIlroy et al.2015). Again, no significant relationship was found between mtDNA levels and the incidence of post-injury MOF. This may have been due to the low incidence of post-injury MOF in the study population. Other authors have demonstrated positive correlation between mtDNA levels after trauma and the development of MOF (Simmons et al. 2013). DNase has been safely administered to humans in nebulised form to digest NETs in the sputum cystic fibrosis, reducing sputum

viscosity (Mogayzel et al. 2013). It has also been successfully used to ameliorate pulmonary atelectasis via the endotracheal tube in neonates ventilated in intensive care (Hendriks et al. 2005). Perhaps more promisingly, intravenous DNase administration in a mouse model of sepsis reduced incidence of MOF and overall mortality in the treatment group (Mai et al. 2015). It has also been safely administered intravenously in humans on a daily basis for a month, with no observed deleterious side effects, in the treatment of SLE nephritis (Davis et al. 1999). There is therefore a real possibility that exogenous DNase could be administered to trauma patients as a direct treatment to digest circulating mtDNA and mtDNA-NETs which are drivers of deleterious inflammation in SIRS (McIlroy et al. 2015) and MOF (Simmons et al. 2015).

In summary, our work has yielded valuable insight into the dynamics affecting mtDNA release, both as a result of the initial injury and the tissue disruption associated with post-injury surgery. The persistent sustained elevated levels of mtDNA in the 5-day peri-operative period after trauma surgery coincides with ongoing abundant mtDNA-NET formation is consistent with the notion that neutrophils are a likely principal source of ongoing mtDNA release. In essence, this body of work alludes to an ongoing vicious cycle of deleterious inflammation, which is driven via a positive feedback mechanism where mtDNA released after the initial injury and surgery then drives its own sustained release in the form of mtDNA-NETs. Exogenous DNase treatment may represent a novel and exciting therapeutic agent in the treatment of mtDNA driven post-injury SIRS and MOF.

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Clinical implications and pathological associations of circulating mitochondrial DNA

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1. ABSTRACT

Mitochondria are membrane-enclosed organelles, the energy-producing centers in almost all eukaryotic cells. The evolutionary emergence of mitochondria is a result of the endocytosis of a-proteobacteria. There are several characteristic features which refer to its prokaryotic ancestors including its independent sets of double-stranded mitochondrial DNA, which is uniquely circular in form and contains a significant amount of unmethylated DNA as CpG islands. Resent research has proven that free mitochondrial DNA found in blood was associated with innate immunomodulation in a broad-range of clinical conditions. Upon release, mitochondrial DNA acts as a danger-associated molecular pattern in the circulation, it is recognized by pattern recognition receptors and it facilitates inflammatory responses. Besides its high receptor activation potential, mitochondrial DNA is likely to perform direct crosstalk with activated leukocytes and to be contributed to other anti-microbial activities. Here we highlight the pathological conditions where cell free mtDNA is involved, describe the potential sources and mechanisms of extracellular mtDNA release and explore evidence for its mechanism of action after being excreted and potential therapeutic strategies.

2. INTRODUCTION

Due to its high mutation rate, mitochondrial DNA (mtDNA) has been studied in the context of ageing-related and multifactorial diseases where mitochondrial dysfunction is thought to play a role (1, 2). However, in the last few years, a novel and significant role of mtDNA has emerged, involving its ability to trigger innate immune system responses and drive inflammation when released from mechanically injured cells (3). MtDNA, along with other host molecules released upon cell damage, falls into the category of damage-associated molecular patterns (DAMPs). According to the endosymbiotic theory, the evolutionary emergence of mitochondria is a result of the endocytosis of a-proteobacteria (4, 5). Phylogenetic investigations support this assumption, as mitochondria possess several features characteristic of their prokaryotic ancestors, such as their barrel-shape and diameter of 0.2. to 1.0. μm. Mitochondria have their own independent sets of double-stranded mitochondrial DNA (mtDNA), which is not linear like nuclear DNA, but circular in form and consists of a high number of unmethylated CpG islands, as typically found in bacteria (6). The genome size of mtDNA is significantly smaller than the nuclear (16.5.69 bp vs. 3.2. billion bp) in humans, the number of encoding mitochondrial genes is only 37, which encode no more

than 16 proteins, all belonging to the electron transport-chain (7). The release of mtDNA and its presence in the circulation were described to play a significant role in various clinical conditions; in particular, inflammatory diseases, several types of cancer and in conditions leading to critical illness requiring intensive care unit admission (8-10). Currently, several conditions are being explored where mechanical and chemical stress can lead to cellular necrosis accompanied by mtDNA release. This review will provide a summary of the pathological conditions where cell free mtDNA is involved, describe the potential sources and mechanisms of extracellular mtDNA release and explore evidence for its mechanism of action after being excreted and potential therapeutic strategies.

2.1. Conditions when mitochondrial DNA release is accompanied by mechanical and chemical stress

The release of mtDNA has been described in diverse inflammation and cell necrosis-related clinical conditions, where the loss of cell membrane integrity leads to the release of intracellular content (11). However, it is important to note that in these conditions, the stress destroying the mitochondria can be of either extracellular or intra-mitochondrial origin, with the latter case initiated by oxidative burst inside the mitochondrion, resulting in mitochondrial and cellular disintegration (12). As such, mechanisms of mtDNA release may vary depending on the origin and the type of insult.

2.1.1. Mechanical stress related mtDNA release

Any type of cell death that culminates in lysis and subsequent release of intracellular content into the extracellular environment could conceivably result in mtDNA being released into the circulation (11). MtDNA and other particles are not exposed to the innate immune system following normal apoptosis, but cell death due to mechanical stress and subsequent lysis can mediate their entry into the systemic circulation to provoke immune response (13). Tissue injury is one such example of mechanical stress leading to release of mtDNA (14). In major trauma patients, the initial cell death due to the injury is an important but non-modifiable factor for post-injury inflammation-associated complications (15). The recognition of mtDNA by innate immune cells (primarily neutrophils) plays a pivotal role in the pathophysiology of sterile inflammation after major trauma (16). Our group has hypothesized that mtDNA may have a primary inflammatory source following major trauma rather than as a result of direct tissue injury and subsequent cell necrosis (17). It is supported by our recent findings, where elevated and increasing concentration of mtDNA was shown to be present in the sera of major trauma patients. We also identified sustained high concentrations of cell free mtDNA in the sera of postoperative trauma patients undergoing major orthopedic trauma surgery without association with well-established markers of tissue necrosis (17).

2.1.2. Stress causes oxidative damage and a subsequent DNA release in mitochondria

In addition to its presence in major trauma patients, alterations in mtDNA content of the blood have been measured in a number of diseases relating to oxidative stress. Besides the measurable oxidative damage, the involvement of the mitochondrial genome was described in response to focal or acute myocardial ischemia/reperfusion in animal models and in human cells (18-20). Elevated blood mtDNA content was described to be in association with higher cardiovascular risk or development of coronary heart disease (21, 22). This latter study was conducted on patients with diabetes mellitus and others found hyperglycemia-induced elevation in mtDNA of the peripheral blood in early diabetes (23). Likewise, neurodegenerative diseases such as Alzheimer's or Huntington's disease are also seemed to be associated with alterations in mtDNA concentration (24-26). Also, different types of cancer or critical conditions like sepsis or hemorrhagic shock are considered to be connected with mtDNA copy number changes (16, 27-30). In the context of high-altitude oxygen deprivation, blood mtDNA content was observed to be increased in lowlanders as compared to highlanders, regardless of age and gender, suggesting that the changes in mtDNA concentration might be due to the ROS-stress adaptation mechanisms (31, 32). Both acute and chronic inflammatory diseases have been associated with increased cell-free mtDNA and correlate with elevated free radical production that may have originated from mitochondria (33), but the question of the source of ROS remains open, as the ROS productive capacity of immune cells is also well-known in these conditions. Liu and co-workers published in 2003, that the copy number of mtDNA in human leukocytes was highly affected by alterations in plasma antioxidants/pro-oxidants (34). In addition to its role in diseases itself, mtDNA is also affected by diagnostic and therapeutic tools. Mitochondria are highly susceptible to ionizing radiation at the clinically relevant dosages and oxidative stress resulting from irradiation was found to be accompanied by a rise in extracellular mtDNA release (35, 36). In fact, the natural process of aging is relevant here, since oxidative stress within aging mitochondria can lead to a vicious cycle in which damaged mitochondria produce increased amounts of reactive oxygen species. This could explain the significant increase in the mtDNA mutation rate also found in human clinical studies in healthy older people's plasma, as compared to young volunteers (37, 38). The cut-off for clinically relevant rise in these mutations is likely to be close to the 6^{th} decade of life in humans, highlighting the importance of age-matching in mtDNA concentration-based human studies (39).

Paradoxically, oxidative injury to mitochondria takes place during "reductive stress": when electron acceptors are expected to be mostly reduced, some redox proteins can donate electrons to O_2 instead,

which increases the NADH/NAD+ ratio of mitochondria. Conditions such as high-intensity exercise training, alcohol intake or chronic fatigue syndrome and other forms of hyperglycemia-induced diseases all result in reductive stress and were reported to be accompanied by cellular damage and therefore mtDNA excretion (40-42).

2.1.3. Cell death pathways culminating in lysis

If a cell is directly injured through a physical insult or severely stress it may become necrotic. Necrosis is characterized morphologically by cell rounding, swelling (oncosis), and expansion of organelles and de-condensation of nuclear chromatin (43). This process culminates in cell lysis. Recent evidence suggests that complex signal transduction mechanisms can control necrosis (43). Further cell death essentially occurs as a result of the physiological stress caused by the mass release of cytokines and other cell signaling molecules from injured tissues and the innate immune cells involved in the acute inflammatory response. “Necrosis” in the post-injury state can be triggered through subsequent complex tightly regulated intracellular signaling cascades, not just through the initial mechanical tissue injury. Necrosis can be triggered by exogenous molecules such as TNFα and Fas ligand binding to cell surface receptors (44). The activation of such receptors can lead to a tightly regulated and controlled form of necrosis. This process is mediated through caspase-8 (anti-cell death enzyme) and receptor interacting protein kinases (RIPK family) and the term “necropoptosis” coined for it (11). The role of DAMPs in triggering necropoptosis has also been explored through their activation of pathogen recognition receptors (PRRs) which then trigger intracellular signaling cascades through RIPK1 and RIPK3 (11). Whilst RIPK1 and RIPK3 can play a role, they are not essential in necrosis following ischemia reperfusion (IR) injury (44). This is characterized by exposure to high levels of hydrogen peroxide (H_2O_2) and is dependent on the activity of a different enzyme poly (ADP-ribose) polymerase (44). Free intracellular iron redox reactions with H_2O_2 appear to play a pivotal role in this modality of cell death by inducing lysosomal permeability (43). Intracellular chelation of free iron was demonstrated to be cell-protective in such conditions (43). Regardless of the initiating stimulus, loss of membrane continuity and lysis leads to the extravasation of the intracellular contents, including mtDNA and associated mtDAMPs into the extra-cellular environment.

2.1.4. Oxidative cellular damage pathways initiated by mitochondria

The majority of ROS are products of mitochondrial respiration, as the electrontransport-chain contains several redox centers that may leak electrons to O_2, serving as the primary source of $O_2^{\cdot -}$ production in most tissues (12). A major threat to this controlled equilibrium is hypoxia, since the absence of the electron acceptor O_2 leads to a shift in reducing potential to a higher than normal reducing power, which results in progressive structural and functional cell damage. It is therefore widely accepted that several disease states are linked to “oxidative stress” and a subsequent mtDNA release (45). The effect of abnormal Ca^{2+} is also inevitable in response to stress, but the mechanisms of the harmful effect of Ca^{2+} on mitochondria is not well characterized (46). ROS and Ca^{2+} constitute important mediators of the propagation of the necrotic signal from the mitochondrial matrix towards the outside and can cause damage to all of the major classes of biological macromolecules, including nucleic acids, proteins, carbohydrates, and lipids (47). Mitochondrial calcium has been described to stimulate oxidative phosphorylation, thereby promoting more ROS generation (48). In addition calcium-mediated activation of calpain can lead to cleavage and inactivation of caspases (49) whereas ROS can target the active site of caspases and render them inactive, promoting necrosis (50). Likewise, mitochondrial H_2O_2 can cause the release of cytochrome c from mitochondria into the cytosol and H_2O_2 may also activate nuclear transcription factors, like NF-κB, AP-1, and p53, which may upregulate death proteins or produce inhibitors of survival proteins (51).

3. CIRCULATING MITOCHONDRIAL DNA IN INNATE IMMUNE RESPONSES

Despite their seemingly independent existence within the cell, mitochondrial transcription and replication are co-dependent on nuclear encoded factors transported into mitochondria (52). The aforementioned similarity to bacterial DNA makes mtDNA highly immunostimulatory to cells of the innate immune system (6). The latest evidence suggests that it does not only facilitate antibacterial immune responses, but significantly contributes to further adverse effects and may have important roles in inflammatory diseases and complicated outcomes following cellular damage or oxido-reductive stress (8). MtDNA has been shown to bind PRRs, namely to the Toll-like receptor (TLR) superfamily members or nucleotide oligomerization domain (NOD)-like receptors (NLRs) and more recently it has been shown to be linked with the stimulator of interferon genes (STING) pathway (53, 54).

3.1. MtDNA and TLR interaction

MtDNA has been demonstrated to induce neutrophil activation and facilitates adverse immune reactions through activation of TLR 4 (55) and TLR 9 (56), mediated through MAP kinases p38 (16, 57) and p44/42 (58). MtDNA triggers activation of the nuclear factor kappa B pathway (NFκB) via TLR9, resulting in upregulation of pro-inflammatory cytokine production including TNF-α (59), IL-1β (60) and IL-6 (61). MtDAMPs have been shown to potentiate inflammatory lung injury when introduced into healthy rats in a landmark paper by Zhang and colleagues (9). One possible contributory factor is that mtDNA triggers increased neutrophil

expression of matrix metalloprotease 8 (MM8) through p38 activation, which is a collagen cleavage enzyme that potentiates tissue degradation (57).

3.2. MtDNA-Nod-like receptor 3 relationship

Of the NLR receptors NLR pyrin domain 3 (NLRP3) inflammasome is the most widely studied mainly due to its affinity for a wide variety of ligands (62). Mitochondria have been implicated in the recruitment of NLRP3 in a variety of different ways including through direct activation with mtDNA (63). The assembly of the NLRP3 inflammasome in complexes containing caspase-1 has now been directly implicated in triggering a novel form of cell death termed “pyroptosis” (64). Interestingly when cells lack mtDNA (induced by treatment with ethidium bromide) NLRP3 inflammasome formation was completely inhibited (65). Conversely, NLRP3 inflammasome formation releases mtDNA (65). This indicates a possible positive feedback loop where mtDNA potentiates its own release by stimulating further NLRP3 inflammasome formation.

3.3. MtDNA-STING pathway relationship

MtDNA has the ability to stimulate the innate immune system through stimulation of interferon genes (STING) pathway, resulting in interferon release. The STING pathway was recently mechanistically dissected to reveal an intricate relationship demonstrating how mtDNA triggers interferon release (54). The study showed that through depletion of mitochondrial transcription factor A (TFAM) during a herpes viral infection, mtDNA stability was disturbed, causing enlargement of the mitochondrial nucleoid. Subsequently, fragmented mtDNA was released, activating peri-mitochondrial cyclic GMP-AMP synthase (cGAS) causing increased cGAMP formation. The second messenger cGAMP then activates the endoplasmic reticulum bound STING pathway which ultimately upregulates type I interferon (IFN I) expression which inhibits viral propagation. Interestingly, pro-apoptotic caspase activation inhibits this response and suppresses downstream interferon production (66).

3.4. MtDNA-neutrophil extracellular trap formation

Neutrophil extracellular trap (NET) formation or “NETosis” was first described by Brinkmann and colleagues in 2004 (67). It is characterized by smooth extracellular filaments-17nm in diameter- which are composed of stacked and probably modified nucleosomes (68). This filamentous chromatin backbone is adorned with globular domains of approximately 50nm diameter containing neutrophilic granular proteins. The principle function of the NET is believed to be to entrap and kill circulating pathogens and this function has been directly shown in both Gram- and Gram+ bacteria, viruses and fungi (68, 69).

The composition of NETs was initially widely believed to be predominantly nuclear DNA (nDNA), however under specific stimulatory conditions NETs composed exclusively of mtDNA were demonstrated (70). Our group described that NETs formed after trauma and subsequent surgery were predominantly composed of mtDNA (71). More recently NETs rich in oxidized mtDNA have been discovered in systemic lupus erythematous (72). The emerging body of evidence suggesting NETs can indeed be composed exclusively or predominantly of mtDNA means NETosis may represent a significant source of circulating mtDNA in certain inflammatory conditions.

In addition to the role of intracellular mtDNA in NET composition, mtDNA may also trigger NET formation as a DAMP. NETosis has widely been considered as a NADPH oxidase (PHOX) dependent process, reliant on mitochondrial release of reactive oxygen species (73). However mtDNA as a trigger for NETosis is a much more recent concept and there is growing evidence that extracellular trap formation takes place independently from pro-oxidant activity (74, 75). MtDNA has been demonstrated to be a trigger for NETosis after major trauma and with signaling mediated through a TLR9-dependent pathway, independent of PHOX (38). The concept of mtDNA as a signaling molecule involved in NETosis suggests it may have a more diverse role in regulating certain inflammatory processes in a novel and previously unstudied way.

4. CLINICAL IMPLICATIONS OF CELL-FREE mtDNA

4.1. Diagnostic application of mtDNA

The number of studies investigating the concentration of mtDNA as a potential biomarker in different human body fluids has grown significantly in recent years. Real-time PCR allows simultaneous detection and quantification of mtDNA using a small amount of sample and a downstream real-time PCR analysis give an accurate reproducible result within 2 hrs. The detection from blood, saliva, urine or sperm is a minimally or non- invasive process for diagnosis and was proven to be valuable for the prognosis of various clinical conditions, such as different types of cancer, type 2 diabetes, sepsis, multiple organ failure, fertility impairment or neurodegenerative disorders (28, 76-81). However, the exact cellular mechanisms and cell-type of origin which cause mtDNA concentration to fluctuate in many conditions remain unclear.

Elevated mtDNA content in peripheral blood has been demonstrated as a diagnostic factor in various types of cancer, including non-Hodgkin lymphoma, lung cancer, pancreatic cancer, breast cancer, colorectal cancer, or glioma (81-86). In contrast, an increased risk of renal cancer or hepatocellular carcinoma was observed to be associated with decreased circulating mtDNA concentrations within the tumor tissues of

cancer patients (87, 88). In other human studies, mtDNA quantity measured in the blood of patients with sepsis, pulmonary embolism or out-of-hospital cardiac arrest was proven to be a more powerful prognostic marker than those conventionally used, including nuclear DNA or other existing semiquantitative score systems (89, 90).

The rapidly elevated concentrations of circulating mtDNA levels that are observed in trauma patients with severe injury suggests that extracellular DNA originates from direct tissue injury and subsequent necrosis. It was described to be a trustworthy prognostic marker either in blood or in cerebrospinal fluid with good prediction for unfavorable outcome, or even mortality (65, 91-93). Although, in some studies, nuclear DNA concentration and well-established markers of tissue necrosis were not found to correlate with mtDNA levels, or mtDNA concentration was observed to have no contribution in the pathophysiology of critical illness (17, 29, 94). These findings together with the above-mentioned dichotomies, raise some concerns regarding the nucleic acid-based diagnosis. The reduced clearance of DNA over time caused by impaired organ function during systemic inflammation may also be a contributing factor (92) and similarly, the limited capacity of inflammatory cells for taking up dying cells, thereby DNA (95). In the near future, investigation of the mtDNA methylation pattern rather than its concentration might be used for the diagnostic purpose for identifying tissue specific origin, as it was successfully performed to predict cardiovascular problems or amyotrophic lateral sclerosis (96, 97) and suggests a promising approach to diagnose health problems caused by environmental pollution exposure, aging, drug treatment, and oxidative stress (98). Moreover, since mitochondria do not contain histones, it is likely that the mtDNA methylation/hydroxymethylation ratio rather than histone modification is important for mitochondrial genome-based diagnostics.

4.2. Therapeutical implications of mtDNA

Major trauma patients often require lifesaving allogenic blood products in which cellular remnants, such as mitochondria and extracellular mtDNA are described to be present and to mediate adverse inflammatory processes, as neutrophil, eosinophil and basophil leukocyte activation (99, 100). It is important to take into account, that platelet units represent a potential reservoir of mtDNA, since unlike leukodepleted red blood cell units, stored platelets contain mitochondria.

Moreover, other therapies might also cause cytolysis and may be accompanied by circulatory mtDNA release. Plasma mtDNA content was proven to be elevated and observed to mediate pro-inflammatory effects in maintained haemodialysis patients (101) and in another study, increased mtDNA amount in the plasma of patients was considered to be related to the overall procedure of artificial kidney therapy and probably was due to the death of leukocytes (102).

The fact that mtDNA has such potent immunostimulatory effects makes it an exciting target for immunomodulation therapy attenuating some of the potentially deleterious effects of excessive innate immune activation. Whether mtDNA is free or conjugated in NETs it is readily digestible with DNAse. There is certainly good evidence to suggest that focally targeting NETs with DNAse has yielded a reduction in associated inflammatory lung damage in a mouse model of transfusion related acute lung injury (TRALI) (103). Human recombinant DNAse therapy has been used to good effect when nebulized in cystic fibrosis patients by enhancing sputum solubilisation (104), however no studies have been performed in humans to treat acute inflammatory conditions. With such an emergent role of mtDNA in NETs associated with trauma (71) and more recently in SLE (105) the investigation of DNAse therapy in different inflammatory conditions would be very reasonable.

Targeting mtDNA receptors may also yield ways to modify its proinflammatory properties. It has a diverse role as a signalling molecule in various inflammatory pathways as a ligand of multiple receptors including mtDNA stimulation of the NLRP3 inflammasome (63), cGAS in mtDNA-STING pathway (54) and TLR9 in mtDNA mediated NETosis (38). Modulation of these receptors may convey benefit in a variety of clinical conditions and attenuate the immunostimulatory effects of mtDNA.

5. FUTURE DIRECTIONS

It is essential to understand the tissue specific origin of circulating mtDNA for both diagnostic and therapeutic considerations. The natural history of free polynucleotides in the circulation in inflammatory conditions is largely unknown. The available active DNAse concentration in physiological and pathological conditions could indicate the potential need for enzyme supplementation as a therapeutic strategy. We believe that our current knowledge on cell free circulating mtDNA is in a rather exploratory phase with a potential for the future to rewrite the pathology of the leading causes of morbidity and mortality such as inflammatory conditions, autoimmune disorders, cancer, heart disease, stroke and injury.

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Letters/Editorials

The origin and the role of mitochondrial DNA in postinjury inflammation

The origin of the eukaryotic cell continues to be debated among evolutionary, cell, and microbiologists. On the other hand, the origin of the mitochondrion—1 organelle that helps define eukaryotic status—is perhaps more settled. It is thought that ancient prokaryotes (archaea) internalized saprophytic bacteria (α-proteobacteria), which evolved into mitochondria within the eukaryotic cell, and became responsible for cellular energy homeostasis [1]. This endosymbiotic theory is supported by the fact that mitochondria are distinct organelles that contain their own circular, double-stranded DNA genomes and transcription and translation machinery, much of which resembles bacterial DNA and their protein synthesis processes.

Recent discoveries further support this theory of bacterial origin as mitochondrial organelles (including their DNA) are proinflammatory and have been associated with postinjury dysfunctional inflammation-related complications such as acute lung injury, acute respiratory distress syndrome, and multiple organ failure [2]. Mitochondrial DNA (mtDNA) and other particles are not exposed to the innate immune system following normal apoptosis, but cell death due to stress caused by shock, trauma, and sepsis can lead to these mitochondrial components being released into the systemic circulation to elicit an immune response. This is certainly an elegant explanation of a potential mechanism for postinjury inflammation, which is a sterile process and can develop without circulatory shock as the result of tissue injury.

Yamanouchi et al [3] identified that both trauma and sepsis patients have higher concentrations of plasma mtDNA than healthy controls. These levels rapidly decreased in trauma patients after day 1, whereas levels in septic patients remained high during the 5-day observation period. Creatinine phosphokinase (CPK) plasma concentrations correlated with mtDNA concentration on day 1 in trauma patients, but this was not observed in septic patients. Injury severity was associated with mtDNA release but not the severity of sepsis.

Although this study highlights some interesting observations regarding the free mtDNA concentrations in severely injured and septic patients, the only definitive conclusion of this article that we should consider further for inflammation research in critical care is that trauma and sepsis patients have elevated plasma mtDNA concentrations compared with healthy controls during the first 5 days postinjury. We do not know if this is dose dependent on the nature of the tissue injury, the shock severity (very few patients had shock in the trauma group) and the severity of sepsis. In addition, the cellular source of mtDNA is unknown, although the good correlation with CPK at day 1 may indicate that it is due mainly to muscle necrosis. Striated muscle is rich in mitochondria; therefore, muscle damage should result in possibly higher concentrations of mtDNA than nuclear DNA (nDNA) being released. If this is the case, CPK measurement, which is already routinely performed, could be a surrogate serum marker for mtDNA release. The landmark paper of Zhang et al [2] proved the hypothesis that mtDNA is proinflammatory, when they showed that injecting mtDNA intravenously into animals subsequently caused acute lung injury. However, it is not known from the current study whether the plasma mtDNA concentrations (4-11.5 times above the healthy controls) would be sufficient to trigger severe inflammation and remote organ damage.

In sepsis (and to a lesser extent in trauma), mtDNA concentrations did not return to normal levels during the observation period. Is this associated with further cell necrosis? It is not possible to know this without understanding and monitoring an individual patient's postinjury course for secondary surgeries, septic complications, fluid balance, and cell necrosis markers. Normally, DNA is rapidly metabolized by nucleases; therefore, its persistence for days in plasma could be associated with decreased nuclease activity or persistent release. During the process of programmed cell death (apoptosis), the nDNA and mtDNA of the apoptotic cell are broken down within the lysosomes of macrophages without being exposed to the circulation. Until recently, it was thought that DNA could enter the plasma only after cell necrosis.

Mitochondrial DNA–driven inflammatory complications in remote organs is a fascinating potentiality, especially considering the evolutionary aspects and the "Trojan horse" analogy of the potentially harmful molecules hidden in vital cell organelles [4]. The recent discovery that mtDNA and nDNA may gain access to enter the circulation without cell necrosis provides a potential explanation for the source of free circulatory DNA. Polymorphonuclear neutrophil leukocytes (PMN) release neutrophil extracellular traps (NETs) in response to infection, interleukin 8, and various other nonspecific stimuli [5]. The process of NET formation called "NETosis" is a newly described mechanism of cell death that is distinct from necrosis, phagocytosis, and apoptosis. The main structural element of the NETs is a DNA framework ("net") compounded by histones, PMN enzymes, and other compounds from PMN granules. It is known that NETs kill bacteria. However, there is paucity of the literature on whether phagocytosis and NETosis can occur simultaneously in the same cell, whether leukocytes can survive the process of expelling their DNA, and what extent of nDNA and/or mtDNA involvement is in the process. Although it has not been convincingly shown from sterile trauma serum, NETosis could be responsible for the consistently high circulating free DNA concentration, well after the initial tissue injury and necrosis. In this scenario, the origin of detected mtDNA is more likely to be inflammation related rather than due to the mechanical tissue injury. In other words, extracellular DNA could play a role as an instigator and effector in innate immune responses (Fig. 1).

In summary, the detection of high concentrations of mtDNA in plasma of trauma and sepsis patients opens up new frontiers of intriguing research. Currently, we have many more questions than answers, considering the fact that mtDNA is just one of the

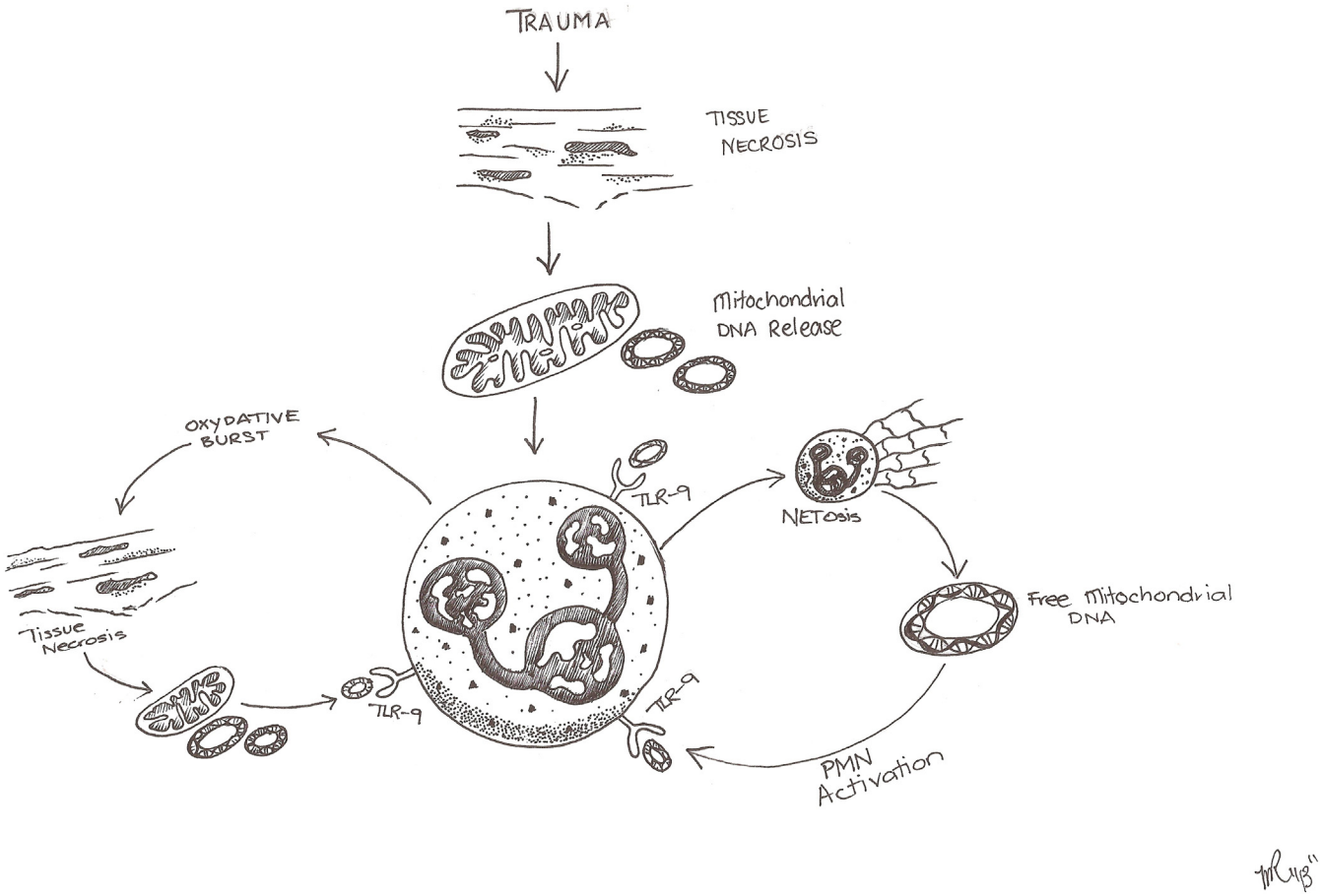

Fig. 1. Possible vicious cycles of mtDNA driven PMN activation.

immunogenic substances released from the mitochondria of the necrotic or NETotic cells.

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