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

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ARTICLE INFO

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

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 900g 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
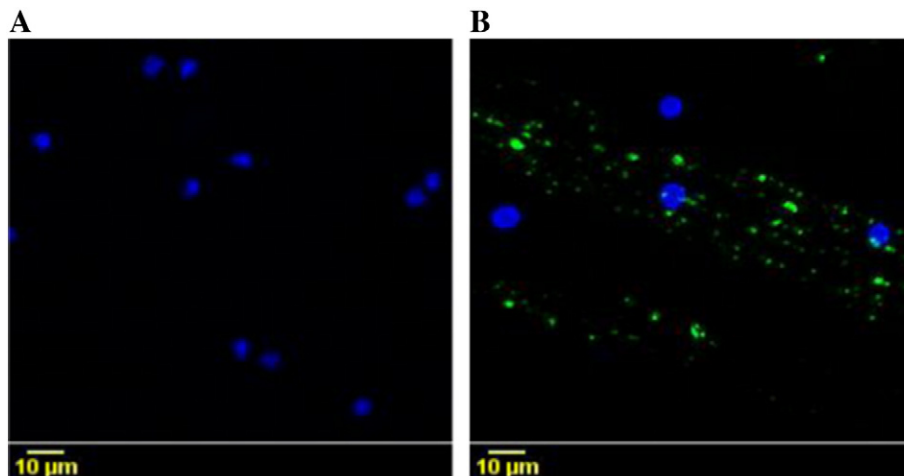

Fig. 1. Viable neutrophil nuclear DNA was stained with membrane permeable Hoechst 33342 (blue), and NETs were visualized by membrane impermeable Sytox Green (green) staining extracellular DNA. Negative control, healthy volunteer without stimulation (A) and positive control, healthy volunteer stimulated with 600 nmol/L PMA for 45 minutes (B).
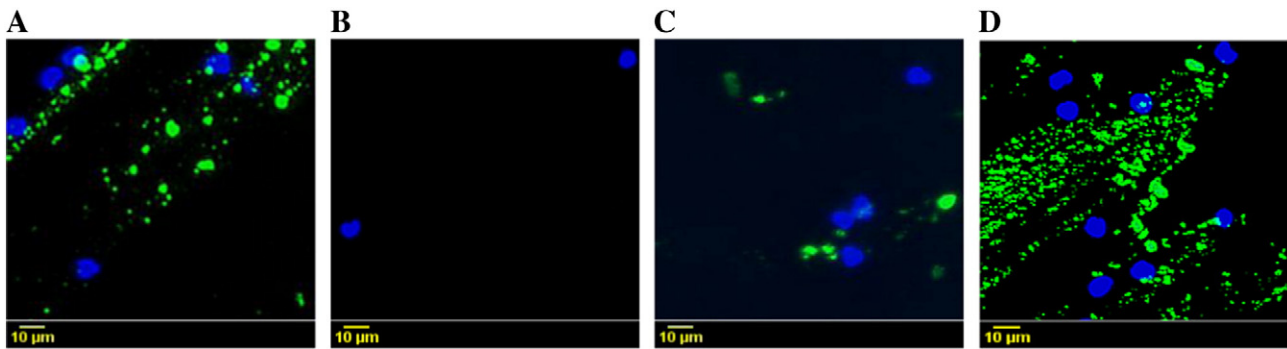
Fig. 2. Viable neutrophil nuclear DNA was stained with membrane permeable Hoechst 33342 (blue), and NETs were visualized by membrane impermeable Sytox Green (green) staining extracellular DNA. Acetabular fracture patient, immediately postinjury (A); 7 days postinjury, immediately preoperative (B); immediately 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
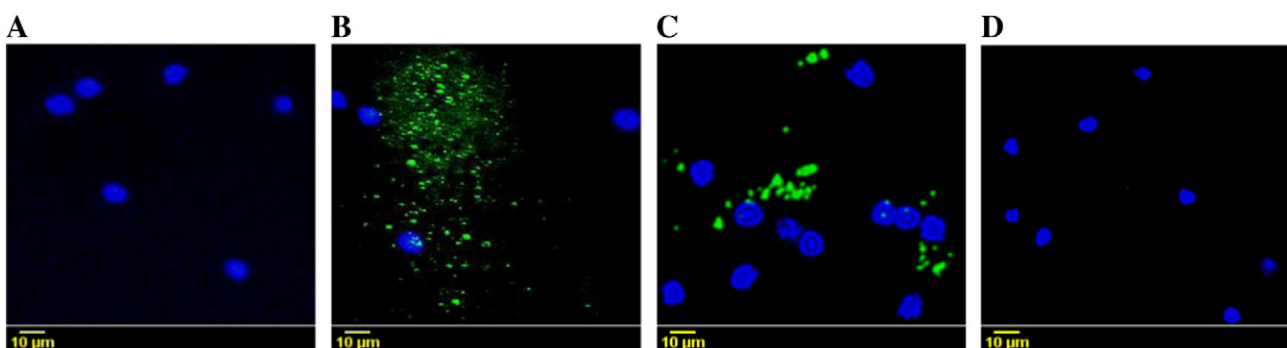

Fig. 3. Viable neutrophil nDNA was stained with membrane permeable Hoechst 33342 (blue), and NETs were visualized by membrane impermeable Sytox Green (green) staining extracellular DNA. Total hip replacement patient immediately preoperative (A), immediately postoperative (B), 3 days (C), and 5 days postoperative (D).
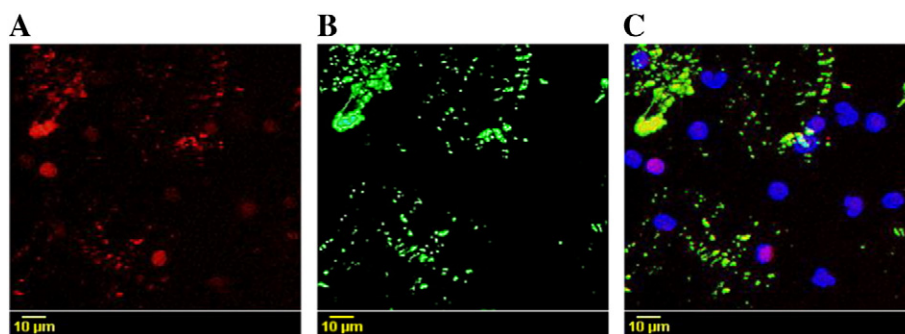
Fig. 4. Neutrophil extracellular traps isolated from a pelvic fracture patient 5 days postoperative. Viable neutrophil nDNA was stained with membrane permeable Hoechst 33342 (blue), and NETs were visualized by membrane impermeable Sytox Green (green) staining extracellular DNA. Extracellullar mtDNA was 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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