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Original Article

Neutrophil extracellular traps were released during intraoperative blood salvage in posterior lumbar surgery

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ABSTRACT

The formation of neutrophil extracellular traps (NETs) has been associated with endothelial damage and severe pulmonary dysfunction. The present study aimed to investigate whether this NETosis occurs during intraoperative blood salvage (IBS), and whether the washing procedures before re-transfusion of autologous blood could remove the NET components, including DNA, histones, and myeloperoxidase. The study was performed using blood samples from 20 patients who underwent posterior lumbar surgery at the Beijing Friendship Hospital. The samples were obtained at three time points/sources: peripheral venous blood prior to surgery (baseline), cell salvage collection reservoir (reservoir), and filtered salvaged blood prior to re-transfusion (pre-transfusion); blood salvage was accomplished with a Cell Saver 5 system. The plasma was collected after centrifugation of the blood sample. Then the DNA amount was measured using SYTOX Green labeling; the integrity and length of the DNA were roughly evaluated by agarose gel electrophoresis; and the levels of nucleosomes (DNA and histones) and myeloperoxidase were detected using commercial ELISA kits. Extracellular DNA, nucleosomes, and myeloperoxidase were found higher in the reservoir samples and pre-transfusion samples, as compared to the baseline samples. The DNA was primarily non–fragmented with high molecular weight (> 15 kb). And lower levels of these NET components were observed in pre-transfusion samples, compared with the reservoir samples. In conclusion, DNA, histones, and myeloperoxidase were released during IBS, indicating the NET formation by activated neutrophils. Pre-transfusion processing could reduce the NET components but the levels remained significantly elevated compared to the baseline.

1. Introduction

The use of autologous transfusion has become a widely accepted medical practice. The reasons for this rapid growth were initially attributed to the high demand of blood in regional blood-banking centers and hospital blood banks that often exceeded the regular supply. In addition, autologous transfusion eliminates potential morbidity and complications that may be observed in recipients from blood donors (such as acute lung injury, transfusion-transmitted acquired immunodeficiency syndrome, hemolysis and post-transfusion hepatitis) [1]. Autologous transfusion has advantages since it excludes the risk of isoimmunization to foreign red blood cell (RBC) and platelet antigens, and it decreases the risk of exposure to infectious agents [2,3]. Furthermore, the possibility of salvaging a major part of RBCs from the patient during surgery is considered a significant advantage, notably when excessive hemorrhage occurs. As an alternative to allogeneic transfusion, autologous transfusion involves the removal and re-transfusion of blood from the surgical field [1–3].

Intraoperative blood salvage (IBS) is performed routinely during posterior lumbar surgery and provides readily available fresh RBCs for autologous transfusion. Although salvaged blood has an excellent clinical safety profile [4,5], some patients have developed complications, including disseminated intravascular coagulopathy and increased vascular permeability in the lungs or systemic circulation (anasarca) following infusion of large amounts of washed autologous blood, resulting in salvaged blood syndrome [6–9].

It has been suggested that this complication could be mediated by neutrophils that have been damaged during the salvage process and activated by platelets [10–12]. Upon contact with activated platelets or inflammatory stimuli, neutrophils and white blood cells (WBCs) release depolymerized DNA chains, histone proteins, and neutrophil enzymes. This process results in the formation of neutrophil extracellular traps (NETs) and causes dramatic changes in the cellular morphology [13,14]. The process is termed NETosis and can be broadly in two types: a) conventional suicidal NETosis and b) vital NETosis [15]. Conventional suicidal NETosis is initiated by ligand binding to neutrophil Toll-

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like receptors and receptors for IgG-Fc [16], whereas vital NETosis can occur following direct exposure to bacteria and lipopolysaccharide conjugates after induction of NETs via Toll-like receptor-mediated activation of platelets. The Toll-like receptor 4 is involved in the activation of NETs by Gram-negative bacteria, while the Toll-like receptor 2 is involved in the activation of NETs by Gram-positive bacteria [15]. The main difference between the two NETosis processes is the destruction of the outer membrane of the polymorphonuclear cells (PMNs) that occurs in conventional NETosis, while in vital NETosis the PMNs continue to function even to the point of becoming anuclear [15]. The activation of NETosis [15,16] requires chromatin unwinding through the action of reactive oxygen species (ROS) [17], elastase, myeloperoxidase (MPO) [18], and histone hypercitrullination by the enzyme peptidylarginine deiminase 4 [19].

Although NETs protect against infection [14,20], excessively high plasma levels of neutrophil components can cause organ damage, [13] such as transfusion-related acute lung injury (TRALI) [21,22], thrombosis [23,24], and autoimmune diseases [25,26]. It has been suggested that in the case of platelet-induced neutrophil NET production that may occur after autologous transfusion, a certain threshold of lipopolysaccharide concentration has to be exceeded in order for the induction of neutrophils to occur [15]. This finding indicates that neutrophils are safeguarded from the production of NETs in the vasculature, unless lipopolysaccharide concentrations reach very high levels, such as in the case of severe infection that in turn can activate the platelets [15]. NET components have been identified in the plasma of patients with deep vein thrombosis [27], systemic thrombotic microangiopathies [23], autoimmune diseases [28], and TRALI [21,22].

Since the neutrophils are activated to release extracellular DNA and histones during posterior lumbar surgery, the present study aimed to evaluate the effect of IBS on the aforementioned components prior to re-transfusion of autologous blood. Accordingly, three types of blood samples derived from patients that were scheduled to undergo posterior lumbar surgery were analyzed. The presence of NET components was examined, in terms of the plasma levels of extracellular DNA, histone, and MPO obtained from the peripheral venous blood of the patients. The levels of these parameters were compared among the following blood samples: prior to surgery, cell salvage collection reservoir, and washed, centrifuged, and filtered salvaged blood prior to re-transfusion into the patient.

2. Material and methods

2.1. Patients and ethical approval

The study was approved by the Institutional Review Board of Beijing Friendship Hospital. Twenty patients provided written informed consent for their participation in the study. Eligibility criteria were: 1) adult patients between 18 and 65 years of age; 2) male or female; 3) American Society of Anesthesiologists (ASA) Class I or II; and 4) scheduled to undergo posterior lumbar surgery. The exclusion criteria were: 1) history of myocardial infarction, stroke, infection, trauma, cancer, or autoimmune disease; or 2) allogeneic transfusion.

2.2. Anesthesia

Posterior lumbar surgery was carried out under general anesthesia. All patients received total intravenous anesthesia with propofol (Propolipid, Fresenius Kabi, Bad Homburg vor der Höhe, Germany) and sufentanil (Ultiva, Glaxo-SmithKline, Middlesex, UK). The patients were monitored by electrocardiogram, arterial oxygen saturation, and invasive blood pressure monitoring during the entire operation.

2.3. Blood salvage device

Blood was salvaged with a Cell Saver 5 system (Harmonetics Corp., Braintree, MA, USA) that is used routinely at the study site. The device collects shed blood mixed with heparinized saline from the surgical field. The blood is stored in a collection reservoir that contains an integral 150-μm filter to remove debris. Following successful salvage of 1 L of blood a centrifugation is conducted. Packed RBCs accumulate in the centrifuge bowl and are detected using a light sensor. The RBCs are washed with 1 L of saline. The process results in a final product of approximately 225 mL of autologous non-leukoreduced RBC units that are pumped to an infusion bag for re-transfusion to the patient. The whole cycle lasts approximately 3 min. All the cell salvage procedures were conducted by a blinded investigator who was an expert in using the Cell Saver 5 system.

2.4. Blood samples

The blood samples were collected at three stages of the procedure: from peripheral veins following anesthesia induction and prior to surgery (baseline); from the blood salvage collection reservoir and prior to debris removal (reservoir); and after washing, centrifugation, filtration, and passage through a standard y-type blood transfusion set (170-μm filter) prior to reinfusion into the patient (pre-transfusion). At each stage, 2 mL of blood were collected into tubes containing EDTA (BD Vacutainer EDTA Tubes, Franklin Lakes, NJ, USA). The blood samples were centrifuged for 10 min at 1500 × *g*. The plasma was collected and centrifuged for 10 min at 3000 × *g*. The plasma was stored at −80 °C.

2.5. DNA quantification

The plasma was diluted in phosphate-buffered saline (PBS; GIBCO, Invitrogen Inc., Carlsbad, CA, USA). A total of 50 μl of diluted plasma were mixed with an equal volume of PBS containing SYTOX Green (final concentration: 1 μM; Invitrogen Inc., Carlsbad, CA, USA) for DNA labeling [20]. Fluorescence was recorded using a fluorometer (Fluoroskan; Thermo Fisher Scientific, Waltham, MA, USA). The autofluorescence was determined from samples mixed with PBS without SYTOX Green for the background determination. The DNA concentrations were calculated based on a standard DNA concentration curve (Invitrogen Inc., Carlsbad, CA, USA).

2.6. Isolation and visualization of DNA

The DNA was isolated from plasma using a DNA isolation kit, according to the manufacturer's instructions (Omega Bio-Tek, Norcross, GA, USA). The DNA was subjected to 1% agarose gel electrophoresis in the presence of ethidium bromide and visualized with a gel scanning system (Bio-Rad, Hercules, CA, USA).

2.7. Quantification of nucleosomes and MPO

The plasma was diluted at a 1:100 ratio in PBS with 0.1% bovine serum albumin. The concentration of nucleosomes was quantified by an enzyme-linked immunosorbent assay (ELISA) kit (Cell Death Detection kit containing antibodies against histones and DNA, Roche, Indianapolis, IN, USA), according to the manufacturer's instructions.

The plasma concentration of MPO was quantified using a commercially available ELISA kit (Zen MPO ELISA, Invitrogen Inc., Carlsbad, CA, USA), according to the manufacturer's instructions.

2.8. Western blot analysis for histone H3

The plasma was analyzed by western blotting for histone H3 detection. A total of 10 μl of plasma were mixed with Laemmli buffer supplemented with 5% β-mercaptoethanol (Bio-Rad, Hercules, CA, USA). Following incubation of the samples at 95 °C for 5 min, sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis was conducted and the proteins were transferred on a polyvinylidene fluoride