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Original article

Haemostatic biomaterial based on expired platelets for medical applications

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Abstract

Blood loss has been a concern especially in surgery and bleeding control and a number of haemostatic agents and tissue sealants have been developed and applied in various surgical disciplines. The first steps to stop bleeding due to a vascular lesion, limit blood loss and allow healing are ensured by platelets that play an essential role in forming the primary haemostatic plug. After this, the clot is consolidated by the formation of a fibrin network organized around platelet aggregates.

In this study we tested for the treatment of external haemorrhagic lesions a new biomaterial based on expired platelets from blood banks immobilized on a collagen support. These platelets are no longer used for transfusions, are safe (tested for viral and bacterial contamination) and still have local haemostatic activity. Collagen is non-toxic, non-antigenic and promotes cell adhesion. Platelets bind to the collagen support and subsequently form clots through the intrinsic coagulation pathway.

Keywords

haemostatic biomaterial, medical use, coagulation, SEM.

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Introduction

The body's ability to clot blood is essential to beings and it is what allows us to survive an injury. The clotting process helps wound close up to prevent the injured person from losing too much blood. A healthy body has a specific and effective defence mechanism to prevent excessive blood loss during injuries. The coagulation system is activated very quickly. This leads to the formation of blood clots which will clump together at the lesion to stop the bleeding (haemostasis) [1, 2, 3].

Haemostasis is a vital step and quick haemostasis is important for trauma and surgical operation and other situations in emergency medical care.

The amount of blood in a normal human is approximately 7-8% of the total body weight and when blood loss reaches 20% of total blood volume, it is difficult to maintain normal blood volume and blood pressure. If the extent of blood loss exceeds 40% in a short time, this is the leading cause of trauma-related deaths. 15% of all trauma deaths are caused by a post-traumatic haemorrhage [4]. Adequate haemostasis is an essential strategy to prevent bleeding to avoid death and is the first step in wound healing. Haemostasis is the process of stopping bleeding at the injury site by a haemostatic system consisted of elements that are always present in the blood in an inactive state (platelets and soluble coagulation factors) that are activated immediately after an injury [5].

Platelets play an essential role in maintaining haemostasis, as they form the first line of haemostatic defence by forming the primary haemostatic plug.

Platelets are small pieces of cytoplasm that are shed from the cytoplasm of mature megakaryocytes and these participate in haemostasis and coagulation. Glycoprotein Ib (GPIb) and phospholipid surface of platelets participate to increasing the speed of the clotting response. Under normal circumstances, rapid haemostasis can be achieved in the presence of a large number of local adherent platelets and clotting factors. During processing and storage, platelet activation markers were observed such as the release of specific granular contents in plasma (β thromboglobulin, platelet factor 4 and RANTES), changes in GP expression on the surface of platelets (GPIB, GPIIb and GPIIIa), protein expression, sequestered granular membranes (P-selectin -CD62P, CD63 and CD40L) and the externalization of phosphatidylserine (determined by annexin V binding) [6]. Platelet, platelet-derived nano-vesicles and synthetic platelet mimics can be used to develop haemostatic materials.

With rapid developments in science and technology, various haemostatic materials have been developed with the hope of enhancing the haemostatic effect by activating different coagulation mechanisms. Therefore, developing safe, effective, and convenient haemostatic materials is important. Active haemostatic agents currently used in the field of haemostasis can be natural polymers, synthetic polymers, inorganic materials, and metal-containing materials based on activation of different coagulation mechanisms [7]. Today, the available natural polymeric haemostatic materials are polysaccharides and collagens. These materials are also widely used to manufacture artificial skin, absorbable sutures and drug carriers. Regarding the research status in this field, the design of composite haemostatic materials should conform to the requirement according to which multiple coagulation mechanisms can be simultaneously activated in order to enhance the haemostatic effect [8].

In this study, we aimed to obtain a new biomaterial based on the use of platelets from blood banks at the end of the storage period (hPL-e), immobilized on a collagen support, with good regeneration and healing properties for the treatment of external haemorrhagic lesions. These platelets are no longer used for transfusions, are safe (being tested for viral and bacterial contamination) and still have local haemostatic activity.

Materials and methods

Biological materials

Blood samples were collected from healthy donors into heparinized tubes and tested immediately after sampling. Cells were sedimented by centrifugation, 350 g at 4°C for 5 min, for the removal of plasma, platelets and leukocytes. Platelets concentrate (CUT) and plasma were provided by Tulcea County Hospital, Tulcea, Romania.

Chemicals

Fluorescein conjugated annexin-V (Annexin-V-FITC), HEPES binding buffer (HEPES buffer pH 7.4 containing 2.5 mM calcium chloride), were obtained from Pharmingen (San Diego, CA, USA).

Methods

Obtaining of haemostatic biomaterial

The first phase of the research consisted in testing the coagulation capacity of expired platelets (hPL-e), preserved for 5 days, at the end of this period. The platelet suspension was deposited in glass plates with wells, in the presence or absence of plasma and the supernatant was removed after sedimentation and fixed in cacodylate (0.1 M, pH of 7.2) and 1.25% glutaraldehyde buffer or 70% ethanol for 4 hours. The addition of freshly collected blood on heparin in the wells with immobilized platelets was analysed, after washing with

PBS, for the attachment of red blood cells to platelets and preservation of the capacity to participate in clot formation by optical microscopy and SEM analysis.

In the stage 2 of research, we proposed to use a collagen film as a support for immobilization of platelets (hPL-e), in order to obtain a haemostatic product applicable to wounds at the dermal level.

Cells may be immobilized by a number of methods for a variety of purposes. Although the study of cell immobilization is comparatively novel, the methods that have been developed are very effective and there are few indications that further, greatly superior techniques are likely to evolve [9, 10]. Cells are best immobilized by aggregation, by adsorption onto a support material or by entrapment within gels, of which the natural supports have proved the most useful.

The immobilization of the sedimented platelets on the collagen gel was done with a buffer containing 2.5% glutaraldehyde, in the presence or absence of plasma, followed by washing the collagen/platelet film product with distilled water. On top of the obtained haemostatic supports, freshly collected blood on heparin was later added and after incubation for 5 minutes, removing by washing with PBS of the red blood cells not attached to the support. In all cases, the samples were analysed by scanning electron microscopy.

Scanning Electron Microscopy (SEM) analysis. Fresh erythrocytes and platelets were fixed for 4 hours in 1.25% glutaraldehyde in cacodylate buffer (0.1 M, pH of 7.2), washed 3 times with distilled water, filtered through 0.2 μm Anodisc filters and analysed at a Hitachi SU 1510.

Flow cytometric analyses

Cells (RBCs and platelets) were analysed by flow cytometry according to Bratosin *et al*., 2007[11], and by Scanning Electron Microscopy. Flow cytometric analyses were performed on a Becton Dickinson FACScan cytometer equipped with an argon-ion excitation laser 488 nm using Cell Quest Pro software for acquisition and analysis of results. Cells were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale.

Morphological changes assessment of platelets by light scattered measurements

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with cell size and the intensity of scattered light measured at a right angle from the laser beam (SSC) correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light, being associated with cell shrinkage. Platelets suspension in isotonic PBS buffer, pH 7.4, was gated under forward and side scatter parameters (FSC versus SSC).

Flow cytometric analysis of phosphatidylserine exposure

Phosphatidylserine exposure on platelets was assessed using Annexin-V-FITC. Platelets were suspended (10^6 cells) in HEPES buffer pH 7.4 containing 2.5 mM calcium chloride with 10 μL (0.1 μg) of Annexin-V-FITC solution and incubated for 15 min at room temperature in the dark. The cells were gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (platelets autofluorescence).

Scanning Electron Microscopy (SEM) analysis

The stages of biological sample preparation for SEM imaging were as follows: Samples of biomaterial were fixed for 4 hours in 1.25% glutaraldehyde in cacodylate buffer (0.1 M, pH of 7.2), washed 3 times with distilled water, filtered through onto 0.2 μm Anodisc filters and analysed directly with a HITACHI SU 1510 scanning electron microscope.

Results

Morphological changes analyses of expired platelets by flow cytometry and scanning electron microscopy (SEM)

Flow cytometric analyses of expired platelets (hPL-e)

Flow cytometry is a widely accepted method for the detection of platelet activation [12]. Expired platelets (hPL-e), preserved for 5 days, were analysed at the end of this period for morphological changes by light scattered, in the mode FSC/SSC that provides information about cell size and structure and for phosphatidylserine (PS) exposure using Annexin-V-FITC compared with fresh platelets. Activation of platelets was observed as a morphological change from the discoid state to activated spherical cells with pseudopods.

As shown in Figure 1, the image given for the dot-plot test contains 2 areas, namely viable platelets and senescent cells whose morphological parameters have changed, having a decrease in size and density, according to cells undergoing apoptosis. This picture evolves during storage, from day 1 to day 5, with the zone of viable platelets rapidly retreating to apoptotic cells for platelets preserved in transfusion centres.

These morphological changes have led to the dot-plot different from that obtained for the control platelets (Fig 1).

It has also been shown that the morphological changes are accompanied by PS externalisation, correlating the results obtained by the dot-plot analysis with the results provided by the Annexin-V-FITC test. A higher percentage of approximately 31% and 44% PS is observed for day 2 and

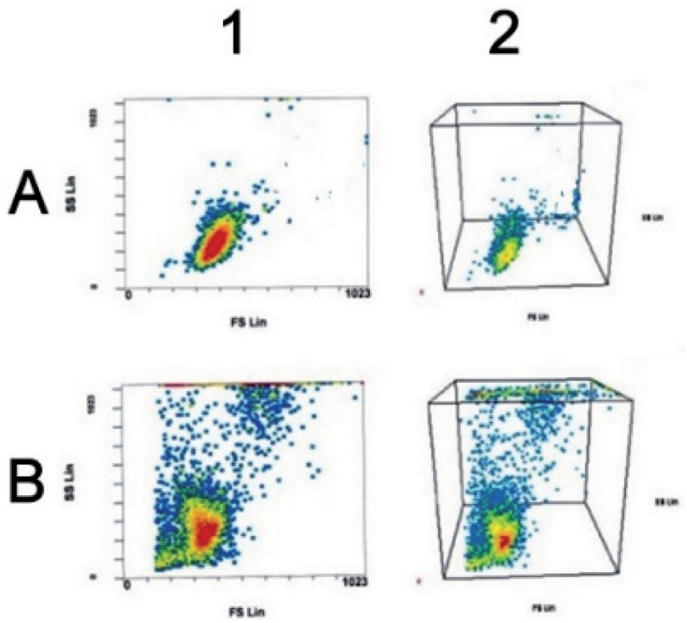

Fig 1. Flow cytometric analysis of fresh (A) and expired platelets *(hPL-e)* (B); (1) Dot-plot analysis Forward Scatter (FSC Lin) versus Side Scatter (SSC Lin); (2) 3D-plot analysis FSC Lin/ SSC Lin /vertically number of cells

day 3, and 65% to 83% PS in platelets preserved on day 5 and 7, compared to 5% - 10% PS in platelets at the time of their collection was obtained.

This morphological change, possibly together with a high percentage of platelets externalizing PS demonstrated that platelet concentrate stored at 20 °C (room temperature) for 5 days storage, (the storage temperature currently used in blood banks), shows a loss of their integrity and functionality, known as the platelet storage lesion.

Scanning Electron Microscopy (SEM) analysis of expired platelets

Scanning electron microscopy showed modification of platelets at the end of the preservation period losing their initial discoidal shape, almost entirely showing the appearance of cells in apoptosis, with vesicles, pseudopodia formation and membrane blebbing. Freshly platelets have a discoid shape, whereas expired platelets are much smaller, mostly with a spherical morphology (Fig 2).

Platelets were activated, since pseudopodia were prominent and interactions, especially platelet–platelet interaction, could be distinguished. This shows that platelets are activated and release their granules. Platelet pseudopodia formation is the initial morphological change associated with adhesion and aggregation. These processes are suggested to aid adherence to other platelets and the forming fibrin strands. The increased pseudopodia formation seen during storage, and the visible interaction of the processes with other platelets, therefore support platelet adhesion and aggregation.

Subsequent granule release will recruit additional platelets and trigger the formation of the fibrin network to form the stable, secondary haemostatic plug. The observed membrane blebbing can therefore be the result of elevated thrombin concentrations. However, no thrombin was added to the platelet samples. It can be deduced that elevated thrombin concentrations were present in the plasma. It is known that elevated thrombin induces platelet activation, resulting in blebbing of the membrane similar to apoptosis. Membrane blebbing is one of the traits of apoptosis. Scanning electron microscopy confirms the results obtained by flow cytometry.

In vitro haemostasis evaluation and characterisation of biomaterial by SEM analysis

Haemostasis analysis obtained in the first stage of the research consisted in testing the coagulation capacities of platelets preserved for 5 days, at the end of this period. (hPL-e) were deposited as a suspension in glass plates with wells, in the presence or absence of plasma. After sedimentation, supernatant was removed and platelets adhered to the glass were fixed with cacodylate/glutaraldehyde buffer or 70% ethanol for 4 hours. Freshly collected blood on heparin it was added for testing of their haemostatic capacities.

The attachment of red blood cells to platelets was measured by measuring the capacity to participate in the clot formation as preservation of haemostatic properties. The best results were obtained when hPL-e were fixed with glutaraldehyde and observed by optical microscopic analysis (data not shown).

If the platelets were fixed on a collagen support, as shown in Figure 3, a first analysis was conducted by scanning electron microscopy of the attachment of red blood

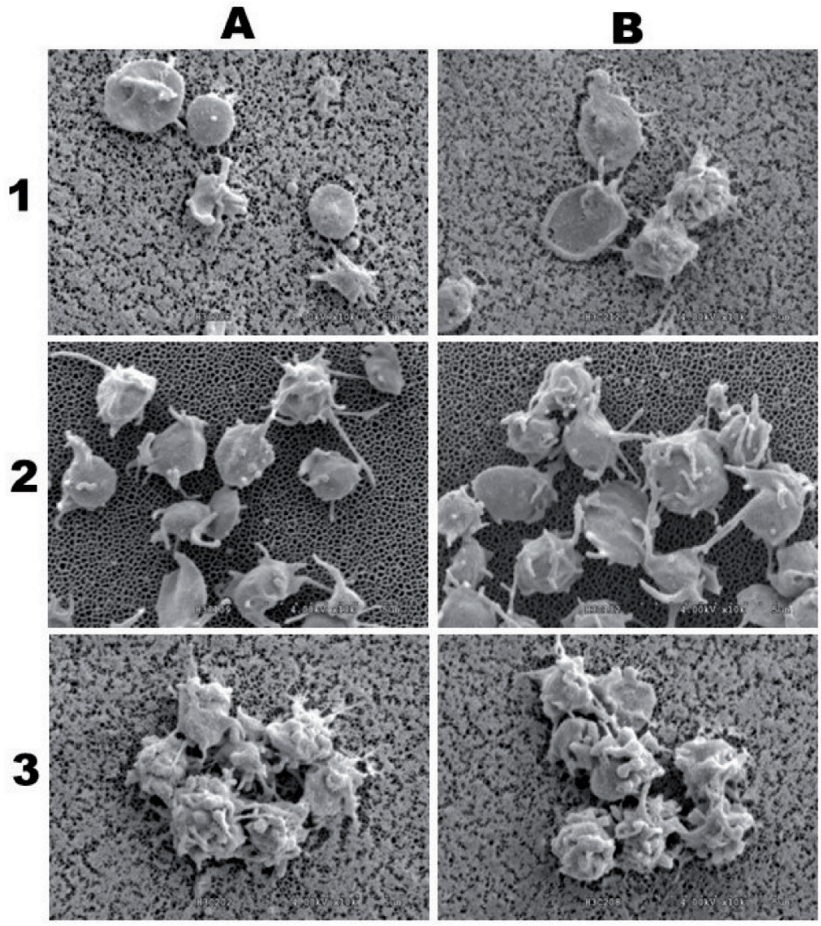

Fig 2. Analysis by Scanning Electronic Microscopy (SEM) of expired platelets (hPL-e), preserved in transfusion centers for 3-day storage (2-A and B) and 5-day storage (3-A and B) compared with freshly normal (hPL) at time zero sampling (1-A and B). Data shown are representative for similar results.

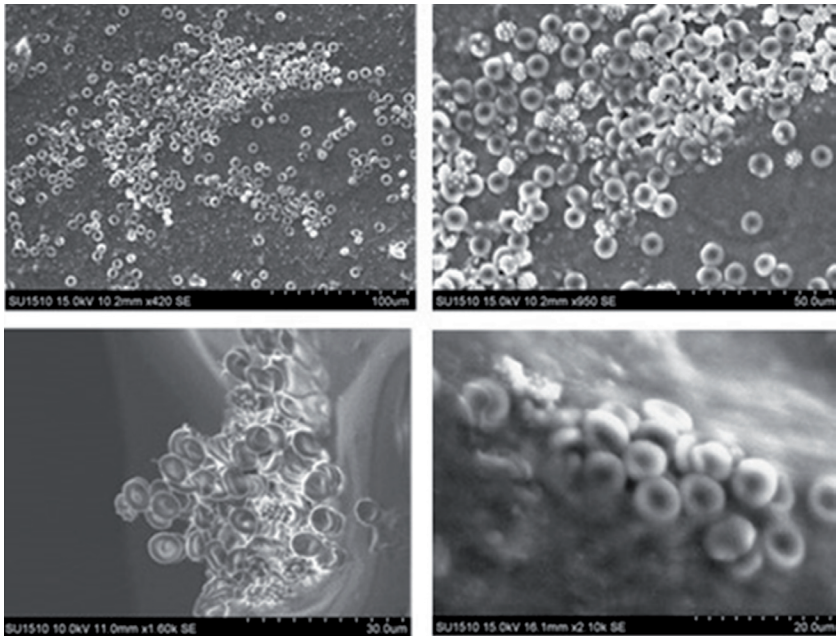

Fig 3. Scanning Electronic Microscopy analysis of *in vitro* haemostasis evaluation and clot formation of expired platelets (hPL-e) fixed on a collagen support. Data shown are representative for similar results.

cells to platelets and clot formation, and the best results were obtained when platelets were fixed with glutaraldehyde in the presence of plasma.

Discussion

Blood loss has always been a concern especially in surgery and bleeding control methods. Over the past years, a number of haemostatic agents and tissue sealants have been developed and are currently used in various surgical disciplines.

The efficacy of some products has been established, but others are awaiting further evaluation and confirmation. Haemostatic treatments include fibrin sealants, microfibrillar collagen, gelatin, haemostatic agents, oxidized regenerated cellulose and cyanoacrylates adhesives [13, 14].

The research and development of haemostatic materials are prerequisite for effective haemostasis. Chitosan with a good biodegradability, biocompatibility and nontoxicity and excellent haemostatic properties led to the appearance of new haemostatic materials based on chitosan widely applied in medicine [8]. Haemostatic agents work by forming blood clots at bleeding site of vessel injury either mechanically or by increasing the coagulation cascade [14]. The first steps to take to stop bleeding due a vascular lesion, limit blood loss and allow healing is ensured by the platelets. After the platelets have formed a first barrier limiting bleeding, the clot is consolidated by the formation of a fibrin network organized around aggregates platelets [15].

Collagen is an extracellular-matrix protein that plays an important role in the formation of tissues and organs and it is non-toxic, non-antigenic, favours cell adhesion, proliferation, and differentiation to mimic the natural cell environment [16]. Erythrocytes and platelets have in their membrane structure Annexin-V, known for its ability to bind to PS. Thus, RBCs recognize the large number of PS residues in the membrane of expired platelets or membrane annexin-V of hPL-e is fixed by PS of RBCs and generate the formation of a thrombus phosphatidylserine [17, 18, 19, 15]. Platelets bind to the collagen framework and subsequently form clots via the intrinsic coagulation pathway. Haemostasis is usually achieved in 2-5 minutes.

The development of medical services requires an increased effort for new haemostatic materials performance, efficiency, security, designed for easy transport.

Conclusions

In conclusion, advances were achieved in the field through our haemostatic biomaterial based on expired platelets, which are safe (platelets were tested for viral and bacterial contamination) and still have local haemostatic activity. Collagen is non-toxic, non-antigenic and promotes cell adhesion. Erythrocytes from blood bind to the expired platelets fixed on collagen support and subsequently form clots through the intrinsic coagulation pathway. Our haemostatic biomaterial can contribute to solving the current needs in medical practice.

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