

Original Article

Identification of mesothelial cells in intraoperative blood salvage

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Received July 24, 2018; Accepted November 16, 2018; Epub March 15, 2019; Published March 30, 2019

Abstract: Intraoperative auto-transfusion with the use of cell saver systems is routinely used to reduce the rate of packed red blood transfusion in major surgery. Nevertheless some concerns have been raised on possible risks of coagulation disorders. The aim of the study was to analyze the blood processed by the cell saver, ready to be re-infused to the patient, in order to individuate unexpected cellular components, that can favor coagulopathy. We tested the blood processed by the cell saver in thirteen patients undergoing coronary bypass surgery with Cellsearch[®], ScreenCell[®], Cytology and Immunofluorescence. Those four methods allowed us to look for the presence of unexpected cells, quantify and characterize them. Furthermore, the blood processed by the cell saver was mixed with the patient's peripheral blood and analyzed with the ROTEM[®] thromboelastography. The Cellsearch[®] revealed and counted a mean number of 1241 unexpected cells/7.5 ml in the blood processed by the cell saver. The ScreenCell[®] and Cytology confirmed the presence of non-hematological cells. Immunofluorescence showed positivity for Calretinin and WT-1, confirming the mesothelial origin. Moreover we detected a peculiar arrangement of the platelets around the mesothelial cells in a "cloud" form, suggesting platelet activation. The ROTEM[®] analysis showed a significantly longer clot formation time, smaller clot amplitude and maximum clot firmness, compared to controls. In conclusion we demonstrated the presence of mesothelial cells in the cell saving blood, ready to be auto-transfused. This finding can contribute to develop a platelet depletion coagulopathy, with coagulation factors consumption.

Keywords: Mesothelial cells, cell saving system, circulating cells, platelet aggregation, coagulation, cardiac surgery

Introduction

The cell saving systems (CSS) are traditionally used in major surgery and routinely in cardiac surgery to increase the postoperative hemoglobin level and to reduce the rate of packed red blood transfusions. The rationale of the cell saving systems is "saving" the red blood cells when a surgical bleeding occurs, therefore, the blood is sucked from the surgical field, filtered, collected in a dedicated reservoir and eventually centrifuged separating the red blood cells from plasma, platelets and white blood cells. As a consequence, the re-infused blood actually lacks platelets and coagulation factors, a condition that may impair the coagulative system leading to a prolonged clot formation time [1] and a need of platelet transfusion [2].

These considerations suggested the need for in-depth evaluations of the cell saving process, analyzing specifically the cellular component, because the coagulative effects highlighted after the blood reinfusion may be caused by something more than just technical issues. During the cardiac surgical maneuvers, in fact, the pericardium and the pleurae are usually opened and the blood coming from the heart chambers and from the vessels comes in contact with the mesothelial surfaces. Moreover, mesothelial cells derived from the pericardium and the pleurae exfoliate during the surgical maneuvers, and may be eventually sucked with blood. We previously demonstrated the presence of mesothelial cells in patient peripheral blood during the cardiopulmonary bypass, after the suction of blood from the surgical field, and their interfering with coagulation [3].

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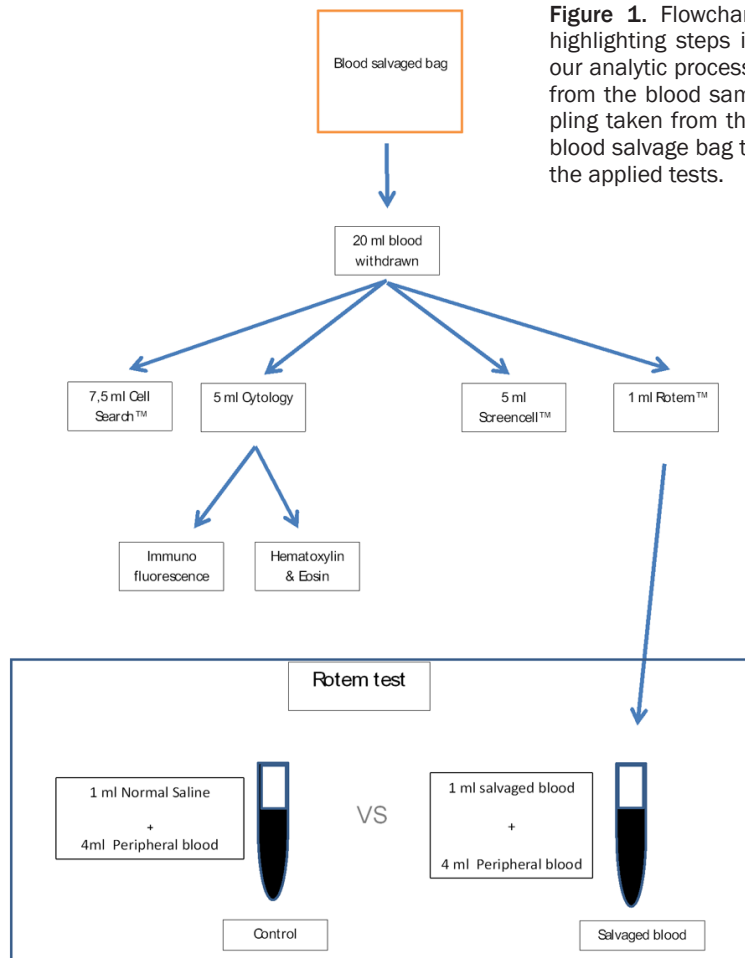


Figure 1. Flowchart highlighting steps in our analytic process: from the blood sampling taken from the blood salvage bag to the applied tests.

closure) are exclusively managed by the institutional perfusionists. Standard set up of the machine have been used for the study: filter in the collection reservoir: 40 microns, pump fill rate: 300 ml/min, pump wash rate: 250 ml/min, empty fill rate: 250 ml/min, final hematocrit 55%, wash solution for every process: 900 ml.

A total of 20 ml of blood was withdrawn from the reinfusion circuit (as recommended by the manufacturer and according to our internal guidelines, when we infuse any blood product we use a specific infusion set with built-in microaggregate filter) and divided in five samples to be analyzed in five different ways: Cellsearch®, ScreenCell®, Cytology, Immunofluorescence, and ROTEM®; test as summarized in **Figure 1**. The first three analyses were performed to achieve a direct observation of cells in the processed blood and to look for the presence of non-hematological cells eventually passed

through the cell saver filters and ready to be transfused into patients. Immunofluorescence was performed in order to define the nature of the non-hematological cells. Finally the ROTEM® analysis, a qualitative and quantitative assessment of the hemostasis, was used to evaluate the effects of the re-infused blood on the coagulation competency.

Therefore, since the origin of re-infused blood from the cell saver is actually the blood from the surgical field, we verified the presence of mesothelial cells in the processed blood and their impact on the coagulation.

Material and methods

We applied multiple methods to demonstrate the presence of mesothelial cells in the blood ready to be transfused and the impact of this cell on coagulation.

After approval by the institutional ethical committee, a written informed consent was obtained by each patient. In thirteen coronary patients, undergoing an elective, off pump, coronary artery bypass graft procedure, we collected the processed blood from the cell saver (Sorin Xtra, LivaNova PLC, UK) system after the lavage.

Blood salvage systems, routinely used during the whole operation (from the cut skin to the

In our center, as a routine, the blood, sucked from the surgical field during the operation, is processed after the protamine administration and re-infused within 6 hours.

CELLSEARCH® Immunomagnetic enrichment and sample staining procedures of circulating epithelial cells

The CellSearch® system (Menarini, Silicon Biosystem) is a FDA-approved automated procedure originally developed to identify and count epithelial cells in blood samples, for diagnosis and follow-up of epithelial tumors [4-6]. We

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Table 1. Characteristics of the patients

Patient n°	Age	Sex	History of smoke	Diabetes	EF (%)	Recent STEMI	Previous PTCA	Number of grafts	ITA use	Bilateral ITA
1	74	Male	Yes	Yes	45	No	No	3	Y	No
2	68	Male	Yes	No	50	No	Yes	2	Y	Yes
3	64	Female	Yes	Yes	38	Yes	No	3	Y	Yes
4	81	Female	No	Yes	40	No	Yes	2	Y	No
5	74	Male	Yes	No	45	Yes	No	1	Y	No
6	56	Male	Yes	Yes	55	No	No	4	Y	Yes
7	63	Female	No	Yes	55	No	No	3	Y	Yes
8	74	Male	Yes	No	40	Yes	Yes	2	Y	Yes
9	77	Male	No	No	50	No	No	2	Y	No
10	61	Male	Yes	Yes	45	No	No	3	Y	Yes
11	65	Female	Yes	Yes	50	Yes	Yes	2	Y	Yes
12	74	Male	No	Yes	40	No	Yes	3	Y	No
13	70	Male	Yes	No	55	Yes	Yes	3	Y	No

EF, Ejection fraction; STEMI, ST elevation myocardial infarction; PTCA, percutaneous transluminal coronary angioplasty; ITA, internal thoracic artery.

recently demonstrated the validity of this method to identify circulating non-hematological cells in cardiac procedures [3]. Therefore, we applied the same technique for the blood processed by the cell saver.

For each patient we collected a blood sample of 7.5 ml directly from the reinfusion bag. Since the hematocrit of the processed blood is generally around 60%, the sample was diluted with the diluent buffer, in order to make it suitable for the red cell detection control of the CellSearch® processing. Standard protocols and reagents were used. Reassuming, blood samples were collected into dedicated tubes and the CellSearch® Epithelial Cell kit (Menarini Silicon Biosystem) was applied for enrichment and enumeration. Epithelial non-hematological circulating cells were captured from blood by anti-EpCAM-antibody-bearing ferrofluid and subsequently identified by cytokeratin positivity, leukocyte common antigen CD45 negativity and 4',6-diamidino-2-phenylindole (DAPI) staining to ensure the integrity of the nuclei.

Cytology

As previously reported we applied a cytologic method to collect and concentrate fluids containing a low number of cells [3]. In brief, blood samples from the reinfusion bag were collected in 5 ml ethylene diamine tetra acetic acid Vacutainer tubes, maintained at 4°C and processed within 2 hours after collection. A dilu-

tion with sterile phosphate-buffered saline and centrifugation at 800× g for 10 min, without brake, were performed. Subsequently, a red blood cell lysis buffer was used, followed by washing with phosphate buffered solution. After 10 min centrifugation, ≈100 µl of sediment samples were smeared on a slide, fixed with an aerosol preparation of a neutral pH-buffered saline that contains 4% w/v paraformaldehyde. Five slides for each case were collected and two of them were stained with hematoxylin and eosin for morphological evaluation at light microscope (Leica DMD108) by a team of pathologists (CM, AA, PZ).

The remaining slides were collected for Immunofluorescence.

ScreenCell® Cyto-kit, filtration system

ScreenCell® is method based on size-exclusion filtering, for isolation of circulating rare cells. It is a filtration mini-device, composed of 8 micron microporous membrane filter, in order to exclude red blood cells and to isolate cells over 8 micron. Hematoxylin and eosin staining of the filters allows the morphologic observation of the cells and their measurement.

All blood samples were collected in 3 ml ethylene diamine tetraacetic acid Vacutainer tubes, maintained at 4°C and processed within 2 hours after the collection, as recommended by the manufacturer (ScreenCell® Cyto kit, Paris,

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Table 2. Number of mesothelial cells automatically counted by the CellSearch® in 7.5 ml of saved blood

	N° cells counted per 7.5 ml
Patient 1	906
Patient 2	1044
Patient 3	1740
Patient 4	850
Patient 5	1532
Patient 6	1630
Patient 7	1952
Patient 8	856
Patient 9	1247
Patient 10	986
Patient 11	1025
Patient 12	896
Patient 13	1475
Mean ± sd. dev	1241.1 ± 379.5

France). Blood was diluted with FC2 buffer (ScreenCell™ Cyto kit, Paris, France) and incubated for 8 min at room temperature, shaking gently after 2 and 6 min. Thereafter the filters were rinsed with 1,6 ml of sterile phosphate buffered solution 1X, pH 7.4, collected from the device, air-dried and immediately stained for 1 min with Hematoxylin Solution S (Merck, Darmstadt, Germany) and then for 20 seconds with Shandon Eosin Y Aqueous Solution (Thermo Fisher Scientific Inc., Waltham, MA, USA), washed in distilled water, air dried and mounted on a glass slide for cytological evaluation by certified pathologists (CM, AA, PZ).

Immunofluorescence

For each patient we prepared slides dedicated to immunofluorescent analysis (Superfrost, Leica Microsystems, Milan, Italy), as previously reported.

Briefly, fixed cells were permeabilized with 1% saponin, and blocked with 10% bovine serum albumin (BSA) in phosphate buffered solution 1X for 30 min at 37°C. Cells were then stained overnight with anti-Calretinin (mouse, 1:100; cod. NCL-L-CALRETININ, clone 5A5; Leica Microsystems, Germany) and anti WT-1 (rabbit, 1:1500; cod. PA5-16879; Life Technologies, California, United States) in 1% BSA, followed by incubation with an anti-mouse FITC-conjugated secondary antibody (1:400; Santa Cruz Biotechnology, Heidelberg, Germany) and anti-

rabbit Alexa Fluor 546-conjugated secondary antibody (1:400; Santa Cruz Biotechnology, Heidelberg, Germany) for 60 min at room temperature. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (2 g/ml; Santa Cruz Biotechnology, Heidelberg, Germany), mounted using an antifade mounting medium (Life Technologies, Monza, Italy).

All nucleated cells in each slide were imaged in 2 fluorescent channels. Calretinin and WT-1 positive cells containing intact DAPI nuclei were identified as mesothelial cells. As positive controls we used cytological slides obtained from pleural washing. Negative controls were also carried out. Microscopic observation was performed at a Laser Microscope (Leica DM2500 Leica Microsystems, Milan, Italy) by three pathologists (CM, AA, PZ).

ROTEM® delta test

To have a qualitative *in vitro* controlled assessment of the effect of intraoperative salvage blood on the coagulation, we used the ROTEM® Delta test (Tem International GmbH) [7, 8]. Briefly, the ROTEM® Delta Test measures kinetic changes of the clot elasticity of whole blood samples, testing both the intrinsic and the extrinsic pathway. It gives qualitative and quantitative evaluation of the clot formation, providing important information on the hemostasis competency.

For each patient we collected two samples of 4 ml of peripheral blood immediately after the skin closure. These specimens were mixed respectively with 1 ml of normal saline (control group), and 1 ml of intraoperative saved blood to simulate a reinfusion of 20% of total circulating blood (reinfusion group). The two samples have been tested with the ROTEM analysis for the INTEM and the EXTEM assay (respectively testing the thromboelastography via the intrinsic or the extrinsic pathway). The assay tested the clotting time (CT), clot formation time (CFT), the alpha angle, the amplitude of the clot 5 and 10 min after the clotting time (A5 and A10), the maximum clot firmness (MCF). An impaired activation of the coagulation is expressed by a prolonged CT; a polymerization disorder is associated to prolonged CFT and/or reduced MCF; on the other hand a reduced MCF with a normal CFT rather indicates a deficiency of clottable substrate (fibrinogen and/or platelets).

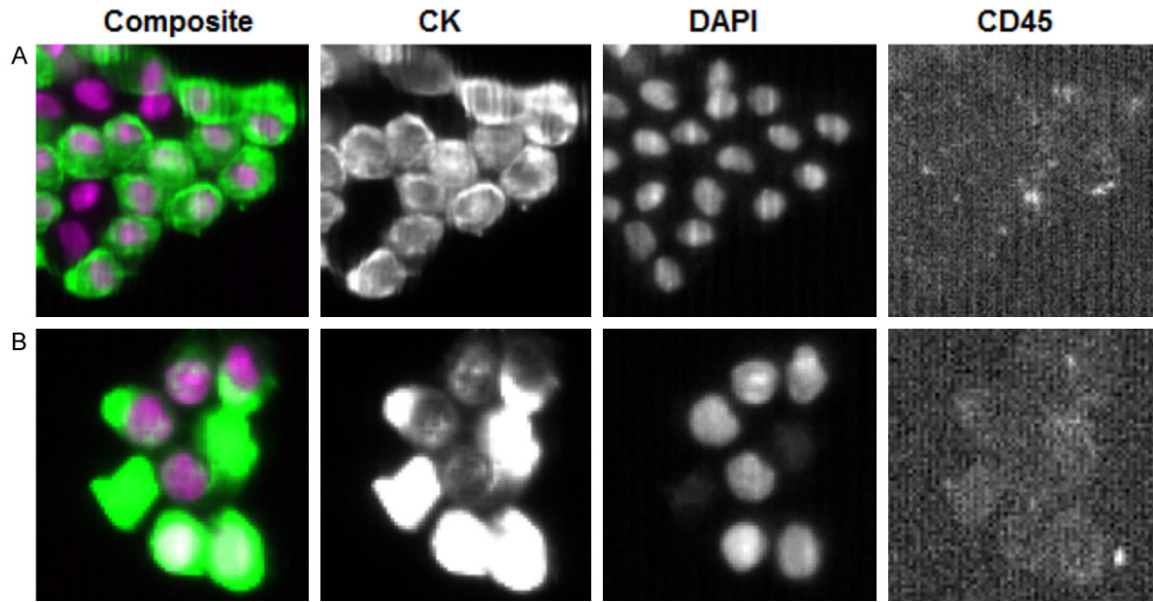


Figure 2. CellSearch® system. Representative images of mesothelial cells macroemboli (A) and microemboli (B) detected by CellSearch™ technologies. Images are automatically taken by the instrument and analyzed by pathologists. Mesothelial cells were recognized as nucleated cells (DAPI, purple staining) positive for cytokeratin 8-18 and 19 (CK, green staining) and negative for CD45, as leucocyte marker. (Magnification 630×).

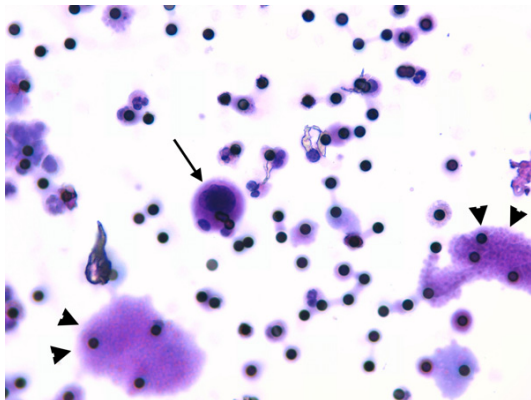


Figure 3. ScreenCell® filtration system. ScreenCell® microporous filter stained with hematoxylin and eosin; the black dots are the pores of the filters (8 µm in diameter). Please note an epithelial cell indicated by an arrow, whereas arrows head indicates platelet aggregates. (Magnification 400×).

Statistical analysis

All statistical analysis were performed using R 3.3 for Windows (R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/> and SAS 9.4 software, by SAS Institute Inc., Cary, NC, USA).

Continuous variables were reported as mean±standard deviation. Categorical variables were expressed as percentages. Comparisons of mean values for the ROTEM tests (CT, CFT, alpha angle, A5, A10, MCF) were performed with Paired T-Test, a P < 0.05 was considered significant.

Results

Patient clinical features are summarized in **Table 1**. Mean age of the patients recruited was 69.3 ± 7.1 years, nine were male.

The Cellsearch® automatically counted a mean number of 1241.4 ± 379.5 non-hematological cells per 7.5 ml, results are showed in **Table 2**. The identified cells were single and arranged in cellular micro-aggregates (clusters) as showed in **Figure 2**.

ScreenCell® filtration system confirmed the presence of non-hematological cells in all samples (**Figure 3**, arrows). Moreover, multiple platelet aggregates were observed on the microporous filter surface (**Figure 3**, arrows head).

Cytology showed single cells with a mean size of 30 µm, and clusters with a mean size of 70

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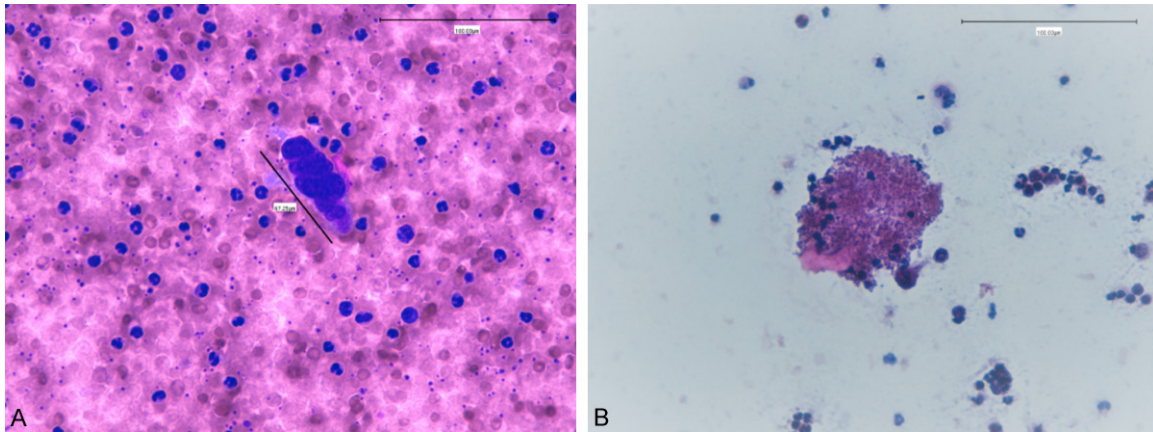


Figure 4. Cytology. Hematoxylin and eosin staining of a cytology smear. A. In the center is present a 67, 25 m macro-embolus (cluster), surrounded by platelets organized in a “cloud form”. (*Magnification 400×*). Hematoxylin and eosin staining of a cytology smear. B. Platelet aggregation in a clot-like organization is showed. (*Magnification 200×*).

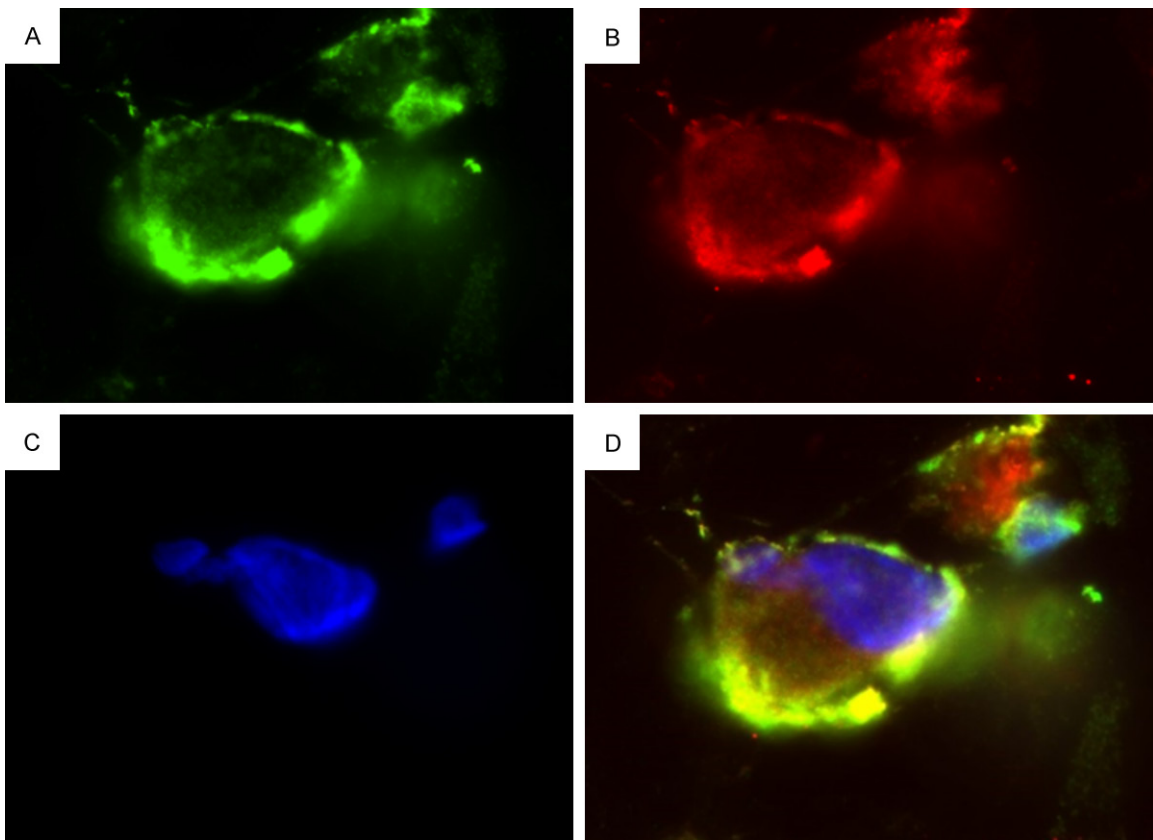


Figure 5. Immunofluorescence analysis. Immunofluorescence images demonstrating Calretinin and WT-1 double positive cells. Please note the specific and strong cytoplasmic staining for Calretinin (shown in green - A), and for WT-1 (shown in red - B). Nuclear counterstaining: 4', 6-diamidino-2-phenylindole (shown in blue - C). A yellow signal by the merging of both channels demonstrating the double positivity of the cells (D). (*Magnification 630×*).

μm , surrounded by platelets in a cloud form (**Figure 4A**). In addition, we revealed the presence of platelet aggregation in a clot-like organization (**Figure 4B**).

Immunofluorescence analysis highlighted the presence of double-positive cells for Calretinin and WT-1, confirming the mesothelial origin of the detected cells (**Figure 5**).

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Table 3. ROTEM® results of the blood re-infused after the cell saving process compared with the controls

	INTEM			EXTEM		
	Control	Reinfusion	<i>P</i> value	Control	Reinfusion	<i>P</i> value
CT (s)	206.9 ± 23.1	207.2 ± 16.9	0.96	73.9 ± 15.9	68.9 ± 12.1	0.08
CFT (s)	81.1 ± 21.4	119.5 ± 34.7	0.005	110.3 ± 16.7	142.9 ± 22.7	< 0.001
A5 (mm)	46.6 ± 7.3	38.4 ± 5.9	0.003	44.4 ± 5.1	37.0 ± 6.4	< 0.001
A10 (mm)	55.9 ± 8.4	48.6 ± 5.5	0.006	55.2 ± 5.1	48.0 ± 5.7	< 0.001
MCF (mm)	64.4 ± 7.9	57.6 ± 3.8	0.008	63.2 ± 4.4	57.1 ± 5.0	< 0.001
Alfa (°)	74.9 ± 1.9	67.1 ± 6.3	< 0.001	68.7 ± 5.6	67.1 ± 4.1	0.008

CT, clotting time, CFT: clot formation time, A5 and A10: amplitude of the clot 5 and 10 minutes after the clotting time; MCF: maximum clot firmness; Alfa: the alfa angle.

ROTEM® analysis showed an altered coagulation process in the reinfusion samples, indeed both the INTEM and EXTEM tests were deranged. The clot formation time was significantly longer in the reinfusion samples than in the controls. Similarly the A5 and A10, the amplitude of the clot at 5 and 10 min respectively, were significantly smaller in the reinfusion compared to controls; as a consequence, the MCF resulted smaller as well (Table 3).

Discussion

Cell salvage (the autotransfusion of shed blood) was first proposed in 1818 and first mentioned in the literature in 1885 [9]. Nowadays, the use of cell saver is largely adopted with the intent of reducing allogeneic blood transfusion and did not appear to impact adversely on clinical outcomes in cardiac surgery [10].

In our study, anyway, we demonstrated for the first time the presence of a considerable number of mesothelial cells, single and micro-aggregates (clusters) in the reinfusion bag of the cell saver, via the application of Cellsearch® technology. The presence of cells was also confirmed by ScreenCell® filtration system and Cytology. Immunofluorescence demonstrated the mesothelial origin of the identified cells. Moreover, cytological analysis highlighted the presence of platelets aggregation around the mesothelial cells and clusters, suggesting that platelet activation occurs early in the reinfusion bag. This new finding demonstrated that the processed blood, ready to be infused, contains isolated mesothelial cells, clusters of aggregated mesothelial cells and activated platelets. It is unclear if the cell aggregates develop when the blood remains in the reservoir or during the

different phases of the procedure, anyway they are present in the blood ready for infusion into patient.

As already shown in our previous study, the surgical maneuvers may lead to exfoliation of mesothelial cells from pericardium and pleura and consequent mixing with the sucked blood [3]; moreover, we demonstrated that, when a sample of mesothelial cells come in contact with the own patient's blood, it cause deranging of the clotting competency [3]. This result is not really surprising, since activated mesothelial cells express the Tissue Factor, therefore they can activate the clotting cascade [11-14]. The morphological manifestation of this occurrence was, probably, the presence of platelet aggregation, also around the cell clusters.

Our observations raised an important question about the use of Cell Saver. How much the re-infused blood can trigger the inflammatory reactions and coagulopathy?

We tried to answer to this question with the ROTEM analysis. This analysis allowed us to evaluate the *in vitro* effects of the re-infused blood on the coagulation, and showed that both INTEM and EXTEM test, in the cell saver samples had significantly longer CFT and smaller MCF, A5 and A10 than controls, suggesting a platelet and fibrinogen depletion. In this scenario, the coagulopathy appeared as a consequence of platelets activation and aggregation around the mesothelial cells rather than a simple dilution of coagulation factors, since the control samples were actually diluted with normal saline.

The recent literature data are difficult to compare to our study due to the different setting

of the published studies and the effects of cardiopulmonary bypass on the results: anyway, the processing of the shed blood from pericardium is widely accepted and recommended [15], with several evidences showing positive effects of the processing of cardiotomy blood [16, 17].

Nevertheless, other authors showed a deranged thromboelastographic profile similar to our results [18], and further evidences on high risk patients showed an impaired blood coagulative function associated with the cell saver use [19]. Moreover, Westerberg and colleagues [20] showed that avoiding cell saver and cardiotomy suction leads to lower activation of tumor necrosis factor alfa (TNF α), interleukin-6 (IL-6) and complement factor C3.

The aim of our study was to analyze specifically the blood processed by the cell saver system, avoiding the detrimental effect of the cardiopulmonary bypass. For this purpose we tested a group of patients undergoing off pump coronary bypass, in order to obtain more reliable results.

Moreover, most of the studies are focused on *in vivo* evaluations, based on clinical outcome of patients re-transfused with saved blood; this experimental approach implies several possible confounding factors, such as the blood losses in the chest drains, transfusions, and the different management of the patients. Our controlled test, on the contrary, supported by the direct microscopic observation of mesothelial cells and activated platelets, was designed to show an impact of the reinfusion on the thromboelastographic profile of the blood of the same patient independently by the perioperative management, since the simulation of the reinfusion has been made *in vitro*.

Some words of caution are necessary. We tested only one cell saving system (Sorin Xtra, LivaNova PLC, UK), nevertheless, since the centrifugal system is similar, we may expect the same results. It would be advisable to test other machines in the future.

We simulated a reinfusion of 20% of circulating blood, therefore we can only suppose the effects of a different rate of reinfusion and the possible therapeutic options (platelet, fibrinogen and coagulation factor transfusion). Finally,

our findings have been obtained on a cardiac setting, where large mesothelial surfaces are clearly open and exposed to surgical maneuvers: it would be interesting to verify whether other surgical environments (liver transplant, orthopedic surgery) with different mesothelial surfaces would lead to similar results.

Conclusions

Intraoperative blood salvage is an important resource in cardiac surgery and in major surgery.

We demonstrated for the time the presence of mesothelial cells in the blood ready to be infused into patient. In addition, we observed activated platelets and alteration of ROTEM[®] test, highlighting a detrimental impact on coagulative competence. These findings open a new scenario on autotrasfusion procedures. Further studies will be needed to better understand the effect of re-infused blood by cell saving systems and consequently ameliorating the existing devices, to improve the perioperative outcome of patients.

Acknowledgements

The authors thank Federica Figuccia and Teresa Mancuso for the valuable technical help.

Disclosure of conflict of interest

None.

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