FLOW CYTOMETRIC ANALYSIS of ERYPTOSIS and HUMAN RED BLOOD CELL-DERIVED EXTRACELLULAR VESICLES (RBC-EVs)

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INTRODUCTION

Red blood cells (RBCs) are anucleated, obligatory glucose consuming cells without an extended intramembranous system. Extracellular vesicles (EVs) are cell derived membrane particles.

Formation of RBC-EVs occur under different circumstances, for example during eryptosis, an apoptosis-like process of RBCs. Eryptosis is comparable to apoptosis in many aspects, such as cell shrinkage, membrane blebbing, phosphatidylserine (PS) externalization, Ca^{2+} signaling and efferocytosis.

Currently, eryptosis is considered the primary source of RBC-EV formation.

AIMS

- Flow cytometry analysis
  - Eryptosis
  - RBC-EVs

MATERIALS and METHODS

Human RBCs were isolated from EDTA-anticoagulated whole peripheral blood of healthy volunteers. Centrifugation and washing steps were used in order to completely eliminate all residual plasma, platelets and nucleated cells.

Eryptosis and RBC-EV formation were induced *in vitro* by A23187 calcium ionophore or cold stress. Modulation of eryptosis was executed by the administration of calcium ions (Ca^{2+}) and glucose. The concentrations of applied chemicals are detailed in Table 1.

RBC-EVs were isolated by differential centrifugation. Characterization of eryptosis and RBC-EVs were recorded on a CytoFLEX S flow cytometer.

Table 1. Concentrations of inducing and modulating agents.

EFFECT	STIMULI	CONCENTRATION
INDUCTION	A23187 (Ca^{2+} ionophore)	1 µM
	cold stress (4 °C)	-
MODULATION	Ca^{2+}	1 mM
	glucose	5 mM

DETECTION of ERYPTOTIC RBCs

PARAMETERS

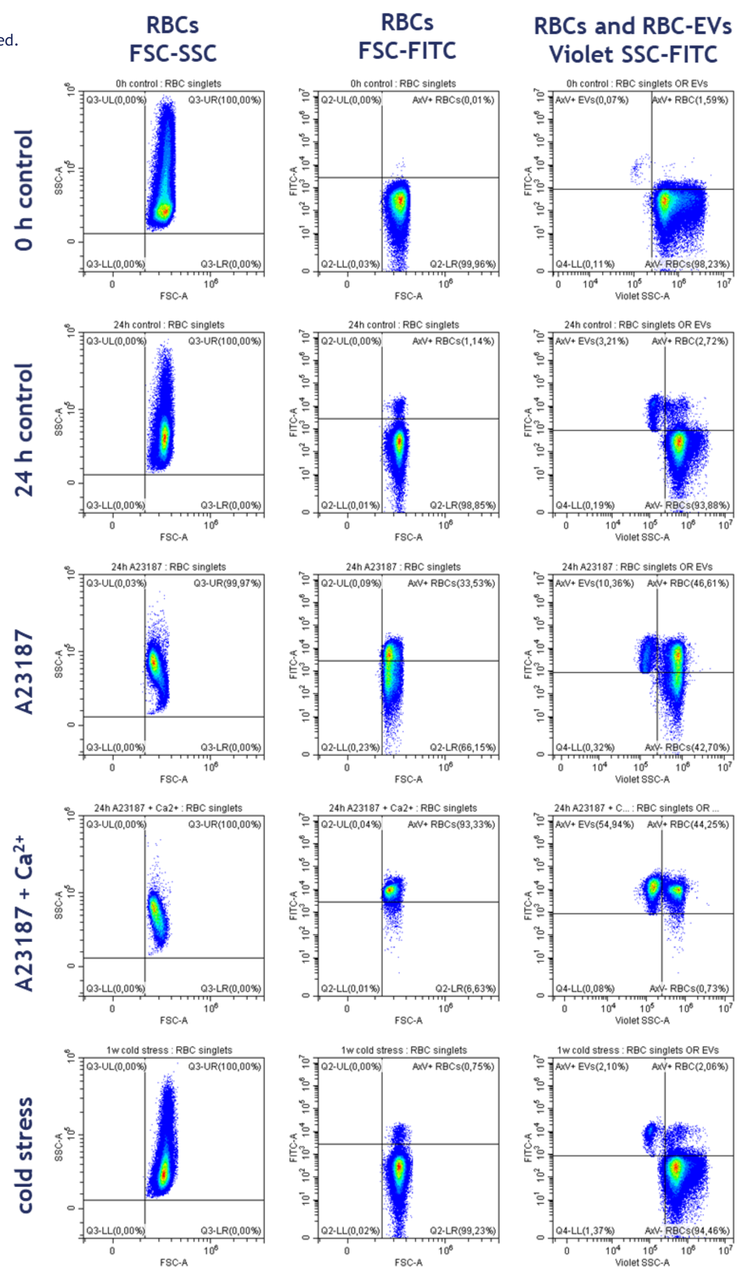
- phosphatidylserine externalization — Annexin V positivity
- cell shrinkage — decreased FSC and SSC profiles

GATING STRATEGY

- all events
  - RBCs → RBC singlets
  - EV size range → EVs

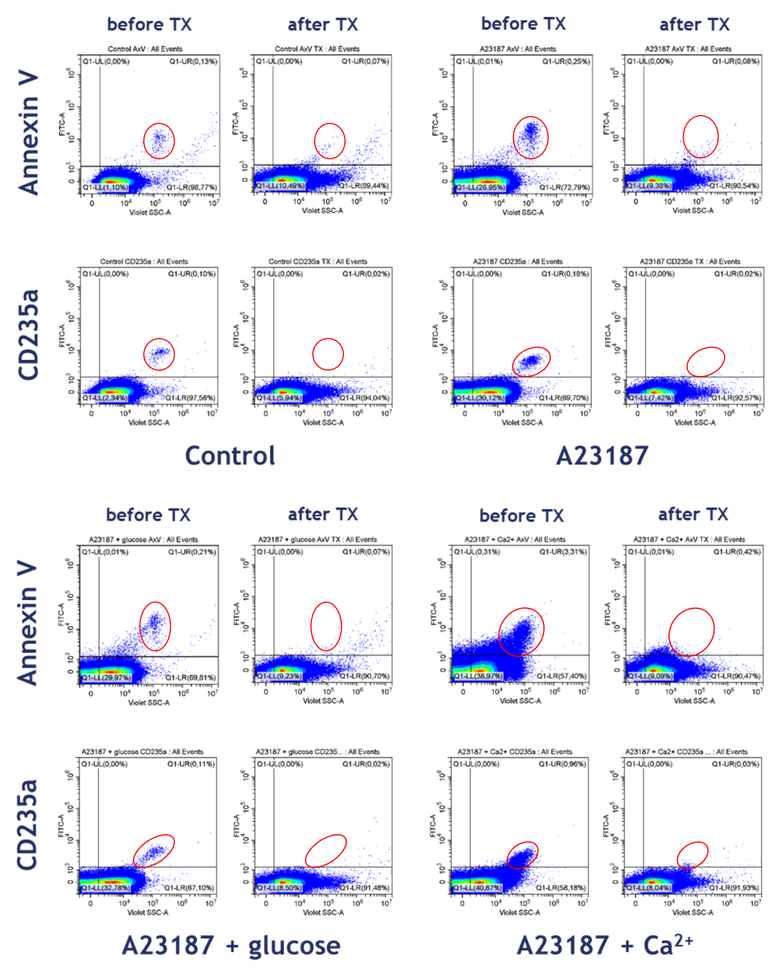
DOT PLOTS

Representative figures.
Annexin V was FITC labelled.
RBCs mean RBC singlets.



- Cell shrinkage and Annexin V positivity were detected, induced by A23187 calcium ionophore and further enhanced by extracellular calcium ions.
- Eryptosis is associated with the mechanism of RBC-EV formation.
- Cold stress can lead to eryptosis and promote RBC-EV production in a non-aggressive manner.

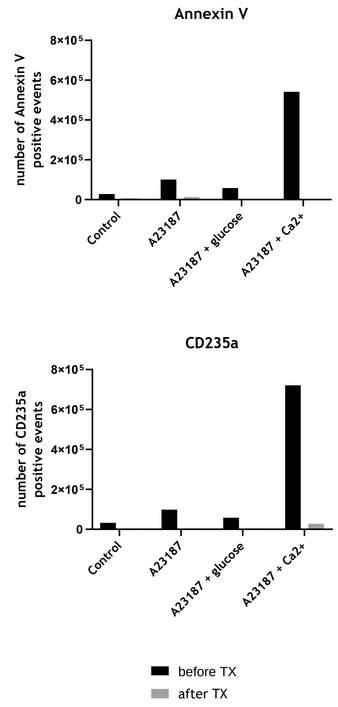
DETECTION of ISOLATED RBC-EVs



TX: Triton X-100

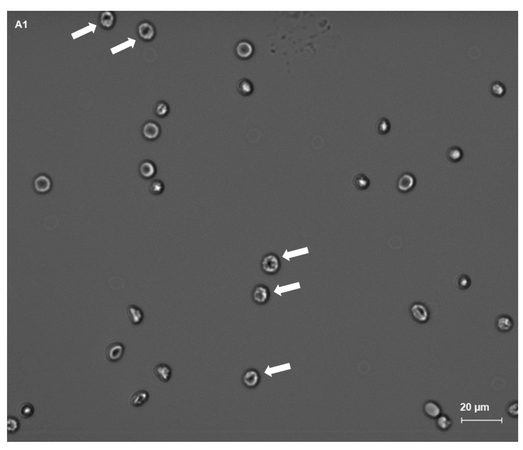
Representative figures.
Annexin V and CD235a were FITC labelled.

NUMBER of POSITIVE EVENTS



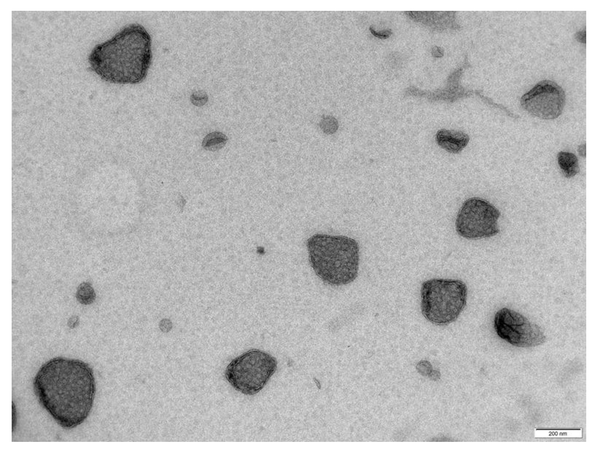
- The detected particles are positive for Annexin V and CD235a (glycophorin A), thus they are eryptosis-related events. (CD235a is a specific RBC marker.)
- Addition of Triton X-100 eliminates Annexin V+ and CD235a+ particles, indicating their vesicular/membraneous nature.
- The formation of RBC-EVs is enhanced by A23187, as expected.
- Elevated concentrations of extracellular Ca^{2+} further evoke the formation of RBC-EVs, induced by A23187.
- Administration of glucose partially reverses the A23187-induced vesicle formation.

MICROSCOPIC ANALYSIS



Representative high content microscopic image of RBCs and eryptosis (control sample) captured by Celldiscoverer 7. White arrows indicate eryptotic cells with membrane blebbing.

Scale bar: 20 µm



Representative TEM image of isolated RBC-EVs (control sample). Membrane-bound, vesicular structures are clearly visible. The vesicles are heterogeneous in size.

Scale bar: 200 nm

CONCLUSION

- Well-managed *in vitro* system
- Detection of eryptosis and RBC-EVs
- RBC-EV concentration differences
- Protective effect of glucose on vesicle biogenesis
- Cold stress induced vesicle production

PERSPECTIVE

- RBC-EVs
  - Validation of RBC-EVs, according to MISEV 2023
  - Further characterization of RBC-EVs: lipidomics, proteomics
  - Effect of lipoproteins (unmodified and oxidized) on the formation of RBC-EVs
  - Interaction of RBC-EVs with cell lines

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