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Cryopreservation of peripheral blood mononuclear cells does not significantly affect the levels of spontaneous apoptosis after 24-h culture☆

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Abstract

Studies performed with malaria patients living in endemic areas are frequently conducted in laboratories located hundreds of kilometer away from research centers, due to the difficulties in performing the assays in field conditions. Thus, we considered the potential indication of cryopreservation of peripheral blood mononuclear cells (PBMC), in most fieldwork, and decided to evaluate the effect of cryopreservation of PBMC on spontaneous apoptosis. The membrane integrity of PBMC was tested using three previously described protocols of cryopreservation. Cell samples were obtained from 19 healthy volunteers. Percentage of apoptotic nuclei in short-term PBMC cultures was determined by a sensitive method using 7-aminoactinomycin D followed by flow cytometry. Our results indicate that although cryopreservation can to some extent affect lymphocyte membrane integrity rates, flow cytometry analysis showed that frequencies of spontaneous apoptosis in cryopreserved cells were not significantly modified after 24-h culture. It is concluded that cryopreserved PBMC could be used for measuring spontaneous apoptosis and therefore, could be employed for the study of populations living in areas distant from research centers, allowing the comparative evaluation of samples obtained at different time.
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Recently, we became interested in studying apoptosis in short-term cultures of peripheral blood mononuclear cell (PBMC)[1] from patients with clinical malaria. Malaria infection induces drastic changes in the immune system such as: an important T-cell activation associated with raised levels of soluble plasma interleukin-2 receptor (sIL-2R) [10,11,15,20], a marked degree of polyclonal B-cell activation [1,2,6,21], a low level of in vitro proliferative response of PBMC to malaria antigens [5], a decreased response to vaccines [12,26] besides a reduction in the number of circulating lymphocytes, particularly of the T-cell population [9,24,29,37–39]. The pathophysiology of the observed lymphopenia is still unclear [13], however, apoptosis may be considered a possible mechanism of decreased T-cell counts since African patients with acute *Plasmodium falciparum* infection have increased ratio of lymphocyte apoptosis levels [27,28]. Therefore, we decided to study the apoptosis profile in *P. falciparum* and *P. vivax* infected individuals. However, due to the difficulties in performing the assays in field conditions, we considered the potential indication of cryopreservation. It has been demonstrated that cryopreservation can affect the results of works aiming the evaluation of the immune response. Representative examples are the enhanced secretion of interferon-γ [34], IL-1 [30], IL-2 [31], and IL-6 [33] by frozen mitogen-activated PBMC. Otherwise, some works have demonstrated that adequately cryopreserved immunocompetent cells can be stored for prolonged periods and then be recovered with comparable functional ability to that of fresh samples when used in assays of cell-mediated immunity [3,4,7,8,18].

The present work was designed in order to setup optimal conditions for successful cryopreservation of human lymphocytes in terms of cell membrane integrity and to study its influence in apoptotic events.

Methods

Experimental design

Nineteen healthy volunteers from the Laboratory of Malaria Research of the Instituto Oswaldo Cruz were enrolled in the study. Cells from three of them were used to evaluate three previously described simple methods for cryopreservation. In this way, PBMC of each one were counted, freeze, and thawed 30 days later. Cell membrane integrity was assessed using trypan blue staining. The reproducibility of the protocol that showed the best performance in terms of membrane integrity was further evaluated using PBMC from other six volunteers.

After standardization of the freezing/thawing protocol, apoptosis levels were comparatively measured in paired fresh and 30 day-cryopreserved PBMC from other 10 healthy volunteers.

Isolation of mononuclear cells

Venous blood (10 ml) was collected from each donor and PBMC were isolated from heparinized whole blood by Ficoll–Hypaque density gradient centrifugation. The cells were washed twice in RPMI-1640 (SIGMA) medium containing 15 mM glutamine (SIGMA), 10 mM Hepes, 200 U/ml penicillin (SIGMA), 200 μg/ml streptomycin (SIGMA), 3 mg/ml gentamycin (SIGMA), and 2 g/L sodium bicarbonate (GRUPO QUÍMICA). PBMC were prepared for cryopreservation by resuspending in RPMI medium supplemented with 10% inactivated fetal calf serum (W.L. IMUNOQUÍMICA). Independently of freezing and thawing procedures PBMC were adjusted to contain 1×10^7 cells/ml and 1 ml sample from each donor was cryopreserved.

Cryopreservation and thawing of PBMC

In order to compare the membrane integrity of PBMC after cryopreservation, freezing and thawing were performed by three different methods (Table 1). Methods 1 [35] and 2 [32] use a small cryocontainer—cryo 1 °C freezing container (Nalgene Catalog N 5100-0001) with isopropyl alcohol, at a temperature rate of 1 °C/min decrease [17]. In both methods the cell aliquots were placed in the container, transferred to a −70 °C freezer, and after at least 4h, the samples were quickly stored in a liquid nitrogen tank.

Method 3 [14] does not use cryocontainer. The freezing method consisted in resuspending PBMC in 4 °C RPMI-1640 supplemented with 40% FCS by gentle shaking with an equal volume of cold RPMI-1640 containing 20% dimethyl sulfoxide (Me_2SO). Shortly, after the cells were transferred to cryotubes they were immersed in a 4 °C cold

[1] *Abbreviations used:* PBMC, peripheral blood mononuclear cells; 7-AAD, 7-aminoactinomycin D; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; FCS, fetal calf serum; FS, forward scatter; SS, side scatter.

Table 1
Conditions of freezing and thawing of peripheral blood mononuclear cells

Methods	Freezing medium	Cryopreservation	Thawing	Freezing rate
M1	500 μl ice-cold FCS + 500 μl ice-cold RPMI-1640 medium supplemented with 20% Me_2SO	Cryocontainer −70 °C—4 h N_2 liquid	37 °C water bath 2 washes	1 °C/min
M2	1000 μl medium consisted of 10% FCS + 10% Me_2SO + 80% RPMI	Cryocontainer −70 °C—4 h N_2 liquid	45 °C water bath 1 wash	1 °C/min
M3	500 μl ice-cold RPMI-1640 medium supplemented with 40% FCS + 500 μl of ice-cold RPMI containing 20% Me_2SO	4 °C ethanol bath −70 °C—overnight N_2 liquid	37 °C water bath 2 washes	Unknown

FCS—fetal calf serum; M1—Vingerhoets, 1995; M2—Venkataraman, 1992; M3—Ichino and Ishikawa, 1985.

ethanol bath and placed in a −70 °C freezer during at least 4 h, and thereafter transferred to a nitrogen liquid storage tank.

The freezing media of the three methods consisted of ice-cold fetal calf serum plus ice-cold Me_2SO solution in 4 °C RPMI-1640.

The thawing and removal of Me_2SO from cells varied in agreement with the method used: in methods 1 and 3 thawing was accomplished by the immersion of the cryopreserved samples in 37 °C water bath, being followed by two washes in 4 °C RPMI-1640 medium; in method 2 to prevent ice recrystallization and cell disruption during this process, the cells were thawed quickly in a 45 °C water bath and washed only 1 time in 4 °C RPMI-1640 medium.

Membrane integrity assay

Trypan blue (GIBCO BRL) was prepared as a stock solution of 1 mg/ml and stored at room temperature. Five microliters of cell suspensions (cells resuspended in 1 ml RPMI medium) plus 45 μl of staining solution diluted in RPMI (v/v) were placed in a Neubauer chamber with a cover slip and the membrane integrity was assessed by counting non-staining-PBMC in an optical microscopy (OLYMPUS mod. BH2), before cryopreservation and immediately after thawing.

Determination of apoptotic cells using 7-aminoactinomycin D (7-AAD) by flow cytometry

We have used a rapid and sensitive method that allows the discrimination of live cells from apoptotic cells or necrotic cells [22]. The recognition of viable, apoptotic or necrotic cells was done using 7-aminoactinomycin D (7-AAD)—a fluorescent cytochemical probe—in a single laser cytometry. While live cells were not stained with 7-AAD, the cells with an apoptotic or necrotic patterns were discriminated based on the low and high 7-AAD incorporation, respectively. Briefly, thawed cells were incubated for 20 min at 4 °C with 10 μg/ml of 7-AAD (SIGMA) in PBS containing 2% fetal calf serum and 0.1% sodium azide. Labeled samples were analyzed with an EPICS XL-MCL Flow Cytometer with a single argon laser at 488 nm (Coulter, Healeah, FL, USA) and red fluorescence from 7-AAD was filtered through a 675 nm long pass filter. More than 10,000 events were analyzed for each sample.

Principles of drawing the regions (gates)

First we have to find the population to be analyzed, or the population that interests us. Since blood cells population have sizes and distinct organelles density and these characteristics can be inferred starting from the parameters of cellular size (forward scatter—FS) versus inner granularity (side scatter—SS), because they generate an image type (region A) that perfectly allows to distinguish the peripheral blood subpopulations (Fig. 1), it was dispensed the leukogate and any antibody was used.

Fresh PBMC were used for defining the size of the gates corresponding to viable (region C), apoptotic (region D), and necrotic (region E) PBMC in flow cytometry (Fig. 1). In order to better define those gates, we used necrotic induced PBMC by heating—65 °C for 5 min—[23] and apoptotic induced PBMC by a known inducer of lymphocyte apoptosis—4 μM staurosporine (Sigma)—isolated alkaloid from *Streptomyces* [19] and inhibitor of kinase C protein [16,25].

Apoptosis assay

Cell cultures were prepared in duplicates in 96-well flat-bottom microtiter plates (FALCON)

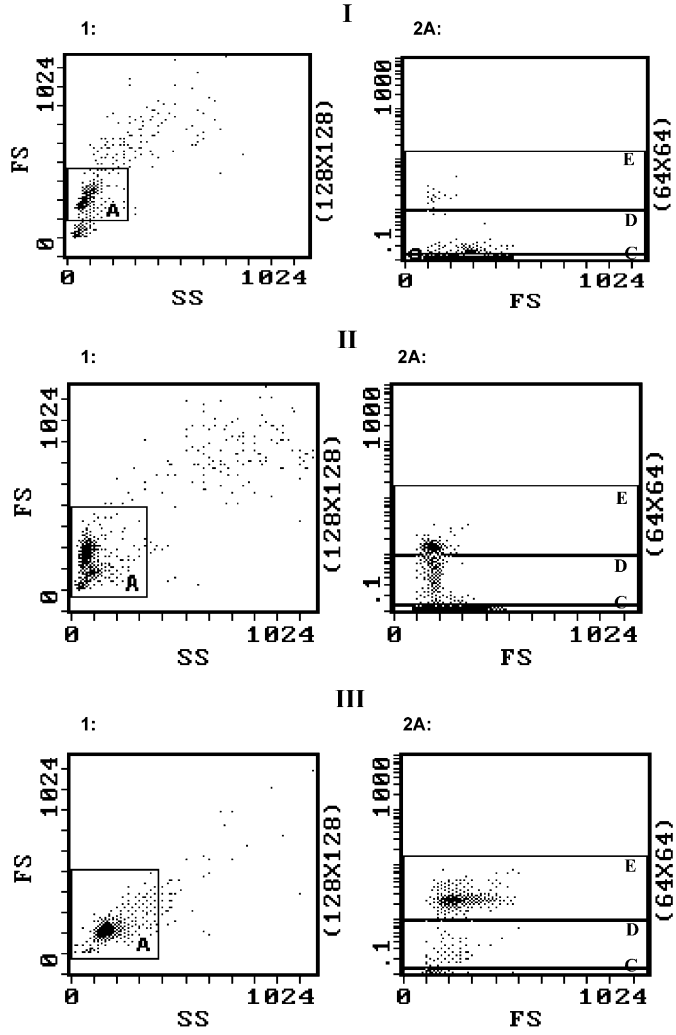

Fig. 1. Cytometric flow analysis of peripheral blood mononuclear cells (PBMC): I, normal PBMC; II, apoptosis-induced PBMC; III, necrosis-induced PBMC. FS, forward scatter; SS, side scatter; A, analyzed region of PBMC; C, viable cells; D, apoptotic cells; and E, necrotic cells.

at 37 °C in 5% CO_2 in a final volume of 200 μl of culture medium alone for 24 h. The cultures were performed at a cell density of 2.5×10^5 cells/well. As a control, non-cryopreserved PBMC from healthy volunteers treated exactly in the same way as the cells from malaria patients or non-infected individuals were used in each experiment.

Gel electrophoresis of fragmented DNA

Cells (4×10^6) were washed three times with PBS and centrifuged (400*g*—5 min) and the pellet was suspended in 400 μl lysis buffer (200 μg/ml proteinase K, pH 7.5 (SIGMA), 100 mM NaCl (MERCK), 1 mM EDTA (SIGMA), 10 mM Tris–HCl, pH 8.0, and 1% SDS (ICN). After incubating at 50 °C for 30 min, DNA samples were extracted with phenol–chloroform and extracted with 100 μg/ml Rnase A (SIGMA) during 30 min at 36 °C. The DNA was precipitated by the addition of 3 M sodium acetate (1/10), 2.5 volumes ETOH (−20 °C) (SIGMA), and 3 μl glycogen 0.02 mg/ml (SIGMA).

Electrophoresis was carried out through a 2% agarose gel in Tris–borate–EDTA (TBE) buffer. DNA bands were visualized by staining with ethidium bromide (0.5 μg/ml) and photographed. Oligonucleosomal fragments appeared as ladders

of band whose molecular sizes are approximate multiples of 200 bp.

Electron microscopy

The morphology was assessed by transmission electron microscopy. The thawed PBMC were washed in PBS and fixed with 2.5% glutaraldehyde in 0.1 M cacodilate buffer for 1 h at 4 °C, washed three times in the same buffer and fixed with 1% osmium tetroxide in 0.1 M cacodilate buffer for 1 h at 4 °C. The cells were washed in buffer, removed with a cell scraper, and then the suspension was dehydrated in graded acetone and embedded in Epon. Ultrathin sections were collected in cooper grids, stained with uranyl acetate and lead citrate and observed with a Zeiss EM-10C transmission electron microscope.

Statistical analysis

The Wilcoxon paired, non-parametric test was performed to study the significance of apoptosis percentage in fresh cells and after cellular cryopreservation. A P value of < 0.05 was considered significant.

Results

Membrane integrity

PBMC of three donors were processed by the three methods. As shown in Table 2, there was a significant drop in membrane integrity following cryopreservation, regardless of the method used. However, the method that consistently presented the best membrane integrity results was the method (M3) described by Ichino and Ishikawa [14]. Membrane integrity rates recorded after freezing and thawing ranged from 57 to 72% for M1 (62%), 52 to 64% for M2 (56%), and from 60 to 85% for M3 (76%). Thus, we choose M3 and the reproducibility of this method was assessed using PBMC from another six blood donors. After freezing and thawing using M3, cell membrane integrity rates were: 62, 69, 75, 82, 85, and 89% (77% ± 10.26 SD), validating the previous results.

Table 2
Membrane integrity of peripheral blood mononuclear cells (PBMC) from healthy donors after freezing and thawing by three different methods

PBMC	Method 1	Method 2	Method 3
Donor 1	5.7×10^6	5.2×10^6	8.2×10^6
Donor 2	7.2×10^6	6.4×10^6	8.5×10^6
Donor 3	5.8×10^6	5.3×10^6	6.0×10^6
Mean (%)	62%	56%	76%

Method 1—Vingerhoets, 1995; Method 2—Venkataraman, 1992; Method 3—Ichino and Ishikawa 1985.

Apoptosis

In flow cytometry the regions (gates) for necrosis and apoptosis were established using necrotic PBMC induced by heating and apoptotic PBMC induced by staurosporine (Fig. 1). Parallel to the experiments of flow cytometry, extractions of DNA were accomplished also. As expected, fragmentation of DNA was not observed in samples containing viable (Fig. 2a), or necrotic-induced cells (Fig. 2b), but in apoptosis-induced cells a typical fragmentation of DNA (Fig. 2c) was observed. The morphologic characteristics were assessed by transmission electron microscopy. Most of non-activated PBMC had normal morphology showing nuclei with normal chromatin appearance (Fig. 3A). The same chromatin profile was observed in necrotic cells. In addition, we also observed loss of membrane and cytoplasmatic organelles integrity (Fig. 3B). In contrast the majority of activated PBMC undergoing apoptosis presented nuclei with highly condensed chromatin, loss of nuclear membrane integrity, and

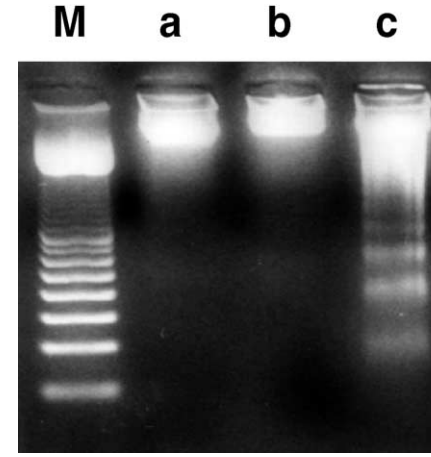

Fig. 2. Analysis of DNA by electrophoresis gel. Intact DNA of viable (a), or induced-necrotic cells by heating (b) and typical fragmentation of DNA in staurosporine-induced apoptosis cells (c), and molecular weight marker (M).

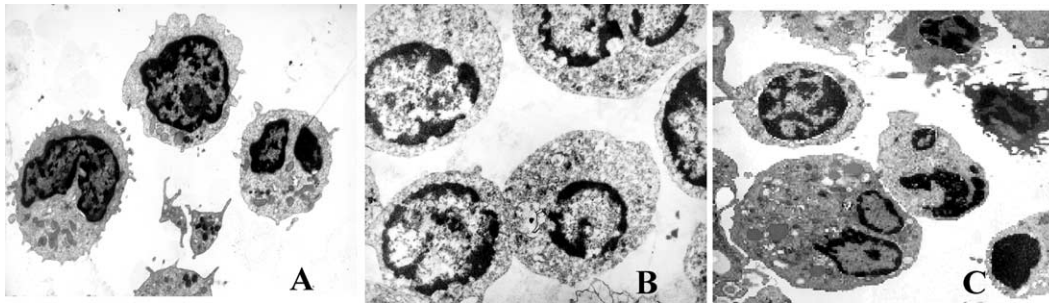

Fig. 3. Electron micrograph of ultrathin sections (uranil acetate staining) of viable cells (A) 12,000×, necrosis-induced cells (B) 14,000×, and cells undergoing apoptosis after staurosporine induction (C) 7500 ×.

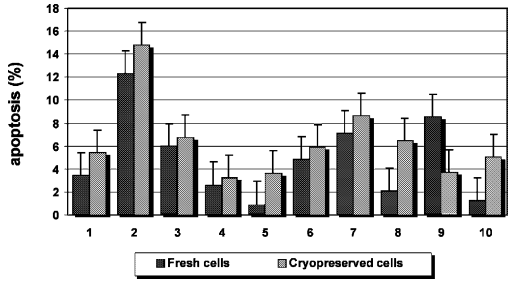

Fig. 4. Comparison of apoptosis in fresh and freezing/thawing peripheral blood mononuclear cells (PBMC) from 10 healthy donors according to the method described by Ichino and Ishikawa [14].

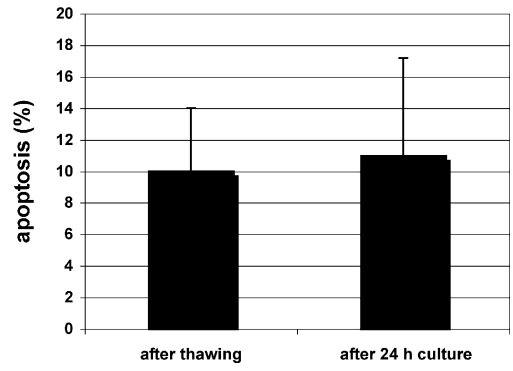

Fig. 5. Comparison of peripheral blood mononuclear cells apoptosis after freezing/thawing and after 24 h culture from 31 healthy donors from malaria endemic area.

cytoplasmatic vacuolization (Fig. 3C). The ultrastructural approach together with DNA fragmentation analysis able us to confirm the gates used for the flow cytometry analysis.

The comparative results of apoptosis in paired fresh and immediately after thawing (30 days freezing) PBMC from the same donors are presented in Fig. 4. No significant difference of apoptosis events in PBMC from 10 volunteers was verified ($p > 0.05$).

Apoptosis was analyzed in cells from healthy individuals directly after thawing (ex vivo) and after 24 h culture without any exogenous stimulus. No significant difference was observed when apoptosis was analyzed after thawing or after 24 h culture (Fig. 5). Thus, these results showed that although freezing injury could result in apoptosis, and cryopreservation-induced apoptosis is known to be a delayed process becoming apparent only several hours after thawing, in the period of 24 h after thawing the apoptosis process was not evident. Consequently, it was excluded the possibility that apoptosis levels were under-estimated.

Discussion

The present study was designed to evaluate the effects of three freezing and thawing procedures on membrane integrity of PBMC populations and on apoptosis.

Previous studies by Venkataraman [31,32] and Vingerhoets et al. [35] compared cryopreservation protocols using an inexpensive freezing container (cryobox), or a conventional controlled rate freezer and concluded that results obtained with both methods were similar. One other report claimed that PBMC viability in a mechanical freezing method in which cell suspensions are placed in plastic tubes and immersed straightly in a cold ethanol bath [14] was as high as that obtained with programmed freezer technique.

Since a controlled rate freezer technique is unavailable in most endemic areas we compared the effect of the mechanical freezing method [14]

with that of protocols described by Vingherhoets et al. [35] and Venkataraman [31,32], using a cryobox on the studies of lymphocytes apoptosis.

Our experiments demonstrating 76% of cell viability, when mechanical freezing was used, are in agreement with those (80%) found by Ichino and Ishikawa [14]. In the method described by Vingerhoets (1995) cells viability was not recorded and Venkataraman [31,32] reported 98% of cells with membrane integrity using cryobox method, contrasting with the lower percentages (56%) of PBMC found by us. This difference could be related to the fact that Venkataraman [31,32] thawed the cells immediately after freezing while we kept them cryopreserved for at least 30 days before thawing, a period usually required to collect the field samples.

Concerning apoptosis, although cryopreservation could to same extent affect lymphocyte and membrane integrity rates, the frequencies of apoptosis ex vivo and after 24 h culture in cryopreserved cells were not significantly modified. In view of these data we are able to conclude that the cryopreservation does not significantly affect the levels of lymphocyte apoptosis, and therefore could be used for the study of populations living in areas distant from research centers, allowing the comparative evaluation of samples obtained at different time.

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