A Method for Introducing Human Blood Leukocytes in Cold Anabiosis at -50°C

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We propose a simple, effective, and economic method for introducing human blood leukocytes in cold anabiosis at moderately low temperature (-50°C).

Key Words: *leukocytes; cold anabiosis; morphofunctional parameters*

The leukocyte concentrate attracts now great attention of physicians and specialists in transfusion medicine. It is a complex transfusion medium containing mainly granulocytes with pronounced phagocytic activity and lymphocytes carrying antigens with high immunogenic (B lymphocytes) and immunoaggressive (T lymphocytes) properties. Transfusions of leukocytic concentrates are an effective method for prevention and treatment of infectious complications in oncological and hematological patients after high-dose chemotherapy, in radiation sickness, sepsis, and agranulocytosis of different origin [5]. Hence, reserves of this blood component should be created. However, preservation of nuclear blood cells at positive temperatures is difficult because of active metabolism and rapid exhaustion of their energy potential. Experience gained in this sphere indicates that viability of leukocytes can be maintained for 24 h at 4°C [3], for 48 h at 0-4°C [1], up to 12 months at low temperatures (-60 to -80°C) [5], and up to 20 months at ultralow temperature (-196°C) [7]. Hence, the most effective method for leukocyte preservation is freezing at ultralow temperature. Unfortunately, this method is very expensive, because it requires cryogenic equipment, permanent supply of liquid nitrogen stored at biological depository, and highly specialized service, which impedes wide practical use of this method.

We propose an effective, simple, and economic method for introducing human blood leukocytes in cold anabiosis at moderately low (-50°C) temperature.

MATERIALS AND METHODS

A total of 25 donor leukocyte concentrates were prepared from the whole blood by cytoferesis (CDF preserving agent). The mean volume of biological object was 20.12±4.30 ml. Leukoconcentrate was mixed (1:1) with one of 5 variants of original solution saving the cells from cold (Table 1) differing by the content of the following ingredients: A-378 substance, antioxidant, and citric acid (for bringing pH to the normal level of 7.2-7.4). The mixture was prepared in a Kom-

TABLE 1. Composition of Solution Saving the Cells from Cold, Used for Freezing Human Blood Leukocytes at Moderately Low Temperature (-50°C)

Cryopreserving agent No.	A-378,%	Antioxidant, %	Citric acid, %	Water for injections, ml
1	24	2.4	0.09	to 100
2	30	2.4	0.06	to 100
3	30	2.8	0.07	to 100
4	30	3.2	0.06	to 100
5	36	3.2	0.09	to 100

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poplast 300 plasticate container and equilibrated at 20°C for 20 min, after which the container was plunged in cold carrier (96% ethanol) filling a 4000-ml bath of a cryostate chamber (special electrical -30°C freezer, developed at Kirov Institute of Hematology and Blood Transfusion and Institute of Cryobiology and Cryomedicine Problems, National Academy of Sciences of Ukraine) cooled to -28°C. After 15-min

TABLE 2. Morphofunctional Parameters of Leukocytes after Cold Anabiosis at -50°C with Different Variants of Cell Saving Solution (*M*±*m*)

Parameter, solution variant	Final concentration of main ingredients, %		Absolute number		Relative number, % of initial
	A-378	antioxidant	before freezing	after defrosting	
Cell number/μl					
1	12	1.2	10 140±3100	8760±3884	83.30±12.79
2	15	1.2	11 530±4020	8967±2684	80.00±14.91
3	15	1.4	12 300±4483	10 550±3621	88.60±14.33
4	15	1.6	11 150±7665	10 430±7477	91.67±6.09
5	18	1.6	11 170±3273	10 980±2570	91.00±4.36
Granulocytes, %					
1	12	1.2	32.0±3.6	18.0±1.7	56.33±2.08*
2	15	1.2	21.6±6.8	13.80±2.59	67.00±15.87
3	15	1.4	19.00±6.25	14.00±2.65	77.00±14.53
4	15	1.6	26.20±6.61	15.40±5.64	59.00±11.38
5	18	1.6	35±1	17.67±0.68	50.57±2.08*
Monocytes, %					
1	12	1.2	5.3±1.5	4.7±1.2	88.67±10.26*
2	15	1.2	3.80±1.09	4.60±0.55	107.50±21.79
3	15	1.4	3.33±1.15	3.33±1.15	100±0
4	15	1.6	4.40±0.55	4.60±0.55	106.00±19.17
5	18	1.6	5±1	4.7±0.6	96.00±25.16
Lymphocytes, %					
1	12	1.2	62.71±4.51	77.33±2.52	123.30±5.13*
2	15	1.2	73.80±8.96	81.6±2.7	111.80±13.22
3	15	1.4	77±7	82.67±3.37	107.70±5.51
4	15	1.6	71.20±3.03	80.60±6.88	113.00±8.97
5	18	1.6	60.00±1.71	77.67±0.68	129.30±4.62*
Eosin resistance, %					
1	12	1.2	97.67±0.58	71.50±14.85	73.50±14.85
2	15	1.2	98.33±0.58	57.33±18.93	58.33±18.93
3	15	1.4	99.0±0.2	63.33±9.45	64.33±9.45
4	15	1.6	98±1	48.00±15.72	48.67±16.17
5	18	1.6	97.67±0.58	40.00±17.74	41.00±17.44*
Phagocytic activity of neutrophils, %					
1	12	1.2	66.00±0.75	31.70±1.53	48.7±4.5*
2	15	1.2	65.2±2.1	46.67±4.16	72.00±3.46
3	15	1.4	65.3±1.2	49.33±1.16	76.30±5.03
4	15	1.6	65±3	45.33±3.51	69.67±2.08
5	18	1.6	65.1±3.2	40±1	61.70±3.79*

Note. *$p<0.05$ compared to variant No. 3.

exposure the biomaterial was transferred into -50°C freezer for further freezing. Thermograms were recorded with a GSP-04 device using a TSM-3-02 pick-up placed in one of the containers (simulator) with the tested bioproduct. Leukocytes were cooled by a rapid two-step protocol: at a rate of 10°/min to the eutectic point (-3°C) and then at a rate of 1-2°/min to -28°C (stage I) and at a rate of 2-3°/min to the storage temperature (stage II). Rapid defrosting of the leukoconcentrate was carried out in a 20-liter water bath at 38°C for 45-60 sec (depending on the volume of biological objects), intensely shaking the container.

The leukocyte count and leukocytic formula were estimated using common clinical laboratory methods.

The integrity of leukocyte membrane was verified by the eosin exclusion test proposed by R. A. Schreck [7]. A small droplet of the leukosuspension was mixed with an equal drop of 1% eosin on a slide and examined without exposure under microscope for 2-3 min (×40). If the membrane was damaged, the cells diffusely colored pink. A total of 100 cells were examined and the percentage of intact cells was recorded.

The neutrophil phagocytic activity was evaluated by the method of S. G. Potapova *et al.* [4], based on evaluation of phagocytosis of inert latex particles (1:200 dilution in Hanks medium, 1.05-μ particles): 0.1 ml of bioobject was mixed with 0.05 ml latex suspension and put into a thermostat (37°C) for 30 min. The mixture was shaken every 10 min, after which smears were prepared, stained with Leishman and Romanowskii stains, and the percentage of phagocytic cells was estimated.

The data were statistically processed using paired Student's test [2].

RESULTS

Analysis of leukocyte concentrate after defrosting showed that all 5 variants of solution preserving the cells from cold decreased the initial number of cells to 80-92%, the differences in the cell-preserving efficiency of solutions were negligible (Table 2).

Analysis of morphological composition of leukocytes after cold anabiosis confirmed high resistance of lymphocytes and monocytes to the damaging freezing factors and lower resistance of granulocytes, which is explained by their more intricate cellular organization [1]. Cell-preserving solution No. 3 preserved morphological intactness of granulocytes significantly ($p<0.05$) better than variants Nos. 1 and 5.

Evaluation of eosin resistance of leukocytes showed that the number of cells with intact membrane in suspensions frozen in the presence of all variants of solution significantly decreased. The highest percentage of eosin-resistant leukocytes after defrosting was observed in experiments with variants No. 1 and No. 3 (74 and 64%, respectively).

The phagocytic activity of neutrophils is a reliable test for evaluating their biological intactness. The highest percentage of neutrophils capable of phagocytosis (76.3%) after defrosting was observed in experiments with solution No. 3. This confirms the efficiency of this cryopreserving solution.

These results confirm high morphofunctional characteristics of human blood leukocytes after cold anabiosis using an original solution No. 3 saving the cells from cold. The method is simple, requires no expensive equipment and reagents, and is recommended for practice.

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