

The method of human fetal liver stem cells cryopreservation

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Introduction

Nowadays, a great variety of cell cryopreservation protocols with different combinations of cryoprotectors and freezing programs exists. One may also find many methods of cell viability evaluation, namely trypan blue exclusion, fluorescent dye staining, in vitro culture and in vivo transplantation. Therefore, the purpose of this study was to develop both a method of cryopreservation and a model of hematopoietic tissue cells viability evaluation. Furthermore, we have examined the cells diversity in crude suspension of human fetal liver cells that was cryopreserved using the developed method.

Materials and methods

Human fetal livers were obtained from aborted fetuses (6 - 12 weeks of gestation) in accordance with a protocol approved by the Coordination Center of Transplantation of Organs, Tissues and Cells of the Ministry of Health of Ukraine. We have used the method of multifactorial experiments 2³ for the developed program of conventional freezing of human fetal liver hematopoietic stem cells. This method includes various parameters, namely the concentration of cryoprotectants, the cooling rate from the initial temperature down to the point of "crystal-formation", the time of holding at the stage of "crystal-formation". The functional cell integrity was evaluated through the cloning method. The quantity of cell-precursors of granulomonocytopenia (GM-CFU) was calculated on a fixed sample. Cryopreserved human fetal liver cells were inoculated in gel-agar medium (upper layer) settled on a solid-agar base with mononuclear cells from peripheral blood.

Results

The analysis of the influence of those factors combination on the cells suspension's ability to generate GM-CFU in the culture of cells after thawing has helped us understand the main mechanisms of cryoinjury in cells and eliminate the factors of cryoinjury to create our method of cryopreservation of hematopoietic stem cells.

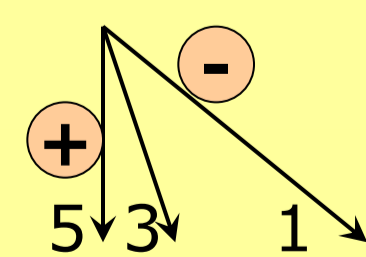
Modeling of cryopreservation process with Me₂SO

Factors

X1 - Me₂SO concentration, mol/l

- 0.4, 0.9
- + 1.4

X2 -cooling rate down to the temperature of adaptation, °C/min



X3 - adaptation temperature, °C

- -9
- 17
- + -25

Storage temperature
-196°C

Matrix of the experiment

Experiment №	Factor's value			Response function	
	X1	X2	X3	y1	y2
1	-	-	-	98.7	58.3
2	+	-	-	46.0	65.0
3	-	+	-	7.0	11.3
4	+	+	-	89.0	46.0
5	-	-	+	107.3	92.3
6	+	-	+	106.3	75.3
7	-	+	+	88.3	65.0
8	+	+	+	91.8	78.7

The equation describing the cryopreservation process

$$Y1=79.25-10.33x2+19.08x3+17.33x1x2-16.33x1x2x3$$

$$Y2=61.5-11.25x2+16.33x3$$

In various experimental groups the GM-CFU recoveries were ranging from 50 to 90 % using the same culture conditions as for the samples prior to freezing. After eliminating the uncontrolled parameters' influence we had the best hematopoietic stem cells viability and GM-CFU recovery between 95 and 98% in the samples cryopreserved with Me₂SO 0,4 - 0,7 mol/ L by slow cooling with 0,5 - 1,50 °C/min with seeding and holding after "crystal-formation". This freezing program was variously tested for cryopreserved suspension of human fetal liver hematopoietic stem cells (Fig. 1, 2).

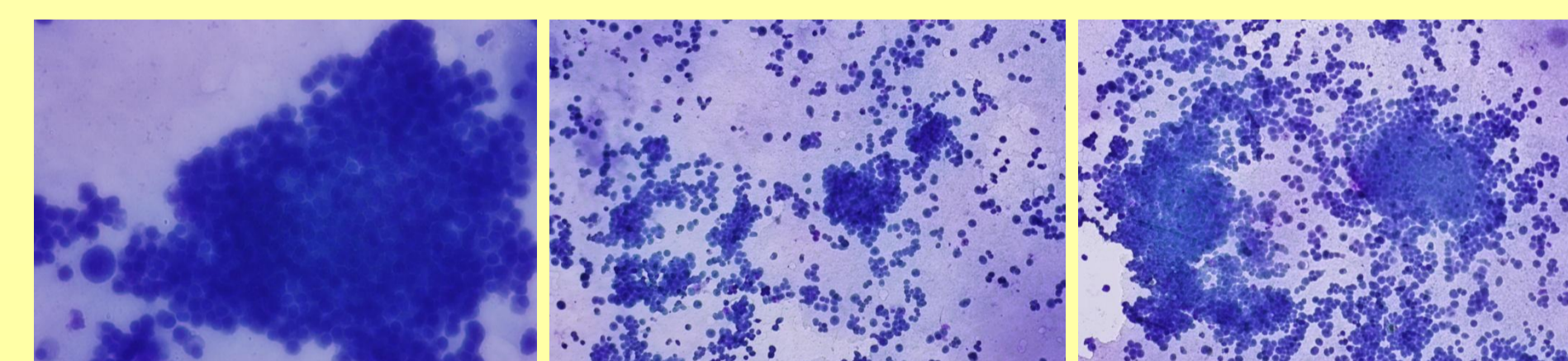


Fig.1. Different hematopoietic colonies of fetal liver cells in agar medium with GM-CSF, G-CSF, SCF, Epo, IL-3. Romanovsky staining, magnification ×100.

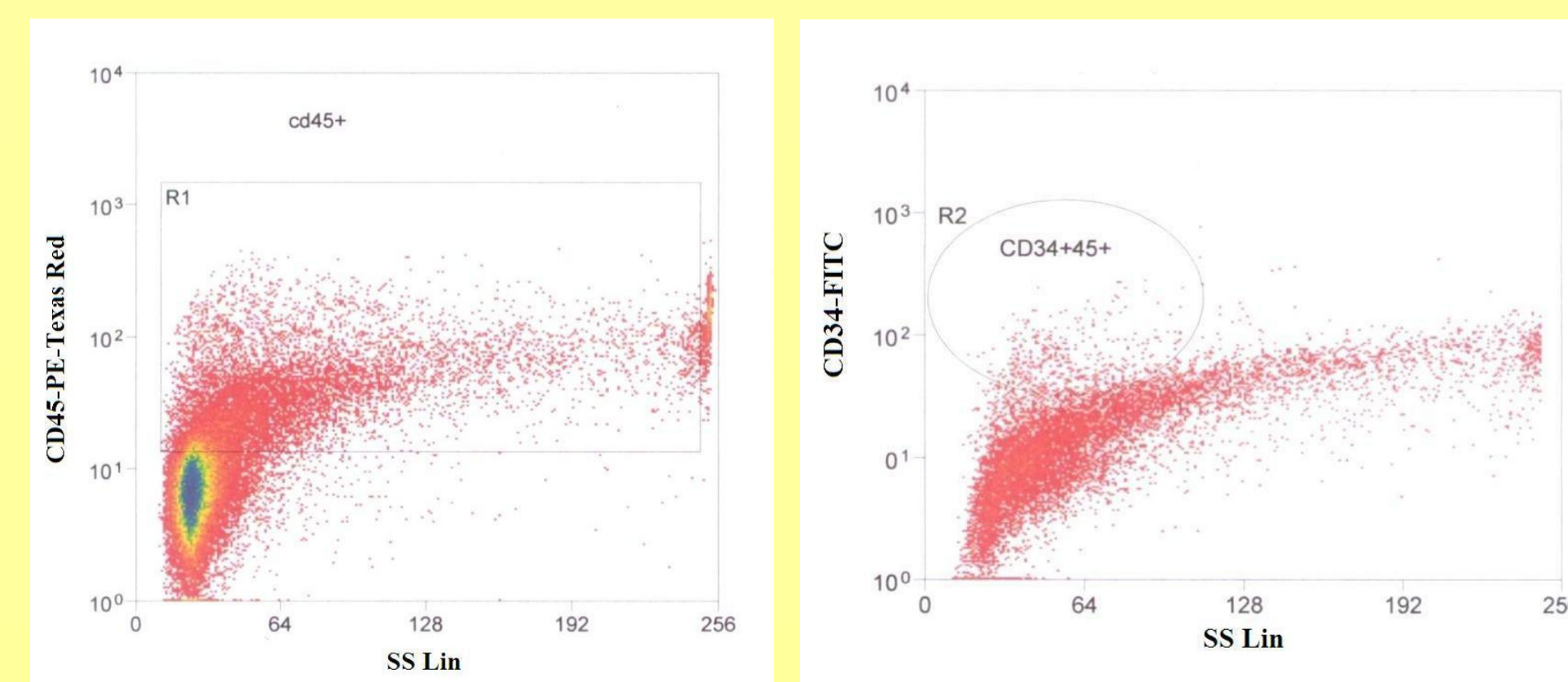


Fig. 2. Dot plot of human fetal liver-derived cells stained with antibodies against CD34 (stem cells marker) and CD45 (pan leukocyte marker).

Non-hematopoietic cells viability after thawing

The suspension of fetal liver cells contained all the cells that form the fetal liver at 6 -12 weeks of gestation (stromal cells, hepatoblasts, endothelial cells, etc.); therefore, the second goal of our study was to evaluate the viability of non-hematopoietic cells, which has shown that all progenitor cells cryopreserved using the developed method have saved their ability to grow in cultures.

During long-term culturing of fetal liver cells on various substrates and in various culture media we have observed growth in colonies of fibroblast-like cells, hepatoblasts, and endothelial-like cells. The culture of fetal liver cells in serum-free medium gave rise to colonies formed of spherical compact cells and hepatoblasts (Fig.3 a, b).

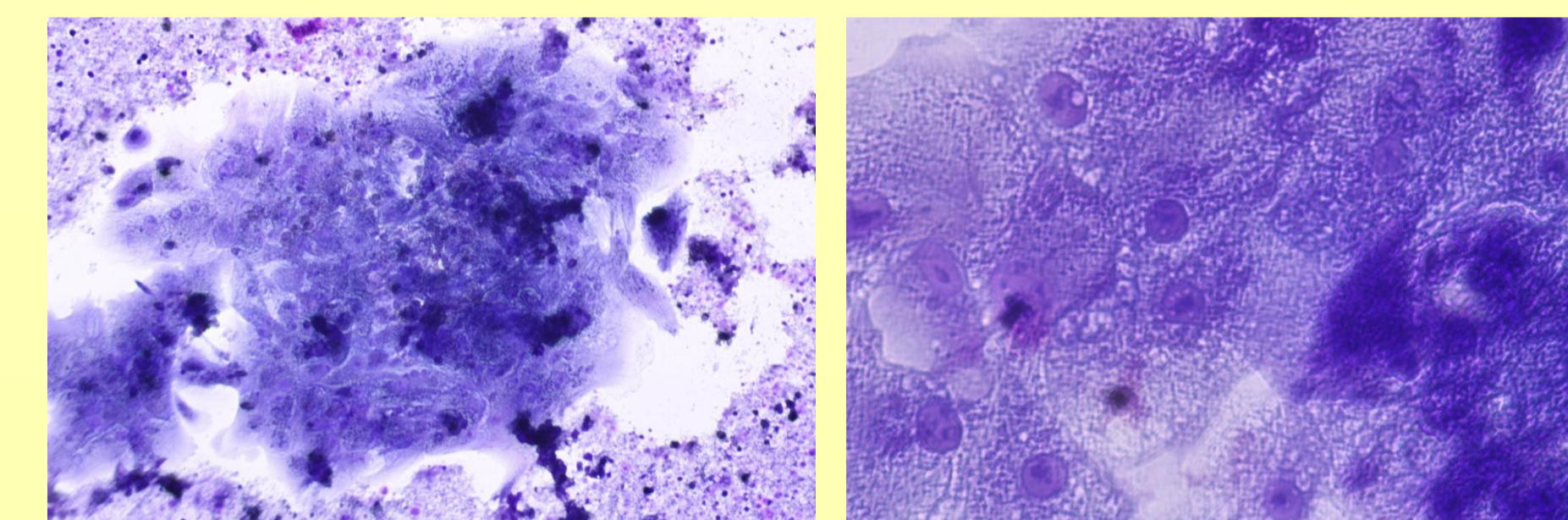


Fig.3 a, b. Hepatic colony forming after inoculating the suspension of cryopreserved cells from human fetal liver of 10 gestation weeks in AIM-V culture medium on plastic, 20 d of culture. Romanovsky staining, a - ×200, b - ×1000.

We have also observed growth of colonies that consist only of small spherical compact cells in DMEM/FBS culture medium. These cells have a lot of lipophilic vacuoles and are positive for cytokeratin 18, with is typical for human fetal liver multipotent progenitor cells* (Fig.4, 5).

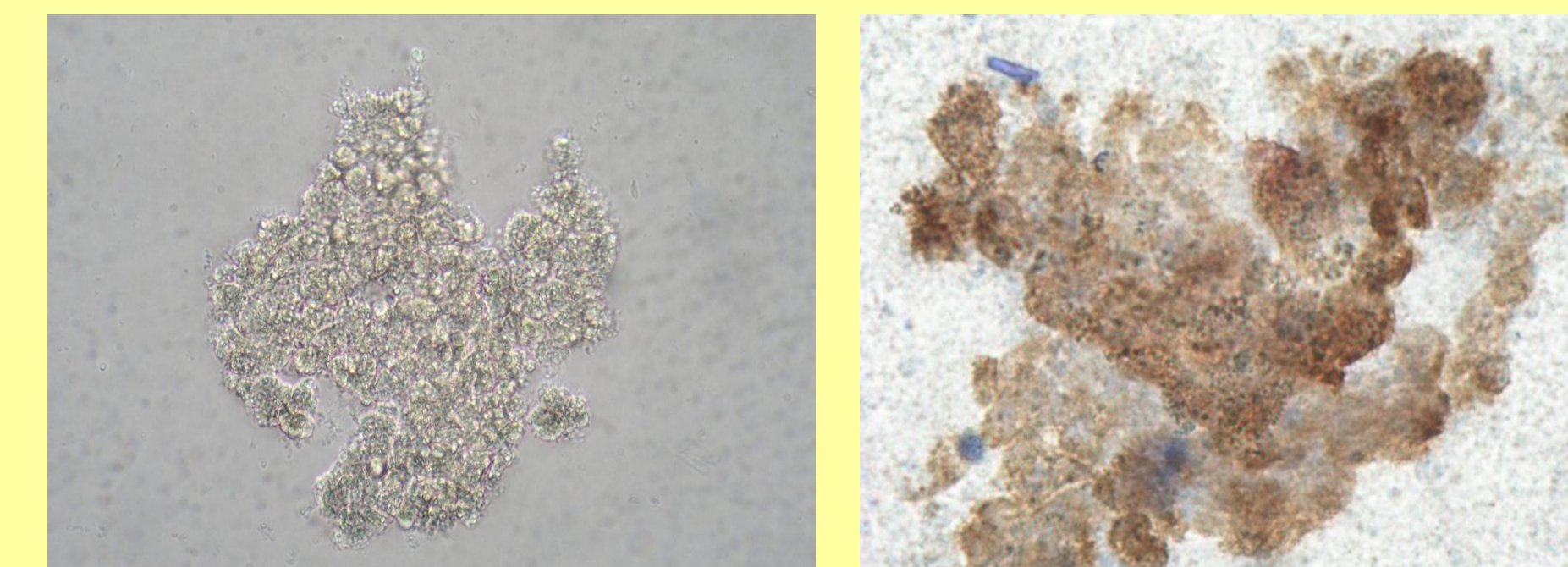


Fig.4. Bright light microscopic appearance of a colony obtained on day 10 of cultivation in DMEM/FBS medium on plastic, magnification ×400.

In addition, we have observed hepatic colonies consisting of at least two types of cells: hepatoblast and endothelial-like cells (by morphological features) (Fig. 6a).

The companion cells in the human hepatic colonies are comparable with endothelial-like cells**. These small compact cells emerge at the periphery of a hepatoblast colony, and spread further when attached to the plastic (Fig. 6b).

The immunocytochemical analysis has shown that endothelial-like cells are positive for cytokeratin 18 (Fig. 7a,b) and in presence of serum form disperse colonies (Fig 7b).

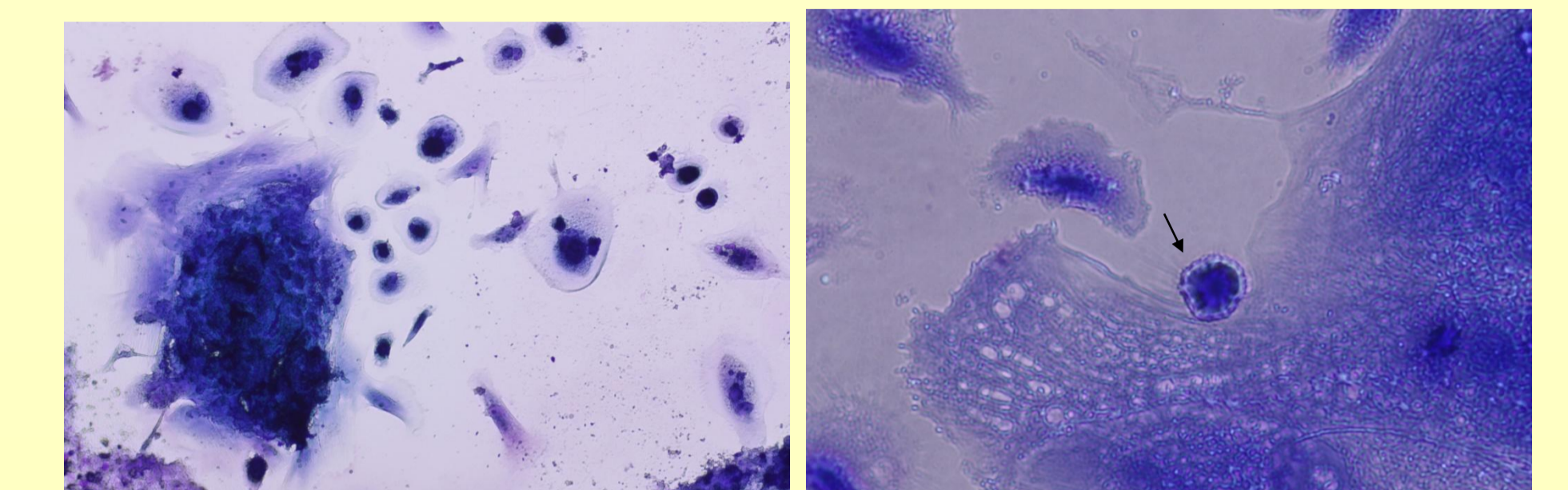


Fig.6 a,b. Hepatic colony consisting of two types of cells: hepatoblasts and endothelial-like cells, a - ×100, b - ×400.

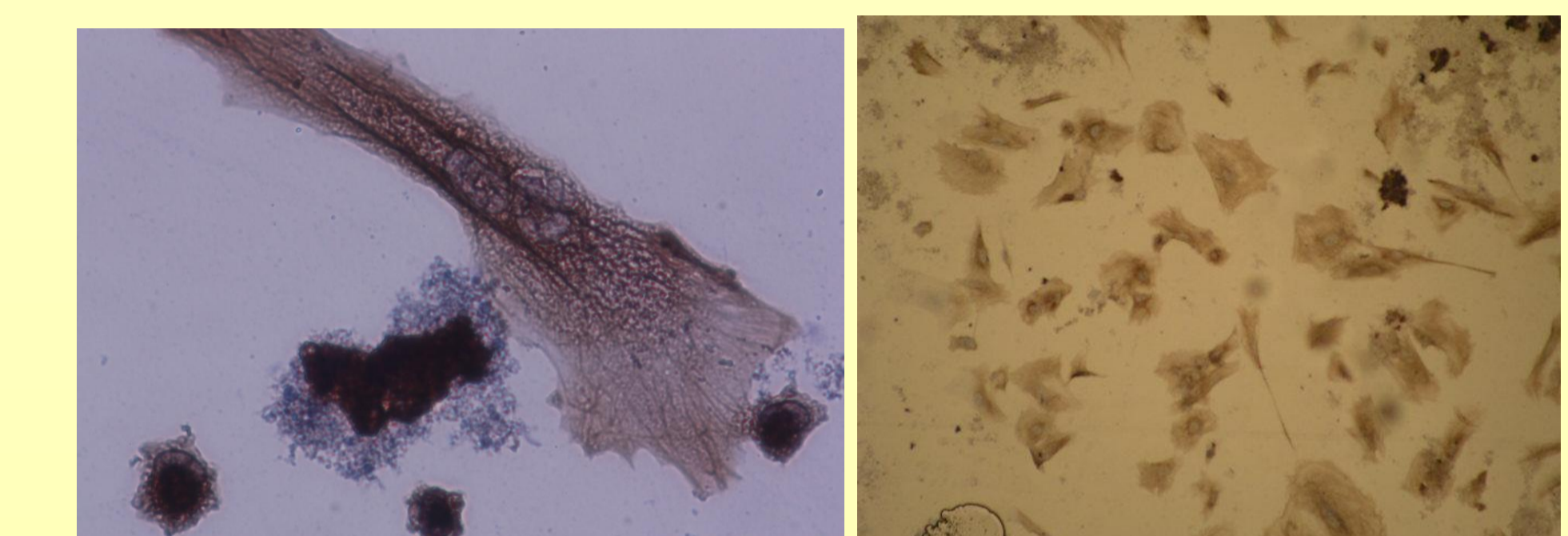


Fig.7 a,b. Companion cells to the hepatoblast colony are positive for cytokeratin 18, a - ×400, b - ×100.

The cultivation of a defrosted suspension of fetal liver cells in DMEM with 15% FBS gave rise to a monolayer of fibroblast-like cells; we have also registered spontaneous adipocytic differentiation (Fig.8,9).

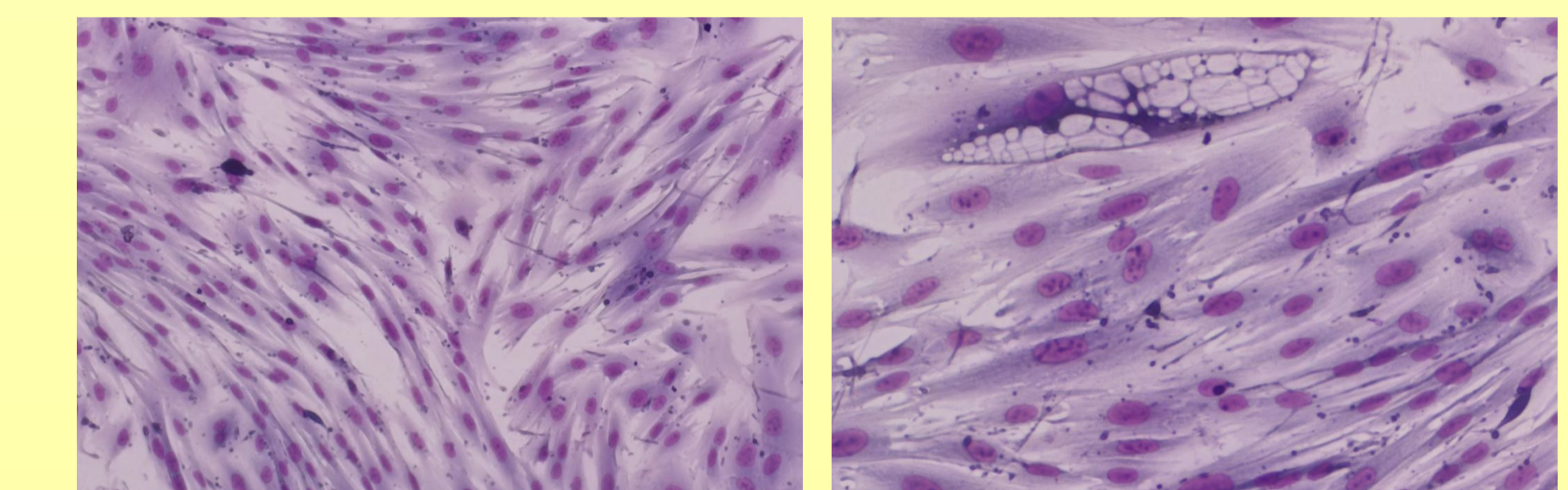
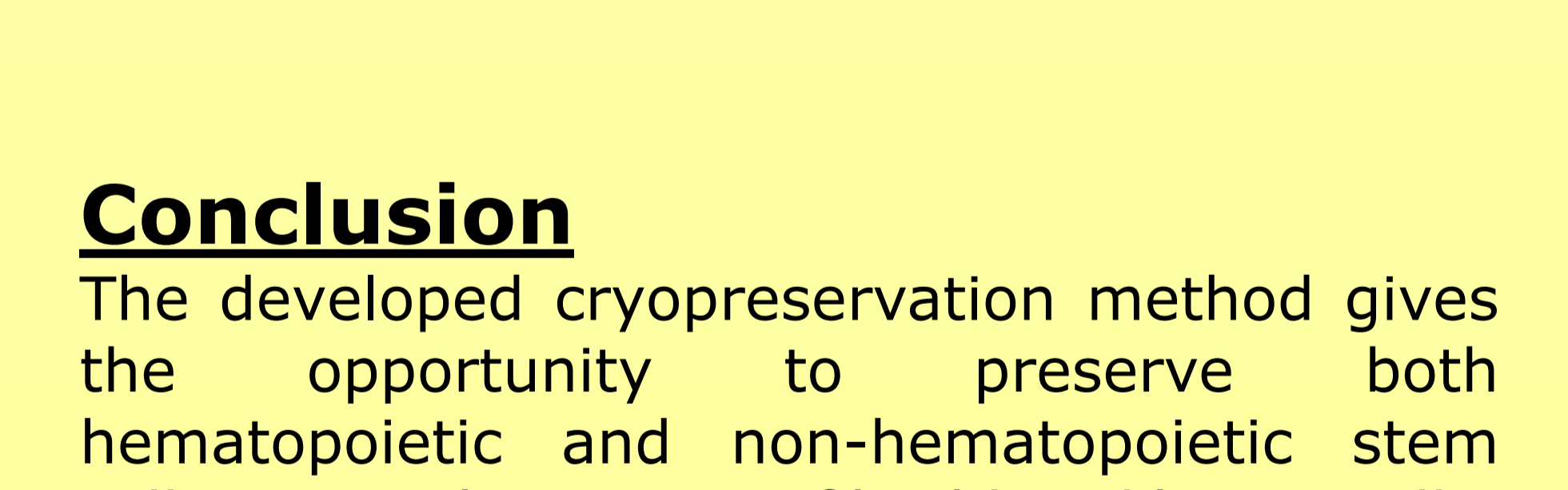


Fig.8. Mesenchymal-like cells, 20 day in DMEM/ FBS. Romanovsky staining, ×200.



Adipocytic differentiation was evidenced based on lipid vacuoles formation, at original magnification ×400.

Conclusion

The developed cryopreservation method gives the opportunity to preserve both hematopoietic and non-hematopoietic stem cells, such as fibroblast-like cells, hepatoblasts, and endothelial cells. We are currently studying the suitability of this cryopreservation method for freezing stem cells from chorion, term placenta and umbilical cord blood.

*Y. Y. Dan, K. J. Riehle, C. Lazaro et al. Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. PNAS. 2006. Vol. 103. No. 26, 9912-9917.

**Eva Schmelzer, Lili Zhang, Andrew Bruce et al. Human hepatic stem cells from fetal and postnatal donors. JEM. 2007. Vol. 204. No. 8, 1973-1987.