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CRYOPRESERVATION OF AMNIOTIC FLUID STEM CELLS DERIVED FROM ZOBOR RABBIT

M. KOVÁČ[1,2]*, B. KULÍKOVÁ[1,3], J. VAŠÍČEK[1,2], P. CHRENEK[1,2]

[1]Faculty of Biotechnology and Food Science, Slovak University of Agriculture in Nitra, Slovak Republic
[2]NPPC – Research Institute for Animal Production Nitra, Slovak Republic
[3]Department of Zoology and Anthropology, Constantine the Philosopher University, Nitra, Slovak Republic

ABSTRACT

The aims of our preliminary study were to evaluate the viability and to investigate the occurrence of early and late apoptosis of fresh, frozen-thawed and thawed-cultured rabbit amniotic fluid stem cells (AFSCs). Rabbit AFSCs were isolated from young (4-6 months old) Zobor rabbit females (n = 3), cultured in endothelial basal medium (EBM-2) to passage 3 (P3) and frozen. One month after cryopreservation AFSCs were thawed and cultured for 72 hours. We identified the apoptotic (Annexin-V - AnV; Yo-Pro-1 - YoP) and dead (propidium iodide - PI) AFSCs using flow cytometry and evaluated the viability of fresh (F-P3), frozen-thawed (F-T) and thawed-cultured (72 hours of post-thaw *in vitro* culture – T-72h) AFSCs. To examine the phenotype of P3 AFSCs we performed an antibody-based staining of surface markers CD29, CD44 and CD45. We have found decreased ($P < 0.05$) viability in F-T cells (77.5 ± 2.2 % and 75.2 ± 0.7 % for AnV^-/PI^- and YoP^-/PI^-, respectively), compared to F-P3 (92.5 ± 2.2 and 89.45 ± 1.3 for AnV^-/PI^- and YoP^-/PI^-, respectively). However, viability of T-72h cells was similar to F-P3 (90.9 ± 1.5 % and 84.6 ± 1.7 % for AnV^-/PI^- and YoP^-/PI, respectively). Our AFSCs were both $CD44^+$ (95.98 ± 1.51 %) and $CD29^+$ (92.33 ± 4.46) positive but $CD45^-$ (0.73 ± 0.55 %) negative, according to defined MSCs phenotype.

Key words: rabbit; amniotic fluid; stem cells; viability; cryopreservation

INTRODUCTION

Stem cells are the focus of great interest because of their potential for therapy in a wide variety of conditions. Conditions possibly treatable by stem-cell transplants include genetic defects, tissue and organ replacement, autoimmune disease and malignancies (Dziadosz *et al*., 2016). These cells with high differentiation capacities are able to give rise to tissue derivatives of three germ layers; ecto-, meso- and endoderm lineages. They can be spontaneously differentiated through the formation of embryoid bodies (Valli *et al.*, 2010), and can also undergo targeted differentiation under appropriate culture conditions (De Coppi *et al*., 2007a).

Amniotic fluid collected routinely via amniocentesis allows us to bank stem cells. These AFSCs can be proliferated in culture as needed and offer a convenient source for autologous transplantations (Abdulrazzak *et al.*, 2013). They can be isolated from mid-term amniotic fluid (AF) of both human and rodents and cultured up to 300 passages (De Coppi *et al*., 2007b; Phermathai *et al.*, 2010). Many authors declare that human AFSC are cultured easily, remain viable over many passages and tolerate cryopreservation very well (Da Sacco *et al*., 2011; De Coppi *et al*., 2007a; Young *et al.*, 2015; Chen *et al.*, 2014). Besides human, they have been isolated from various species including dogs, pigs, horses and buffaloes (Chen *et al.*, 2011; Choi *et al*.,2013; Dev *et al.*, 2013; Filioli Uranio *et al*., 2011; Iacono *et al*., 2012; Yadav *et al.*, 2011).

Stem cells are characterized by the presence of surface markers that are associated with self-renewal without differentiation. The flow cytometry has been a primary technique for the identification of stem cells

***Correspondence:** E-mail: 3mkovac3@gmail.com
Michal Kováč, Institute of Farm Animal Genetics and Reproduction
NPPC – Research Institute for Animal Production Nitra
Hlohovecká 2, 951 41 Lužianky, Slovak Republic
Tel.: +421 37 6546 285 Fax: +421 37 6546 189

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by detection of these markers (Dziadosz *et al.*, 2016). Previously described AFSCs also exhibit intermediate characteristics between embryonic and adult stem cells. They are devoid of ethical controversies and are safer in clinical applications (Chen *et al.*, 2011; De Coppi *et al.*, 2007b; Pozzobon *et al.*, 2010). In the past, scientists classified AFSCs as pluripotent, according to the marker expression. However, the inability to form tumors upon transplantation positioned AFSCs as broad multipotent (Canazi and De Coppi, 2012) or potentially pluripotent stem cells (Gao *et al.*, 2013). Moreover, primary cultures of amniotic fluid contain a heterogeneous population of cells due to the direct contact of the fluid and the fetus (Cremer *et al.*, 1981; Ferdaos and Nordin, 2012; Joo *et al.*, 2012). Paebst *et al.* (2014) showed method for selection of specific cells prior to the cultivation by sorting of $CD44^+$ cells from amniotic fluid.

The use of preclinical models is promising for future therapeutic possibilities (Dziadosz *et al.*, 2016). For example, Klein *et al.* (2011) transplanted AFSCs to fetal lambs and reported more rapid healing time of skin wounds. Sun *et al.* (2015) differentiated human AFSCs into keratinocytes that improved epidermal regeneration of intentionally excised wounds in mouse model. De Konick *et al.* (2015) observed improved lung density and function in rabbit model with congenital diaphragmatic hernia after intratracheal injection of human AFSCs.

The aims of our preliminary study were to evaluate the viability and to investigate the occurrence of early and late apoptosis of fresh, frozen-thawed and thawed-cultured rabbit AFSCs.

MATERIAL AND METHODS

Cells isolation and culture

Amniotic fluid was obtained from humanely sacrificed Zobor rabbits (n = 3) at 23rd day of gravidity using pipette under sterile conditions and mixed with culture medium at the 5:6 ratio. The medium was composed of EBM-2 basal medium (CC-3156, Lonza, USA) supplemented with 20 % fetal calf serum (FCS), EGM-2 SingleQuots™ Kit (CC-4176, Lonza, USA) and 1 % of penicillin and streptomycin (Life Technologies, Slovakia). Five ml of the mixture was used for seeding the cells into T25 culture flasks. Cells were cultured and medium was changed at 5th day, after the cells formed adherent colonies. Subsequently, the culture medium was changed every 2nd day. Cells were dissociated after the colonies reached 80-90 % confluency using Accutase (Invitrogen, Carlsbad, CA, USA) for 5 min at 37 °C and 5 % CO_2. AFSCs were counted and seeded into new culture flasks at density of 4-5 x 10^4 cells/cm^2. Subsequently, the cells were cultured and passaged after 3-4 days, after they reached 80-90 % confluency.

Freezing

Rabbit AFSCs were frozen in freezing solution composed of medium used for culture and 10 % DMSO (D2650, Sigma-Aldrich). 2 x 10^6 cells were mixed with 1.5 ml of freezing solution and placed into cryovials. Cryovials were frozen at slow rate by reducing the temperature by 1 °C per minute in Mr. Frosty container (Thermo Scientific Nalgene, Rochester, NY, USA) and stored for 24 hours at -80 °C. After 24 hours the cryovials were immersed into liquid nitrogen and stored for 1 month. Thawing of the AFSCs was rapid and performed by swirling the cryovials in water bath at 37 °C for 30-60 sec. The suspension was then slowly instilled into pre-warmed culture medium to prevent osmotic shock.

Fluorescent staining

The viability of the cells was evaluated prior to cryopreservation (F-P3), immediately after thawing (F-T) and after additional culture for 72 h (T-72 h). Annexin-V (Roche Slovakia, Slovak Republic) and Yo-Pro-1 (Molecular Probes, Lucerne, Switzerland) staining was used for detection of early and late apoptosis, respectively. The dead cells were identified by propidium iodide (Molecular Probes, Eugene, Oregon, USA) staining. At least 0.5-1 x 10^6 cells were washed in AnV buffer and PBS without magnesium/calcium (Life Technologies, Slovakia) for AnV and YoP staining, respectively. Washed cells were placed in 50 µl of prepared staining solution and incubated in the dark at room temperature for 20 min. At least 1 x 10^4 cells in each sample were analyzed by flow cytometer FACS Calibur (BD Biosciences, USA).

Surface marker detection

Flow cytometry immunostaining was performed to identify the expression of the cell surface markers CD44, CD29 and CD45. Primary antibodies were as follows: anti-rabbit CD29 (mouse IgG1, clone P4G11, Abcam, United Kingdom), anti-rabbit CD44 (mouse IgG1, clone W4/86, AbD Serotec, Germany), anti-rabbit CD45 (mouse IgG1, clone L12/201, AbD Serotec, Germany). Secondary antibodies were as follows: anti-IgG1-PE, mouse (rat IgG1, clone X-56, Miltenyi Biotec, Germany) for CD44 and CD29; anti-IgG1-APC, mouse (rat IgG1, clone X-56, Milteny Biotec, Germany) for CD45. Briefly, F-P3 cells were dissociated, washed with PBS and centrifuged for 5 min at 587 x g at 4 °C. The pellet was resuspended in 50 µl of rabbit serum to block the Fc receptors. Cells were incubated with isotype controls, primary and secondary antibodies for 15 min on ice. At least 0.5 x 10^5 cells were analyzed for each CD marker.

Statistical Analysis

The statistical analysis was performed with One-Way ANOVA (Tukey-Test) for comparison of mean values ± SDs using SigmaPlot software (Systat Software Inc., Germany).

RESULTS AND DISCUSSION

Our present research was focused on isolation of rabbit AFSCs that could serve as a tool for preserving the animal genetic resources in gene bank.

Immunophenotyping of rabbit AFCS in our study revealed that they express surface molecular markers CD44 and CD29 that are commonly used to profile MSCs. The expression of CD44 was 95.98 ± 1.51 %, which fulfil criteria of the International Society for Cellular Therapy for positive MSCs marker expression (Dominici *et al.*, 2006). The expression of CD29 was slightly lower (92.33 ± 4.46 %). Nevertheless, such positivity was considered as sufficient for accepting these cells as $CD29^+$ according to other studies, which declared AFSCs as CD29 positive, without quantification (You *et al*., 2009; Paebst *el al*., 2014; Filioli Uranio *et al*., 2011).

Moreover, AFCSs in our study were more positive to CD44 marker than AFSCs isolated by Slamečka and Chrenek (2013). We suggest that more than the two-fold higher proportion of cells expressing CD44 (95.98 ± 1.51 % vs 47.8 %) might be attributed to antibodies specificity. We have used anti-rabbit antibodies, whereas Slamečka and Chrenek (2013) used antibodies designed for human AFSCs. It is necessary to say, that the markers required for defining a MSC were mainly determined for human cells, and the lack of specific antibodies for different animal species led to a great variability of approaches and results for molecular characterization of animal MSCs (Iacono *et al*., 2015). Furthermore, hematopoietic lineage marker CD45 was used as a negative control. The cells fulfilled the criteria of Dominici *et al*. (2006) for negative marker expression (0.73 ± 0.55 %).

Viability of AFSCs was evaluated by flow cytometry fluorescent staining. AnV^-/PI^- and YoP^-/PI^- cells were gated to evaluate the overall viability. We have found decreased ($P < 0.05$) viability in F-T cells (77.5 ± 2.2 % and 75.2 ± 0.7 % for AnV and YoP, respectively), compared to F-P3 control (92.5 ± 2.2 and 89.45 ± 1.3 for AnV and YoP respectively). However, viability of T-72 h cells was similar to fresh passage (90.9 ± 1.5 % and 84.6 ± 1.7 % for AnV^-/PI^- and YoP^-/PI, respectively).

Our results showed that freezing of stem cells increased the apoptotic and dead cell proportion. It has been reported that hematopoietic and other stem cells, particularly human embryonic stem cells, undergo apoptotic transformation during the cryopreservation process (Sangeetha *et al*., 2010; Stroh *et al*., 2002). MSCs also lose their viability very soon post-thaw, what can most likely be attributed to the rapid development of apoptotic processes (Berz and Colvin, 2012). Our F-T AFSCs showed more than two-fold increase in apoptotic or PI^+ cells compared to F-P3 AFSCs (9.8 ± 1.8 % vs 3.7 ± 1.2 %; 10.5 ± 4.8 % vs 4.0 ± 1.8 % for AnV^-/PI^- and YoP^-/PI^-, respectively). Pal *et al.* (2008) investigated the post-thaw behavior of MSCs and reported a rapid decrease in viability from > 80 % at 2 hours to < 40 % at 8 hours, while the cells were maintained at 4 °C.

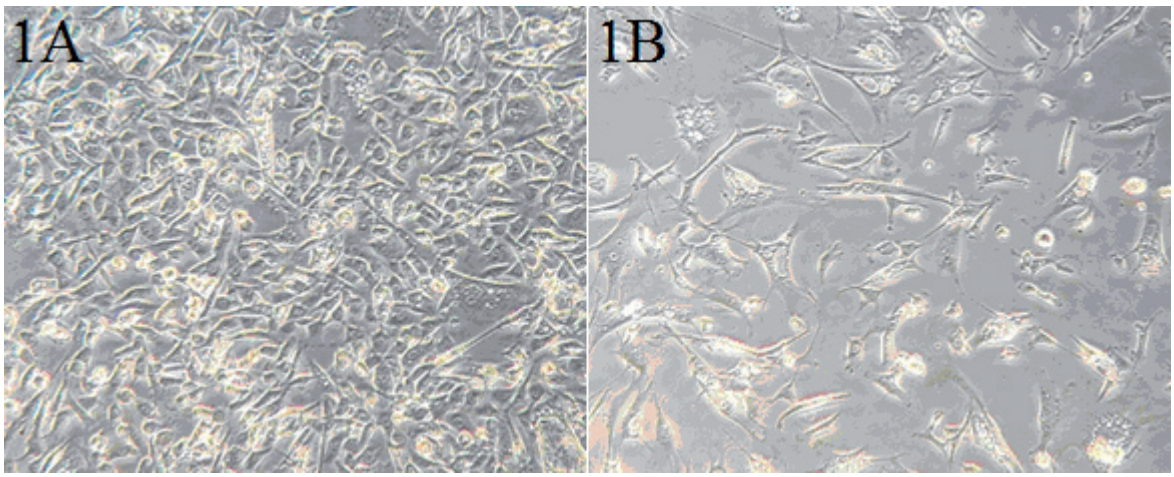

Fig. 1: Phase contrast images of the post-thaw cultured rabbit AFSCs (Zeiss Primo Vert Inverted Microscope). 1A – endothelial-like shape (10x magnification) 1B – mesenchymal-like shape (20x magnification).

Table 1: Detection of Annexin-V, Yo-Pro-1, and propidium iodide positive^{+}/negative^{-} cells expressed in %

Amniotic fluid stem cells	AnV^{-}/PI^{-}	AnV^{+}/PI^{-}	AnV^{-}/PI^{+}	AnV^{+}/PI^{+}	YoP^{-}/PI^{-}	YoP^{+}/PI^{-}	YoP^{-}/PI^{+}	YoP^{+}/PI^{+}
Fresh	92.5 ± 2.2^{a}	0.6 ± 0.3	4.1 ± 1.7^{a}	3.7 ± 1.2^{a}	89.45 ± 1.3^{a}	2.6 ± 0.9	4.6 ± 1.7	4.0 ± 1.8^{a}
Thawed	77.5 ± 2.2^{b}	2.9 ± 0.9	8.5 ± 0.9^{b}	9.8 ± 1.8^{b}	75.2 ± 0.7^{b}	3.8 ± 1.2	6.2 ± 0.8	10.5 ± 4.8^{b}
Thawed 72 h	90.9 ± 1.5^{a}	1.2 ± 1.4	4.9 ± 1.9^{ab}	4.0 ± 1.9^{a}	84.6 ± 1.7^{a}	3.6 ± 0.3	3.9 ± 1.0	5.9 ± 2.4^{ab}

Results are expressed as means ± SEM; a vs b within the same column were statistically significant at $P < 0.05$.

The decrease in viability may be associated also with DMSO addition. It is a small amphipathic molecule that penetrates via plasma membrane of cells and acts as a strong hydrogen bond disrupter and hence exerts colligative effects (Ruiz-Delgado *et al*., 2009). It affects multiple organ systems with wide spectrum of toxicities. However, a DMSO concentration of 10 % in stem cell concentrate is still considered to be standard in most centers around the world. Interestingly, the maximal possible cryopreservation time span is still unknown (Berz and Colvin, 2012).

CONCLUSION

Although the freezing of rabbit AFSCs negatively affects the viability and lead to increase in apoptotic processes, our AFSC were able of post-thaw proliferation. Moreover, after additional 72 hours of culture the proportion of dead and apoptotic cells was reduced. The AFSCs morphology did not change post-thaw and following culture. These preliminary results could potentially serve for cryopreservation of these cells with subsequent use for gene bank purposes as genetic resources or for tissue engineering.

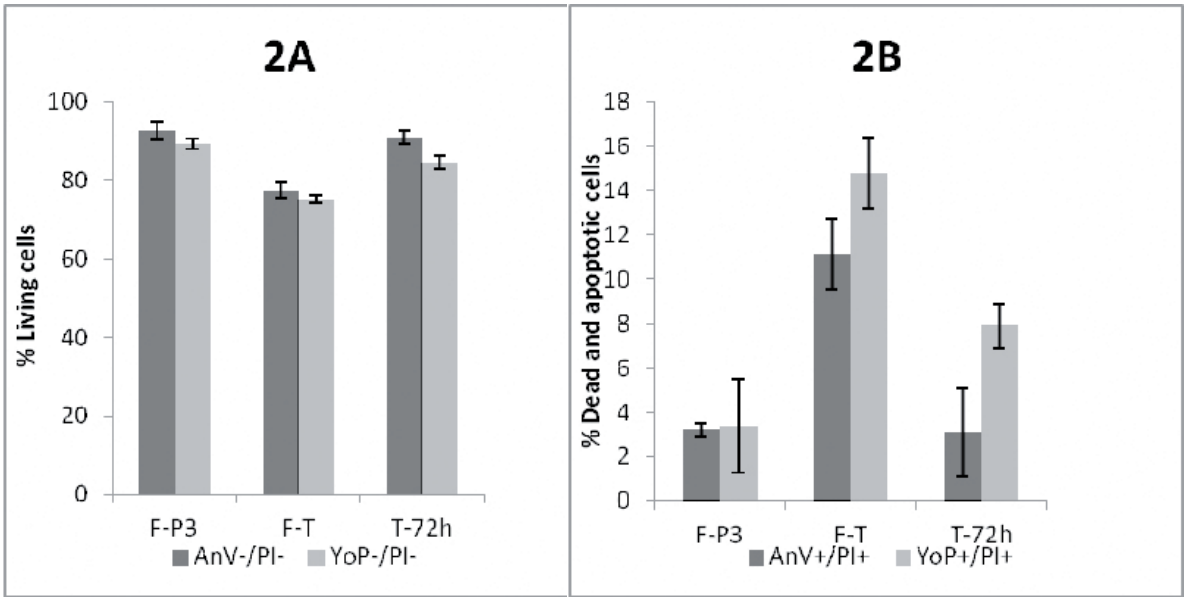

Fig. 2: Proportion of apoptotic, dead and viable AFSCs - P3 fresh (F-P3); frozen-thawed (F-T); thawed-cultured for 72 hours (T-72 h). 2A – proportion of living AnV^{-}/PI^{-} and YoP^{-}/PI^{-} AFSCs. 2B – Necrotic and apoptotic AnV^{+}/PI^{+} and YoP^{+}/PI^{+} AFSCs.

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