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Neural differentiation potential of sympathoadrenal progenitors derived from fresh and cryopreserved neonatal porcine adrenal glands

G.A. Bozhok*, O.S. Sidorenko, E.M. Plaksina, T.M. Gurina, A.N. Sukach, V.S. Kholodnyy, V.D. Ustichenko, S.B. Bilyavskaya, T.P. Bondarenko, E.I. Legach

Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkov, Ukraine

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Stem/progenitor cells are thought to have the potential in the treatment of severe neurodegenerative diseases. Recently, sympathoadrenal progenitors expressing specific markers of neural crest derivatives and capable to differentiate into neurons were discovered in adult bovine and human adrenal glands, but there was no reported data on cryopreservation of sympathoadrenal progenitors. The aim of the present study was to examine the neural differentiation potential of sympathoadrenal progenitors derived from fresh and cryopreserved neonatal porcine adrenal glands. Considering impact of various initial state of frozen biomaterial on cell recovery, we carried out a comparative estimation of cryopreservation outcome both for adrenal tissue fragments and isolated primary cells. The estimation consisted of determining cell yield, viability, ability to adhere, proliferate and differentiate in vitro.

Cells isolated from the fresh adrenal glands were cultured until confluence. A formation of sympathoadrenal progenitors-embedded spherical cell colonies, whose cells are differentiated then into βIII-tubulin-positive cells with neuron-like morphology, was observed on the monolayer. The colonies were well preserved after cryopreservation of cell culture with a cooling rate of 1 °C/min in the cryoprotectant media containing 5–15% of dimethylsulfoxide. Adrenal tissue fragments were cryopreserved in the presence of 10% dimethylsulfoxide at the cooling rates of 0.3; 1: 5; 40 and > 100 °C/min. Sympathoadrenal progenitors were recovered after cryopreservation with 0.3 °C/min cooling rate but not higher.

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1. Introduction

During embryonic development adrenal glands are formed from different anlagen: cortex from mesoderma and medulla from ectoderma [4,24]. Perhaps, such heterogeneity of the origin causes the presence of progenitor cells belonging to both mesenchymal [36] and neuronal [10,17] derivatives in adrenal glands.

Stem/progenitor cells are known to possess a great potential for treating severe neurodegenerative diseases [39,47,48,58,65]. Even though there are the protocols of neural stem cells isolation from neural tissues [5], olfactory epithelium [43,50], induced pluripotent stem cells [8,22] the search for the appropriate source of neural stem cells remains a topical task.

Cryopreservation as one of the main technologies of cell-based therapy enables banking, testing and transportation of biomaterial. Previous studies have shown the possibility of human and animal adrenal tissue cryopreservation to maintain its functional potential [2,56,63].

Cell culture techniques are often used as standard procedures for pretransplant cell processing. During culture, many types of stem/progenitor cells form spherical colonies either free-floating or adherent to the monolayer [52]. The spherical cell colonies, which formed in the primary cell cultures derived from fetal, neonatal and adult adrenal glands of human, rat and bull were demonstrated in previous studies [6,9,10,25,49,55,71]. The presence of specific markers of SAP in the adrenal gland derived cell colonies and its ability to differentiate into neurons have been discovered by several authors [6,9,55].

Until now, there was no research carried on cryopreservation of adrenal gland derived SAP. Obviously, the parameters for successful cryopreservation of multicellular colonies differ from those of single cell suspension. Furthermore, feasible modifications in cell volume or permeability during culture [26,35] cause changes in the filtration rate, which ultimately determines the rates of cell

* Corresponding author.
E-mail address: bozhokgaru@mail.ru (G.A. Bozhok).

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dehydration and rehydration during the phase transitions from water to ice and from ice to water during freezing-thawing. As a result, at the same cooling rate the probability of intracellular ice formation is increasing when cultured cells are exposed to freezing comparing with fresh-isolated ones [26–28].

Pigs are one of the species possessing genomic, anatomical and physiologic parameters similar to humans [37]. That makes it possible to study a lot of issues associated with peculiarities of organ development and function in the model system. Moreover, obtaining induced pluripotent stem cells from pigs [18] reflects the increased interest of the researchers in this species as potential donors for transplantable organs, tissues and cells for humans.

Basing on the previous data about presence of sympathoadrenal progenitors (SAP) in human [55] and bovine [9] adrenal glands we made the attempt to isolate such cells from pig adrenals. Taking into consideration that decline in the stem cell pool and its differentiation potential is age-dependent [21,38], neonatal glands were taken as an object of research.

The aim of the present study was to examine the neural differentiation potential of SAP derived from fresh and cryopreserved neonatal porcine adrenal glands.

2. Materials and methods

2.1. Preparation of tissue fragments and cell suspensions

Adrenal glands were obtained from freshly slaughtered 1–2 day old Yorkshire piglets (n = 22). Both adrenal glands were removed, immersed in an ice-cold DMEM/F12 medium (Sigma, USA) with 100 U/ml penicillin (Sigma), 200 μg/ml streptomycin (Sigma), 5 μg/ml amphotericine B (PAA, Austria), and cleaned from adjacent fat. Adrenals were cut into small pieces (1–2 mm^3), washed 3–4 times with the medium and kept on ice before collagenase digestion or cryopreservation.

Collagenase digestion of adrenal tissue fragments (ATFr) was carried out in the DMEM/F12 medium with collagenase type V (1 mg/ml, Sigma, USA) and DNase I (0.1 mg/ml, Sigma) at 37 °C in a shaking water bath in three stages (30; 10; 10 min correspondingly). Supernatant of each digestion step was collected, and 0.5 ml aliquots of fetal bovine serum (FBS, Sigma) was added. The tissue after a three-stage digestion was dissociated by gentle pipetting, put together with supernatants and washed twice with medium supplemented with 0.2% bovine serum albumin (BSA, Sigma). Then cell suspensions were filtered through 100 μm nylon mesh and washed again.

2.2. Primary porcine adrenal cell culture

Suspended cells were seeded at a density of 0.3–0.4 × 10^6 cells/sm^2 into culture flasks (Corning, USA) or 24-well plates (PAA) in the DMEM/F12 medium, supplemented with 10% FBS, 100 U/ml penicillin, 200 μg/ml streptomycin and 5 μg/ml amphotericine B at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every 3 days.

For cryopreservation or subculture, cells of primary culture were detached by 3 min incubation with a 1:1 mixture of Versene (PAA) and 0.25% trypsin (Sigma) solutions and washed.

For evaluation of nerve growth factor (NGF) impact on cell differentiation, the culture medium was supplemented with 100 ng/ml NGF (Sigma).

2.3. Cryopreservation of ATFr and primary adrenal cell culture

Two sets of experiments were conducted (Fig. 1). In the first set, cell culture was obtained from ATFs, which were cryopreserved in the DMEM/F12 medium containing 10% dimethylsulfoxide (Me2SO, v/v). Fresh fragments were placed with 0.5 ml medium in a 1.5 ml cryovial (Thermo Fisher Scientific, USA) and an equal volume of 2× concentrated cryoprotectant medium was added. Samples were incubated with a cryoprotectant for 20 min at 22 °C, and then cryopreserved using different cooling rates: i) 0.3, 1 and 5 °C/min in automatic controlled rate freezer Cryoson (Germany) from room temperature down to −70 °C and then plunged into liquid nitrogen (LN); ii) 40 °C/min in LN vapor for 3.5 min, then plunged into LN; iii) >100 °C/min by vertical stepwise immersion into LN for 1.5 min.

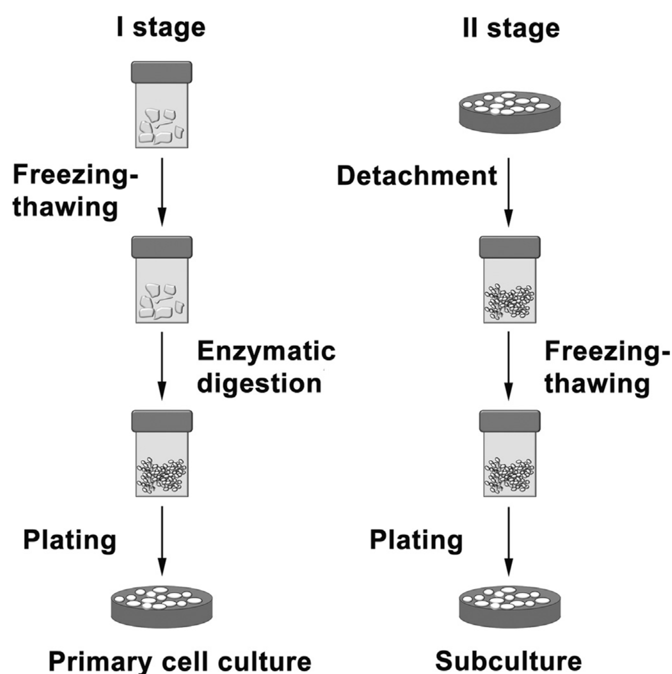

Fig. 1. Cryopreservation study design.

In the second set, cells of primary culture were detached by enzymatic method and washed. The pellets were resuspended in DMEM/F12 medium and aliquoted at a density of 0.5–1 × 10^6 cells/cryovial. Equal volume of 2× concentrated cryoprotectant solution was added to obtain final concentrations of Me2SO at 5, 7.5, 10 and 15%. A portion of cells was frozen in a combination of Me2SO with 10 or 25% FBS. Cryopreservation was carried out using a cooling rate of 1 °C/min in an automatic freezer from room temperature down to −70 °C without initiation of ice nucleation, and then cryovials were plunged into LN.

Thawing of the samples was performed in a water bath at 37 °C. Cryoprotectant was removed by doubling the volume until 4 ml of the DMEM/F12 medium had been added to 1 ml of sample during step by step dilution.

2.4. Evaluation of cell viability, post-thaw recovery and cell yield

The viability of cells was determined by a trypan blue dye-exclusion test. For staining, 0.05 ml of cells was mixed with an equal volume of 0.4% trypan blue solution and counted using a hemacytometer. Cell viability was calculated as a percentage of the number of unstained cells divided by the total number of cells. Cell recovery after cryopreservation was calculated as percentage of the total number of cells after thawing divided by the total number of frozen cells.

Cell yield was defined as the number of cells, which was obtained from one adrenal gland after all experimental procedures

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