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

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ABSTRACT

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.

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

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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.

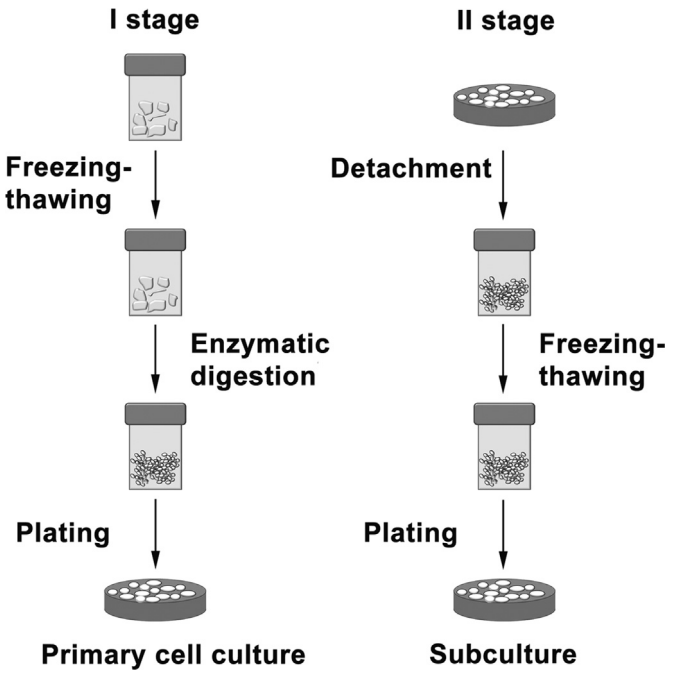

Fig. 1. Cryopreservation study design.

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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including collagenase digestion, any sedimentation and dilution.

2.5. Cell migration assay

Fluorescent dye 3,3′-dioctadecycloxacarbocyanine perchlorate (DiO, Molecular Probes, USA) was used to evaluate cell migration. Spherical cell colonies were labeled in the DMEM/F12 medium with 5 μM DiO during 2 h at 37 °C. The dye was removed, and cell colonies were seeded into aforesaid culture conditions. Cell fluorescence was detected on 5–7th days of culture under an inverted fluorescent microscope at 485 nm excitation and 501 nm emission maxima.

2.6. Measurement of cortisol levels

Culture supernatants were collected during primary adrenal cell culture, subculture and after cryopreservation. Cortisol levels in the culture were measured by radioimmunoassay kit (Immunotech, France) according to the recommendations of the manufacturer.

2.7. Immunocytochemistry

Primary mouse anti-βIII-tubulin (TU-20) monoclonal antibodies (1:500, Abcam, UK), primary mouse anti-S100 (B32.1) monoclonal antibodies (1:500, Abcam), secondary goat anti-mouse FITC-conjugated antibodies (1:1000, Abcam) were used for immunocytochemistry. Cell cultures were fixed in 4% paraformaldehyde (Sigma) for 15 min and permeabilized with 0.2% Triton. Blocking the nonspecific binding was carried out by incubation of samples with 1% BSA overnight. Then cultures were incubated with primary antibodies for 2 h and, after washing with PBS, for 30 min with secondary antibodies at room temperature. Finally, either 2 μg/ml propidium iodide (PI, Sigma) or 5 μg/ml Hoechst 33342 (Sigma) was added to the samples for identification of cell nuclei. Negative controls included cultures in which primary antibodies were omitted. Samples images were collected with a confocal laser scanning microscope Carl Zeiss LSM510 META and analyzed using AxioVision Rel.4.7 and LSM Image Examiner software (Carl Zeiss).

2.8. Morphometric study of βIII-tubulin-positive cells

Well-stained βIII-tubulin-positive cells were analyzed. Diameter of cell body and length of processes of the βIII-tubulin-positive cells were microscopically determined at twenty high-power fields (x 400). Cell body diameter was measured in a direction perpendicular to the axis of processes growth.

2.9. Statistical analysis

All data are presented as mean ± SD. Statistical significance of differences was calculated with a two-tailed unpaired Student's t-test or a one-way ANOVA in case of multiple comparisons. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Formation of spherical cell colonies (CCs) in the primary cell culture

After the 1st day of culture the majority of cells adhered, spread and started to proliferate. Due to the entire adrenal gland digestion, the primary culture consisted of different cell types: fibroblast-like cells; chromaffin cells, which were round-shaped and bright in appearance under phase-contrast microscopy; and slightly flattened brown adrenocortical cells (Fig. 2a). Fibroblast-like cells formed a confluent monolayer on average by 3–4 days of culture (Fig. 2b). CCs, consisting of several cells, arose on the monolayer in the next few days (Fig. 2c). During the first week, the average density of CCs was 1.2 ± 0,5 per mm^2, and diameter was <50 μm. After two weeks in culture CCs increased in size up to 100–300 μm.

3.2. CCs subculture

CCs were detached by mechanical or enzymatic methods after 14–15 days of culture. Mechanical detachment was performed by gentle pipette aspiration, while enzymatic detachment was carried out with 3 min incubation with EDTA/trypsine solutions. CCs were reseeded in the DMEM/F12 medium supplemented with 10% FBS. When mechanically detached, CCs subcultures contained a few contaminated fibroblast-like cells, while in the case of enzymatic detachment the CCs were plated with cells, which constituted the monolayer. The main characteristics of CCs subcultures did not differ depending on the method of detachment.

CCs became attached during first day of subculture (Fig. 3 a), thereafter neuroblast-like cells appeared around CCs (Fig. 3 b). Cell processes became extended, forming a network on the monolayer during the 4^{th}–5th day post-plating (Fig. 3 c, d). Along with the formation of networks by cells with neuronal morphology, the proliferation of fibroblast-like cells was observed. On the 8^{th}–11th day after reseeding, CCs became flattened to form a new monolayer together with fibroblast-like cells (Fig. 3 e, f). Upon continued culture, the neuron-like cells expanded on the monolayer of fibroblast-like cells.

Direct evidence of cells migration was obtained in experiments with DiO labeling (Fig. 4). Aspirated CCs were incubated with DiO and seeded. After 5–7 days, the presence of DiO-labeled cells migrating out of the CCs was prominent. Among migrating cells, the four abundant morphological types were determined: i) cell body only; ii) cell body with growth cone; iii) cell with unipolar process; iv) cell with bipolar processes (Fig. 5). Cells with multiple dendritic processes were rare.

Cell cultures were immunocytochemically labeled against β-III-tubulin as an early neuron-specific marker [61], and S100 as a marker for glial elements of the nervous system including sustentacular cells of adrenal medulla [32].

CCs and neuron-like cells were positive for the β-III-tubulin, while staining with anti-S100 antibodies revealed the absence of positively labeled cells (Fig. 6). Strong cell labeling with β-III-tubulin (data not shown) and S100 (Fig. 6) was observed in the culture of neonatal rat brain cells, which was used as a positive control.

NGF is known to be a main factor of chromaffin cell transdifferentiation into neuron-like cells in vitro [66]. Furthermore, in common stem cell protocols NGF is widely applied for differentiation into neuronal cell types [10,57]. Hence, NGF was added to the culture medium immediately after reseeding of CCs.

The appearance of neuron-like cells with processes forming the network was observed regardless of exogenous NGF. The number of neuron-like cells, cell body diameter and process length did not depend on the presence of NGF (Table 1).

3.3. Efficiency of CCs formation in the cell cultures obtained from frozen-thawed ATFr

10% of Me2SO has been chosen for cryopreservation of ATFr, because the efficacy of such cryoprotectant concentration was demonstrated in our previous study on freezing of porcine adrenal gland tissue [67]. ATFr were cryopreserved with a cooling rate of 0.3; 1; 5; 40 and > 100 °C/min. After thawing and cryoprotectant removal, cell suspension was prepared by collagenase digestion (as

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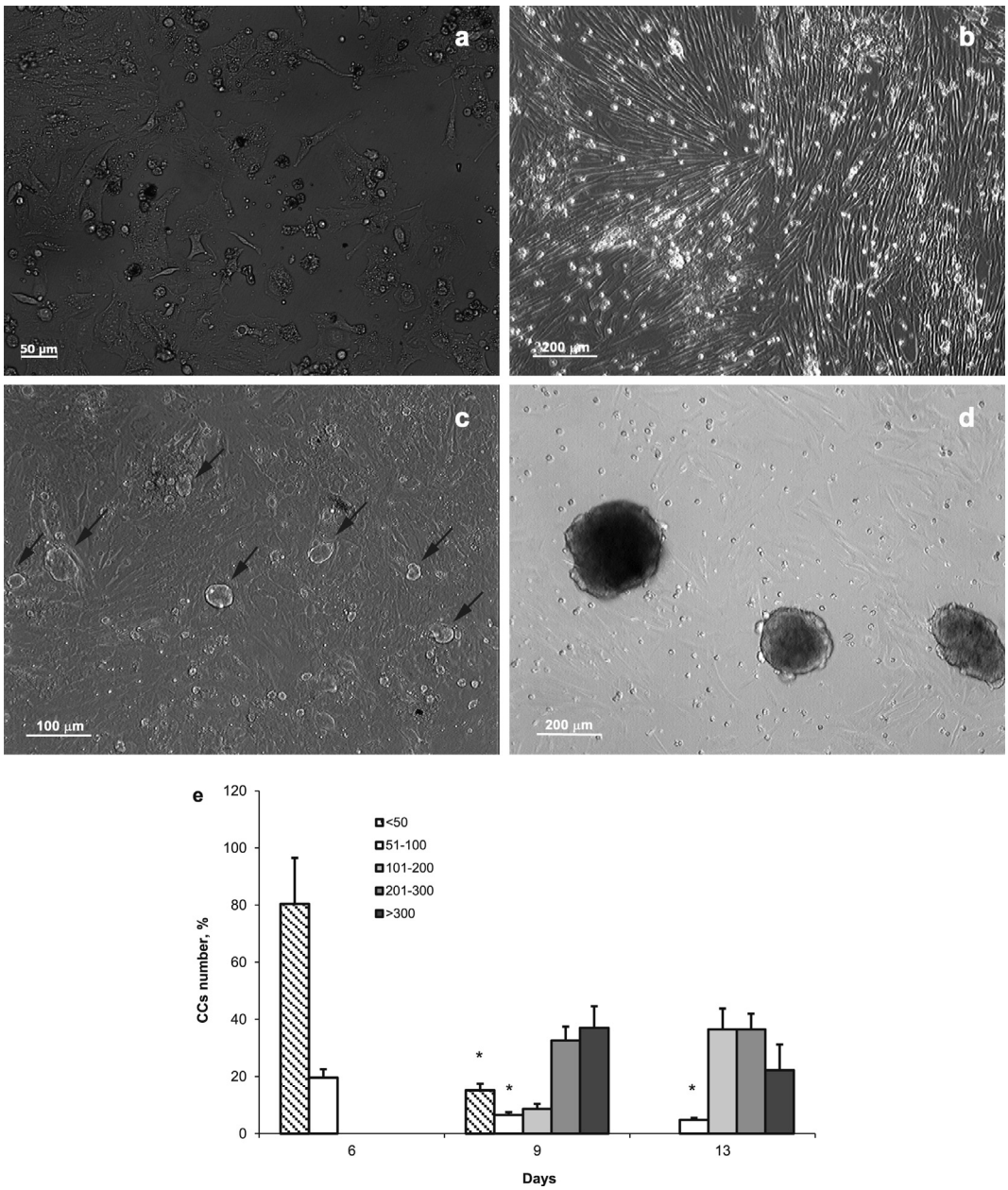

Fig. 2. Representative images of primary cell culture obtained from neonatal porcine adrenal glands: a – different types of cells, 2nd day after plating; b – monolayer formation, 4th day after plating; c – formation of spherical CCs (arrows), 5th day after plating; d – large CCs, 21st day after plating; e – diagram shows a decrease in number of CCs with a diameter of <50 and 50–100 μm as well as an appearance of CCs with a diameter of 100–300 and > 300 μm during culture. The data were obtained from 15 independent experiments performed in triplicates. (*) – indicates significant increase of CCs diameter on 9th and 13th day compared to 6th day at $P < 0.05$.

described in the *Materials and methods*).

Viable cell yield after ATFr freezing-thawing was significant lower compared to the cells derived from fresh ones (Table 2). High rates of cells viability were observed at the cooling rate of 0.3 and 1 °C/min, and the minimal – at 40 and > 100 °C/min.

Cell attachment and spreading, as well as the formation of a monolayer, occurred only after the plating of cells that were obtained from the ATFr frozen at the cooling rate of 0.3 °C/min. The same cell types were present in the culture as in the non-frozen control: fibroblast-like cells, chromaffin cells and adrenocortical cells. Monolayer formation after cryopreservation was slightly delayed. CCs appeared in the culture on the 4^{th}–5th day (Fig. 7a). Their number was higher than in the non-frozen control (1461 ± 199 vs. 404 ± 160 CCs/10^6 seeded cells). Differentiation of cells derived from the CCs into neuron-like cells was also observed after subculture (Fig. 7b).

In cell cultures obtained from the ATFr, which were cryopreserved using cooling rates of 1; 5; 40 and > 100 °C/min, there was neither cell proliferation nor formation of CCs. Round-shaped and slightly flattened cells were noted during the observation period.

3.4. Efficiency of CCs formation after cryopreservation of primary adrenal cell culture

The conventional regime, which is commonly used for cryopreservation of primary cultures of mammalian cells, includes a slow cooling rate (1–3 °C/min), Me2SO as a cryoprotectant and the

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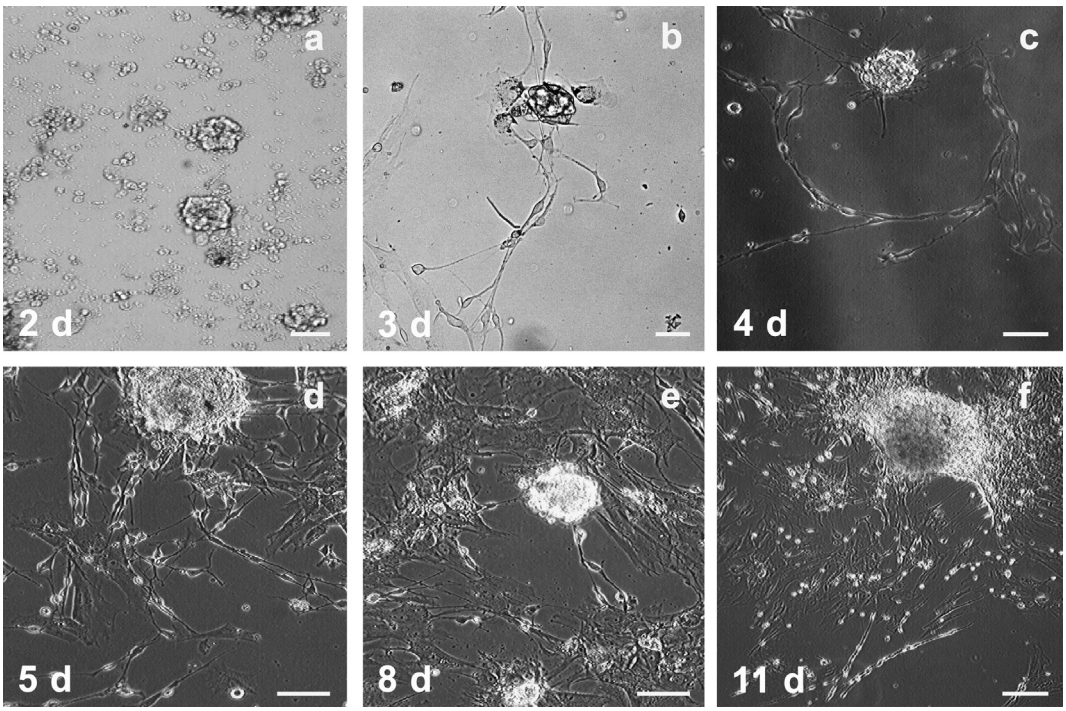

Fig. 3. Representative images of CCs: a – attached colonies and single rounded cells, 2nd day after plating; b – appearance of cells with processes around colony, 3[d] day after plating; c – neuron-like cells begin to form a network, 4th day after plating; d – expansion of neuron-like cells, 5th day after plating; e – proliferation of fibroblast-like cells along with the formation of networks by neuron-like cells, 8th day after plating; f – formation of new monolayer of fibroblast- and neuron-like cells, 11th day after plating. Scale bar: a, b – 50 μm; c, d, e – 100 μm; f – 200 μm.

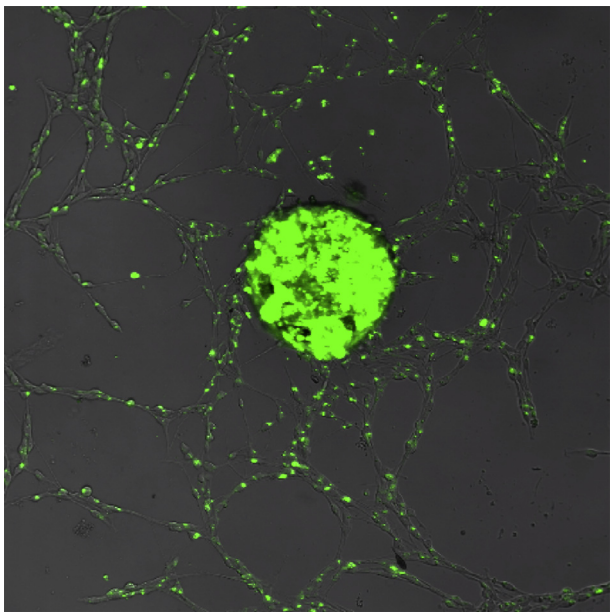

Fig. 4. DiO labeling of CCs. Neuroblast-like cells with fluorescent dye inclusions migrate out of the CCs during culture. CCs were labeled in the DMEM/F12 medium with 5 μM DiO during 2 h at 37 °C. The dye was removed, and cell colonies were seeded into culture conditions.

FBS as a stabilizing agent [20,46]. The choice of cooling rate for primary porcine adrenal cell culture cryopreservation was made in favor of 1 °C/min, because this cooling rate is widely used in commercial benchtop freezing devices [59]. Moreover, previously we have established that along with the increase of the cooling rate above 1 °C/min the number of nucleated cells obtained from the rat adrenal glands decreased [15].

For cryopreservation of primary adrenal cell culture we applied several cryoprotectant media containing Me2SO at concentrations of 5, 7.5, 10 and 15% alone or in combination with 10% or 25% of FBS. The beneficial effect of serum addition to the cryoprotectant medium was confirmed during cryopreservation of cell suspension obtained from neonatal murine adrenal glands [62].

Monolayers with early CCs were detached on the 5th day of culture and cryopreserved. After thawing, cells with quite high recovery (62–96%) and viability (70–91%) were obtained for all the combinations of cryoprotectant media (Fig. 8 a, b). No significant difference in cell recovery was observed between 10 and 25% FBS in the presence of all concentrations of Me2SO. Cell viability was significantly increased ($p < 0.05$) with increasing concentration of FBS in the cryoprotectant medium up to 25%.

Most of the cells attached to the substrate during the first days of culture. Monolayer formation in the frozen-thawed culture was observed for 4–5 days. Spherical CCs were found in all cryopreserved samples on the 2nd day after plating. The number of CCs after cryopreservation was not significantly different among the cryoprotectant media as well as when compared with the non-frozen control (Fig. 8 c). The ability of CCs to differentiate into β-III-tubulin-positive cells with neuron-like morphology remained undamaged.

3.5. Cortisol content in the adrenal cell cultures

Glucocorticoid hormones are present at very high levels in the adrenal gland. Since the porcine adrenal cell culture included not only chromaffin cells, but also steroid-producing cells of adrenal cortex, cortisol as a main glucocorticoid hormone of pig was measured before and after cryopreservation.

In the non-frozen culture the hormone content in the medium

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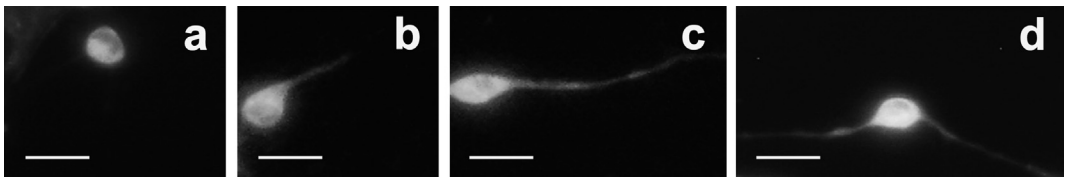

Fig. 5. Morphology of cells migrating from CCs: a – cell body only; b – cell body with growth cone; c – cell with unipolar process; d – cell with bipolar processes. Scale bar = 20 μm.

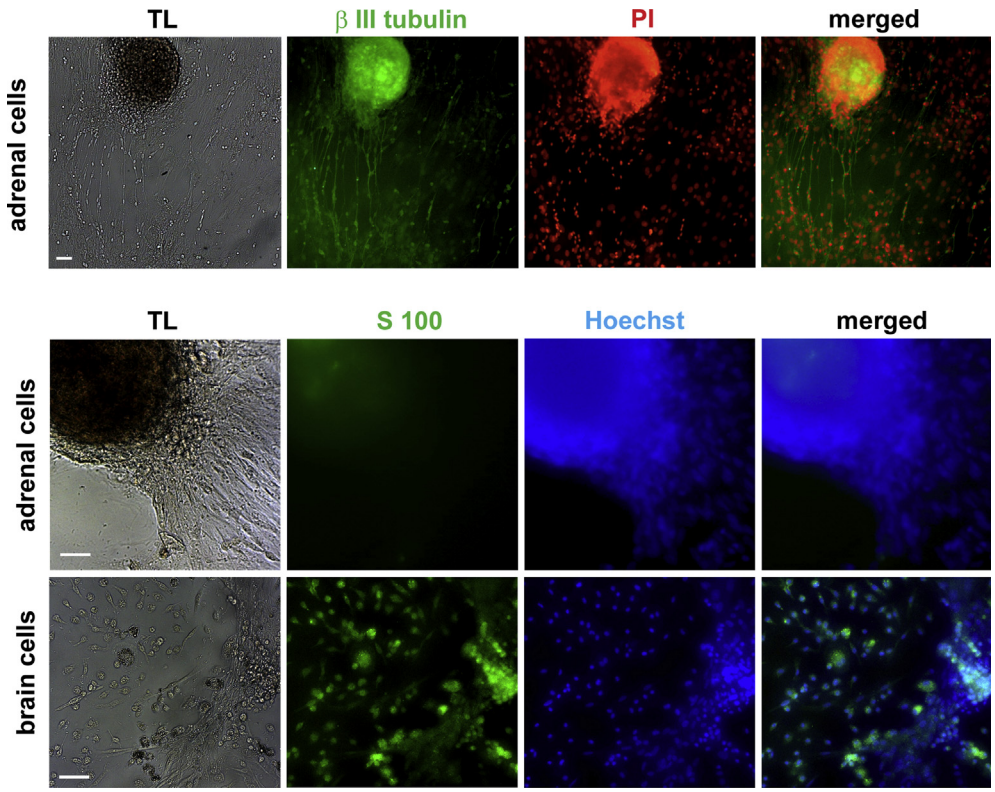

Fig. 6. Representative images of immunocytochemical staining of CCs (12th day after replating). The neuron-like cells migrated from CCs were positive for β-III-tubulin, but negative for S100. A strong cell labeling with S100 was observed in the culture of neonatal rat brain cells, which was used as a positive control. Nuclei were counterstained with PI or Hoechst 33342. TL – transmission light. Scale bar = 50 μm. The data were obtained from 9 independent experiments performed in duplicates.

Table 1
Indices of neuron-like cells in the CCs subculture (4–5 days after reseeding).

Samples	Cell number/1 mm^2[a]	Cell body diameter, μm	Processes number, %		
			<50 μm	51–100 μm	>100 μm
Control	165 ± 31	9,3 ± 1,7	63 ± 7,7	30 ± 8,0	7 ± 2,9
With NGF	158 ± 22	8,9 ± 2,4	59 ± 8,5	33 ± 6,4	8 ± 3,4

[a] Cells have been calculated around the CCs.

Table 2
Indices of the adrenal cell cultures obtained from ATFr.

Samples	Viable cell yield, 10^6/gland	Monolayer formation	CCs		
			Availability	CCs number/10^6 seeded cells**	Time of appearance
0.3 °C/min (n = 12)	1.54 ± 0.11*	on 4–5 day	+	1461 ± 199*	on 4–5 day
1 °C/min (n = 15)	1.70 ± 0.41*	–	–	–	–
5 °C/min (n = 10)	1.20 ± 0.47*	–	–	–	–
40 °C/min (n = 12)	0.60 ± 0.46*	–	–	–	–
>100 °C/min (n = 12)	0.60 ± 0.28*	–	–	–	–
Non-frozen (n = 22)	4.19 ± 1.71	on 3–4 day	+	404 ± 160	on 4–5 day

+ indicates presence of CCs; – indicates absence of cell growth or CCs; * indicates a significant difference compared with control at $P < 0.05$; ** CCs were calculated at 15 day postplating.

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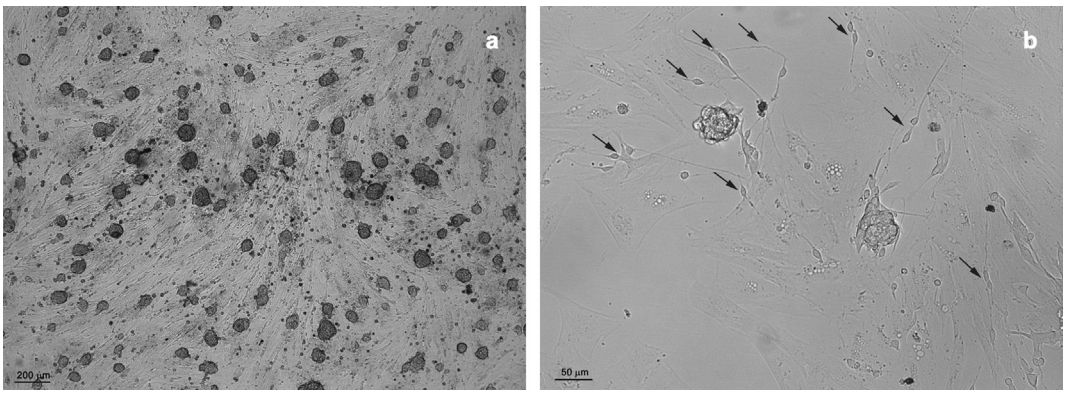

Fig. 7. Representative images of CCs formation in the cell culture obtained from ATFr cryopreserved in the presence of 10% ME2SO at a cooling rate of 0.3 °C/min: a – formation of CCs on the 5th day after plating; b – the appearance of neuron-like cells after subculture (arrows). The data were obtained from 6 independent experiments performed in duplicates.

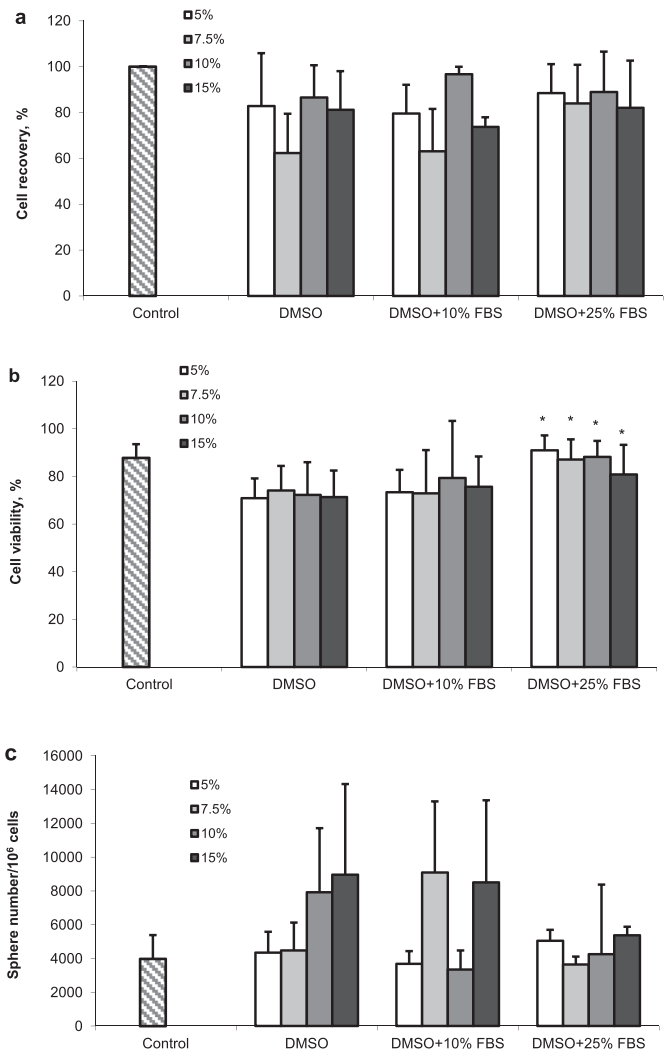

Fig. 8. Cell recovery (a), viability (b) and CCs number (c) after cryopreservation of primary adrenal cell culture using different ME2SO and FBS concentration in the cryoprotectant media. Monolayer with early CCs was detached on the 5th day of culture and cryopreserved. Number of CCs was calculated on the 2nd day of post-thaw plating. Control represents the CCs number on appropriate day of culture (7th day). Data are presented as mean ± SD, n = 15 batches of cell preparation. (*) – indicates a significant difference from the non-frozen control at $P < 0.05$.

did not change significantly during the first 8 days (Fig. 9). The cortisol concentrations ranged from 171.3 to 842.8 nmol/l (median 454.9 nmol/l). After cryopreservation using all regimes, the cortisol levels in the medium were reduced, and ranged from 14.1 to 176.1 nmol/l (median 30.1 nmol/l).

Decreased hormone levels ranged from 39.5 to 203.7 nmol/l (median 85.12 nmol/l) were observed in subculture without cryopreservation.

4. Discussion

Until now, very few studies have been performed to obtain a cell culture from porcine adrenal glands. The secretion of enkephalins and catecholamines by adult porcine chromaffin cells, which were cultured for 1 week, was demonstrated by Lu Y. et al. [40]. An automated method for cell isolation from neonatal porcine adrenal glands was described by Vizzardelli C. et al. [68]. Unfortunately, the authors did not perform a study on cell morphology and growth in long-term culture.

In our present research we have obtained primary cell culture from neonatal porcine adrenal glands and established its ability to form spherical CCs. After reseeding and attachment, the CCs gave rise to neuron-like cells.

Spherical cell colonies can be produced from many stem/

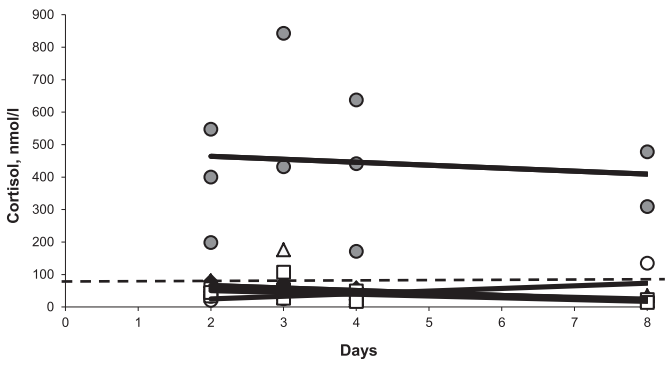

Fig. 9. Cortisol levels in the culture supernatants in the control (non-frozen) and cryopreserved primary adrenal cell cultures. The cortisol levels in the cryopreserved samples were significantly ($P < 0.05$) decreased compared with those found in the control. Data are presented as individual values for non-frozen control (open circle) and samples frozen in the presence of 5% ME2SO (closed circles), 7.5% ME2SO (closed triangles), 10% ME2SO (closed diamonds), 15% ME2SO (closed squares). Trend lines indicate best linear fit. Dashed line indicates the mean cortisol level during subculture without cryopreservation.

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progenitor cell cultures and cancer cell lines. Recently, isolation and characterization of SAP from adult human and bovine adrenal glands, which grew as spherical free-floating structures named "chromospheres", were described by Chung K. et al. and Santana M. et al. [10,55].

SAP originate from neural crest, and during the embryonic development migrate from it to differentiate into neurons of sympathetic ganglia and chromaffin cells of adrenal glands [3].

According to Zhou H. et al., a formation of two types of spheroid colonies, adherent and neurosphere-like free-floating, were observed in human fetal adrenal gland culture [71]. The adherent colonies were found in adrenal gland cultures of fetal humans and neonatal rats by several authors [6,25,49].

In our study, colony formation in the form of sphere may suggest the existence of colony-forming SAP in the neonatal porcine adrenal glands. These SAP grew in culture as adherent CCs and were able to differentiate in the neuronal direction and express a marker of early neurons β-III-tubulin.

Another specific feature of CCs obtained in the primary culture of porcine adrenal glands is the lack of an NGF effect on appearance of neuron-like cells expressing β-III-tubulin. Contrary to our data, in the research of Chung K. et al. treatment of murine chromospheres with NGF induced development of neurite-like extensions with strong immunofluorescent staining for βIII-tubulin, while the presence of glucocorticoid dexamethazone stimulated expression of phenylethanolamine-N-methyltransferase as a specific marker of chromaffin cells and suppressed the origin of cells with neuron-like morphology [10].

For many years in the study of the biology of sympathoadrenal neural crest derivatives, the key role of glucocorticoids and NGF in maintaining/transdifferentiating chromaffin and neuron cells phenotype has been widely accepted [3,4,13,14,66]. However, there are certain contradictions concerning the role of these two factors in SAP development in the current scientific literature. Indeed, dexamethazone administration considerably decreased NGF-induced neurite outgrowth in the culture of rat chromaffin cells [13]. However, there was no significant change in the number and phenotypical characteristics of chromaffin cells in glucocorticoid receptor knockout mice [19]. Tischler A. et al. did not observe any stable dependence of activation or suppression of neurite formation on NGF or dexamethazone presence in the cell culture of adult murine adrenal glands [64]. SAP isolated from human fetal adrenal glands were insensitive to NGF administration for neurite outgrowth, mitotic rate, and survival [7].

Beyond the glucocorticoids/NGF paradigm, recently there has been a search for other potential factors determining development of sympathoadrenal lineage. In particular, the expression of bone morphogenetic protein 4 (BMP-4) during embryogenesis was found to differ greatly in the place of adrenal anlagen and sympathetic ganglia location [29]. Neural crest cells cultured with BMP-4 formed ganglion-like clusters, but neurite outgrowth took place only after BMP-4 removal from the culture medium [53]. Taking into consideration that adrenal cells express BMP-4 during the entire period of embryonic development and after birth [33], there is a supposition that this factor can inhibit neuronal differentiation of SAP in the adrenals [29]. Besides, bFGF was proposed as another important factor inducing neuronal differentiation and neurite outgrowth [23,60].

Indirectly, we observed the dependence in the differentiation into neuron-like cells on glucocorticoids present in the cell culture of neonatal porcine adrenal glands. Formation and growth of CCs did not depend on the levels of cortisol in the culture supernatants, while active migration of β-III-tubulin-positive cells with neuron-like morphology was observed after subculturing or cryopreservation, when the levels of cortisol decreased significantly. However, this effect can probably be induced by the presence of other factors excreted by cells in culture. Therefore this issue requires further study.

Sustentacular cells possessing antigen specificities for glial marker S100 are located in the medulla of fetal, neonatal and adult adrenal glands [11,41]. They are believed to play a significant role in the migration and differentiation of cells of the sympathetic nervous system. The possibility of differentiation of neural crest derivatives into glial elements was shown in previous studies [16,69]. Due to the abovementioned prerequisites, we considered it necessary to analyze the expression of S100 in the CCs subculture. However, no such labeling was found. That indicated a lack of cell differentiation into glial elements in the culture of neonatal porcine adrenal glands derived SAP.

Over the last few decades, much research has been done on stem cell cryopreservation resulting in an accumulation of certain knowledge concerning their cryoresistance. Many researchers note high susceptibility of human embryonic stem cells to freezing [30,54]. As for other stem cells, there is no common point of view, perhaps, due to the diversity of their sources and stemness. Some authors believed that adult stem cells are rather resistant to cryopreservation [34,44,45]. In the research conducted mainly with hematopoietic stem cells, cryoresistance of more primitive stem cells was found to be higher than committed progenitors [42,51].

Cryopreservation of biomaterial in the form of fragments may be useful for tissue preservation when resecting organs or taking biopsies. Moreover, advances in tissue engineering make possible the generation of multicellular structures, whose long-term storage involves cryopreservation.

Tissue fragment cryopreservation outcome is less predictable than single cell suspension one, due to involvement of additional physical and chemical factors reviewed in detail by Karlsson J. et al. [35]. In brief, the parameters of liquid and heat transfer in tissue have a great influence on cell viability. A tissue fragment usually consists of several types of cells, which have different coefficients of penetration for water and cryoprotectants, and, consequently, the optimal cooling rate at the stage of crystallization of the main bulk of water will be different. Moreover, cells in the tissue are joined in the functional system with gap junctions, which enable intracellular ice formation at the stage of freezing [1,31].

In our research maximum viable cell yield and functional integrity were observed in the cell culture derived from the ATFr cryopreserved at the cooling rate of 0.3 °C/min but not higher (1; 5; 40; >100 °C/min). During tissue freezing, the dehydration of interior cells occurs more slowly than in surface layer cells [12,35]. Hence, the interior cells need a slower cooling rate for achieving a dehydration level sufficient to prevent intracellular ice formation. Furthermore, due to the temperature gradient existing between the surface and inner layers of the fragment during freezing-thawing, the cooling rate through the fragment will be different. Based on these considerations, a low cooling rate facilitates preservation of all cell types in the tissue fragment. This is confirmed by our study, which showed that the integrity of several types of cells (fibroblast-like cells forming a monolayer; SAP forming the CCs; adrenal medulla and cortical cells) was observed only when the minimal cooling rate was applied. Moreover, this cryopreservation mode enabled an increase in the number of CCs as compared to the non-frozen control (Table 2), which may testify to a certain cryoselectivity of the chosen regimen.

Cryopreservation of the primary cell culture of porcine adrenal glands using 1 °C/min and the cryoprotectant media with Me2SO concentration ranging from 5 to 15% does not significantly affect the total cell recovery and number of CCs. As the culture initially containing CCs was used for cryopreservation their presence after thawing testifies to their resistance to used regimen. Stem cells are

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known to be sensitive to increased Me2SO concentration [45]. In our study, Me2SO in the concentration of 15% did not decrease number of CCs in the post-thaw cell culture of porcine adrenal glands.

Cryopreservation is known to have an effect on differentiation potential of stem cells [45,70]. Our results demonstrate that CCs ability to differentiate into neuronal cells remained undamaged both after cryopreservation of ATFr and primary adrenal cell culture.

In summary, the results of the study indicate that the main properties of the spherical CCs derived from primary cell culture of neonatal porcine adrenal glands are: i) growth in the form of adherent colonies; ii) differentiation into neuronal but not glial cells; iii) independence of CCs differentiation from exogenous NGF; iv) possible dependence of neuronal differentiation on the concentration of glucocorticoids; v) sufficient cryoresistance. For CCs generation and neuronal differentiation the biomaterial can be cryopreserved in the form of tissue fragments or primary cell culture.

In the current study, no attempts were made to separate the chromaffin cells from the adrenocortical cells, as well as to characterize the colony-forming progenitor cells. Preservation of the adrenal cortex as component of the microenvironment may be important for this type of progenitors. Further research should elucidate the questions of phenotype, commitment, and differentiation potential of neonatal porcine adrenal gland progenitor cells.

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Conflict of interest declaration

We have no conflict of interest to declare.

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