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EFFECT OF SERUM STARVATION AND CONTACT INHIBITION ON DERMAL FIBROBLAST CELL CYCLE SYNCHRONIZATION IN TWO SPECIES OF WILD FELIDS AND DOMESTIC CAT*

Wiesława Młodawska[1♦], Patrycja Mrowiec[1], Michał Bochenek[2], Katarzyna Wnęk[1], Joanna Kochan[1], Agnieszka Nowak[1], Wojciech Niżański[3], Sylwia Prochowska[3], Marcin Pałys[4]

[1]Department of Animal Reproduction, Anatomy and Genomics, University of Agriculture in Krakow, Al. Mickiewicza 24/28, 30-059 Kraków, Poland
[2]Małopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7 A, 30-387 Kraków, Poland
[3]Department of Reproduction and Clinic of Farm Animals, Wrocław University of Environmental and Life Sciences, Pl. Grunwaldzki 49, 50-366 Wrocław, Poland
[4]Foundation Municipal Park and the Zoological Garden in Cracow, 30-232 Kraków, Poland
♦Corresponding author: rzmlodaw@kinga.cyf-kr.edu.pl

Abstract

Cell cycle synchronization of donor cells is an important step in mammalian somatic cell nuclear transfer (SCNT). This study was designed to compare the efficiency of serum starvation (Ss) and contact inhibition (cI) on cell cycle synchronization of jaguarundi, manul, and domestic cat skin fibroblasts, in the production of G0/G1 cells suitable for SCNT in felids. Ss was performed after the growing (G) cells reached 40–50% (G50+Ss), 60–70% (G70+Ss) and full confluency (Fc), i.e. in association with cI (cI+Ss). Frozen-thawed cells were cultured to the given state of confluency (d0; controls), and subjected to Ss or cI for 1, 3, and 5 days (d). In manul, the effect of Ss on arresting fibroblasts in the G0/G1 phase was noted after just 1d of culture at G70 confluence, while G50+Ss and cI+Ss were effective after 5d of treatment. In jaguarundi, 1–5d of G50+Ss and 5d of G70+Ss increased the percentage of G0/G1 cells versus d0 ($P<0.01$), with 5d of G70+Ss producing more ($P<0.05$) quiescent cells than after the same period of G50+Ss, cI+Ss and cI. In the domestic cat, Ss was efficient only after 3 and 5d of G50+Ss. In all species, cI alone failed to increase the proportion of G0/G1 cells compared to d0, however in the domestic cat, 5d of cI was more efficient than the same period of G50+Ss. In jaguarundi, >93% of cells were already in G0/G1 phase at d0 of Fc, suggesting that culture to Fc could be also a valuable method for fibroblast cell cycle synchronization in this species. In contrast to cI, prolonged Ss generated cell loss and could induce apoptosis and/or necrosis. In conclusion, Ss was the more efficient method for skin fibroblast cell cycle synchronization at the G0/G1 phase in manul, jaguarundi and the domestic cat. The response of cells to the treatments was species-specific, depending on cell confluence and duration of culture. This research may find application in preparing donor karyoplasts for SCNT in felids.

Key words: fibroblasts, cell cycle, contact inhibition, serum starvation, feline

The first attempts to generate feline somatic cell nuclear transfer (SCNT) embryos were conducted at the beginning of the 21st century (Fahrudin et al., 2001; Shin et al., 2002; Skrzyszowska et al., 2002), with the birth of the first SCNT-derived kitten reported in 2002 (Shin et al., 2002). Despite a further 20 years of research, the efficiency of somatic cloning in felids, as in other mammals, is still low, with generally fewer than 5% of transferred SCNT embryos developing into healthy live offspring (Wilmut et al., 1997; Shin et al., 2002; Gómez et al., 2004; Yin et al., 2007; Loi et al., 2016). The success of the SCNT procedure is affected by a variety of factors, the most important of which are: i) appropriate synchronization of cell-cycle stage between the donor cell nucleus and the enucleated recipient oocyte (cytoplast/ooplast); ii) the ability of the ooplast to reset the epigenetic memory of the cell nucleus inherited from the differentiated donor cell, in order to restore its totipotent status and iii) the epigenomic reprogrammability of the donor cell nucleus in the SCNT-derived oocyte and embryo (Campbell et al., 1996; Wilmut et al., 1997; Samiec and Skrzyszowska, 2005; Loi et al., 2016). It appears that when the recipient cytoplast originates from a metaphase II stage oocyte, the transferred donor nucleus should be in the G0 or G1 phase of the cell cycle, as the chromatin of the somatic nucleus is likely to be more amenable

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to proper reprogramming by the recipient cytoplast and maintenance of the correct ploidy of the resultant embryo (Campbell et al., 1996; Wilmut et al., 1997; Rideout et al., 2001). Incomplete/abnormal epigenetic reprogramming leads to developmental anomalies in the resultant embryos/fetuses and/or placenta, stillbirth or the birth of animals with different types of malformations and high perinatal mortality (Blelloch et al., 2006; Cho et al., 2007; Bang et al., 2011; Imsoonthornruksa et al., 2012; Kallingappa et al., 2016; Samiec and Skrzyszowska, 2018 a, b; Veraguas et al., 2020).

Somatic cloning allows the generation of genetically identical copies of an individual, thus representing a universal tool for asexual reproduction. It is believed that SCNT creates the possibility of preserving species threatened with extinction, and so might be useful for the conservation of genetic biodiversity as well as for various types of basic, biomedical, agricultural, and phylogenetic research (Gómez et al., 2003, 2006; Holt et al., 2004; Loi et al., 2016; Samiec and Skrzyszowska, 2021; Skrzyszowska and Samiec, 2021). According to the currently revised taxonomy of the Felidae, this mammalian family is represented by 14 genera, 41 species and 77 subspecies (Kitchener et al., 2017), with most of these species listed in the IUCN Red List of Threatened Species as Endangered, Vulnerable or Near Threatened, depending on their region of habitat. Some of them, such as the European wildcat (*Felis silvestris*) or African wildcat (*Felis silvestris lybica*), are further threatened by the loss of genetic purity due to crossbreeding with domestic cats (Pierpaoli et al., 2003; Kochan et al., 2019). Others, such as the Caspian Tiger (*Panthera tigris virgata*), *Panthera tigris balica*, or *Panthera tigris sondaica* became extinct in the 20th century (Jackson and Nowell, 2008 a, b, 2011). Increasing SCNT efficiency as well as other assisted reproductive techniques (ART) such as oocyte *in vitro* maturation and *in vitro* fertilization, artificial insemination, biobanking of gametes and somatic cells, are therefore of particular importance for maintaining these animal species (Prochowska et al., 2017; Młodawska et al., 2019).

In felids, as in other mammals, serum starvation and contact inhibition are commonly used methods for the synchronization of donor cell nuclei in the G0/G1 phase (Gómez et al., 2003; de Barros et al., 2010; Wittayarat et al., 2013; Veraguas et al., 2017), and their use in SCNT protocols has resulted in live born kittens (Shin et al., 2002; Gómez et al., 2004; Yin et al., 2005, 2007). However, it remains unclear which of these methods of cell cycle synchronization produces a higher proportion of G0/G1cells (Gómez et al., 2003; Hayes et al., 2005; Khammanit et al., 2008; de Barros et al., 2010; Ma et al., 2015; Veraguas et al., 2017), or whether the reconstructed embryos have an equal capacity to cleave and create a functional genome and/or to develop to term (Gómez et al., 2003, 2006; Hayes et al., 2005; Samiec et al., 2013 a, b). The available literature on fibroblast culture and cell cycle synchronization does not contain data concerning Pallas's cat (Otocolobus manul; Felis manul) or jaguarundi (Puma yagouaroundi; Harpailurus yagouaroundi). Pallas's cat, commonly referred to as manul, is distributed widely, but unevenly and fragmentarily, across Central and Western Asia. The core populations are in Mongolia and China, but it can be found along the border of Russia-China, Russia-Mongolia, and in Transbaikal regions of Russia, as well as in Kazakhstan, Western Iran, Afghanistan and the eastern Himalayan region; it is possibly extinct in Armenia and Azerbaijan. On the IUCN Red List of Threatened Species, manul is currently qualified as Least Concern (Ross et al., 2020). Jaguarundi is also a widespread species with low population density, inhabiting South America, from Argentina through Brazil up to Venezuela and Columbia, across Central America, and up to Mexico in North America. Jaguarundi is also listed as Least Concern, however, it is considered Vulnerable in Brazil, Near Threatened in Argentina, Threatened in Mexico, and is probably extinct in the United States (south Texas) (Caso et al., 2015). Therefore, the aim of this study was to compare the effects of serum starvation and contact inhibition on cell cycle synchronization and survival of dermal fibroblasts from manul and jaguarundi – the two representatives of wild Felidae species – and domestic cat, as research models in terms of increasing the efficiency of somatic cloning of endangered felids.

Material and methods

All chemical reagents were purchased from Sigma-Aldrich Poznań, Poland, unless otherwise indicated.

Skin biopsy

Skin biopsies were obtained from jaguarundi (n=1), Pallas's cat (manul; n=2) and domestic cats (n=4), with the consent of the owners and according to the guidelines issued by the Ethics Committees (Kraków, Wrocław). Wild cats were sourced from the Zoological Garden in Kraków, and domestic cats from private owners. The skin biopsies (0.25–0.4 cm^2) were collected from the inguinal area following anesthesia of the animal, and were then submerged individually in phosphate-buffered saline (PBS; Polfa, Lublin, Poland) supplemented with 1% antibiotics (AAS: Antibiotic-Antimycotic-Solution) and transported on ice to the laboratory.

Fibroblast cultures and freezing

After rinsing in 70% ethanol and in PBS containing AAS (3 times), the skin samples were cut into small (~1 mm^2) pieces, seeded on the bottom surface of 25-cm^2 tissue culture flasks and cultured in DMEM (Dulbecco's Modified Eagle's Medium) or in DMEM/F12 (Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham's Medium) supplemented with 10% Fetal Bovine Serum (FBS), at 37°C in a humidified atmosphere containing 5% CO_2, as previously described (Młodawska et al., 2019). Briefly, after reaching 70–80% confluence

around the explants, the cells were routinely trypsinized in 0.25% trypsin-EDTA solution, centrifuged (467 × g; 7.5 min), suspended in the appropriate medium, counted using a hemocytometer, stained with 0.4% trypan blue (for viability estimation using the trypan blue exclusion test) and passaged in equal numbers to the new flasks. The cells were then cultured, passaged 3–4 times and/or frozen and stored in liquid nitrogen (Młodawska et al., 2019).

Cell cycle synchronization

After thawing, the cells were suspended in 10 ml DMEM supplemented with 10% FBS and centrifuged to remove the freezing medium. The supernatant was then discarded, and the cells were resuspended in culture medium, counted, seeded in 6-well plates (100 000 viable cells/well; trypan blue negative) and cultured as described above. The cells were exposed to cell cycle synchronization using two methods: serum starvation (Ss) and contact inhibition (cI). Ss was performed when the growing (G) cells reached: i) 40–50% (G50; G50+Ss), ii) 60–70% (G70; G70+Ss) and iii) full confluency, i.e. in association with cI (cI+Ss). When the cells reached the appropriate confluency (day 0 for each treatment), the culture medium was replaced with serum-deprived medium (DMEM supplemented with 0.5% FBS) and the cells were cultured for a further 1, 3, or 5 days. For cI, after reaching full confluency (day 0), the cells were cultured in regular culture medium for an additional 1, 3 or 5 days. The media were changed every 1–2 days, if required. For each treatment, cell cycle analysis was estimated by flow cytometry at day 0 (control for each treatment), and after 1-, 3-, and 5-days' culture. At least two trials were performed for the cells from each animal.

Cells fixation

On the designated days, the medium and any floating, detached cells were removed prior to trypsinization, and the cells from each animal were then harvested separately from each culture well, counted, and their viability estimated (using the trypan blue test). After centrifugation (467 x g for 7.5 min), the supernatant was removed, and the cells were resuspended in 200 µl culture medium and then fixed by gradual (dropwise) addition of 800 µl cold methanol, according to method proposed by Khammanit et al. (2008). The fixed cells were stored at –20°C until the day of analysis.

Flow cytometry cell cycle analysis

Before analysis, the fixed cells were centrifuged (500 × g for 5 min) to remove the methanol and resuspended in 1 ml PBS; 30 µl RNAse stock solution (10 mg/ml) was then added, and the cells were incubated for 30 min at room temperature. Subsequently, 30 µg propidium iodide (PI) was added and incubation was continued for a further 30 minutes. Cell cycle analysis was performed using a CytoFLEX flow cytometer (Beckman Coulter) and at least 12000 cells were analyzed per sample. Kaluza 2.1.1 (Beckman Coulter) software was used to calculate the G0/G1, S and G2/M cell cycle phase distribution. Cell doublets were gated out on a *PI Peak* vs. *PI Width* dotplot.

Statistical analysis

Data were analyzed with SigmaStat 3.5 Software, using ANOVA followed by Tukey's test. For data not normally distributed, the Kruskal-Wallis ANOVA was used. The results are presented as means ± SEM. Differences between means were considered statistically significant at $P<0.05$.

Results

Cell cycle synchronization of feline fibroblasts by serum starvation and contact inhibition

The results of the flow cytometry cell cycle analysis of feline fibroblasts are presented in Table 1. For all three species, the percentage of G0/G1 cells was dependent on the degree of cell culture confluency and treatment. In jaguarundi and domestic cat, the lowest proportion of G0/G1 cells occurred on the day that the growing cells reached 40–50% confluence (i.e. day 0 – G50 vs. day 0 – G70+Ss, cI+Ss and cI; $P<0.01$; Table 1), when there was also a higher percentage of cells in the S and G2/M phases than during the remaining days of culture.

In jaguarundi, 1 day of G50+Ss was sufficient to increase significantly the proportion of cells arrested in the G0/G1 phase (to over 89%), while prolonged Ss did not further increase the percentage of quiescent cells compared to day 0. For G70+Ss, 5 days of culture were required to achieve a significantly higher percentage of G0/G1 cells than was observed at day 0, yet this was the most efficient treatment, generating a significantly higher proportion of quiescent cells (~ 96%) than G50+Ss, cI+Ss, or cI alone after the same period of culture (Table 1; Figure 1). On the day of reaching full confluency (cI+Ss and cI – day 0), over 93% of jaguarundi fibroblasts were in the G0/G1 phase, and neither 1–5 days of cI+Ss nor cI alone increased the percentage of quiescent cells. In manul, 1–5 days of G70+Ss resulted in a higher percentage of G0/G1 cells compared to the control ($P<0.01$), while for G50+Ss and cI+Ss, 5 days were needed to increase the proportion of G0/G1 cells significantly; cI alone had no effect on fibroblast cell cycle synchronization (Table 1; Figure 2). For domestic cat fibroblasts, only 3 and 5 days of G50+Ss resulted in a higher proportion of cells arrested at the G0/G1 stage compared to day 0 ($P<0.01$), however, this treatment was less efficient than 1 day of cI+Ss, and 5 days of cI alone (Table 1; Figure 3).

Morphology and viability of feline fibroblasts under different culture conditions

After thawing, the cell lines of each species varied in terms of the time needed to reach 40–50%, 60–70% and full confluence, requiring 3–4, 4–5 and 6 days of culture, respectively, for jaguarundi, 3, 3–4 and 6–7 days for manul, and 3, 3–6 and 7–8 days for domestic cat.

Table 1. Effect of serum starvation and contact inhibition on cell cycle synchronization of jaguarundi, manul and domestic cat dermal fibroblasts

Treatment	Cell cycle phase	Jaguarundi				Manul				Domestic cat			
		Days of culture/mean (±SEM) percentage of cells				Days of culture/mean (±SEM) percentage of cells				Days of culture/mean (±SEM) percentage of cells			
		0 (control)	1	3	5	0 (control)	1	3	5	0 (control)	1	3	5
G50	G0/G1	66.3±0.3 aX	89.4±0.8 cY	92.8±1.2 cXY	91.5±0.2 cX	74.5±6.5 a	94.1±1.1 ab	94.2±1.5 ab	97.0±1.1 b	74.7±1.9 aX	83.6±1.3 acX	89.2±0.8 c	87.6±1.0 cX
+Ss	S	16.9±0.5 aX	3.7±0.1 cX	2.3±0.7 cX	2.4±0.3 cX	12.0±3.4 a	3.3±0.9 b	3.5±1.2 b	3.0±1.1 b	14.3±1.0 aX	5.5±0.6 c	4.7±1.2 c	3.2±0.5 c
	G2/M	16.8±0.8 aX	6.9±0.7 cX	4.9±0.5 cX	6.1±0.5 cX	13.5±3.2 aX	2.6±1.0 ac	2.2±0.8 ac	0.0±0.0 c	11.0±1.8 a	10.9±0.8 abX	6.0±1.0 b	9.2±1.0 abX
G70	G0/G1	92.5±0.2 aZ	92.5 ±0.6 aXY	93.2±0.2 abX	95.9±0.2 cY	78.0±3.4 a	92.9±1.5 c	94.3±0.9 c	96.6±1.1 c	88.8 ±1.6 Z	89.4±1.6	88.8±1.0	91.7±0.9
+Ss	S	4.9±0.5 aZ	4.5±0.7 abX	3.6±0.3 abXY	2.2±0.2 bX	14.4±2.8 a	6.0±1.5 bd	5.3±1.0 bd	3.1±1.0 cd	5.5±0.8 Z	7.2±1.0	8.6±1.1	6.1±0.9
	G2/M	2.6±0.3 Z	3.0±0.01 Z	3.2±0.6	1.9±0.01 Z	7.6±3.2 aXY	1.1±0.3 ab	0.4±0.1 b	0.3±0.2 b	5.6±1.3	3.4±0.9 Z	2.6±0.6	2.2±0.5 Z
cI+Ss	G0/G1	93.4±0.7 aZ	94.1±0.7 aX	89.1±0.5 bY	91.6±0.3 aX	86.0±3.8 a	94.6±1.2	92.8±0.8	95.0±1.4 b	86.1±2.2 Z	91.6±1.0 Y	89.9±1.4	91.4±1.2
	S	4.3±0.5 aZ	4.6±0.6 aX	7.6±0.03 bZ	5.2±0.0 aY	8.1±2.6	4.4±0.6	5.7±1.2	3.5±0.6	6.9±0.8 Z	5.9±1.0	7.5±1.1	5.8±0.9
	G2/M	2.3±0.1 Z	1.3±0.1 Z	3.3±0.5	3.2±0.4 YZ	5.8±1.3 aY	1.0±0.7 b	1.5 ±0.6	1.5± 0.8	7.0±1.7 a	2.4±0.5 bZ	2.6±0.6 ab	2.8±0.6 Z
cI	G0/G1	93.4±0.7 aZ	81.3±0.4 cZ	93.5±0.2 aX	92.7±0.3 aX	86.0±3.8	92.8±1.5	92.9±0.8	93.3±1.4	86.1±2.2 Z	87.8±2.1	90.7±1.3	92.1±1.4 Y
	S	4.3±0.5 aZ	11.7±0.5 cZ	5.0±0.3 aY	5.5±0.3 aY	8.2±2.6	5.0±0.9	4.4±1.0	5.2±1.4	6.9±0.8 Z	6.4±1.7	5.3±0.7	5.3±1.2
	G2/M	2.3±0.1 aZ	7.0±0.1 cX	1.5±0.2 aY	1.8±0.6 aZ	5.8±1.3 Y	2.1±1.0	2.7±0.7	1.5±0.7	7.0±1.7	5.8±1.3 Z	4.0±1.0	2.6±0.4 Z

Growing cells at 40–50% (G50) and 60–70% (G70) confluency: Ss – serum starvation; cI+Ss – contact inhibition in association with serum starvation; cI – contact inhibition alone. The differences between the means marked with different letters differ significantly: in rows: a, b; b, c = $P<0.05$; a, c = $P<0.01$ (for comparison between the days of culture, separately for each species); in columns: X, Y; Y, Z, = $P<0.05$; X, Z = $P<0.01$ (for comparison within the same phase of the cell cycle, between the treatments).

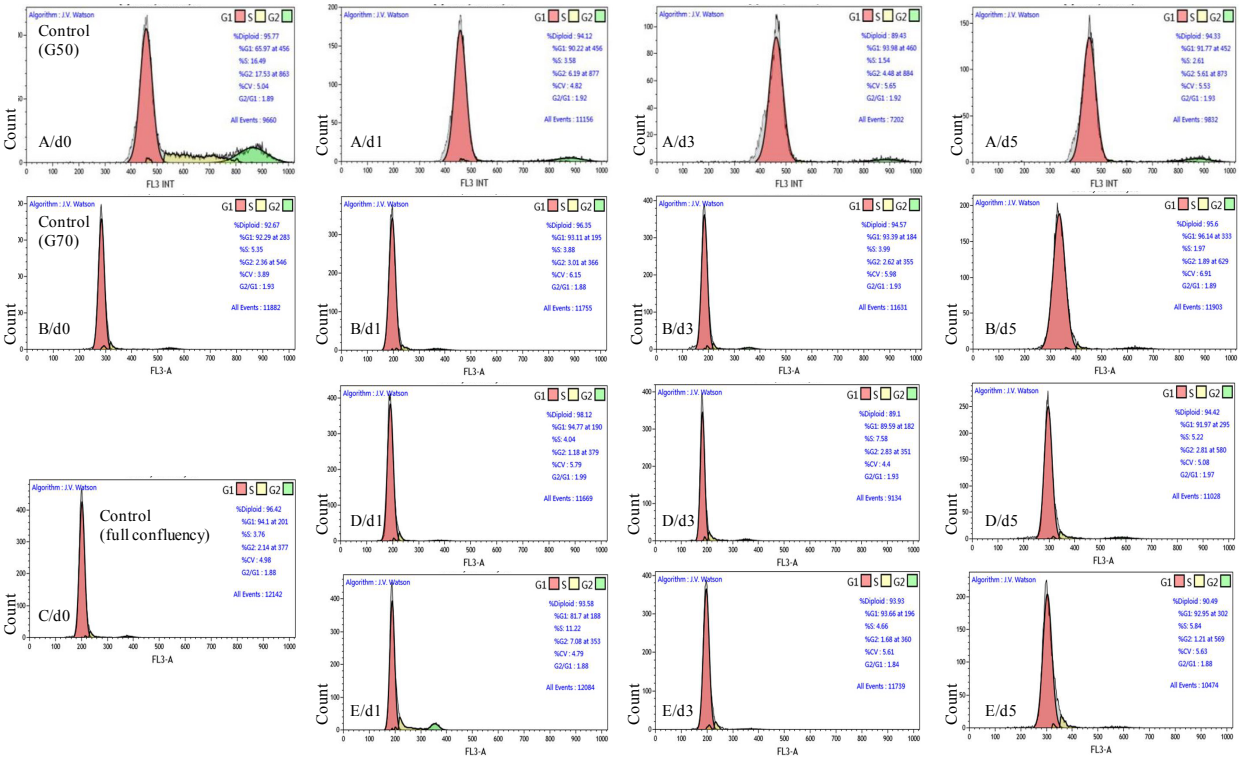

Figure 1. Representative histograms of the flow cytometry analysis of jaguarundi dermal fibroblast cell cycle obtained at day 0 (d/0; control) and after 1 (d1), 3 (d3) and 5 (d5) days of culture in different condition: serum starvation at 40–50% (G50; A/d0–d5), 60–70% (G70; B/d0–d5) and 100% (C/d0, D/d1–d5) confluency and in contact inhibition alone (C/d0, E/d1–d5); Phases of the cell cycle: G0/G1 (G1), S (S) and G2/M (G2)

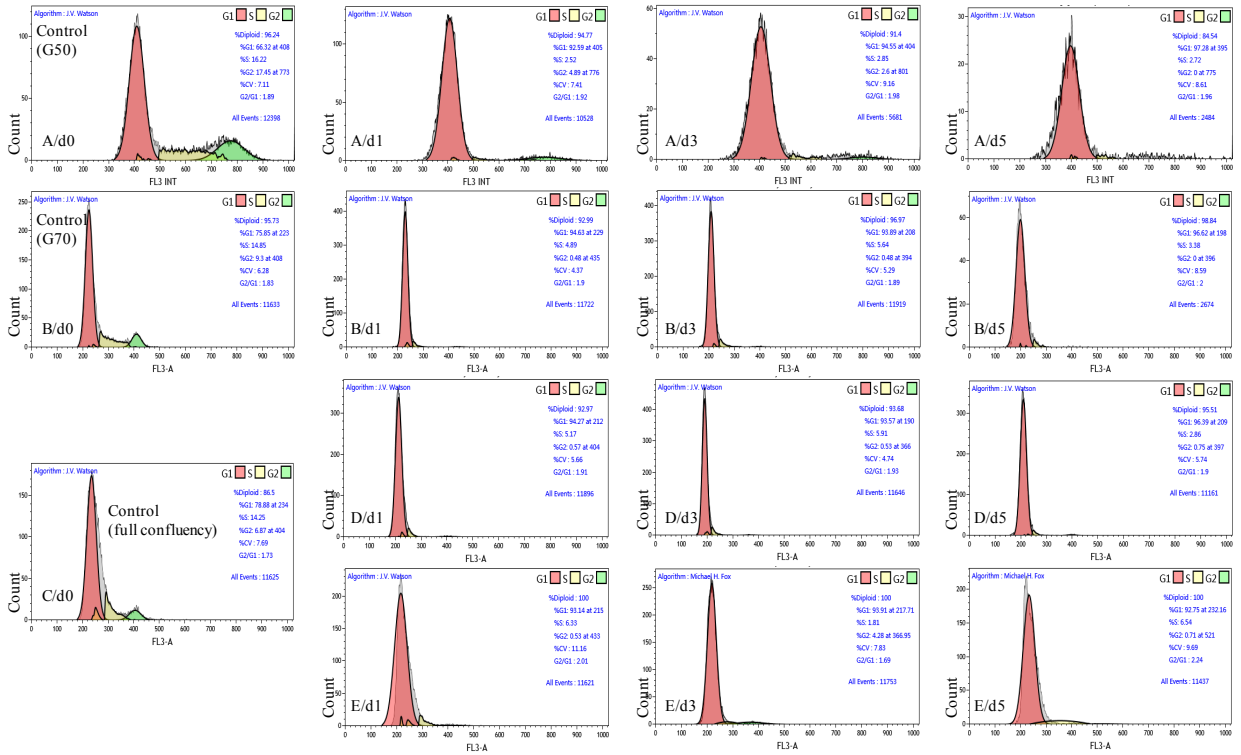

Figure 2. Representative histograms of the flow cytometry analysis of manul dermal fibroblast cell cycle obtained at day 0 (d/0; control) and after 1 (d1), 3 (d3) and 5 (d5) days of culture in different condition: serum starvation at 40–50% (G50; A/d0–d5), 60–70% (G70; B/d0–d5) and 100% (C/d0, D/d1–d5) confluency and in contact inhibition alone (C/d0, E/d1–d5); Phases of the cell cycle: G0/G1 (G1), S (S) and G2/M (G2)

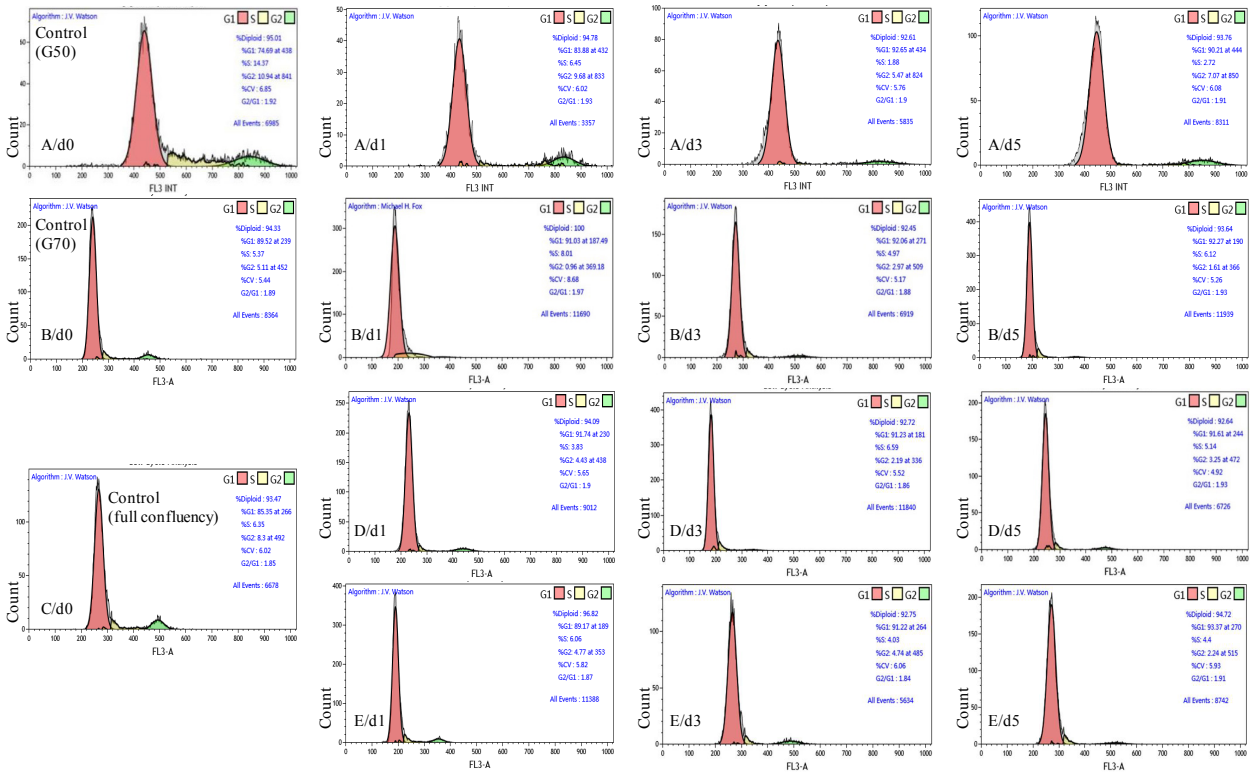

Figure 3. Representative histograms of the flow cytometry analysis of domestic cat dermal fibroblast cycle obtained at day 0 (d/0; control) and after 1 (d1), 3 (d3) and 5 (d5) days of culture in different condition: serum starvation at 40–50% (G50; A/d0–d5), 60–70% (G70; B/d0–d5) and 100% (C/d0, D/d1–d5) confluency and in contact inhibition alone (C/d0, E/d1–d5); Phases of the cell cycle: G0/G1 (G1), S (S) and G2/M (G2)

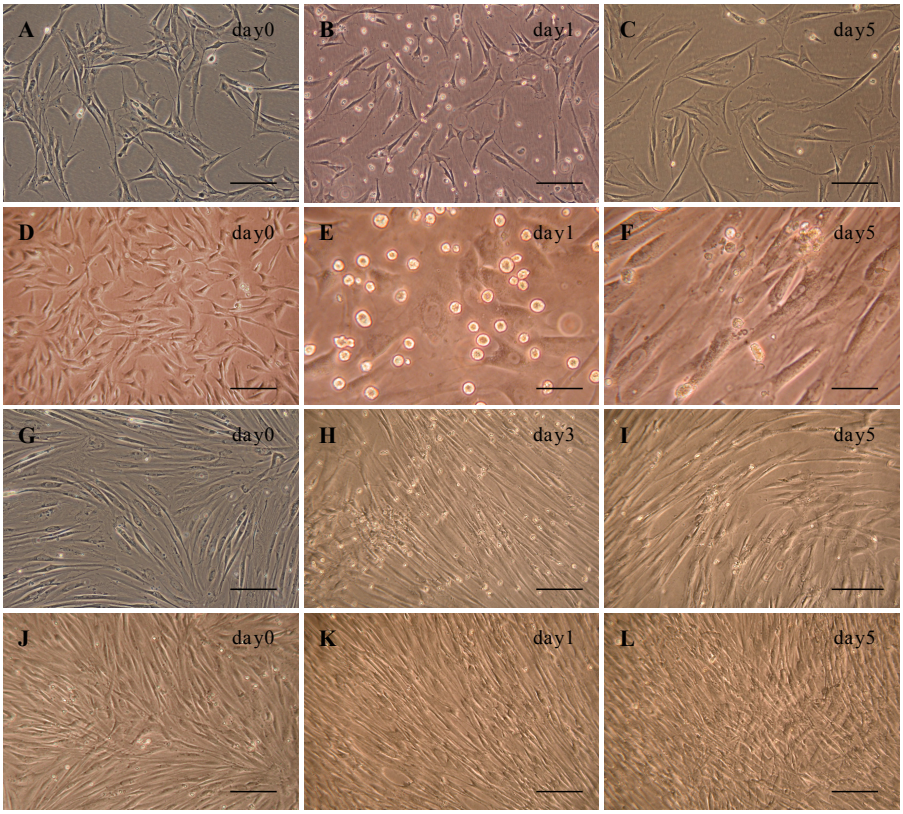

Figure 4. Representative microphotographs of feline dermal fibroblasts after 0 (control for each treatment) to 5 days of culture in different condition: serum starvation at 40–50% (A–C), 60–70% (D–F) and 100% (G–I) confluency, and contact inhibition alone (J–L); scale bars = 100 µm (A–C; D, G–L) and 50 µm (E,F)

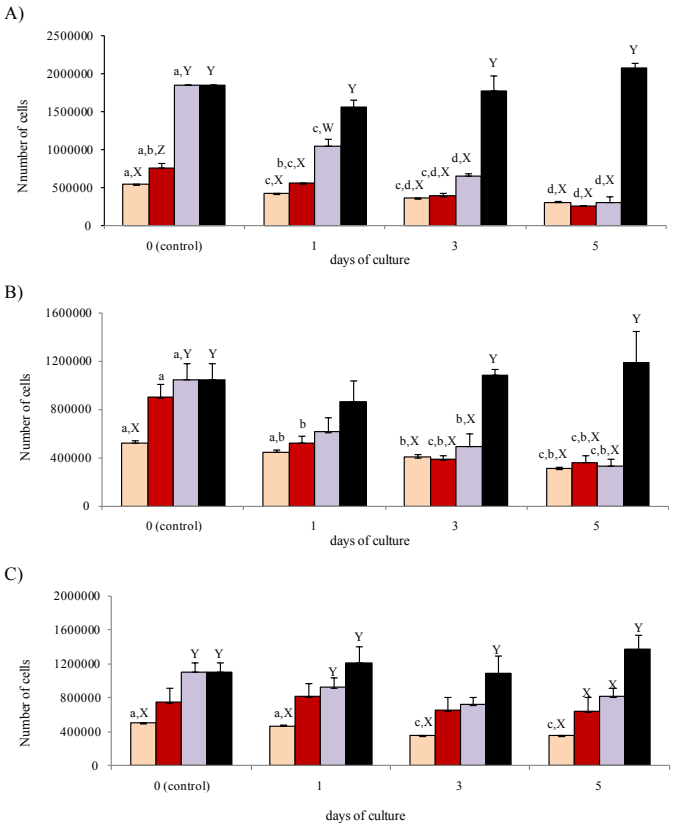

Figure 5. Mean (±SEM) number/ml of jaguarundi (A), manul (B) and domestic cat (C) dermal fibroblast at day 0 (control for each treatment) and after 1–5 days of culture in different condition: serum starvation at 40–50% (□), 60–70% (■) and 100% (▢) confluency, and contact inhibition alone (■); The differences between means marked with different letters differ significantly: a,b; c,d = P<0.05; a,c; a,d; b,c = P<0.01 (for comparison within a treatment, between the days of culture); X,Z; X,W; W,Y = P<0.05; X,Y; Z,Y = P<0.01 (for comparison within a given day of culture, between the treatments)

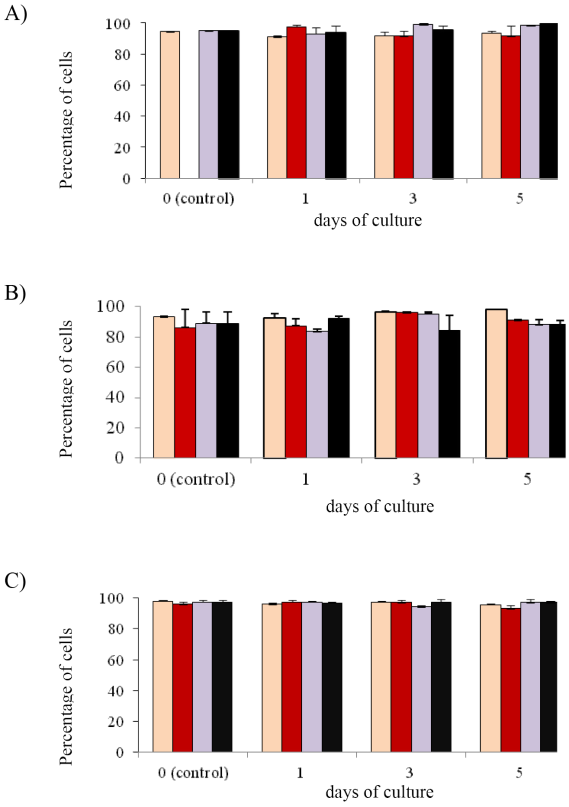

Figure 6. Mean (±SEM) percentage/ml of viable (trypan blue negative) jaguarundi (A), manul (B) and domestic cat (C) dermal fibroblast at day 0 (control) and after 1–5 days of culture in different condition: serum starvation at 40–50% (□), 60–70% (■) and 100% (▢) confluency, and contact inhibition alone (■)

Regardless of the species or cell culture confluency, a change in cell morphology and detachment of the cells from the bottom of the culture wells was observed after the first day of Ss. The cells were shrunken and were slightly less elongated than under cI conditions. At the same time, the regular medium cultures had considerably fewer cells floating in the culture wells (Figure 4). In all serum-deprived media, the mean number of cells/ml harvested from the culture wells after the first day of Ss was lower than for day 0, and continued to decrease gradually over the following days of culture (Figure 5 A–C). In jaguarundi, the number of cells/ml at days 3 and 5 of cI+Ss were 64.3% and 83.4%, respectively, lower than at day 0 ($P<0.01$), while for G70+Ss these values ranged from 49.0% to 65.7%, respectively ($P<0.01$; Figure 5 A). In manul, the loss of cells under cI+Ss and G70+Ss conditions was comparable and at days 3 and 5, the mean cell counts/ml were 53–57% and 62–68% lower, respectively, than at day 0 ($P<0.05$ vs. day 0; Figure 5 B). In the case of the domestic cat, a significant loss of cells was observed only for G50+Ss (day 3 and 5), compared to day 0 (Figure 5 C). For all three species, there was no significant change in the mean number of cells/well in regular medium throughout the culture period (Figure 5 A–C), nor was there any significant impact of either method on the proportion of trypan blue negative cells (Figure 6).

Discussion

In this study, serum starvation and contact inhibition were used to synchronize manul, jaguarundi and domestic cat dermal fibroblast cells in the G0/G1 phase, in order to increase their suitability for SCNT in felids, with Ss imposed after the cells reached 40–50% (G50), 60–70% (G70), and full confluency. Flow cytometric analysis revealed that Ss was a more effective method for cell cycle synchronization than cI, although the response of the fibroblasts was species-specific, and depended on the degree of cell confluency and duration of treatment. A species-specific response of feline dermal fibroblasts to different methods of cell cycle synchronization has also been reported by other investigators (Gómez et al., 2003; Wittayarat et al., 2013; Veraguas et al., 2017). In manul and jaguarundi, culture of growing cells at both G50 and G70 confluence under Ss conditions resulted in the arrest of a high proportion of fibroblasts in the G0/G1 phase, while in the domestic cat, this treatment was efficient only at G50 confluence. In manul, the faster effect of Ss on the fibroblast cell cycle (after just 1 day of treatment) was observed at G70 confluence, while in jaguarundi, it was noted at G50 confluence. A differential response of growing cells to Ss, depending on the level of cell confluency and duration of treatment, has also been reported for giant panda fibroblasts (Han et al., 2003).

Generally, Ss has been reported to have a rapid effect on the fibroblast cell cycle, with even a short (1–2 day) treatment successfully arresting a high proportion of cells (~75–91%, depending on species) at the G0/G1 phase (pig: Kues et al., 2000; giant panda: Han et al., 2003; dog: Khammanit et al., 2008; cattle: Miranda et al., 2009; Felidae family: Wittayarat et al., 2013), which is in agreement with our observations. Extending the starvation period by a few days usually does not increase the proportion of quiescent cells (Kues et al., 2000; Han et al., 2003; Khammanit et al., 2008), while Ss negatively affects cell viability in just the first days of application, leading to cell loss and/or DNA fragmentation (Kues et al., 2000; Yu et al., 2003). In a study using canine skin fibroblasts, there was no increase in the percentage of apoptotic cells after 1–3 days of Ss compared to control (Khammanit et al., 2008), while in the case of feline fibroblasts, an increasing incidence of apoptosis was observed after 4–5 days of Ss for Siamese cat and marbled cat fibroblasts, but not for leopard or Asian golden cat cells (Wittayarat et al., 2013). In our study, the greatest loss of cells (>83%, compared to day 0) was noted for jaguarundi cells after 5 days of cI+Ss. Such a huge number of detached cells floating in the culture wells suggests that this treatment had a drastic and detrimental effect on jaguarundi fibroblasts, increasing the incidence of apoptosis and/or necrosis. Furthermore, this treatment failed to increase the proportion of G0/G1 cells, and thus could not be recommended for jaguarundi fibroblast cell cycle synchronization. In contrast, 5 days of cI+Ss effectively arrested a high proportion (95%) of manul cells in the G0/G1 phase with a concomitantly less drastic effect on cell survival, suggesting that it could be useful for cell cycle synchronization in this species. Based on these results, we can infer that the cell lines of some species or individuals might be more sensitive to a lack of nutrients in culture medium at full confluency than during the logarithmic phase of growth. The synergistic effects of Ss and full confluency on increasing the percentage of G0/G1 cells were observed for domestic cat fetal (de Barros et al., 2010) and sheep dermal fibroblasts (Ma et al., 2015). In sheep, a negative effect of prolonged Ss, both at 70–80% confluency and in conjunction with cI was also noted (Ma et al., 2015). There is evidence that apoptotic cells used as karyoplast donors could negatively influence the efficiency of SCNT (Yu et al., 2003; Park et al., 2004; Miranda et al., 2009; Samiec et al., 2013 a, b), and in cattle, culture of donor fibroblasts in the presence of putative apoptosis inhibitors (such as β-mercaptoethanol or hemoglobin) improved the quality and early development of reconstructed embryos (Park et al., 2004). Interestingly, the live birth of one cloned calf reconstructed with an apoptotic (annexin-positive) cell implies, according to the authors, that the recipient cytoplast is to some extent capable of reversing apoptotic changes in the donor cell generated by serum starvation (Miranda et al., 2009).

It should be emphasized that under our culture conditions, a vast majority (>86%) of fibroblasts in all three Felidae species were already in the G0/G1 phase on the day of reaching full confluency (day 0: cI+Ss and cI),

with the highest proportion (>93%) found in jaguarundi cells, and that further culture in cI conditions did not increase the efficiency of cell cycle synchronization in any of the species. A high percentage of fibroblasts in the G0/G1 phase (85–87.4%) has also been reported in pigs (Boquest et al., 1999) and cattle (Cho et al., 2005) at full confluency, and in giant panda at just 90% confluency (Han et al., 2003). It is well known that when the cells form a monolayer and reach full confluency, they enter the so-called plateau/stationary phase of cell growth (Liu et al., 2008; Guan et al., 2010; Młodawska et al., 2019). Under high density conditions, the contact surface between adjacent cells gradually increases (Curto et al., 2007), leading to contact inhibition which causes most cells to cease dividing and remain at the early G1 phase, despite the availability of nutrients and growth factors (Levine et al., 1965; Davis et al., 2001). In the present study, jaguarundi cells (like most other cell lines) reached full confluency after 6 days of culture following thawing. Such a high percentage of G0/G1 cells by day 0 suggests that in this species, the inhibition of proliferation and entry into the state of cI may have occurred before full confluency was achieved. It may also be indicative of an inherently long G1 phase of the jaguarundi fibroblast cell cycle, like other non-transformed mammalian fibroblast cells (Gadbois et al.,1992). At the same time, the lack of a negative impact of cI on cell viability and on their number in culture wells leads us to conclude that in the case of jaguarundi, achieving full confluency and possibly a few days' cI can be also a valuable method of obtaining a high proportion of skin fibroblasts in the G0/G1phase. It should be noted that in human fibroblasts, contact inhibition may be achieved when the cells reach ~90% confluency (Davis et al., 2001).

The available literature does not contain unambiguous results as to the effectiveness of cI for generating G0/G1 quiescence in felids. In the domestic cat, 3–5 days of cI was effective at eliciting a higher percentage of G0/G1 fibroblasts (~80–85%) compared to growing cells, however in kodkod, cI was efficient after 1–3 days but not after 5 days of treatment (Veraguas et al., 2017). In other studies, 5 days of cI was sufficient to induce quiescence in fibroblasts of three species of the Felidae family, but not in marbled cat cells (Wittayarat et al., 2013), and significantly more G0/G1 cells in the fibroblasts of the domestic shorthaired cat than in those of the African wild cat (88% vs. 61%, respectively; Gómez et al., 2003). In our study, after the same period of cI, the percentage of G0/G1 cells in all species was higher, ranging from ~92 to 94%. In the case of the domestic cat, this treatment was more efficient than 5 days of G50+Ss, and so could be also used for fibroblast cell cycle synchronization. These variations between our and other authors' findings might be due to individual characteristics of the animal (species, breed, sex, age) from which the cells were obtained, as well as the cell types and the culture conditions used.

It is believed that Ss and cI are not functionally equivalent, as they differ with respect to the mechanisms (signaling pathways) by which they affect the cell cycle and the extent to which they modulate different gene expression profiles (Gos et al., 2005; Coller et al., 2006; Shin et al., 2008; Swat et al., 2009; Ma et al., 2015; Kallingappa et al., 2016). Research implies that the inhibition of cell proliferation resulting from cell-to-cell contact involves upregulation (accumulation) of p27 (cyclin-dependent kinase 2 inhibitor) via the p38α-Spry2-EGFR-p27 network (Swat et al., 2009), while the mitogen/growth factor depletion associated with culture in a serum-deprived medium induces quiescence in cells through suppression of the Skp2-CDK2 and CDK4 pathway (Shin et al., 2008). It is unclear whether embryos reconstructed from cells subjected to Ss or cI have the same quality and developmental potential. For example, in cattle, the cleavage rate was higher for embryos derived from serum-starved than from confluent fibroblast cells, but blastocyst formation did not differ between groups (Hayes et al., 2005). In contrast, the morula/blastocyst formation yields in pigs were higher when cI instead of Ss was applied in SCNT procedures (Samiec et al., 2013 a, b). It is believed that in felids (domestic shorthaired cat and African wild cat) the method of cell cycle synchronization has no influence on the frequency of fusion, and cleavage of reconstructed embryos or their development to the blastocyst stage (Gómez et al., 2003, 2006).

In conclusion, serum starvation of growing cells could be used successfully for manul, jaguarundi and domestic cat dermal fibroblast cell synchronization in the G0/G1 phase. The response of the cells is species-specific and depends on initial cell culture confluence and duration of treatment, and therefore the treatment should be customized. In all three species, contact inhibition alone did not elicit an important shift in the proportion of quiescent cells, nevertheless in the domestic cat prolonged cI was more efficient than the same period of Ss at 40–50% confluency. In jaguarundi, culture of cells to full confluence could also be a valuable method of obtaining a high proportion of skin fibroblasts in the G0/G1phase, without causing damage to the cells. In contrast to cI, prolonged trophic deprivation may generate cell loss and could induce apoptosis. This research may find application in preparing donor karyoplasts for somatic cell nuclear transfer in felids.

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Conflict of Interest

The authors declare no conflict of interest.

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