EFFECTS OF INCUBATION TIME AND METHOD OF CELL CYCLE SYNCHRONIZATION ON COLLARED PECCARY SKIN-DERIVED FIBROBLAST CELL LINES*

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Abstract

The success of cloning by somatic cell nuclear transfer depends on the efficiency of nuclear reprogramming, with the cycle stage of the donor cell playing a crucial role. Therefore, the aim was to evaluate three different approaches for cell cycle synchronization: (i) serum starvation (SS) for 1 to 4 days, (ii) contact inhibition (CI) for 1 to 3 days, and (iii) using cell cycle regulatory inhibitors (dimethyl sulfoxide, cycloheximide, cytochalasin B, or 6-dimethylaminopurine) for 1 and 2 days, in terms of their effects on synchronization in G_0/G_1 phases and viability of collared peccary skin fibroblasts. Flow cytometry analysis revealed that SS for 4 days ($79.0\% \pm 1.6$) and CI for 3 days ($78.0\% \pm 1.4$) increased the percentage of fibroblasts in G_0/G_1 compared to growing cells GC ($68.1\% \pm 8.6$). However, SS for 3 and 4 days reduced the viability evaluated by differential staining ($81.4\% \pm 0.03$ and $81.6\% \pm 0.06$) compared to growing cells (GC, $95.9\% \pm 0.06$). CI did not affect the viability at any of the analyzed time intervals. No cell cycle inhibitors promoted synchronization in G_0/G_1 . These results indicate that CI for 3 days was the most efficient method for cell cycle synchronization in peccary fibroblasts.

Key words: G_0/G_1 phase, karyoplast, somatic cell nuclear transfer, wild mammals

The collared peccaries are wild mammals endemic to the American continent, where they play a crucial ecological role in maintaining their habitats (Desbiez et al., 2012). Among the various interactions of these animals with the ecosystem are their functions as seed dispersers and prey for larger mammals (Desbiez and Keuroghlian,

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2009). Although their population is not considered to be of concern internationally (Gongora et al., 2011), in some biomes, such as Caatinga and the Atlantic forest, these individuals' populations are declining (Desbiez et al., 2012), thus requiring conservation strategies that ensure their maintenance in biodiversity.

Fortunately, this species' high adaptation capacity and zootechnical performance in captivity (Briceño-Méndez et al., 2016) suggest that assisted reproductive technologies (ARTs) such as cloning by somatic cell nuclear transfer (SCNT) may be successfully used for generation and multiplication of genetically identical specimens of collared peccary (*Pecari tajacu*) as well as for research focused on elucidation of molecular and epigenetic mechanisms underlying embryonic development in this mammalian species. The efficiencies of intra- and interspecies (intrafamily, interfamilial, intragenus and intergenus) somatic cell cloning of non-ruminating ungulates from the family *Suidae* that encompass domestic pigs (the most closely related to peccaries from the family *Tayassuidae*) are largely affected by: 1) the provenance of *in vitro* cultured nuclear donor cells (Lee et al., 2019 a; Loi et al., 2011; Opiela et al., 2017; Samiec and Skrzyszowska, 2010 a, 2013); 2) the method used for artificial synchronization of their mitotic cycle at G₀/G₁ phases (Samiec et al., 2013 a, b; Zhang et al., 2018; Zhao et al., 2016); and 3) the quality of *ex vivo* expanded donor cell lines, which is measured by incidences of DNA aneuploidy (Opiela et al., 2013) and apoptotic or autophagic cell death (Lee et al., 2019 b; Samiec et al., 2013 a, 2019).

The efficiencies of intra- and interspecies SCNT techniques in pigs are also dependent on the capabilities of donor cell nuclei to be epigenetically reprogrammed in a cytoplasm of nuclear-transferred oocytes and the blastomeres of cloned embryos (Borges and Pereira, 2019; Jin et al., 2018; Samiec and Skrzyszowska, 2018 a, b; Song et al., 2017; Wang et al., 2018). Moreover, the above-mentioned efficiencies seem to be biased, to a high extent, by intergenomic crosstalk between nuclear and mitochondrial compartments in activated SCNT-derived oocytes (nuclear-cytoplasmic hybrids) and developing cloned embryos (Samiec, 2005 a, b; Samiec and Skrzyszowska, 2014; St John et al., 2005; Takeda et al., 2006). Additionally, it is noteworthy that fetal or adult dermal fibroblast cell lines are the genetic resources that seem to be the most frequently used for research programs aimed at the somatic cell cloning in domestic pig (*Sus scrofa domesticus*), which exhibits a very close taxonomic/phylogenetic consanguinity with wild pig species (peccaries) stemming from the family *Tayassuidae* (Guo et al., 2018; Samiec and Skrzyszowska, 2010 b, 2012 a, b; Samiec et al., 2013 b; Wu et al., 2019).

In recent years, our group has developed steps to implement the SCNT technique in collared peccaries. This path has been developed since the establishment of the ideal environment for receptor oocytes (cytoplasts, Borges et al., 2018 b), and the development of an efficient oocyte artificial activation protocol to provide the development of the reconstructed embryo (Borges et al., 2020 b). In parallel, we established somatic tissue banks (Borges et al., 2017, 2018 a; Queiroz Neta et al., 2018), somatic cell banks (Lira et al., 2020), and, recently, we established skin-derived fibroblast lines (Borges et al., 2020 a). Therefore, now, we have evaluated the different cell cycle synchronization protocols of these lines, aiming at a higher percentage of cells in G₀/G₁.

Several cell cycle synchronization protocols in G_0/G_1 stages have been proposed. However, the cellular response to these protocols has been variable in domestic (Goissis et al., 2007) and wild mammals (Mahesh et al., 2012), necessitating the evaluation of each protocol for a given cell type and species (Veraguas et al., 2017; Wittayarat et al., 2013). Three main approaches have been employed for cell synchronization: serum starvation (SS, Kues et al., 2000), contact inhibition (CI, Boquest et al., 1999), and using cell cycle regulatory inhibitors (Goissis et al., 2007). While SS and CI act on the checkpoints by depriving the cells of adequate environmental or nutritional conditions (Kues et al., 2000), cell cycle inhibitors regulate specific biosynthetic processes such as repression or induction of cyclins (dimethyl sulfoxide, DMSO), protein synthesis inhibition (cycloheximide, CHX), cytoskeleton inhibition (cytochalasin B, CB), and protein kinase inhibition (6-dimethylaminopurine, 6-DMAP) to cause prolongation of the G_1 phase (Koo et al., 2009; Kretz et al., 2019).

The aim of this study was to evaluate SS, CI, and cell cycle regulatory inhibitors (DMSO, CHX, CB, or 6-DMAP) on synchronization in G_0/G_1 phases as well as the viability of collared peccary skin fibroblasts, in order to develop efficient cell cycle synchronization protocols for this species. We want to achieve this goal by defining appropriate methodologies for the last stage of the preparation of karyoplast to be used in the cloning of this species.

Material and methods

Chemicals and bioethics

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, and antibiotic-antimycotic solution were obtained from Gibco-BRL (Carlsbad, CA, USA). The experiments were conducted in accordance with the Animal Ethics Committee (CEUA/UFERSA, no. 23091.001072/2015-92) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 48633-2).

Establishment and culture of fibroblasts

Skin tissue samples were obtained from the ear notch of four adult male collared peccaries at the Wild Animal Multiplication Center (CEMAS/UFERSA, no. 1478912, BR). The skin samples were cultured, and four fibroblast lines were previously established (Borges et al., 2020 a). Subsequently, cells from four lines frozen in 10% DMSO, 10% FBS and 0.2 M sucrose were thawed, and 4th and 5th passage cells were used for this study.

Initially, cells were cultured in DMEM supplemented with 10% FBS and 2% antibiotic-antimycotic solution in a humid atmosphere containing 5% CO_2 at 38.5°C. Prior to initiating cell cycle synchronization protocols, the cells were evaluated for their proliferative activity by obtaining the growth curve and determining the population doubling time (PDT). Cells were seeded in 12-well plates at a concentration

of 1.0×10^4 cells/mL. Cells from each well were counted at 24 h intervals for up to 216 h of culture. After each interval, the mean cell count was recorded; finally, the cell growth curve was generated, and the PDT was estimated based on these measurements (Roth, 2006).

Additionally, the percentage of living cells was determined by trypan blue exclusion assay (Borges et al., 2018 a). Briefly, the cells were stained with 0.4% trypan blue in phosphate buffered saline (PBS) and counted using a hemocytometer.

Cell treatments

Evaluating three cell cycle synchronization methods, we examined the effects of serum starvation (SS), contact inhibition (CI), and cell cycle regulatory inhibitors (DMSO, CHX, CB, and 6-DMAP) treatments on the cell cycle stages of collared peccary fibroblasts in different incubation times. In each treatment group, cells without any treatment and with 70% confluence were used as a control (growing cells, GC). All treatments were performed in duplicate for each cell line, producing a total of eight repetitions for each treatment and each incubation time.

Experiment 1 – Effect of serum starvation

SS was performed by replacing the culture medium with DMEM containing 0.5% FBS after the cell confluence had reached 70% in DMEM with 10% FBS (Caamaño et al., 2008). After the start of the SS treatment (day = 0), the stage of fibroblasts from each animal was analyzed on days 1, 2, 3 and 4. The culture medium was changed every 2 days.

Experiment 2 – Effect of cell contact inhibition

The effect of CI was studied for 3 days in culture after confluence had reached 90–100%. During the treatment of CI, the culture medium composed of DMEM and 10% FBS was changed every 2 days (Gómez et al., 2018).

Experiment 3 – Effect of cell cycle regulatory inhibitors

DMSO (0.5%, inhibitor of cytokinesis), CHX (7.5 µg/mL, protein synthesis inhibitor), CB (7.5 µg/mL, inhibitor of cytokinesis), and 6-DMAP (2 mM, protein kinase inhibitor) treatments were performed for 2 days of cell culture after the cell confluence reached 70%. After the start of treatment (day = 0), the stage of fibroblasts from each animal was analyzed on days 1 and 2 (Mahesh et al., 2012).

Cell cycle synchronization analysis

After the treatments, cells were trypsinized, centrifuged at $600 \times g$ for 10 min and resuspended in 1.0 mL of cold 70% ethanol for fixation. The cells were then maintained at -20°C for 5 days. The fixed cells were washed in PBS for ethanol removal and each sample was centrifuged at $400 \times g$ for 10 min. Subsequently, cells were stained with 20 µg/mL propidium iodide, 50 µg/mL RNase were added, and samples were incubated at 4°C for 50 min. After that, the samples were analyzed using Guava Easycyte flow cytometer (Guava Technologies, Stamford, Lincolnshire, United Kingdom). Data were obtained from 15,000 events from each sample. Histograms of red fluo-

rescence vs counts were generated to evaluate the percentages of cells for each cell cycle phase (G_0/G_1 , S, G_2/M) as well as the levels of sub G_0/G_1 . The proportion of cells in each phase of the cell cycle and sub G_0/G_1 levels was assessed using MOD-FIT software version 5.0 (Verity, <https://www.vsh.com/products/mflt/index.asp>).

Cellular viability assessment

A cell aliquot was resuspended in 2 $\mu\text{g/mL}$ acridine orange and 10 $\mu\text{g/mL}$ ethidium bromide. Subsequently, the cells were evaluated using fluorescence microscopy at 480 nm, where 300 cells were counted at 200 $\times$. The cells were then classified into (i) viable cells, with a uniform light green nucleus; (ii) cells in initial apoptosis, with a non-uniform green nucleus; (iii) cells in late apoptosis, with a non-uniform bright orange nucleus, and (iv) necrotic cells, with a uniform orange nucleus (Kosmider et al., 2004). A fluorescence microscope was used to observe apoptotic changes in the stained cells, which were quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

Data were expressed as mean $\pm$ standard error and analyzed using the Graph-Pad software (Graph-Pad Software Incorporation, La Jolla, CA, USA). All results were verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by Tukey test. Significance was set at $P < 0.05$.

Results

Initially, thawed cells were morphologically normal (Figure 1 A), with a percentage of $91.2\% \pm 1.2$ live cells after the thawing. Moreover, the cells showed a sigmoidal curve with the lag phase of adaptation up to day 2 followed by exponential and stationary growth, indicating that these cells were going through various phases of growth. Additionally, the decreasing phase was not observed (Figure 1 B). The growth kinetics of the cells was demonstrated by counting total cells during 216 h of culture, resulting in an estimated PDT of 15.3 ± 1.4 h.

Experiment 1 – Effect of serum starvation

After 4 days of SS, the percentage of G_0/G_1 phase cells was significantly higher ($P < 0.05$) when compared to GC, which was the control (Figure 2 A–B, Table 1). Moreover, there was no change in the other phases of the cell cycle. Although the SS did not cause differences in sub G_0/G_1 , when the levels of viability were evaluated by differential staining, 3 and 4 days of SS caused cell damage (Figure 3 A).

Experiment 2 – Effect of cell contact inhibition

CI for 3 days significantly increased the proportion of fibroblasts in G_0/G_1 phase when compared to GC (Figure 2 C, Table 2) and decreased the proportion of cells in G_2/M phase when compared to GC ($P < 0.05$). However, neither evaluated time inter-

val promoted modifications in S cell cycle phase or levels of sub G_0/G_1 ($P>0.05$). Additionally, CI did not affect the cell viability at any of the analysed intervals ($P>0.05$, Figure 3 B).

Figure 1. Culture of collared peccary fibroblast cells. (A) Fibroblasts at 70% confluency showing normal morphology. (B) Growth curve of fibroblasts

Figure 2. Representative histograms of the flow cytometry analysis of collared peccary fibroblasts. (A) growing cells (GC, control), (B) serum starvation (SS), (C) contact inhibition (CI), (D) dimethylsulfoxide (DMSO), (E) cycloheximide (CHX), (F) cytochalasin B (CB), (G) 6-dimethylaminopurine (6-DMAP)

Table 1. Effect of serum starvation (SS) on the percentage of collared peccary fibroblasts in the G_0/G_1 , S and G_2/M phases of the cycle

Conditions	Cell cycle phase (%)			
	G_0/G_1	S	G_2/M	Sub G_0/G_1
Growing cells (GC)	68.1±8.5 a	17.6±10.3 a	14.3±1.8 a	15.9±7.2 a
1 day SS	78.7±2.3 ab	6.7±1.5 a	14.6±0.9 a	7.0±1.8 a
2 day SS	78.1±1.7 ab	7.2±0.9 a	14.7±1.5 a	10.5±3.0 a
3 day SS	75.8±2.9 ab	6.8±2.2 a	17.4±0.7 a	19.0±11.3 a
4 day SS	79.0±1.6 b	5.0±0.8 a	16.0±1.1 a	5.9±2.2 a

Within a column, values with different letters differ ($P<0.05$).

Figure 3. Effect of different synchronization methods on the viability of collared peccary fibroblasts. (A) and (A') effect of SS on fibroblast viability, (B) and (B') evaluation of the viability of fibroblasts subjected to CI, (C) and (C') effect of cell cycle inhibitors on the viability of synchronized fibroblasts. Within a bar at the same time, the values of a, b differ ($P < 0.05$). Within a bar at different times, A, B differ ($P < 0.05$). Fine arrow: viable cell; arrowhead: cell in initial apoptosis; fat arrow: cell in late apoptosis; triangle: cell in necrosis

Table 2. Effect of contact inhibition (CI) on the percentage of collared peccary fibroblasts in the G_0/G_1 , S and G_2/M phases of the cycle

Conditions	Cell cycle phase (%)			
	G_0/G_1	S	G_2/M	Sub G_0/G_1
Growing cells (GC)	70.8±1.4 a	5.9±0.8 a	23.3±1.3 a	1.1±0.2 a
1 day CI	75.6±3.2 ab	7.3±1.0 a	17.1±2.2 ab	2.0±0.7 a
2 day CI	75.5±1.8 ab	6.9±0.5 a	17.6±1.5 ab	5.5±3.5 a
3 day CI	78.0±1.4 b	6.9±1.2 a	15.1±1.8 b	7.6±5.3 a

Within a column, values with different letters differ ($P < 0.05$).

Experiment 3 – Effect of cell cycle regulatory inhibitors

Cell cycle inhibitors did not promote cell synchronization in G_0/G_1 at the time tested when compared to GC for 1 and 2 days (Figure 2 D–G, Table 3). While DMSO did not change any of the evaluated parameters, CHX increased sub G_0/G_1 levels on both days and reduced the percentage of cells in S phase after 2 days when compared to CG ($P < 0.05$). After 2 days of treatment with CHX, the proportion of fibroblasts in G_0/G_1 phase was higher when compared to that after 1 day ($P < 0.05$).

Table 3. Effect of treatment with dimethyl sulfoxide (DMSO), cycloheximide (CHX), cytochalasin b (CB), or 6-dimethylaminopurin (6-DMAP) on the percentage of collared peccary fibroblasts in the G₀/G₁, S and G₂/M phases of the cycle

Conditions	Cell cycle phase (%)							
	treated for 1 day				treated for 2 days			
	G ₀ /G ₁	S	G ₂ /M	Sub G ₀ /G ₁	G ₀ /G ₁	S	G ₂ /M	Sub G ₀ /G ₁
Growing cells (GC)	75.4±0.8 a	7.1±0.7 b	17.5±0.3 b	10.9±2.9 b	75.4±0.8 a	7.1±0.7 a	17.5±0.3 b	10.9±2.9 b
0.5% DMSO	75.7±0.9 a	5.9±1.5 b	18.4±1.0 b	10.9±0.5 b	74.1±0.5 a	8.0±0.6 a	17.9±0.3 b	17.7±0.7 b
7.5 µg/mL CHX	72.7±0.6 abB	9.1±0.5 abA	18.2±1.0 b	24.3±2.7 c	77.4±1.0 aA	5.4±0.6 bB	17.2±0.9 b	25.7±3.3 a
7.5 µg/mL CB	68.3±1.6 bB	12.5±1.2 a	19.2±0.7 bA	5.8±2.9 d	74.4±0.1 aA	9.8±0.5 a	15.8±0.5 bB	17.2±1.3 b
2 mM 6-DMAP	73.5±1.5 a	0.0±0.0 cA	26.5±1.5 aA	46.7±1.1 aA	74.6±1.5 a	3.7±0.6 bB	21.7±1.2 aB	27.3±5.7 aB

Within a column, a, b values with different letters differ (P<0.05). Within a row, A, B values with different letters differ (P<0.05).

Moreover, CB reduced the percentage of cells in G_0/G_1 after 1 day, increasing the percentage of cells in S phase when compared to GC ($P<0.05$). Additionally, treatment with 6-DMAP for 1 and 2 days significantly reduced the proportion of fibroblasts in S phase and caused an increase in the proportion of cells in G_2/M phase. 6-DMAP also caused an increase in sub G_0/G_1 levels when compared to GC (Table 3). Finally, after 1 day of treatment of 6-DMAP, a reduction of viable cells was observed when compared to GC, although no reduction was observed after 2 days of treatment ($P<0.05$, Figure 3 C).

Discussion

An appropriate cell synchronization protocol in G_0/G_1 is a crucial step in the development of cloning by SCNT in collared peccaries. Previously, our group developed somatic resource banks aiming at their application in the conservation of this species (Borges et al., 2017, 2018 a, 2020 a, b; Lira et al., 2020; Queiroz Neta et al., 2018). With this new study, we have developed a suitable protocol for somatic cell synchronization, the last step in the preparation of karyoplasts, i.e., somatic nucleus donor cells. To our knowledge, this is the first work to elucidate nuclear reprogramming in cells of this species.

Here, we identified that using CI for 3 days was more efficient for cell cycle synchronization when compared to SS for 1 to 4 days and treatment with cell cycle inhibitors (DMSO, CHX, CB, or 6-DMAP) for 1 and 2 days. This result may be related to a high cell density where CI favors the regulation of reactive oxygen species (ROS) as well as activates the coactivator-1 α (PGC1 α), which functions as a key regulator of energy expenditure, involved in reduction of ROS and protection of cells from oxidative stress (Yang et al., 2018). In studies with the Asian golden cat (*Catopuma temminckii*), leopard (*Panthera pardus*), and Siamese cat, researchers observed that CI for 5 days promoted an increase of more than 80% in the percentage of cells in G_0/G_1 when compared to non-synchronized cells, without increase in apoptotic cells (Wittayarat et al., 2013). In swine, domestic species phylogenetically close to the collared peccaries, cells were efficiently synchronized by CI for 3 to 4 days and resulted in 20.4% of zygotes developing into blastocysts after cloning by SCNT (Lee et al., 2019). Moreover, for wood bison (*Bison bison*), the CI was adequate for cell synchronization in G_0/G_1 phase, which resulted in 19.2% of developed blastocysts (Seaby et al., 2013). Therefore, the CI has been efficient in producing clone embryos.

Although SS promoted cell synchronization after 4 days in collared peccaries, this treatment had a negative effect on cell viability after 3 and 4 days. In general, the synchronization of the G_0/G_1 cell cycle of mammalian cells via SS is due to the response to absence or presence of mitogens to continue the cell cycle during the onset of G_1 phase, so when these cells are in absence of mitochondrial growth factors that would be offered by the serum, they accumulate in a state of a 2n DNA content (Coller et al., 2007). In porcine cells, the SS for 1 to 3 days increased the proportion of cells at G_0/G_1 phase up to 77.9–80.2% (Kues et al., 2000). However, these authors

also observed cell damage by visualization of DNA fragmentation. Similar to our study, goat fibroblasts were found to be efficiently synchronized in G_0/G_1 by both CI and SS (Dalman et al., 2010); the number of cells in apoptosis increased by 39.8% in 3 days in SS whereas in cells at CI there was no increased apoptosis (8.7%).

The negative effect of SS was also observed on embryonic development. The cell synchronization using SS resulted in embryos with a higher degree of apoptosis and increased ROS when compared to CI (Lee et al., 2016). Likewise, it was observed that SS led to high Bak (pro-apoptotic) and low Bcl-xL (anti-apoptotic) expression while the CI showed equilibrium in the increase in Bak and Bcl-xL expression (Park et al., 2010). Hence, the increase in apoptosis in cells synchronized in G_0/G_1 by 4 days with SS may have a negative effect on the later embryonic development in collared peccaries.

In the present study, no cell cycle inhibitors promoted synchronization in G_0/G_1 in collared peccaries and 6-DMAP had negative effects on cell viability with increase in sub G_0/G_1 levels and reduction of viable cells. A study with goral (*Naemorhedus caudatus*) fibroblasts also indicated no cell cycle synchronization in G_0/G_1 using CHX (60.1%), 6-DMAP (3.9%) and CB (10.8%) in cultures for 1 day (Hashem et al., 2006). Porcine fibroblasts treated with CHX after 2 days showed an increased percentage of dead cells when compared to that after 1 day (Goissis et al., 2007). In our study, we observed an increase in cells in sub G_0/G_1 at both times. Also, evaluation was made of the synchronization in G_0/G_1 for porcine fibroblasts in different passages (5th, 10th and 15th) and it was observed that CHX when used in cells from the 5th passage was unable to synchronize cells when compared to that observed with cells from the 10th and 15th passages, presenting higher number of cells in G_0/G_1 only in 10th passage fibroblasts (Goissis et al., 2007). Possibly, 10th passage presented a higher proportion of senescent cells, becoming more susceptible to synchronization by this inhibitor because of the lower levels of cyclin D, the target mechanism of CHX in the cell cycle (Goissis et al., 2007). The treated cells in this study were from the 4th and 5th passage; this was probably why the cells did not present a positive synchronization result using CHX.

Furthermore, DMSO did not change any of the evaluated parameters in collared peccary cells. Studies in porcine, brown bear (*Ursus arctos pyrenaicus*), and goral cells have shown that cell cycle synchronization with DMSO is concentration dependent. While for porcine cells, 1% DMSO (88.7%) was more efficient than 0.5% (86.5%) and 2.5% (86.0%) DMSO after 2 days (Boquest et al., 1999), brown bear cells were efficiently synchronized with DMSO concentrations of 1–3% (79.0–79.1%) after 2 days (Caamaño et al., 2008). Nevertheless, in goral cells, while 0.5% (74.8%) and 1% DMSO (75.2%) caused synchronization in G_0/G_1 after 1 day, no effect was observed using 2.5% DMSO (Hashem et al., 2006). Therefore, these results show that the effect of DMSO is linked to the concentration used and the sensitivity of the species to this chemical. Additionally, although DMSO may be efficient for synchronization in G_0/G_1 , the use of high concentrations of this agent may cause negative effects on cells (Caamaño et al., 2008).

In summary, this study identified that both CI for 3 days and SS for 4 days promoted the synchronization of somatic cells in collared peccaries; however, the per-

centage of viable cells was maintained only in cells synchronized by CI. Therefore, CI for 3 days was considered the most efficient method. With the cells being subjected to less chemical exposure and metabolic stress, CI can be efficiently applied to cell cycle synchronization in collared peccary fibroblasts. Thus, we established the last step of the preparation for the use of these fibroblast as karyoplast for application in somatic cell nuclear transfer of collared peccary, with potential application to conservation of the species.

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