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Communication

[*Comunicação*]

Viability of fibroblasts from "Curraleiro Pé Duro" cattle after different cryopreservation protocols

[*Viabilidade de fibroblastos de bovinos da raça Curraleiro Pé Duro submetidos a diferentes protocolos de criopreser*vação]

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The breed *Curraleiro Pé-Duro* was named after research revealed the genetic equality of the *Curraleiro* and the *Pé-Duro* cattle (Carvalho *et al*., 2012). It is one of the most important cattle breeds in the history of the northeastern hinterland and the São Francisco valley, present for almost 500 years in the Northeast of Brazil (Santiago, 1975). The conservation of native breeds directly reflects on the environment and culture, making up a genetic, historical, and cultural heritage. Crossing between breeds for new strains can subdue the real potential of a native breed (Fioravanti, 2015), which with a relatively small number of individuals, the loss of genetic material can occur even with the death of a single individual, reducing the gene pool and even genes important for the maintenance of the species (Martins *et al*., 2007).

For the conservation of genetic material and biodiversity, it is necessary to develop and improve techniques, such as the cultivation of somatic cells that can be cryopreserved and stored in cryobanks to later be used in reproductive biotechnologies (Wani and Hong, 2018). Briefly, somatic cells are diploid cells of the animal's body, such as fibroblasts, that is, all cells that have a complete gene set, therefore, all cells of the individual except for reproductive cells (Strachan and Read, 2012). In general, somatic samples for cell cryopreservation are obtained from skin-derived tissues and cultured in vitro in the form of fibroblasts, which are stored in cryobanks and can be used for biotechnologies such as cloning (Mestre-Citrinovitz *et al*., 2016).

Cryopreservation can bring forth a reduction in cell metabolism for maintenance of functional and structural aspects for long periods in liquid nitrogen, enabling the future use of samples in reproductive biotechniques such as cloning and Somatic Cell Nuclear Transfer (Costa *et al*., 2016).

Germplasm banks or cryobanks are a highly viable alternative for the maintenance of biological and genetic diversity (Silva *et al*., 2012). Therefore, the objective of this work is to evaluate the different cryopreservation protocols for maintaining the cell viability of fibroblasts isolated from the *Curraleiro Pé Duro* cattle breed.

The experiment was carried out in the city of Teresina-PI, at the Laboratory of Animal Reproduction Biotechnology – LBRA, at the Center for Agricultural Sciences at UFPI. For this purpose, a cattle breeder of the breed *Curraleiro Pé-Duro*, healthy, fed on pasture and complementary fed, with access to water and mineral salt at will. The animal underwent clinical evaluation as well as additional evaluation.

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The procedures were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under registration number AD94098 and approved by the Ethics Committee on Animal Experimentation of the Federal University of Piauí (CEEA-UFPI), registered under number 673/21.

After the initial evaluations, the animal was placed in a containment trunk, where the animal's ear was cleaned and anesthetized, from which a skin sample of about 1cm² was taken and transported in a sterile tube containing PBS solution plus antibiotic, in a thermal box with monitored temperature, reaching the laboratory in up to 2 hours.

In the laboratory, the sample was dissected, removing cartilage, fat, hair, and other remaining tissue. The skin fragment was then chopped into smaller fragments of approximately 2mm². These new fragments were placed in 35mm dry and sterile Petri dishes, with a total of 5 fragments in each plate and 4 ml of Dulbecco MEM culture medium (DMEM Cell Culture Medium, Vitrocell Embriolife, Brazil) was added and incubated in a controlled atmosphere of 5% carbon dioxide (CO2) in air, at a temperature of 39ºC and high humidity of CO2 (HF151UV, Heal Force, China) where they remained in cultivation for one week.

On D7, the biopsies were removed, and the entire contents of the solution and culture of each plate were washed with 1mL of the DMEM culture medium and then added with another 3mL of the same. The plates again remained in cultivation for 1 week.

On D14, the cell culture plates were transferred to culture bottles after removing all the culture medium from the plates and detaching the plate using Trypsin/EDTA (Trypsin 0.05x, LGC Biotecnologia, Brazil) for 5min at 39ºC and centrifuged at 200G for 5 minutes and after centrifugation, the supernatant was removed and the pellet resuspended with 1mL of DMEM medium and transferred to two culture bottles, remaining in the incubator under the same atmospheric conditions. On D21, the cells, already in the confluence stage, began to decrease the meiotic process and then the first subculture was performed, initially with the removal of all the culture medium from the flasks and then trypsinized, centrifuged, resuspended, and transferred to two new culture bottles to be incubated for another 7 days. On D28, D35, D42 and D49, the 2nd, 3rd, 4th and 5th passes were performed respectively, using the same protocol.

Cryopreservation was performed with cells from the 4th and 5th passage, which during the subculture process and after centrifugation, half of the sample was poured into 0.25mL straws. A small aliquot of 10 uL was taken for cell concentration counting in a Neubauer chamber. Cells were poured into straws (1.0x106 Cel) with culture medium plus 10% DMSO. After the straws were sealed and identified, they were subjected to 3 cryopreservation protocols, where in Treatment 1 (T1), the samples went through a freezing curve by storing them in a freezer (Frost Free, electronic 280, Brastemp, Brazil) at -20°C for 24h and immersion in Liquid Nitrogen (NL2). Treatment 2 (T2) underwent a freezing curve by storing the straws in a freezer at -80°C (CL 347-86v, Cold Lab, Brazil) for 24h and immersion in NL2. Treatment 3 (T3) passed through an automated machine (TK 3000, TK Tecnologia, Brazil) and a cryopreservation curve normally used for embryos was used, in which the straws with cells were placed in a container previously stabilized at -6ºC, where they were crystallized and then passed through a negative ramp of -0.5ºC/min until reaching a temperature of -32°C where they stabilized for 5min and immersed in NL2. The cells were stored for approximately 10 days in the cryobank and then they were thawed by removing the straws from the cryogenic cylinders and then immersing them for 20s in a water bath previously heated to 38°C.

During all cell passages of the experiment, morphological evaluations were carried out in a binocular microscope (BX41, Olympus) observing their size, appearance, opacity, shape, and adhesion patterns. The viability analysis was performed with Trypan Blue Dye (0.4% Trypan Blue in PBS, LGC Biotecnologia, Brazil). Post-cryopreservation growth capacity, confluence and morphology were performed with cell culture for an additional 7 days after thawing. In the different analyses, cell confluence and cell viability data were analyzed by ANOVA and the means compared by Tukey's test (5% probability).

All cells had their morphology monitored during growth, presenting a fusiform shape accompanied by cellular extensions, with a round, large and centralized nucleus in a well-filled cytoplasm. The *Curraleiro Pé Duro* cattle fibroblasts derived from ear biopsy had a uniform growth, presenting a regular confluence during the culture days, reaching 90% of confluence in approximately 7 days of culture.

After cryopreserved in 0.25mL straws at a concentration of approximately 1x10^6, the cells maintained the same morphological pattern of preserved fibroblasts, which reinforces work, where it was already possible to verify that at a concentration of 1x10^6 cells per straw, it is possible to obtain good results for cryopreservation of cells of ear origin in cattle, when compared to other concentrations such as 3x10^6 and 5x10^6 (Urio, 2012). It was also possible to observe a statistically non-significant decrease in the confluence capacity, expressed as a percentage, in the cells of *Curraleiro Pé Duro* (83.33±5.16), when compared to the same pre-freezing (96.67±5.77), which may slightly affect cell viability, since in the work of Munhoz and Costa (2012), it was found that the best viability index of cryopreserved cattle cells was reached when cell confluences reached the closest to 100%, those with 73.6% of cell viability, while cells with 70 to 80% of confluence showed 69.9% of cell viability.

Curraleiro Pé Duro somatic cells managed to maintain good levels of cell viability (Table 1) in all treatments that used freezers (T1 and T2), observed by the technique of staining in Trypan Blue and corroborating the results of Urio (2012) which he used in his work with 10% DMSO and 0.25mL straws containing cattle somatic cells, observing a cell viability of 72.9%±11.7. At the same time, the treatment using a freezing machine with automatic temperature control (T3) proved to be a method as efficient as the others, as shown in the work by Cetinkaya and Arat (2011), who found good viability of somatic cells frozen in automatic curves of -0.5, 1 and 2°C/min.

Table 1. Cell viability (%) and confluence (%) in treatments (p>0.05)

Treatment	Viability	Confluence
T1	68.00±3.94[a]	80±7.53[a]
T2	72.14±3.13[a]	90±5.16[a]
T3	70.99±2.62[a]	85±5.48[a]

No statistical difference was observed on the viability and confluence of 4th and 5th passage cells (Table 2), thus both passages were viable for cryopreservation. According to Garfield (2010), the use of well-differentiated cells from the 1st to the 4th passage preserves the normal structure and function of the cells since this replication process is limited in addition to reducing the risks of apoptosis of the generated embryos.

Table 2. Viability (%) and cell confluence (%) in relation to the passage of origin of the cell (p>0.05)

Treatment	Viability		Confluence	
	4ª Cell pass	5ª Cell pass	4ª Cell pass	5ª Cell pass
Pre-freezing	96.34±1.50[a]	92.90±0.56[a]	90±5.77[a]	90±5.77[a]
T1	68±2.97[a]	67.96±5.45[a]	80±5.77[a]	70±5.77[a]
T2	72.93±3.45[a]	69.56±3.02[a]	90±5.77[a]	90±5.77[a]
T3	72.06±3.20[a]	69,86±0.71[a]	80±5.77[a]	90±5.77[a]

The results of this work showed the possibility of cryopreserving fibroblasts from *Curraleiro Pé Duro* cattle, even with less specialized equipment, which is an important step for several reproductive biotechnologies assisted in the preservation of local and/or endangered species, as occurred in other studies, such as that of Srirattana *et al*. (2012), where, through the use of somatic cells, an interspecific cloning was performed, with a donor animal of genetic material from the Gauro breed (Bos gaurus) and a recipient female cattle (Bos taurus).

In the present work, it was possible to verify that there is no significant difference in the cryopreservation protocols tested for cells from the ear tissue of *Curraleiro Pé Duro* cattle, which maintained both their viability and their capacity for growth and confluence at good levels, even in cells from different passages, showing their ability to resist cryopreservation protocols and to be used as a source of genetic material in cryobanks for research and for use in assisted reproduction biotechniques, even if coming from protocols that used simpler equipment with lower cost.

Keywords: somatic cells, germplasm bank, cell culture

RESUMO

A raça tropicalmente adaptada Curraleiro Pé Duro (CPD) possui grande rusticidade e capacidade de produção. A técnica de criopreservação de células somáticas permite estocar, por tempo indeterminado, o material genético. O objetivo deste trabalho é avaliar a viabilidade de fibroblastos, pós-criopreservação, em protocolos diferentes. Foi utilizada uma biópsia auricular de um bovino CPD, que passou por antissepsia e anestesia local. Posteriormente o material foi processado e incubado para ser observado quanto à confluência e à morfologia. Em seguida, os fibroblastos foram criopreservados em três tratamentos (T1, T2 e T3). Após serem criopreservados, foram descongelados e analisados quanto à viabilidade celular, à capacidade de crescimento e à morfologia. Na análise de variância e das médias, foram comparados pelo teste de Tukey com significância de 5%. Não foram observadas, nos protocolos, diferenças estatísticas entre a viabilidade celular (T1 = 67,98%, T2 = 71,42% e T3 = 69,93%), a capacidade de confluência (T1 = 80%, T2 = 90%, T3 = 85%) ou a passagem de origem das células. A criopreservação de fibroblastos auriculares de bovinos CPD não mostrou diferença entre os três métodos, sugerindo até que o método que demanda equipamentos menos especializados (T1) é tão eficiente quanto os demais.

Palavras-chave: células somáticas, banco de germoplasma, cultivo celular

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