TISSUE PRESERVATION AND CELL CULTURE OF PRZEWALSKII´S HORSE (*Equus przewalskii)*: AN ENDANGERED SPECIES

*PRESERVAÇÃO DE TECIDOS E CULTURA DE CÉLULAS DO CAVALO DE PRZEWALSKI (*Equus przewalskii*): UMA ESPÉCIE EM EXTINÇÃO*

Rodrigo PUENTES[1]; Agustín FURTADO[1]; Maria José ESTRADÉ[1]; Rafael ARAGUNDE[2]; Nicolás CAZALES[2]; Gabriela COSTA[2]; Jorge ESTÉVEZ[2]; Fernando CIRILO[3]; Cecilia GALOSI[4,5]

1. Departamento de Ciencias Microbiológicas; Facultad de Veterinaria - Universidad de la Republica, Montevideo, Uruguay. rpuentes@adinet.com.uy ; 2. Departamento de Reproducción, Facultad de Veterinaria, Montevideo, Uruguay; 3. Zoológico Municipal Villa Dolores, Montevideo, Uruguay; 4. Cátedra de Virología, Facultad de Ciencias Veterinaria, Universidad Nacional de La Plata, Argentina; 5. Comisión de Investigaciones Científicas de la Pcia de Buenos Aires, Argentina.

ABSTRACT: The Przewalskii´s horse or Mongolian wild horse (*Equus przewalskii,* Poljakov, 1881) is presently the only species of wild horse in existence. Originally from Asia, it is, classified as in extremely high risk of extinction which puts the species in the seriously threatened category. The aim of this work was the preservation of tissues and the development of cell cultures from tissue samples obtained from a Przewalskii´s horse after its death. Biopsies of skin, skeletal and cardiac muscle, and ear cartilage were removed from a recently dead horse, added to Phosphate Buffer Saline (PBS) and refrigerated until processing. Some of the samples were frozen in liquid nitrogen and the other was grown as explants to generate fibroblast cell monolayers. The cell cultures obtained, were subsequently propagated with low passages, and frozen in liquid nitrogen, thus avoiding genetic and phenotypic alterations. The tissues and cell cultures were thawed to ascertain their viability by checking its progressive grow in a flask. It was not possible to obtain cultures from cardiac muscle. A bank of tissues and cells from the single Przewalskii´s horse that existed in Uruguay was generated, and can be used for scientific purposes and for the conservation of the species in the future.

KEYWORDS: Species conservation. Tissue bank. Wild horse.

INTRODUCTION

Preservation of biodiversity is a main key for the maintenance of life (WILSON, 1992). However, the rapidly growing human population puts considerable pressure on the ecosystem, destroying and displacing many species from their natural habitats. A direct consequence is the need for developing conservation strategies for the species concerned (WILDT, et al. 1997). According to Soulé (1991), the strategy with the greatest potential impact is the protection or the ecosystem in its entirety (protection *in situ*), followed by protection of the communities, species and populations (*ex situ* breeding programs). And finally, another strategy is the preservation of biological materials by cryopreservation, generating genetic resource banks that may provide gametes, embryos, tissues and cell cultures as sources of genetic information (BENIRSCHKE, 1984).

The preservation of viable cells *in vitro* is an essential technology for conservation biology (TOVAR, et al. 2008). However, it is well known that the establishment of *in vitro* cell culture is costly, susceptible of contamination (LINCOLN; GABRIDGE, 1998), and prone to genetic and phenotypic drift (FRESHNEY, 2005; SIMIONE, 1992). In practice, it is common to use skin tissue as a source of somatic cell lines for different applications, obtaining samples directly from fresh or frozen tissues (TOVAR, et al. 2008).

Przewalskii's horse (*Equus przewalskii*), is native to Asia. It was an endemic species in Mongolia, becoming extinct in nature in the 60's (VAN DIERENDONCK; WALLIES DE VRIES, 1996). It differs genetically from the domestic species of horse, as *Equus przewalskii* has 66 chromosomes but *Equus cabalus* (domestic horse) has only 64 (BENIRSCHKE, et al. 1965). Currently, there remains only a small isolated population in captivity in various countries (MONFORT, et al. 1991). Today all living Przewalskii´s horses descend from 13 individuals, the last captured in Mongolia in 1947 (BOWLING; RYDER, 1987). There are breeding programs for the species in various parts of the world, as is the case of Przewalskii's Horse Species Survival Plan (SSP) in North America; Przewalskii´s horse European Endangered Species Programme (EEP) and the Foundation for the Preservation and Protection of the Przewalskii´s Horse (FPPPH). In Uruguay there was only one specimen originally brought from Poland in 1985, allocated to the Zoológico Municipal Villa Dolores (Montevideo). This

Received: 16/09/10
Accepted: 03/04/11

individual was registered number 1106 in the Studbook of the species, under the name of Warsaw 18. The animal was euthanized in May 2009, at 26 years old, due to complications of advanced age.

The aim of this work was to generate a bank of genetic material and cells of the species, by cryopreserving tissues and cells obtained from the Przewalskii´s horse that existed in Uruguay. This can be used for future research and for genetic conservation.

DEVELOPMENT

Samples

Tissues were obtained from an adult male Przewalskii´s horse (*Equus przewalskii*) from the Zoológico Municipal Villa Dolores (Montevideo, Uruguay), 15 hours after the animal's death. Samples of approximately 1 cm^3 of skin, skeletal muscle, cardiac muscle and ear cartilage were collected. The samples were then submerged in phosphate buffer saline solution (PBS) with antibiotics (100 mg / ml penicillin and 100 U / ml streptomycin) and chilled at 4 °C during 5 hours until processing in the laboratory.

Cryopreservation of tissues

Approximately 5 to 10 pieces of 2-4 mm^3 of each sample were washed by immersion in PBS three times and were then immersed in 1 ml of cryopreservation solution prepared with 70% (v/v) of Minimal Essential Medium with Eagle salts (E-MEM, Sigma-Aldrich, USA), 20% (v/v) fetal calf serum (FCS, Probiomont ®, Montevideo, Uruguay) and 10% (v/v) dimethyl sulfoxide (DMSO, Sigma Co., USA). Each special tubes containing the samples were stored at -80 ° C for 24 h, and then transferred to liquid nitrogen (-196 ° C) for long-term preservation.

Analysis of the viability of the frozen tissue

Twelve months after being frozen in nitrogen, samples of each tissue were thawed quickly (in about 3 min) at 37°C in water bath, washed twice by immersion in E-MEM and then directly cultivated as explants on 24 well-plates (Nunc ®) with E-MEM supplemented with 20% (v/v) of FCS (E-MEM-20%FCS), under a humid atmosphere with 5% CO_2 at 37ºC. The cultures were daily monitored until the appearance of a monolayer of cells progressively growing around the explants. They were maintained for four weeks under the same culture conditions and the medium was replaced every week. During the first two weeks E-MEM-20% FCS was used for cell growth, and then E-MEM-5% FCS was used as maintenance medium. The presence of bacterial and fungal contamination was evaluated microscopically. In addition, every 4 days, samples of the supernatant of the cultures were sown in nutrient broth, thioglycollate broth and Saboureaud medium.

Development of primary cultures from fresh tissue, cell cultures and cryopreservation

Small fragments of tissue removed from each specimen were cut into pieces and directly cultured, as explants, in 24-well plates with E-MEM-20% FCS under a humid atmosphere with 5% (v/v) CO_2 at 37ºC. The growth medium was replaced every week. Then, samples were kept for four weeks or until the appearance of a monolayer of cells around the explants.

The cell cultures generated from each tissue were propagated in bottles of 25 cm^2 (Greiner Cellstar®) and preserved in liquid nitrogen using a method similar to the one described above for freezing tissues. When the monolayer of cells was formed, cells were removed with trypsin (0.25% w/v) – EDTA (0.2% w/v) (Sigma-Aldrich, USA) and frozen at a range of 5 x 10^6 cells/ml with E-MEM-20% FCS and 10% (v/v) DMSO. The vials were kept conventionally at -80 ° C for 24 h, and then transferred to liquid nitrogen (-196ºC) for long-term preservation.

Twelve months after freezing 1 derived tube of each cell culture was thawed and cultured on disposable plates. The growth of cells and the absence of contamination according to the previously described method were monitored every day.

The extraction of tissue samples of Przewalskii´s horse was successful. The fact that the animal had been dead for 15 hours was not detrimental to the samples being processed favorably.

After thawing, the tissues developed homogenous cultures. These cultures showed no bacterial or fungal contamination after four weeks of culture at 37°C. Cells migrated around the fragments after approximately two weeks, which confirmed that the pieces of frozen tissues were viable and able to proliferate after thawing.

Development of primary cultures from skin tissue, ear cartilage, and skeletal muscle was performed. A monolayer was generated around each explant after two weeks of culture, and then was propagated for subsequent freezing. The day in which each cell type reached the confluence was similar: around five days at 37 ºC. After five passages, it was possible to freeze cells by standard methods and to preserve them in liquid nitrogen. It

was not possible to obtain cultures from cardiac muscle. Although in a first stage some development of these cells began, it was unsuccessful afterwards, and after 5 days the cells began to detach. Probably this cell type would have required a richer culture medium.

The generation of cell cultures from frozen tissue fragments and primary cultures was also successful, with similar methodologies as those previously used in other species. However, Tovar et al. (2008) found differences in the morphology and cell type of primary cultures depending from which treatment the cells were generated. Collagenase treatment yielded cells of a more fibroblast-like structure while after trypsin treatment more epithelial-like cells were usually found (TOVAR, et al. 2008). By contrast, in our experience, which was only used trypsin treatment, as judged by cell morphology and shape, we believe that most of the cells are fibroblasts. In addition, there was no apparent difference between growth from fresh and frozen tissues. This properly allows the saving of frozen tissue samples, until they may be used for cell culture (LEEK, 2006; FOWLER, 1984).

In a next step we will check the presence of any virus that could be infecting these cultures, like Herpesvirus, Adenovirus, Retrovirus, among others. Also we will perform genetic studies to deepen the information about the Przewalskii´s horse that existed in Uruguay.

A wide range of frozen tissue samples of endangered animal species could preserve biodiversity for future generations (ACKERS, et al. 2006). *In situ* conservation programs and / or *ex situ* conservation are essential for allowing the expression of the frozen genomes in members of the same gender in the future (BENFORD, 1992). In this way, the results of this work constitute an important contribution to the biodiversity conservation of this species (GLENISTER, et al. 1990). There was no previous tissue preservation of the individual in Uruguay. These tissues and cells, now conserved in liquid nitrogen can contribute to the gene pool of *Equus przewalskii* in the world.

In this manuscript, we described: a) a method for successful conservation of living tissues, with samples taken many hours after the animal has died. This method may also be used for the preservation of specimens of other species, given that one of the main constraints to achieve conservation of viable tissue for the development of cell culture, is the time elapsed between the animal's death and sampling (TOVAR, et al. 2008) and b) the generation of a bank tissues and cells of Przewalskii´s horse, preserved in liquid nitrogen, which may be maintained indefinitely and used for future research.

RESUMO: O Cavalo de Przewalski ou Cavalo selvagem da Mongólia (*Equus przewalskii*, Poljakov, 1881) é o único cavalo selvagem que existe na atualidade. Originário da Ásia é uma espécie seriamente ameaçada, classificada em risco elevado de extinção. O objetivo deste trabalho foi à preservação de tecidos e o desenvolvimento de culturas celulares a partir de amostras teciduais de um Cavalo de Przewalski. Biopsias da pele, músculo esquelético e cardíaco e cartilagem auricular foram coletadas de um cavalo recentemente morto, colocadas em tampão fosfato salino e refrigeradas até o processamento. Uma parte destas amostras foi congelada em nitrogênio líquido e a outra foi utilizada para a cultura em forma de explantos, visando à obtenção de uma monocamada celular de fibroblastos. A geração de culturas celulares foram obtidas e subseqüentemente propagadas e congeladas em nitrogênio líquido com baixas passagens, evitando assim alterações genéticas e fenotípicas. Posteriormente os tecidos e as culturas celulares foram descongelados para testar a viabilidade por meio da visualização do crescimento progressivo em frascos de cultura celular. Não foi possível a obtenção de cultura de músculo cardíaco. Gerou-se um banco de tecidos e células do único Cavalo de Przewalski que existia no Uruguai que poderá ser utilizado com fins científicos e para a conservação da espécie no futuro.

PALAVRAS-CHAVE: Banco de tecidos. Cavalo selvagem. Conservação da espécie.

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