UNIVERSIDADE DE BRASÍLIA
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA

EFFECTS OF LONG COOLING PERIODS OF THE EAR SKIN ON THE ISOLATION AND CULTIVATION OF BOVINE FIBROBLASTS FOR USE IN NUCLEAR TRANSFER TECHNIQUE.

JÉSSICA MARESCH DE ARAÚJO

DISSERTAÇÃO DE MESTRADO EM CIÊNCIAS ANIMAIS

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DISSERTAÇÃO DE MESTRADO SUBMETIDA AO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS ANIMAIS, COMO PARTE DOS REQUISITOS NECESSÁRIOS À OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS ANIMAIS.

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1. Clonagem 2. Refrigeração 3. Reprogramação Nuclear
4. Transporte 5.Viabilidade Celular

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ÍNDICE

RESUMO

O armazenamento e transporte de amostras de animais que vieram a óbito ou estão localizados em fazendas distantes de laboratórios por longos períodos de tempo constitui uma realidade. A necessidade de um protocolo eficiente de armazenamento e transporte destas amostras para a manutenção da viabilidade tecidual do animal de interesse existe, e para tal, as orelhas de oito fêmeas bovinas foram coletadas no momento do óbito, lavadas, tricotomizadas e armazenadas por 30 dias a 5ºC. Nos dias 0, 2, 4, 7, 14, 21 e 30, o cultivo de explantes de pele foi realizado. Em comparação aos diferentes períodos de refrigeração, o tempo para o aparecimento das primeiras células ao redor dos explantes, tempo até confluência, concentração celular no momento do congelamento, taxa de contaminação e viabilidade celular foram observados. Um aumento no tempo de aparecimento dos primeiros fibroblastos foi observado com o aumento do tempo de refrigeração, onde no dia 0, as células levaram 4±0 para aparecer ao redor dos explantes enquanto no dia 30, elas cresceram apenas com 33.5±1.5 dias. Um aumento também ocorreu no tempo até confluência celular com períodos de refrigeração mais longos. Células do dia 0 levaram 24±2 dias para atingir confluência enquanto células do dia 30 atingiram confluência com 31±0 dias. Contaminação foi mais prevalente em períodos posteriores (14, 21 e 30) e a concentração celular caiu drasticamente com o aumento do período de resfriamento, caindo de 1.334.375±131.375cel/mL no dia 0 para 311.250±0cell/mL no dia 30. De igual maneira, a viabilidade celular diminuiu de 85,6±1,7% no dia 0 para apenas 28,72±4,81% no dia 30. Quando células refrigeradas a 5°C por 30 dias foram utilizadas como doadoras de núcleo a técnica de transferência nuclear, uma taxa de blastocisto de 26.05% em D7 foi obtida, uma boa taxa de blastocisto para um longo períodos de refrigeração. Foi observado que a refrigeração de tecido viabiliza a cultura celular para transferência nuclear por longos períodos de refrigeração de amostra. No entanto, o aumento no tempo de armazenamento acarreta danos celulares, dificultando o cultivo, diminuindo consideravelmente a viabilidade celular pós-descongelamento e a concentração celular, mas mantendo a capacidade celular como doadora de núcleo para a produção de embriões clones.

Palavras-Chave: Clonagem, Refrigeração, Reprogramação Celular, Transporte, Viabilidade Celular.

ABSTRACT

Storage and transportation for long periods of time of samples from animals that died or are housed in properties located far away from laboratories is a reality. The need for an efficient protocol of storage and transportation of such samples for maintenance of the animal of interest's tissue viability exists, and for such, ears of eight bovine females were collected on the moment of death, washed, trichotomized and stored for 30 days at 5°C. On days 0, 2, 4, 7, 14, 21 and 30, skin explant culture was performed. In comparison between the different time points, the time for the first fibroblasts to outgrow skin explants, time to confluence, cell concentration on freezing moment, contamination rates and cell viability were observed. An increase in fibroblast outgrowth time was observed with increased storage time, where on day 0, cells took 4±0 days in order to outgrow explants while on day 30, they outgrew with 33.5±1.5 days. An increase also occurred on time to confluence with longer refrigeration periods. Cells from day 0 reached confluence in 24±2 days while day 30 cells took 31±0 days in order to reach confluence. Contamination was more prevalent in posterior periods (14, 21 and 30 days) and cell concentration dropped drastically with increased storage time, reducing from 1.334.375±131.375cell/mL on day 0 to 311.250±0cell/mL on day 30. Likewise, cell viability was reduced with increased time, dropping from 85.6±1.7% on day 0 to only 28.72±4.81% on day 30. When cells cooled at 5°C for 30 days were used as nuclei donors in nuclear transfer technique, a blastocyst rate of 26.05% at D7 was obtained, a good embryonic development rate for a long period of storage. It was found that tissue refrigeration enables viable cell culture for nuclear transfer use for long periods of sample cooling. However, increase in sample storage time brings cell damage, making cultivation more difficult, lowering considerably cell viability post thawing and cell concentration, but maintaining cell capacity as nuclei donor for cloned embryo production.

Keywords: Cell Viability, Cloning, Nuclear Reprogramming, Refrigeration, Transportation

CHAPTER I

1 INTRODUCTION

Animal cloning emerged in science as an important discovery that enabled genetic manipulations such as gene-knockout by homologous recombination (Yang et al., 2000) and transgenic animal production aiming the production of human therapeutic proteins and other proteins. The first recombinant pharmaceutical protein, antithrombin, which was produced in the mammal glands of transgenic goats had its commercialization approved by the European Medicine Agency in 2006 (Kues and Niemann, 2011).

The technique also appears as a tool in cellular pluripotency studies (Verma et al., 2012) and exploration of mechanisms involving embryonic development and nucleus-cytoplasm interactions (Lanza et al., 2000). In addition, cloning also has an important role in endangered species conservation where reproduction by normal means is not possible (Moulavi et al., 2017).

Cloning also has a direct role in agroindustry, helping in the maintenance and multiplication of high genetic merit animals. Some high genetic value stallions and bulls may lose their reproductive capacity due to old age, pathologies, accidents involving the reproductive tract or others and in that moment a clone may take their place. In addition to all above, there is the most common occurrence that leads high genetic merit animal's owners to resort to cloning: death.

The first step in cloning is the recovery of the genetic material from the animal of interest through somatic cell cultivation. The most commonly used cells are somatic cells present on skin, called fibroblasts (Kubota et al., 2000; Kasinathan et al., 2001; Okonkwo e Singh, 2014). In countries with continental proportions like Brazil, it is common for the animal to die on a property and its material only be taken to the lab many hours or days after it's death. For this reason, it is necessary to know the maximum period *post-mortem* that allows the isolation and cultivation of cells from this material and its real potential for use in NT. One of the biggest difficulties faced by the people responsible for the animal of interest's cell cultivation is the lack of an efficient transport and storage protocol on the transfer from the farm to the laboratory. Frequently, the material arrives frozen to the lab, which makes cell cultivation difficult, in other cases the material arrives already in putrefaction state, which makes cell isolation and culture unfeasible.

In that case, this project sought to develop an efficient methodology for bovine skin biopsies transportation and storage for long periods of time in order to obtain viable fibroblasts to compose a cell cryobank for posterior use in nuclear transfer.

Hypothesis

It is possible to isolate ear skin fibroblasts for use in NT, even after long periods of time after the animal's death, when the samples are kept refrigerated.

Objectives

Isolate, cultivate and cryopreserve ear skin fibroblasts refrigerated for long periods of time at 5°C;

Verify the biggest interval between the animal's death and refrigerated ear fragment storage for the isolation of viable fibroblasts;

Study the potential of TN embryo production from cells obtained in long periods of storage.

2 LITERATURE REVIEW

2.1 Cloning history and evolution

The first experiments with animal cloning consisted of embryo bipartition, being the sea urchin the first species to be cloned by this technique in 1891. Hans Driesch managed to separate the blastomeres of a two cell sea urchin embryo by mechanic action, shaking the embryo in a recipient with salt water. The separated cells developed in two distinct sea orchids (Vajta and Gjerris, 2006). In 1902, Hans Spemann cloned the first vertebrae using a hair from his son in order to separate a salamander embryo, obtaining a similar result to that of Driesch (Vajta and Gjerris, 2006).

It was only in 1979 that Willadsen reported the production of two monozygotic sheep produced via micromanipulation, marking then the production of the first clones of domestic animals by embryo splitting (Willadsen, 1979). In 1982 Ozil et al reported the production of Charolais cows monozygotic embryos obtained after superovulation. 6 and 7 day embryos were split in half by micromanipulation and each halve was then put into an empty pellucid zone. The micromanipulated embryos were then transferred to 14 recipient cows, that presented a pregnancy rate of 64.2%. Six recipients carried twins, and at the end of the experiment, 15 fetuses were reported from the 14 pairs of transferred embryos (Ozil et al., 1982).

However, cloning via embryo splitting in addition to presenting a numeric restriction, because embryos can only be divided up to two times originating 4 identical embryos, also presents a problem on the point of view for the replication of high genetic merit animals: because it is still an embryo, it is unknown if such embryo is carrying or not the desired traits that raised interest for cloning in the first place. It is known that even progeny from parents with excellent genetic lineage may not inherit desired characteristics, turning cloning via embryo splitting a mystery in relation to the generated clones.

In 1894, Loeb accidently made a revolutionary discovery that consisted in the basis of all future cloning experiments. His experiment originally consisted in trying to induce parthenogenesis in sea urchin embryos using different salt concentrations on the water. However, Loeb observed the formation of a big blob in some of the early developmental stage embryos. While the embryo developed, the blob remained unchanged. However, he observed that occasionally, when a nucleus entered the blob, it started to develop with the rest of the embryo, and if separated from the embryo, the blob

containing the nucleus developed independently. This was the first evidence that embryos could be formed moving nucleus between different cells (Vajta and Gjerris, 2006).

The first birth of cloned mammals from nuclear transfer was reported by Karl Illmensee and Peter C. Hoppe (1981) that were able to produce three mice using embryonic cells as donors. After mechanical isolation of the trophectoderm and internal cell mass of blastocysts, the internal mass forming cells was separated and their nucleus were injected individually in fertilized mice oocytes (zygotes). After the injection of the new nucleus in each zygote, the male and female pronuclei were removed. Sixteen reconstructed structures were transferred to females in order to observe if gestation went to term. Eventually, the birth of two females and one male occurred, from which, one male and one female later had normal progeny that presented genomic and phenotypical characteristics from the original embryonic cells donors used in nuclear transfer (Illmensee and Hoppe, 1981).

After the results obtained by Illmensee and Hoppe (1981), many experiments tried to replicate the experiment, but without success, until 1986, when Willadsen repeated the experiment using non-fertilized oocytes instead of a zygote as recipient cell for the new nucleus. The non-fertilized oocytes were combined with 8 and 16 cell embryos and transferred to sheep in diestrus. The result was the first cloning in domestic mammals by nuclear transfer (Willadsen, 1986).

However, all these experiments used embryonic cells as nucleus donors and it was only in 1952 that Briggs and King (1952) were able to materialize nuclear transfer cloning of differentiated adult cells. Using frogs in their experiment, donor cells initially came from 4 day morula, tadpoles and eventually from the intestinal epithelium of adult animals. However, it was observed that the more differentiated the donor nuclei cell, the smaller the developmental level of the resulting embryos.

Embryos prevenient from tadpole donor cells resulted in adult frogs, while embryos prevenient from adult frog cells generated tadpoles that did not develop into adult animals (Briggs e King, 1952; Vajta e Gjerris, 2006).

Finally, in 1997 with the birth of Dolly, the sheep, it was demonstrated that adult somatic cells might be successfully used in the production of a cloned adult animal (Wilmut et al., 1997). Since then, the production of clones was already reported in several species such as bovines (Kato et al., 1998), swines (Onishi et al., 2000; Polejaeva et al., 2007), cats (Shin et al., 2002), camels (Wani et al., 2010) and others.

2.2 Somatic Cell Nuclear Transfer

Despite presenting considerable variations around the globe, somatic cell nuclear transfer (SCNT) basically consists in obtaining oocytes that will be the new nucleus recipients, cultivation of nuclear donor cells from the animal of interest, oocyte enucleation, its reconstruction with the genetic material donor cell and the posterior activation of the reconstructed structure that will then be cultivated until it becomes an embryo that may be transferred to a recipient female (Vajta and Gjerris, 2006).

The success of SCNT depends on the obtaining of undamaged DNA prevenient from cells from the animal of interest (animal that is going to be cloned). For such, cell lab cultivation appears as an excellent alternative (Okonkwo and Singh, 2014). The cell type, as its form of cultivation have a direct impact on the success rates of the technique for influencing in the adult cell's DNA reprogramming to an embryonic stage (Wani and Hong, 2018).

Many cell types were already used in NT procedures, showing that cells from different tissues and from donors of different ages may be used. Cells prevenient from ovarian cumulus cells (Wakayama et al., 1998), fibroblasts (Kubota et al., 2000; Kasinathan et al., 2001), Sertoli cells (Ogura et al., 2000), immune system cells (Inoue et al., 2005), mature B and T cells, olfactory, neural stem cells, myoblasts (Gao et al., 2003) adipocytes and amniotic fluid cells (Silva et al., 2016), blastomeres and embryonic stem cells (Campbell et al., 2007) were already successfully used in cloned embryos generation.

It was found that the less differentiated the nucleus donor is, the more easily can the cell be reprogrammed to an embryonic state by the enucleated oocyte (cytoplast) (Silva et al., 2016), for this reason, multipotent cells as blastomeres and embryonic stem cells show a better cloned embryo development when compared to somatic cells (Campbell et al., 2007). However, until the present, despite different somatic and mesenchymal cells being already used in the technique and the higher efficiency of mesenchymal cells being already confirmed, skin fibroblasts are still the most commonly used cells for their facility of their obtainment and cultivation.

2.3 Somatic cells obtained from dead animals

For the obtainment of the nucleus donor cell, it is necessary the obtainment of viable material from the animal of interest. After the animal's death, the survival and proliferation of cells drops due to pH changes, the accumulation of toxins such as oxygen

reactive species (Neta et al., 2018) and the progressive RNA degradation, consequently reducing the genic expression rate leading to tissue death and the unfeasibility of the cultivation of cells present in the tissue (Birdsill et al., 2011).

With the objective of trying to solve this problem, the use of sample refrigeration of such animals was already tested and applied in many species with the intent of slowing down the metabolism and cellular death. Experiments using techniques such as slow freezing (Caamaño et al. 2008; Moulavi et al., 2017), vitrification (Borges et al., 2017) and refrigeration at 4-6^{o}C (Caputcu et al, 2013; Okonkwo & Singh, 2014; Neta *et al*. 2018) of samples were conducted.

Experiments involving samples from humans (Bravo et al., 2000; Turhan-Haktanir et al., 2011; Boekema et al., 2015) and domestic and wild species as bears (Caamaño et al. 2008), camels (Wani and Hong, 2018), goats (Okonkwo and Singh, 2014), pigs (Silvestre et al., 2003; Ge et al., 2010) collared peccaries (Borges et al., 2017), cheetah (Moulavi et al., 2017), sheep (Singh et al., 2011), rabbits (Silvestre et al., 2003) and bovines (Silvestre et al., 2004; Caputcu et al., 2013; Walcott and Singh, 2017) were conducted. Their objective was to investigate the maximum survival period of somatic cells present in those samples when they remain refrigerated for long periods of time and investigate possible differences between cells that were cultivated few hours or days after death and cells cultivated many days or even weeks after death.

It was observed in those experiments that the cooling of the samples showed itself to be efficient in the maintenance of tissue viability for long periods of time, enabling cell isolation and culture for up to 41 days *post-mortem* in goat tissue (Okonkwo and Singh, 2014), 65 days in sheep (Singh and Ma, 2016) and 30 days in collared peccaries (Neta et al., 2018). In bovine, Silvestre et al., (2004), Caputcu et al. (2013) and Walcott and Singh (2017) obtained viable fibroblasts up to 12, 9 and 49 days *post-mortem* respectively. Silvestre et al. (2003) also obtained viable rabbit and pig cells up to 10 and 14 days after their deaths.

However, increase in refrigeration time affected growth rate, time to confluence, concentration and cell viability. In more recent periods in relation to death (up to one week), the first cells took around 4 days in order to outgrow skin explants (Okonkwo and Singh, 2014; Aoued and Singh, 2015) while in posterior periods, time to outgrowth increases significantly. In goat skin biopsies, cultivation performed with material refrigerated for 37 and 41 days took 25 and 32 days respectively to present the first fibroblasts (Okonkwo and Singh, 2014).

An increase in time needed to cell confluence was also observed with increased time of sample cooling (Silvestre et al., 2003; Birdsill et al., 2011; Kim et al., 2014; Boekema et al., 2015; Singh e Ma, 2016; Neta et al., 2018). Cells obtained from rabbit and pig biopsies 14 days after death presented a growth rate of only 33% when compared to cells obtained on the day of death (Silvestre et al., 2003). Goat skin biopsies refrigerated for 41 days presented 19% of growth rate when compared to day of death biopsies (Okonkwo and Singh, 2014) and bovine ear biopsies kept for 49 days at low temperatures presented growth of 16,7% (Walcott and Singh, 2017).

Cells from posterior periods also presented an increase in size and a decrease in final concentration on the moment of freezing, possibly because of the increase in the number of vacuoles present on the cytoplasm of older cells (Walcott and Singh, 2017; Neta et al., 2018).

However, the obtaining and cultivation of fibroblasts for long periods of time alone is not enough for nuclear transfer. The nucleus donor cell must be viable for micromanipulation success. Time of cooling also had great influence in cell viability rates, presenting an inverse proportion between time of refrigeration and cell viability. Collared peccaries cells presented a drop of approximately 60% with 10 days of cooling to approximately 40% with 30 days (Neta et tal., 2018) and pig cells cultivated 15 days *post mortem* had 20% of viable cells per sample (Ge et al., 2010). In human skin culture, at 9 days of refrigeration cells presents approximately 20% of viability, dropping even further at 29 days of cooling, striking only 6% of viability (Bravo et al., 2000). In human skin culture, at 21 days of refrigeration, cells reached 30% of viability (Boekema et al., 2015).

Therefore, sample refrigeration presents itself as a strategy to the conservation of tissues and cell isolation for posterior use in nuclear transfer, however, prolonged refrigeration time has a negative impact in obtained cell quality, therefore, such strategy must only be used in case of need.

2.4 Cell reprogramming and epigenetics

Even though cloning via nuclear transfer is a technique that is increasingly progressing, the success rates of NT are still very low. Currently the success rate of cloning spins around 0-10% of born animals per transferred embryos, and even many

born animals still present a number of diseases and developmental malformations characterized as *large offspring syndrome* (Silva et al., 2019). High abortion and fetal mortality rates exist, and such problems have been attributed to incomplete reprogramming of the somatic cell nuclei on cloning process (Tian et al., 2003).

In order to figure out the causes of such low success rates and how to address them, it is important to understand first the epigenetic mechanisms involved in cell reprogramming (Silva et al., 2019), which consists in the reversion of a differentiated nucleus back to its pluripotent status (Tian et al., 2003).

Epigenetic is defined by Iager et al (2008) as genic expression modifications that are not caused by DNA sequencing alterations. This definition partly explains why in a living organism that has the same DNA inside all cells, there are so many different cell types and tissues (Silva et al., 2019).

DNA methylation is characterized by epigenetic markings that control gene expression in every cell type and is one of the most important mechanisms that control epigenetic modifications. Despite having the same DNA, different cells express different genes and produce different proteins. Methylation marks prevent some gene group's transcription, silencing those genes and defining what genes that particular cell can and cannot express. Methylation also guides embryo development, determining in which cell type that particular pluripotent cell is going to differentiate into (Tian et al., 2003). In vertebrates, methylation occurs by binding of a methyl group to the position 5 of cytosine (5-methylcytosine). This binding happens especially in cytosine-guanine dinucleotides (Silva et al., 2019). Because they are already differentiated and already present specific epigenetic markings for their specific cell type, adult cells present a bigger challenge for nuclear reprogramming than less differentiated cells (Silva et al., 2019).

Another important epigenetic control mechanism is histone tail acetylation. The fundamental unit of chromatin is the nucleosome, formed by histone proteins H1, H2A, H2B, H3 and H4. The N-terminal extremities of all histones with exception of H1 extend on the nucleosome surface, being susceptible to modifications, altering chromatin structure. One of the biggest changes that embryonic chromatin is going to suffer during development is histone acetylation (Silva et al., 2019). Two families of enzymes regulate histone tail acetylation levels: histone acetyltransferases (HATs), that catalyze the addition of acetyl groups to lysine residues and histone deacetylases (HDACs) that remove them (Iager et al., 2008). HATs can be divided in two families, and one of them, known as KAT2A (or GCN5) is found inside the nucleus and plays an important role in

gene expression regulation acting as a transcriptional coactivator. In general, deacetylation impairs gene expression and its consequent transcription while acetylation enables gene expression, unzipping the chromatin and giving access to DNA transcriptional apparatus (Silva et al., 2019).

The enucleated oocyte (karyoplast) is responsible for receiving, completely deprogramming and then reprogramming the cell nuclei to a pluripotent state, capable of generating a new individual, firstly with the embryonic and posteriorly with tissue specific epigenetic markings. An abnormal epigenetic reprogramming may lead to an equally abnormal genic expression, entailing fetal and post-natal problems (Silva et al., 2019). In natural fertilization, the oocyte rapidly demethylates paternal DNA and maternal DNA also undergoes a slow and passive process of demethylation. Therefore, the oocyte already has the mechanisms to induce DNA demethylation, however, for being an artificial "fertilization" technique that uses a far more methylated DNA in its composition, DNA demethylation is far more difficult in SCNT (Iager et al., 2008).

The ideal status to achieve a correct nuclei reprogramming is of low DNA methylation and histone hyperacetylation (Silva et al., 2019), similar to that of naturally fertilized embryos (Iager et al., 2008).

With the intent of solving this reprogramming issues, a number of different strategies have been used, being among them the modification of epigenetic marks of donor cells with drugs (Tian et al., 2003). One of the mostly used drugs to improve histone acetylation levels in SCNT embryos is Trichostatin A (TSA). TSA consists in a HDAC inhibitor, inhibiting therefore the removal of acetyl groups from histones and favoring gene expression and DNA transcription, increasing SCNT blastocyst rates and resulting in less problematic offspring (Tian et al., 2003).

3 REFERENCES

AOUED, H. S., SINGH, M. Recovery of fibroblast-like cells after 160 days of postmortem storage of goat skin tissues in refrigerated media. *J. Veterinar. Sci. Techolo* v.6, 1000236, 2015.

BIRDSILL, A. C., WALKER, D. G., LUE, L., SUE, L. I., BEACH T. G. Postmortem interval effect on RNA and gene expression in human brain tissue. *Cell Tissue Bank* v.12, p.311-318, 2011.

BOEKEMA, B. K. H. L., BOEKESTIJN, B., BREEDEVELD, R. S. Evaluation of saline, RPMI and DMEM/F12 for storage of split-thickness skin grafts. *Burns* v.41, p.848-852, 2015

BORGES, A. A., LIMA, G. L., NETA, L. B. Q., SANTOS, M. V. O., OLIVEIRA, M. F., SILVA, A. R., PEREIRA, A. F. Conservation of somatic tissue derived from collared peccaries (Pecari tajacu Linnaeus, 1758) using direct or solid-surface vitrification techniques. *Cytotechnology* v.69, p.643-654, 2017

BRAVO, D., RIGLEY, T. H., GIBRAN, N., STRONG D. M., NEWMAN-GAGE, H. Effect of storage and preservation methods on viability in transplantable human skin allografts. *Burns* v.26, p.367-378, 2000

BRIGGS, R., KING, T. J. Nuclear transplantation studies on the early gastrula (*Rana pipiens*). *Dev. Biol.* v.2, p.252–270, 1952

CAAMAÑO, J. N., RODRIGUEZ. A., MUÑOZ, M., FRUTOS, C., DIEZ, C., GOMEZ, H. Cryopreservation of brown bear skin biopsies. *Cell Preservation Technology* v.6, p.83-86, 2008

CAMPBELL, K. H. S., FISHER, P., CHEN, W. C., CHOI, I., KELLY, R. D. W., LEE, J. H., XHU, J. Somatic cell nuclear transfer: Past. Present and future pespectives. *Theriogenology* v.68, p.214-231, 2007

CAPUTCU, A., AKKOC, T., CETINKAYA, G., ARAT, S. Tissue cryobanking for conservation programs: effect of tissue type and storage time after death. *Cell Tissue Bank* v.14, p.1-10, 2013.

GAO, S., MCGARRY, M., LATHAM, K. E., WILMUT, I. Cloning of mice by nuclear transfer. *Cloning Stem Cells* v.5, p.287-294, 2003

GE, L., SUN, L., CHEN, J., MAO, X., KONG, Y., XIONG, F., WEI, H. The viability change of pigskin in vitro. *Burns* v.36, p.533-538, 2010

IAGER, A. E., REGINA, N. P., ROSS, P. J., BEYHAN, Z., CUNNIFF, K., RODRIGUEZ, R. M., CIBELLI, J. B. Trichostatin A improves histone acetylation in somatic cell nuclear transfer early embryos. *Cloning and Stem Cells* v.10, p.371-379, 2008)

ILLMENSEE, K., HOPPE, P. C., 1981. Nuclear transplantation in *Mus musculus*: developmental potential of nuclei from preimplantation embryos. *Cell* v.23, p.9–18, 1981

INOUE, K., WAKAO, H., OGONUKI, N., MIKI, H., SEINO, K., NAMBU-WAKAO, R., NODA, S., MIYOSHI, H., KOSEKI, H., TANIGUCHI, M., OGURA, A. Generation of cloned mice by direct nuclear transfer from natural killer T cells. *Curr. Biol.* v.15, p.1114-1118, 2005

KASINATHAN, P., KNOTT, I. G., WANG, Z., JERRY, D. J., ROBJ, J. M. Production of calves from G1 fibroblasts. *Nat. Biotechnol.,* v.19, p.1176-1178, 2001.

KATO, Y., TANI, T., SOTOMARU, Y., KUROKAWA, K., KATO, J., DOGUCHI, H., YASUE, H., TSUNODA, Y. Eight calves cloned from somatic cells of a single adult. *Science* v.282, p.2095–2098, 1998.

KIM, B. Y., NAM, B. M., LEE, K. M., JO, Y. H., NEMENO, J. G., YANG, W. LEE, S., KIM, I. J., JANG, T., TABEKE, J. I. Effect of preservation conditions on cartilage tissue for cell transplantation, *Transplantation Proceedings* v.46, 1.139-1.144, 2014.

KUBOTA, C.; YAMAKUCHI, H.; TODOROKI, J.; MIZOSHITA, K.; TABARA, N.; BARBER, M.; YANG, X. Six cloned calves produced from adult fibroblast cells after long-term culture. *PNAS* v.97, p.990-995, 2000.

KUES, W. A.; NIEMANN. H. Advances in animal transgenesis. *Preventive Veterinary Medicine* v.102, p.146-156, 2011.

LANZA, R. P., CIBELLI, J. B., DIAZ F., MORAES, C. T., FARIN, P. W., FARIN, C. E., HAMMER C. J., WEST, M. D., DAMIANI, P. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. *Cloning* v.2, p.79-90, 2000.

MOULAVI, F., HOSSEINI, S. M., TANHAIE-VASH, N., OSTADHOSSEINI, S., HOSSEINI, S. H., HAJINASROLLAH, M., ASGHARI, M. H., GOURABI, H., SHAHVERDI, A., VOSOUGH, A. D., NASR-ESFAHANI, M. H. Interspecies somatic cell nuclear transfer in Asiatic cheetah using nuclei derived from post-mortem frozen tissue in absence of cryo-protectant and *in vitro* matured cat oocytes. *Theriogenology* v.90, p.197-203, 2017.

NETA, L. B. Q., LIRA, G. P. O., BORGES, A. A., SANTOS, M. V. O., SILVA, M. B., OLIVEIRA, L. R. M., SILVA, A. R., OLIVEIRA, M. F., PEREIRA, A. F. Influence of storage time and nutrient medium on recovery of fibroblast-like cells from refrigerated collared peccary (*Pecari Tajacu* Linnaeus, 1758) skin. *In Vitro Cell. Dev. Biol. Anim.* v.54, p.486-495, 2018.

OGURA, A., INOUE, K., OGONUKI, N., NOGUCHI, A., TAKANO, K., NAGANO, R., SUZUKI, O., LEE, J., ISHINO, F., MATSUDA, J. Production of male cloned mice from fresh, cultured and cryopreserved immature Sertoli cells. *Biol. Reprod.* v.62, p.1579-1584, 2000.

OKONKWO, C., SINGH, M. Recovery of fibroblast-like cells from refrigerated goat skin up to 41 d of animal death. *In Vitro Cell. Dev. Biol. Anim.* v.51, p.463-469, 2014.

ONISHI, A., IWAMOTO, M., AKITA, T., MIKAWA, S., TAKEDA, K., AWATA, T., HANADA, H., PERRY, A. C. Pig cloning by microinjection of fetal fibroblast nuclei. *Science* v.289, p.1188–1190, 2000.

OZIL, J. P., HEYMAN, Y., ENARD, J. P., Production of monozygotic twins by micromanipulation and cervical transfer in the cow. *Vet. Rec*. v.110, p.126–127, 1982.

POLEJAEVA, I. A., CHEN, S. H., VAUGHT, T. D., PAGE, R. L., MULLINGS, J., BALL, S., DAI, Y., BOONE, J., WALKER, S., AYARES, D. L., COLMAN, A., SHI, D., LU, F., WEI, Y., CUI, K., YANG, S., WEI, J., LIU, Q. Buffalos (*Bubalus bubalis*) cloned by nuclear transfer of somatic cells. *Biol. Reprod*. v.77, p.285–291, 2007.

SHIN, T., KRAEMER, D., PRYOR, J., LIU, L., RUGILA, J., HOWE, L., BUCK, S., MURPHY, K., LYONS, L., and WESTHUSIN, M. A cat cloned by nuclear transplantation. *Nature* v.415, p.859–859, 2002.

SILVA, C. G., MARTINS, C. F., CARDOSO, T. C., CUNHA, E. R., BESSLER, H. C., MARTINS, G. H. L., PIVATO, I., BAO, S. N. Production of bovine embryos and calves cloned by nuclear transfer using mesenhymal stem cells from amniotic fluid and adipose tissue. *Cellular Reprogramming* v.18, p.127-136, 2016

SILVA, C. G., MARTINS, C. F., BESSLER, H. C. C., MORAES, A. F. N., CARDOSO, T. C., FRANCO, M. M., MENDONÇA, A. S., LEME, L. O., BORGES, J. R. J., MALAQUIAS, J. V., BÁO, S. N. Use of trichostatin A alters the expression of HDAC3 and improves in vitro development of bovine embryos cloned using less methylated mesenchymal stem cells. *Reproduction in Domestic Animals*, v.54, p. 289-299, 2019

SILVESTRE, M. A., SAEED, A. M., CERVERA, R. P., ESCRIBA, M. J., GARCIA-XIMENEZ. F. Rabbit and pig ear skin sample cryobanking: effects of storage time and temperature of the whole ear extirpated immediately after death. *Theriogenology* v.59, p.1469-1477, 2003

SILVESTRE, M. A.; SANCHEZ, J. P., GOMEZ, E. A. Vitrification of goat, sheep and cattle skin samples from whole ear extirpated after death and maintained at different storage times and temperatures. *Cryobiology* v.49, p.221-229, 2004

SINGH, M., MA, X., AMOAH, E., KANNAN, G. In vitro culture of fibroblast-like cells from post mortem skin of Katahdin sheep stored at 4◦C for different time intervals. *In Vitro Cell. Dev. Biol. Anim*. v.47, p.290-293, 2011

SINGH, M., MA, X. Recovery of fibroblast cells up to 65 d of postmortem storage of sheep ear skin at 4°C. *J Anim Sci* v.94, p.837–838, 2016

TIAN, X. C., KUBOTA, C., ENRIGHT, B., YANG, X. Cloning animals by somatic cell nuclear transfer – biological factors. *Reproductive Biology and Endocrinology* v.98, online issue, 2003

TURHAN-HAKTANIR, N., DILEK, F. H., KÖKEN, G., DEMIR, Y., YIMAZ, G. Evaluation of amniotic fluid as a skin graft storage media compared with RPMI and saline. *Burns* v.37, p.652-655, 2011

VAJTA, G., GJERRIS, M. Science and technology of farm animal cloning: State of the art. *Animal Reproduction Science* v.92, p.211-230, 2006.

VERMA, R., HOLLAND, M. K., TEMPLE-SMITH, P., VERMA, P. J. Inducing pluripotency in snow leopard (*Panther uncia*) an endangered felid. *Theriogenology* v.77, p.220-228, 2012.

WALCOTT, B., SINGH, M. Recovery of proliferative cells up to 15- and 49-day postmortem from bovine skin stored at 24◦C and 4◦C respectivelly. *Cogent Biology* v.3:1333760, 2017.

WANI, N. A., WERNERY, U., HASSAN, F. A. H., WERNERY, R., SKIDMORE, J. A. Production of the first cloned camel by somatic cell nuclear transfer. *Biol. Reprod*. v.82, p.373–379, 2010.

WANI, N. A., HONG, S. B. Source, treatment and type of nuclear donor cells influences in vitro and in vivo development of embryos cloned by somatic cell nuclear transfer in camel (*Camelus dromedaries*). *Theriogenology* v.106, p.186-191, 2018.

WAKAYAMA, T., PERRY, A. C., ZUCCOTTI, M., JOHNSON, K. R. YANAGIMACHI, R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* v.394, p.394-374, 1998.

WILLADSEN, S. M. A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature* v.277, p.298–300, 1979.

WILLADSEN, S. M. Nuclear transplantation in sheep embryos. *Nature* v.320, p.63-65, 1986

WILMUT, I., SCHNIEKE, A. E., MCWHIR, J., KIND, A. J., CAMPBELL, K. H., Viable offspring derived from fetal and adult mammalian cells. *Nature* v.385, p.810–813, 1997.

YANG, Y., ISAAC, C., WANG, C., DRAGON, F., POGACIC, V., MEIER, U. T. Conserved composition of mammalian box X/ACA and box C/D small nucleolar ribonucleoprotein particles and their interaction with the common factor Nopp140. *Mol. Biol. Cell* v.11, p.567-577, 2000.

CHAPTER II

Effects of long cooling periods of the ear skin on the isolation and cultivation of bovine fibroblasts for use in nuclear transfer technique.

1 RESUMO

O objetivo deste estudo foi determinar até quando após a morte de um animal ainda é possível obter fibroblastos viáveis com o objetivo de formar um criobanco para posterior uso em transferência nuclear. Muitos animais com alto valor genético morreram subitamente sem ter tido seu germoplasma conservado in vitro. Com o intuito de simular o transporte da fazenda ao laboratório e determinar até quando após o óbito de um animal ainda e possível isolar e cultivar fibroblastos, orelhas de 8 fêmeas bovinas foram obtidas no momento da morte, tricotomizadas, lavadas com detergente neutro, borrifadas com álcool 70% e preservadas por 30 dias a 5°C em geladeira de temperatura controlada. Nos dias 0, 2, 4, 7, 14, 21 e 30 (nomeados D0 a D30), o isolamento e cultivo de fibroblastos foi realizado em meio Dulbecco's modification of minimum essential media (DMEM) com 10% de soro fetal bovino (SFB). Quando em confluência celular, células foram criopreservadas com 10% de Dimetilsulfóxido (DMSO). As seguintes analises foram realizadas: períodos de tempo que permitiram cultivo celular, tempo até o aparecimento das primeiras células ao redor dos explantes de pele, tempo até confluência (contando a partir do dia que as primeiras células cresceram ao redor dos explantes), taxa de contaminação, concentração celular no momento do congelamento e viabilidade celular pós-descongelamento através de integridade de membrana por citômetro de fluxo (utilizando-se Anexina e iodeto de propídio para identificar células viáveis, necróticas e apoptóticas). Teste de médias e teste de Tukey foram utilizados para comparar dados a 5%. Todos os períodos permitiram cultivo celular. Contaminação foi mais prevalente nos dias 14, 21 e 30. O tempo para crescimento celular inicial aumentou com o aumento do tempo de armazenamento onde células do dia 0 (dia do óbito) levaram 4±0 dias para começar a crescer enquanto células do dia 14 levaram 19,6±2,19 dias e células do da 30 precisaram de 33,5±1,5. Periodos posteriores também levaram mais tempo para atingir confluência, onde células do dia 0 levaram 24±2 dias enquanto nos dias 7, 14, 21 e 30 as células precisaram de 36±4, 36±0, 34,5±4,04 e

36,0±0 dias respectivamente. A concentração celular reduziu drasticamente nos dias 14, 21 e 30 assim como viabilidade nos dias 21 e 30, caindo de 1.334.375±131.375 cel/mL no dia 0 para 304.166±47.604 e 311.250±0 nos dias 21 e 30. Foi concluído que a refrigeração da pele de orelha de bovinos a 5°C consiste em uma importante estratégia para o transporte de tecido bovino por longas distancias e obtenção de células viáveis por até 30 dias após o óbito de um animal. No entanto, o aumento no tempo de refrigeração interfere na taxa de proliferação até o congelamento e padrão de viabilidade após criopreservação. Apesar dos efeitos negativos acarretados por longos períodos de refrigeração, fibroblastos obtidos de biopsias refrigeradas por 30 dias consecutivos permitiram a produção de uma taxa de blastocistos clonados de 26,05%, indicando que esta metodologia pode ser usada em casos extremos de transporte por longas distancias.

Palavras-chave: Clonagem, refrigeração, reprogramação nuclear, transporte, viabilidade celular.

2 ABSTRACT

The purpose of this study was to determine how long after an animal's death it is possible to obtain viable fibroblasts in order to form a cryobank for further use in nuclear transfer. Many animals with high genetic value have died suddenly without having their germplasm conserved in vitro. In order to simulate tissue transportation from the farm to the lab and to determine how long after an animal's death it is possible to isolate and cultivate fibroblasts, ears of 8 bovine females were obtained on the moment of death, trichotomized, washed with neutral detergent, sprayed with 70% alcohol and preserved for 30 days at 5°C on a temperature controlled refrigerator. On days 0, 2, 4, 7, 14, 21 and 30 post mortem (named D0 to D30), isolation and cultivation of fibroblasts were performed on Dulbecco's modification of minimum essential media (DMEM) medium with 10% of Fetal Bovine Serum. When in cellular confluence, cells were cryopreserved in DMEM with 10% of Dimethylsulfoxide (DMSO). The following analyses were performed: Periods of time that allowed cell culture, time until initial cell outgrowth around skin explants, time until confluence was reached (counting from when the first cells outgrew skin explants until they were confluent), contamination rates, cell concentration on freezing moment and cell viability through membrane integrity via flow cytometry (using anexin and propidium iodide in order to verify viable, necrotic and apoptotic cells). Test of means and Tukey's test were used to compare data at 5%. All time points allowed cell isolation and culture. Contamination was more prevalent on days 14, 21 and 30. Cell initial outgrowth time increased with increasing time points where cells from D0 (day of death) took 4±0 days in order to begin outgrowing explants while D14 cells took 19.6±2.19 days and D30 cells needed 33.5±1.5 days. Later time points also needed more time in order to reach confluence, where D0 cells took 24±2 days while D7, D14, D21 and D30 cells took 36±4, 36±0, 34.5±4.04 and 36.0±0 days respectively. Concentration decreased drastically on days 14, 21 and 30 and so did cell viability on days 21 and 30, dropping from 1.334.375±131.375 cells/mL on D0 to 304.166±47.604 and 311.250±0 cells/mL on days 21 and 30. It was concluded that cooling of the bovine ear skin at 5°C is an important strategy for transporting bovine tissue for long distances and obtainment of viable cells up to 30 days after an animal's death. However, the increase in cooling time interferes on the proliferation rates until freezing and viability cell patterns after cryopreservation. Despite negative effects entailed by long periods of cooling,

fibroblasts obtained from 30 consecutive day cooling biopsies allowed 26.05% of cloned blastocysts production, indicating that this methodology can be used in extreme cases of long distance transportation.

Keywords: Cell viability, cloning, nuclear reprogramming, refrigeration, transportation

3 INTRODUCTION

The nuclear transfer (NT) produced blastocyst's competence has been demonstrated firstly by the production of live animals (Wilmut, 1997) and secondly by the derivation of embryonic stem cells prevenient from the embryo's internal cell mass (Wakayama et al., 1998). These observations provided definitive proof that a somatic cell nucleus may be reprogrammed to a pluripotent state by the oocyte's cytoplasmic factors, and that the reprogrammed nucleus may develop into a full individual.

It is known that epigenetics plays a major role in nuclear reprogramming, and mechanisms such as DNA methylation and histone acetylation are currently on the radar of a great number of researchers due to their importance in NT success (Tian et al., 2003)

Aberrant epigenetic patterns directly affect nuclear reprogramming and embryo development, as such epigenetic marks are strictly correlated to gene expression and are responsible for orchestration of embryonic development and tissue differentiation (Iager et al., 2008).

Most experiments aiming epigenetic improvement focused on the nuclei donor cell (Iager et al., 2008), showing that the cell selected to be the nuclei donor is of major importance, because it carries the desired DNA.

The isolation and conservation of viable cells is the first step before using preserved material in nuclear transfer technique aiming the multiplication of farm or wild animals. Cloning via nuclear transfer using somatic cells as nuclei donors allows the isolation of cell lines to become an alternative to the conservation of genetically valuable animals (Okonkwo e Singh, 2014). A critical aspect for the establishment of such lineages is the period between biopsy collection and sample processing to initiate cultivation. In continental countries, animals may be in faraway properties or farms with difficult access, which may greatly extend this period.

Genome integrity is essential for successful cloning of animals (Hoshino et al. 2009). The quality of nuclear donor cells including their viability and proliferation in culture ensures their genomic integrity and enhances the success rate of full-term development of animals in a cloning experiment (Mastromonaco et al. 2006).

Different researchers have showed in vitro culture of bovine cells from live or dead animal tissue conserved at low temperatures. Silvestre et al. (2004) have shown bovine fibroblasts obtained up to 12 days of storage at 4°C. Bovine muscle and cartilage cells were cultivated for up to 9 days at 4°C storage (Caputcu et al., 2013) and Walcott

and Singh, 2017 reported that bovine fibroblasts outgrew skin samples up to 49 days *post mortem* also at 4°C. However, none of those studies used those cells in SCNT in order to produce embryos.

After sample removal from the donor animal, the survival and proliferation of cells drops due to pH changes, the accumulation of toxins such as oxygen reactive species (Neta et al., 2018) and the progressive RNA degradation, consequently reducing the genic expression rate leading to tissue death and the unfeasibility of the cultivation of cells present in the tissue (Birdsill et al., 2011).

The use of low temperatures as a strategy for longer tissue viability conservation had its efficiency tested by a great number of experiments in different species, whether it was simple sample cooling at low temperatures of 4-6°C (Silvestre et al., 2004; Caputcu et al, 2013; Okonkwo & Singh, 2014; Walcott and Singh, 2017; Neta et al. 2018), slow freezing (Caamaño et al. 2008; Moulavi et al., 2017) or vitrification (Borges et al., 2017).

All of these experiments verified that refrigeration offered good results regarding cell isolation after long periods of time when refrigerated samples were compared with fresh samples.

The objective of this study was to determine if refrigerated bovine skin fragments could still be used for fibroblast isolation and cultivation up to 30 days *post mortem*, verify possible differences in different time point cultivated cells and discover if cells cultured many days or weeks after the animal's death could be successfully used in the production of cloned embryos.

4 MATERIALS AND METHODS

4.1 Experimental Design

The experiment was delineated in randomized blocks with seven treatments (refrigeration time points) and eight blocks (ears). The effects of treatments over outgrowth time, confluence, concentration, contamination, cell viability, apoptosis and necrosis post thawing were evaluated via variance analyses (ANOVA) and comparison of medium by Tukey's test with significance level of $p<0.05$.

4.2 Ethics and animal welfare

All ears were excised from abattoir cattle or animals that died of natural causes in nearby farms, so no animals were slaughtered for this experiment, creating no ethical or moral issues.

4.3 Skin sample obtainment

For this experiment, ears of 6 Nelore bovine females, 24 months of age prevenient from the same farm were collected on the moment of slaughter. Ears of another 2 Gir cows over 15 years of age were also collected after natural causes obit in local farms. All samples were transported to the lab in a container with recyclable ice and a thermometer that marked 5ºC. Transportation lasted around 1 hour.

In the lab, ears were trichotomized, washed 5 times with neutral detergent, rinsed off with water, dried with sterilized tissues, sprayed with 70% alcohol and left to dry completely before being stored in individual sterilized *Ziplock*® type bags. All ears were kept in a temperature-controlled fridge at 5ºC for 30 days.

On days 0 (day of death), 2, 4, 7, 14, 21 and 30 (named D0 through D30), cell cultivation was conducted.

4.4 Fibroblast isolation, culture and freezing

A small piece of each ear (3cm x 3cm) was cut off, washed with neutral detergent, dried with sterile tissue, sprayed with 70% alcohol and left to dry into a sterilized petri dish. The sample was then taken to the laminar flow chapel where with scalpel and tweezers the skin was separated from the cartilage and 9 small pieces of skin (0,5cm x 0,5cm) were fabricated and placed on the bottom of a cell cultivation dish.

After 10 minutes waited so that the skin fragments could dry and attach to the bottom of the dish, the dish was then completed with 2,5mL of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Life Science, Rockville, MD, USA) with 10% of bovine fetal serum (Silva et al., 2016) (Figure 1). Dishes were observed every two days in order to locate and register initial cell outgrowth, identify and control possible contaminations, observe how long it took for cells to reach confluence and ultimately freeze those cells and calculate cell concentration on freezing moment. DMEM was changed for nutrient renovation every 4 days when no contamination was present and every 2 days when contamination was detected.

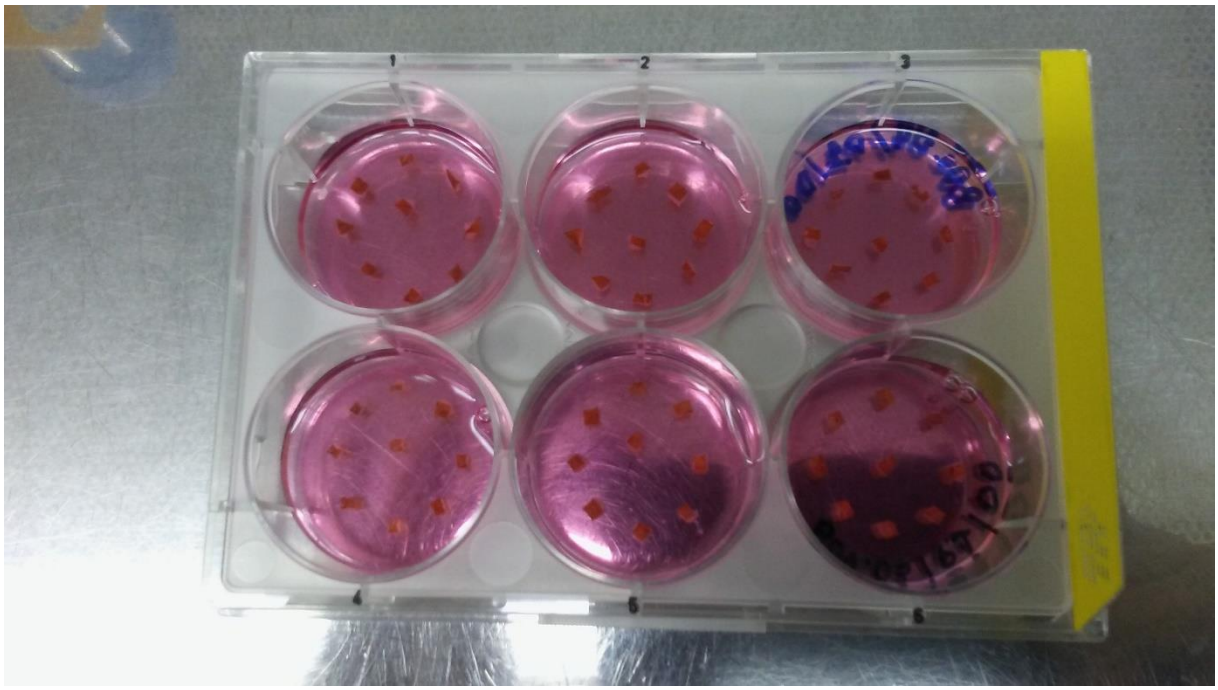


Figure 1 – Cultivation dish of D0 samples from two different bovines (3 wells per animal per time point in total)

When in 70-90% confluence, cells were detached from the bottom of the dish with 0.25% Trypsin (Sigma-Aldrich) and incubated for 6 minutes, transferred to 1,5mL Eppendorf tubes and centrifuged for 6 minutes at 5.000rpm. Once centrifuged, the supernatant was discarded leaving only the cell pellet at the bottom of the tube. The pellet was then resuspended with a solution composed of 90% DMEM (with the standard 10% bovine fetal serum and antibiotics in its composition) and 10% Dimethyl Sulfoxide (DMSO) as cryoprotectant. Once resuspended and homogenized, the solution was then pulled into 0,25mL identified straws and sealed. Straws were then stored for 24h into a -80^{o}C freezer and then dipped into liquid nitrogen, being stored in liquid nitrogen canisters (Arantes et al., 2020) (Figure 2).

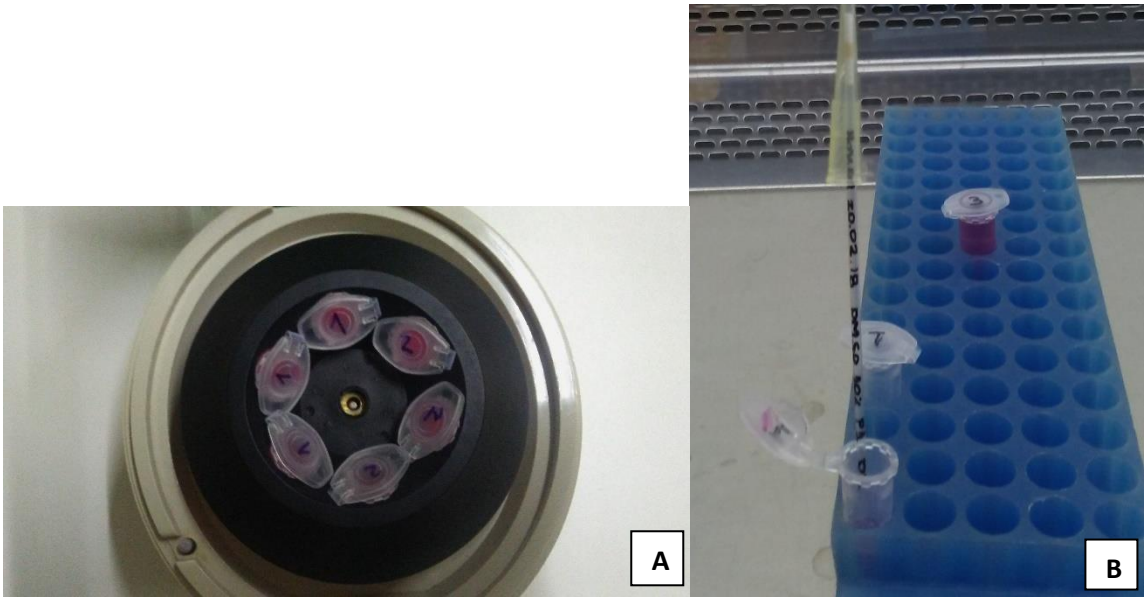


Figure 2 – Centrifuged eppendorffs from two different animals ready for freezing after detachment from the bottom of the plate with trypsin (A) and resuspended cells begin pulled into 0,25mL straws for cooling and feerzing (B).

The remaining solution in the Eppendorf tubes was used in order to calculate cell concentration on freezing moment in Neubauer chamber. In total, 64 squares were counted in each sample. The result was divided by four and the resulting number multiplied by 10 in order to obtain cell concentration per mL (Arantes, et al., 2020) (Figure 3).

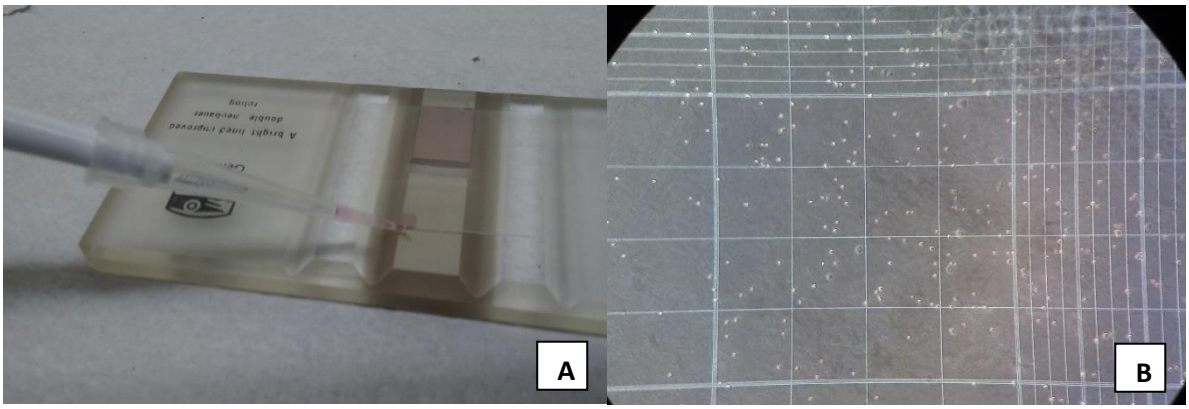


Figure 3 – Mounting of Neubauer chamber (A) and cell concentration count under 20x lens inverted microscope (B).

4.5 Somatic Cell Nuclear Transfer (SCNT)

When in confluence for approximately 2 days (when cells had proliferated and covered completely the bottom of the cultivation dish), D30 fibroblasts were used in Nuclear Transfer as donor nuclei in order to investigate if they could be successfully used to produce cloned embryos.

For the cloning process, a Narishige micromanipulator was used (model IM-9B, Narishige, East Meadow, NY, USA) comporting a Nikon inverted microscope (model Ti-

S, Nikon Instruments, Melville, NY, USA). The protocol used in this experiment was according to Silva et al. (2019).

Oocytes from abattoir ovaries were obtained, selected according to cumulus cells and cytoplasm integrity and only degrees 1 and 2 oocytes were maturated for 18 hours. After the 18h mark, cumulus cells were removed by pipetting for 2 minutes after oocytes remained for 3 minutes inside the incubator in hyaluronidase. Oocytes that presented the first apparent polar body were used for enucleation. Using the first polar body as reference, both polar body and metaphase plate were removed and one fibroblast was injected into the periviteline space. For confirmation of metaphase plate removal, oocytes were stained with Hoechst 33342 (bisBenzemide H33342 trihydrochloride) (Figure 4).

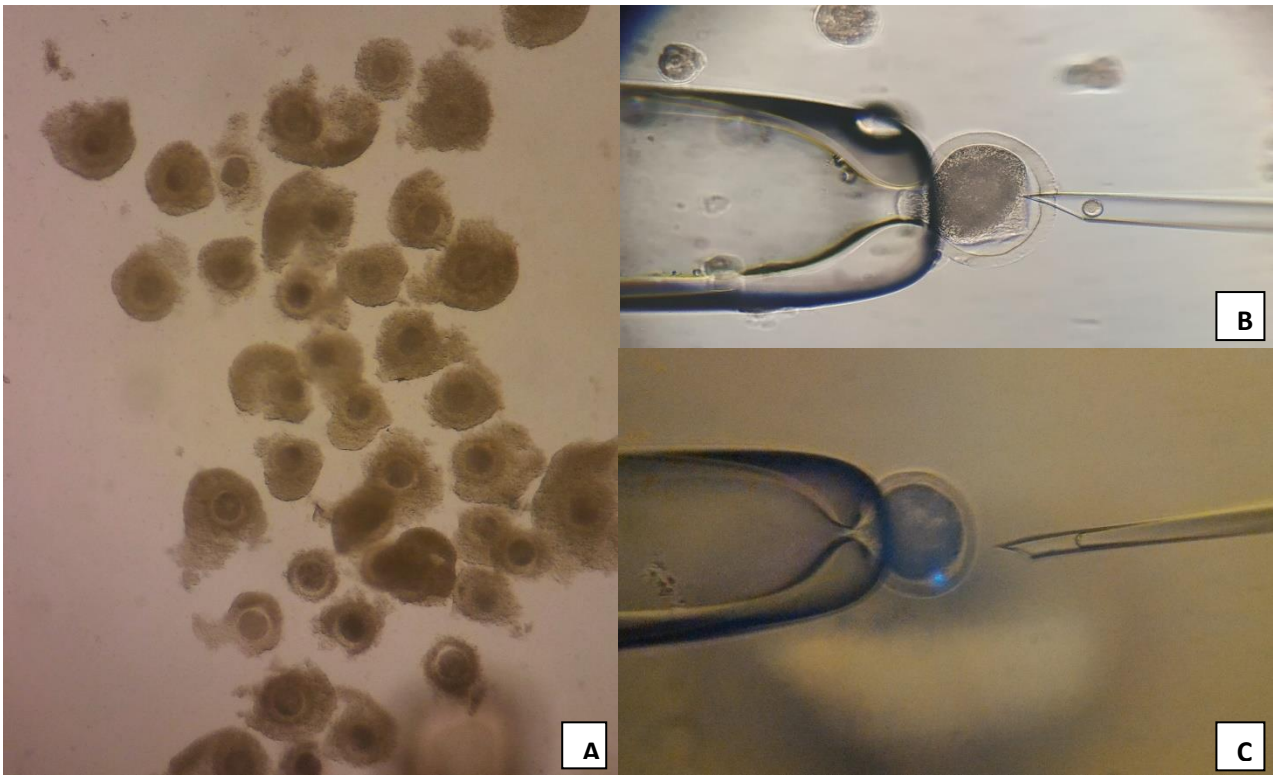


Figure 4 – Degrees I and II oocytes selected for maturation (A), fibroblast injection into a cytoplast's periviteline space (B) and Hoechst DNA coloring to cofirm oocyte DNA removal (C).

After reconstruction, karyoplast-cytoplast couplets were fused in a microslide fusion chamber containing stainless steel electrodes with a 1mm gap between them. An electron cell fusion/activation system (Voltain EP-1, Cryologic, Blackburn, Victoria, Australia) was used in couplet fusion. The fusion solution used was a 0.3M mannitol that filled the chamber allowing the couplet to be placed between the two electrodes and conducted electric chock appropriately.

Karyoplast-cytoplast couplets were fused in two consecutive DC pulses (pulse, 140 volts; alignment frequency, 100 kHz; pulse time, 20 ls; amplitude, 0.7–14 peak volts). After a minimum of 20 minutes, couplets were evaluated for fusion by microscopic examination (Figure 5).

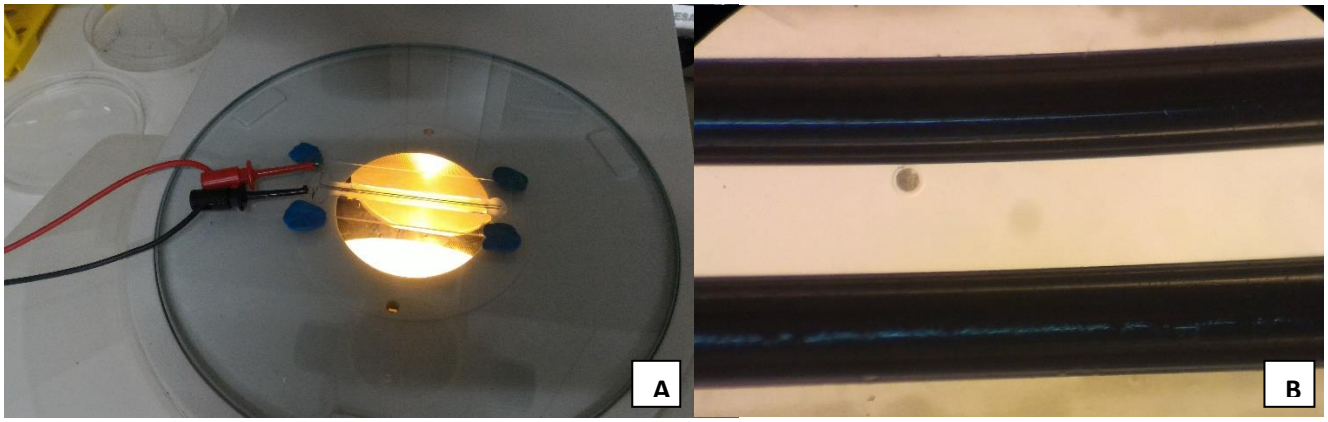
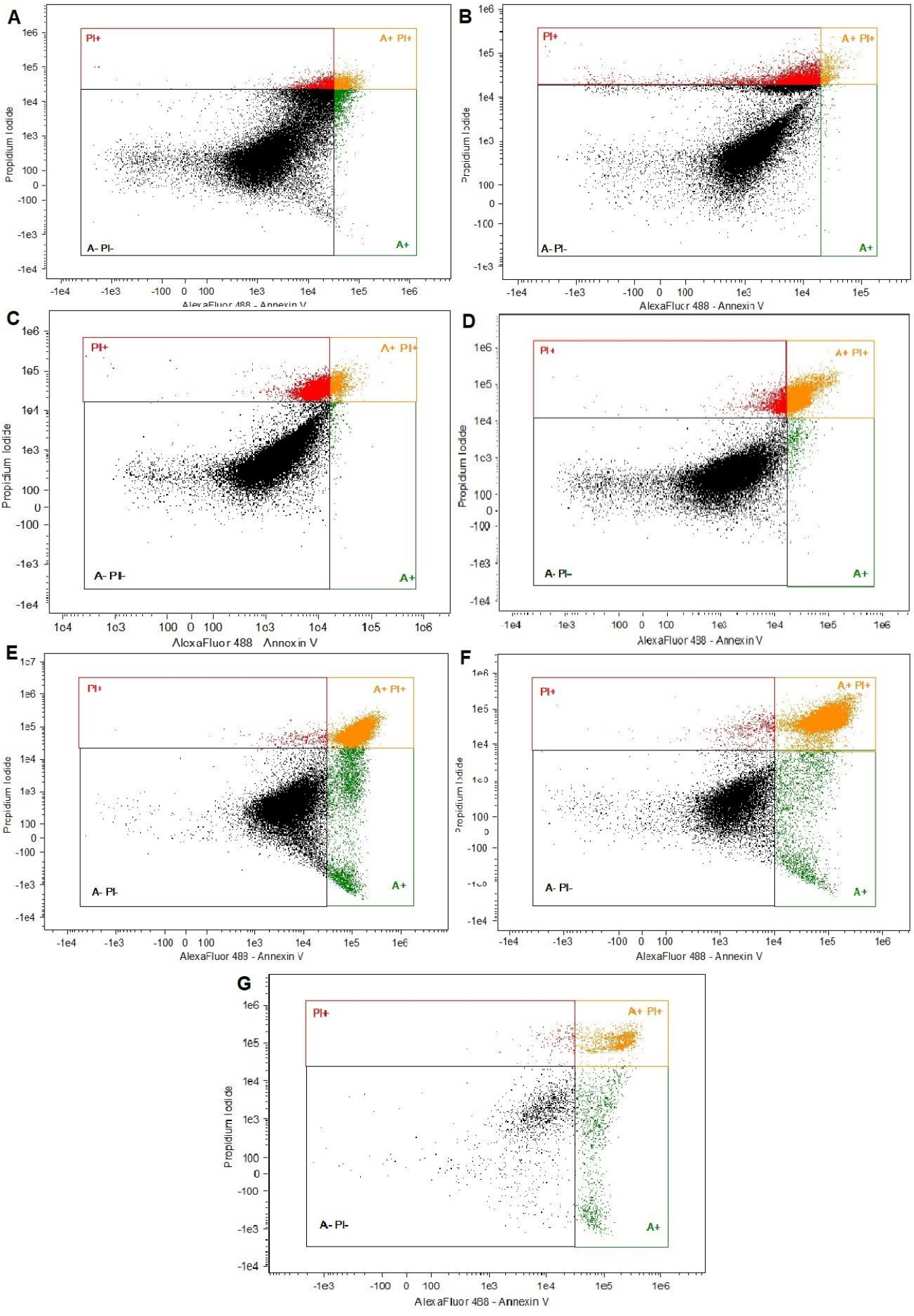


Figure 5 – Mounted electrofusion chamber (A) and optic microscope vision of structure (60x lens) (B).

For chemical activation, couplets were exposed for 5 minutes to 5µM of ionomycin and for 4 hours in 1.9mM of 6 dimethylaminopurine (6DMAP). Mature oocytes presenting the first polar body were activated at the same time as fused couplers to be used as parthenogenetic control. All activated couplets were cultured in synthetic oviductal fluid (SOF) with 5% FBS until 7-10 days.

4.6 Flow cytometry analyses

Plasmatic membrane integrity, cell apoptosis and necrosis were evaluated through fluorescent colorant combination in cells from all periods after thawing. Dead Cell Apoptosis kit (AlexaFluor® 488 Annexin V, Molecular Probes Inc., Eugene, OR, EUA) (AN), propidium iodide (IP) and nuclear colorant Hoechst 33342 (bisBenzemide H33342 trihydrochloride – in order to eliminate possible debris and other grime) were the colorants of choice. Viable cells were registered as An- IP-, as they did not color with any of the probes. Cells that colored with AlexaFluor (A+) were at initial apoptosis, cells that colored with both propidium iodide and AlexaFluor were at final apoptosis and initial necrosis estate and were identified as An+ IP+ and cells that were at final necrosis estate colored with propidium iodide and were identified as IP+ (graphic 1).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Propidium Iodide
AlexaFluor 488 - Annexin V

Graphic 1 – Comparison between day's 0 (A), 2 (B), 4 (C), 7 (D), 14 (E), 21 (F) and 30 (G) viability, apoptosis and necrosis. X axis marks cells stained with Alexa Fluor (A+). Y axis marks cells stained with propidium iodide (PI+). Viable cells did not stain with either colorant, being marked as A-IP-. Cells presenting initial apoptosis stained with Alexa Fluor and were marked as A+. Cells in final apoptosis and initial necrosis stained with both fluorescent probes, being identified as A+ IP+ and final necrosis estate cells appear as PI+, as they stained only with propidium iodide.

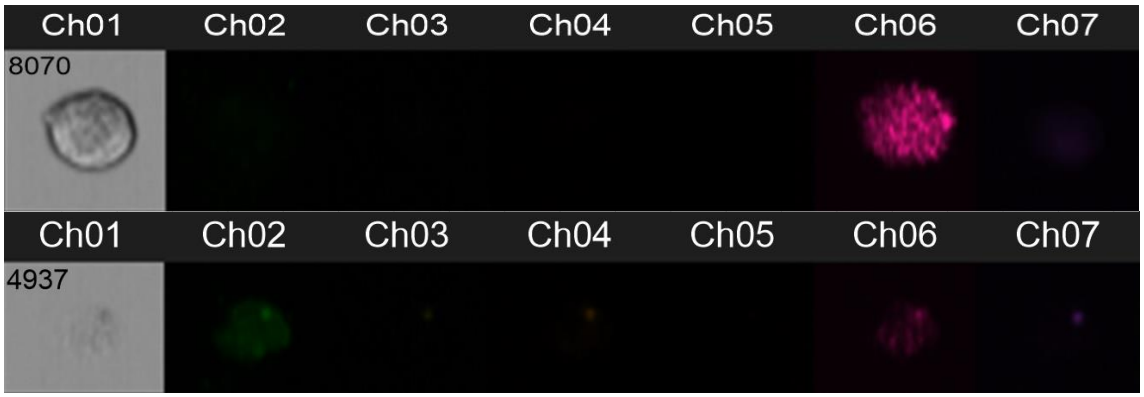
Fibroblasts from all periods were evaluated in order to investigate possible differences in cell viability after thawing. Each straw contained approximately $1x10^6$ cells, and 3 repetitions from each sample were analyzes.

Samples were brought to the lab frozen inside a cryogen canister. They were thawed at 37◦C water for 30 seconds. Cells were then placed in 1.5mL Eppendorf tubes and centrifuged for 4 minutes at 1.300rpm. After centrifugation, excessive supernatant was discarded and samples (20µL) were incubated for 15-30 minutes at 37°C in work solution (20µL) prepared according to fabricant instructions.

All samples were divided in two, forming one control group and one treatment group for each sample analyzed. Control groups had 10.000 single cells evaluated and were not treated with fluorescent probes. Treatment groups had 40.000 single cells analyzed after being treated with fluorescent probes. Images were obtained with an objective 20x lens.

A specific gate was created with the following coordinates: X axis (area) = 155 to 1944 and Y axis (aspect ratio) = 0.371 to 0.997 for fibroblast only selection. Samples were then analyzed thorough IDEAS v6.1, AMNIS FlowSight (AmnisCorp., Seattle, WA) software.

AlexaFluor (An) emissions were caught through channel 2 (505-560 nm/Figure 6 "B") and excited using 488nm at 30mW laser. Propidium iodide (IP) emissions were caught in channel 4 (595-642 nm/Figure 6 "C") and excited with a 488 at 30 mW laser. Signals emitted from Hoechst 33342 were detected at channel 7 (435-505 nm/ Figure 6 "A", "B", "C" and "D") after laser excitement of 405 nm at 45mW. SSC laser was used at 10mW. Data was then analyzed with IDEAS V5.0 software.



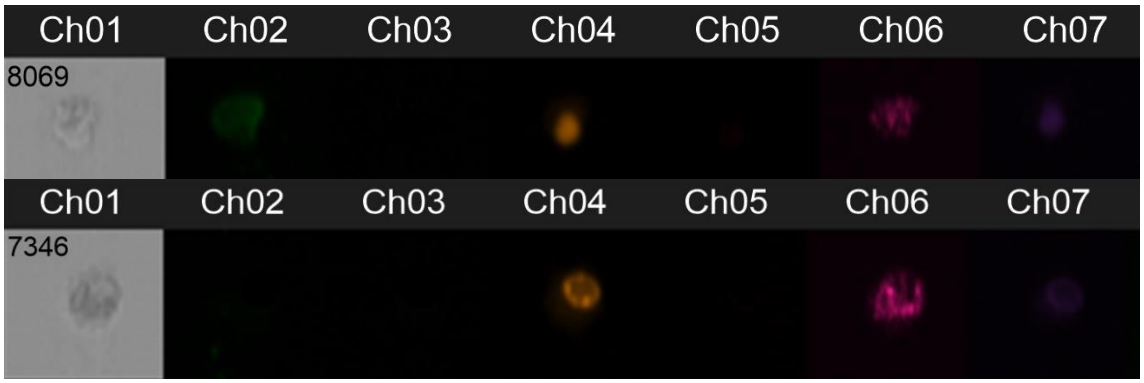


Figure 6 – Flow cytometry image of cell conformation (Ch01) and different emissions captured according to cell status. Viable cells did not color with any of the fluorescent probes, therefore no emission was caught except for Hoechst on Ch07 and the cell's own glow on Ch06 (A). Cells in initial apoptosis colored with Anexin (An), and were caught by Ch02 (B). Cells in final apoptosis/initial necrosis colored with both probes and were caught by both channels, Ch02 and Ch04 (C). Cells in final necrosis colored with Propidium Iodide (IP), being caught by Ch04 (D).

4.7 Statistical Analyses

Statistical analysis was performed on Sigma Plot 12 software. ANOVA was employed in order to verify the effect of cooling time in cell parameters before and after sample cryopreservation. Mean comparison was verified by Tuckey's test at 5%. T test was applied in cloned and parthenogenetic embryo mean production comparison.

5 RESULTS

5.1 Cell culture, time to confluence and concentration

All time points allowed cell culture (D0 through D30), but a significant difference in initial cell outgrowth was observed. The more distant to the day of death (D0), the longer it took for the first cells to outgrow skin explants, especially from D7 forward. A growing pattern was observed. While D0, D2 and D4 cells took around 4-5 days in order to start proliferating, D7, D14, D21 and D30 took a significant larger amount of time, with D30 fibroblasts taking up to 35 days in order to start proliferating around skin explants (Figure 7). Complete information is shown in Table 1.

Table 1. Mean and standard deviation of days taken for detection of initial cell outgrowth, time needed for cells to reach confluence and cell concentration on freezing moment from cells cultivated in different time points.

Time Point	Outgrowth (Days)	Confluence (Days)	Concentrations (Cell/mL)
D0	4±0[a]	24±2[a]	1.334.375±131.375[a]
D2	5.33±1.5[a]	28.0±3.1[a]	1.645.050±252.639[a]
D4	4.33±1.03[a]	36±4[b]	1.571.656±234.462[a]
D7	10.0±1.78[b]	36±0[b]	1.509.400±347.860[a]
D14	19.6±2.19[c]	34.5±4.04[b]	698.125±131.203[b]
D21	24.5±3.5[c]	36±0[b]	304.166±47.604[c]
D30	33.5±1.5[d]	31±0[b]	311.250±0[c]

Different letters represent statistically significant differences ($p<0.05$).

The same growing pattern appeared regarding the amount of time it took for cells to reach confluence; however, statistical difference can be seen even earlier, starting from D4. Cells isolated in posterior periods took a larger amount of time in order to reach confluence when compared to cells isolated in earlier time points.

Contrary to initial outgrowth and time to confluence that rose as storage time increased, cell concentration suffers an inverse proportion, decreasing sharply in posterior time points. From D0 to D7, cell concentration remains stable, but at D14, cell concentration drops significantly, and days 21 and 30 present even lower concentration rates (Figure 8).

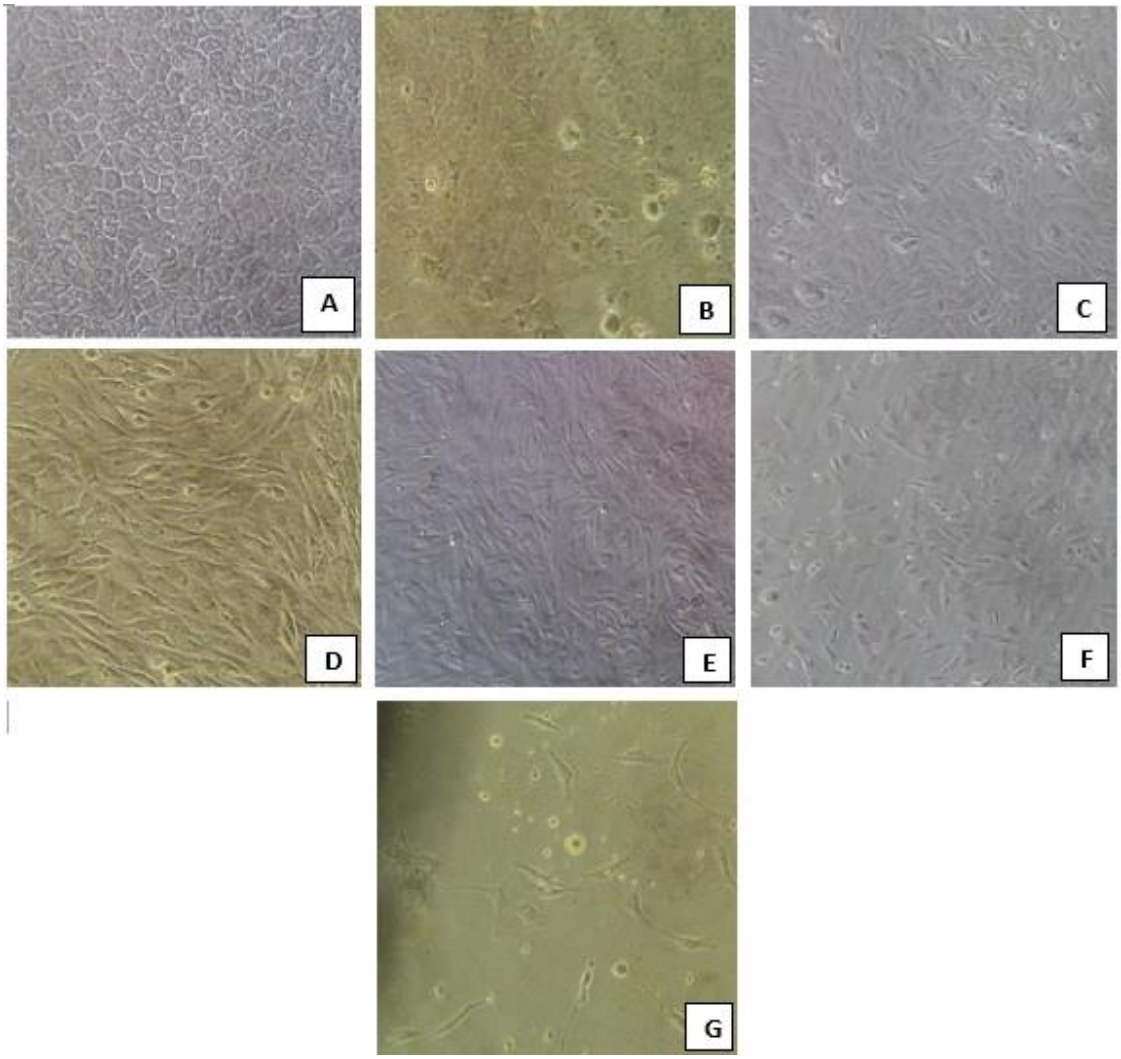


Figure 7 – Table showing the difference between different time points in fibroblast outgrowth around skin explants with 30 days after first cultivation. Cells from D0 (A), D2 (B), D4 (C), D7 (D), D14 (E), D21 (F) and D30 (G) with 30 days of cultivation where cells from earlier time points (D0, 2, 4, 7, and 14 – A, B, C, D and E) already reached confluence while later time points (D21 and 30 – F and G) are still outgrowing.

Figure 8: Comparison of D0 (left) and D30 (right) pellets after centrifugation post cellular confluence

As expected, contamination was more prevalent on days 14, 21 and 30, particularly given the fact that even inside the refrigerator, ears started to decompose, as cold temperatures slowed down but did not stop autolysis. The longer the ears remained inside the refrigerator, the harder it became to fabricate skin explants, given their friable condition. Despite such difficulties, fibroblast isolation and culture were possible, but with constant monitoring due to persistent contamination and the loss of a great number of culture dishes.

5.2 Flow cytometry analyses (viability)

Up to D14, viability percentage remained at approximately 80%, suffering a sharp decrease in D21 and D30 to 33.7±4.3 and 28.72±4.81 respectively. Full information is shown in Table 2,

Table 2: Viability, apoptosis and necrosis (average±standard error) comparison between different time points post thawing of bovine fibroblasts obtained from refrigerated tissue for up to 30 days of sample storage.

Time Point	Viability	Apoptosis	Necrosis
D0	85,6±1,86 [a]	4,11±1,6 [a]	4,32±2,06 [a]
D2	80.35±4.67[a]	0.47± 0.15[a]	17.07±3.70[a]
D4	80.79±6.40[a]	1.01±0.47[a]	18.14±5.92[a]
D7	74.96±3.47[a]	0.79±0.36[a]	24.06±3.57[a]
D14	71.62± 4.93[a]	3.40±1.59[a]	22.16±3.70[a]
D21	33.70±4.3[b]	10.50±2.89[b]	55.81±1.33[b]
D30	28.72±4.81[b]	47.18±4.56[c]	34.05±5.27[b]

Different letters in the columm[a,b] indicate statistical difference (P<0.05).

5.3 Embryo production

Table 3 presents the results regarding electrofusion, cleavage and D7 blastocyst production in comparison to parthenogenetic embryos. A 26.05% blastocyst rate was achieved from all 119 fused couplets. While P value for Cleavage presented a statistical

difference of 0.0491 (P>0.05), D30 blastocyst NT rate was similar to parthenogenetic embryo rate (P value was 0.0906, a non-significant difference with P>0.05).

Table 3: Electrofusion, cleavage and blastocyst rate comparison between D30 cell cloned and parthenogenetic embryos.

Treatments	N° oocytes	Electrofusion	Cleavage (D2)	Blastocyst (D7)
NT D30	267	119/212 (55,24%)	65/119 (54.62%)[b]	31/119 (26.05%)[a]
Parthenogenetic	162	---	130/162(80.24%)[a]	95/162 (58.64%)[a]

Different letters in the columm[a,b] indicate statistical difference (P<0.05).

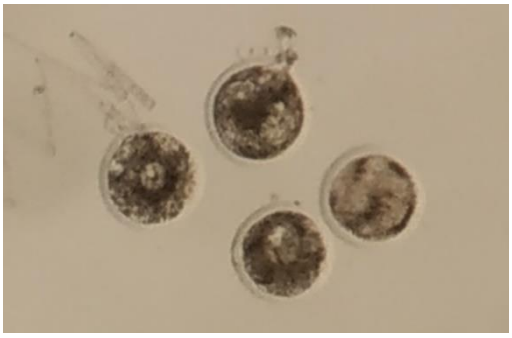


Figure 9 – Four cloned blastocysts made from D30 nuclei donor fibroblasts

6 DISCUSSION

The first step of cloning via somatic cell nuclear transfer (SCNT) is the isolation and cultivation of the desired DNA. Nowadays this is achieved through cultivation of skin fibroblasts prevenient from the ear, tail or other skin fragment, being the ear the most commonly used because of its facility in separating skin from cartilage. One of the biggest issues in farm animal cloning is that sometimes the animal dies or is housed in a property located far away from a laboratory, or the material can only be sent a few days after the animal's death. Another problem faced in this reality is that because of the lack of an appropriate storage and shipping protocol, it is common to for the lab to receive frozen samples, which makes cell cultivation difficult. On the other hand, sometimes the skin fragments arrive at the lab already on putrefaction state, which makes cultivation

impossible. We have tested the conservation of skin tissue at 5°C for long periods of time before fibroblast isolation and its use in nuclear transfer.

In this study, we observed that cooling of the bovine ear skin at 5°C is an important strategy for transporting bovine tissue for long distances and recovery of viable cells up to 30 days after sample storage. However, the increase in cooling time interferes on the proliferation patterns until freezing and cell viability after thawing from cryopreservation.

Despite the negative effect of cooling for long periods of time, viable fibroblasts were obtained from refrigerated biopsies for 30 consecutive days. Blastocysts were produced by nuclear transfer, indicating that this methodology can be used in extreme cases of transport over long distances.

In this experiment all time points allowed cell culture, however, the increase in cooling time had a direct impact on the time it took for fibroblasts to outgrow skin biopsies. On days 0, 2 and 4, fibroblasts took 4±0, 5.33±1.5 and 4.33±1.03 days in order to outgrow biopsies, when in day 7 a significant increase in that time was observed (10.0±1.78 days). Such increase in time appeared in all periods, with days 14, 21 and 30 presenting 19.6±2.19, 24.5±3.5 and 33.5±1.5 days respectively. Silvestre et al. (2004), Caputcu et al. (2013) and Walcott and Singh (2017) also observed bovine fibroblast, muscle and cartilage outgrowth up to 12, 9 and 49 days *post mortem.*

A proportion between time of cooling and time to reach confluence was also observed in our experiment. In earlier time points (D0 and D2), cells needed 24±2 and 28.0±3.1 days in order to reach confluence, while D4, D7, D14, D21 and D30 cells took significantly larger amounts of time. Walcott and Singh (2017) observed a growth rate of 16.7% of 49 day cells when compared to cells isolated from fresh biopsies.

This increase in time to confluence may be due to the reduction of stem cells present in tissue with increased storage time (Aoued and Singh, 2015, 2015; Neta et al., 2018). Cell survival and proliferation rate decrease may be attributed to pH changes and accumulation of toxins such as reactive oxygen species (Neta et al., 2018) and RNA progressive degradation, reducing the rate of gene expression and confluence (Birdsill et al., 2011). During low temperature storage, a fast consumption of cellular energy reservoir settles, primarily ATP followed by a gradual decline in total adenine nucleotide content, and a consequent accumulation of lactate happens (Salehi et al., 2004).

Regarding cell concentration, in our study, the longer the storage period, the lower the concentration of cells at the time of freezing. Days 0, 2, 4, and 7 presented a concentration of approximately 1,515,120.25±241.584 cells/mL while days 14, 21 and 30

showed and abrupt drop to approximately 437.847±59.602 cells/mL. Moreover, in addition to reducing in quantity, later time point cells (D14, D21 and D30) also presented a larger size when compared to D0 to D7 cells. Such increase in cell size may be explained by the increase in the number of halos in "older" cells. The presence of halos may have taken more space within the cell, making the cell occupy more space on the dish, giving the dish an appearance of confluence when in reality the dish presented a significant lower number of cells when compared to dishes that were in actual confluence from earlier time points, where cells showed a higher percentage of viable cells with consequently less apoptotic cells. Halos consist in structures that signal the onset of apoptosis and are formed after the separation of the nucleus from the cytoplasm (Walcott and Singh, 2017; Neta et al., 2018). This observation was also made by Walcott and Singh (2017) on their experiment with 11 different bovine ear skin explants.

In our experiment, just like concentration, post thawing viability rates suffered a great descend in later time points, dropping from around 78.66±4,23% on days 0, 2, 4, 7 and 14 to 33.7±4.3 on D21 and 28.72±4.81 on D30. This abrupt drop in cell viability may also be due to the number of halos formed in older cells, signaling the onset of apoptosis (Walcott and Singh, 2017; Neta et al., 2018). Besides, we observed an increase in cell apoptosis with increase of time, where D0, D2, D4, D7 and D14 cells presented 4.11±1.6, 0.47±0.15, 1.01±0.79, 7.9±0.36 and 3.40±1.59% of apoptotic cells respectively while D21 cells already went up to 10.50±2.89% and D30 presented a total of 47.18±4.56% dead cells because of apoptosis.

Studies with other species achieved similar results. Okonkwo and Singh (2014) observed outgrowth around goat skin explants up to 41 days *post mortem*, and reported that earlier time point cells took 4 days in order for fibroblasts to outgrow, while later time point (days 37 and 41) cells took 25 and 32 days respectively. Aoued and Singh (2015) also reported earlier period goat samples taking 4 days in order to fibroblasts to outgrow. Singh and Ma (2016) achieved successful sheep outgrowth up to 65 days and Neta et al. (2018) showed collared peccary fibroblast outgrowth up to 30 days without nutrient medium. Silvestre et al. (2003) verified that rabbit and pig fibroblasts could be obtained up to 10 and 14 days respectively. All these results show that skin fibroblasts from different species can be successfully isolated after long periods of sample cooling.

Regarding time to confluence, Birdsill et al. (2011), demonstrated that human brain cells derived from refrigerated tissue require longer time to reach cellular confluence when compared to cells derived from non-refrigerated tissue. This proportion

between time of storage and time taken for cells to reach confluence was also observed by Silvestre et al. (2003) with rabbit and pig samples. With sheep, Singh and Ma (2016) too detected slower growth rates in refrigerated sample cells. Furthermore, Boekema et al. (2015) identified a slower growth rate between human skin explants cultivated 3 and 7 days of storage at 4ºC. Neta et al. (2018) reported slower collared peccary fibroblast growth rates on samples stored at 4ºC for 30 days without nutrient medium. Kim et al. (2014) observed that the proliferation rates of rabbit chondrocytes was compromised after 7 days *post mortem* culture. Cells obtained from rabbit and pig explants 14 days *post mortem* presented a growth rate of only 33% in comparison to cells obtained from non-refrigerated skin explants fabricated on the day of the animal's death (Silvestre et al., 2003). Goat tissue refrigerated for up to 41 days presented a 19% growth rate (Okonkwo and Singh, 2014).

As to cell viability, Neta et al. (2018) also observed a drop in fibroblast viability with increased storage time, going from approximately 60% on day 10 to less than 50% in day 30. Ge et al. (2010) also observed this phenomenon with pig skin fibroblasts cultured up to 15 days *post mortem*, that presented only 20% of viability and Bravo et al. (2000) reported a drop in viability from human skin fibroblasts stored for 9 days, that presented a rate of also 20%. Such rate dropped even further on day 29, striking only 6% of viability. Boekema et al. (2015) also observed this, with human skin in the absence of medium that presented 30% of viability at the 21 day mark.

In general, in this study we have found that tissue refrigeration enables viable cell culture for nuclear transfer use for long periods of time after an animal's death. However, increase in sample storage time brings cell damage, lowering considerably cell viability, making culture and nuclear transfer success more difficult.

Despite the negative effects of long cooling periods, fibroblasts obtained from 30 consecutive day cooling biopsies allowed 26.05% of cloned blastocyst production, indicating that this methodology can be used in extreme cases of long distance transportation. This blastocyst rate was similar to others papers that used fibroblast obtained from fresh biopsies (Mello et al., 2003, Silva et al., 2016; Silva et. al., 2019).

7 CONCLUSION

Somatic cell nuclear transfer is an important tool in endangered species conservation, nuclear-cytoplasm interactions, scientific and biomedical research and

superior animal replication. For consisting in the first step in the technique, efficient cell isolation and cultivation is of major interest, and the need to obtain viable cells from samples stored and transported for long distances is a reality.

Nuclear reprogramming is one of the most important steps in nuclear transfer, and one of the steps that brings most difficulties. Because of that, the assessment of techniques that enable cell culture and sample storage that allow cell viability to remain after long periods of time is essential.

Low temperatures of 5ºC have shown to be efficient in maintaining viable tissue for up to 30 days. However, significant changes in cell attachment and proliferation, concentration, contamination rates until freezing and viability after cryopreservation can be observed the longer the tissue is stored. Despite such obstacles, it is clear that the protocol presented in this experiment enables genetic obtainment and storage for posterior use in cloning via somatic cell nuclear transfer when long storage periods are necessary.

It was also demonstrated that despite the problems entailed by longer storage periods, viable cells can be obtained and a satisfactory blastocyst rate can be achieved with such cells, demonstrating that the protocol is indeed efficient for long periods of sample storage.

8 REFERENCES

AOUED, H. S., SINGH, M. Recovery of fibroblast-like cells after 160 days postmortem storage of goat skin tissues in refrigerated media. *J. Veterinar. Sci. Technolo.* V.6, 1000236, 2015

ARANTES, L. G., TONELLI, G. S. S. S., MARTINS, C. F., BAO, S. N. Cellular characterization and effects of cryoprotectant solutions on the viability of fibroblastos from three brasilian wild cats. *Biopreservation and Biobanking* Published online 9 oct 2020, DOI: 10.1089/bio.2020.0059. <https://www.liebertpub.com/doi/full/10.1089/bio.2020.0059> last accessed in 12/08/2020.

BOEKEMA, B. K. H. L., BOEKESTIJN, B., BREEDEVELD, R. S. Evaluation of saline, RPMI and DMEM/F12 for storage of split-thickness skin grafts. *Burns* v.41, p.848-852, 2015

BORGES, A. A., LIMA, G. L., NETA, L. B. Q., SANTOS, M. V. O., OLIVEIRA, M. F., SILVA, A. R., PEREIRA, A. F. Conservation of somatic tissue derived from collared peccaries (Pecari tajacu Linnaeus, 1758) using direct or solid-surface vitrification techniques. Cytotechnology v.69, p.643-654, 2017

BIRDSILL, A. C., WALKER, D. G., LUE, L., SUE, L. I., BEACH T. G. Postmortem interval effect on RNA and gene expression in human brain tissue. *Cell Tissue Bank* v.12, p.311-318, 2011

BRAVO, D., RIGLEY, T. H., GIBRAN, N., STRONG D. M., NEWMAN-GAGE, H. Effect of storage and preservation methods on viability in transplantable human skin allografts. *Burns* v.26, p.367-378, 2000

CAAMAÑO, J. N., RODRIGUEZ. A., MUÑOZ, M., FRUTOS, C., DIEZ, C., GOMEZ, H. Cryopreservation of brown bear skin biopsies. Cell Preservation Technology v.6, p.83-86, 2008

CAPUTCU, A., AKKOC, T., CETINKAYA, G., ARAT, S. Tissue cryobanking for conservation programs: effect of tissue type and storage time after death. *Cell Tissue Bank* v.14, p.1-10, 2013

GE, L., SUN, L., CHEN, J., MAO, X., KONG, Y., XIONG, F., WEI, H. The viability change of pigskin in vitro. Burns v.36, p.533-538, 2010

IAGER, A. E., REGINA, N. P., ROSS, P. J., BEYHAN, Z., CUNNIFF, K., RODRIGUEZ, R. M., CIBELLI, J. B. Trichostatin A improves histone acetylation in somatic cell nuclear transfer early embryos. *Cloning and Stem Cells* v.10, p.371-379, 2008)

HOSHINO, Y., HAYASHI, N., TANIGUCHI, S., KOBAYASHI, N., SAKAI, K., OTANI, T., IRITANI, A., SAEKI, K. Resurrection of a bull by cloning from organs frozen without cryoprotectant in a -80^{o}C freezer for a decade. *PloS one* v.4 e4142, 2009

KIM, B. Y., NAM, B. M., LEE, K. M., JO, Y. H., NEMENO, J. G., YANG, W., LEE, S., KIM, H., JANG, I J., TAKEBE, T., LEE, J. I. Effect of preservation conditions on cartilage tissue for cell transplantation. *Transplantaion Proceedings* v.46, p.1.139-1.144, 2014)

MASTROMONACO, G. F., PERRAULT, S. P., BETTS, D. H., KING, W. A. Role of chromosome stability and telomere length in the production of viable cell lines for somatic cell nuclear transfer. *BMC Developmental Biology* v.6, article n. 41, 2006

MELLO, M. R. B., CAETANO, H. V. A., MARQUES, M. G., PADILHA, M. S., GARCIA, J. F., MILAZZOTTO, M. P., ASSUMPÇÃO M. E. O. A., LIMA, A. S., NICÁCIO, A. C., MENDES, C. M., OLIVEIRA, V. P., VISINTIN, J. A. Production of a cloned calf from a fetal fibroblast cell line. *Braz. J Med. Biol. Res.* v.36, p. 1485-1489, 2003

MOULAVI, F., HOSSEINI, S. M., TANHAIE-VASH, N., OSTADHOSSEINI, S., HOSSEINI, S. H., HAJINASROLLAH, M., ASGHARI, M. H., GOURABI, H., SHAHVERDI, A., VOSOUGH, A. D., NASR-ESFAHANI, M. H. Interspecies somatic cell nuclear transfer in Asiatic cheetah using nuclei derived from post-mortem frozen tissue in absence of cryo-protectant and *in vitro* matured cat oocytes. *Theriogenology*, v.90, p.197-203, 2017

NETA, L. B. Q., LIRA, G. P. O., BORGES, A. A., SANTOS, M. V. O., SILVA, M. B., OLIVEIRA, L. R. M., SILVA, A. R., OLIVEIRA, M. F., PEREIRA, A. F. Influence of storage time and nutrient medium on recovery of fibroblast-like cells from refrigerated collared peccary (*Pecari Tajacu* Linnaeus, 1758) skin. *In Vitro Cell. Dev. Biol. Anim.* v.54, p.486-495, 2018

OKONKWO, C., SINGH, M. Recovery of fibroblast-like cells from refrigerated goat skin up to 41 d of animal death. *In Vitro Cell. Dev. Biol. Anim.* v.51, p.463-469, 2014.

SALEHI, P., SPRATLIN, J., CHONG, T., CHURCHILL, T. A. Beneficial effects of supplemental buffer and substrate on energy metabolism during small bowel storage. *Cryobiology* v.48, p.245-253, 2004

SILVA, C. G., MARTINS, C. F., CARDOSO, T. C., CUNHA, E. R., BESSLER, H. C., MARTINS, G. H. L., PIVATO, I., BÁO, S. N. Production of bovine embryos and calves cloned by nuclear transfer using mesenhymal stem cells from amniotic fluid and adipose tissue. *Cellular Reprogramming* v.18, p.127-136, 2016

SILVA, C. G., MARTINS, C. F., BESSLER, H. C. C., MORAES, A. F. N., CARDOSO, T. C., FRANCO, M. M., MENDONÇA, A. S., LEME, L. O., BORGES, J. R. J., MALAQUIAS, J. V., BÁO, S. N. Use of trichostatin A alters the expression of HDAC3 and improves in vitro development of bovine embryos cloned using less methylated mesenchymal stem cells. *Reproduction in Domestic Animals*, v.54, p. 289-299, 2019

SILVESTRE, M. A., SAEED, A. M., CERVERA, R. P., ESCRIBA, M. J., GARCIA-XIMENEZ. F. Rabbit and pig ear skin sample cryobanking: effects of storage time and temperature of the whole ear extirpated immediately after death. *Theriogenology* v.59, p.1469-1477, 2003

SILVESTRE, M. A.; SANCHEZ, J. P., GOMEZ, E. A. Vitrification of goat, sheep and cattle skin samples from whole ear extirpated after death and maintained at different storage times and temperatures. *Cryobiology* v.49, p.221-229, 2004.

SINGH, M., MA, X. Recovery of fibroblast cells up to 65 d of postmortem storage of sheep ear skin at 4°C. J Anim Sci v.94, p.837–838, 2016.

TIAN, X. C., KUBOTA, C., ENRIGHT, B., YANG, X. Cloning animals by somatic cell nuclear transfer – biological factors. *Reproductive Biology and Endocrinology* v.98, online issue, 2003.

WAKAYAMA, T., PERRY, A. C., ZUCCOTTI, M., JOHNSON, K. R., YANAGIMACHI, R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* v.394, p.374-394, 1998

WALCOTT, B., SINGH, M. Recovery of proliferative cells up to 15- and 49-day postmortem from bovine skin stored at 24◦C and 4◦C respectively. *Cogent Biology* v.3:1333760, 2017.

WILMUT, I., SCHNIEKE, A. E., MCWHIR, J., KIND, A. J., CAMPBELL, K. H. Viable offspring derived from fetal and adult mammalian cells. *Nature* v. 385, p.810-813, 1997

CHAPTER III

FINAL CONSIDERATIONS

Somatic cell nuclear transfer is an important tool in scientific research and in the biomedical field, however, the low success rates achieved with the technique must be addressed in order to better take advantage of its potential. Cell reprogramming is an extremely important aspect of the technique, and one of the steps that entails more difficulties in nuclear transfer. The cryopreservation of valuable genome is the first step in nuclear transfer. The nuclei donor cell carries the desired DNA, and its origin, as its culture and preservation directly impact in the technique's success. This experiment showed that refrigeration of skin biopsies at 5^{o}C is a great strategy for maintaining ear skin tissue viable for fibroblast isolation and culture for up to 30 days after an animal's death.

It was also shown that 30 day *post mortem* cultivated cells are still viable and can be successfully used to produce cloned embryos.

However, increase in cooling time affects directly initial cell growth time, time to confluence, cell concentration, contamination rates and cell viability, and the strategy should be used only when necessary.