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Potential role of intraspecific and interspecific cloning in the conservation of wild mammals

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Summary

Intraspecific and interspecific cloning via somatic cell nuclear transfer (iSCNT) is a biotechnique with great possibilities for wild mammals because it allows the maintenance of biodiversity by recovering species, nuclear reprogramming for the production of pluripotency-induced cells, and studies related to embryonic development. Nevertheless, many areas in cloning, especially those associated with wild mammals, are still in question because of the difficulty in obtaining cytoplasmic donor cells (or cytoplasts). Conversely, donor cell nuclei (or karyoplasts) are widely obtained from the skin of living or post-mortem individuals and often maintained in somatic cell banks. Moreover, the creation of karyoplast–cytoplast complexes by fusion followed by activation and embryo development is one of the most difficult steps that requires further clarification to avoid genetic failures. Although difficult, cloning different species, such as wild carnivores and ungulates, can be successful via iSCNT with embryo development and the birth of offspring. Thus, novel research in the area that contributes to the conservation of biodiversity and knowledge of the physiology of species continues. The present review presents the failures and successes that occurred with the application of the technique in wild mammals, with the goal of helping future work on cloning via iSCNT.

Introduction

The decrease in the biodiversity of wild mammals has been caused mainly by human activity, resulting in an increase in research aimed at the development of conservation strategies (Pereira *et al.*, 2016). In general, several techniques to help with the conservation of wild animals are available, including the formation of biobanks (León-Quinto *et al.*, 2009), artificial insemination (Howard *et al.*, 2016), embryo transfer (Goeritz *et al.*, 2012), *in vitro* fertilization (Herrick *et al.*, 2010), and cloning using somatic cell nuclear transfer (SCNT, Folch *et al.*, 2009). Because of the low availability of oocytes for SCNT, interspecific cloning using intraspecific and interspecific nuclear transfer techniques (iSCNT) has been shown to be an important tool in conservation (Wani *et al.*, 2017).

The main argument for the application of iSCNT is the rapid decrease in the number of species. Any tool that can avoid this decrease is important. iSCNT preserves and even expands genetic variability when somatic cells of different individuals representative of the original biodiversity of a population are collected for its use (Loi *et al.*, 2001). In addition, interest in cloning has increased not only for the conservation of endangered species, but also for the multiplication of reproducers with better genetic characteristics (Saini *et al.*, 2015), basic research on cell epigenetic status (Saragusty *et al.*, 2016), embryonic development (González-Grajales *et al.*, 2016), and the production of induced pluripotent cells (Sukparangsi *et al.*, 2018).

Therefore, in all applications of cloning, studies related to the improvement of iSCNT, as well as its wide use in different individuals, are important.

Overview of the iSCNT technique and its limitations

The iSCNT technique involves embryo reconstruction by fusing a nucleus of a donor cell (karyoplast) derived from a wild mammal with an enucleated oocyte (cytoplast) from a domestic mammal of a different species, family, order, or class (Do & Taylor-Robinson, 2014). The nucleus in G0/G1 is exposed to reprogramming by the oocyte, followed by the fusion and activation of the reconstructed embryo (Loi *et al.*, 2011). Subsequently, the resultant embryo can be transplanted into the uterus of a recipient for term development (Pereira & Freitas, 2009).

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Different steps are involved in the production of clones via iSCNT. Therefore, it is interesting to highlight the steps of the technique and its peculiarities that can define the success of cloning by iSCNT.

Preparation of cytoplasts

Whether using the oocyte from a domestic or a wild mammal, some fundamental criteria must be met to obtain a cytoplast suitable for cloning, such as oocyte selection, *in vitro* maturation, and enucleation systems (Loi *et al.*, 2011). In general, follicular size, the oocyte collection method, and the culture environment are factors that can affect the quality of mature oocytes, and different responses to these factors can be observed in wild mammals. In some cervid species, Brahmasani *et al.* (2013) observed that low maturation rates could probably be caused by slicing. In this method, non-competent oocytes can be recovered, as the technique can result in the recovery of structures of small diameter follicles (Rho *et al.*, 2001). Additionally, the quality of ovaries obtained post-mortem may have been one of the factors that reduce the quality of oocytes in these species.

Therefore, studies have shown that enriched culture medium and ovarian transport conditions may result in good results using ovaries from post-mortem animals for recovery of immature oocytes. Macías-García *et al.* (2018) verified that oocytes of Iberian red deer (*Cervus elaphus hispanicus*) obtained from ovaries maintained for 16 h in a holding medium increased the oocyte meiotic competence. Moreover, these authors observed that the epidermal growth factor (EGF) demonstrated a differential effect depending upon oocyte grading and conditions of ovary transportation. Additionally, for ovaries derived from Hokkaido sika deer (*Cervus nippon yesoensis*), maturation rates of oocytes were highest when ovaries were kept for 12 h at 20–25°C, when compared with 24 h (Tulake *et al.*, 2014).

Specifically, with respect to the culture environment, the requirements for both composition and maturation time should be established for the *in vitro* maturation of each species. In the Indian blackbuck (*Antilope cervicapra*), oocytes cultured in the presence of gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) showed higher rates of expansion of the cumulus oophorus (79.3%) and extrusion of the first polar body (46.1%) compared with oocytes cultured without gonadotropins (60.4% and 33.3%, respectively) (Rao *et al.*, 2010). In the sika deer (*Cervus nippon hortulorum*), oocytes cultured in medium supplemented with fetal bovine serum (FBS), FSH, LH, cysteamine and EGF resulted in a higher maturation rate (75.4%) compared with medium without supplementation (30.1%; Yin *et al.*, 2013). Already, different hormonal combinations of FSH, LH and 17β-estradiol did not alter the maturation rates in oocytes derived from lions (*Panthera leo*; Fernandez-Gonzalez *et al.*, 2015). In the collared peccary (*Pecari tajacu*), we proved that oocytes need 48 h to achieve maturation instead of 24 h, according to the expansion of the cumulus cells (100% vs. 38.1%), the presence of first polar body (90.5% vs. 52.4%), and the status of the nucleus in the second metaphase (76.2% vs. 52.4%), respectively (Borges *et al.*, 2018c).

In addition to obtaining mature oocytes, the preparation of cytoplasts depends on the method of enucleation of these structures. The amount of ooplasm present in the reconstructed embryo is related to the enucleation technique that removes the nucleus from the oocyte. Matured oocytes can be enucleated in different ways, including squeezing the first polar body and the surrounding cytoplasm through a cleft in the zona pellucida of the oocyte (Lee *et al.*, 2003). Another method is manual removal in which zona-free oocytes are enucleated with a bisection blade that hand bisect the metaphase II chromosomes along with a small volume of the surrounding cytoplasm. Oocytes can also be aspirated using a micromanipulator at the location of the metaphase II chromosomes and the polar body via brief exposure to ultraviolet light (Pereira *et al.*, 2015).

Selection of karyoplasts

To obtain karyoplasts appropriate for cloning, their type and age and the manipulation techniques used are important for their future reprogramming (Kim *et al.*, 2007). Karyoplasts can be obtained from fresh or cryopreserved somatic tissues (Folch *et al.*, 2009, Pan *et al.*, 2014), from an adult (Moulavi *et al.*, 2017) or a fetus (Liu *et al.*, 2018), and *in vivo* or post-mortem (Pereira *et al.*, 2014). Although the recovery of these cells is not a difficult task, their processing and preservation until use in iSCNT require attention (Pereira *et al.*, 2014). In general, skin cells have been the most used cell type for karyoplasts (Song *et al.*, 2007). The skin has an abundance of cells of interest that may have different efficiencies in cloning, as observed in wild buffalo (*Bubalus arnee*). Saini *et al.* (2015) detected that fibroblasts of this species are easier to reprogram than epithelial cells.

After harvest, cells used as nuclei donors need to be characterized with respect to their culture conditions, cryopreservation, and cell cycle synchronization (Pereira *et al.*, 2014). For these steps, cells are evaluated for the number of passages, nutritional requirements during *in vitro* culture (Santos *et al.*, 2016), and the damage done during cryopreservation (Song *et al.*, 2007). Thus, karyoplasts have been established *in vitro* in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS and growth factors (Santos *et al.*, 2016).

Karyoplasts have been routinely cryopreserved by slow freezing (Sharma *et al.*, 2018) using a combination of dimethyl sulfoxide (DMSO), FBS, and sucrose as the cryoprotectant, as observed with Iberian lynx (*Lynx pardinus*, León-Quinto *et al.*, 2014). Although it is more desirable to use a somatic cell bank after tissue culture, the absence of *in vitro* culture conditions sometimes makes these banks unfeasible, resulting in the immediate formation of the targets for those somatic tissues (Borges *et al.*, 2017a,b; Queiroz Neta *et al.*, 2018). The three somatic tissue conservation techniques used for wild animals are slow-freezing cryopreservation (Mestre-Citrinovitz *et al.*, 2016), vitrification (Borges *et al.*, 2018a,b), and cooling at 4–6°C (Queiroz Neta *et al.*, 2018). In collared peccaries, we compared two techniques of vitrification and we observed that solid-surface vitrification was found to be a more efficient method for vitrifying skin tissue when compared with direct vitrification in cryovials, probably due to tissues not being involved in large amounts of cryoprotectants before passing through a drastic change in temperature during the solid-surface vitrification (Borges *et al.*, 2017b).

Finally, the third step in the preparation of the karyoplasts is cell synchronization in the G0/G1 stage (Gómez *et al.*, 2003; Yelisetti *et al.*, 2016). In general, nuclear reprogramming is controlled by epigenetic modification. For this to occur, the somatic cells must be in G0/G1 to allow the removal of reversible epigenetic changes acquired during cell differentiation (Song *et al.*, 2007). Therefore, cells can be subjected to different treatments for synchronization during culture. Inhibition by contact (Moulavi *et al.*, 2017), serum deprivation (Wani *et al.*, 2017), and chemicals that inhibit the cell cycle (Gómez *et al.*, 2003) are methods used for synchronization. Serum deprivation and inhibition by contact are the most commonly used (Moulavi *et al.*, 2017).

Under high confluence or serum privation, fibroblast cells derived from the skin of adult argali (*Ovis ammon*) were efficiently

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Table 1. iSCNT in some wild mammals

Scientific name	IUCN Category*	Country	Karyoplast	Cytoplast	Outcome	Authors
Carnivores						
Canis lupus	Least Concern	Seoul, Korea	Fibroblast	*Canis lupus familiaris*	17% pregnancies, 2 pups	Kim *et al.* (2007)
Canis lupus	Least Concern	Seoul, Korea	Fibroblast	*Canis lupus familiaris*	23.5% pregnancies, 4 pups	Oh *et al.* (2008)
Felis margarita	Least Concern	USA	Fibroblast	*Felis catus*	3 pups	Gómez *et al.* (2008)
Felis silvestris lybica	Least Concern	USA	Fibroblast	*Felis catus*	28% blastocyst	Gómez *et al.* (2003)
Felis silvestris lybica	Least Concern	USA	Fibroblast	*Felis catus*	75% pregnancies, 17 pups	Gómez *et al.* (2004)
Ungulates						
Acinonyx jubatus	Vulnerable	Argentina	Fibroblast	*Felis catus*	27.4% blastocyst	Moro *et al.* (2015)
Acinonyx jubatus venaticus	Vulnerable	Iran	Fibroblast	*Felis catus*	5.9% morula	Moulavi *et al.* (2017)
Bos gaurus	Vulnerable	USA	Fibroblast	*Bos taurus*	25% pregnancies, none term	Lanza *et al.* (2000)
Bos javanicus	Endangered	USA	Fibroblast	*Bos taurus*	17% pregnancies, none term	Sansinena *et al.* (2005)
Bubalus arnee	Endangered	India	Fibroblast	*Bubalus bubalis*	38.7% blastocyst	Priya *et al.* (2014)
Bubalus arnee	Endangered	India	Fibroblast/Epithelial cell	*Bubalus bubalis*	50.6 *vs* 20.5% blastocyst	Saini *et al.* (2015)
Capra ibex	Least Concern	China	Fibroblast	*Capra hircus*	11% blastocyst	Wang *et al.* (2007)
Capra pyrenaica	Absente	Spain	Fibroblast	*Capra pyrenaica*	1 born	Folch *et al.* (2009)
Ovis ammon	Near Threatened	China	Fibroblast/Cumulus cell	*Ovis aries*	22.1% blastocyst	Pan *et al.* (2014)
Ovis orientalis musimon	Vulnerable	Italy	Granulosa cell	*Ovis aries*	1 pup	Loi *et al.* (2001)
Tragelaphus eurycerus isaaci	Critically Endangered	USA	Fibroblast	*Bos taurus*	24% blastocyst	Lee *et al.* (2003)
Others species						
Balaenoptera bonaerensis	Data deficient	Japan	Cumulus cells	*Bos taurus/Sus scrofa domesticus*	27.5–52.8% cleavage	Ikumi *et al.* (2004)
Macaca fascicularis	Least Concern	Thailand	Fibroblast	*Bos taurus*	33% blastocyst	Lorthongpanich *et al.* (2008)

*IUCN: International Union for Conservation of Nature and Natural Resources. USA: United States of America.

synchronized at G0/G1; nevertheless, cells were in lower proportion in the growing stage (Pan *et al.*, 2014). Authors observed that the highest proportion of cells from the African wild cat (*Felis silvestris lybica*) at G0/G1 was obtained by serum deprivation compared with that obtained by inhibition by contact and the inhibitor roscovitine (Gómez *et al.*, 2003). Leopard (*Panthera pardus*) skin cells treated with chemical inhibitors such as sodium butyrate have a greater propensity to undergo alterations (Yelisetti *et al.*, 2016).

Embryonic reconstruction stages

After the transfer of the nucleus into the enucleated oocyte, the cytoplast–karyoplast complex is subjected to an electric pulse that not only induces the fusion of the somatic cell nucleus with the enucleated oocyte to form a new complex, but also promotes the release of intracellular calcium that initiates cellular activation (Pereira & Freitas, 2009). In general, the successful development of a reconstructed embryo depends on the complex interactions between the cytoplast and the nuclear structure during embryonic development; failures in this interaction can cause problems during early cleavage and embryonic development (González-Grajales *et al.*, 2016).

The activation of the cytoplast–karyoplast complexes guarantees adequate embryonic reconstruction (Yamochi *et al.*, 2013). Because the iSCNT technique reprograms the nucleus of a somatic cell of one species using the oocyte cytoplasm of another species, it is essential that the activation protocol be able to activate the reconstructed embryo (Zhao *et al.*, 2006). Physiologically, a mammalian oocyte is activated during fusion with a sperm, releasing meiotic cell cycle arrest and enabling the resumption of the oocyte meiotic cell cycle (Sparman *et al.*, 2010). Therefore, a well developed protocol allows a high rate of blastocyst formation by promoting good embryonic development through activation.

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Activation protocols, including physical methods such as electrical pulses and alteration of osmolarity, and chemical methods such as calcium-mobilizing compounds like strontium chloride, ionomycin, and ethanol, to promote the initial release of calcium have been evaluated in different species, as sika deer (Yin *et al.*, 2013), alpaca (*Vicugna pacos*) and llama (*Lama glama*, Ruiz *et al.*, 2015), with blastocyst rates of 32.4%, 22.5% and 18.7%, respectively. In general, calcium mobilizers are used in combination with kinase protein inhibitors or protein synthesizers such as cycloheximide and 6-dimethylaminopurine (6-DMAP). In addition, a cytostatic factor inactivator and microfilament inhibitor such as cytochalasin B are used to prevent extrusion of the second polar body and maintain the diploidy of the presumed embryo (Ruiz *et al.*, 2015).

For red deer (*Cervus elaphus*), electrical activation before chemical activation with ionomycin and 6-DMAP was efficient for the production of clone embryos (32–44%), obtaining genetically healthy calves (Berg *et al.*, 2007). Nevertheless, the same protocol resulted in a low developmental rate (5.7%) of activated oocytes in swamp deer and 0.0% embryos in spotted deer, sambar deer, and brow-antlered deer after oocyte parthenogenetic activation (Brahmasani *et al.*, 2013). Blackbuck (*Antilope cervicapra*) oocytes activated with ionomycin and 6-DMAP resulted in 58% cleaved embryos and 13% blastocysts (Rao *et al.*, 2010). Therefore, the artificial activation method (chemical, electrical protocols or your combination) can result in different responses among species. In this sense, it is necessary to evaluate the type of artificial activation that promotes the best rates of embryonic development in the species of interest.

In vitro culture systems are essential for early embryonic development and nuclear reprogramming (Gómez *et al.*, 2008; Pereira *et al.*, 2013). Choosing the appropriate culture medium for each species is considered the initial step in proper embryonic development (Zhao *et al.*, 2006). Lee *et al.* (2003) used somatic cells of the mountain bongo (*Tragelaphus eurycerus isaaci*) and domestic cow (*Bos taurus*) oocytes and observed that a chemically defined, protein-free medium of TCM199 supplemented with FBS supported embryonic development. Nonetheless, there is no one culture medium suitable for all species that allows better embryonic development for a given species under study.

Finally, the effect of epigenetic reprogramming is a very relevant factor in the success of iSCNT (Gómez *et al.*, 2008). Some epigenetic markers were characterized with respect to their function during embryonic reprogramming and their influence on the chromatin structure from post-translational modifications (Song *et al.*, 2007). The overall level of the acetylation of histone H3 at lysine 18 (H3K18ac) and trimethylation of histone H3 at lysine 27 (H3K27me3), and the expression level of some important apoptosis proteins (caspase 3 and caspase 7), and p53 were evaluated. The hyperacetylated state of histones is associated with transcriptionally active domains, while the hypoacetylated state is associated mainly with silenced chromatin regions of histone acetyl transferases and histone deacetylases. The methylation pattern of the DNA is determined by DNA methyltransferases. OCT3/4, NANOG, and CDX2 are very important because of their close association with pluripotency and early embryonic development (Saini *et al.*, 2015).

Advances and perspectives of iSCNT in wild mammals

Several works aimed at cloning different wild mammals have been conducted (Table 1). Among these studies, those that obtained offspring were on wild bovine (Lanza *et al.*, 2000), sheep (Loi *et al.*, 2001), felid (Gómez *et al.*, 2004; Li *et al.*, 2007), canid (Kim *et al.*, 2007; Oh *et al.*, 2008), and goat (Folch *et al.*, 2009). Therefore, several families have proven the success of using iSCNT for the recovery and reintroduction of wild mammals.

An important point to remember is that as the taxonomic distance between donor and recipient species increases, the production of blastocysts decreases because of the decreased ability of somatic cells to be reprogrammed (Priya *et al.*, 2014). In general, enucleated oocytes are from a domestic species that is phylogenetically close to the wild species that donates the nucleus. For example, domestic sheep cytoplasts were able to reprogram me argali fibroblast nuclei (Pan *et al.*, 2014) and domestic buffalo cytoplast was able to reprogram me wild buffalo karyoplast (Priya *et al.*, 2014).

Carnivores

Some works have shown the advances achieved by iSCNT in wild canine species. These species, including the grey wolf (*Canis lupus*), have gradually become endangered or extinct. Therefore, in 2007, with the goal of canid conservation, Kim *et al.* (2007) cultured fibroblasts derived from the ear of an adult female grey wolf that were then used as donor cells of nuclei. Using domestic canine oocytes, the authors produced a pregnancy with cloned embryos of two genetic identities of the cloned wolves, but there were no births. In 2008, Oh *et al.* (2008) obtained three wolf pups from cloned embryos using cells obtained from a male grey wolf 6 h after death and domestic canine oocytes. These studies demonstrated the successful cloning of endangered wild canines.

In felid species, the main oocyte source has been the domestic cat. In species from the Felidae subfamily, some progress has been achieved. Therefore, synchronized nuclei were donated by the African wild cat and transferred to enucleated domestic cat oocytes resulting in a high rate of blastocyst formation but no pregnancies (Gómez *et al.*, 2003). In another study in which embryos were constructed using somatic cells derived from the African wild cat and domestic cat oocytes, 75% of the embryos developed to term and 25% underwent fetal resorption or abortion (Gómez *et al.*, 2004). Of the 17 cloned kittens born, seven were stillborn, eight died within hours of delivery or up to 6 weeks of age, and two are currently alive and healthy. Additionally, some studies on wild felids have shown the establishment of somatic resource banks. There is a bank with somatic samples of 69 individual Iberian lynx, considered the most endangered felid in the world, with the aim of future cloning (León-Quinto *et al.*, 2009; 2014).

In addition, works on the cheetah (*Acinonyx jubatus*), a species of the Pantherinae subfamily, have been performed in South America and Asia. Somatic cells from a cheetah raised in South America were transferred to domestic cat oocytes, and, after embryo aggregation during *in vitro* culture, high blastocyst formation rates were obtained (16.7–28.3%) (Moro *et al.*, 2015). Moulavi *et al.* (2017) used non-viable frozen cells derived from frozen tissue from an Asiatic cheetah (*Acinonyx jubatus venaticus*) and *in vitro*-matured domestic cat oocytes and obtained morula rates of 5.9%. Although no blastocyst was obtained, this study demonstrated that enucleated cat oocytes can partially remodel and reactivate nonviable nuclei of the Asiatic cheetah and support its reprogramming back to the embryonic stage.

Ungulates

Some studies with ungulates have been performed with significant success, especially for species already extinct. The first animal

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derived from an extinct subspecies was obtained using fibroblasts from skin biopsies collected before the death of the last female *Capra pyrenaica pyrenaica*. After a year under cryopreservation, these cells were used as karyoplasts and fused with the cytoplasts of a domestic goat to reconstruct embryos. The rate of cleaved embryos after 36 h was 47.3%, of which 65.5% were transferred. Five recipients were pregnant at 45 days but only one pregnancy went to term. Unfortunately, a few minutes after birth the animal died from pulmonary complications (Folch *et al.*, 2009).

Experiments were carried out with wild yak (*Bros grunniens*) with the goal of evaluating the parameters that affect the success of iSCNT (Li *et al.*, 2007). Fibroblasts and cumulus cells were used as donor cells, but the cell type and different ages were found to have no significant effect on iSCNT.

In 2017, the birth of a Bactrian camel cloned by iSCNT was first reported (Wani *et al.*, 2017). The fibroblasts used to donate nuclei were obtained from ear skin biopsy samples from an adult male Bactrian camel (*Camelus bactrianus*) and the cytoplast of dromedary camel (*Camelus dromedaries*) was the oocyte recipient. Twenty-six blastocysts were transferred to 23 synchronized dromedary recipients yielding five pregnancies with one going to term. This work has great importance because the Bactrian camel is the eighth most endangered large mammal on Earth.

Finally, the woolly mammoth (*Mammuthus primigenius*) is perhaps the one wild mammal of the ungulates whose cloning arouses the greatest interest. This animal became extinct about 10,000 years ago. However, epithelial and muscular cells from 14,000–15,000-year-old mammoth tissues were cryopreserved, with the goal of producing embryos of this species (Kato *et al.*, 2009). In this study, the authors injected cell nucleus-like structures into mature mouse enucleated oocytes; however, the oocytes did not form pronuclear-like structures at 7 h after injection.

Other species

The ability of bovine enucleated oocytes to support dedifferentiation of nuclei from monkey fibroblasts in interspecies cloned monkey embryos has been observed (Lorthongpanich *et al.*, 2008). These embryos were cultured in conditions different from the medium used for cattle with monkey-specific alterations, but the embryos were not able to develop past 16 cells under any culture condition. Nevertheless, OCT-4 was detected, demonstrating the ability of bovine ooplasm to support dedifferentiation but not embryonic development. Therefore, the culture medium promotes dedifferentiation but is not able to support complete embryonic development (Lorthongpanich *et al.*, 2008). In another work that used porcine cytoplasts and donor cells from a rhesus monkey, it was possible to obtain blastocysts despite the low rate (2.04%) (Zhu *et al.*, 2014). Although being a SCNT study, the cloning of cynomolgus monkeys (*Macaca fascicularis*) is cited here because of recent advances in this species. Thus, in a study on cynomolgus monkeys using SCNT, Liu *et al.* (2018) applied histone demethylase Kdm4d mRNA and histone deacetylase inhibitor trichostatin A after activation. Embryonic development improved followed by a greater number of pregnancies, which resulted in the birth of two monkeys via the SCNT technique using fetal fibroblasts and oocytes of cynomolgus monkeys.

With respect to aquatic mammals, a study performed on the minke whale (*Balaenoptera bonaerensis*) compared different conditions of iSCNT, including the ability of porcine and bovine ooplasms to produce reconstructed embryos and the effects of different donor cell types (viable or nonviable cells) on whale SCNT embryos (Ikumi *et al.*, 2004). The authors concluded that whale iSCNT embryos can develop to at least the four-cell stage, regardless of the survivability of the donor cells and the porcine or bovine ooplasm.

Final considerations

Although cloning has several technical limitations that require greater attention to improve the technique, iSCNT has been applied to numerous species of wild mammals and has achieved positive results with respect to embryonic stages in pregnancies and offspring born. The works cited in this paper have made it possible to analyze the state of the art and to perform specific studies the problems in the technique that can be fixed according to the species being studied.

This review has shown that there is no rule that says several species should be cloned following the same protocol, but that each species has different needs at each stage of the technique. In addition, all the papers referred to in this review point to the need for improvement and study at a certain stage, which will lead to improvement of the technique. Thus, to achieve a satisfactory result with iSCNT, each step involved in cloning must be suitable for the species being studied.

Although iSCNT is not the main tool for the reestablishment of endangered wild mammals, its use to increase the possibilities of reproduction and multiplication of individuals has been proposed. It should be refined so that it can be an alternative when traditional techniques cannot be applied. In addition, cloning helps elucidate the embryonic development of a wild species and the subsequent application of this knowledge.

Finally, this biotechnology can help generate more ways to maintain individual species. Therefore, the improvement of protocols to potentiate this technique is of interest because although it has low efficiency rates, iSCNT shows promise because of the pups of different species that have been born.

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Conflicts of interest. None of the authors has any conflict of interest to declare.

Ethical standards. Not applicable

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