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Applied Biotechnologies in the Conservation of Wild Felids: \textit{In vitro} Embryo Production and Cellular Regenerative Therapies

Daniel Veraguas, Diana Echeverry, Fidel Ovidio Castro and Lleretny Rodriguez-Alvarez

Abstract

This review consists of a complete recompilation of advances made in \textit{in vitro} embryo production (IVP) and cellular therapies in the domestic cat and wild felids species. Actually, the domestic cat is considered a valuable model for the development of assisted reproductive techniques and cellular regenerative therapies that might be used in the conservation of endangered felids. The \textit{in vitro} embryo production technologies such as \textit{in vitro} fertilization (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT) have been applied in different species of small wild felid and big felids, resulting in live birth in some cases. However, more studies are needed to improve the efficiency of these techniques and maximize their use in the reproduction and conservation of wild felids. On the other hand, the mesenchymal stem cells (MSCs) therapies have been increasingly used in the veterinary medicine. Recent studies had reported the use of MSCs in the treatment of some chronic diseases in the domestic cat. For these reasons, the MSCs therapies are projected as a promising alternative for the treatments of chronic diseases that might affect wild felids that live in zoos, which could be caused by their captive environment conditions.

Keywords: wild felids, domestic cat, \textit{in vitro} fertilization, somatic cell nuclear transfer, mesenchymal stem cells

1. Introduction

Actually, the domestic cat is considered a valuable model for the development of assisted reproductive technologies (ARTs) and cellular regenerative therapies that might be used in the conservation of wild felids.
conservation of endangered felids [1, 2]. At present, according to the Red List of Endangered Species of the IUCN, 17 species of wild felid are classified as endangered and 8 as near threatened [3]. This has led to the implementation of programs based in ARTs for the genetic preservation of endangered felids in zoological institutions from the USA and countries of Latin America like Mexico and Brazil [4, 5]. The in vitro embryo production (IVP) systems are a valuable tool that allow obtaining information regarding the developmental biology and might help the genetic preservation of endangered felids. The in vitro fertilization (IVF) is the most used IVP technique in the domestic cat and it has been used to produced embryos from several wild felid species [2, 6, 7]. The intracytoplasmic sperm injection (ICSI) has been used in the domestic cat with successful live birth [8, 9]. In some wild felid species, the ICSI has been used as a tool to help the embryo production in the cases of low sperm motility [8, 10]. The somatic cell nuclear transfer (SCNT) is a complex technique with a reduced efficiency compared to the techniques previously described. However, the SCNT allows the generation of genetically identical individuals and might help to preserve animals with a high genetic value [11]; the first domestic cat generated by SCNT was born in 2002 [12]. Since then, several research groups have tried to replicate the same achievement in wild felids. In 2003, the first African wildcat (Felis silvestris lybica) was born by interspecific somatic cell nuclear transfer (iSCNT), which proved the efficacy of this technique for the conservation of endangered felids [13, 14]. In general, the IVP techniques in felids still have low efficiency compared to other species [15]. For this reason, more studies are needed to improve these problems.

Regarding cellular therapies, cellular resources that might be potentially used in cell therapy, have been obtained in different species. The use of methodologies for cellular reprogramming has allowed the production of pluripotent and multipotent cells. However, it has been preferred that those methods that do not implicate a dependence on the expression of exogenous genes or changes at the cellular level may affect the viability of the cell for the use in therapy.

The use of stem cell for regenerative therapy in cats has been stimulated by the possibility to treat severe diseases such as chronic renal failure that is the main cause of death in felines, asthma and chronic feline gingivostomatitis. The domestic cats also represent a good model for the study of the efficacy of stem cell therapies for human diseases. Furthermore, it has been postulated that the use of a less differentiated cell type, as the mesenchymal stem cells, like nucleus donor, can improve the efficiency of the SCNT technique, which could be applicable in the conservation of endangered felid species.

2. In vitro embryo production systems in the domestic cat and wild felids

The studies related to the use of ARTs in felids have increased considerably during the last years [16]. In 1990s, several studies reported the live birth of different big felid as the cheetah (Acinonyx jubatus), Siberian tiger (Panthera tigris altaica), clouded leopard (Neofelis nebulosa) and the Puma (Puma concolor) by artificial insemination (AI) [17–20]. However, scares information has been published regarding the in vitro embryo production in big felids. On the other hand, several studies of IVF, ICSI and SCNT have been made in different species of small wild
felids [16]. The in vitro embryo production systems are a valuable tool that allows the study of the gamete interaction, the early embryonic development and the rescue of genetic material. Based on gathered information, it seems that the improvement of IVP technologies in felids might have a significant impact to preserve the endangered big felids.

2.1. In vitro fertilization (IVF)

The first IVF report in felids was made in the domestic cat in 1970, in which cat oocytes were co-cultured in vitro with spermatozoa that were previously incubated in the uterus [21]. Subsequently, Goodrowe et al. described the first live offspring of domestic cat by IVF after the transfer of embryos at 2–4 cell stage [22]. Actually, the IVF is the most used technique for the in vitro embryo production in the domestic cat and several wild felids species [2].

2.1.1. In vitro fertilization in the domestic cat

A series of studies have been made to improve the efficiency of the IVF. However, the domestic cat embryos generated by IVF have a reduced developmental capacity compared to the in vivo produced embryos [23]. In the domestic cat, the percentage of blastocysts produced from oocytes matured and fertilized in vitro is low compared to those matured and fertilized in vivo (10–50 vs. 50–66%, respectively) [15]. The domestic cat embryos generated in vitro suffer a developmental block at the morula stage, which is attributable to deficient conditions in the oocyte in vitro maturation and in the in vitro embryo culture [24, 25]. However, some research groups have overcome this inconvenience, reporting blastocyst rates of 50–70% approximately after 6, 7 or 8 days of culture [2, 16, 26–28] (Table 1).

One of the biggest problem of the IVP systems in the domestic cat, is the low competence of the in vitro matured oocytes. For this reason, some research groups have decided to use gonadotrophins to induce the in vivo maturation of cat oocytes. Pope have used porcine FSH (pFSH), to induce follicular recruitment and porcine LH, (pLH) to induce oocyte maturation, which have allowed the successful embryo generation by IVF, ICSI and SCNT [16]. Likewise, Yu et al. have described the use of eCG and hCG for the in vivo maturation of cat oocytes and their subsequently use in parthenogenetic activation, IVF and SCNT [29].

Our research group evaluated the individual effect of both gonadotrophins (pFSH and eCG) in the morphological quality and gene expression pattern of cumulus-oocyte complexes (COCs). Regarding the pFSH treatment, our study demonstrated that pFSH enhanced the morphological quality of COCs and increased the expression of the gonadotrophin receptor LHCGR. Furthermore, these results were related to an improved in vitro embryo development with an increased blastocyst and hatching blastocyst rates compared to the control untreated group (30.5 vs. 13.1% and 13.2 vs. 1%, respectively) [30]. Likewise, the treatment of domestic cat with eCG enhanced the morphological quality and the expression pattern of the COCs. The eCG treatment increased the relative expression of gonadotrophin receptors (FSHR and LHCGR) and gonadotrophin-induced genes (EGFR, EGR1 and ESR2) in the COCs, which might be related to an enhanced oocyte competence [31]. Subsequently, we observed that the oocytes recovered from cats treated with eCG had an enhanced developmental capacity after
parthenogenetic activation, which was reflected in a higher blastocyst rate compared to the control untreated group (32.8 and 16.9%, respectively) [unpublished data]. No differences on oocyte competence were observed between pFSH and eCG treatments. For this motive, we choose the eCG treatment for the ovarian stimulation in domestic cats. The eCG has a longer half-life than pFSH and only a unique dose is required, which results in a more practical protocol and less stress for the animals.

Actually, our research group has used the mild ovarian stimulation with eCG for the in vitro embryo production in the domestic cat. The domestic cat was treated only with a unique dose of 200 IU of eCG and then the recovered COCs were in vitro matured. We choose this protocol because no additional improvement was observed in the developmental competence using in vivo matured oocytes after eCG and hCG treatment. After parthenogenetic activation, no differences were observed in the blastocyst rate between the eCG and IVM group (32.8%) and the eCG and hCG group (31.4%) [unpublished data].

This protocol was used in the production of embryos by IVF (Table 1). Additionally, we evaluated the in vivo development of the generated blastocyst. Three embryo transfers were made, 23 day-7 blastocysts were transferred to two domestic cat (15 and 13 blastocysts, respectively). In both cats pregnancy was established, but only the cat that received 15 blastocysts gave birth to one female kitty after 64 days of gestation. Additionally, 8 day-8 blastocysts were transferred to one domestic cat but no pregnancy was established (Table 1).

In general, the transfer of felid embryos has been successfully made at earlier stages, at the 1–2 cell stage [14] and morula or early blastocyst stages [16, 32]. Kanda et al. evaluated the transfer of cat embryos at the morula stage, early blastocyst stage after 4–6 days of IVC and blastocyst stage after 7 days of IVC. All the recipients that received embryos at the morula stage (4/4, 100%) and three recipients that received early blastocyst (3/5, 60%) became pregnant.

### Table 1. In vitro and in vivo development of domestic cat embryos after IVF using oocytes from different sources.

<table>
<thead>
<tr>
<th>Oocyte source</th>
<th>In vitro embryo production by IVF</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cleavage rate (%)</td>
<td>Morula rate (%)</td>
</tr>
<tr>
<td>In vivo maturation</td>
<td>62.9–88.2</td>
<td>40–47</td>
</tr>
<tr>
<td>In vitro maturation</td>
<td>50.2–71.5</td>
<td>52.2–62.7</td>
</tr>
<tr>
<td>eCG + IVM</td>
<td>64.9</td>
<td>74.2</td>
</tr>
</tbody>
</table>

The ranges showed were derived from several results of the respective studies cited.

- Pregnancy rate was derived from the total pregnancies established/total transferred recipients, according to each reference.
- Live birth rate correspond to the transferred recipient that delivered healthy kittens/total transferred recipient, according to each reference.
However, no pregnancies were established after the transfer of 7-days blastocysts (0/3) [33]. It has been postulated that this might be due to the zona pellucida integrity of 7-days cat blastocysts, most of the blastocysts begin to hatch the zona pellucida after day 6 and this might affect negative in vivo development [16, 33].

2.1.2. In vitro fertilization in small wild felids

The first live birth report was made in the Indian desert cat (*Felis silvestris ornata*). After IVF, 4 Indian desert cat morulae and 10 domestic cat morulae were transferred to the uterine horn of a domestic cat recipient, which gave birth to one live Indian desert cat [6, 34]. This demonstrated that the domestic cat can be successfully used as recipient for small wild feld embryos. The same research group reported later, the live birth of an African wildcat generated by IVF; the embryos were cryopreserved and subsequently transferred to a domestic cat recipient [35]. In subsequent years, the live birth from IVF-derived embryos was reported in the Serval cat (*Leptailurus serval*), Caracal (*Caracal caracal*), Fishing cat (*Prionailurus viverrinus*), Sand cat (*Felis margarita*) and Black-footed cat (*Felis nigripes*) [7, 32, 36, 37]. In these studies, the embryos were transfer at the morula stage after 5 or 6 days of IVC to domestic cat recipients [7] or intra-species recipients [7, 32, 36, 37]. Additionally, the interspecific IVF has been described in the bobcat (*Lynx rufus*) and in the Iberian lynx (*Lynx pardinus*) as a method to prove the fertilization capacity of cryopreserved spermatozoa from these species using domestic cat oocytes [38, 39] (Table 2).

2.1.3. In vitro fertilization in big felids

Actually, only a few studies have reported the embryo production by IVF in big felid. The first successful report of IVF was made in tiger (*Panthera tigris*). After IVF, 86 tiger embryos at the 2–4 cell stage were transferred into the oviduct of four recipients. One pregnancy was established and three cubs were delivered 107 days after embryo transfer [40]. Other study demonstrates that the cryopreservation of tiger spermatozoa does not affect the fertilization rate and developmental competence after IVF [41]. Regarding embryo cryopreservation, vitrification is the most efficient method for the cryopreservation of tiger embryos generated by IVF [42]. No more studies of IVF in the tiger have been published.

IVF have been performed in the Puma as well; the semen samples collected from male specimens and used for the IVF showed high degree of pleomorphism (82–99%). Despite that, an overall fertilization rate of 43.5% was obtained [43]. Subsequently, the IVF in the Cheetah was described; after IVF an overall fertilization rate was only 26.6%. However, the fertilization rate varied from 0 to 73.3% among males [44]. Previous reports have described a high proportion of abnormal spermatozoa/ejaculate in the Cheetah (31–97%) [45, 46]. A decade later, it was described the IVF in the lion (*Panthera leo*), being the first report that described the early embryo development in this specie (Table 2 and Figure 1).

In resume, the IVF has been used more in small wild felids than in big felids, this is mainly due to the easier manipulation of small wild felids during the laparoscopic oocyte retrieval and semen collection procedure. Furthermore, in the case of some small felids species, the
generated embryos can be transferred to a domestic cat recipient producing live offspring. Additionally, the high pleomorphism incidence in the spermatozoa of some big felid species, reduces the efficient of IVF in those species.

### 2.2. Intracytoplasmic Sperm Injection (ICSI)

Actually, the fertilization techniques assisted by micromanipulation are used widely in the human fertility clinics, allowing the in vitro embryo production in the cases of low sperm quality or reduced motility [48]. The ICSI is the direct injection of a single spermatozoon into the cytoplasm of a matured oocyte, eliminating the negative effects of low sperm motility [48]. ICSI is projected as a possible alternative for the in vitro embryo production in wild felids, where a high degree of morphological abnormality and low motility in the collected sperm have been observed [49].

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**Table 2. Efficiency of the embryo production by IVF in wild felids.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Fertilization rate (%)</th>
<th>Cleavage rate (%)</th>
<th>Morula rate (%)</th>
<th>Blastocyst rate (%)</th>
<th>Pregnancy rate (%)</th>
<th>Live birth rate (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indian desert cat</td>
<td>67.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>25.0</td>
<td>25.0</td>
<td>[34]</td>
</tr>
<tr>
<td>African wildcat</td>
<td>74.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>66.6</td>
<td>33.3</td>
<td>[2, 35]</td>
</tr>
<tr>
<td>Sand cat</td>
<td>—</td>
<td>76.9</td>
<td>—</td>
<td>—</td>
<td>25.0</td>
<td>25.0</td>
<td>[37]</td>
</tr>
<tr>
<td>Serval</td>
<td>33.0–68.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>16.6</td>
<td>16.6</td>
<td>[36]</td>
</tr>
<tr>
<td>Caracal</td>
<td>—</td>
<td>66.0</td>
<td>—</td>
<td>—</td>
<td>33.0</td>
<td>33.0</td>
<td>[32]</td>
</tr>
<tr>
<td>Fishing cat</td>
<td>—</td>
<td>60.0</td>
<td>—</td>
<td>—</td>
<td>9.0</td>
<td>9.0</td>
<td>[32]</td>
</tr>
<tr>
<td>Black-footed cat</td>
<td>—</td>
<td>47–70</td>
<td>—</td>
<td>—</td>
<td>40.0</td>
<td>40.0</td>
<td>[7]</td>
</tr>
<tr>
<td>Bobcat</td>
<td>46*</td>
<td>—</td>
<td>27*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>[38]</td>
</tr>
<tr>
<td>Iberian lynx</td>
<td>11.5–20.5*</td>
<td>44.7–87.5*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>[39]</td>
</tr>
<tr>
<td>Tiger</td>
<td>63.4</td>
<td>39.0</td>
<td>43.5</td>
<td>30.4</td>
<td>25.0</td>
<td>25.0</td>
<td>[40]</td>
</tr>
<tr>
<td>Puma</td>
<td>43.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>[43]</td>
</tr>
<tr>
<td>Cheetah</td>
<td>0–73.3</td>
<td>17.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>[44]</td>
</tr>
<tr>
<td>Lion</td>
<td>—</td>
<td>53.0</td>
<td>50.0</td>
<td>30.0</td>
<td>—</td>
<td>—</td>
<td>[47]</td>
</tr>
</tbody>
</table>

*Interspecific embryos generated using domestic cat oocytes.

The ranges showed were derived from several results of the respective studies cited.

- Pregnancy rate was derived from the total pregnancies established/total transferred recipients, according to each reference.
- Live birth rate correspond to the transferred recipient that delivered healthy kittens/total transferred recipient, according to each reference.
2.2.1. Intracytoplasmic sperm injection in the domestic cat

The first ICSI-derived domestic cat embryos were produced using *in vivo* matured oocytes. After the transfer of 10–11 morulae at 5 day of culture into four recipients, two pregnancies were established and three kittens were born [8]. It was described that the embryos generated by ICSI using *in vitro* matured cat oocytes had a decreased developmental competence compared to those derived from *in vivo* matured oocytes [9]. Despite this, the ICSI embryos generated from *in vitro* matured cat oocytes are able to produce live births [9]. Subsequently, the production of cat embryos by ICSI using *in vitro* matured oocytes and frozen epididymal spermatozoa was described. The generated embryos were capable to reach the blastocyst stage [50]. However, the blastocyst rate was low (6.6%) compared to the previously reported by Pope et al. (42.9%) and Gómez et al. (19%) [8, 9, 50].

2.2.2. Intracytoplasmic sperm injection in small wild felids and big felids

No many studies related to the ICSI in wild felids have been published. The first report was made in the jaguarundi (*Herpailurus yagouaroundi*). Nine jaguarundi embryos that reached the early morula stage at day 5 of culture were transferred into two domestic cat recipients, but none of them developed to term [8]. Subsequently, it was described the generation of Fishing cat embryos by ICSI, 19 day-2 cleaved embryos were transferred into the oviduct of one fishing cat but no pregnancy was established [32] (Table 3).

It has been described that the generation of lion embryos by ICSI using sperm collected by percutaneous epididymal sperm aspiration (PESA) from two vasectomized male lions. After ICSI, all the cleaved embryos reached the morula stage at day 5 of culture [51]. Subsequently,
it was described that the lion oocytes are able to mature in vitro and after ICSI the embryos are able to reach blastocyst stage. However, the embryos reached the morula stage at day 6 and the blastocyst stage at day 9, which indicated a slow in vitro development [52]. More recently, the interspecific ICSI in the leopard (Panthera pardus) and Cheetah using domestic cat oocytes was described. The interspecific embryos were able to develop in vitro until the blastocyst stage with a similar rate compared to domestic cat embryos. This demonstrates that the developmental capacity of big felid spermatozoa can be evaluated by interspecific ICSI [10] (Table 3 and Figure 1).

More research is needed to evaluate the ICSI in big felids. The ICSI allows the in vitro embryo generation in the cases of poor sperm quality, which is consisted in some big felid species. Furthermore, the interspecific ICSI using domestic cat oocyte offers the possibility to study the in vitro developmental capacity in these species.

### 2.3. Somatic cell nuclear transfer (SCNT)

During the SCNT, the oocyte cytoplasmic factors are capable to reprogram the expression pattern of a somatic cell into a pluripotent embryonic state, allowing it to initiate the early development [53]. The fact that a differentiated cell must be reprogrammed to a pluripotent stage made the SCNT a complex technique with a reduced efficiency. Different factors may affect the efficiency of the SCNT, some of these are: the quality of the donor nucleus or the recipient oocyte and the synchronization between the donor nucleus and the oocyte. These factors can lead to an inadequate reprogramming of the donor genome and to a reduced embryo development [11]. Despite this low efficiency, the SCNT is the only technique that may generate genetically identical individuals, which bring the possibility to rescue the genetic material of endangered species.

<table>
<thead>
<tr>
<th>Species</th>
<th>In vitro embryo production by ICSI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domestic cat</td>
<td>Cleavage rate (%)</td>
<td>57.0–81.6</td>
</tr>
<tr>
<td>Fishing cat</td>
<td>22.0–70</td>
<td>0</td>
</tr>
<tr>
<td>Jaguarundi</td>
<td>55.6</td>
<td>0</td>
</tr>
<tr>
<td>Cheetah</td>
<td>66.3–73.6</td>
<td>0</td>
</tr>
<tr>
<td>Lion</td>
<td>30.8–60.0</td>
<td>0</td>
</tr>
<tr>
<td>Leopard</td>
<td>35.3–73.7</td>
<td>0</td>
</tr>
</tbody>
</table>

*Interspecific embryos generated using domestic cat oocytes.
The ranges showed were derived from several results of the respective studies cited.

• Pregnancy rate was derived from the total pregnancies established/total transferred recipients, according to each reference.

• Live birth rate correspond to the transferred recipient that delivered healthy kittens/total transferred recipient, according to each reference.

Table 3. Efficiency of the in vitro embryo production by ICSI in domestic and wild felids.
2.3.1. Somatic cell nuclear transfer in the domestic cat

The first domestic cat produced by SCNT was made using cumulus cells as nucleus donors for metaphase II enucleated oocytes. A live kitten was born 66 days after the embryo transfer [12]. Subsequently, it was described the use of cat fetal fibroblasts and adult fibroblasts as nucleus donors for SCNT. No statistical differences were observed in the fusion, cleavage and blastocyst rates when fetal or adult fibroblasts were used. Furthermore, live birth was obtained using both type of cells [54].

In addition, the advances of the SCNT in felids have led the production of transgenic animals. A domestic cat that expresses the red fluorescent protein was generated using a retroviral vector for the incorporation of the transgene in the somatic cells used as nucleus donor [55]. A year later, it was reported the birth of a transgenic cat that express the green fluorescent protein, which was generated using a lentiviral vector to modify the donor cells [56]. The objective of these studies was to potentiate the use of the domestic cat as a biomedical model for the study of analogues diseases in humans. This proves that these techniques could be implemented to generate genetically identical animals that have integrated coding genes for specific human diseases.

2.3.2. Somatic cell nuclear transfer in small wild felids

The scares availability of gametes is one of the biggest problem for the in vitro embryo production in endangered animals due to their reduced population. The interspecific somatic cell nuclear transfer (iSCNT) is proposed as alternative for this problem. It has been demonstrated that the domestic cat oocyte can be used as recipient cytoplasm for somatic cells of wild felids, generating embryos of these species [13]. It was reported the live birth of African wildcats by iSCNT using the domestic cat oocyte as recipient cytoplasm and a female domestic cat as recipient for the cloned embryos [14]. Subsequently, the same research group described the live birth of three Sand cats by iSCNT [57]. Different studies have tried to replicate those results in other felid species, but no live births have been reported. This may be because the African wildcat and the Sand cat are subspecies closely related to the domestic cat, which might improve the efficiency of cell reprograming during iSCNT. However, it seems when somatic cells from felid species, phylogenetically more distant from the domestic cat are used, the efficiency of the iSCNT tend to decrease.

In the leopard cat (Prionailurus bengalensis), the embryo generation by iSCNT was reported, using domestic cat oocytes as cytoplasm recipients. The cloned embryos reached the blastocyst stage. However, after embryo transfer, pregnancy was established but no live births were obtained [58]. Subsequently, the generation of embryos of rusty-spotted cat (Prionailurus rubiginosus) and black-footed cat by iSCNT was described. The cloned embryos were capable to develop until the blastocyst stage. After embryo transfer, the rusty-spotted cat embryos were not able to implant. Meanwhile, the transferred black-footed cat embryos were able to establish pregnancy but all the fetuses were reabsorbed later [59]. Similarly, in the marbled cat (Pardofelis marmorata) and flat-headed cat (Prionailurus planiceps); after iSCNT, the embryos were able to develop in vitro until the blastocyst stage. However, no pregnancies were established after embryo transfer into domestic cat recipients [60].
2.3.3. Somatic cell nuclear transfer in big felids

There are scarce reports regarding to the iSCNT in big felids. The first report was made in the tiger, in which pig oocytes were used as recipient cytoplast. However, a blastocyst rate of only 0.7% was obtained [61]. More recently, leopard, tiger and lion cloned embryos were generated by iSCNT, using rabbit oocytes as cytoplast recipients. This study described that the rabbit oocytes were capable to reprogram big felid somatic cells. However, the blastocyst rate after in vitro culture was 5–6% in the three species [62].

On the other hand, several methods have been implemented to improve the low efficiency of the SCNT such as embryo aggregation. This consists of the in vitro culture of two or more zona-free embryos together in the well of the well system (WOW) [63]. It was reported that the aggregation of cloned cheetah embryos and cloned tiger embryos generated by iSCNT improves the in vitro development in these species. However, the developmental capacity of cheetah and tiger cloned embryos was reduced compared to domestic cat cloned embryos [63, 64] (Table 4 and Figure 1).

In resume, the iSCNT have a reduced efficiency and it seems that these problems are more evident when the species used as nucleus donor are more distant phylogenetically from the domestic cat. This might be related to an incomplete donor nucleus reprogramming. Regarding the big felids, it has been proved that pig and rabbit oocytes are able to reprogram big felid donor cells, but they are not as efficient as domestic cat oocytes. The embryo aggregation has been postulated as a method that improves the developmental capacity of big cat cloned embryos. However, the developmental capacity of these embryos is reduced compared to domestic cat cloned embryos. More studies are needed to improve the reprogramming events that allow the development of iSCNT-derived embryos in big felids.

2.4. Gene expression analysis as indicator of developmental capacity in felid embryos

During the early development, the expression of specific genes allows the embryo progress from one stage to the next [65]. For this reason, the relative quantification of mRNA from crucial genes during the early development is considered an adequate indicator of the embryo quality [66]. The pluripotency markers OCT4, SOX2 and NANOG and the differentiation markers CDX2 and GATA6 play a crucial role during the embryo development in the domestic cat [67]. According to this, an aberrant expression pattern of these genes might be related to a reduced developmental competence of the produced embryos [67].

It has been described that the gonadotrophin treatments in the domestic cat improve the gene expression pattern in the COCs and in the produced embryos. The ovarian stimulation of domestic cat with pFSH enhances the embryo developmental capacity and increases expression of OCT4 and GATA6 at the blastocyst stage [30].

Furthermore, in the domestic cat, it has been described that the in vitro-produced blastocysts had a decreased relative expression of OCT4 compared to the in vivo-produced blastocysts [67]. This demonstrated that the in vitro culture affect negatively the gene expression pattern of domestic cat embryos, which is reflected in their reduced developmental competence.
The technique used for the embryo generation might also affect the gene expression pattern. A decreased OCT4 expression was related to a reduced developmental capacity of feline embryos generated by iSCNT [68]. Moreover, it has been described that cheetah embryos generated by iSCNT have an aberrant expression of OCT4, SOX2, NANOG and CDX2 compared to domestic cat embryos generated by SCNT and IVF. This was in accordance with a reduced developmental competence of the cheetah cloned embryos, which might be due to an inadequate reprogramming of the cheetah donor cell by the cat oocyte [63].

### 3. Cellular regenerative therapies in felids

The challenge in feline cell therapy is to offer treatment options to different diseases and to use stem cells in endangered animals is another challenge. This latter challenge is even more
complicated because of the low availability of tissue to isolate stem cells in these species. However, biotechnology can bring us closer to the possibility of improving techniques for obtaining stem cells for therapy and tissue regeneration that may also contribute to the conservation of these individuals. This review tries to cover the main aspects of stem cells in domestic cats and its future application in wild cats, as well as their obtaining and characterization.

3.1. Induced pluripotent feline stem cells

The induced pluripotent stem cells (iPSCs) are generated by the induced expression of pluripotency transcription factors in cells that normally do not express. In this way, stem cells from somatic and differentiated cells can be obtained. Takahashi and Yamaka were the first to develop and implement this technique in 2006, who induced somatic cells mouse in iPSC by transfection of the cells with the transcription factors “OSKM” corresponding to transcription factor binding octamer 3/4 (Oct3/4), high protein group mobility-related gene Sry2 (Sox2), Kruppel factor 4 (Klf4) and c-Myc oncoprotein [69].

To date, there are reports of iPSC generated from endangered feline species such as snow leopard, tiger, jaguar and African serval [70, 71]. Protocols for induction to pluripotency in wild cats conclude that Nanog is a key factor in reprogramming [68]. For this reason, reprogramming cocktails included the four Yamanaka factors plus Nanog and use culture medium supplemented with LIF and SFB. When Nanog was removed from the cocktail, the reprogramming efficiency was greatly reduced and the colonies reached only up to the seventh pass (P7) [70]. On the other hand, the inclusion of Nanog made that the iPSCs colonies could be expanded in vitro, were positive for alkaline phosphatase and for OCT-4, NANOG and SSEA-2 proteins at the passage 14 (P14) [70]. Oct4 and Nanog endogenous were detected by RT-PCR at passage 4 and 14, indicating reprogramming and reactivation of endogenous pluripotency genes and the transgenes of Oct4, Sox2 and Nanog were silenced at the passage 14 [70]. These cells showed a good set of stem cell characteristics like teratoma formation and genetic markers for the differentiation to ectoderm, endoderm and mesoderm. A curious fact of these protocols is the use of mouse embryonic fibroblasts (MEF) as feeder and the absence of FGF2 in the culture medium. In general, regarding the culture medium, this is simpler than the used in other species and in the domestic cat embryonic stem cell (ESC) culture [72]. This could be considered advantageous to the use of the resources, but may affect the obtaining of a “naïve” phenotype in these feline iPSCs. However, there remains doubt about the pluripotency of these cells because the chimera formation could not be assessed. There is a great interest for the feline iPSCs formation because they represent a novel opportunity for the preservation of species through assisted reproduction. The reason why there are no publications of iPSCs in domestic cats is unknown. According to some researchers, there is not enough funding for research in cell therapy in cats or it is not probably an area in demand [73]. However, the results obtained in the published studies gave the impression that the techniques in this species are still complex and require a high level of standardization.

Despite all these difficulties, the therapeutic and conservation potential that can be reached with cellular reprogramming in domestic and wild felids is promising.
3.2. Mesenchymal stem cells (MSC)

Mesenchymal stem cells (MSC), also called by some authors, as mesenchymal stromal cells are unspecialized cells that have the ability to self-renew by cell division and differentiate into specialized cells [74]. The MSCs are involved in the tissue regeneration by two different mechanisms. They may contribute directly to tissue repairing by differentiation into specific cell phenotypes such as, ligaments, tendons or alternatively fibroblasts, but not necessarily exclusive. These adult stem cells have the ability to produce extracellular matrix and bioactive proteins such as growth factors, anti-apoptotic and chemotactic agents that have an important effect on the dynamic cellular, production of anabolic effects, stimulate neovascularization and additional recruitment of stem cells the site of injury. Stem cells recruited at these sites can differentiate and/or produce biologically active peptides [75]. MSCs have a fibroblast-like morphology, must be adherent to plastic, express surface molecules like CD105, CD73 and CD90 and have a negative expression of CD45, CD34, CD14 o CD11b, CD79α o CD19 and HLA-RD. In addition, the MSCs are capable to differentiate in vitro into osteoblasts, adipocytes and chondroblasts [74, 76]. However, characterization of MSCs in veterinary species is more difficult due to the lack of specificity or low affinity antibodies, which are generally made against human or murine antigens. These differences in the surface markers may be due to specific features of the tissue from which the MSCs were collected. In the domestic cat, some authors report differences in the expression of surface markers in the MSCs depending of the tissue from which they were isolated (Table 5 and Figure 2). An additional characteristic reported in cat MSCs derived from adipose tissue is the expression of pluripotency markers as NANOG, KLF4 and OCT4 [77].

3.2.1. Tissues source of mesenchymal stem cells (MSCs) in cats

In animals and humans, different tissues represent a potential resource for obtaining MSCs. The bone marrow was the first tissue explored; one of the best sources of production and so far the most widely used in cell therapy. However, other sources such as adipose tissue, have been investigated and successfully used. In the field of veterinary medicine, depending on the target species, the tissue from which these cells are obtained is crucial and can be influenced by the weight, size and the handling of the patient. However, the method for obtaining these tissues does not always correlate with a good source of MSCs. Therefore, the investigation of other resources for obtaining MSCs in different species is important, considering that the results between species cannot be extrapolated in all cases.

3.2.1.1. Bone marrow

MSCs isolated from bone marrow (BM-MSCs) has been described in many animal species such as mice, rabbits, horses, pigs, cattle, dogs and cat [78–83]. Maciel and colleagues conducted a study of the morphology of BM-MSC in felines and they noted the predominance of two types of cells, spindled and elongated [84]. It has been reported that feline BM-MSCs have the potential to differentiate toward neuronal lineage [85]. Feline BM-MSC after the fourth passage becomes more homogeneous and express surface markers like CD44, CD105 and CD29.
Table 5. Expression markers of domestic cat mesenchymal stem cells derived from different tissues.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Adipose tissue</th>
<th>Bone marrow</th>
<th>Blood</th>
<th>Amniotic membranes</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>POS</td>
<td>CD90</td>
<td>CD90</td>
<td>CD90</td>
<td>CD90</td>
<td>[77, 86-90]</td>
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<tr>
<td></td>
<td>CD44</td>
<td>CD44</td>
<td>CD44</td>
<td>CD44</td>
<td>[86-90]</td>
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<td></td>
<td>CD105</td>
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<td>CD105</td>
<td>[77, 86-90]</td>
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<td></td>
<td>CD146</td>
<td></td>
<td></td>
<td></td>
<td>[77]</td>
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<tr>
<td>NEG</td>
<td>CD4</td>
<td>CD4</td>
<td>CD4</td>
<td></td>
<td>[86, 90]</td>
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<td></td>
<td>CD14</td>
<td></td>
<td>CD14</td>
<td></td>
<td>[77, 87, 88]</td>
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<td></td>
<td>CD18</td>
<td></td>
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<td>[89]</td>
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<td>CD34</td>
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<td>CD45</td>
<td>CD45</td>
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<td></td>
<td>CD73</td>
<td>CD73</td>
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<td>[77, 87]</td>
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<tr>
<td></td>
<td>CD271</td>
<td></td>
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<td></td>
<td>[77]</td>
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<tr>
<td></td>
<td>MHC II</td>
<td>MHC II</td>
<td>MHC II</td>
<td></td>
<td>[77, 86, 87, 92]</td>
</tr>
</tbody>
</table>

Figure 2. Representative graphic of the surface markers expression in the domestic cat mesenchymal stem cells.
3.2.1.2. Peripheral blood

Peripheral blood is another tissue of which has been documented obtaining of MSCs. Compared to bone marrow, it is a safer and less painful resource with less subsequent complications. Feline peripheral blood (fPBMSCs) has been isolated from venous blood of cats and expanded in culture [90]. The morphology and capacity for differentiation into mesoderm lineages of these MSCs is similar to others obtained from different feline tissues. However, these MSCs require a special culture medium containing 5% autologous plasma (AP) and 10% fetal bovine serum (FBS), showing a similar growth curve that others reports because its proliferation is limited to the seventh passage [90]. These cells express CD44 and CD90 surface markers and are negative to major histocompatibility class II and CD4.

3.2.1.3. Fetal membranes

The interest of MSCs derived from fetal and extra-embryonic tissues has increased because of its usefulness in the field of regenerative medicine [75, 92–94]. In felines, these MSCs are obtained from tissues discarded after birth or from procedures such as cesarean operation and ovariohysterectomy. These cells have been isolated and successfully expanded. It was reported that these cells have an adequate level of homogeneity at the passage 3, as it was found in canines [95]. Unlike other tissues, the cell population of the feline AMSC increases considerably after the passage 7, with higher values than those reported in horses and dogs, the cell number tends to increase along with number passages [95]. Additionally, the viability of these cells after cryopreservation was similar to the viability of fresh cells, which is ideal for cell banks and future applications in cell therapy [95]. AMSCs feline express CD73 and CD90 surface markers but did not express hematopoietic specific markers CD34, CD45 and CD79 [95].

3.2.1.4. Adipose tissue

Adipose tissue is abundant and more accessible than other tissues and is the most commonly used in cellular veterinary therapies [89]. Cellular therapies for animals using cells derived from adipose tissue (AMSC) have been used to treat osteoarthritis, injuries of ligaments and tendons in canine and equine, feline gingivostomatitis with good results and other pathologies that are being studied as chronic renal failure, asthma and chronic enteropathies in domestic cats [91, 96–98]. Feline AMSCs have been isolated and characterized from black-footed cat, an endangered feline [77]. Both cat and black-footed cat AMSCs showed potential adipogenic, osteogenic and chondrogenic differentiation, but the surface marker expression of black-footed cat AMSCs is unknown and it could be difficult to evaluate because of the specificity of the antibodies. However, these results obtained from endangered felines can be used in ARTs for their conservation such as SCNT, or potentially be used in cell therapy of individuals of the same species.

3.2.1.5. Potential sources of MSCs

Brain, muscle, synovial fluid, tendon, placenta and dental pulp are others potential sources for obtaining MSCs in several species [99–101]. Some of these tissues could eventually be
used to isolate mesenchymal stem cells in felines to perform extensive characterization procedures, which could define if these cells have any potential to be employed in cell therapy. Considering that stem cells can be isolated for other purposes such as species conservation, spermatogonial stem cells (SSCs) can be isolated from testicular tissue. In domestic cats, these cells were successfully isolated and cultured in vitro, but its viability was compromised and the culture only reached 57 days [102]. Interestingly, SSCs can differentiate into sperm and be available for transplantation in testicular tissue. The first successful xenotransplant study was performed using ocelot SSCs in domestic cat testicular tissue [103]. Ocelot (Leopardus pardalis) spermatozoa were observed in the cat epididymis 13 weeks following transplantation suggesting that the domestic cat may be a useful species to produce gametes from another close species. This study along with others demonstrates the importance of the domestic cat as a research model for the conservation of endangered species [103].

3.3. Doses and routes of administration for MSC treatment in domestic cats

To date, mesenchymal stem cells are the only ones that have been evaluated in treatments of certain pathologies in domestic cats. The establishment of the treatment doses of MSCs in cats has been a main point to obtain the expected therapeutic effect and to reduce the adverse effects that may occur. After the adequate characterization of MSCs depending on their place of origin, the challenge is to evaluate the dose response and the route of administration. It has been reported that the intravenous route is the most chosen in the treatments with MSCs. Allogenic and autologous administration of MSCs in cats have not shown any side effects [96, 98, 104]. More studies are still required to determine alternative administration routes that may increase the treatment efficacy. Furthermore, the doses used are still a topic of discussion, but some doses are proposed for certain treatments (Table 6). The effects of reported treatments suggest that they can be seen since the third dose of MSCs administration [96].

4. Conclusions

Great advances have been made in the in vitro embryo production and cellular therapies in felids. The studies first made in the domestic cat have allowed the subsequent application of these techniques in endangered felids. Regarding the in vitro embryo production, several
live birth reports have demonstrated the efficiency of these techniques in the conservation of endangered felids. However, more studies are needed to improve their efficiency especially in the case of the SCNT. On the other hand, the results in cell therapy applied to felids set the background for future research. There is still need to identify the potential of stem cells isolated from different felid tissues. Additionally, the tools for the isolation and characterization of these cells in wild felid species must be improved considering that it is a very limited resource. However, there are unexplored alternatives for the use of stem cells, not only as therapy for different pathologies but also for obtaining gametes or other cell types from which is possible to rescue genetic material.

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