Chapter 2

Assisted Reproductive Techniques on South American Wild Mammals

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http://dx.doi.org/10.5772/62427

Abstract

For South American wild mammals, assisted reproductive techniques (ARTs) have been established in accordance with the procedures used for domestic mammals. However, often, it is not possible to infer the experimental conditions from one species to another. In general, the use of these ARTs aims the conservation of the animal genetic material, using gamete manipulation, embryo technology by *in vivo* or *in vitro* methods, and cloning. In all animals, the previous knowledge of reproductive physiology becomes the starting point for the use of ARTs in the expansion of all species of interest. In this sense, the purpose of this chapter is to develop an approach of theoretical, technical, and applied aspects of the ARTs in South American wild mammals, with emphasis on the state-of-the-art and its progress and perspectives.

Keywords: Wildlife, gamete, embryo technology, germplasm bank

1. Introduction

South America, the fourth largest continent, presents an extreme geographic variation that contributes to its large number of biomes, which includes the marine rainforests, alpine, deserts, savannahs, grasslands, chaparral, desert scrub, fresh water, marine, and deciduous desert. Due to such biome diversity, the South American fauna consists of various unique animals.

In the past decade, a rapid and continuous decline in mammalian species has been documented, indeed affecting South American countries. Therefore, the worry put on the development of conservation strategies, as well as on the rapid expansion of the commercial interest on wild

species around the world, requires an equally rapid development and adoption of assisted reproductive techniques (ARTs). These techniques allow the conservation and multiplication of genetic valuable individuals, as well as facilitate the transport of the germplasm among distant regions. However, the application of ARTs is still a challenge, since data related to basic reproductive information is scarce, and it is indispensable for the genetic management of rare species. Because of the high diversity in reproductive mechanisms among mammals, it is not possible to directly apply protocols developed from one species to another, hindering the extrapolation of developed ARTs. In this sense, this chapter highlights the importance of applying ARTs for the South American wild mammals, showing the most recent studies in this area and the perspectives for its use in conservation programs.

2. Manipulation of spermatozoa

Technology of male gamete consists of various forms for sperm processing, since its collection until its storage by cooling or cryopreservation. In wild animals, the gamete recovery is generally conducted by electroejaculation (EEJ) [1]; nevertheless, for animals unable to ejaculate, or immediately *postmortem*, one possibility is to obtain epididymal sperm or testicular tissue, which can be cultivated and cryopreserved [2]. After collection, samples could be stored in cryobanks, which maintains the sperm quality for an indefinite time, providing valuable genetic material to be used for ARTs destined to the wild mammals' conservation and multiplication.

2.1. Collection of ejaculated sperm

The EEJ is a safe and effective method for semen collection, especially in wild animals, as it allows handling live animals under anesthesia. It consists of a controlled electrical stimulation of the ejaculatory reflex. A transrectal probe coupled with a specific voltage-producing unit [1] is used to apply the stimulus. The types and disposition of the electrodes, the electric stimulation protocol, and the anatomical characteristics of each species exert important influences on the efficiency of the method [3]. Due to the large species diversity, it is necessary to establish adequate species-specific stimulation protocols using appropriate anesthetic procedures, according to animal responses and well-being [4]. Spermatozoon from different species is presented in Figure 1.

In general, most researchers use adaptations of the original protocol developed for felid collection [5], consisting of a total of 80 electrical stimulations divided into three series: 30 stimuli (10 stimuli, 2 to 4 V, series 01), 30 stimuli (10 stimuli, 3 to 5 V, series 02), and 20 stimuli (10 stimuli, 5 and 6 V, series 03), with a 5-minute interval among series. This serial EEJ protocol was adapted for various South American felids such as the ocelot (*Leopardus pardalis*), the margay (*L. wiedii*), the tigrina (*L. tigrinus*) [6], the jaguar (*Panthera onca*) [7], and other carnivores such as coatis (*Nasua nasua)* [8] (Table 1) and maned wolves (*Chrysocyon brachyurus*) [9]. In addition, this protocol has been adapted for other animals such as sloths [10], giant anteaters

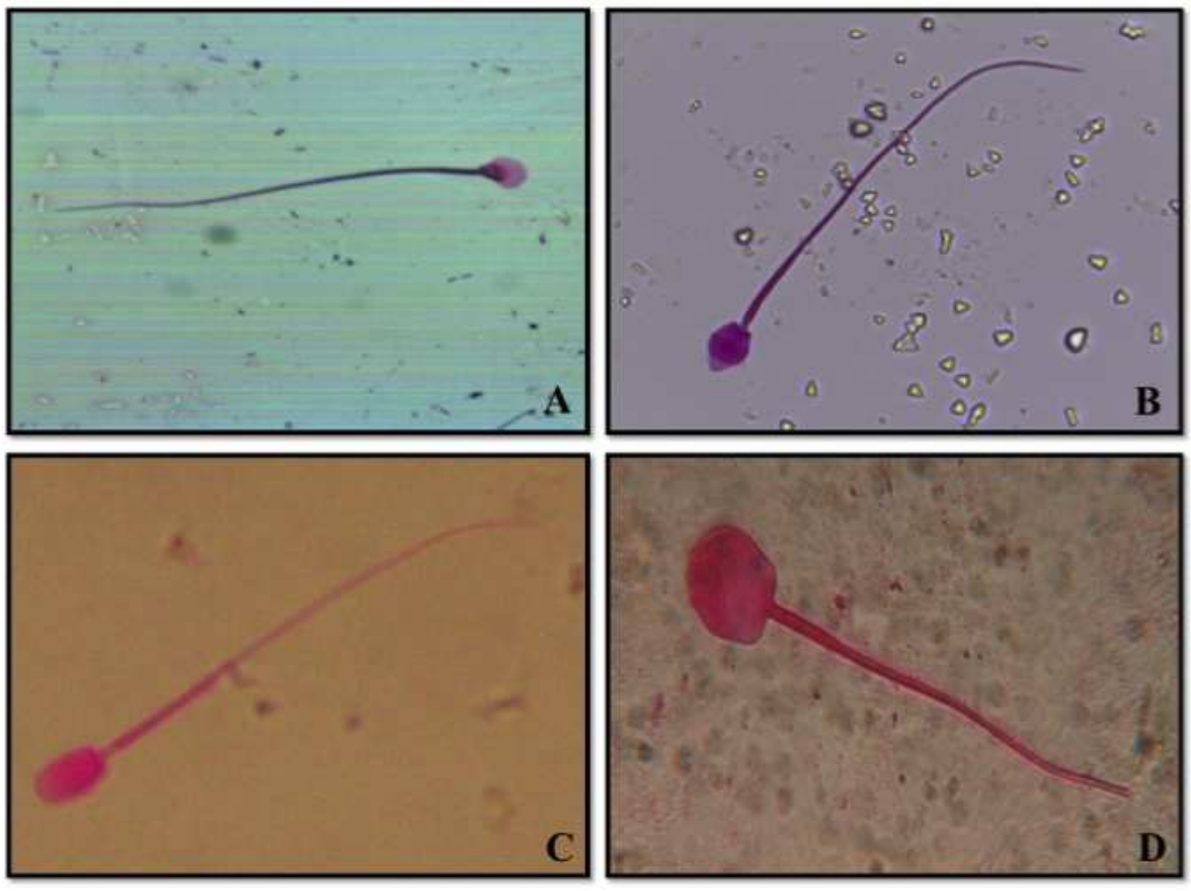

Figure 1. Sperm images of some animals in Latin America, (A) Spix's yellow-toothed cavy—*Galea spixii*, (B) Coati—*Nasua nasua*, (C) Collared peccary—*Pecari tajacu*, (D) Six-banded armadillos—*Euphractus sexcinctus*.

[11], six-banded armadillos (*Euphractus sexcinctus*) [12], tapirs (*Tapirus bairdii*) [13], and agoutis (*Dasyprocta leporina*) [14] (Table 1).

A totally different serial protocol was described for primate species. For spider monkey (*Ateles geoffroyi*), stimulation series begins at 1 V, followed by 1 V increments up to a maximum of 10 V. Each series lasts 5 minutes, with 3-minute rest periods between series [15]. For capuchin monkey (*Sapajus apella*), stimulation session is comprised of six series, 10, 15, 20, 25, 30, and 35 electrical stimuli (12.5–100 mA), with a 30-second interval between consecutive series [16].

In some species, however, a better response is achieved using continuous electrical stimulation. For collared peccaries (*Pecari tajacu*) EEJ (Table 1), the stimulatory cycle consists of 10 stimuli in each voltage, starting from 5 V, followed by voltage increases of 1 V up to 12 V, then, the voltage remains being applied during 10 minutes, continuously [17].

2.2. Epididymal sperm retrieval

Obtaining viable sperm directly from the epididymis tail is an additional option for the conservation of genetic material of South American wild animals, particularly for those accidentally killed. However, different factors such as the epididymis size and vas deferens

Parameters	*Collared peccary* [18]	*Agouti* [14]	*Ring-tailed coati* [8]	*Six-banded armadillo* [12]
Sperm concentration (x10^6)	765.0 ± 313.7	357.0 ± 61.2	197.5 ± 204.9	450.0 ± 14.0
Motility (%)	86.7 ± 2.6	80 ± 29.6	91.3 ± 9.2	61.0 ± 7.0
Vigor (0-5)	4.4 ± 0.3	3.5 ± 1.9	4.5 ± 0.6	2.0 ± 0.2
Viability (%)	92.3 ± 1.6	65.5 ± 26.1	73.1 ± 14.5	55.0 ± 7.0
Membrane functional integrity (%)	75.3 ± 2.3	68.5 ± 17.6	74.3 ± 12.0	46.0 ± 6.0
Normal morphology (%)	83.2 ± 2.2	83.9 ± 8.6	81.2 ± 11.8	86.0 ± 2.0

Table 1. Characteristics of semen obtained by electroejaculation from various South American species.

diameter [18] as well as the possibility for contamination of samples with blood or epithelial cells are considered as limiting factors [19].

Epididymal sperm can be retrieved by flotation or retrograde flushing. In the flotation method, the epididymis is cut into small pieces in a diluent solution for the removal of sperm; this is one of the preferred techniques for small species due to the reduced size of the epididymis [20]. On the other hand, the retrograde washing is accomplished by injecting a buffered solution at the vas deferens, as it was described for the agouti [21]. In some species, such as collared peccaries [22] and yellow-toothed cavies (*Galea spixii*) [23], both recovery methods can be conducted with no notorious differences on sperm quality.

2.3. Sperm preservation

The research on gamete preservation needs to focus on the species-specific variations in sperm physiology, as well as the changes that gametes undergo during sperm processing [24]. The factors most often cited as crucial for success include the sperm processing and packaging, type and composition of extender, duration of equilibrium time, freezing rate, storage, and thawing rate [25].

The short-term preservation of sperm on a liquid form is rarely applied for South American species. In capuchin monkey, for instance, dilution of semen in coconut water–based extender allowed its preservation for 24 hours at 33°C [16]. Moreover, the use of a TRIS extender containing egg yolk or *Aloe vera* extract was efficient in preserving the collared peccary semen for 36 hours at 5°C [26].

Nowadays, intensive research focuses on the development of effective methods for sperm cryopreservation. In general, methods are adapted from the most closely related phylogenetic domestic species; for example, the domestic pig serves as an experiment model for wild pigs; however, such adaptation is not always effective. This fact is verified for six-banded armadillos that present unique semen characteristics, such as high viscosity, large sperm dimensions, and *rouleaux* formation, which hinders the success of freezing protocols [27].

http://dx.doi.org/10.5772/62427

Extenders based on TRIS plus egg yolk and glycerol were reported for collared peccary semen cryopreservation [17]. Other compounds have been described, such as the INRA® extender for tapir [13], the lactose-egg yolk extender for jaguars [28], and a combination of TES and TRIS extenders for capuchin monkey [16]. Recently, a coconut water–based extender was reported for semen cryopreservation in collared peccaries [29], agoutis [30], and squirrel monkeys (*Saimiri collinsi*) [31].

Although most researchers use egg yolk and glycerol as main cryoprotectant, a recent study demonstrated that the dimethyl sulfoxide (DMSO) would be more appropriate for the preservation of maned wolf semen than that of glycerol. Moreover, low-density lipoprotein [32] and *Aloe vera* extract [33] could effectively substitute the egg yolk for the cryopreservation of collared peccary semen.

Regarding preservation of epididymal sperm from South American wild mammals, studies are scarce. For collared peccaries [34] and cavies [35], epididymal sperm was efficiently cryopreserved using TRIS extender added to egg yolk and glycerol. In agoutis, however, epididymal sperm is better cryopreserved in coconut water–based extenders [36].

2.4. Artificial insemination

Artificial insemination (AI) is the single most important technique ever devised for genetic improvement of animals. It refers to the artificial process of sperm deposition into the female genital system, at the appropriate time, seeking the fertilization of the oocyte. The main factors that determine the fertility rates derived from AI include the individual fertility of players, the way in which sperm is collected and manipulated, the ability of the inseminator, the female management (the time of insemination), the type of sperm used (fresh, chilled, or frozen), the insemination dose, and the site for the semen deposition [37].

In spite of AI popularity among domestic animals breeders, its use remains limited in South American wild animals. Initial studies include the successful production of offspring derived from laparoscopic intrauterine AI in ocelots. One female was inseminated with 7.5×10^6 frozen-thawed spermatozoa after receiving 500 IU equine chorionic gonadotropin (eCG) and 225 IU human chorionic gonadotropin (hCG), giving birth to a male offspring after 78 days of gestation [38]. Moreover, deposition of fresh or cryopreserved semen, as well as cryopreserved epididymal sperm, into the cervix of marmoset (*Callithrix jacchus*) resulted in pregnancy and the production of offspring [39].

2.5. Testicular tissue processing

The preservation of testicular tissue for prolonged periods is one big challenge in the field of cryobiology, because spermatogonia, Sertoli, and Leydig cells contain large amounts of water, therein increasing the risk for intracytoplasmic crystal formation [40]. If the testicular tissue has preserved active spermatogenesis, it can be used for the extraction of sperm or elongated spermatids that could be used for oocyte fertilization using intracytoplasmic sperm injection (ICSI) [31]. Moreover, testicle freezing can be used in cases where the animal dies suddenly [41].

Although this is a promising area, studies on the collection of sperm or even on the preservation of testicular tissues are very scarce. In South American individuals, the unique published results can be found in collared peccaries, in which the first successful xenotransplantation of fresh testicular tissue was also documented [42]. In addition, its testicular tissue was efficiently vitrified using ethylene glycol (EG) as cryoprotectant at 3.0 or 6.0 M [43].

3. Estrous and ovulation control

For wild females exhibiting irregular or regular cycles of sexual activity, estrus synchronization support assisted breeding procedures particularly to maximize the chances of conception and the use of fresh or cryopreserved semen [44]. The administration of exogenous hormones, whether or not in association, can artificially synchronize the estrous and ovulation of wild females, altering its endogenous endocrine environment [45]. Research has dramatically increased the number of synchronization protocols for wild animals, being the prostaglandins, progestins, or gonadotropins the most available, each with specific functions and peculiar mechanism of action.

One of the most ancient methods for estrus synchronization is the use of a luteolytic agent such as the prostaglandin F2α (PGF2α) or its analogues, which provokes the *corpus luteum* degeneration [46]. Another approach is by using gonadotropin-releasing hormone (GnRH), which represents the final common pathway where by internal and external relevant stimuli converge to control reproduction. GnRH neurons, thus, stimulate pituitary gonadotropin secretion to appropriately regulate gametogenesis and sex steroid secretion [47]. On the other hand, progestins inhibit the synthesis and secretion of GnRH that subsequently inhibits follicular growth and development as well as the ovulation [48]. Additionally, gonadotropin-based protocols include the use of equine and human chorionic gonadotropins (eCG and hCG, respectively), as well as the application of follicle stimulating hormone (FSH) and luteinizing hormone (LH), as a synthetic or extract form [49]. The action of eCG is similar to that of FSH at stimulating the ovary follicles to produce mature oocyte, thus promoting the outward signs of estrus [50]. In contrast, the hCG action is similar to that of LH, causing the release of mature oocyte at ovulation, and promoting corpora lutea formation [51]. Currently, the majority of studies focus on the use of hormonal associations. This is acceptable because the results of studies have shown that such protocols increased rate, occurrence, and ovulation speed, therefore increasing the fertility rate [52].

Various attempts have been made to establish effective protocols for estrous control in South American wild felines, mainly due to the variety of manifestations of reproductive cycles. For example, the margay present spontaneous ovulations during normal estrous cycles, but ocelot and tigrina are induced ovulators. Luteal control in feline is not a feasible option, due to several factors: (1) although some individuals ovulate spontaneously at unpredictable intervals, many felids are obligated to induce ovulations and (2) the diestrus *corpus luteum*, when present, is refractory to prostaglandins and dopamine agonists up to day 40 postovulation [50].

http://dx.doi.org/10.5772/62427

The use of porcine FSH (pFSH) with minimal LH activity is reported for inducing follicular growth in wild felines [53]. This protocol was effectively applied for pumas (*Puma concolor*) [54], jaguars (*Panthera onca*) [55, 56], and jaguarundis (*Puma yagouaroundi*) [49]. Recently, the ability of an oral progestin, altrenogest (0.192 mg/kg, 14 days), to suppress the ovarian follicular activity in tigrina was demonstrated. This protocol was also effective in reducing hyperstimulation and hyperestrogenism after hCG and eCG administration. However, the authors affirmed that not all females responded uniformly [45]. In fact, studies with both conventional gonadotropins showed diversity of response in different feline species. For example, the ovarian response to a combined treatment with eCG injection followed 80-84 hours later by a hCG administration resulted in a conception rate lower than 30% in ocelots (400 IU eCG and 200 IU hCG) [38] and tigrina (200 IU eCG and 150 IU hCG) [51]. Exogenous gonadotropins are immunologically complex foreign peptides, and thereby, the development of antibodies against gonadotropin is expected sequelae in wild feline submitted to short-term repeated treatments [57]. For this motive, the isolation, characterization, and production of recombinant gonadotropins has been suggested to avoid these complications. On the other hand, it is important to remember that felid species present a large variation in body sizes, a factor that directly influences the synchronization protocols, as well as the individual ovarian response to gonadotropin stimulation [58].

A considerable effort has been made to develop a method to induce estrus and ovulation in the maned wolf, the biggest South American canids. An implant of deslorelin (2.1 mg), a GnRH analogue, was 100% effective for induction of ovary activity, with ovulation occurring between days 9 and 16 (mean: 12.5 ± 1.4 days) after implant when paired with a male, a physiological condition required for the effective ovulation. Alternatively, a single injection of equine recombinant LH (reLH), associated with the deslorelin removal effectively induced ovulation in unpaired females, without apparent adverse impact on fertility in subsequent breeding seasons [59]. Regarding the coati, a carnivore from the Procyonidae family, the unique attempt of estrous control was made by using melengestrol acetate, a progestin, but treatments induced purulent vaginal discharge and uterine adenocarcinoma, and consequently the authors recommend caution when using this drug [60].

In ungulates, as the brown brocket deer (*Mazama gouazoubira*), a monovulatory nonseasonal breeder, estrous synchronization can be achieved using an intravaginal progesterone device (CIDR®) for 8 days, associated with an injection of 265 μg cloprostenol at the removal of the device [61]. In collared peccaries, on the other hand, the use of two injections of 60 μg cloprostenol administered at a 9-day interval was effective for estrous synchronization [62].

In primates, it is known that PGF2α can act on luteal cells to inhibit the luteotrophic actions of LH or hCG *in vitro* [63]. In this context, the administration of cloprostenol has a marked and rapid luteolytic action (0.5–0.8 μg) in the marmoset monkey [64]. In this species, the daily use of recombinant human FSH (rehFSH; 50 IU) during the first 6 days of the follicular phase resulted in superovulation [65]. Moreover, when associated, the protocol using rehFSH 25 IU/hCG 500 UI proved to be efficient to collect oocytes [66].

Despite the difficulties in using a great number of individuals for experimentation, there is notably a need for the development of estrous synchronization protocols for South American wild mammals, especially focused on dosage and efficiency of drugs.

4. Female gametes manipulation

As with other ARTs, the main limitation for wild species is the difficulty in obtaining viable oocytes, being the recovery of preovulatory oocytes for *in vitro* culture (IVC), an available alternative [67]. The possibility to store frozen female gametes as ovary tissue fragments, isolated follicles, or either mature or immature oocytes represents an attractive alternative to cryopreservation [68]; however, the technique is not yet well-established, presenting several challenges that need to be addressed in order to be routinely used.

4.1. Recovery of female gametes

The first step in the manipulation of female gametes is to obtain viable oocytes; however, such procedures are still uncommon in South American wild mammals and are most frequently conducted in Neotropical primates. Oocyte can be collected *in vivo*, or postmortem.

The recovery of oocytes by mechanical preantral follicle (PF) isolation in ovariectomy specimens [69], the collection of ovarian biopsies by exploratory laparoscopy [70], and the puncture of antral follicles by laparotomy [71] have been described studies in capuchin monkeys (*Sapajus apella*). In marmoset monkeys, oocyte recovery was achieved through follicle aspiration after laparotomy [72], laparoscopy [65], or uni- or bilateral ovariectomy [73]. The latter was also the method described in squirrel monkeys (*Saimiri boliviensis boliviensis*) [74] for oocytes collected by ultrasound-guided puncture, after monitorization of the follicular development, in superstimulated females [75]. Finally, in *Saimiri sciureus*, follicle aspiration was conducted after laparoscopy [76] or laparotomy [77].

Regarding other South American species, in particular, wild felids, ovary collection is often performed immediately after the animal death, as it was reported for Geoffroyi and tigrina cats [78]. Also in puma and jaguar, isolation of small PFs (40-90 μm) was performed after animal death [79]. The oocyte aspiration by laparoscopy was also described for tigrina and ocelot [51], puma [80], and jaguar [55]. In vicunas (*Vicugna vicugna*), a South American wild camelid, oocytes also were recovered after follicle aspiration [81].

4.2. Characterization of ovarian follicles population

The rescue and manipulation of matured oocytes represent an important genetic source from wild species; nevertheless, it faces many obstacles, particularly the limited knowledge on the species reproductive physiology [82]. Thus, the use of immature oocytes from antral follicles or PFs allows the recovery of gametes from prepubertal, pregnant, old, or even dead animals [83, 84] The characteristics of the ovarian follicles population in various South American wild mammals are described in Table 2 and illustrated in Figure 2.

Specie	Preantral follicles population			Preantral follicles category									
				Primordial			Primary			Secondary			Authors
	Right ovary	Left ovary	Total	Total number	%	Diameter (μm)	Total number	%	Diameter (μm)	Total number	%	Diameter (μm)	
Sapajus apella	56.9 ± 21.9	49.1 ± 26.9	-	-	30.0 ± 4.3	22.1± 0.5	-	6.0 ± 1.0	27.3 ± 0.5	-	4.0 ± 0.7	61.2 ± 4.0	[85]
Saimiri sciureus	-	-	-	915.0 ± 78.8	73.3 ±1.3	20.5 ± 0.8	230.5 ± 20.8	18.6 ± 0.7	33.7 ± 2.4	115.9 ± 15.7	8.1 ± 0.9	512.5 ± 67.1	[86]
Dasyprocta leporina	4419.8 ± 532.3	5397.5 ± 574.9	-	-	86.6	18.6 ± 3.4	-	13.0	23.8 ± 5.7	-	0.4	88.6 ± 17.6	[87]
Galea spixii	220.8 ± 175.1	195.2 ± 182.8	416.0 ± 342.8	-	32.2	16.6 ± 0.3	-	63.7	28.3 ± 2.1	-	4.1	123.7 ± 18.3	[88]
Pecari tajacu	-	-	33273.5 ± 579.0 (per ovary)	30 466.6 ± 5194.8	91.6	31.8 ± 1.1	2 093.9 ± 595.0	6.3	40.9 ± 2.1	712.9 ± 95.4	2.2	196.2 ± 17.1	[89]

Table 2. Characterization of ovarian follicles population from different South American wild species

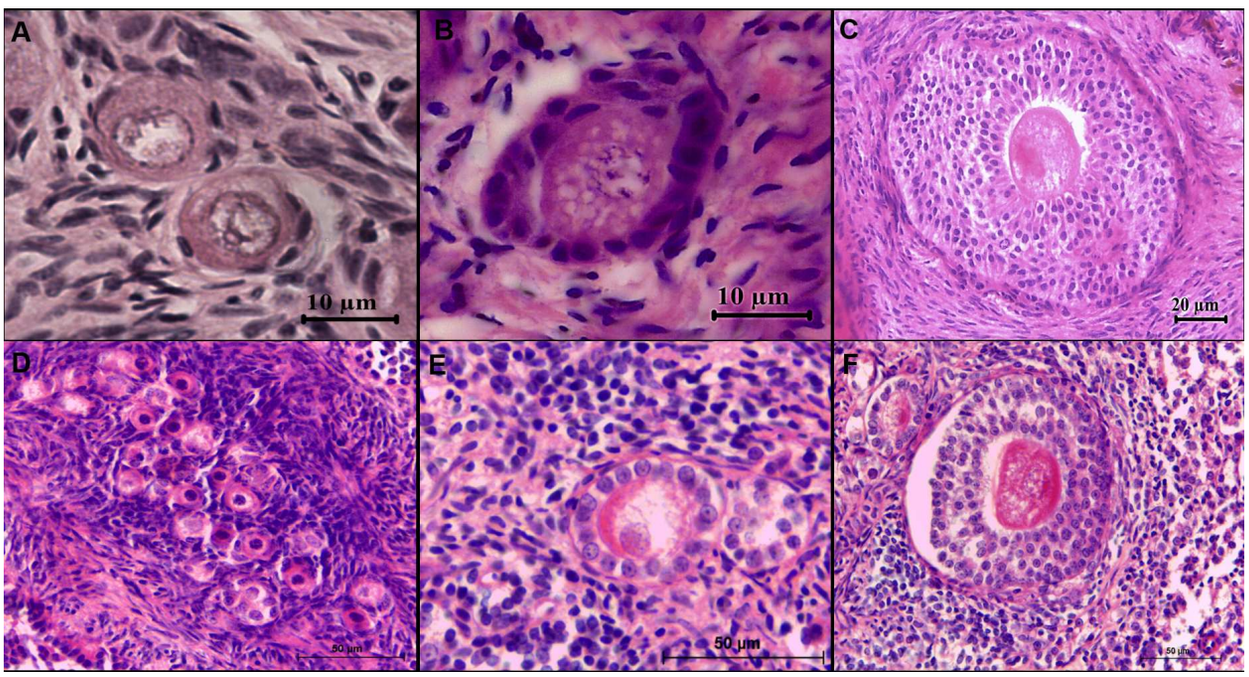

Figure 2. Photomicrograph of the ovary cortex. (A-C) Preantral follicles from *Pecari tajacu* and (D-F) *Dasyprocta leporina*. Primordial (A,D), primary (B,E), and secondary (C,F) follicles.

4.3. In vitro culture and maturation of oocytes

The IVC and *in vitro* maturation (IVM) of oocytes has highlighted some of the mechanisms underlying the folliculogenesis in particular wild species, particularly among the South American wild mammals, offering new opportunities for the use of many other ARTs [85]. Oocyte IVM is a technique that provides material for the study of the final steps of oocyte meiosis. Nevertheless, the practical application of this procedure remains relatively inefficient, and the embryo production rate is much lower compared with *in vivo* matured oocytes [73]. Some of the researches that have been developed using IVC and IVM are revised below.

The competence of oocytes retrieved from antral follicles in capuchin monkeys was achieved after 36 hours of IVM, the highest maturation rate occurring in oocytes collected from dominant follicles [71]. It has also demonstrated that the IVC of ovarian cortical strips in TCM199 supplemented of β-mercaptoethanol (BME), bone morphogenetic protein 4 (BMP4), or pregnant mare serum gonadotrophin (PMSG) promoted a follicular viability similar to that of controls (89.3%) for this species, while it also increases the rate of secondary follicle formation (44.9%) [70].

The oocyte meiotic competence and the *cumulus* cell function were positively associated with the follicle size in marmoset monkeys [86], as it was demonstrated in IVM studies. It was shown that chromosome quality is crucial for cytoskeletal organization allowing a correct meiosis in IVC and IVM of the immature oocyte [73]. Moreover, abnormal spindle formation was observed in oocytes derived from small antral follicles failing to complete meiosis, contrasting almost 90% matured oocytes, surrounded by expanded *cumulus* cells at the time of isolation [66]. For this species, an alternative two-step culture system consisting in the culture of oocytes within stromal tissue fragments for 2 days has been described. Afterwards, follicles were mechanically isolated and transferred to a culture system where they grew for up to further 12 days. This process produces full-sized matured oocytes from primary and early secondary follicles [87].

An immunohistochemical assay conducted in squirrel monkey ovaries during IVC demonstrated that growth differentiation factor 9 (GDF-9) and c-Kit protein were detectable in oocyte cytoplasm from primordial to secondary follicles, whereas the Kit Ligand expression was observed in oocytes and granulosa cells from primordial to secondary follicles. On the other hand, the anti-Müllerian hormone was expressed in primary and secondary follicles, but not in the primordial ones [88]. In a different species from the same gender, the *S. boliviensis*, IVM of follicles obtained by laparoscopy requested the use of a medium containing high-energy source [89]. Moreover, oocyte IVM was also described for *S. scierus* [90].

Regarding South American wild felids, the yield of the IMV technique varies greatly among species. Immature oocytes were recovered from Geoffroyi cat, jaguar, and puma after the animal death and further matured *in vitro*. In Geoffroyi cat, from a total of 45 oocytes retrieved, 23% succeed maturation, similarly to that described for jaguar, from whose oocytes (n=21) only one-third advanced to metaphase II during IVM. In the puma, more than 100 oocytes were recovered from 8 females, from which 43.8% of the oocytes matured *in vitro* [91]. IVM has been previously described in this species after oocyte recovery using laparoscopy and transabdo-

minal aspiration [92]. In a recent study, 42 mature oocytes were collected by laparoscopic ovum pick up from pumas, after the administration of eCG (750 UI) with hCG (500 UI). In this species, oocytes were also matured *in vitro* using TCM199 supplemented with LH, FSH, and 17β-estradiol [80].

Studies on ovarian gene expression have been conducted to better understand folliculogenesis in plains viscacha (*Lagostomus maximus*), an animal showing the highest ovulation rate among all mammals, releasing between 400 and 800 oocytes per estrous cycle [93]. The expression of germ cell–specific VASA protein, apoptotic proteins BCL2 and BAX, as well as DNA fragmentation revealed an unrestricted proliferation of germ cells in this species, without apoptosis-driven elimination, contrary to what is normally found in other mammals [94, 95].

In ungulates, IVC of collared peccaries ovarian tissue reveals that more than 50% follicles remain morphologically intact when the TCM199 medium was used whether supplemented or not with FSH. Moreover, the activation of collared peccaries PFs was stimulated by the addition of FSH to the medium during IVC, the proportion of growing PFs increased from only 31.2 ± 0.7% in the control group to more than 90% after a 7 day in TCM199+FSH [96].

4.4. Female gametes cryopreservation

The conditions required for the complete *in vitro* development of oocytes remain incompletely established in wild species; therefore, cryopreservation emerges as a promising tool for female germplasm conservation, allowing its future use in ART. It is possible to store ovarian tissue, isolated follicles, and mature or immature oocytes, for which purpose two methods are routinely used: the slow-freezing procedure and vitrification. The value of vitrification has been highlighted to avoid damages caused by conventional freezing protocols, such as the risk of ice crystal formation. It is considered a cheap method that can be performed under field conditions.

Another major factor on the success of cryopreservation of female gametes is the cryoprotectant used. In marmoset monkeys, DMSO was advantageously used as a cryoprotectant for slow freezing of ovarian tissue. It provided a higher percentage of morphologically normal primordial (26.2 ± 2.5%) and primary follicles (28.1 ± 5.4%) compared with propanediol (PROH) (12.2 ± 3.0 and 5.4 ± 2.1%, respectively) [97]. Furthermore, the development up to secondary PFs [98] and the formation of antrum after xenografting cryopreserved tissue in this species were also described [99].

Regarding South American wild felids, short-term IVC of ovarian cortex slices collected from Geoffroyi cat was performed to assess the success of the slow freezing protocol, using EG and sucrose. The results showed an increase in the number of viable PFs in frozen–thawed samples after 7 days of IVC (48%) compared with fresh cultured pieces (31%) obtained in the same sampling day. In tigrina cats, the percentage of primordial follicles after freezing and culture (44%) indicated a normal population of viable follicles [78].

Regarding Neotropical rodents, the conventional freezing was adopted to preserve ovarian fragments from agoutis. After freezing, no differences were found among groups using different cryoprotectants: DMSO (60.6 ± 3.6%), EG (64.0 ± 11.9%), or PROH (62.0 ± 6.9%).

However, only follicles cryopreserved with PROH presented a normal ultrastructure, similar to that of the control group [100]. A reduction on the morphologically normal PFs (69.5%) compared with the control group (91.2%) was also observed, after solid surface vitrification of ovarian tissue from yellow-toothed cavies. The preservation of oocytes and granulosa cell membranes and the morphological aspect of follicles were acceptable, and the transmission electronic microscopy showed that the presence of vacuoles in the oocyte and granulosa cells cytoplasm and turgid mitochondria remained the main alteration observed in some vitrified follicles [101].

In collared peccaries, the refrigeration of ovaries for up to 36 hours allowed supporting the morphological integrity and viability of PFs. Further, it was demonstrated that powdered coconut water media (66.7%) was more effective than the phosphate buffered saline (PBS, 49.4%) to preserve the morphological integrity after 36 hours storage, although without statistical differences on respect to the follicular viability [102]. Moreover, the solid surface vitrification of ovarian tissue using EG, DMSO, or dimethylformamide (DMF), at 3.0 or 6.0 M, preserved the morphological integrity of more than 70% PFs using any of these cryoprotectants [103].

5. Embryo technology

ARTs using *in vivo* embryo production by applying hormonal superovulation protocols, as well as those obtaining *in vitro* blastocyst through *in vitro* fertilization (IVF) and ICSI, allowed to establish the adequate conditions for embryonic development in different species. Furthermore, ARTs associated with embryo cryopreservation foster the preservation of genetic material, contributing to conservation of biodiversity. In general, ARTs applied to wild mammals in South America are established in accordance with procedures used in domestic mammals, despite that some wild species may have complex reproductive characteristics, compromising the rapid progress of ARTs in particular species.

5.1. In vivo embryo production

Soon after the recognition of the gametes and embryo physiology, application of ARTs in each new species begins with *in vivo* embryo production, defined as the selection of female donors, AI or natural mating, embryo collection, cryopreservation, or direct transfer into synchronized recipients. Inevitably, the number of individuals is a crucial point in research, and individual variations nurture the development of *in vitro* ARTs.

In general, superovulatory protocols consist of a single dose of eCG and/or multiple injections of FSH [104]. The status of follicular development at the moment of superovulation, as well as the stress induced by intense manipulation during ARTs, is the main factor ascribed for the variability on response [105]. In an attempt to provide efficient superovulation protocols for embryo collection, different studies have been developed in various wild mammals. An interesting example corresponds to brown brocket deer [106], in which eCG induced a good response to superovulatory protocol, promoting the formation of functional corpora lutea (7.0

± 1.8) although 66.7% (4/6) of the females showed premature luteal regression. In comparison, FSH administration resulted in a low formation of corpora lutea (2.6 ± 0.8) and lower proportion premature luteal regression (33.3%; 2/6).

There are few studies on *in vivo* embryo production protocols in wild camelids. In vicunas, two studies [107, 108] proposed ovarian superstimulatory protocols using eCG with or without CIDR® during 5 days; unfortunately, the results involved only data for follicular development, with no information on embryo recovered or transferred.

5.2. In vitro embryo production

The IVF is still the main *in vitro* technique for the reproduction of wild mammals, although this technique demands a wide quantity of viable oocytes to be fertilized. Briefly, IVF steps involve the oocyte recovery, the *in vivo* or IVM and selection of oocytes, the IVF using capacitated spermatozoa, and the preimplantation embryonic development. The aim is to develop high-quality embryos and obtain normal pregnancies, resulting in the birth of healthy offspring. In this sense, Table 3 summarizes the results of *in vitro* ARTs applied in some South American wild mammals. In general, the protocols used for domestic animals were gradually adjusted to wild species.

Although IVF embryos have been produced from capuchin monkeys [109], marmoset monkeys [66, 110], pumas [92], and tigrinas [51], modifications are needed to increase the efficiency of protocols. Currently, *in vitro* embryo production depends primarily on two major products: meiotic viable oocytes (meiotic competence) and capacitated viable spermatozoa (sperm competence). Domingues et al. [71] reported the viability of *in vitro* matured oocytes collected from nonstimulated capuchin monkeys. Lima et al. [109] established 40 hours as the optimal IVM length and showed the positive effect of FSH/LH (0.5 μg/mL/50.0 μg/mL) on *IVF* of oocytes collected also in nonstimulated females. In addition, spermatozoa obtained from semen diluted in coconut water were able to fertilize oocytes. However, the establishment of experimental conditions is still a requirement for this species.

Another interesting example of the use of IVF in wild felids [111] is that of jaguars; a stimulation protocol using FSH/LH resulted in approximately 25 follicles/female, with more than 80% of high-quality oocytes but the fertilization rates were fairly low (<25%) [55]. Contrastingly, ocelot and tigrina each treated with eCG/hCG produced an average of approximately 10 follicles with ~7–9 high-quality oocytes/female that resulted in 76 ocelot and 52 tigrina embryos [51].

Alternatively, ICSI offers a precise control of sperm competence for *in vitro* procedures. ICSI was first used in marmoset monkeys in 2007, by Grupen et al. [66], using *in vivo* matured oocytes. Still, no blastocyst was produced by ICSI, despite that 47% of blastocysts were achieved after IVF. In 2014 [110], ICSI embryos derived from *in vitro* matured marmoset oocytes developed into neonates, through the use of two strategies: the evaluation of the most suitable timing for ICSI and establishing the *in vitro* and *in vivo* embryo developmental potential. In this study, the blastocyst rate tended to be low when ICSI was performed 1–2 hours after the extrusion of the first polar body. For *in vitro* developmental competence, a

Species	Oocyte source	Maturation type	Spermatozoa		ART applied	Embryo production					Authors
			Collection/ selection	Motility (%)		Media[a]	Cleavage rate[b]	Blastocyst rate[c]	Stage reached	Embryo destination	
Sapajus apela	Without stimulation	*In vitro*	EEJ, cooled to 4°C/ Swim up	~80	IVF	SOF	20	---	4 cells	Cell staining	[114]
Callithrix jacchus	Hormonal stimulation	*In vivo*	FertiCare vibrator, artificial vagina/ Pure gradient	">70	IVF/ICSI	G1.2, G2.2	44.7/94.2	46.7/0.0[d]	Blastocyst	Cell staining	[66]
Callithrix jacchus	Hormonal stimulation	*In vitro*	FertiCare vibrator, artificial vagina/ Swim up	NI	IVF/ICSI	ISM1, ISM2	93.2/97.6	39.2/35.4	Blastocyst	Embryo transfer	[115]
Felis concolor	Hormonal stimulation	*In vivo*	EEJ/ Swim up	40-50	IVF	mKRB	---	---	1 cell	NI	[97]
Pecari Tajacu	Without stimulation	*In vitro*	Testis cell suspension xenografts/---	NI	ICSI	NI	37.5	---	4 cells	Xenograft assay	[42]

Abbreviations: EEJ, electroejaculation; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; ARTs, Assisted reproductive technique; NI, not informed.

[a]More information about the composition of the media see reference.

[b]Cleavage rate represents the number of cleaved oocytes in relation to the number of oocytes entering to maturation in D2.

[c]Blastocyst rate represents the number of blastocyst in relation to the number of zygotes.

[d]Blastocyst rate represents the number of blastocyst in relation to the number of cleaved embryos.

Table 3. Results of IVF and ICSI techniques applied in some South American wild mammals.

greater fertilization rate was observed in ICSI embryos (93.2%) compared with IVF embryos (82.2%). However, no differences in developmental rate (blastocyst/fertilized oocytes) were observed between ICSI (35.4%) and IVF (39.2%). In addition, for *in vivo* development of ICSI embryos, an offspring rate of 28.6% (6/21) and 2.7% (1/37) was obtained after the transference of 6 to 8 cell embryos and blastocysts, respectively.

In collared peccary [42], ICSI was used to evaluate the quality of sperm cells derived from testis cell suspension xenografts. The collared peccary presents an interesting Leydig cell cytoarchitecture, and therefore, it represents an interesting mammalian model for investigating cellular roles in male gonads. In the study, the sperm recovered from the xenografts originated 75.0% (6/8) of two-cell embryos and 37.5% (3/8) of four-cell embryos produced in 24 and 48 after ICSI, respectively.

For South American wild camelids, information on oocyte recovery and maturation are only available for vicuna: using the surgical oocyte aspiration from superstimulated females, a

recovery rate of 55.4% (46/83) and an IVM rate of 41% were obtained, characterized by extrusion of the first polar body and cytoplasmic maturation [81], but no information was presented on IVF or ICSI.

5.3. Embryo cryopreservation and transfer

In recent years, advances in the embryo cryopreservation were achieved with studies in slow freezing conventional method or vitrification. Currently, embryo cryopreservation targets the establishment of a consistent vitrification protocol to be applied for the conservation of preimplantation embryos in different stages of development. Protocols developed for other species appear to be adequate for the purpose of embryo cryopreservation in wild felids. Offspring were obtained in ocelot following transfer of cryopreserved embryos [111].

In marmoset monkey, due to its small body size, the technical development of an efficient embryo transfer is being delayed. Different factors are being studied, such as nonsurgical approaches, embryo cryopreservation, use of late-stage embryos, and volume medium [112] to overcome these gaps. Using embryos in different developmental stages (10- to 16-cell, morula, and blastocysts) after vitrification in Cryotop, Ishibashi et al. [112] defended that reducing the transfer volume to 1 μL or less is essential for successful embryo transfer this species. This procedure provides pregnancy and birth rates of 80% (8/10) and 75% (9/12), while the use of larger volumes (2–3 μL) results in pregnancy and birth rates of 50% (5/10) and 27% (3/11), respectively.

6. Somatic cell processing and cloning

Somatic cell nuclear transfer (SCNT, cloning) consists of the transfer of the nucleus of donor cells into enucleated oocytes, resulting in the production of an individual genetically identical to the nucleus donor animal. In general, SCNT is performed according to the following steps: (i) preparing cytoplast receptors from oocyte recovery, selection, and maturation (cytoplast); (ii) isolation, characterization, *IVC*, and cellular cycle synchronization of the somatic cells to be used as nuclear donors (karyoplast); (iii) embryonic reconstruction by nuclear transfer, fusion of karyoplast–cytoplast complex, cellular activation, and preimplantation embryo development *in vitro;* and (iv) transfer of embryos for previously synchronized recipients, establishment of pregnancies, fetal development, and birth of offspring. In all these steps, there are determining factors for the success of the technique, including the media composition, the donor cell type and the oocyte source, and the timing of all steps down the success of the reproductive output of viable clones [113].

The use of cloning for conservation is often questionable for the risk of reducing the genetic variability. However, it is now known that SCNT not only provides the production and multiplication of a group of individual species of interest, as well as it allows additional studies in the field of reprogramming and cryopreservation of somatic tissue and cells, providing a larger scene in the reproduction of these species. Up to now, there are no studies on clone embryo production in South American wild mammals.

An important step in cloning is the selection of the cells donating a nucleus. In general, fibroblasts derived from skin biopsies are the cell model used. Numerous reasons justify its use for embryo reconstruction, such as the ease of obtaining and handling for the primary culture or subcultures, freezing patterns, and suitable transfection, in addition to obtaining inexhaustible quantities from a single explant. Moreover, the preservation of cells and tissues of the animal to be cloned allows the preservation of the genetic material of an individual indefinitely.

6.1. Generation and establishment of somatic cell primary cultures

The karyoplast preparation derived from skin biopsies involve multiple steps, harvesting of the biological material, through the isolation, establishment and characterization of cell cultures, and the cryopreservation conditions of cell populations after subculture cycle. Routinely, karyoplasts may be derived from skin biopsies of adult or fetal auricular region, according to established standards of asepsis and sterilization procedures [113]. After processing tissue samples into smaller fragments, these are distributed in Dulbecco Modified Eagles Medium (DMEM) supplemented with sodium pyruvate, sodium bicarbonate, antibiotics, amphotericin B, and fetal bovine serum. The primary cultures are monitored every 24 hours to evaluate the cell growth and total medium replacement. When 70% cell confluence is purchased on the plates, the cultures are subcultured. In general, explants are cultured under these conditions for 7–14 days [114]. To confirm the cells' characteristics, particular antibodies are used, like vimentin [113].

In native Chilean species, the establishment of donor cell was already observed in Chilean Shrew opossum (*Rhyncholestes raphanurus*) and chinchilla (*Chinchilla lanigera*) [115]. In the study, tissue fragments were subjected to culture systems and somatic cells were obtained for future cloning.

6.2. Somatic tissue conservation

The tissue cryopreservation involves some major issues, such as the conservation of cellular functionality, extended storage of the samples, and the easiness of the procedures performed outside the laboratory [115]. Tissue sample cryopreservation of wild species is an interesting step for biodiversity conservation, when there is not an appropriate culture system.

In collared peccary [114, 116], vitrification techniques (conventional or solid-surface vitrification) may be used for cryopreservation of somatic tissue, allowing the isolation of viable cells. In this study, ear fragments (9.0 mm^3) were vitrified in a solution containing DMEM plus 3.0 M DMSO, 3.0 M EG, 0.25 M sucrose, and 10% fetal calf serum. The histological analysis and IVC showed that few tissue damages were associated with solid-surface vitrification [116]. Moreover, all cells resulting in vitrified and noncryopreserved fragments showed the characteristics of suitable fibroblasts: fusiform features with oval nucleus in the center. The growth curves showed clear log and lag phases of development, with no difference between treatment and nonvitrified for population doubling time (conventional vitrification directly: 66.1 hours; solid-surface vitrification: 66.3 hours; and nonvitrified: 54.0 hours) [114].

6.3. Cloning and its perspectives

Although some domestic mammals have already been cloned, information on cloning of South American wild animals is scarce. As with other animal species, cloning allows to know the principles of nuclear reprogramming, conservation of cells and tissues for different purposes (reproductive and therapeutic), and clones can also be used as models in several studies. Thus, the efficient production of pluripotent stem cells (iPSC) allows the generation of genetic preservation by different ways, such as gamete production and embryo complementation. Dermal fibroblasts derived from jaguars were transduced using genes encoding the human transcription factors, in an experiment that highlighted the homeobox protein NANOG as crucial to the cell reprogramming in this species, and demonstrated that the technique may represent an efficient method for iPSC production from endangered felids [117].

7. Final considerations

The ARTs have applications in different productive and scientific sectors, whether or not aiming at the conservation of species. As occurred for the domestic species, the use of ART sequence followed the order of complexity and evolution of biotechnologies. Accordingly, the routine use of AI and embryo transfer in different species of mammals is evident. Moreover, *in vitro* ARTs continue to grow, using a parallel between the protocols applied to domestic species and individual characteristics of each wild ones. With the progress of *in vivo* or *in vitro* ARTs, it is expected to know, to manipulate, and to conserve the genetic material, promoting the preservation of biodiversity, especially in South American wild mammals.

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