We are IntechOpen,
the world's leading publisher of
Open Access books
Built by scientists, for scientists

4,800

Open access books available

122,000

International authors and editors

135M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities

BOOK CITATION INDEX
CLARIVATE ANALYTICS
INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Assisted Reproductive Technologies in Safeguard of Feline Endangered Species

Natascia Cocchia, Simona Tafuri, Lucia Abbondante,
Leonardo Meomartino, Luigi Esposito and
Francesca Ciani

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61004

Abstract

The growth of the human population and the escalating consumption of natural resources have reduced wild habitats, modifying the existing balance of biological cycles. Therefore, *ex situ* conservation efforts have received renewed attention as a potential safeguard for species with an uncertain future in the wild. Most wild felid species are classified as rare, vulnerable, or endangered due to poaching and habitat loss. Any directed action taken by humans to enhance animal reproduction results in assisted reproductive technologies (ART) development. These technologies have been included in programs for the conservation of endangered species. Therefore, ART provide a new approach in the safeguard programs of felid biodiversity. Currently, ART mainly include Artificial Insemination (AI); *In Vitro* Embryo Production (IVEP) consisting of *In Vitro* Maturation (IVM), *In Vitro* Fertilization (IVF), *In Vitro* Culture (IVC), Embryo Transfer (ET), and Intra Cytoplasmic Sperm Injection (ICSI); gamete/embryo cryopreservation; gamete/embryo sexing; gamete/embryo micromanipulation; Somatic Cell Nuclear Transfer (SCNT); and genome resource banking.

The domestic cat is used as a model for the ART development in *Felid* species and as a successful recipient of embryos from closely related, small, nondomestic cats. The Indian desert cat and African wildcat kittens have been born after IVF-derived embryo transfers.

The creation of the biological resource bank represents a complementary support tool for the application of ART in the *in situ* and *ex situ* conservation of endangered felids. Its chief purpose in the protection of endangered species is to preserve the maximum

INTECH
open science | open minds

current genetic and biological diversity of the population by the processing and cryopreservation of germinal cells and tissues from dead animals so that these genetic recourses may be used in future reproductive projects. In humans and domestic species, it is usually possible to plan the place and time for gonad explants to recover germplasm, thereby enabling a reduction in the gonad storage time in the transport medium. In wild species, it is impossible to predict when and where the gonads can be collected. The gonads can be recovered postmortem, which entails the possibility that the collection place could be distant from a laboratory for IVEP.

In the present chapter, we will make an overview of the data from detectable literatures and focus our attention on analysis of methods utilized in ART for maximizing their efficiency in feline species.

Keywords: Assisted Reproductive Technologies (ART), In Vitro Embryo Production (IVEP), endangered feline species

1. Introduction

The growth of the human population and the escalating consumption of natural resources have reduced wild spaces, modifying the existing balance of the biological cycles. Therefore, *ex situ* conservation efforts have received renewed attention as a potential safeguard for species with an uncertain future in the wild. Assisted Reproductive Technologies (ART), which consist of various techniques such as Artificial Insemination (AI), In Vitro Fertilization (IVF), Embryo Transfer (ET), and cryopreservation of embryos, have greatly promoted animal reproductive efficiency and have become a potential means for the conservation and management of wildlife populations threatened with extinction. [1]. Several species, such as domestic cats, dogs, and ferrets are the most popular pets, while other carnivores, like minks and foxes, have an economic value to the industry of fur farming. Tigers, bears, and other large predators have a major effect on the health of natural ecosystems. Any directed action taken by humans to enhance animal reproduction has resulted in assisted reproductive technologies (ART) development. These technologies have been included in programs for the conservation of species threatened with extinction. ART, therefore, affords investigators a new approach that they can include in the safeguard programs of felid biodiversity. Although this technique has greatly improved animal reproduction, it has not advanced beyond the rudimentary stages for use in the conservation of felines threatened with extinction [1]. As several other more widely studied species, the earliest descriptions of successful production of embryos using in vitro fertilization (IVF) in the cat occurred in the 1970s [2, 3]. Meanwhile, as IVF studies in the most popular laboratory animals, economically important domestic animals, and humans literally exploded during the last two decades of the twentieth century, the field of cat IVF experienced no comparative proliferation in publications. Publications in human IVF area represent approximately 50% of the reports from 1970 through 2000 and approximately 50% from 2001 through 2012. The majority of the total citations listed in the search on "IVF in cats",

were published in the current century [4]. Most wild felid species are classified as rare, vulnerable, or endangered due to poaching and habitat loss. A great deal of progress has been made in recent years toward the development of assisted reproductive techniques (ART) for species conservation [5, 6] In fact, ART have been included in the programs for the conservation of species threatened with extinction, but the effectiveness of this application to semi domestic, not domestic, and particularly endangered species such as felines, remains consistently low [4]. These ART tools are potentially important for the captive breeding programs of selected felid species. The domestic cat is often used as a model for developing these techniques in the felid species [5, 6]. In the last few years, the ART application in the domestic cat has allowed researchers to obtain 70% metaphase II oocytes after *in vitro* maturation (IVM) [7], and 80% cleaved embryos after IVF and 70–80% after Intra Cytoplasmic Sperm Injection (ICSI) [8]. However, only 10% cleaved embryos could develop to blastocyst [8-11]. Kittens have been born after embryo transfer [5]. *In vivo* embryo collection is the most popular technique for embryo production, in spite of the fairly rapid development and adoption of *in vitro* embryo production. The domestic cat could also be used as a successful recipient of embryos from closely related small nondomestic cats. In fact, some evidences have confirmed the birth of African wildcat and Indian desert cat kittens after the transfer of IVF-derived embryos in female domestic cats [12]. Recently, African wildcat kittens were produced after the transfer of embryos derived by fusion of adult somatic cells from one species with enucleated oocytes of a closely related species (domestic cat) [13]. However, the application of ART, which tries to produce a single viable offspring, unfortunately, cannot justify the expense, labor, and the handling of animals which is associated with stress. Thus, "ART" must be applied within the programs of population management established to have a real impact on conservation. The immediate value of ART is to assist those responsible for the maintenance of viable populations of felines in captivity. Its wider application will require the creation of a global network of qualified scientists and veterinarians willing to perform these procedures as a reproduction service for keeping cats themselves [1].

2. Reproductive cycles of wild felids

Knowledge of anatomical features and hormones and the cycles of wild reproductive feline is the ability to track feline reproductive activity. The hormone measure is a key technique to develop successful ex situ breeding programs to determine the reproductive activity of domestic feline. The ovaries in the wild felid and the domestic cat are caudal to the respective kidneys and connected proximally by the suspensor ligament and dorsally by the mesovaria. The oviducts are covered by the mesosalphinx that forms, laterally to the ovaries – an ovarian bursa. Each oviduct cranially is localized in the medial aspect of the ovarian bursa; caudally it is located in the lateral aspect before terminating at the uterotubal junction. The mesometrium suspends dorsally the horns of the uterus bicornuate. The uterine body is divided internally by an incomplete septum. The cervix is short, but it opens at an angle close to the vaginal orifice. The lips of the vulva are located just below the anus. After fertilization, the blastocysts are distributed evenly along the uterine horns, with an efficient result of transu-

terine migration. Cats have a zonary endotheliochorial type placenta. Regarding the hormone profile of wild felines, reasonable results were obtained by noninvasive monitoring of steroid [14]. Reproductive cycle models of ovarian steroids have now been published about half of nondomestic felid species, by analysis of fecal steroid metabolites. There are four phases of the oestrous cycle in the cat: proestrus, oestrus, diestrus, and anestrus (or interestrus) [15]. Proestrus usually lasts less than a day, and is associated with the presence of ovarian follicles, increased circulating estrogens, no sexual interest but occasionally there could be copulation with the male. Oestrous has maximum concentrations of follicular estradiol. It is characterized by coitus and, depending on the species, by special and typical behaviors such as vocalization, rubbing, rolling, lordosis, and foot stamping. The release of gonadotrophins-releasing hormone (GnRH) from the basal medial hypothalamus and successive waves of luteinizing hormone (LH) from the anterior pituitary gland, are considered necessary in most felines [16, 17]. This cascade of events will result in ovulation after mating. Surges of estrogens distinguish oestrous from interestrus periods, with cycles ranging from 2 to 4 weeks and oestrus lasting 3–10 days. Cats have historically been categorized as having "induced ovulation," that is, requiring mating to stimulate ovulation. Nevertheless, we now know that felids exhibit a range of ovulatory patterns, from almost exclusively induced to manifold combinations of induced and spontaneous ovulation. There are differences not only across species, but also between individuals within a species [14]. In fact, spontaneous increase in progestogens after oestrogen surges is rare or inexistent in the tiger (*Panthera tigris*), snow leopard (*Panthera uncia*), ocelot (*Leopardus pardalis*) puma (*Felis concolor*), tigrina (*Leopardus tigrinus*), cheetah (*Acinonyx jubatus*), and lynx (*Lynx pardinus, Lynx canadensi, Lynx lynx*). It happens, at least occasionally, in the lion (*Panthera leo*), Pallas'cat (*Otocolobus manul*), leopard (*Panther pardus*), fishing cat (*Prionailurus viverrinus*), and regularly in the margay (*Leopardus wiedii*), clouded leopard (*Neofelis nebulosa*), and domestic cat. In some species of certain taxonomy, the spontaneous ovulation occurs in a more prevalent way when the females are kept together, while in others the provoked ovulation occurs if they are kept in individual housings. Thus, within the same taxonomy, ovulatory mechanisms are regulated to different degrees depending on species and individual-specific responses to psychosocial and/or physical stimuli.

Several studies report the domestic cat be seasonally poly oestrous animal with positive photoperiod under natural [16]. In general, ovarian cyclic activity and reproductive functions are reduced under decreasing photoperiod and starts again after exposure to increasing light. In the cat, melatonin seems to regulate photoperiod-induced seasonality. The highest concentrations happen during the dark phase [17]. Reproduction is in someway seasonal in many nondomestic felids like the tiger, pallas'cat, clouded leopard, snow leopard, and lynx (Table 1). The follicular activity, conversely, is not influenced by season in lions, bobcats, pumas, leopards, margays, tigrinas, ocelots, jaguars, and fishing cats [14]. Progestogen concentrations during pregnant and nonpregnant luteal phases are quantitatively similar in nondomestic felids and domestic cats [15].

Felids express marked variations in reproductive mechanisms among species. Two characteristics impact both natural and assisted breeding efforts: effect of seasonality on reproduction and identifying the type of ovulation (induced *vs* spontaneous). Developing ovulation

	Puberty	Seasonality	Induced Ovulation	Oestrus Length	Pregnancy Length
Domestic cat	6–9 months	YES	YES	3-16 days	58–65 days
Cheetah	2–3 years	NOT	YES	2–6 days	94 days
Pallas cat	1 year	YES	OCCASIONALLY NOT	3–14 days	66–77 days
Clouded leopard	2 years	YES	NOT (40%)	3–6 days	90 days
Tiger	3–4 years	YES	YES	7 days	108 days
Leopardis species	1–2 years	YES /NOT	NOT	2–7 days	75–85 days
Linx	1–2 years	YES	YES	7 days	60–70 days
Lion	3 years	NOT	NOT	5–6 days	108 days

Table 1. Particularity of reproductive cycles of *domestic cat* and some *wild felids*.

induction protocol, with consistent responses, is high priority. Furthermore, it is important to ensure an optimal maternal environment for fertilization and embryo development. Down-regulating endogenous ovarian activity and synchronizing time of oestrus are steps of reproductive cycle that need to be controlled and reinforced [1, 18]. We also need a quick and reliable test for diagnosing pregnancy, preferably a noninvasive method.

3. Oestrus induction in felids

In the late of 1970s, various doses and single versus multiple treatments with either a pituitary extract of porcine FSH (approximately 10–20 mg) or eCG for stimulation of follicular development and induction of oestrus was evaluated [15]; 2.0 mg FSH per day until oestrus was observed to be the optimal dose of FSH despite the elevated average ovulation rate and the presence of residual follicles observed after treatment. Cats in the latter group were given the optimal dose of FSH as determined previously [15]: 2.0 mg/ day for 5 days ¼ 10 mg FSH. In the mid-1980s, the Center for Reproduction of Endangered Wildlife of the Cincinnati Zoo established a domestic cat colony model for developing assisted reproductive technologies to apply in conservation efforts for endangered species. The domestic cat, in addition to its prototypical role, was envisioned as a potential recipient of embryos from other species of similarly sized nondomestic cats, of which most are classified as threatened or vulnerable to extinction. In view of previous results in exogenous gonadotropins for oestrous induction, initial emphasis was directed at determining optimal FSH treatment regimes for ovarian follicular stimulation. In a 1987 published article [19] on ovarian response and embryo recovery after treatment with various doses of FSH (2.75–8.0 mg total) and hCG (0–1500 IU) and natural mating, the greatest average number of viable embryos (15.8 morulae and blastocysts) was recovered from the group receiving 4.0 mg FSH/750 IU hCG. Unexpectedly, there was no difference in the average number of viable embryos recovered from donors given the least amount of FSH (2.75 mg total) versus the greatest amount (8.0 mg total): 6.9 versus 7.9. Also,

in 1988, we made our one and only attempt to apply the same methods to a species of nondomestic cat, the serval (*Leptailurus serval*). After daily FSH treatment, at the time of ovulation induction (with hCG), the female was paired with a male. Seven days later, both uterine horns were flushed, but only degenerating ova (>30) were recovered. The ova were examined microscopically, but no sperm were seen, either attached to or penetrating into the zona pellucida. The mating failure was persuasive evidence that, to achieve our goal of applying assisted breeding technology to nondomestic cats, a program to develop methods for in vitro fertilization/embryo culture in cats would be essential. Coincidently, the first report on the birth of kittens after transfer of IVF-derived embryos to recipient females was published at this time [20]. Moreover, repeated treatment of domestic cats with eCG and hCG may cause an immune-mediated refractoriness to ovarian stimulation, dictating that the suitability of these hormonal combinations should be further investigated [1]. Similarly, protocols using porcine FSH and LH resulted in reduced numbers of follicles at the second treatment as compared with the first, possibly due to a humoral immune response [4]. By considering the feasibility of fecal steroid analyses with radioimmunoassay [14] combined with sexual behavior and ultrasonographic images, it is possible to determine the more ideal time for oocyte recovery by laparoscopy, without the use of exogenous gonadotropins.

4. Gamete recovery from nondomestic felids

The first step for ART development is the gamete recovery. Several methods have been reported for semen collection in animals, such as the use of an artificial vagina [21], digital masturbation of the penile bulb and electroejaculation [22], but only electroejaculation method may be used for gamete recovery from wild felids. In any case, the application on nondomestic cats is based on learning how to use these methods in the domestic cats. Electroejaculation is to obtain both epidydimal spermatozoon and spermatogonial germ cells. In female, oocytes are retrieved and recovered from both antral and preantral follicles in ovarian tissue transplantation [23].

5. Male gamete recovery in felids

With wild carnivores, electroejaculation is the method of choice due to the difficulty and risks involved in handling these animals. Electroejaculation occurs after introducing of a transrectal probe with three electrodes, connected to an electric stimulator that provokes a controlled electric stimulation to allow the ejaculatory reflex to work. The nerves that supply the reproductive organs are stimulated by a weak electric current. The probe is inserted 7–9 cm into the rectum and the electrodes are directed ventrally. It is necessary to take care to evacuate any feces from the rectum for this kind of manipulation. [24]. Different protocols of electroejaculation have been used by many researchers [15]. The authors reported three series for a total of 80 electric stimulations. The three series were divided in: 30 stimuli (10 stimuli at 2–4 V series 01), 30 stimuli (10 stimuli at 3–5 V series 02), and 20 stimuli (10 stimuli at 5 and 6 V

series 03) for the collection of semen from South African cheetahs (*Acinonyx jubatus*), with 5 min intervals between the series. The animal responds to the stimuli with a rigid extension of the hind legs. If this reaction is not seen in series 01 or if stronger stimulation is observed, the electrode may not be in the proper position in the rectum, or there may be interference in the current transmission due to the presence of feces. To collect semen, a gentle pressure applied at the penile base should allow for penile extrusion, and the ejaculate is collected into a prewarmed test tube that has been placed over the glans penis. Using electroejaculation has collected the semen from more than 28 cat species [25]. Moreover, some researchers have reported successful semen collection from wild felids by using electroejaculation, such as tigers (*Panthera tigris*), snow leopards (*Panthera uncia*), Indian leopards (*Panthera pardus*), caracals (*Caracal caracal*), jaguars (*Panthera onca*), ocelots (*Leopardus pardalis*), margays (*L. wiedii*), and tigrinas (*L. tigrinus*). The electroejaculation has been used to collect semen from nondomestic felids [4] and the semen has been cryopreserved. After thawing, in lions (*Panthera leo*), jaguars (*P. onca*), leopards (*Neofelis nebulosa*), cheetahs (*A. jubatus*), and leopard cats (*Felis bengalensis*) 25–50% sperm motility was preserved, and in the latter, a 70% sperm motility was maintained. Furthermore, they reported finding lesser values of sperm motility, ranging between 1 and 20% post-thaw, for Geoffroy's cats (*Felis geoffroy*), Indian tigers (*P. tigris*), and ocelots (*Felis pardalis*), but unfortunately, the spermatozoa from gold cats (*Felis aurata*) did not survive cryopreservation. In addition, 40% sperm motility post-thaw in Siberian tigers (*P. tigris*) was obtained, in semen collected with electroejaculation by [4]. The epididymis is an anatomical component of the male reproductive tract and is connected to the testicle. One of its main functions is the storage of spermatozoa for ejaculation [25]. Current technologies allow semen to be collected directly from the epididymis and this seems to be a viable alternative method for obtaining gametes from animals that have recently died or from animals unable to ejaculate (Figs. 1, 2, 3). It has been suggested that viable epididymal spermatozoa from Iberian deer (*Cervus elaphus*) could be collected in the 10–20 h postmortem period. However, it must be noted that this could vary depending on the temperature conditions and the weather where the procedure is being executed [26]. Comparing epididymal spermatozoa from domestic cats and ejaculated spermatozoa, it was verified that epididymal spermatozoa require less capacitating time as compared with those ejaculated and are able to penetrate feline oocytes 20 min after in vitro insemination [27, 28]. Fresh feline epididymal spermatozoa were able to fertilize oocytes in vitro, promoting 40.7% cleavage rate. After freezing, a 26% cleavage rate was obtained. After intracytoplasmic sperm injection (ICSI) using feline frozen epididymal spermatozoa, 34.9% of embryos have developed to the morale stage, indicating that spermatozoa with minimal motility could be used in assisted reproductive techniques [29]. Also, the unilateral intrauterine artificial insemination with frozen–thawed epididymal semen from cats may obtain 23% conception rate [30]. For nondomestic cats, [31] were able to collect spermatozoa from the finely minced cauda epididymus of leopards (*P. pardus*), tigers (*P. tigris*), lions (*P. leo*), pumas (*F. concolor*) and jaguars (*P. onca*). The samples were treated as described by [32], washing the spermatozoa in Hank's balanced salt solution and extended in medium M199 supplemented with 2.5 mmol/l sodium lactate and 0.4% bovine serum albumin. Progressively motile spermatozoa were 60–85% depending on the various felids. In the same study, the epididymal semen was frozen, and thawing motility is between 25 and 65% for the different

species. The frozen semen was then submitted to in vitro fertilization and 18.5% developed to 8-cell embryo. Similarly, some evidences showed that frozen epididymal spermatozoa from jaguars were able to penetrate heterologous zona-free oocytes.
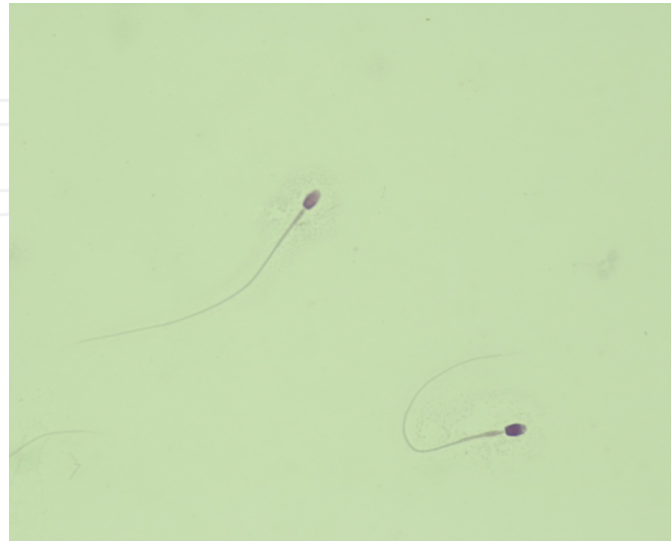

Figure 1. Epididymal sperms from dead *Panthera pardus.*

Spermatogenesis is a complex and very efficient process with the mitosis and the differentiation of spermatogonial stem cells in the basal membrane of seminiferous tubules where they are supported by Sertoli cells [33]. The spermatogonial stem cells in mammals are unique, and thus they can maintain their proliferation in adults: the genetic material can be passed from a generation to the subsequent one. Therefore, these cells are a valuable source for medical research, biological experimentation, agricultural biotechnology, and genetic modification of the species [34]. Recent studies on their recovery and cryopreservation showed the perspective of application in the conservation of genetic material from endangered animal species. Present methods described for spermatogonial isolation from fragments of collected testis consists of elutriation or sedimentation rate in a gradient of bovine serum albumin under gravity force action [35]. Some other isolation techniques have been proposed as immunological markers for posterior magnetic cellular separation [36]. After collection, germ cells can remain for several months in tissue culture media, only resuming spermatogenesis afterward in an environment that provides favorable conditions for their expansion and differentiation [37]. The favorable conditions are generally provided by transplant to other organisms [34]. The first success in the spermatogonial transplant was described by [38]. They showed that the microinjection of a cell heterogeneous suspension of mouse testis into the seminiferous tubules of a recipient sterile mouse resulted in spermatogenesis in the injected animal. After this study, several other researchers showed real possibilities such as the spermatogonial culture among different species: the xenograft [39]. It seems that cryopreservation of testis cell suspensions could be the greatest promise for the storage of germ cells to be used later in transplants. Indeed, after cryopreservation, spermatogenesis can continue [39]. In spite of the progress in this field, some elements remain to be controlled, such as the quantity of germ cells to be

Figure 2. Testis and epididymus (a) and excised epididymus (b) of *Panthera pardus* collected 6 h postmortem; testis and epididymus of domestic cat and *Panthera onca* (c).

transplanted, formation of antibodies against spermatogonial cells by the recipient [40], and poor quality of cells that have developed using these procedures [41]. There is also a problem concerning xenograft related to the different time of spermatogenesis in each species [26, 42]. However, the complete spermatogenesis was observed after transplantation of testicular tissue fragments from species that are phylogenetically more distant, such as pigs and goats, into castrated immunodeficient mice. The new reproductive technologies on stem cells offer several potential advantages for carnivorous species. For example, the development of lines of embryonic stem cells in cats and dogs would allow the creation of a generation of transgenic animal models, which could be used to improve the health of both animals and humans. Techniques such as testis xenografting spermatogonial and stem cell transplantation offer new approaches to diffuse genetically valuable individual males, even if they should die before producing sperm. Therefore, these techniques could be applied to biomedical research, as well as to the programs for the conservation of endangered carnivore species. Recently, spermatogonial stem cell transplantation has been performed in a recipient able to produce sperm of donor genetic origin [26]. Sperm production, from prepubertal testis tissue from both ferrets and cats, was obtained from testis xenografting. These first steps reinforce the need for research on stem cell technologies and for complementary technologies of carnivore assisted reproduction, so clinical benefits and the largest array of research can be achieved [26].

Figure 3. Ultrasound guided epididymal sperms collection in *Panthera pardus*.

6. Female gamete collection in felids

The ovarian follicular population seems to be made up of thousands of follicles in different mammalian females. Therefore, oocyte retrieval represents a rich source of genetic material to be used for genetic bank and assisted reproductive techniques in endangered species preservation, mainly in relation to the possibility of collecting material originated from postmortem or convalescent animals. The development of efficient methods for in vitro maturation (IVM) or fertilization (IVF) of oocytes collected postmortem or through ovariectomy is an important tool to prevent the species extinction [43-45]. Thus, IVM and IVF techniques are adjusted for several nondomestic animals [5] based on systematic studies in domestic animals [46] including wild carnivores. Moreover, application of oocyte and ovary tissue cryopreservation will help in the conservation of several animal species, with the objective of maintaining biodiversity [47]. Further, ultrasonographic images of the reproductive tract offer new opportunities for induction of sexual cycles and ovulation, adoption of superovulating regimens, as well as the ovum pickup application. Ovarian follicles are then visualized on a monitor, allowing oocyte collection by puncturing the follicles with a fine needle connected to a tube collector. The collected oocytes could be used in IVM and IVF [48, 49]. This technique is extensively used for oocyte collection in cattle and the findings indicate the possibility of repeated collections in both pregnant and nonpregnant females [50]. Concerning carnivorous species, ovum pickup using ultrasonography has yet to be reported. This may be due to the difficulty of ovarian visualization, because in bitches the ovary is surrounded by a pouch rich in conjunctive tissue [23]. Furthermore, there are no commercial probes developed for intravaginal use in either canids or felids. However, in spite of this difficulty, the presence of antral ovarian follicles can be detected by the fluid accumulation in the antral cavity [51]. A success in the follicular and corpora luteal visualization in ovaries of female African wild dogs (*L. pictus*) was reported by transrectal ultrasonography, suggesting the possibility of oocyte puncture in carnivores too [52]. The adaptation of this technique would be an important alternative, because it is a noninvasive procedure and it could allow oocyte collection without the risks involved with surgical procedures. The potential of ultrasonography is underestimated by researchers for assisted reproduction in endangered canid and felid species. Another possibility for oocytes retrieval is laparoscopy. It is the lowest invasive procedure commonly used for intrauterine deposition of frozen–thawed semen in domestic dogs [23] and cats [54]. For domestic cats, [27] reported the laparoscopic collection of oocytes, which were subject to fertilization in vitro with ejaculated semen. In this study, when the developing embryos reached the 4-cell stage, they were transferred to the oviduct of oocyte donors. Thus, five of the six cats receiving embryos became pregnant. According to [53], laparoscopy is effective in the evaluation of reproductive status, particularly the ovarian anatomy and function, direct visual biopsy of internal organs, and as a surgical means of fertility control. In wild felids, [55] reported the laparoscopic visualization of changes in the reproductive tract during ovarian stimulation by gonadotropins in the ocelot (*F. pardalis*). Moreover, the multiple laparoscopic oocyte retrievals was successfully performed in caracal (*C. caracal*) after repeated ovarian stimulation with equine (eCG) and human (hCG) chorionic gonadotropin [4]. Embryos could also be reliably produced in vitro using cryopreserved spermatozoa and live offspring could be produced after embryo

transfer. It was suggested that the collection of ovaries from tigers (*P. tigris*), lions (*P. leo*), pumas (*F. concolor*), cheetahs (*A. jubatus*), leopards (*P. pardus*), and jaguars (*P. onca*) could be accomplished by ovary dissection up to 8 h after the death of these animals, by mechanical follicle isolation [56]. The best results were obtained with lion oocytes, fertilized by lion sperm, with a 31.6% (18/44) conception rate. It was demonstrated that leopard oocytes can be fertilized by domestic cat sperm and used in IVF procedures to produce 22% (2/9) 8-cell embryos. Otherwise, domestic cat oocytes can be fertilized by leopard spermatozoa, producing 19.5% (8/41) 8-cell embryos. Also oocyte collection from domestic and nondomestic cats by laparotomy and posterior ovary dissection was successfully performed. These oocytes were submitted to IVF and then transferred to recipient females [57]. The main result obtained in this study was the interspecies embryo transfer from an Indian desert cat (*Felis silvestris ornata*) embryo to a domestic cat (*F. catus*), which resulted in the birth of two kittens. Afterward, the oocyte collected from domestic cat ovaries after ovariectomy were used to demonstrate that morphology of the oocyte ooplasm can affect in vitro maturation, as well as the gonadotropin supplementation [12]. According to the morphological aspect by stereomicroscopic exam, cumulus–oocyte complexes were classified as mature, immature, or degenerated. Besides the successful embryo production by IVF using this approach, light and electron microscopic evaluations revealed that ovarian stimulation followed by follicular aspiration resulted in a heterogenous oocyte population with respect to meiotic maturation. The correct assessment of the oocyte maturation status is difficult to perform through stereomicroscopical exam [58]. Oocytes can be preserved if they are not immediately submitted to IVF. However, [59] observed that cooling could cause chromosomal anomalies in mature oocytes, as a consequence of the temperature decrease on meiotic fusion. [60] performed the IVM of domestic feline oocytes, previously kept under refrigeration at 4 °C for 24 h, and they did not observe deleterious effects of storage on oocyte meiotic progression. Moreover, [61] demonstrated that even brief (2–3 weeks) salt storage significantly affects cat oocyte penetration rate, and the penetration continues to decline as storage duration increases to 2–3 months. However, the authors hypothesized that the composition of the solution may have contributed to reduce sperm penetration. For canine species, [62] found that oocyte storage in hypertonic salt solution damages the zona pellucida, reducing the sperm penetration rates. In mice [63], rabbits [64], and bovines [65], it was possible to obtain the birth of normal offspring following IVF after thaw. In domestic felines, [66] demonstrated that the mature oocyte could be cryopreserved and, soon after, fertilized in vitro with success. The maturation of oocyte recovered from antral follicles is an efficient method for the use of haploid female material and the oocyte activation in initial phases of development is a possible tool that also increases the efficiency of the oocyte utilization [56]. The preantral ovarian follicles (PAF) represent 90% of the follicular population in mammals [67]. Small PAF recovered from the ovaries collected from postmortem animals or through ovariectomy, therefore, are a rich oocyte source, because they can mature in vitro (Fig. 4). [68] reported that feline PAF are capable of developing in vitro to the antral phase. Moreover, [69] demonstrated the isolation of PAF from domestic cats by mechanical ovary dissection. By adapting the methods described for domestic cats to nondomestic felid species, [70] accomplished the isolation and the ultrastructural characterization of PAF from cheetahs, jaguars, lions, and Sumatran, Siberian, and Bengal tigers that had died at local zoos. The

similarity among domestic and nondomestic felid PAF was verified. The PAF collection was performed from ovary of several species of nondomestic felids [56] with a recovering of 1867±1144 PAF from each ovary, observing that the follicle growth is possible in the culture media for up to 14 days, with a 20% increase (40–50 mm) on the diameter of preantral follicles of the puma. These promising results suggest the possibility of future use of preantral follicles as a source of oocytes to be used in other biotechniques, and the foundation for germplasm banks. [70] reported that it is possible to maintain the viability of PAF from domestic cats after cryopreservation procedures.
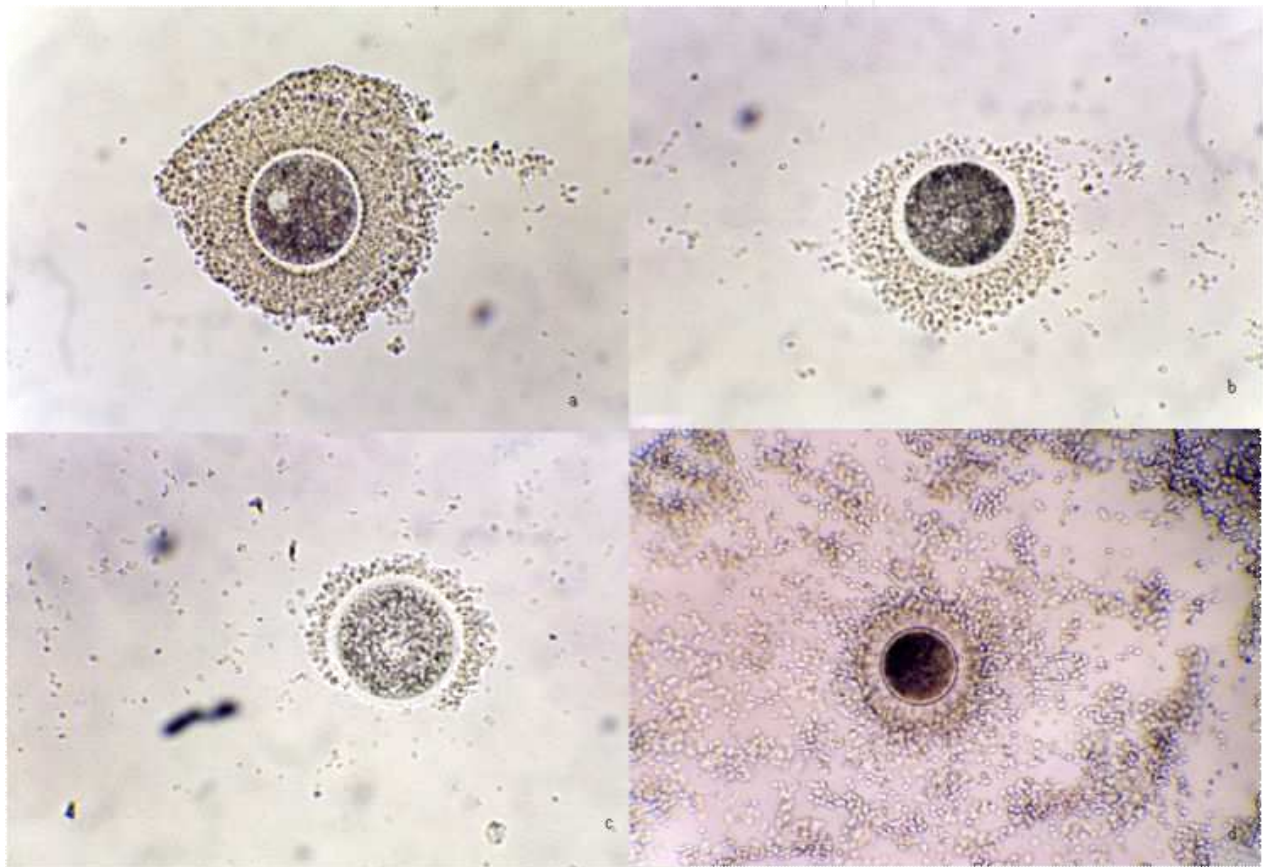

Figure 4. Cat Cumulus Oocyte Complex (COC) collected from Preantral Follicle (PAF); Cat COC grade: a) grade I: compact and integral and multistratified cumulus and dark ooplasm; b) grade II: compact but not integral and paucistratified cumulus and not homogeneous dark pigmentation of ooplasm; c) grade III: interrupted and incompact cumulus and clear ooplasm; d) expanded cumulus.

A further alternative is represented by the ovarian tissue transplantation. [71] was the first to report an ovarian transplantation. Only in the twentieth century was a significant improvement of the vascular anastomosis techniques of several transplanted organs including the ovary achieved [72]. According to [73], both whole ovary and ovarian fragment transplantations could be used for ovarian follicle cultures. Moreover, [74] was reported that a great advantage for the preservation and culture of ovarian tissue is due to the possibility of material collection not dependent upon the age or reproductive status of the donor. Moreover, [75]

suggested that the term allotransplantation refers to the transplantation of an organ originating from one individual to another that is genetically different, but belonging to the same species. Ovarian cortex fragments transplantation was successfully performed from domestic cats to the renal capsules of severely immunedeficient infertile mice [76]. After 9 months, the necropsy of the recipient mice was accomplished, when the presence of follicles was verified in the grafts. These ovarian follicles reached a 3 mm diameter, had a normal antral cavity, and appeared to be cytologically normal as follicle in integer cat ovary (Fig. 5). However, ovulation was not observed in any of the grafts. Furthermore, [77] reported that xenotransplanting into the kidney capsule from severe combined immunedeficient mice freeze–thawed of cat ovarian cortex did not allow its surviving, but the follicles containing gonadotropin responsive granulosa cells were able to grow to antral stages. Conversely, [78] declared that oocyte and ovarian tissue cryopreservation is not yet fully established. There are still several obstacles to overcome for this technology to be routinely used. Even so, improvement in the cryopreservation techniques is seen as an important tool for the formation of ovarian tissue banks, with the purpose of conserving precious genetic material of endangered species [79].
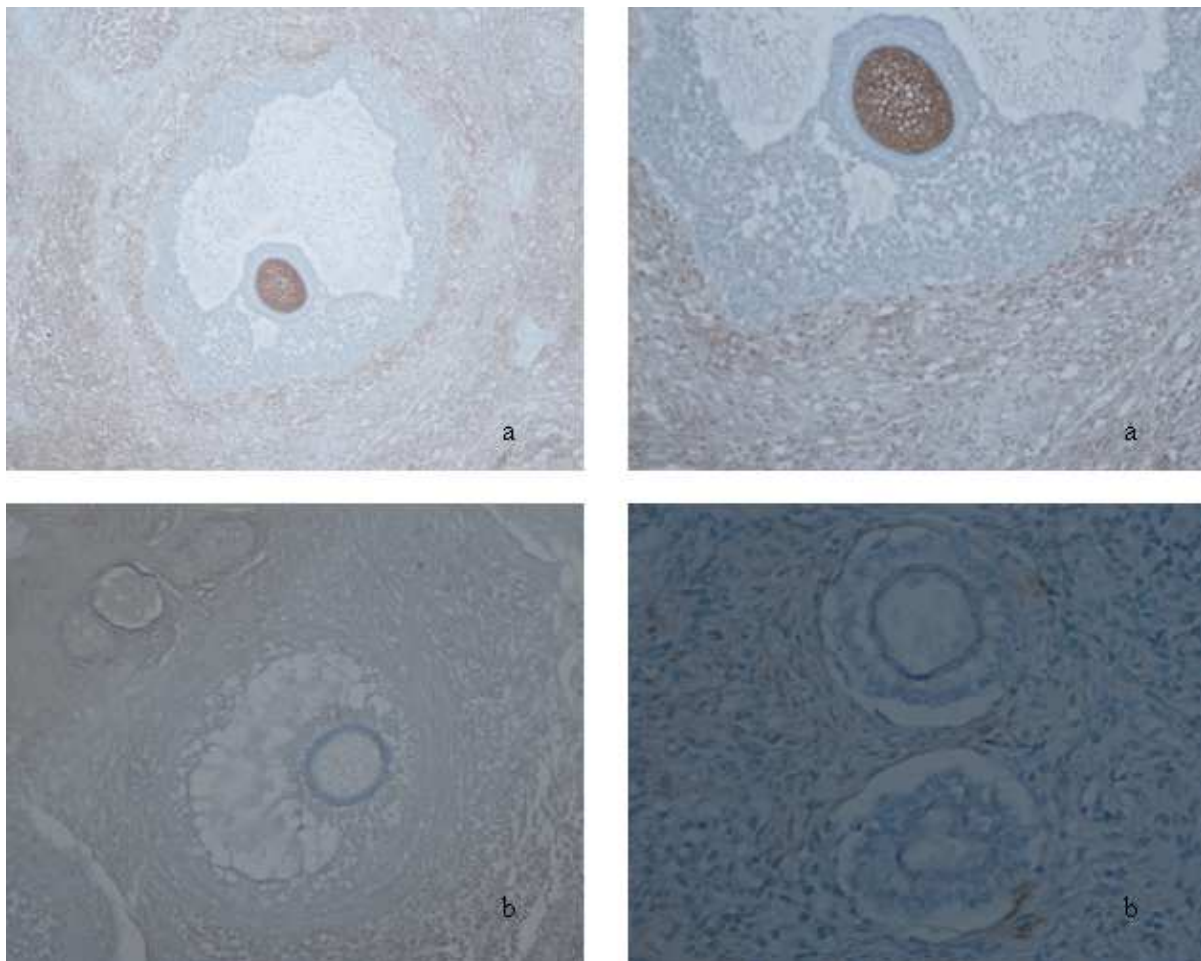

Figure 5. Histological section (O.M. 200X) of explanted cat ovarian tissue with follicle at different developmental stage: a) and a1) antral follicle; b) preantral follicle; b1) primary follicle.

7. Gamete cryopreservation in felids

Cryopreservation of gametes is an important tool in assisted reproduction programs. In fact, long-term storage of oocytes or spermatozoa is necessary for in vitro fertilization (IVF) or artificial insemination (AI) in the future. When geographical or temporal distance between donors and recipient results in nonsimultaneous availability of male and female gametes, cryopreservation is the only option. Maintenance of biodiversity has intrinsic value for the genetic preservation of valuable domestic cat breeds and an extrinsic value for conservation management of taxonomically related nondomestic feline species. New knowledge about felid reproductive physiology will enhance the development of techniques that are potentially applicable to nondomestic cats. Domestic cat spermatozoa and oocytes have peculiar physical characteristics that increase the difficulty of developing successful cryopreservation methods as compared to gametes of some other species. Therefore, even though a variety of procedures have been investigated, optimal cryopreservation techniques, either for spermatozoa or oocytes, are yet to be realized [80]. Cat semen was successfully cryopreserved, and kittens were born after AI with frozen–thawed semen [81]. Achievements in cryopreservation of felid semen and different protocols of freezing–thawing ejaculated and epididymal cat semen have been reviewed [80]. In our laboratory we cryopreserved epididymal sperm felines with the following protocol: epididymides were collected from 20 domestic cats during routine neutering procedure and from two wild felines at autopsy. The sperm samples, diluted with 4% glycerol/Tris/egg yolk, were loaded into 0.25 ml ministraws, exposed to nitrogen vapor and stored in liquid nitrogen. After 4 weeks, samples were thawed and reevaluated. The quality of each fresh and frozen–thawed sperm sample was tested by determining the motility (54.7±11.3% and 32±13.1%, respectively, for cat spermatozoa; 38.3±18.7% and 21.5±16.8%, respectively, for tiger spermatozoa), viability (74.3±8.6% and 45.2±9.4%, respectively, for cat spermatozoa; 42.4±14.5% and 33.5±12.9%, respectively, for wild felid spermatozoa), morphology, and acrosomal status. The present study showed that feline epididymal spermatozoa can be frozen in egg-yolk extender with 4.0% glycerol in 0.25 ml straws. The procedure used in the present study for epididymal cat sperm cryopreservation may be applied to bank for genetic resources of wild felid species. [82] Protocols for freezing/cryopreservation of cat oocytes [80] are established; nevertheless, this technology is still considered "experimental" because the survival rates of cat oocytes after freezing procedures are still low, but to date, there is evidence that some preantral follicles extracted from cat ovaries remain structurally intact and physiologically active after freezing/cryopreservation and subsequent thawing [80]. However, there is evidence that some preantral follicles from cat ovaries remain structurally intact and physiologically active after freezing/cryopreservation and subsequent thawing [70]. Domestic cat oocytes have high lipid droplet content in the ooplasm [83]; thus, oocyte permeability to cryoprotectant solutions may be lower than in oocytes of other species [84-87]. Only a few studies have investigated cat oocyte cryopreservation, and the few successes were only obtained for mature oocyte cryopreservation [80]. In the first study [80], mature and immature oocytes were cryopreserved by slow cooling, but no blastocysts were obtained after in vitro fertilization (IVF). In

the second study [88], matured cat oocytes were vitrified in straws and, after IVF with frozen–thawed epididymal spermatozoa, the first two blastocysts were obtained [80]. In a recent study, the first attached cat blastocysts were obtained from matured cat oocytes that were vitrified using a cryo-loop system [89]. Another very recent study reported blastocyst production from vitrified germinal vescicle (GV) cat oocytes exposed to resveratrol (Res) in order to compact the decondensed chromatin contained in the large GV of cat oocytes [90]. Despite the importance of cryoprotectant penetration to avoid intracellular ice crystal formation, the greater cryoprotectant concentrations in vitrification solutions are toxic and may cause osmotic injury [91]. Suggestions for minimizing the toxicity of vitrification solutions include the use of less toxic substances, association with different cryoprotectants, previous exposure to lesser concentrations of cryoprotectants, and reduction of exposure time to vitrification solutions [92, 93]. The major penetrating cryoprotectants for oocyte cryopreservation are ethylene glycol (EG), glycerol (GLY), dimethylsulfoxide (Me_2SO), propylene glycol (PrOH), and acetamide [94]. Another common permeating CPA, 1,2-ethanediol (EG) [94], is also suitable for less permeable immature oocytes, as demonstrated in cattle [95]. A recent study investigating bovine oocyte vitrification demonstrated that a solution of EG + Me2SO is a favorable cryoprotectant combination, as the Me2SO (MW = 78.13) molecule is smaller and consequently more permeable than the glycerol molecule (MW = 92.1) [96]. In our laboratory for the first time we obtained blastocysts from egg vitrified at GV stage from cat [97]. The vitrification was performed in OPS into sucrose medium (1 M sucrose in HSOF + 6% BSA) containing dimethyl sulfoxide (DMSO) (16.5% final concentration) and ethylene glycol (EG) (16.5% final concentration) as cryoprotectants. This vitrification protocol ensured a development to blastocyst stage and it is the first report of development of vitrified GV COC and confirmed that the selection of an appropriate cryoprotectant mixture and sample volume reduction are two simple but important parameters in the study of a successful vitrification method for feline species. Ovarian tissue cryopreservation combined with the subsequent transplantation into immunocompromised recipients, in order to resuming follicular development, is considered to be a promising approach for cryobanking female gametes in nondomestic felid species [81].

8. *In vitro* embryo production in felids

Several laboratories have independently assessed the potential of maturing and fertilizing domestic cat oocytes, mainly using IVM /IVF. The biological competency of IVM/IVF domestic cat embryos has been demonstrated after embryo transfer. Nonetheless, it has become apparent that IVM/IVF success in the cat is generally less than that reported for other commonly studied species like the cow [4]. For example, it is not unusual for 60–80% of cow antral follicular oocytes to be fertilized and to cleave in vitro [9]. Techniques in the mouse have progressed even further to allow the growth, maturation, and successful fertilization of oocytes from primordial ovarian follicles. In contrast, only about 50–60% of cultured cat oocytes achieve nuclear maturation in culture and, after insemination, usually <40% oocytes are

fertilized on the basis of embryo cleavage [9]. Even under optimal culture conditions, <20% of these cleaved embryos grow into blastocysts *in vitro* [9]. Nevertheless, in vitro embryo production has also been successful in felids. In vitro-derived embryos of the domestic cat were successfully frozen and developed to term kittens after cryopreservation and transfer. Various aspects of in vitro maturation of felid oocytes and in vitro culture of felid embryos have been comprehensively reviewed [81]. Cat oocytes can be collected from ovaries from sexually mature queens recovered following routine ovariohysterectomy. Feline oocytes can be collected from ovary explanted postmortem within 6 h from death. Within 3–6 h of excision, each ovary was sliced longitudinally with a scalpel blade followed by lateral mincing of the ovarian cortex in a 35 x 0.7 mm Petri dish, flushed by different media (e.g., HEPES synthetic oviductal fluid HSOF or HMEM or TCM199). Collected oocytes were graded (only oocytes exhibiting uniform, darkly pigmented ooplasm and an intact cumulus cell investment were chosen for culture), gently rinsed in a fresh dish of culture medium and immediately placed in 50 µl drops of culture medium under mineral oil or in 500 µl 4WD. In vitro maturation may be performed (25–50 oocytes/ml) in different media, for example, SOF synthetic oviductal fluid added with amino acids and 6 mg/ml BSA containing 0.1 IU of porcine follicle-stimulating hormone and porcine luteinizing hormone supplemented with 25 ng/ml EGF, 25 ml/ml insulin–transferrin–sodium selenite (ITS) and 1.2 mmol/l L-cysteine or Eagle's minimum essential medium containing 0.026 g pyruvate, 0.292 g L-glutamine, 0.4% (w/v) BSA, 100 IU penicillin, 100 IU streptomycin, 1 pg LH ml, 1 pg FSH and 1 pg oestradiol or MEM minimal essential medium containing Earle's salts and bicarbonate buffer supplemented with 0.4% (w/v) BSA, 2.0 mmol glutamine, 1.0 mmol pyruvate, 1 µg FSH, 1 µg LH ml, and 1 µg oestradiol (EMEM)] in a 5% CO_2 incubator at 38.5° C for 24 h. In vitro matured COC can be in vitro fertilized with fresh/cryopreserved epididymal or ejaculated spermatozoa. Briefly, in order to perform IVF, semen was diluted 1:1 in different media (SOF or Ham's FIO medium (HF10) supplemented with 1.0 mmol pyruvate, 2.0 mmol glutamine, and 5% (v/v) fetal calf serum or Tris extender (3.025% Tris(hydroxymethyl)aminomethane, 1.7% citric acid, 1.25% fructose, 0.06% Sodium Benzyl penicillin, 0.1% streptomycin sulphatee) and centrifuged at 300 g for 8 min. Supernatant was discarded and the remaining pellet overlaid with 100 µl HFlO and the sample maintained at room temperature undisturbed for 1 h. Next, 50 µl was removed from the top layer and evaluated for sperm motility, forward progression, and concentration. Cultured oocytes were washed twice in 90 µ fertilization drops of IVF media or in 500 µl FWD under oil in 5% CO_2 in air at 38°C. Processed sperm samples were diluted in IVF media in order to obtain a final concentration of 1×10^6 spz/ml. After coincubation for 18 h, oocytes were washed to remove cumulus cells and loosely attached spermatozoa and returned to fresh 50 µ drops of IVC media (SOFaaBSA or F10). At 30 h after insemination, oocytes were evaluated at a stereomicroscope for survival, and those showing cytoplasmic degeneration were discarded. The cleavage to the two-cell stage was assessed as an index of fertilization. Subsequent embryonic development was assessed at intervals of 24 to 48 h. As explained, despite some past difficulties achieving in vitro development beyond the morale stage after IVF, more recently, several reports have shown blastocyst rates from approximately 50% to 80% on Day 6, Day 7, or Day 8 [98-100]. The frequency of blastocyst development is universally considered to be a useful measure of embryo developmental potential. In favorable in vitro culture

conditions, cat blastocysts, like those of more widely studied species, undergo further growth, in cell numbers and in overall size. Thus, another widely accepted indication of blastocyst developmental potential is their ability to undergo expansion and "hatch" from the zona pellucida in vitro. Most of Day 8 blastocysts show noticeable expansion of the blastocyst cavity such that the zonal diameter is larger and its thickness is much thinner than at earlier stages. In addition, some of them are "hatching" because the embryonic cells are gradually extruded through one or more small apertures in the zona, rather than popping out through a large crack [4].
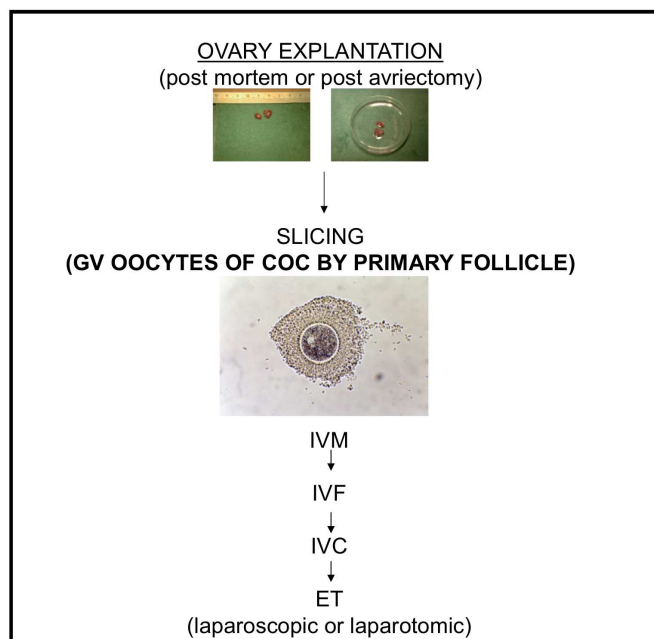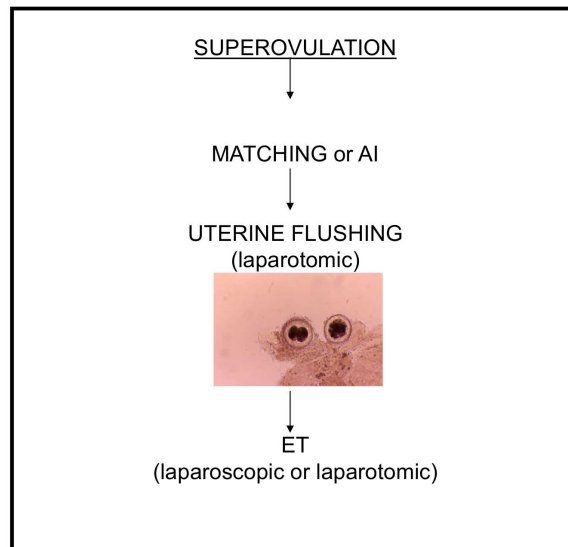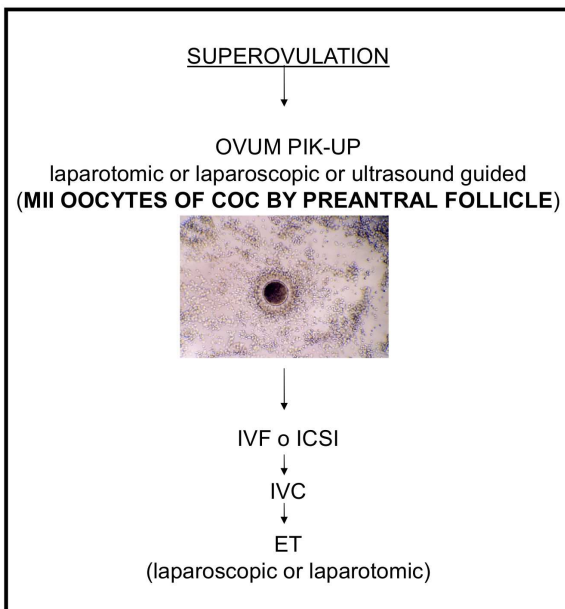
9. Artificial Insemination (AI) and Embryo Transfer (ET) in felids

Embryo transfer (ET) and artificial insemination (AI) are potentially important techniques for the propagation and management of genetically valuable domestic cat and endangered nondomestic cat populations. There are different AI techniques for cats [101]. In early studies, intravaginal insemination was exploited, but the success rate has not exceeded 43%. Later, this technique has been used with more effectiveness and a better success rate [102]. Another approach is intrauterine insemination, either surgical [103] or nonsurgical with the use of specially designed catheters [104]. They performed the first transcervical insemination with fresh or frozen semen in cats. This can be considered the method of choice in almost all cases; it is less invasive than the surgical approach, and a much smaller amount of semen is needed with respect to the intravaginal insemination. Recently, AI with semen cryopreservation has been applied in a number of wild felid species. Unfortunately, the teratospermia problem aggravates freezing/cryopreservation in many felid species [4]. In vitro embryo production has also been successful in felids. In vitro-derived embryos of the domestic cat were successfully frozen and developed to term kittens after cryopreservation and transfer. Various aspects of in vitro maturation of felid oocytes and in vitro culture of felid embryos have been comprehensively reviewed [4]. In 1979, the first successful embryo transfer (ET) in cats was reported [105]. The embryos, recovered from donors after mating during a natural cycle, were transferred into like recipients. Three litters of kittens were born from four pregnancies established in seven recipients. Nonetheless, in the following decade, in most ensuing reports on ET of in vitro-produced cat embryos, mixed morale and early blastocysts were deposited into the uterus of Day 4, 5, or 6 recipients [106-108]. This interval was used because it is the approximate length of time required for cat embryos to be transported through the oviduct and enter the uterus [109, 110]. To examine the effect of developmental stage, morula versus blastocyst, on pregnancy rate after ET, [111] recovered 1–4-cell embryos from gonadotropin-treated donors and cultured them in vitro (in 20% fetal calf serum) for 3–7 days before transfer into synchronous recipients. All four recipients of morulae (6–12 each) established pregnancies; two aborted before term, and two delivered a total of 10 kittens. Three of five recipients of blastocysts cultured for 4–6 days delivered a total of nine kittens, but none of the three recipients of blastocysts cultured for 7 days became pregnant. Although the zonal status of the blastocysts transferred after 7 days in vitro was not provided, most morulae had developed to the blastocyst stage by Day 7 of culture, with hatching starting to occur on Day 6 in blastocysts that were not fully expanded, which would suggest that most were either hatching or hatched when transferred. In their comments on failure of later-stage blastocysts (Day 8, 1 day in vivo, and 7 days in vitro) to establish pregnancies, the authors noted that further studies were needed "on in vivo development and hatching of transplanted embryos." [112] found that separate transfer of in vitro-produced Day 5 late morulae and Day 5 early blastocysts into synchronous gonadotropin-treated recipients resulted in equally high pregnancy rates of 71% (5/7) and 80% (8/10), respectively. Each recipient received six morulae or six blastocysts and the average litter size was 2.0 (1–3) and 3.0 (1–3), respectively. Possibly, the only pregnancy/birth after ET of in vitro-derived (IVM/IVF/IVC) Day 7

blastocysts is the single kitten born from 21 embryos transferred into two synchronous recipients [113]. All of the blastocysts were completely zona-intact when transferred. The transfer of fresh or frozen in vitro-derived embryos has proved to be successful in some wildlife felids. In the lion (*Pantera leo*), in vivo-derived oocytes were inseminated in vitro with fresh semen, and some of these embryos developed up to the blastocyst stage. In the tiger (*Panthera tigris*), term kittens developed after the transfer of in vitro-derived embryos [43], and one live kitten was born after the transfer of African wildcat (*Felis silvestris lybica*) frozen–thawed in vitro-derived embryos into three recipients [5]. Although the rate of success was low (4.5%), this result is important as this was the first kitten born after embryo cryopreservation in a wildlife felid species. Two term kittens born after transferring frozen–thawed embryos of ocelot (*Leopardus pardalis*) have also been reported [110]. Cleavage stage ocelot embryos were conventionally frozen with ethylene glycol and kept in liquid nitrogen [110]. More recently, three live ocelot kittens were born in Cincinnati Zoo after the thawing of ocelot embryos from a cryobank and the transfer of nine frozen–thawed embryos into eight synchronized recipients. The ocelot is an endangered species at least in some countries, and a cryobank is needed to secure its biodiversity [110]. The birth of live kittens produced by intracytoplasmic sperm injection of domestic cat oocytes matured in vitro has been reported [4]. Also noteworthy, there are experiments on "interspecies in vitro hybridization," when oocytes of nondomestic felid species were successfully fertilized in vitro by heterologous (domestic cat) spermatozoa. Cleavage stage "hybrid" embryos have resulted from in vitro fertilization of leopard (*Panthera pardus*) and puma (*Felis concolor*) oocytes with frozen–thawed domestic cat semen [4].

10. Cloning of domestic and wild cats and interspecies of felide

Along with these achievements with cryobanking, the domestic cat has been cloned by two independent groups [114,115]. Recently, a domestic cat recipient female has been reported to have given birth to African wildcat (*F. silvestris lybica*) cloned kittens and sand cat (Felis margarita) kittens [13]. Fibroblast nuclei of African wildcat were fused to domestic cat oocytes (interspecies nuclear transfer) and the cloned embryos were transferred into recipient domestic cat females; 17 kittens were born, but only 8 survived, after birth, up to 1 month. These African wildcat kittens represent the first wild Carnivora species to be produced by nuclear transfer [13]. This study showed the possibility of cloning other felid species beside the domestic cat, but it is also a big success in interspecies nuclear transfer/embryo transfer in felids. Earlier, an interspecies ET was performed between the Indian desert cat (*Felis silvestris ornata*) and the domestic cat [4]. Recently, transgenic clones have also been produced in the cat. Genetically modified adult or fetal fibroblasts have been used as donors of nuclei. These nuclei were moved into cat oocytes and then embryos were developed in vitro. After these embryos were transferred into appropriate recipients, three alive transgenic kittens were obtained [81].

11. Laparoscopic oviductal embryo transfer and artificial insemination in felids

The application of laparoscopy to reproductive studies in felids has been invaluable for helping to alleviate some concerns of animal welfare: sowing reproductive organs through the intraabdominal access through a minimally invasive and traumatic approach [116]. Likely, the extrapolation of ART to the genetic management of wild cats would be unattainable in the future without laparoscopy. The latter, for oocyte collection and intrauterine insemination, has been used largely with numerous cat species over the past 20 years. Recently, laparoscopic approaches have been developed and applied in cats for accessing the oviduct precisely to perform laparoscopic oviductal embryo transfer (LO-ET) and artificial insemination (LO-AI) procedures [117,118]. To our knowledge, just in eight cat hereditary disease models and two nondomestic cat species, the ocelot and sand cat, it has been possible to get viable offspring following LO-ET of nonfrozen and frozen–thawed IVF-derived embryos. LO-AI with low sperm numbers and LO-ET have been demonstrated to be similar in efficacy, resulting in high pregnancy percentages (50–70%) following insemination of domestic cats treated with gonadotrophins. Following LO-AI, multiple kittens have been produced in some hereditary disease models with frozen semen, and both Pallas' cat and ocelot kittens were born after LO-AI with freshly collected semen. The application of LO-ET and LO-AI to felids has brought important and effective improvement in the efficiency of ART for genetic management of these invaluable wild and domestic cat populations [119].

12. Conclusions

In the present chapter, we made an overview of the data and methods detectable in literature and focused our attention on analysis of methods utilized in ART for maximizing their efficiency in feline species. ART include mainly Artificial Insemination (AI); *In Vitro* Embryo Production (IVEP) consisting of IVM (*In Vitro* Maturation), IVF (*In Vitro* Fertilization), IVC (*In Vitro* Culture), ET (Embryo Transfer), and ICSI (Intra Cytoplasmic Sperm Injection); gamete/embryo cryopreservation; gamete/embryo sexing; gamete/embryo micromanipulation; Somatic Cell Nuclear Transfer (SCNT), and genome resource banking, which has been widely used in genetic improvement and industry in livestock animals. The domestic cat is used as a model for the development of ART in Felidae species and can serve as a successful recipient of embryos from closely related, small, nondomestic cats, as shown by the birth of the Indian desert cat and African wildcat kittens after in vitro fertilization (IVF)-derived embryo transfer. The creation of the Biological Resource Bank represents a complementary support tool for the application of ART in the in situ and ex situ conservation of endangered felids. The chief purpose of ART in the protection of endangered species is to preserve the maximum current genetic and biological diversity of the population by the processing and cryopreservation of germinal cells and tissues from dead animals, which can later be used in future reproductive projects. In humans and domestic species, it is usually possible to plan the place and time for gonadal explants to recover germplasm, thereby enabling a reduction in the gonadal storage

time in the transport medium. In wild species, it is impossible to predict when and where the gonads can be collected. The gonads can be recovered postmortem, which entails the possibility that the collection place could be distant from a laboratory for IVEP.

Author details

Natascia Cocchia, Simona Tafuri, Lucia Abbondante, Leonardo Meomartino, Luigi Esposito and Francesca Ciani[*]

*Address all correspondence to: ciani@unina.it

Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Naples, Italy

References

[1] Swanson WF: Application of assisted reproduction for population management in felids: The potential and reality for conservation of small cats. *Theriogenology*. 2006;66:49–58.

[2] Hamner CE, Jennings LL, Sojka NJ: Cat (Felis catus) spermatozoa require capacitation. *J Reprod Fertil*. 1970;23:477–480.

[3] Bowen RA: Fertilization in vitro of feline ova by spermatozoa from the ductus deferens. *Biol Reprod*. 1977;17:144–147.

[4] Pope CE: Aspects of in vivo oocyte production, blastocyst development, and embryo transfer in the cat. *Theriogenology*. 2014;81:126–137.

[5] Pope CE: Embryo technology in conservation efforts for endangered felids. *Theriogenology*. 2000;53:163–174.

[6] Spindler RE, Crichton EG, Agca Y, Loskutoff N, Crister J, Gardner DK, Wildt DE: Improved felid embryo development by group culture is maintained with heterospecific companions. *Theriogenology*. 2006;66:82–92.

[7] Pope CE, Gómez MC, Dresser BL: In vitro production and transfer of cat embryos in the twenty-first century. *Theriogenology*. 2006;66:72–81.

[8] Comizzoli P, Wildt DE, Pukazhenthi BS: In vitro development of domestic cat embryos following intracytoplasmatic sperm injection with testicular spermatozoa. *Theriogenology*. 2006;66:1659–1663.

[9]   Wood TC, Wildt DE: Effect of the quality of the cumulus–oocytes complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts in vitro. *J Reprod Fertil*. 1997;110:355–360.

[10]   Wood TC, Bayers AP, Jennette BE, Wildt DE: Influence of protein and hormone supplementation on in vitro maturation and fertilization of domestic cat eggs. *J Reprod Fertil*. 1995;104:315–323.

[11]   Freistedt P, Stojkovic M, Wolf E: Efficient in vitro production of cat embryos in modified synthetic oviductal fluid medium: Effects of season and ovarian status. *Biol Reprod*. 2001;65:9–13.

[12]   Pope CE, McRae MA, Plair BR, Keller GL, Dresser BL: In vitro and in vivo development of embryos produced by in vitro maturation and in vitro fertilization of cat oocytes. *J Reprod Fertil*. Suppl. 1997;51:69–82.

[13]   Gomez MC, Pope CE, Giraldo A, Lyons LA, Harris RF, King AL, Cole A, Godke RA, Dresser BL: Birth of African wildcat cloned kittens born from domestic cats. *Clon Stem Cells*. 2004; 6:247–258.

[14]   Brown JL: Female reproductive cycles of wild female felids. *Anim Reprod Sci*. 2011;124:155–162.

[15]   Wildt DE, Brown JL, Swanson WF: Reproduction in cats. In: Knobil, E., Neill, J. (eds.), *Encyclopedia of Reproduction*, 1st edn. Academic Press, 1998:497–510.

[16]   Shille VM, Munro C, Farmer SW, Papkoff H: Ovarian and endocrine responses in the cat after coitus. *J Reprod Fertil*. 1983;69:29–39.

[17]   Leyva H, Madley T, Stabenfeldt GH: Effect of light manipulation on ovarian activity and melatonin and prolactin secretion in the domestic cat. *J Reprod Fertil*. 1989;39:125–133.

[18]   Pelican KM, Wildt DE, Pukazhenthi B, Howard JG: Ovarian control for assisted reproduction in the domestic cat and wild felids. *Theriogenology*. 2006;66:37–48.

[19]   Dresser BL, Sehlhorst CS, Wachs KB, Keller GL, Gelwicks EJ, Turner JL: Hormonal stimulation and embryo collection in the domestic cat (Felis catus). *Theriogenology*. 1987;28:915–27.

[20]   Goodrowe KL, Wall RJ, O'Brien SJ, Schmidt PM, Wildt DE: Developmental competence of domestic cat follicular oocytes after fertilization in vitro. *Biol Reprod* 1988;39:355–372.

[21]   Jalkanen L: Embryonal development and embryo losses during the preimplantation period in the silver fox. *Norw J Agric Sci*. 1992;9:108–114.

[22]   Wildt DE, Bush M, Morton C, Morton F, Howard JG: Semen characteristics and testosterone profiles in ferrets kept in a long-day photoperiod, and the influence of hCG

timing and sperm dilution medium on pregnancy rate after laparoscopic insemination. *J Reprod Fertil*. 1989;86:349–358.

[23]   Silva A: The potential for gamete recovery from non-domestic canids and felids. *Anim Reprod Sci*. 2004;81:159–175.

[24]   Platz CC, Wildt DE, Seager SWJ: Pregnancy in the domestic cat after artificial insemination with previously frozen spermatozoa. *J Reprod Fertil*. 1978;52:279-282.

[25]   Howard JG, Brown JL, Bush M, Wildt DE: Teratospermic and normospermic domestic cats: ejaculate traits, pituitary–gonadal hormones, and improvement of spermatozoal motility and morphology after swim-up processing. *J Androl*. 1990;11:204–215.

[26]   Travis AJ, KimY, Meyers-Wallen V: Development of new stem cell-based technologies for carnivore reproduction research. *Reprod Domest Anim*. 2009;44(Suppl 2):22–28.

[27]   Goodrowe KL, Hay M: Characteristics and zona binding ability of fresh and cooled domestic cat epididymal spermatozoa. *Theriogenology*. 1993;40:967–975.

[28]   Ciani F, Cocchia N, Rizzo M, Ponzio P, Tortora G, Avallone L, Lorizio R: Sex determining of cat embryo and some feline species *Zygote*. 2008;16:169 – 177.

[29]   Bogliolo L, Leoni G, Ledda S, Naitana S, Zedda M, Carluccio A, et al.: Intracytoplasmic sperm injection of in vitro matured oocytes of domestic cats with frozen–thawed epididymal spermatozoa. *Theriogenology*. 2001;56:955–967.

[30]   Tsutsui T, Tanaka A, Nakagawa K, Fujimoto Y, Murai M, Anzai M, et al.: Unilateral intrauterine horn insemination of frozen semen in cats. *J Vet Med Sci*. 2000;62:1247–1251.

[31]   Jewgenow K, Penfold LM, Meyer HHD, Wildt DE: Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation. *J Reprod Fertil*. 1998;112:39–47.

[32]   Lengwinat T, Blottner S: In vitro fertilization of follicular oocytes of domestic cat using fresh and cryopreserved epididymal spermatozoa. *Anim Reprod Sci*. 1994;35:291–301.

[33]   Schlatt S, Kim SS, Gosden R: Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. *Reproduction*. 2002;124:339–346.

[34]   Brinster CJ, Ryu BY, Avarbock MR, Karagenc L, Brinster RL, Orwig KE: Restoration of fertility by germ cell transplantation requires effective recipient preparation. *Biol Reprod*. 2003;69:412–420.

[35]   Bellvé AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M: Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J Cell Biol*. 1977;74:68–85.

[36] Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K, Honjo T, Ogura A: Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. *Hum Reprod*. 2002;17:3039–3045.

[37] Nagano M, McCarrey JR, Brinster RL: Primate spermatogonial stem cells colonize mouse testes. *Biol Reprod*. 2001;64:1409–1416.

[38] Brinster RL, Zimmermann JW: Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA.* 1994;91:11298–11302.

[39] Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL: Rat spermatogenesis in mouse testis. *Nature*. 1996;381:418–421.

[40] Ogawa T, Dobrinski I, Avarbock MR, Brinster RL: Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. *Biol Reprod*. 1999;60:515–521.

[41] Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED: *Histological and Histopathological Evaluation of the Testis*. Cache River Press, Inc., St. Louis, MO 1990.

[42] Honaramooz A, Megee SO, Dobrinski I: Germ cell transplantation in pigs. *Biol Reprod*. 2002;66:21–28.

[43] Donoghue AM, Johnston LA, Seal US, Armstrong DL, Tilson RL, Wolf P, Petrini K, Simmons LG, Gross T, Wildt DE: In vitro fertilization and embryo development in vitro and in vivo in the tiger (Panthera tigris). *Biol Reprod*. 1990;43:733–744.

[44] Wildt DE: Fertilization in cats. In: Dunbar, BS, O'Rand, MG (eds.), *A Comparative Overview of Mammalian Fertilization*. Plenum Press, New York 1991;299–328.

[45] Luvoni GC, Oliva O: Effect of medium-199 and fetal calf serum on in vitro maturation of domestic cat oocytes. *J Reprod*. Fertil. Suppl. 1993;47:203–207.

[46] Wolfe BA,Wildt DE: Development to blastocysts from in vitro maturation and fertilization of domestic cat oocytes following prolonged cold storage ex situ. *J Reprod Fertil*. 1996; 106:135–141.

[47] Rodrigues APR, Amorim CA, Brasil AF, Figueiredo JRF: Criopreservação de oócitos mam´ıferos: Importˆancia, estado atual, limitações e perspectivas. *Ci Anim*. 2001;11:101–112.

[48] Hildebrandt TB, Hermes R, Jewgenow K, Góritz F: Ultrasonography as an important tool for the development and application of reproductive technologies in non-domestic species. *Theriogenology*. 2000;53:73– 84.

[49] Nibart M, Marquant Le Guienne B, Humblot P, Guerin B: The application of new reproductive technologies in France. *Arq FacVet*. UFRGS. 1997;25:21–35.

[50] Lacaze S, Marquant Le Guienne B, Delalleau N, Richef L, Maunas S, Nibart M, Humblot P: Centralized in vitro embryo production after ultrasound guided bovine oo-

cyte collection: Effects of parity and superovulation treatment. *Theriogenology*. 1997;47:161.

[51] Hayer P, Gunzel-Apel D, Luerssen D, Hoppen HO: Ultrasonographic monitoring of follicular development, ovulation and the early luteal phase in the bitch. *J Reprod Fertil Suppl*. 1993;47:93–100.

[52] Hermes R, Goritz F, Maltzan J, Blottner S, Proudfoot J, Fritsch G, Fassbeder M, Quest M, Hildebrandt TB: Establishment of assisted reproduction technologies in female and male African wild dogs (*Lycaon pictus*). *J Reprod Fertil Suppl*. 2001;57:315–321.

[53] Bush M, Wildt DE, Kennedy S, Seager SW: Laparoscopy in zoological medicine. *JAVMA* 1978;173:1081–1087.

[54] Farstad W, Hyttel P, Hafne AL, Nielsen J: Maturation and fertilization of blue fox (*Alopex lagopus*) oocytes in vitro. *J Reprod Fertil Suppl*. 2001;57:161–165.

[55] Swanson WF, Howard JG, Roth TL, Brown JL, Alvarado T, Burton M, Starnes D, Wildt DE: Responsiveness of ovaries to exogenous gonadotrophins and laparoscopic artificial insemination with frozen-thawed spermatozoa in ocelots (Felis pardalis). *J Reprod Fertil*. 1996;106:87–94.

[56] Jewgenow S, Blottner S, Lengwinat T, Meyer HHD: New methods for gamete rescue from gonads of non-domestic felids. *J Reprod Fertil Suppl*. 1997;51:33–39.

[57] Pope CE, Keller GL, Dresser BL: In vitro fertilization in domestic and non-domestic cats including sequences of early nuclear events, developments in vitro, cryopreservation and successful intra- and interspecies embryo transfer. *J Reprod Fertil Suppl*. 1993;47:189–201.

[58] Gjorret JG, Crichton EG, Loskutoff NM, Armstrong DL, Hyttel P: Ultrastructure of oocyte maturation, fertilization, and early embryo development in vitro in the Siberian tiger (Panthera tigris altaica). *Mol Reprod Dev*. 2002;63:79–88.

[59] Aman RR, Parks EJ: Effects of cooling and rewarming of the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. *Biol Reprod*. 1994;50:103–110.

[60] Rodrigues FHG: Biologia e conservação do lobo guará na estação ecológica de Águas Emendadas, DF. Thesis, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, Brazil 2002;96.

[61] Herrick JR, Swanson WF: Gonadotropin exposure, salt storage and storage duration affect penetration of domestic cat oocytes by homologous spermatozoa. *Theriogenology* 2003;59:1503–1513.

[62] Mastromonaco GF, Hay MA, Goodrowe KL:The effect of oocyte storage and cummulus cell presence on canine zone penetration by domestic dog spermatozoa. *Theriogenology*. 2002;57:1123–1134.

[63] Carrol J, Gosden RG: Transplantation of frozen-thawed mouse primordial follicles. *Hum Reprod*. 1993;8:1163–1167.

[64] Vicent C, Garnier V, Heyman Y, Renard JP: Solvent effects of cytoskeletal organization and in vivo after freezing rabbit oocytes. *J Reprod Fertil*. 1989;87:809–820.

[65] Fuku E, Kojima T, Shioya Y, Marcus GJ, Downey BR: In vitro fertilization and development of frozen-thawed bovine oocytes. *Cryobiology* 1992;29:485–492.

[66] Luvoni GC, Pellizzari P: Embryo development in vitro of cat oocytes cryopreserved at different maturation stages. Theriogenology 2000;53:1529–1540.

[67] Figueiredo JR, Rodrigues APR, Amorim CA: Manipulação de oócitos inclusos em fol´ıculos ovarianos. In: Gonsalves, PBD, Figueiredo JR, Freitas, VJF (eds.), *Biotécnicas Aplicadas à Reprodução Animal*. Varella, São Paulo, 2002;227–260.

[68] Jewgenow K, Pitra C: Hormone-controlled culture of secondary follicles of domestic cats. *Theriogenology*. 1993;39:527–535.

[69] Jewgenow K, Stolt M: Isolation of preantral follicles from non-domestic cats-viability and ultrastructural investigations. *Anim Reprod Sci*. 1996;44:183–193.

[70] Jewgenow S, Penfold LM, Meyer HH, Wildt DE: Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation. *J Reprod Fertil*. 1998;112:39–47.

[71] Bert P: De la greffe animale. Thesis Medicale, Paris, France.1863.

[72] Carrel A, Guthrie CC: Technique de la transplantation homoplastique de l'ovarie. *C.R. Séances Soc* Brinster RL, Zimmermann JW: Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA*. 1994;91:11298–11302. *Biol*. 1906;6:466–468.

[73] Salle B, Demirci B, Franck M, Rudigoz RC, Guerin JF, Lornage J: Normal pregnancies and live birth after frozen-thawed hemi-ovaries autograft in ewes. *Fertil Steril*. 2002;77:403–408.

[74] Shaw JM, Oranratnachai JM, Trounson AO: Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology*. 2000;53:59–72.

[75] Bogliolo GBG, Pereira FEL, Vitela JEH, Bambirra EA, Barbosa AJA: Degenerações. In: Brasileiro GF, Pereira FEL, Pittela JEH, Bambirra EA, Barbosa AJA (eds.), *Patologia Geral*. Guanabara-Koogan, Rio de Janeiro. 1993; 51–59.

[76] Gosden RG, Boulton M, Grant K, Webb R: Follicular development from ovarian xenografts in SCID mice. *J Reprod Fertil*. 1994;101:619–623.

[77] Bosch P, Hernandez-Fonseca HJ, Miller DM, Wininger JD, Massey JB, Bracket BG: Cryopreservation and development of antral follicles in cat ovarian tissue after transplantation into Nod-Scid mice. *Theriogenology*. 2002;57:362.

[78] Ledda S, Leoni G, Bogliolo L, Naitana S: Oocyte cryopreservation and ovarian tissue bank. *Theriogenology*. 2001;55:1359–1371.

[79] Paris MCJ: Xenotransplantation and its potential role in assisted reproduction and animal conservation. In: *Proceedings of the Second International Symposium on Assisted Reproductive Technology for the Conservation and Genetic Management of Wildlife, Nebraska*. 2002;233–246.

[80] Luvoni GC: Gamete cryopreservation in the domestic cat. *Theriogenology*. 2006;66:101–111.

[81] Amstislavsky S, Lindeberg H, Luvoni GC: Reproductive technologies relevant to the genome resource bank in carnivora. *Reprod Domest Anim*. 2012;47:164–175.

[82] Cocchia N, Ciani F, ElRass R, Russo M, Borzacchiello G, Esposito V, Montagnaro S, Avallone L, Tortora G, Lorizio R: Cryopreservation of feline epididymal spermatozoa from dead and alive animals and its use in assisted reproduction. *Zygote*. 2010;18:1–8.

[83] Guraya SS: A histochemical analysis of lipid yolk deposition in the oocytes of cat and dog. *J Exp Zool*. 1965;160:123–136.

[84] Luciano AM, Franciosi F, Lodde1 V, Perazzoli F, Sleza′ kova M′, Modina S: Cryopreservation of immature bovine oocytes to reconstruct artificial gametes by germinal vesicle transplantation. *Reprod Dom Anim*. 2009;44:480–488.

[85] Massip A: Cryopreservation of bovine oocytes: Current status and recent developments. *Reprod Nutr Dev*. 2003;43:325–330.

[86] Papis K, Shimizu M, Izaike Y: Factors affecting the survivability of bovine oocytes vitrified in droplets. *Theriogenology*. 2000;54:651–658.

[87] Van der Elst J: Oocyte freezing: Here to stay? *Hum Reprod*. Update. 2003;9:463–470.

[88] Murakami OM, Karja NWK, Wongsrikeao P, Agung B, Suzuki T: Blastocysts derived from in vitro-fertilized cat oocytes after vitrification and dilution with sucrose. *Cryobiology*. 2004;48:341–348.

[89] Merlo B, Iacono E, Regazzini M, Zambelli D: Cat blastocysts produced in vitro from oocytes vitrified using the cryoloop technique and cryopreserved electroejaculated semen. *Theriogenology*. 2008;70:126–130.

[90] Comizzoli P, Wildt DE, Pukazhenthi BS: In vitro compaction of germinal vesicle chromatin is beneficial to survival of vitrified cat oocytes. *Reprod Domest Anim* (Suppl. 2) 2009;44:269–274.

[91] Arav A, Shehu D, Mattioli M: Osmotic and cytotoxic study of vitrification of immature bovine oocytes. *J Reprod Fertil*. 1993;99:353–358.

[92] Hotamisligil S, Toner M, Powers RD: Changes in membrane integrity, cytoskeletal structure, and developmental potential of murine oocytes after vitrification in ethylene glycol. *Biol Reprod*. 1996;55:161–168.

[93] Vajta G: Vitrification of the oocytes and embryos of domestic animals. *Anim Reprod Sci*. 2000;61:357–364.

[94] Pedro P, Yokoyama E, Zhu SE, Yoshida N, Valdez Jr DM, Tanaka M, Edashige K, Kasai M: Permeability of mouse oocytes and embryos at various developmental stages to five cryoprotectants. *J Reprod Dev*. 2005;51:235–246.

[95] Agca Y, Liu J, Peter AT, Critser ES, Critser JK: Effect of developmental stage on bovine oocyte plasma membrane water and cryoprotectant permeability characteristics. *Mol Reprod Dev*. 1998;49:408–415.

[96] Yamada C, Simoes R, Nicacio AC, Feitosa WB, Ortiz ME, D'A´ vila Assumpcao, Visintin JA: Immature bovine oocyte cryopreservation: Comparison of different associations with ethylene, glycol, glycerol and dimethylsulfoxide. *Anim Reprod Sci* 2007;99:384–388.

[97] N. Cocchia N, Ciani F, ElRass R, Russo M, Borzacchiello G, Esposito V, Montagnaro S, Avallone L, Tortora G, Lorizio R: Immature cat oocyte vitrification in open pulled straws (OPSs) using a cryoprotectant mixture. *Cryobiology*. 2010;60:229–234.

[98] Herrick JR, Bond JB, Magarey GM, Bateman HL, Krisher RL, Dunford SA, et al.: Toward a feline-optimized culture medium: impact of ions, carbohydrates, essential amino acids, vitamins, and serum on development and metabolism of in vitro fertilization derived feline embryos relative to embryos grown in vivo. *Biol Reprod*. 2007;76:858–870.

[99] Gómez MC, Pope CE, Harris RF, Davis A, Mikota S, Dresser BL: Births of kittens produced by intracytoplasmic sperm injection of domestic cat oocytes matured in vitro. *Reprod Fertil Dev*. 2000;12:423–433.

[100] Karja NW, Otoi T, Murakami M, Yuge M, Fahrudin M, Suzuki T: Effect of protein supplementation on development to the hatching and hatched blastocyst stages of cat IVF embryos. *Reprod Fertil Dev*. 2002;14:291–296.

[101] Zambelli D, Cunto M: Transcervical artificial insemination in the cat. *Theriogenology*. 2005;64(3):698–705.

[102] Tanaka A, Takagi Y, Nakagawa K, Fujimoto Y, Hori T, Tsutsui T: Artificial intravaginal insemination using fresh semen in cats. *J Vet Med Sci*. 2000;62(11):1163–1167.

[103] Howard FM: Breaking new ground or just digging a hole? An evaluation of gynecologic operative laparoscopy. *J Gynecol Surg*. 1992;8(3):143–158.

[104] Zambelli D, Buccioli M, Castagnetti C, Belluzzi S: Vaginal and cervical anatomic modifications during the oestrus cycle in relation to transcervical catheterization in the domestic cat. *Reprod Domest Anim*. 2004;39(2):76–80.

[105] Kraemer DC, Flow BL, Schriver MD, Kinney GM, Pennycook JW: Embryo transfer in the nonhuman primate, feline and canine. *Theriogenology*. 1979;11:51–62.

[106] Pope CE, Keller GL, Dresser BL: In vitro fertilization in domestic and nondomestic cats including sequences of early nuclear events, in vitro development, cryopreservation and successful intra- and interspecies embryo transfer. *J Reprod Fertil Suppl*. 1993;47:189–201.

[107] Pope CE, McRae MA, Plair BL, Keller GL, Dresser BL : Successful in vitro and in vivo development of in vitro-fertilized two- to four-cell cat embryos following cryopreservation, culture and transfer. *Theriogenology*. 1994;42:513–525.

[108] Kanda M, Miyazaki T, Kanda M, Nakao H, Tsutsui T: Development of in vitro-fertilized feline embryos in a modified Earle's balanced salt solution: Influence of protein supplements and culture dishes on fertilization success and blastocyst formation. *J Vet Med Sci*. 1998;60:423–431.

[109] Denker HW, Eng LA, Mootz U, Hamner CE: Studies on the early development and implantation in the cat: I. cleavage and blastocyst formation. *Anat Anz*. 1978;144:457–468.

[110] Swanson WF, Roth TL, Wildt DE: In vivo embryogenesis, embryo migration, and embryonic mortality in the domestic cat. *Biol Reprod*. 1994;51:452–464.

[111] Kanda M, Oikawa H, Nakao H, Tsutsui T: Early embryonic development in vitro and embryo transfer in the cat. *J Vet Med Sci* 1995;57:641–646.

[112] Kanda M, Miyazaki T, Kanda M, Nakao H, Tsutsui T: Development of in vitro-fertilized feline embryos in a modified Earle's balanced salt solution: influence of protein supplements and culture dishes on fertilization success and blastocyst formation. *J Vet Med Sci*. 1998;60:423–431.

[113] Pope CE, Gomez MC, King AL, Harris RF, Dresser BL: Embryos produced in vitro after recovery of oocytes from cat ovaries stored at 4 °C for 24 to 28 hours retain the competence to develop into live kittens after transfer to recipients. *Theriogenology*. 2003;59:308.

[114] Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M: A cat cloned by nuclear transplantation. *Nature*. 2002;415(6874):859. Epub 2002 Feb 14.

[115] Yin XJ, Lee Y, Lee H, Kim N, Kim L, Shin H, Kong I: In vitro production and initiation of pregnancies in inter-genus nuclear transfer embryos derived from leopard cat (Prionailurus bengalensis) nuclei fused with domestic cat (Felis silverstris catus) enucleated oocytes. *Theriogenology*. 2006;66(2):275–282. Epub 2005 Dec 27.

[116] Wildt DE, Kinney GM, Seager SWJ: Laparoscopy for direct observation of internal organs in the domestic cat and dog. *Am J Vet Res*. 1977;38:1429–1432.

[117] Conforti VA, Bateman HL, Vick MM, Lyons LA, Grahn RA, Deddens JA, Swanson WF : Improved fertilization success using laparoscopic oviductal artificial insemination with low sperm numbers in domestic cats. *Proc Soc Study Reprod*. 2011:40.

[118] Lambo C, Grahn RA, Lyons LA, Bateman HL, Newsom J, Swanson WF: Comparative fertility of freshly-collected versus frozen-thawed semen with laparoscopic oviductal artificial insemination in the domestic cat. International Symposium on Canine and Feline Reproduction. 2012.

[119] Swanson WF: Laparoscopic oviductal embryo transfer and artificial insemination in felids – challenges, strategies and successes Reprod Domest Anim. 2012;47(6):136–140.