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The potential for gamete recovery from non-domestic canids and felids

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Abstract

Species are becoming extinct at a rate 100 times the natural background rates. Considering all mammalian orders, 24% of all Carnivora species are threatened. The goal of carnivore conservation is to reverse the decline in populations and to secure remaining populations in ways that will assure enduring public support. In this context, biotechnology is a tool with tremendous potential for assisting the conservation of endangered canid and felid species. As the first step for biotechnology development is the gamete obtainment, this review will discuss the potential of gamete recovery from non-domestic canids and felids, based on learning how to apply these procedures in the domestic carnivores. Thus, electroejaculation and obtaining both epididymal spermatozoon and spermatogonial germ cells are indicated as techniques for male gametes recovery. In the female gametes retrieval, different methods for oocyte recovery from both antral and preantral follicles, and the possibility for ovarian tissue transplantation are discussed. Furthermore, the study discusses the responsibilities involved in the use of assisted reproduction in endangered species conservation. © 2003 Elsevier B.V. All rights reserved.

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1. Introduction

The constant search for widening territory has led to the destruction and fragmentation of ecosystems. As a consequence, some species have disappeared while some forest islands have been formed. These islands isolate populations and, consequently, prevent the

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interchange of genetic information, which can lead to the decrease of genetic variability of the involved populations (O'Brien et al., 1985; Roelke et al., 1993). Furthermore, a large number of animals are suffering due to the transformation of their habitats into agricultural centers. It is then that humans systematically eliminate species because they are causing economic damage to farmers. Another consequence is that a large number of animals have been victims of traffic accidents on roads, increasing the number of convalescent or dead animals (Rodrigues, 2002). Because a great part of these animals belong to endangered species, the loss of a single individual could be deleterious for the maintenance of a species.

Species are becoming extinct at a rate 100 times the natural background rates. With only around 5% of the land surface of the planet protected in some way, continued habitat loss will produce much greater extinction rates, with a potential disappearance of up to half of the world's species. Of all mammalian orders, 24% of all Carnivora species are threatened (Gittleman et al., 2001). Carnivores represent extremes of problems in conservation biology. In one lineage, some canid species such as the red fox (*Vulpes vulpes*) have virtually no risk of extinction—indeed, many now consider them as pests in our largest cities—while other canids such as the Ethiopian wolf (*Canis simensis*) are rapidly proceeding toward inevitable extinction (Purvis et al., 2001). Moreover, among the 37 felid species, only the domestic cat (*Felis catus*) is not in risk of extinction (Pope et al., 1993).

Otherwise, there are many questions concerning the conservation of non-domestic carnivores. Gittleman et al. (2001) classify carnivores as “umbrella species”, since they are predators and require large areas to compound their territory. Thus, if carnivores are conserved, a large number of species and ecosystems will be protected also. Carnivores are also classified as indicator species—those that reflect critical environmental damage; keystone species—those that play a pivotal role in ecosystems; flagship species—popular species that attract much attention; and vulnerable species—species most likely to become extinct.

The goal of carnivore conservation is to reverse declines in populations and to secure remaining populations in ways that will gain enduring public support. In this context, biotechnology has a tremendous potential as a tool for assisting conservation of endangered canids and felids (Wildt, 1997; Bainbridge and Jabbour, 1998; Howard, 1999). Goodrowe et al. (2000) suggest that the Carnivora order has numerous representatives that are imperiled and could benefit from reproductive biotechnology. Due to the phylogenetic similarities between the domestic and non-domestic carnivorous species, the first are used as an experimental model for the others, mainly because of the lack of availability of non-domestic carnivores for use in experiments (Pope et al., 1993). Because the first step for biotechnology development is gamete obtainment, this review will discuss the potential of gamete recovery from non-domestic canids and felids, based on learning how to apply these procedures in the domestic carnivores.

2. Male gamete recovery

2.1. Semen characteristics and storage

Semen is a cellular suspension containing spermatozoa and secretions originating from accessory organs of the masculine reproductive tract. In addition, semen technology refers

to the various forms of sperm quality maintenance, including expansion of the seminal volume for fresh use and storage by cooling and cryopreservation. Cryopreservation is an important tool for the formation of cryobanks, as it maintains the sperm viability for an indefinite time. Cryobanks can serve as a source of genetic material to be used through utilizing the reproductive biotechniques in conservation of the non-domestic carnivores. Thus, particular attention will be given to the methods of gamete recovery from canids and felids.

2.2. Electroejaculation

Several methods are reported for semen collection in animals, as the use of an artificial vagina (Jalkanen, 1993), digital masturbation of the penile bulb (Forsberg et al., 1989) and electroejaculation (Watson, 1978; Wildt et al., 1983). With wild carnivores, electroejaculation is the method of choice due to the difficulty and risks involved in handling these animals. Electroejaculation would facilitate the gamete recovery particularly from convalescent animals, allowing sperm preservation for an indefinite period of time followed by the application of several other biotechniques such as artificial insemination and in vitro embryo production.

For its execution, anesthesia using the tiletamine-zolazepan or ketamine-xilazine in proportions that vary with the species is needed. However, some drugs seem to interfere with the sperm quality, such as promazine, which allows urine contamination (Morato and Barnabé, 1998). Furthermore, it is important to be aware of the risks involved in the anesthetic procedure. According to Osofsky et al. (1996), the factors that must be considered in selecting a drug include efficiency, safeness, existence of an antagonist, and availability and cost of the drug.

Electroejaculation is based on the controlled electric stimulation of the ejaculatory reflex, through the introduction of a trans-rectal probe with three electrodes, connected to an electric stimulator. The probe is inserted 7–9 cm into the rectum with the electrodes directed ventrally. Care should be taken to evacuate any feces from the rectum. A weak electric current stimulates the nerves supplying the reproductive organs (Platz and Seager, 1978). Different EEJ protocols have been reported but many researchers are using the Wildt et al. protocol (1983). The authors reported a total of 80 electric stimulations divided in three series: 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 pre-warmed test tube that has been placed over the glans penis.

Using electroejaculation, Howard et al. (1984) reported semen collection from more than 28 cat species. Moreover, some researchers have reported successful semen collection from wild felids by using electroejaculation, such as tigers (*Panthera tigris*—Donoghue et al., 1992), snow leopards (*Panthera uncia*—Roth et al., 1994), Indian leopards (*Panthera*

pardus—Jayaprakash et al., 2001), caracals (*Caracal caracal*—Pope et al., 2001), jaguars (*Panthera onca*—Morato et al., 2001; Silva et al., 2003), ocelots (*Leopardus pardalis*—Queiroz et al., 2002), margays (*L. wiedii*) and tigrinas (*L. tigrinus*—Morais et al., 2002).

Graham et al. (1978) reported the use of electroejaculation to collect semen from non-domestic felids. The authors cryopreserved the samples and found that 25 to 50% sperm motility was preserved after thawing in lions (*Panthera leo*), jaguars (*P. onca*), leopards (*Neofelis nebulosa*), cheetahs (*A. jubatus*) and leopard cats (*Felis bengalensis*), and in the latter a 70% sperm motility was maintained. The same authors reported finding lesser values of sperm motility, between 1 and 20% post-thaw, for Indian tigers (*P. tigris*), Geoffroy's cats (*Felis geoffroy*) and ocelots (*Felis pardalis*), but the spermatozoa from gold cats (*Felis aurata*) did not survive cryopreservation. Furthermore, using electroejaculation to collect the semen, Byers et al. (1989) obtained 40% sperm motility post-thaw in Siberian tigers (*P. tigris*).

In canids, Goodrowe et al. (1998) collected semen by electroejaculation from the red wolves (*Canis rufus*) and obtained semen of acceptable quality. They followed the cryopreservation technique commonly used for the domestic dog (*Canis familiaris*), which results in significant damages to wolf spermatozoa, suggesting that further studies on cryoinjury in these species must be performed. Afterwards, Goodrowe et al. (2001) collected semen from 11 adult male red wolves by the same method and used cryopreservation. After thawing, spermatozoa motility was 23.7% and the fertilizing capacity was examined by using a penetration assay in domestic dogs oocytes, with a 18.1% penetration rate. Moreover, Leibo and Songsasen (2002) reported the collection of semen from Mexican gray wolves (*Canis lupus*), followed by cryopreservation. It appeared as if the gray wolf spermatozoa were also sensitive to freezing and thawing, as only a very low percentage of spermatozoa survived the freezing process.

Hermes et al. (2001) collected semen from African wild dogs (*Lycaon pictus*) by electroejaculation and performed cryopreservation. These authors found 40% of motile spermatozoa post-thaw and suggested cryopreservation could be used in conservation efforts for gamete rescue of genetically distinct populations threatened by extinction. Recently, successful semen collection in maned wolves (*Crysocyon brachyurus*) has been performed by electroejaculation (Wildt, unpublished; Songsasen, personal communication). However, in some occasions, urine contamination is a barrier to obtaining good sperm quality (Morato, personal communication).

The blue (*Alopex lagopus*) and silver (*V. vulpes*) foxes are commercially valuable canids and are both farmed for their pelts. In these species, semen collection is usually performed by using an artificial vagina (Jalkanen, 1993) or digital masturbation (Forsberg et al., 1989). However, Barta and Jakubicka (1989) reported semen collection from foxes by electroejaculation under halothane anesthesia, which suggests the possibility of using this technique in those canids.

2.3. Collection of epididymal spermatozoa

The epididymus is a component of the male reproductive tract and is attached to the testicle. One of its main functions is the storage of spermatozoa for ejaculation. Present technologies allow for semen collection directly from the epididymus and this seems to

be a viable alternative method for obtaining gametes from animals unable to ejaculate or from animals that have recently died. Furthermore, Garde et al. (1998) suggested that viable epididymal spermatozoa from Iberian deers (*Cervus elaphus*) could be collected in the 10–20 h post mortem period. However, it must be noted that this period would vary according to the weather and temperature conditions where the procedure is being executed.

In studies accomplished with epididymal spermatozoa from domestic cats, it was verified that these spermatozoa require less capacitating time as compared with ejaculated spermatozoa and are able to penetrate feline oocytes 20 min after in vitro insemination (Niwa et al., 1985; Goodrowe and Hay, 1993). Fresh feline epididymal spermatozoa were able to fertilize oocytes in vitro, promoting 40.7% cleavage rate. After freezing, a 26% cleavage rate was obtained (Lengwinat and Blottner, 1994). Bogliolo et al. (2001) reported that after intracytoplasmic sperm injection using feline frozen epididymal spermatozoa, resulted in 34.9% embryos that developed to the morula stage, indicating that spermatozoa with minimal motility could be used in reproductive biotechniques. Moreover, Tsutsui et al. (2002) performed the unilateral intrauterine artificial insemination with frozen-thawed epididymal semen from cats and obtained a 23% (3/11) conception rate.

For non-domestic cats, Jewgenow et al. (1997) demonstrated that spermatozoa were collected from the finely minced cauda epididymus of lions (*P. leo*), tigers (*P. tigris*), leopards (*P. pardus*), pumas (*F. concolor*) and jaguars (*P. onca*). The samples were treated as described by Lengwinat and Blottner (1994), washing the spermatozoa in Hank's balanced salt solution and extended in medium M199 supplemented with 2.5 mmol/l sodium lactate l^{-1} and 0.4% bovine serum albumin. Progressively motile spermatozoa varied between 60 and 85% for the various felids. In this same study, the epididymal semen was frozen and motility between 25 and 65% for the different species that were utilized were obtained after thawing. The frozen semen was then submitted to in vitro fertilization and 18.5% 8-cell embryos developed. Similarly, Morato et al. (1999) provided evidence that frozen epididymal spermatozoa from jaguars were able to penetrate heterologous zona-free oocytes.

Unfortunately, there is no information concerning epididymal semen collection from wild canids. However, procedures developed for domestic dogs (*C. familiaris*) could be adapted to wild dogs. Thus, Yu and Leibo (2002) obtained canine testes by orchiectomy, followed by dissection of the epididymus, which was kept under refrigeration before the isolation of epididymal sperm. These authors found that epididymal spermatozoa could be stored at 4 °C for 8 days, while still maintaining motility, intact morphology and the ability to bind to oocytes. Sirivaidyapong (2002) reported that the sperm collection technique could influence post-thaw results suggesting that canine epididymal spermatozoa should be collected by inserting a fine needle in the vas deferens, injecting bovine fetal serum, TCM 199 or Tris-buffer extender. This procedure facilitates washing the epididymal tail with consequent collection of spermatozoa of improved quality. Hewitt et al. (2001) observed that the conventional cryopreservation method for ejaculated canine semen could be adopted for canine epididymal spermatozoa. In addition, Fahrning (2003) reported collection of canine epididymal semen 20 h after orchiechotomy if the testes were kept at 15–25 °C. Epididymal semen was cryopreserved in pellets and had 1–54% sperm motility following thawing.

2.4. Obtaining spermatogonial stem cells

Spermatogenesis is a complex and very efficient process that starts with the division and differentiation of spermatogonial stem cells located in the basal membrane of seminiferous tubules sustained by Sertoli cells (Schlatt, 2002). Mammals have unique spermatogonial stem cells; because they can maintain their proliferation in adults so genetic material can be passed to subsequent generations. Consequently, these cells are a valuable source for biological experimentation, medical research, agricultural biotechnology and genetic modification of the species (Brinster and Nagano, 1998). 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 recently collected testis consists on elutriation (Bucci et al., 1986) or sedimentation rate in a gradient of bovine serum albumin under gravity force action (Bellvé et al., 1977). Some other isolation techniques have been proposed as immunological markers for posterior magnetic cellular separation (Shinohara et al., 1999). After collection, germ cells can remain for several months in tissue culture media, only resuming spermatogenesis after in an environment that provides favorable conditions for their expansion and differentiation (Nagano et al., 1998). Such favorable conditions are generally provided by transplant to other organisms (Brinster and Zimmermann, 1994).

Brinster and Zimmermann (1994) described the first success in the spermatogonial transplant. They demonstrated that the microinjection of a mouse testis cell heterogeneous suspension into the seminiferous tubules of a recipient mouse, previously confirmed to be sterile, could result in spermatogenesis in the injected animal. After this finding, several other researchers began studies in this area, showing possibilities such as the spermatogonial culture among different species, i.e. the xenograft (Clouthier et al., 1996). Cryopreservation of testis cell suspensions seems to hold the greatest promise for the storage of germ cells to be used later in transplants, as Avarbock et al. (1996) showed that spermatogenesis can continue after cryopreservation.

In spite of the progress in this area, some factors remain to be controlled, such as the ideal number of germ cells to be transplanted, formation of antibodies against spermatogonial cells by the recipient (Ogawa et al., 1999), and poor quality of cells that have developed using these procedures (Russell and Brinster, 1996). There is also a problem concerning xenograft related to the different time of spermatogenesis in each species (França et al., 1998). However, Honaramooz et al. (2002) recently observed complete spermatogenesis after transplantation of testicular tissue fragments from species that are phylogenetically more distant, such as pigs and goats into castrated immunodeficient mice.

As for carnivores, Dobrinsky et al. (1999) were the first to report spermatogonial transplantation from domestic dogs to mice. These authors achieved the dissociation of seminiferous tubules by enzyme digestion with collagenase, DNase and trypsin in pieces of testis from mongrel dogs. Viable spermatogonial germ cells were selected through Trypan blue exclusion. Some seminiferous tubules were cryopreserved using dimethylsulfoxide as cryoprotectant. Fresh or frozen canine testicular tissue was transplanted to several immunodeficient infertile mice and success was determined by immunohistochemistry, which detected the presence of dividing canine germ cells in the recipient mice. Although countless

divisions and cellular transformations have been observed, the development of such cells to spermatozoa was not verified.

By using molecular biology techniques and genetics, testicular activity is better understood and provides important data for the development of germ cell recovery methods. Phenomena involved in spermatogenesis are studied by analyzing spermatozoa through flow cytometry (Jewgenow et al., 1997). As relationships between spermatogenic activity and age, season or the inbreeding among non-domestic carnivores are not known, the information obtained with the study of the testicular activity will be very helpful in the future application of reproductive biotechniques in programs of animal conservation.

3. Female gamete recovery

3.1. Ovarian follicles and oocytes

The follicle is the ovarian morphofunctional unit constituted by the oocyte surrounded by somatic cells. When performing its gametogenic function, the follicle is an essential element for maintenance of oocyte viability, seeking to assure growth and maturation of immature oocyte and, finally, to release a mature oocyte by the ovulation process (Figueiredo et al., 2002). It is known that the ovarian follicular population seems to be made up of thousands of follicles in different mammalian females (Driancourt, 1991). Thus, oocyte retrieval represents a rich source of genetic material to be used as a foundation for genetic banks, aiding in endangered species preservation, mainly in relation to the possibility of collecting material originated from post mortem or convalescent animals.

Donoghue et al. (1990), Wildt (1991) and Luvoni and Oliva (1993) suggested that the development of efficient methods for in vitro maturation (IVM) or fertilization (IVF) of oocytes collected post mortem or through ovariectomy is important to prevent the species extinction. Thus, IVM and IVF techniques are adapted for several non-domestic animals (Johnston et al., 1991; Pope et al., 1993), based on systematic studies in domestic animals (Wood et al., 1995; Wolfe and Wildt, 1996), including wild carnivores. Moreover, Rodrigues et al. (2001) suggest that application of oocyte and ovary tissue cryopreservation will help in the conservation of several animal species, with the objective of maintaining biodiversity.

3.2. Recovery of oocytes included in antral follicles

According to Hildebrandt et al. (2000), ultrasonographic images of the reproductive tract offers new opportunities for induction of sexual cycles and ovulation, adoption of super-ovulating regimens, contraception programs, semen collection and techniques of sperm extraction directly from the testicle, as well as the ovum pick up application.

Pieterse et al. (1988) were the first to describe the ovum pick up technique, which consists on the insertion of an ultrasonographic probe in the female vagina or rectum. 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 oocytes could be used in IVM and IVF (Nibart et al., 1997). This technique is extensively used for oocyte collection

in cattle and results indicate the possibility of repeated collections in both pregnant and non-pregnant females (Lacaze et al., 1997; Guyader-Joly et al., 1997).

Concerning carnivorous species, ovum pick up 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 (Silva et al., 1996). 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 (Hayer et al., 1993). Hermes et al. (2001) were thus able to visualize the follicles and corpora lutea in ovaries of female African wild dogs (*L. pictus*) by transrectal ultrasonography, suggesting the possibility of oocyte puncture in carnivores. Researchers could be underestimating the potential of ultrasonography for assisted reproduction in endangered canids and felids species. The adaptation of this technique would be an important alternative, because it is a non-invasive procedure and it could allow oocyte collection without the risks involved with surgical procedures.

Another possibility for oocytes retrieval is laparoscopy. According to Bush et al. (1978), laparoscopy was effective in the evaluation of reproductive status, particularly ovarian anatomy and function, direct visual biopsy of internal organs, and as a surgical means of fertility control. Laparoscopy is a minimally invasive procedure commonly used for intrauterine deposition of frozen-thawed semen in domestic dogs (Silva et al., 1995) and cats (Farstad, 2000).

For domestic cats, Goodrowe et al. (1988) reported the laparoscopic collection of oocytes, which were inseminated in vitro with ejaculated semen. 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. In wild felids, Swanson et al. (1996) reported the visualization of changes in the reproductive tract during ovarian stimulation with exogenous gonadotropins in the ocelot (*F. pardalis*) by laparoscopy. Moreover, Pope et al. (2001) reported that multiple laparoscopic oocyte retrievals could be successfully performed in caracal (*C. caracal*) after repeated ovarian stimulation with equine (eCG) and human (hCG) chorionic gonadotropin. Embryos could also be reliably produced in vitro using cryopreserved spermatozoa and live offspring could be produced after embryo transfer.

Unfortunately, in addition to the individual variability in ovarian response universally observed following gonadotropic hormone administration, there are huge species-specific differences in sensitivity and the optimal dosage determination is not simply a matter of extrapolation (Pope, 2000). 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 (Swanson et al., 1995). Similarly, protocols using porcine FSH and LH resulted in reduced numbers of follicles at the second treatment as compared with the first (Morato et al., in press), possibly due to a humoral immune response. By considering the feasibility of fecal steroid analyses with radioimmunoassay (Brown et al., 1994) 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.

In addition, Jewgenow et al. (1997) 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 8 h after the death of these animals, followed by mechanical follicle isolation. By performing this procedure, the authors obtained 16 ± 2 cumulus–oocyte complexes with acceptable quality, which were submitted to IVM and IVF using homologous semen or heterologous epididymal domestic cat semen. The most desirable results were obtained with lion oocytes fertilized by lion sperm, with a 31.6% (18/44) conception rate. This research also provides evidence that leopard oocytes can be fertilized by domestic cat sperm and used in in vitro fertilization 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.

Pope et al. (1993) performed oocyte collection from domestic and non-domestic cats by laparotomy and posterior ovary dissection. These oocytes were submitted to IVF and then transferred to receiving females. 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. Afterwards, Pope et al. (1997) recovered ovaries from domestic cats by ovariectomy and demonstrated that morphology of the oocyte ooplasm can affect in vitro maturation, as well as the gonadotropin supplementation. According to the morphological aspect by stereomicroscopical 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 (Gjorret et al., 2002).

Domestic bitches have ovulations of an immature oocyte in a germinal vesicle stage and they require around 2 days meiotic maturation to be complete (Silva et al., 2002). Hay et al. (1997) reported that canine oocytes could be collected by ovariectomy and be immediately used in IVF, or after freezing at -18°C . However, as for laparotomy, this collection technique is an invasive procedure and should be considered as the last alternative for convalescent animals due to the risks and stress it causes to the animals. Moreover, Rodrigues and Rodrigues (2003) reported that oocyte quality is a more reliable indicator of its potential for meiotic maturation in vitro as compared with the hormonal environment of the donor female at the time of oocytes retrieval, as the in vitro nuclear maturation of dog oocytes is not influenced by the in vitro reproductive status of the female.

The use of IVF in canine species was not particularly successful in facilitating reproduction. Metcalf (1999) supplemented the culture medium with gonadotropins and observed its beneficial effect on the nuclear maturation of canine oocytes. Furthermore, the formation of canine 8-cell embryos was obtained after 96 h of IVF and culture, representing 2.4% of the total oocytes fertilized in vitro. Otoi et al. (2000) reported 13% in vitro fertilized canine oocytes, but only 0.5% oocytes used in the experiment reached the phase of initial blastocyst. According to these authors, canine oocytes could be developed to the blastocyst stage after IVM, IVF and culture, although the competence of the oocyte developing is significantly reduced after these procedures.

Unfortunately, oocyte recovery studies for wild canids are rare. Farstad et al. (1993) collected the ovaries from six blue foxes (*A. lagopus*) immediately after their death. They performed oocyte isolation and selection by stereomicroscopy. This study provided evidence

that vulpine oocytes could be fertilized and reach the initial embryonic development stage, because 5 of 13 ova had cleaved into the 2-cell stage at 24 h after insemination, but only one developed into a morula by 144 h. Afterwards, Farstad et al. (2001) collected ovaries from seven blue foxes after ovulation from the dominant follicles and obtained the oocytes by blind aspiration from subordinate antral follicles. MIV and FIV were performed and 75 of 125 oocytes (60%) underwent germinal vesicle breakdown within 48 h after insemination, suggesting that immature oocytes from subordinate follicles are able to mature and be fertilized.

Oocytes can be preserved if they are not immediately submitted to IVF. However, Aman and Parks (1994) observed that cooling could cause chromosomal anomalies in mature oocytes, as a consequence of the temperature decrease on meiotic fusion. Rodrigues et al. (2002) 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, Herrick and Swanson (2003) 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 reduced sperm penetration. For canine species, Mastromonaco et al. (2002) found that oocyte storage in hypertonic salt solution damages the zona *pellucidae*, reducing the sperm penetration rates. These authors also observed that the integrity of cumulus cells is necessary for an enhanced interaction between the oocytes and the canine spermatozoa.

It has already been demonstrated that the cryopreservation of oocytes originating from antral follicles is successful in mice (Carrol and Gosden, 1993), rabbits (Vicent et al., 1989) and bovine (Fuku et al., 1992), followed by IVF after thaw, resulting in the birth of normal offspring in all of these species. In domestic felines, Luvoni and Pellizzari (2000) demonstrated that the mature oocyte could be cryopreserved and, soon after, fertilized in vitro with success.

3.3. Recovery of preantral follicles

Although the maturation of oocyte recovered from antral follicles is an efficient method for the use of haploid female material, it may be possible to also increase the efficiency of the oocyte utilization by activation in initial phases of development (Jewgenow et al., 1997). The preantral ovarian follicles (PAF) represent 90% of the follicular population in mammals (Figueiredo et al., 2002). Small PAF recovered from the ovaries collected from post mortem animals or through ovariectomy, therefore, are a rich oocyte source, because they can mature in vitro.

In canids, PAF retrieval studies are extremely scarce. Telfer and Gosden (1987) observed that the domestic bitch present a great number of PAF containing several oocytes. Durrant et al. (1998) accomplished canine PAF isolation and characterization, and suggested the use of the enzymatic digestion associated to the mechanical isolation. Bolamba et al. (1998) verified that after the enzyme method that uses collagenase and DNase for the isolation, PAF could be cultivated in vitro and oocytes would be capable of reinitiate the meiotic divisions.

Jewgenow and Pitra (1993) reported that feline PAF are capable of developing in vitro to the antral phase. Moreover, Jewgenow and Goritz (1995) demonstrated the isolation of PAF

from domestic cats by mechanical ovary dissection. By adapting the methods described for domestic cats to non-domestic felid species, Jewgenow and Stolt (1996) accomplished the isolation and the ultra-structural characterization of PAF from cheetahs, jaguars, lions and Sumatran, Siberian and Bengal tigers that had died at local zoos. These authors verified the similarity among domestic and non-domestic felid PAF. Later on, Jewgenow et al. (1997) recovered 1867 ± 1144 PAF from each ovary from several species of non-domestic felids, observing that the follicle growth is possible in the culture medium for up to 14 days, with a 20% increase (40–50 mm) on the diameter of preantral follicles from 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, as the foundation for germoplasm banks, as Jewgenow et al. (1998) reported that it is possible to maintain the viability of PAF from domestic cats after cryopreservation procedures.

3.4. Ovarian tissue preservation and culture

Bert (1863) was the first to report an ovarian transplantation. A significant impact on the vascular anastomosis techniques of several transplanted organs, including the ovary, was only achieved in the twentieth century, with the studies by Carrel and Guthrie (1906). According to Salle et al. (2002), both whole ovary and ovarian fragment transplantations could be used for ovarian follicle cultures. Moreover, Shaw et al. (2000) 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.

Bogliolo et al. (1993) 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. McCone (1899) was the first to report a canine ovarian tissue allotransplant to the broad ligament of another bitch, which resulted in pregnancy. Metcalf et al. (2001) obtained a canine ovarian tissue xenografting to the renal capsule of several immunodeficient infertile mice. The mice necropsy was done after 56 days, when the follicular development was confirmed, but the antral formation of follicles was not visualized.

Gosden et al. (1994) performed the transplant of ovarian cortex fragments from domestic cats to the renal capsules of severely immunodeficient infertile mice. 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. However, ovulation was not observed in any of the grafts. Furthermore, Bosch et al. (2002) reported that freeze-thawed cat ovarian cortex not only survives xenotransplantation into the kidney capsule from severe combined immunodeficient mice but also contains follicles able to grow to antral stages containing gonadotropin responsive granulosa cells.

According to Ledda et al. (2001), oocytes 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 endangered species (Paris, 2002).

4. Final Considerations

As reviewed, there are several methods available for recovering gametes from non-domestic canids and felids, including healthy, convalescent animals or animals that recently died. However, even if these gametes would be a valuable resource in the formation of cryobanks by contributing to the preservation of genetic material essential for the maintenance of the species, there are many obstacles to overcome, such as the improvement of the cryopreservation protocols for both male and female gametes, hormone supplements in the medium for IVM and IVF, compatible recipients for the embryos produced in laboratory, and suitable habitats to receive the animals that are produced by assisted techniques.

According to Wildt et al. (2001a), effective techniques in domestic species are not necessarily applicable to wild species. Reproductive mechanisms are highly varied among animal groups. Without understanding the fundamentals of reproductive processes, assisted breeding can never become consistently successful. There is no doubt that the reproductive sciences can contribute to carnivore conservation, but the first priority must always be the production of new basic knowledge that eventually may have applications in the management of these animals. Once this information is known, wildlife can be propagated by using improved natural or assisted breeding.

In addition, the use of stored gametes requires further studies. Sub-specific populations can become independent from other populations through natural or historical events. The non-studied introduction of gametes or new individuals into a population can produce undesirable hybrids, which can accelerate the genetic extinction of the native population in just a few years (Wayne and Brown, 2001).

Reproductive science is only one component of an abundantly complex conservation puzzle and can contribute to wildlife conservation, but only in the context of problems being simultaneously addressed by wildlife and habitat management, ecology, population biology, behavior, nutrition, genetics, veterinary medicine, sociology and conflict resolution. Genuine conservation is achieved only when the reproductive knowledge and technologies are integrated into multidisciplinary programs that conserve species integrity *ex situ* and preferably *in situ* (Wildt et al., 2001b).

Currently, several ecosystems are being destroyed by human actions, beginning the process of extinction of several species. Scientific research, however, improves or develops new reproductive biotechniques. Thus, the application of these techniques could minimize the effects of the devastation. Even so, there is no sense in using them just to multiply animals in the laboratory. It is necessary to have a habitat prepared to receive these animals after they are produced. There needs to be an awareness of actions and their consequences by trying to develop ways of expanding the land mass used by humans, but still allowing a peaceful coexistence with all of the ecosystems.

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