

Workshop 4: Reprodução em pequenos animais e animais silvestres

Reproduction in small and wild animals



Conservation of Germplasm from Wild Animals of the Caatinga Biome

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ABSTRACT

Background: The degradation of caatinga requires the development of strategies for its conservation and for the animals that inhabit in it. The need for studies on the conservation of germplasm from those animals led to the creation of the Center of Multiplication of Wild Animals of the Universidade Federal Rural do Semi-Árido (CEMAS/UFERSA). This work presents data regarding the status of the conservation of the caatinga, and the main results related to the acquirement and conservation of germplasm from the animals that live in CEMAS.

Review: The Caatinga biome is one of the most inhospitable landscapes of Brazil, and is considered the unique biome that is exclusively Brazilian due to a series of physical factors. This biome is the most altered by human action, with approximately 45.3% of the areas modified and only 1% protected by conservation units. Despite this, little attention has been given to its conservation and negligence is evident when investments in research on biodiversity and conservation of this biome are examined. The conservation of Caatinga biome is important for the maintenance of regional and global climate, the availability of drinking water, adequate soil for agriculture, and as an important part of the biodiversity of the planet. However, the caatinga remains as one of the lesser-known ecosystems in South America from a scientific point of view, which favors the process of its extinction. In order to preserve the wild species of the Brazilian northeast semi-arid, the Centre of Multiplication of Wild Animals – CEMAS/UFERSA not only promotes research, preservation, and conservation of wild species, but also develops technologies capable of producing animal protein, at low-cost for low-income familiar populations and meets producers interested in the creation of wild animals, using the criterion of sustainability. Nowadays, research projects aiming the obtaining of information regarding reproductive physiology and conservation of male and female germplasm from several species have been conducted. In collared peccaries (*Tayassu tajacu*), it was standardized that semen could be collected by electroejaculation under anesthesia using Propofol, followed by the cryopreservation in Tris supplemented by fructose or glucose and added by egg yolk and glycerol. In the female peccaries, studies on reproductive cycle and protocols for the conservation of ovarian preantral follicles are now being conducted. For the agouti (*Dasyprocta aguti*), a protocol for the obtaining and cryopreservation of epididymal sperm was established and now, we are trying to develop methodologies for their oocyte conservation. For the six-banded armadillos (*Euphractus sexsinctus*) and the coatis (*Nasua nasua*), several studies on semen technology, including protocols for collection and evaluation of sperm physiology, were conducted.

Conclusions: In spite of our efforts to develop strategies for the conservation of animal germplasm, a genuine program of conservation for the Caatinga Biome will only be achieved when the knowledge and reproductive technologies be integrated into multidisciplinary programs for the preservation of the integrity of species *ex situ* and, preferably, *in situ*.

Keywords: Caatinga, endangered species, germplasm conservation.

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I. INTRODUCTION

Studies show that caatinga is, among Brazilian biomes, the one that suffers most alterations by the humans, and the least that is conserved. Given the current situation of environment degradation, it becomes necessary to search for alternatives for the conservation of the remaining natural areas based on the animals that inhabit them.

The creation of germplasm banks is an alternative for the conservation of endangered species. Therefore, information on the conservation status of caatinga biome, as well as some studies on the conservation of germplasm from the animals that compose its fauna is presented in this study.

II. THE CAATINGA BIOME

The Caatinga biome is one of the most inhospitable landscapes of Brazil, and is considered the unique biome that is exclusively Brazilian due to a series of physical factors. It occupies an area of approximately 750,000 km², covering approximately 11% of the national territory, embracing a continuous part of the states of Maranhão, Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia, and parts of the north of Minas Gerais (Brazilian southeast). Its place is in the semi-arid region, which presents high temperature and high rainfall irregularity and the irregularity in space and in time is the primary brand of semiarid climate [13].

This biome is the most altered by human action, with approximately 45.3% of the areas modified and only 1% protected by conservation units. Despite this, little attention has been given to its conservation and negligence is evident when investments in research on biodiversity and conservation of this biome are examined. The caatinga is unaware of strong legal and institutional framework that would promotes its preservation, so it is threatened by extinction due to deforestation [5].

In the caatinga, 187 species of bees [14], 240 of fish [9], 167 of reptiles and amphibians [8], 510 of birds, and 148 species of mammals [6] have already been registered. Several of these species have been hunted by the local population to be used as an alternative source of animal protein due to historical-cultural habits, and due to the great acceptance in the international market for its meat and pelts [2].

The conservation of Caatinga biome is important for the maintenance of regional and global climate, the availability of drinking water, adequate soil for agriculture, and as an important part of the biodiversity of the planet. However, the caatinga remains as one of the lesser-known ecosystems in South America from a scientific point of view, which favors the process of its extinction.

III. THE CENTRE OF MULTIPLICATION OF WILD ANIMALS (CEMAS)

In order to preserve the wild species of the Brazilian northeast semi-arid, the Universidade Federal Rural do Semi-Árido – UFERSA – created the CEMAS with scientific purposes on September 12, 1989, and registered it at the Brazilian Institute for Environment and Natural Resources – IBAMA – with the registry number 12.492-0004. This center not only promotes research, preservation, and conservation of wild species, but also develops technologies

capable of producing animal protein, at low-cost for low-income familiar populations and meets producers interested in the creation of wild animals, using the criterion of sustainability.

The CEMAS occupies an area of 20 ha and is located between geographic coordinates 5°11'S and 37°20'W, at an altitude of around 16 m. It is divided into 11 separate sectors, in which some projects are developed for the conservation of collared peccaries (*Tayassu tajacu*), agouti (*Dasyprocta aguti*), ostrich (*Rhea americana americana*), and honey bees, with pretensions to be expanded to include species such as the six-banded armadillos (*Euphractus sexcinctus*) and coatis (*Nasua nasua*) arising out of the Zoobotanical Park, Onélio Porto, Mossoró-RN, whose activities were recently closed.

IV. CONSERVATION OF MALE GERMLASM

Among the animals that live in CEMAS/UFERSA, collared peccaries deserve more attention because of their ecological importance and the possibility of their rational exploitation on a production system. Therefore, studies have been conducted in order to develop efficient protocols for the semen conservation of this species. Our first challenge was to improve the protocols used for semen obtaining in collared peccaries. Sousa *et al.* [12] showed that the anesthetic, propofol, promotes a better and quicker recovery from the anesthetic plan after the electroejaculation for 5 min and promotes the procurement of ejaculates in 80% of the attempts, while the association acepromazine-tylamine-zolazepam promotes only 40% of efficiency. Recently, Castelo [3] demonstrated that increasing the duration of the electroejaculatory procedure for 10 min promotes the obtaining of an ejaculate presenting higher volume and sperm concentration, without affecting the animal health. This author also verified that the dilution and processing of semen can be performed by using Tri-based extenders supplemented with glucose or fructose; it is also not necessary to centrifuge the samples before freezing, and samples can be thawed at 37 °C/1 min or at 55 °C/7s. However, we emphasize that a sperm motility of only 31% was obtained from that freezing protocol adapted from the swine species. In order to improve the methodology, new studies are being conducted for the determination of the ideal concentration of egg yolk and glycerol in extenders based on Tris and coconut water (ACP®, ACP Biotecnologia, Fortaleza, Brasil), and also, different freezing protocols are being adapted to the collared peccaries' semen.

The agoutis are terrestrial rodents commonly used as a source of alternative protein by local communities. As a way to ensure the maintenance of the species and leverage the genetic material of those newly slaughtered animals, Silva [11] obtained the epididymal spermatozoa from agoutis through retrograde washing and froze it in Tris-based and ACP® extenders, both plus egg yolk (20%) and glycerol (6%), verifying a better efficiency for ACP®, when samples were stored in 0.25 mL plastic straws, and thawed at 37 °C/1 min, instead of 70 °C/8 s.

Coatis (*Nasua nasua*) are carnivores that inhabit the caatinga, and their reproductive physiology is still less known. Barros *et al.* [1] found better efficiency for anesthetic association ketamine-xylazine that promotes the obtaining of ejaculates in 100% of the electroejaculatory attempts, while tylamine-zolazepam was efficient in only 33.3% procedures. In addition, Lima *et al.* [4] described semen characteristics in this species, including the sperm morphometry and ultrastructural appearance. Queiroz *et al.* [7] verified that no correlations exist between testicular biometry and semen characteristics in coatis.

Armadillos (*Euphractus sexcinctus*) are mammals found in caatinga and are hunted by many local communities that use them as food, contributing for the reduction of these individuals in natural environment. Serafim *et al.* [10] demonstrated that semen from armadillos could be obtained in the use of electroejaculation without using anesthesia. These authors, for the first time, described the semen characteristics of the armadillos. Nowadays, studies on semen technology of armadillos are conducted by the adaptation of different tests for the evaluation of seminal quality. Both in armadillos and coatis, these initial studies serve as basis for the future development of protocols for the conservation of their germplasm.

V. CONSERVATION OF FEMALE GERMLASM

Recently, the team of the LCGA/UFERSA, in association with the team of the *Laboratório de Manipulação de Oócitos em Folículos Pré-antrais* – LAMOFOPA – of the *Universidade Estadual do Ceará*, began studies in order to know the reproductive physiology of wild females of caatinga, especially collared peccaries and agouti, and also for the development of protocols for the conservation of their germplasm.

In this sense, a study regarding the reproductive cycle of collared peccaries by hormonal measurements, vaginal cytology, and evaluation of ovarian dynamics by ultrasound is being conducted. Besides, ovaries from collared peccaries and agoutis have been collected for the characterization and estimation of the ovarian follicular population and for the development of protocols for oocyte conservation by cooling and freezing.

VI. CONCLUSION

In addition to the results presented here, other teams have also endeavored to develop strategies for the conservation of wild fauna of caatinga. As an example, the National Centre of Investigations for Conservation of Natural Predators – CENAP/*Instituto Chico Mendes para Conservação da Biodiversidade* – ICMBio recently initiated a project, which aims to preserve the jaguar (*Panthera onca*) in the margins of São Francisco river, gathering information about the ecology of this animal with future prospects of collection and conservation of genetic material of the same (Morato, personal communication, 2010). Furthermore, the National Research Council (CNPq) approved the creation of the Institute for the Research of the Semi-Arid Biodiversity (PPBio-Semi-arid 2010) that gathers 30 research institutions in order to study and preserve the fauna and the flora of the Caatinga Biome.

Regardless of biome, knowledge about the reproduction of fauna is just one component of the complex puzzle of conservation, contributing to the conservation of wild life when incorporated into a context associated with the management, ecology, behavior, nutrition, genetics, and resolution of conflicts inherent to animals and their habitats. In spite of our efforts to develop strategies for the conservation of animal germplasm, a genuine program of conservation for the Caatinga Biome will only be achieved when the knowledge and reproductive technologies be integrated into multidisciplinary programs for the preservation of the integrity of species *ex situ* and, preferably, *in situ*.

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Chemical Castration of Male Dogs... Myth or Reality?

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ABSTRACT

Background: Fertility control of animals by nonsurgical methods has been pursued to prevent unwanted pregnancies and suppress testosterone-dependent traits in farm and companion animals. In the latter, injection of sclerosing agents into the testis had considerable potential as a candidate for chemical sterilization of dogs. This article aims to review the potential of this new non surgical technology for sterilization of male dogs.

Review: Investigations of contraceptive agents for male dogs where initiated only in the last decade. Chemical sterilization is a solution for pet overpopulation and for non surgical methods of male contraception. A variety of compounds have been tested, some of which were either safe but not effective or vice versa. The ideal chemical sterilization needs to meet three key criteria to be regarded as a good alternative to surgical sterilization. First, it has to be effective in a high percentage of treated animals. Secondly, it should have a high margin of safety for treated animals and the environment. Third, it has to be permanent and irreversible following a single treatment. The first product obviously fulfilling these criteria was zinc gluconate.

Conclusion: Intratesticular injection of a zinc gluconate-based solution has great potential as a permanent contraceptive for dogs.

Keywords: Chemical sterilization, zinc, testis, semen, dog

I. INTRODUCTION

II. EFFECTS OF ZINC IN MALE REPRODUCTION

III. ZINC AS A STERILANT AGENT

IV. INTRATESTICULAR INJECTION OF ZINC GLUCONATE

V. CONCLUSIONS

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I. INTRODUCTION

In the last years, methods for prevention or interruption of reproductive cycle have been described for population control of dogs and cats. Fertility control includes surgery, hormonal therapy and, more recently, immunologic and chemistry control [3]. The focus has been the reproductive control of the female and few has been studied about male contraception. The sterilization of a large number of males contributes for the reduction of pregnant females.

Many researches have been developed to make contraceptive methods more accessible for population to reduce the number of dogs that are euthanized every year. Surgery needs time and is more expensive, besides, it is inconvenient for the owner who needs to submit their animal to a surgical procedure. Pharmacological contraception needs continue administration of medicine/treatment once animal does not lose the capability of reproduction, and also needs continue attention of its owner. Less invasive techniques have been studied [7]. However, researches about male sterilization are still limited and the reason is related to the results obtained with major sclerosing agents, which does not result in azoospermia and most cause skin irritation and ulceration of the scrotum [6].

A technology to sterilize dogs or cats needs to meet some key criteria to achieve the same results of surgical sterilization. First it has to be as close to 100 percent effective as possible. Secondly, it is going to be safe not only for the pet but also for the technician or veterinarian who is administering it. Third, it has to be irreversible after one treatment. The first product to fulfill both the safety and effectiveness criteria was zinc gluconate.

II. EFFECTS OF ZINC IN MALE REPRODUCTION

Zinc is essential for the normal growth, reproduction and life expectancy of animals and has a beneficial effect on the process of tissue repair and wound healing. It is considered noncarcinogenic, nonteratogenic and nonmutagenic [13]. The element is an essential component of a number of enzymes present in animal tissues, including alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase, procarboxypeptidase and cytosolic superoxide dismutase [10]. Also, it is an important component of semen and affects sperm motility [10]. In low concentrations, zinc is important for spermatogenesis (it incorporates in the flagellum in late spermatids and is also localized in the outer dense fibers) [6], but in high concentrations it prevents the replication of germ cells and causes fragmentation of cell membrane and nucleus [2,6]. The reproductive and developmental toxicity of zinc has been investigated in several animal studies [5,20].

Adult male Sprague-Dawley rats were exposed to 20mg/kg-day in the diet for three weeks and to 28mg/kg-day for six weeks [20]. Zinc intake significantly affected enzyme activities in tissue of the male reproductive system. Histological examination of the gonads of rats consuming increased levels of zinc for three weeks revealed meiotic arrest at the primary spermatocyte stage, degeneration of secondary spermatocytes, fluid accumulation within the seminiferous tubules, and reduced epithelial cell height in the epididymides. After six weeks of exposure, histological examination of the testes revealed additional evidence of arrested spermatogenesis. The germinal epithelium contained only spermatogonia, one layer of primary spermatocytes and a few pyknotic secondary spermatocytes; Necrotic nuclei were observed among Sertoli cells and Leydig cells. No mature spermatozoa was presented in the cauda epididymides.

In another study, 10 male Sprague-Dawley rats were fed with a diet containing deficient, adequate or excessive amounts of zinc (4, 12, or 500 mg total Zn/kg food) for eight weeks [5]. Flow cytometric data revealed that excess zinc caused abnormalities in the chromosome structure of sperm. The authors suggested that excess zinc, represented by the highest dose group, destabilizes disulfide bonds and complexes with protamine (a basic protein in the sperm) molecules, leading to a destabilization of sperm chromatin quaternary structure and greater susceptibility to DNA denaturation.

Leatham (1970) cited by Kellokumpu and Rajanien [10] suggested that zinc controls androgen metabolism at the cellular level. Supported for such a hypothesis is that 5 α -reductase activities can be inhibited completely by zinc at a concentration of 10^{-4} M and greater, whereas lower concentration of zinc stimulates reduction of testosterone to dihydrotestosterone (DHT). In high concentration zinc inhibits the binding of testosterone to the 5 α -reductase enzyme and DHT production is also inhibited. Besides, zinc inhibits testosterone biosynthesis in the testes by the formation of a receptor-hormone complex with gonadotrophins [11].

The role of zinc was evaluated in the conversion of testosterone into the biologically more potent androgen 17 α -hydroxy-5 α -androstane-3-one (DHT) [12]. In low concentrations, conversion of testosterone to DHT increases but in high concentration the metabolism is inhibited significantly. The decrease is mediated by both a non competitive inhibition of the binding of testosterone to the 5 α -reductase enzyme and by reduction in the formation of the NADPH cofactor produced by a competitive inhibition of both G6P and NADP binding to the G6PD enzyme.

III. ZINC AS A STERILANT AGENT

Zinc is neutral in pH (7.0) and is part of composition of the male reproductive fluid and tissue, and both have the highest concentration of zinc than any other organ in the body [6].

Zinc plays a role in the normal development and maintenance of the immune system, by the fact that immune system cells have high proliferation, and this mineral is involved in traduction, transport and replication of DNA [1]. Direct effect of zinc in immune system is the stimulation of activity of enzymes involved in mitosis, such as DNA and RNA polymerase, timidin kinase, desoxiribonucleotidol terminaltransferase and ornithine descarboxilase [4,8,21]. Zinc can affect the process of phagocyte response of macrophages and polymorphonuclear leucocytes and interfere in cellular lyses mediated by natural killer cells and the cytolytic action of T cells [1].

Exposure to high zinc concentrations increased the number of polymorphonuclear leucocytes, macrophages and all types of lymphocytes [24]. The injury inflicted by intratesticular injection of zinc is thought to be similar to an auto-immune orchitis (due to production of antibodies against testicular antigens). Spermatogenesis is seriously affected, with seminiferous epithelium lesions and destruction of spermatocytes, spermatids and spermatozoa, resulting in sterility [14].

Intratesticular injection of zinc tanate in rats and dogs result in the suppression of spermatogenesis and a significant reduction of serum testosterone concentration to values observed after surgical castration. Also, it reduced weight of the prostate gland in 85%, 24 months after injection [6].

A study conducted by Wang [25] who evaluated the effect of intratesticular injection of a zinc gluconate solution (Neutersol[®]) in dogs from 2 to 10 months of age, revealed aspermia, azoospermia or necrospermia after 60 days post injection in 95% of animals. In a later study, it was showed that plasma testosterone concentration reduced by 41 to 52% in dogs treated with zinc gluconate, however, some animals preserved androgen concentration similar to control group [9,16]. Neutersol[®] became commercially available in 2003 in USA, distribution was halted in 2005 and, in 2008, a Latin American version was launched in Mexico (Esterisol[®], Ark Sciences).

In March 2009 a new injectable sterilant for male dogs was introduced in Brazil (Infertile[®], Bio Pharma). Infertile[®] is similar to Neutersol[®]/ Esterisol[®], but with some differences in formulation. One difference is the addition of DMSO (dimethyl sulfoxide) as a "carrier" to aid in the distribution of the drug within the testicle. Another difference is that Infertile[®] uses approximately two times the concentration of zinc gluconate. Intratesticular injection of Infertile[®] in young adult male dogs does not resulted in azoospermia, although it was observed reduction of motility and sperm concentration 12 months post injection. Histological evaluation of testes revealed testicular degeneration, reduction of the number of germ cells, atrophy, architecture disruption of seminiferous tubules and Sertoly cell injury [23]. About testosterone concentration, it was not evaluated [23].

Since 2003, another product made up of zinc gluconate neutralized by arginine (Testoblock, BioRelease Technologies, Birmingham, AL, USA) has been evaluated for chemical castration of male dogs by intratesticular injection. Its efficacy was evaluated in animals from 8 months to 4 years old [17,18]. The results reported by Oliveira et al [18], in a six months study suggests that zinc based solution (Testoblock) is effective in prevent spermatogenesis. It was observed azoospermia 60 days post injection and histological changes suggested irreversibility. A long duration study has been developed in the Laboratory of Andrology (Androlab) at DMV/UFRPE for the establishment of the long term safety and effectiveness of intratesticular injection of zinc gluconate [15,22].

IV. INTRATESTICULAR INJECTION OF ZINC GLUCONATE

Dogs are premedicated with atropine (0.044 mg/kg, subcutaneous) and xylazine (1.0 mg/kg, intramuscularly). After sedation, they are restrained in a supine position and the scrotum is cleaned with an antiseptic solution (Polyvinylpyrrolidone, 10% Povidone Iodine). Testes are injected using a 0.5 mL U100 insulin syringe with a 28-gauge,

12-mm needle (separate needle for each testis). The injection is given in the cranial area of the testis, lateral to the caput of the epididymides (as close as possible to the ductuli efferentes), with the needle inserted in a parallel plane relative to the testis. Testoblock is given into each testis, in accordance with testicular width (Table 1), which is determined by measuring (with calipers) each testis at its widest point.

Table 1. Volume of zinc-based solution injected into each testis in dogs according to testis width

Testis width(mm)	Volume per(mL)	TestisZinc content (mg)
10-12	0,2	2,6
13-15	0,3	3,9
16-18	0,5	6,6
19-21	0,7	9,2
22-24	0,8	10,5
25-27	1,0	13,1

V. CONCLUSIONS

Each day, new researches have been developed for the improvement of techniques for fertility control. In addition to conventional surgery, there is a new possibility of sterilization, safe and as effectiveness as the classical orchiectomy. Intratesticular injection of zinc gluconate is faster, less complicated, less invasive and more affordable method of sterilization; it can be used in sterilization programs to contribute to the control of zoonoses and reduction of euthanasia. Besides, it can provide to the owner a less invasive technique of sterilization which is in agreement to the animal welfare.

About feline population, this has been growing in the urban centers and occupying its place in the family. This species has been revealing a great importance in social life, including the preservation of mental health of our society, providing mental stability. The evaluation of effectiveness of an intratesticular injection of zinc gluconate based solution in male cats was reported for the first time by Oliveira et al [19], and preliminary data suggests an alternative contraception for this species. Studies have been developed to support this hypothesis.

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Reproductive technology in domestic carnivorous

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ABSTRACT

Background: The delay in development of artificial reproduction techniques on carnivorous could be due to countless reasons, but the lack of commercial interest is probably the most important one. The majority of canines are small structures, canidae are extremely fertile and a great number of species are adapted to domestication or captivity. Finally, the canine gamete physiology presents a difficult adaptation of technology knowledge obtained from other species. Furthermore, domestic felines are animals of company and there is no interest in reproducing them in a large scale, as it has been observed in other domestic animals, however, besides of being a valuable model for the development of in vitro techniques, the domestic cat is also used as an embryo receptor for different species of small wild felines due to physiological similarities among them, in vitro embryonic development,

Review: It was reviewed the main insights about the reproductive physiology in female dogs, in vitro oocyte maturation (IVM), pregnancy and conception rate with dogs' frozen/unfrozen semen and PIV in domestic cats. The majority of mammal oocytes restart meiosis spontaneously after ovulation and reaches MII in artificial environment; in an in vitro maturation system in bovines, around 90% of oocytes complete their maturation, although its development capacity can be reduced subsequently. The success of IVM in canidae have been limited, with maturation rate varying from 0 to 58%, usually around 20%. The greatest difficulties include oocyte quality, hormonal environment, protein supplementation, cumulus / oocyte cell interaction, donor breed and age, culture systems, oxygen tension, amino acids, growth factor and sequential means. The freezing process reduces the quality of the semen, firstly because it reduces the number of living sperms and secondly because freezing produces cell modifications that could alter the sperm motility, longevity, integrity of membranes and its fertilizing capacity.

Conclusion: Nowadays, several researches are being performed with the aim of increasing viability after dogs' and cats' semen is unfrozen, using extenders, cryoprotectors, freezing and unfreezing curves, addition of antioxidant substances. The aim of this text is to inform about the improvements obtained on the artificial reproduction techniques, emphasizing the oocyte maturation in female dogs, semen cryopreservation and artificial insemination in domestic dogs and cats.

Keywords: oocyte maturation, semen cryopreservation, artificial insemination, dog and cat.

- I. INTRODUCTION
- II. REPRODUCTIVE PHYSIOLOGY IN FEMALE DOGS
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I. INTRODUCTION

The first Artificial Insemination (AI) with satisfactory results was performed on dogs by Abbé Lazaro Spallanzani, in 1780 and the first description of a mammal oocyte was done by Karl Genst Von Bôer, in 1827. Both basic research and commercialization of reproduction techniques have been developed in a slow pace in carnivorous, especially if compared to other domestic species or human beings [2,6,24,25,29].

The delay in development of artificial reproduction techniques on carnivorous could be due to countless reasons, but the lack of commercial interest is probably the most important one. The majority of canines are small structures, canidae are extremely fertile and a great number of species are adapted to domestication or captivity. Finally, the canine gamete physiology presents a difficult adaptation of technology knowledge obtained from other species.

Furthermore, domestic felines are animals of company and there is no interest in reproducing them in a large scale, as it has been observed in other domestic animals [6], however, besides of being a valuable model for the development of *in vitro* techniques, the domestic cat is also used as an embryo receptor for different species of small wild felines due to physiological similarities among them [21,22].

In this context the assisted reproductive techniques on the domestic cat has proven to be valuable tools. They promote the development of highly efficient *in vitro* culture techniques; exchange of genetic material among captivity animals and animals living in the wild; reproduction of animals showing physical or behavioral deficiency; increase in certain population; male and female proportion rectification in determined place and specie and gamete bank formation [20].

The aim of this text is to inform about the improvements obtained on the artificial reproduction techniques, emphasizing the oocytary maturation in female dogs, semen cryopreservation and artificial insemination in domestic dogs and cats.

II. REPRODUCTIVE PHYSIOLOGY IN FEMALE DOGS

Dogs are the kind of monoestric, poliovulatory, non-seasonal species. The estral cycle is divided into three phases: estrus (proestrus and estrus), diestrus and anestrus. The female dogs present a particular reproductive endocrinology: The progesterone concentration in the blood increases few days before the LH pre-ovulatory wave and ovulations. This pre-ovulatory luteinization is typical of canidae. The female dog's oocytes are released during an immature diploid state period (germinative vesicle) and complete its meiotic maturation in the uterine tubes. The oocytes and the canine embryos remained in the in the uterine tubes for a long period of time, when compared to other species (8 to 10 days). The embrionary development is slow and the pre-implantation period is particularly long (8 to 10 days) [24].

The basic pre-requirement for the use of reproductive biotechniques is the determination of ovulations. Several methods are used to identify ovulations, but the safety applied on these methods may vary – vulva edema, reproductive behavior, proestrus and estrus duration. Ovulations, however, are related to the LH peak and progesterone

concentrations. Serum concentrations and ovulations are relatively stable; Five to seven ng/mL are serum progesterone concentrations that correspond to ovulations [14]. The serum/plasmatic progesterone are frequently used to predict ovulation. Recently, ovarian transabdominal ultrasonography has also been used to monitor ovulations, and has demonstrated its importance. It is a non-invasive method and can be used to monitor ovulations, but requires 2 or 3 daily observations before the ovulation and during its process.

According to Bicudo et al. [3], after the beginning of follicular growth, it was observed a crescent ovarian follicles rise that reached from 3.2 to 6.7 mm diameter (10 Mhz transducer). Fontbone & Malodaim [7] concluded that no matter the breed, the pre-ovulatory follicles dimensions vary from 6 to 9 mm and ultrasonography normally underrates the number of follicles.

The average ovulation rate in dogs can be estimated after ovariectomy and corpora lutea count and varied from 5.7 ± 0.3 ; 6.0 ± 0.1 ; 7.7 ± 0.6 ; in mongrel female dogs [6,30,33]. According to Luz et al. [12] the ovulation rate in female dogs whose weigh varied from 5 to 16 kg, was 5.5 in pregnant females and 5.5 in diestrus females. The ovulation rate, as well as the litter size varies and depend on the animal breed.

Tsutsui [33] and Reynoud et al. [23] studied the *in vivo* dogs oocyary maturation, and started the study from 17 to 24 hours after the ovulation; the oocytes average diameter, including pellucid zone varied from 86 to 195 μm with an average of 110 μm ; the follicle core showed periphery localization and was surrounded by countless mitochondria's. The oocytes restarted the meiosis and MI (metaphase I) were observed within 48 hours; the oocytes with MII cromossomic configuration (metaphase II) were made evident in the uterine tubes approximately 48-54 hours after ovulations.

III. *IN VITRO* OOCYTARY MATURATION (IVM)

The majority of mammal oocytes restart meiosis spontaneously after ovulation and reaches MII in artificial environment; in an *in vitro* maturation system in bovines, around 90% of oocytes complete their maturation, although its development capacity can be reduced subsequently. The success of IVM in canidae have been limited, with maturation rate varying from 0 to 58%, usually around 20%. The greatest difficulties include oocyte quality, hormonal environment, protein supplementation, *cumulus* / oocyte cell interaction, donor breed and age, culture systems, oxygen tension, amino acids, growth factor and sequential means.

Information regarding the connection between reproductive cycle state and female dogs oocytes meiotic competence are contradictory. Some authors did not demonstrate the association between estral cycle phases and oocyte competence [19,27,31], while other researchers, on the contrary, have shown that the reproductive stage correlates in a significant way to the oocytes development capacity [11,15]. According to Rodrigues et al. [28] the pro nucleus formation is more frequently observed in oocytes collected from female dogs during follicular phase, when compared to the luteal and anestrus phases.

Songsasen & Wildt [31] showed that the ovarian follicle size influences oocyary maturation rates. Around 80% of oocytes originating in $\approx 2\text{mm}$ diameter follicles complete their *in vitro* nuclear maturation when compared to 16-38% of 0.5 to 2mm diameter follicles. Many researchers recover the oocytes through the slice technique, resulting in a heterogeneous oocyte population, which will vary according to its development and ability to complete maturation; according to Songsasen & Wildt [32], this can at least partially explain the contradictory results obtained by the researchers concerning the reproductive stages and the oocyary competence, and the unsatisfactory results of canine species IVM.

Most of papers about oocyary maturation in female dogs use the means TCM199 and OSF (oviduct synthetic fluid). TCM199 contains vitamins, cysteine and ascorbic acid that protect the cell from oxidative stress. The addition of *thiol* compounds in the culture media increases the oocyary maturation rate [10]. According to Songsasen & Wildt [32] the inclusion of antioxidant substances in the maturation mean is important due to the great amount of intraoocyary lipids, which makes the oocytes susceptible to the oxidative stress.

Another important agent to the *in vitro* oocyary maturation is the donator's ovarian follicle's size. Oocytes collected from follicles bigger than 2 mm diameter present 80% rate of meiotic competence, when compared to a 16.9% rate of meiotic competence in follicles smaller than 0.5mm and 26.1% in follicles from 0.5 to 1mm and 38.4% in 1 to 2 mm follicles [31].

The ovaries donor's age also influences the oocytes quality. Hewitt & England [9] and Rocha et al. [26]

showed that oocytes that were collected from female dogs with ages between 1 to 6 years showed higher maturation potential than those collected from older or younger females.

The epidermal growth factor (EGF) is a supplement that has received special attention on IVM on female dogs. The addition of EGF to the culture media works as stimulus to the nuclear oocytyary maturation and cumulus expansion, but did not significantly affect the percentage of oocytyary maturation.

Serum protein supplementation or bovine serum albumin have been described in several researches [4,17,18]. This kind of supplementation has been associated with high percentages of canine oocytes with non identified nuclear material.

Another factor that has been calling researchers' attention regarding canine oocyte maturation is the sperm penetration. The immature oocytes ovulation in dogs could be due to its interaction with the sperm before the maturation has been completed, and it is likely to happen. This could really occur considering the sperm longevity in the female dog's reproductive tract. A great deal of sperm concentration remains still inside the womb (4 to 6 days after mating) with less quantities that can remain in the womb up to 288 hours after mating. The result of Saint-Dizier et al. [29] demonstrated the sperm penetration has a connection with oocytyary maturation, showing a high percentage of fertilized oocytes hitting MI or MII phases. However, further research contradicts this result, and it documents only 3 out of 112 immature oocytes penetrated by sperms after ovulation and artificial insemination [24]. This research team suggested the immature oocyte penetration by sperm *in vitro* can be an inferred finding through culture suboptimal condition.

In a study conducted by Wesselowski [34] about dogs oocyte metabolic activity, scientists discovered that dogs' naked oocytes used mainly the glucose, via glucolysis, as energy source during a 3 hour period, when *in vitro* maturation was evaluated. When compared to other species, this condition seems to be unique. Cows' naked oocytes require pyruvate as primary energy source during its 14 hour maturation *in vitro* [8]. Other carnivorous naked oocytes such as the cats, use mainly pyruvate during the IVM, even more than glucose.

Specifically, *cumulus* cells metabolize glucose to pyruvate or any other Krebs cycle mediator that could transfer themselves through *gap junctions* to the oocytes in order to act as energy source. As it has been stated before, the dogs' oocyte, on the other hand, is able to provide energy by itself and metabolize glucose without *cumulus* cells assistance [34]. Rodrigues et al. [28] showed that only 4% of canine oocytes cultivated for 48 hours develop until MII. However, when cultivated for 48 hours and inseminated *in vitro*, more oocytes were fertilized (24% of them formed pro-nucleus and even developed initial stage embryos). It's still unknown whether sperms exert any influence on IVM.

During mammal fertilization, sperms induce oscillatory changes in intra-oocytyary Ca^{++} levels. It has been suggested that cellular intra and extra Ca^{++} play a very important role on the meiosis restart and progression; therefore it is possible that the oocyte penetration by the sperm induces the meiosis' new start via Ca^{++} dependent.

Apparicio-Ferreira [1] researched about the use effect of sequential mediums and co-cultivated with sperms on *in vitro* maturation rates on female dogs. The following means have been tested: *TCM 199 + HCG + P4 + E2 for 48 hours followed by de TCM 199 + HCG + P4 + E2 for 24 more hours; TCM 199 + HCG for 48 hours and TCM 199 + P4 e TCM 199+ HCG + P4 + E2 for 24 more hours + P4 e TCM 199+ HCG + P4 + E2 for 48 hours and TCM 199 + P4 and TCM 199 for 72 hours*. The moving sperms were diluted HF-10 medium in a 1×10^7 spz/MI concentration. 50 μ l from the sperm solution was added in the oocytes culture mean after 48 hours and remained for the next 24 hours. The results showed that the use of sequential systems was benefic to the meiotic competence, but the presence of sperms did not influence the meiotic competence of matured oocytes.

IV. IN VITRO EMBRYONARY DEVELOPMENT

In vitro embryo development hasn't been a successful practice on dogs. Songsasen et al. [31] inseminated the oocytes cultivated *in vitro* and obtained 34% of these oocytes fertilized. The normal fertilization obtained by the presence of two pro-nucleus and a single sperm tail inside the cytoplasm has been verified in only 4% of the oocytes.

Rodrigues et al. [28] made evident a fertilization rate of 30%, however, half of oocytes were fertilized by more than one sperm (polyspermy). The embryony development in this study was 10%, and no embryo developed

further than 8 cells. Therefore, none of the puppies have been produced in dogs nor canidae by embryo transfer originated from *in vitro* oocytary maturation.

V. PREGNANCY AND CONCEPTION RATE WITH DOGS' FROZEN/UNFROZEN SEMEN

The freezing process reduces the quality of the semen, firstly because it reduces the number of living sperms and secondly because freezing produces cell modifications that could alter the sperm motility, longevity, integrity of membranes and its fertilizing capacity.

Nowadays, several researches are being performed with the aim of increasing viability after dogs' and cats' semen is unfrozen, using extenders, cryoprotectors, freezing and unfreezing curves, addition of antioxidant substances, etc.

A paper conducted by Rota et al. [25] evaluated and compared the fertility results between the 5% ethylene glycol frozen semen and 5% glycerol with intra-vaginal insemination. The semen quality after unfreezing was similar concerning both cryoprotectors. All the inseminated dogs became pregnant despite the semen that had been used; the concept number varied from 1.0 to 10.0 on the group inseminated with glycerol and from 1.0 to 9.0 on the group inseminated with ethylene glycol. The concept relations: the luteous body varied from 0.14 to 1.0 on the female dogs belonging to the glycerol group and from 0.20 to 0.67 on the ethylene glycol group. The inseminations were made between the 4th and 5th days after the LH stimulated wave.

When the extenders effects containing LDL (low density lipoprotein) were compared, yolk and Equex over frozen/unfrozen dogs' semen longevity, motility and fertility, the LDL and Equex means showed superiority over yolk means concerning motility and other sperm moving parameters. Concerning membrane integrity, the three means showed similar results; the Equex mean showed higher acrosome integrity percentage evaluated by FITC/PSA. The DNA integrity didn't show any alterations in the different means; the same results were observed in the acrosome integrity evaluated by the use of Spermac. Only the LDL mean was tested for fertility, out of 6 inseminated females, all of them showed positive results for pregnancy [2]. According the authors, the LDL mean eliminates the possibility of bacterial contamination that would endanger the quality of the frozen semen.

Neves et al. [16] compared 4 extenders: 1. *egg yolk + glycerol*. 2. *LDL (8.9 mg) + glycerol*. 3. *LDL + dimethylformamide* 4. *LDL + ethylene glycol*, evaluated the membranes integrity, differences among the means were observed concerning progressive motility and spermatid morphology; the means containing LDL with glycerol or ethylene glycol were better than the others.

Magalhães et al. [13] evaluated liofilization technical viability in dogs' sperms, allowing its transportation and storing in a practical and economical way. The sperms, after liofilization, kept the structure of the head intact, allowing its use in assisted reproduction techniques. After analysis and cryopreservation, HTF and SOF cryopreservation the semen underwent liofilization (-40°C/48 hours). The samples were resuspended in its respective means and showed 84.5% of DNA integrity evaluated through the *acridina orange* technique; the integrity of the membrane presented 100% of damaged cells and 70% of acrosome integrity using the JC fluorescent probes, propidium iodide and PSA. Despite the membrane integrity results, the liofilization proved to be a promising technique, allowing its usage in artificial reproduction. The same liofilization procedure was performed on domestic cats and showed the following results: 87.5% of DNA integrity, 75% of acrosome integrity and 100% of damaged spermatid membranes.

VI. PIV IN DOMESTIC CATS

In a study performed by Martins [15] about the seasonability influence and ovarian condition over IVP in domestic cats, the COC average number obtained in the females' ovaries in the months January, February and March – fotoperiod I – was 23.65 and in the fotoperiod II (August, September and October) 48.2 COC/cat. Cats whose ovaries were presented in the follicular condition produced a lower number oocytes/ovary; however, the majority of oocytes were classified Degree I. The oocytary maturation level in this study was 75% in both periods; cleavage rate was 64.26%, morula rate was 40.37 and blastocyste rate was 23.89%, independently of ovarian condition.

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