CURRENT ASPECTS OF REPRODUCTION BIOTECHNOLOGY IN CARNIVORES

ASPECTOS ATUAIS DAS BIOTECNOLOGIAS DA REPRODUÇÃO EM CARNÍVOROS

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SUMMARY

The development of assisted reproductive techniques (ART) in dogs and cats is an area of increasing interest because ART can be potentially applied to non-domestic carnivores threatened with extinction. Much progress has been achieved in ART of the feline species. However, advances on assisted reproduction in dogs are hampered by its low efficiency. A major obstacle lies on the particular characteristics of canine gamete physiology, for instance, the highly complex requirements for oocyte maturation and development, which makes difficult to apply protocols/techniques that have been successful in other species. This review aims to depict the advances made on assisted reproductive techniques in dogs and cats, focusing on those features that may be relevant for applying these techniques to wild endangered carnivores.

KEY-WORDS: Bitch. Cat. *In vitro* embryo production.

RESUMO

O desenvolvimento de técnicas de reprodução assistida no cão e no gato desperta grande interesse, pois poderiam ser utilizadas para a conservação de espécies de carnívoros selvagens ameaçadas de extinção. Nos últimos anos houve um grande avanço das biotecnologias da reprodução aplicáveis aos felinos domésticos. No entanto, o seu desenvolvimento nos caninos domésticos tem sido dificultado pela baixa eficiência. Um dos maiores problemas reside na fisiologia única dos gametas caninos, que exigem meios complexos para a maturação e desenvolvimento, o que dificulta a implementação de protocolos e técnicas que têm sido eficazes em outras espécies. O objetivo desta revisão é descrever os avanços nas biotecnologias da reprodução que se aplicam aos cães e gatos, com enfoque sobre os aspectos que podem ser importantes para o uso em espécies protegidas de carnívoros

PALAVRAS-CHAVE: Cadela. Gata. Produção *in vitro* de embriões.

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INTRODUCTION

From the 90's and on, when the scientific community took interest in the preservation of endangered species, the biotechnology of carnivore reproduction saw great development (LUVONI, 2000). Since then, domestic cats and dogs are used as experimental models (DURRANT et al., 1998).

The *in vitro* production (IVP) consists of the *in vitro* steps: maturation (IVM), fertilization (IVF) and embryo culture (IVC). The IVM in dogs has limited success, with values that range from 0 to 58% (FARSTAD, 2000). Luvoni (2000) cites rates around 20%, while in cats, about 75% of oocytes complete the maturation step (NAGANO et al., 2008).

The objective of this work is to show the advances in IVP of domestic dogs and cats, since these are important experimental models of wild species under extinction threat.

IVP EMBRYOS IN DOGS

Considerations on the reproductive physiology of the bitch

Dogs are known for their peculiar reproductive physiology. These animals are considered monoestrous, predominantly non-seasonal and ovulation occurs one or two days after the LH surge, the onset of estrus. In bitches and foxes, luteinization of pre-ovulatory follicles occurs and plasma progesterone increases during proestrus (REYNAUD et. al., 2005), opposite to what occurs in other species of domestic mammals, where estrogen dominates the pre-ovulatory follicular environment (FARSTAD, 2000).

Further, while in most mammals, the oocyte in the germinal vesicle stage (prophase) undergoes meiosis in the later stages of follicle maturation and is ovulated in metaphase II, already with the extrusion of the first polar body; in dogs, the oocytes are released still immature during the germinal vesicle stage. Thus, both full maturity and fertilization occur inside the oviduct and meiotic competence acquisition happens in the intra and extra-follicular environment (FARSTAD et. al., 1989). This peculiarity justifies the importance of the oviductal environment for canine oocyte maturation, because the oviduct is responsible for sustaining over an extended period, the survival of immature oocytes released to complete their development, become fertilized and reach the blastocyst stage (FARSTAD et. al., 1989; LUVONI et al., 2005).

IVM: Oocyte retrieval

Oocytes are obtained mainly by ovary slicing (NICKSON, et al., 1993) and enzymatic digestion (BOLAMBA et. al., 2002). The number of retrieved oocytes by either technique is greater than the number by the aspiration technique, which is very difficult to execute since they are visible only few days before ovulation due to ovary and follicle small size. The enzymatic digestion technique, despite the low number of degenerate oocytes, causes a lot of damage to the granulosa cells (BOLAMBA et. al., 2002), thus making slicing the technique of choice in the laboratory of FCAV – UNESP - Jaboticabal.

Strom Holst et al. (2001) and Rodrigues & Rodrigues (2003) reported that there is no significant difference in the number of oocytes obtained from purebred or mixed breed animals and among bitches with different body weights. Rodrigues & Rodrigues (2003) showed that the older the bitch, the lower the rate of retrieved oocytes. Regarding the influence of the estrous cycle on oocyte retrieval, the results are controversial. Some authors suggest that there is a tendency towards higher rate of oocyte retrieval during the follicular phase of the estrous cycle (STROM HOLST et al., 2001); however, Ribeiro (2007) showed no significant difference and reported such correlation only in animals where estrus was hormonally induced.

Oocyte Selection

Canine oocytes can resume spontaneously *in vitro* meiosis when subjected to adaptations of maturation techniques used for cattle and pigs, albeit at much lower rates, compared to the aforementioned species and cats as well (FARSTAD, 2000). Thus, in an attempt to improve these ratios, the selection of oocytes for dog IVM is attentive not only to aspects related to canine oocyte, but also to factors inherent to the female donor, such as age and stage of the estrous cycle.

According to Ebner et al. (2006) the morphology of the oocyte is regarded as a prerequisite for the development of embryos. The integrity of the cumulus-oocyte complex (COC) phenotype is considered not only an indicator of its viability, but also a potential marker of gene transcription ability involved in the maturation and early embryonic development (RODRIGUES et al., 2009). For this reason, in dogs, the selection of oocytes for *in vitro* culture relies on morphological criteria that selects for IVM only grade I cumulus-oocyte complexes (Figure 1), that is, oocyte with uniformly dark cytoplasm, zona pellucida intact and surrounded by two or more layers of cumulus cells (BOLAMBA et al. 1998).

Another evaluated parameter is oocyte diameter. For some years meiotic competence of canine oocyte was related to the growth level achieved by the oocyte before it was released from the follicle; therefore, the larger the oocyte, the greater the ability to reach the germinal vesicle breakdown stage and reach metaphase II (ENGLAND & HEWITT, 1998; OTOI et al. 2001). However, recent studies have shown that meiotic competence is influenced by follicular diameter and not by oocyte diameter (SONGSASEN et al. 2009). This assertion was based on the fact that although oocytes had reached the ideal maximum diameter, they were unable to complete nuclear *in vitro* maturation, since they originated from follicles smaller than 2-mm diameter (SONGSASEN et al. 2,009). According to these researchers, in order for the canine oocyte to be able to complete nuclear maturation and become a fertilized embryo, it is necessary intracellular

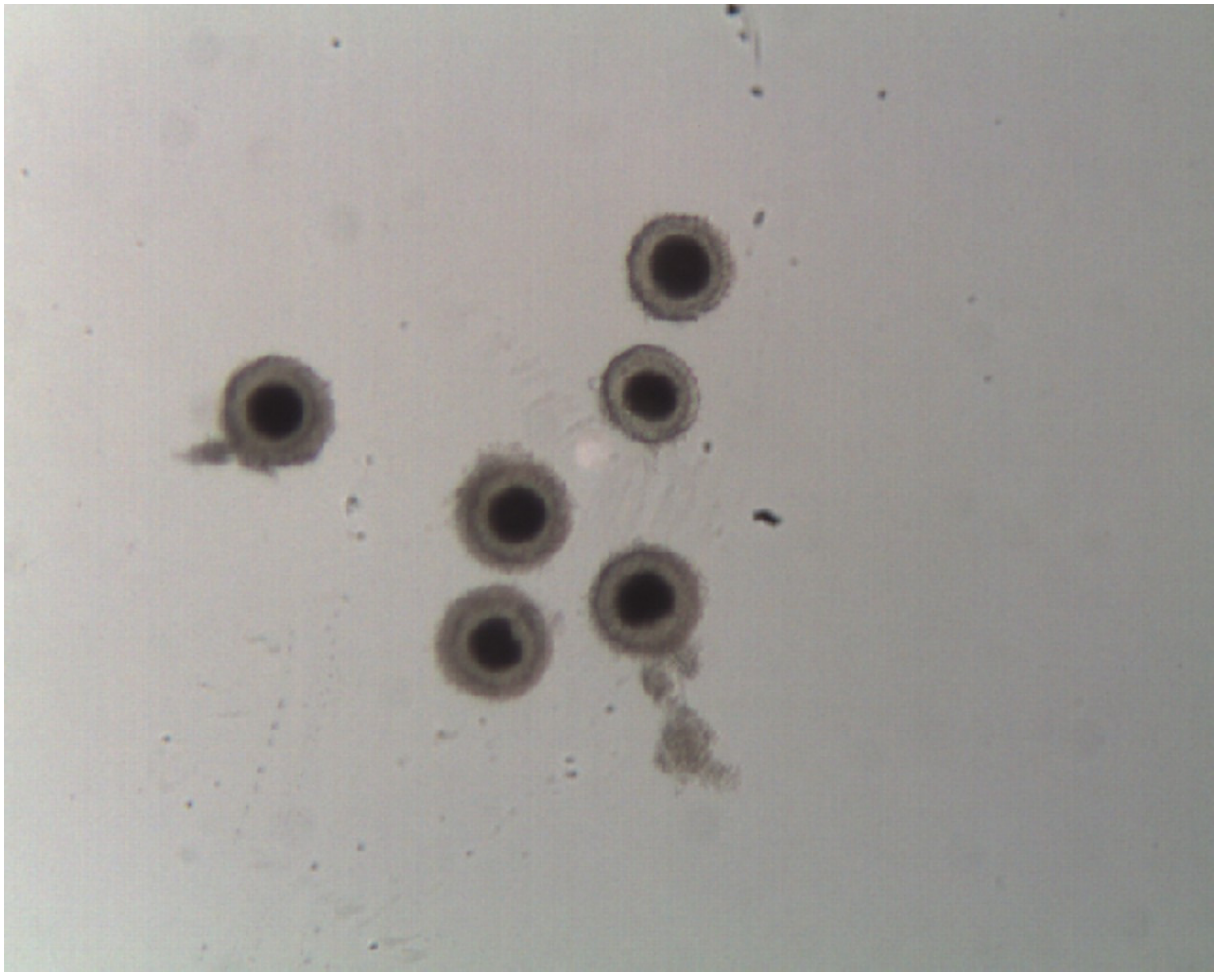

Figure 1 - Photomicrograph of Grade 1 COCs bitch. It should be noted that the ooplasm is uniformly dark with intact zona pellucida, surrounded by one or more layers of cumulus cells and with a diameter greater than 100 µm. Stereoscopic microscope, magnification 4x. Jaboticabal-SP, 2012.

modifications, which possibly occur in the late stages of follicular development.

In addition to the morphological factors mentioned above, other factors associated with female donor, such as age and stage of the estrous cycle are important in the selection of oocytes with the ability to resume meiosis *in vitro*. According to Dolezel et. al. (2004), the ovaries of prepubertal female dogs have a lower proportion of follicles in advanced stages of development and higher rate of follicle atresia. Hewitt and England (1998) observed that the ability to mature *in vitro* is compromised in females over the age of seven.

The influence of estrous cycle phase on oocyte recovery rate and nuclear maturation frequency is still controversial. While some researchers report no association (ROBERTS & RODRIGUES, 2003; APPARÍCIO et al., 2011), others show that bitch reproductive status influences the ability of oocyte development (OTOI et al., 2001; KIM et al. 2004). Such that, oocytes obtained during the follicular phase (proestrus and estrus) show better maturation indexes compared to oocytes retrieved from animals at other reproductive stages (YAMADA et al. 1993; OTOI et al. 2001; KIM et al., 2004; SONGSASEN & Wildt, 2007).

There are many explanations for the influence of estrous cycle phase on meiotic competence. According Luvoni et al. (2005), the influence of the estrous cycle stage is related to the GAPjs functional status between oocyte and cumulus cells. Low rates of maturation during anestrus would be caused by the lack of communication between the cumulus cells and oocyte. On the other hand, Buttler-Pires (2010) while studying the morphological characteristics of canine cumulus-oocyte complex, stated that although oocytes from anestrous females in follicular phase have the same communication intensity between cumulus cells, suggesting that these oocytes have the same ability to resume meiosis, the best *in vitro* maturation rates are achieved in oocytes from animals in the follicular phase. Probably, this fact arises from the greater degree of cytoplasm maturity reached by the oocyte during this reproductive stage.

The controversies and the different explanations about the influence of estrous cycle phases on the meiotic competence reiterate the need for more studies on this subject.

Maturation medium

The maturation media are adaptations of those commonly used in cattle, the most commonly used are the SOF, Synthetic Fluid oviduct, (BOLAMBA et al. 2002; MACHADO et al., 2007) and TCM 199, Tissue Culture Medium 199 (OTOI et al., 2001; SONGSASEN et al., 2001; & RODRIGUES RODRIGUES, 2003; APPARICIO et al., 2011) plus various substances, such as hormones, growth factors and antioxidants, aiming to improve the rates of maturation processes and reduce degeneration.

Based on the peculiar reproductive physiology of bitches, where the oocytes are exposed to high concentrations of progesterone in the preovulatory follicle, many studies about supplementation of the maturation media with steroid hormones (estrogen and progesterone) were conducted. Vannucchi et al. (2009) found that the addition of estrogen and progesterone to the maturation medium had a beneficial effect on IVM of bitches; the same conclusion was reported by Apparício et al. (2011) when hCG, estradiol and progesterone were added to the medium. In contrast, Ribeiro (2007) reported that progesterone addition had no positive effect.

Regarding growth factors, IGF-I plays an essential role on folliculogenesis. This growth factor stimulates the proliferation of the granulosa cells, enhances the biological effect of FSH and LH on theca and granulosa cells since it is responsible for stimulating the synthesis of receptors through which these hormones act (CHASTANT-MAILLARD et al., 2011).

Alhaider & Watson (2009) studied the effect of different growth factors association on canine oocyte maturation and found that the combination of IGF-1, human GH, TGF-α and FGF favored meiotic competence. Likewise, Cui et al. (2006) found that supplementation of the culture medium with EGF had a beneficial effect on IVM of bitches. From these data, it is believed that the addition of growth factors to the culture medium is a prerequisite for canine oocyte to complete *in vitro* maturation, although one research has shown that addition of IGF-1 or EGF to the TCM 199 medium was not beneficial for IVM of canine oocytes (CARDILLI et al., 2011).

A major concern during *in vitro* maturation and fertilization is the damage caused by free radicals (ROS) produced usually by the oxidative metabolism (DEW, 2001). Although most cells present an efficient defense system against free radicals, represented by glutathione; oocyte manipulation during *in vitro* maturation, removing it from the intra-follicular environment and inevitably exposing it to high oxygen levels, promotes high oxidative stress, which causes the mobilization of large glutathione amounts and consequent decline in its levels (LUBERDA, 2005). As a consequence, meiotic competence decreases due to rising degeneration rates and cell death caused by free radicals present in the maturation medium (PIRES, 2006). Because of this, supplementation with compounds that act as substrates or glutathione precursor such as cysteine, cysteamine and β-mercaptoethanol, which have been successfully used in different species, has been proposed (DE MATOS et al. 1996).

Kim et al. (2004) concluded that the addition of β-mercaptoethanol to the culture medium improved IVM rates in bitches; however, Pires (2006) while studying the effects of cysteine and cysteamine, reported no positive effect.

Aiming to mimic oviductal environment, some researchers have studied the co-culture of oocytes in the oviduct cells of bitches. Bogliolo et al. (2002) showed that this procedure has a positive effect on IVM of bitches.

Some researchers believe that sperm has an important role in the resumption of oocyte meiosis during IVM in bitches. Studies with added sperm to the culture media have been conducted; however, no positive effect was observed on IVM (SAINT DIZIER et al., 2001).

Cultivation time

The cultivation time may reach up to 120 hours. The longer the IVM phase, the smaller the findings of intact germinal vesicle and the bigger the findings of metaphase and of the percentage of degenerated oocytes. Some studies have reported that 72 hours would be ideal for bitch oocyte IVM (LUVONI et al. 2,005).

Evaluation methods

IVM efficiency should be evaluated based on nuclear and cytoplasmic oocyte maturation. Assessment of nuclear maturation should be made using the fluorescence technique due to the large amount of lipids present in canine oocytes (BOLAMBA et al. 2002). After the cultivation time, cumulus cells are removed through successive passages in 0.2% hiluronidase, the denuded oocyte is then stained with bisbenzimide (APPARÍCIO et al., 2011).

The following pattern is recommended to classify maturation degree: germinal vesicle (presence of vesicular nuclei with slightly condensed chromosomes), germinal vesicle breakdown (with some degree of chromosome condensation and scattered distribution, but also with vesicular-aspect core), metaphase I (chromosomes reach the most advanced level of condensation, it is not possible to visualize individual chromosomes), metaphase II (dense group of chromosomes forming the first polar body and another group further away, characterized by metaphyseal plate) and degenerate that cannot be identified (HEWITT & ENGLAND, 1998).

The evaluation of cytoplasmic maturation relies on some features of cellular organelles, such as the position of cytoplasmic granules, which are located at the periphery and in the central region of mature and immature oocytes, respectively (MACHADO et al., 2007; APPARÍCIO et al., 2011).

***In vitro* fertilization (IVF)**

Due to the difficulties incurred in the IVM phase, IVF cleavage and blastocyst rates in dogs are still very low. Yamada et al. (1993) reported that only 2% of inseminated oocytes reached the eight-cell stage. Otoi et al. (2000) obtained one blastocyst out of 217 inseminated oocytes and England et al. (2001) obtained a single IVF pregnancy in this species.

Cloning

Despite all the aforementioned difficulties with *in vitro* production of canine embryos, in 2005 the first clone of the species obtained from ear cells of a three-year-old Afghan Hound was announced (LEE et al. 2005). The same research group obtained the first commercial clone in 2008.

Hong et al. (2008) obtained a clone through nuclear transfer technique using fetal fibroblasts and concluded that this model is more efficient than the method described above.

EMBRYO IVP IN CATS

Considerations on the reproductive physiology of the cat

Female cats are seasonal polyestrous, characterized by presenting ovulation induced by coitus, although some authors report that in animals that do not have street access, ovulation can occur spontaneously. In this species, oocytes are already ovulated in metaphase II stage, and are quite dark (similar to dogs) due to large lipid amounts (LUVONI, 2000).

Pope et al. (2006) report that seasonality greatly influences the IVP of feline embryos. Oocytes from functional ovaries, that is, the time of year when days are longer, have better quality and higher cleavage and blastocyst rates compared with oocytes from quiescent ovaries, ie, the time of year when days are shorter. In countries like Brazil, seasonality effect is not as striking, especially in regions closer to the equator.

IVM of feline oocytes

IVM of this species presents better results than in canine, and the rates can reach 75% of oocytes in metaphase II, but the results are still worse than those reported for production animals (NAGANO et al., 2008).

Oocyte selection follows the same patterns used in bitches and maturation media are also adapted from another species. Luvoni et al. (2000) reported that the addition of gonadotropins to maturation media and antioxidant compounds have a positive impact on IVM. Regarding growth factors, Kitiyanant et al. (2003) concluded that the addition of 100ng/mL IGF-1 to the medium has a beneficial effect on IVM, the same effect was observed by Merlo et al. (2005) when adding 25 ng/mL EGF to the culture medium.

Nagano et al. (2008) reported that the ideal period for IVM of feline oocytes in TCM 199 is 30 hours.

***In vitro* fertilization**

Recently, many researches have been performed regarding IVP of feline embryos, and the results were satisfactory. The rates of *in vitro* produced embryos that reach blastocyst stage range from 40-60%. Some authors report that the rate of embryos that reach blastocyst stage after IVF of *in vivo* matured oocytes is greater than those obtained from *in vitro* matured oocytes. One explanation for this would be the higher activity of MPF and MAPK factors in oocytes matured *in vivo* (POPE et al., 2006).

Armstrong et al. (2004) achieved a rate of 30% blastocysts when performing IVF of *in vivo* matured oocytes obtained by laparoscopy from wild cats (*Panthera Leo*).

Cryopreservation of feline oocytes and embryos

Some research has been conducted about cryopreservation of feline oocytes and embryos with the objective of storing genetic material from endangered species. Births from frozen embryos produced *in vitro* have been reported, for both the domestic cat and some wild species (POPE et al., 2006).

The cryopreservation of oocytes has shown less satisfactory results. The IVF of cryopreserved mature oocytes has 38.7% cleavage rate, when following the slow method and ethylene glycol as a cryoprotectant; while IVF of cryopreserved immature oocytes presents 6.8% cleavage rate (LUVONI et al., 2000).

Intracytoplasmic sperm injection (ICSI)

Births resulting from the ICSI technique applied to *in vivo* and *in vitro* matured oocytes have already been reported for the domestic cat (POPE et al., 2006). Gómez et al. (2000) compared cleavage and blastocyst rates obtained by applying the IVF and ICSI techniques to *in vivo* and *in vitro* matured oocytes. The best results were reported for *in vivo* matured oocytes.

For wild cats, ICSI embryos resulted from *in vivo* matured oocytes only obtained by laparoscopy, however, no pregnancy has been reported in the literature (DAMIANI et al., 2004).

Cloning

The first feline clone was obtained by Shin et al. (2002) using the nuclear transfer technique, where the genetic material of fibroblastic cells from the oral mucosa of a male, adult domestic cat was transferred to an oocyte of a domestic cat which had undergone *in vitro* maturation and had its genetic material removed by micromanipulation.

FINAL CONSIDERATIONS

IVM of canine oocyte is complex and poorly efficient, which limits the IVP of embryos. On the other hand, IVP of embryos of cats and production animals, such as cattle and pigs produce satisfactory results.

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