Anim Reprod, v.9, n.3, p.205-209, Jul./Sept. 2012

Oocyte maturation in bitches

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Abstract

The canine species has been used as an experimental model for preservation of endangered species. Biotechnologies of reproduction, such as *in vitro* maturation (IVM), have been used to meet this objective. Several protocols for *in vitro* embryo production (IVEP) in swine and bovine species have been adapted for canids. However, the highest rate reported for *in vitro* maturation in canids is only 39%, which is still lower than those in other species. Therefore, current research on assisted reproduction in canids have focused on several IVM protocols, including the addition of proteins, hormones, meiosis inhibitors, growth factors and antioxidants to the maturation media and the determination of suitable timing for culture, so that variables involved in the process can be fine-tuned. This review has the main objective of describing major developments and limitations in the process of oocyte maturation in bitches.

Keywords: canine, fertilization, IVEP, IVM.

Introduction

Most authors who work in the field of biotechnologies, involving *in vitro* maturation as well as culture and fertilization of oocytes from bitches, unanimously agree that there is a great difficulty to obtain satisfactory results.

The difficulty has been attributed to the lack of basic understanding of reproductive events in bitches as well as the specific characteristics of this species, which include the hormonal environment, meiotic resumption and progression, lack of a defined maturation medium for the species and the role of the oviduct in oocyte maturation (Rodrigues and Rodrigues, 2003; Luvoni *et al*., 2005).

Ovulation in bitches occurs 1 or 2 days after the preovulatory peak of the luteinizing hormone (LH), still in the beginning of the estrus stage of the estrous cycle, and the ovarian follicles start luteinization before ovulation. Oocytes are ovulated still immature, in the beginning of the first meiotic division (GV). Therefore, the oocytes require 2 to 5 days to reach maturation after ovulation (Rocha *et al*., 2007). The environment in the uterine tubes plays an important role in canine oocyte maturation. The uterine tubes in canids, unlike other species, is responsible for maintaining, during an extended period of time, the survival of still immature oocytes until they complete their development, are fertilized and reach the blastocyst stage (Luvoni *et al*., 2005). Because of this characteristic, several studies have been conducted aiming to establish efficient protocols for maturation followed by fertilization of oocytes in this species.

The time of culture recommended for IVM of canine oocytes varies widely, but the results of complete maturation have been controversial and unsatisfactory. Therefore, the evaluation of resumption of meiosis becomes of great importance in this species to assess the progression of nuclear maturation throughout the time of culture (Silva *et al*., 2008). Many stages have still not been standardized within the maturation process, such as the acquisition and selection of oocytes, age and stage of the estrous cycle of the donors. Furthermore, *in vitro* media to mature oocytes of other species are considered inadequate for IVM of canine oocytes (Ribeiro *et al*., 2010).

The clear understanding of oocyte metabolic needs in culture systems for *in vitro* maturation requires new studies to establish an ideal condition for oocytes to acquire competence, be fertilized and be able to maintain the initial embryonic development (Barreto, 2007). This review aimed at discussing the main factors related to IVM in bitches.

In vitro maturation

The process of oocyte maturation encompasses several molecular and structural alterations in the cytoplasm (cytoplasmic maturation), which culminate with the chromosomal configuration in metaphase II (nuclear maturation) before the sperm penetration and its activation at fertilization (Roth and Hansen, 2005).

According to Viaris de Lesegno *et al*. (2008), oocytes and *cumulus* cells in dogs show cell-to-cell communications before ovulation. However, 3 days after ovulation, this communications are disrupted. According to Rodriguez and Farin (2004), the reduction in GAP junctions is chronologically related with oocyte maturation, and therefore, these junctions play an important role in the coordination of nuclear and cytoplasmic maturation.

Studies have shown that while COCs (cumulus oocyte complex) from bitches in anestrus have closed GAP junctions, COCs from bitches at the end of proestrus have 89% of opening in communicating junctions (Luvoni *et al*., 2001). This finding could

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Received: July 4, 2012
Accepted: August 5, 2012

justify low maturation rates of oocytes from bitches in anestrus, even after a culture period of 72 or 96 h.

According to Otoi *et al*. (2000), better oocyte maturation rates during proestrus and estrus, may be a result of greater oocyte diameters found in these stages of the reproductive cycle, as this factor is directly associated with meiotic competence.

The morphology of the COCs and the oocyte diameter are parameters used to evaluate competence level. The oocyte diameter is an important characteristic that significantly affects the development of oocyte competence in canine species. Otoi *et al*. (2000) classified oocytes in three groups of different diameters (>100 μm; 100 μm; <100 μm), and reported that only oocytes >100 μm (20%) advanced towards MII phase as compared to smaller oocyes (4-10%). According to Hewitt and England (1999), the larger the oocyte, the higher its ability to advance towards metaphase I, anaphase I and metaphase II, by breaking the germinal vesicle. In addition, diameters higher than 120 μm have higher competence to reach the final stages of maturation, which is similar to findings reported for other domestic species.

Oocyte density may also affect meiotic competence. A study by Otoi *et al*. (2002) reported that inhibition of resumption of meiosis occurs under conditions of high numbers of oocytes per volume of maturation medium. According to these authors, this fact could be attributed to the presence of inhibitory factors produced and secreted by cumulus cells.

The influence of the stage of the estrous cycle on oocyte maturation is still being investigated and results have been controversial. Studies have shown that oocytes from preovulatory follicles complete nuclear maturation more successfully than those from other stages of the cycle (Songsasen and Wildt, 2007). According to Martins *et al*. (2007), the high percentage of oocytes collected from females during estrus to progress through meiosis is probably due to exposure of these oocytes to a follicular environment conditioned by estradiol, progesterone and other factors still unknown. Goretti *et al*. (2011) reported that, unlike results of previous studies, supplementing culture medium with serum of bitches in estrus, the oocytes of bitches in anestrus reached metaphase II, and a high maturation rate (47.7%) was obtained. According to Feldman and Nelson (2004), the physiological anestrus is not a quiescent stage of the estrous cycle regarding endocrine variables. During this phase there are occasional pulses of LH secretion, increases in FSH concentration, and fluctuations in estrogen levels, while progesterone serum concentrations remained low. This condition may suggest that oocytes from bitches in anestrus are also under the influence of critical substances for their development, and therefore, they are likely to succeed during the process of *in vitro* maturation.

The age of the oocyte donor bitch has a direct effect on the number of recovered oocytes, and the mean rate of recovery decreases about 4 COCs per year (Rodrigues and Rodrigues, 2003). According to Durrant *et al*. (1998), the ovaries of prepubertal canine females have a decreased number of follicles in advanced stage of development and an increased number of degenerating follicles. Females aged more than 7 yr have impaired ability of oocyte maturation as compared to younger animals. According to Lopes *et al*. (2007), both the number and the morphological quality of recovered oocytes are influenced by animal age, and the most suitable donors are females aged between 1 and 3 yr.

The number of parturitions in bitches has also been considered as one of the parameters to evaluate the potential of oocyte recovery. According to Hossein *et al*. (2007), the recovery rate of oocytes is higher in multiparous as compared to nulliparous bitches (94% *vs*. 86%), suggesting that this factor could be used to select donors.

Health conditions of donor females seem to have no influence on maturation rates. Studies by Rodrigues and Rodrigues (2003) reported that oocytes from bitches with pyometra reached MII with rates similar to healthy bitches. Similarly, Hishinuma *et al*. (2004) reported that the severity of clinical symptoms presented by females with pyometra had no influence on the number of oocytes recovered.

Concerning *in vitro* time of culture, no conclusive findings have been reached. Studies on IVM in bitches reported periods of maturation varying from 24 to 120 h (Luvoni *et al*., 2005).

Culture systems and media

The composition of a maturation medium has to meet some requirements such as, to contain substances that are alike in nature and concentration to those secreted by the uterine tube cells, to contain a source of supplemental protein, to have hormones such as gonadotropins, and occasionally growth factors, to contain antibacterial and/or antifungal substances. Today, the most used media are the synthetic oviduct fluid (SOF; Bolamba *et al*., 2002), and the tissue culture medium TCM-199 (Rodrigues and Rodrigues, 2003) supplemented with proteins, hormones, antioxidants and growth factors (Ribeiro *et al*., 2010).

The TCM-199 medium has to be supplemented with steroids and other substances that favor oocyte development, such as bovine fetal serum (BFS), bovine serum albumin (BSA) and serum from estrus bitches. According to Bolamba *et al*. (2002), the use of these protein sources increases overall survival of oocytes, as they undergo maturation and subsequent fertilization, while undesirable changes in the zona pellucida are prevented, and adherence of *cumulus* cells to the oocyte is favored.

The SOF medium is not very successful in IVM of canine oocytes (Machado, 2007). Hewitt and England (1999) reported unsatisfactory results obtained

from oocytes of bitches cultured in this medium. According to these authors, the composition of SOF medium, that was developed based on the characteristics of other species, may be inadequate for bitches. Unique characteristics that may influence the success of maturation include the level of proteins, carbohydrates and bicarbonate ions as a source of carbon dioxide to maintain the pH. In contrast, Evecen *et al*. (2010) compared TCM-199 and SOF and reported a relative increased MI/MII rate when SOF medium was used (42.3 *vs.* 15.2%).

Hormonal factors

Luvoni *et al*. (2005) reported that oocyte development is dependent on hormonal stimuli. Oocytes used in IVM are generally from immature follicles, which were not exposed to the effects of hormones. Therefore, according to these authors, the presence of gonadotropins in the medium is essential. Goretti *et al*. (2011) used the serum of estrus bitches associated with TCM-199 medium and found a high rate of oocytes reaching the metaphase II stage (47%) after 96 h of culture.

Several authors studied the effects of supplementation with estrogen, progesterone or the combination of both (Hewitt and England, 1999), addition of serum from estrus bitches (Nickson *et al*., 1993), equine chorionic gonadotropin (Hanna *et al*., 2008, and gonadotropins (Hewitt and England, 1999) on oocyte maturation.

In dogs, the immature oocytes inside the follicles are exposed to high levels of estrogen and progesterone due to the preovulatory luteinization of follicles. Following ovulation, the canine oocytes are yet exposed to high levels of progesterone and estrogen in the bursa and tube. This fact suggests that these hormones play an important role in the resumption of meiosis and progression to the stages of MII (Metcalfe, 1999).

Growth factors

The epidermal growth factor (EGF) and the insulin-like growth factor (IGF) are among the main growth factors studied (Ribeiro *et al*., 2010). Other factors such as fibroblast growth factor (FGF), growth differentiation factor- 9 (GDF-9) and bone morphogenic protein 15 (BMP-15) have already been tested during *in vitro* maturation, and oocyte competence was improved (Gonçalves, 2007). The insulin-like growth factors I and II are members of a family of peptides related to insulin and may participate in oocyte maturation, ovulation, implantation and embryogenesis. In addition to mitogenic effects on cell metabolism and growth, these factors have also paracrine and autocrine effects on cell proliferation. However, despite the effects described, the increase in IGF in the maturation media of canine oocytes had no positive effects, as shown in a study by Oliveira *et al*. (2009), in which TCM-199 medium supplemented with 100 ng/ml of IGF-I was used. In this study MII rates were about 3.4%.

Use of antioxidants

A study by Pires (2006) reported that cysteine and cysteamine supplementation had no positive effect on canine oocyte maturation. Cysteine used as a substrate for glutathione is a very unstable amino acid and is rapidly oxidized to cysteine. Therefore, the 0.1 μm concentration of cysteine added to medium, either isolated or associated to cysteamine, could have not been enough to maintain glutathione production, due to the high oxidative stress during the maturation process. Hossein *et al*. (2007) evaluated the effects of adding cysteine and cysteamine on IVM of canine oocytes. Although these compounds have no effect on the resumption of meiosis, they provided better culture conditions because of their antioxidant properties. Moreover, they influence positively the synthesis of DNA in the cumulus cells, which could in turn aid oocytes that resumed meiosis to reach the MII stage.

Meiosis inhibitors

The mitogen-activated protein kinase *(*MAPK*)* pathway is activated during meiotic maturation, and acts on the phosphorylation of several substrates including transcription factors and proteins of the cytoskeleton (Roux and Blenis, 2004). It is believed that extending meiotic arrest by temporary blocking nuclear maturation, using a technique named "prematuration culture", it is possible to promote synchronization between nuclear and cytoplasmic maturation (Chaves *et al*. 2010). Keeping the oocytes blocked would allow mimicking the period of capacitation. This blockage would allow time for the oocyte to undergo structural and biochemical changes (transcription of mRNA, protein changes, relocation and modification of organelles), which are needed for the oocyte fertilization, and successful embryo development. The mechanisms involved in the process of arrest and restarting of meiotic divisions have not been elucidated, but studies have demonstrated that they are modulated by several molecules (Tosti, 2006), which have either an inhibitory or stimulatory effect on oocyte maturation (Aktas *et al*., 2003). Thus, meiosis may be arrested either by drugs that maintain high concentrations of cAMP in the oocyte, by non specific inhibitors of protein synthesis and activity of protein kinases (Meinecke *et al*., 2001) or by specific inhibitors of Maturation-Promoting Factor (MPF; Hashimoto *et al*., 2003).

A study by Hanna *et al*. (2008) reported that butyrolactone proved to be effective and reversible in canine oocytes resuming meiosis. In this study,

roscovitine was also found to be successful as a meiosis inhibitor and in synchronizing the germinal vesicle breakdown of canine oocytes. According to Ponderato *et al*. (2001) the reversible inhibition of meiosis by butyrolactone-I and roscovitine could be explored using a double system of prematuration culture with inhibitors followed by maturation, so that oocyte competence would be increased.

Final considerations

In vitro maturation and fertilization are biotechnologies for embryo production and preservation of domestic and endangered species. Current protocols used for canine species are adaptations from research conducted for other species. Although several attempts have been made to improve culture conditions, maturation rates are still low. Studies are being conducted to find combinations of protein, hormone, growth factor and meiosis inhibitor supplements to establish an ideal composition of a maturation medium specifically designed for the canine oocyte, with the ultimate aim of increasing oocyte development competence.

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