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Review

The *in vitro* culture supplements and selected aspects of canine oocytes maturation

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Abstract

The maturation of oocytes is one of the most important steps determining their developmental competence. Due to the low percentage of oocytes of bitches that reach the MII stage, searching for reagents that may stimulate the growth and maturation of oocytes is still present in this species of mammals. The most important media supplements include gonadotropins (LH, FSH, hCG), growth factors (IGF, TGF, EGF, FGF), progesterone and follicular fluid. It is suggested that the supplement of EGF, and/or follicular cells may have an important influence on the percentage of cells that reach the MII stage. Despite plenty of research based on the improvement of bitch oocytes *in vitro* culture, the results obtained are still unsatisfactory. Moreover, in the long stages of canine oocytes maturation many molecular and morphological modifications (including changes in mitochondria structure and configuration in the cytoplasm) are involved.

In this article, the influence of selected media supplements on the efficiency of bitch oocytes in vitro maturation was described. The molecular and morphological modifications during canine oocytes maturation were also considered in the text.

Key words: *In vitro* maturation, canine, IVM, IVF, oocyte.

Introduction

Domestic bitches belong to non-seasonally monoestrus species of mammals. The normal estrous cycle is divided into several stages, such as: proestrus, estrus, diestrus and anoestrus with a specific feature of dominant role of progesterone. The inter-estrus intervals include the time between the onset of proestrus to the next proestrus and averages 6-7 months with a range of 5-12 months (Concannon 2011). In case the interestrus intervals are longer than average the bitch has low possibility to be pregnant. The nor-

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mal (physiologic) estrous cycle with proper interestrus intervals has a beneficial role in breeding programs in a domestic bitch which is of increasing economic significance. Moreover, well prepared IVC programs will help to save many endangered species including native lineages (Castroviejo-Fisher et al. 2011).

In vitro culture (IVC) systems differ between each species of mammals in the type of various media application as well as with regards to different supplements (Hewitt et al. 1998, Rodrigues et al. 2007, Bukowska et al. 2008). Various media supplements lead to different efficiency of *in vitro* maturation (IVM), which is presented by different species specific number of oocytes that reach the MII (metaphase II) stage. In some species of mammals, the IVM efficiency is quite satisfactory, although in others, including dogs, the efficiency, regardless of supplements used, is still very low. Since IVM is the most important step before successful monospermic fertilization and proper embryo growth, there is high requirement to improve IVC systems, following *in vitro* production (IVP) procedures.

For over 30 years, since Mahi and Yanagimachi (1976) first described IVM and IVF systems using canine oocytes, increasing evidence has been provided for improving higher maturation efficiency. However, there is a pancity of data indicating the production of canine blastocyst *in vitro* as well as only one paper reports not a full term pregnancy in canine after IVF (Otoi et al. 2000, England et al. 2001, Mrazek and Fulka 2003). Dogs with regard to the specificity of stages of oestrus cycle (a dominant role of progesterone) as well as the morphology of female gamete (dark cytoplasm, compact cumulus) are recognized as highly specific species. Therefore, specific IVC conditions must be used to successful treatment of canine oocytes following IVM, IVF and IVP procedures (Rodrigues et al. 2006). The most frequently used media supplements that may be used in canine oocytes cultivation to modify the IVM condition are: luteinizing hormone (LH), follicle- stimulating hormone (FSH), human chorionic gonadotropin (hCG), progesterone and growth factors: insulin-like growth factor 1 (IGF-1), transforming growth factor alpha (TGFα), transforming growth factor beta (TGFβ), epidermal growth factor (EGF), fibroblast growth factors (FGF), activin (Lim and Hansel 2000, Dadi et al. 2007, Lin et al. 2009). It has been suggested that applying these supplements may improve the IVM efficiency, although there are many contradictory reports which suggesting that not always application of a single supplement may increase the number of oocytes that reach the MII stage. In many of these cases, only the combination of several media supplements may have a positive effect on maturation. However, taken together, results of many studies that present the use of a single or a combination of supplements are often contradictory in canine species as compared to other mammals. It may be suggested that the application of these media supplements may has an effect on IVM of oocytes in species specific manner, which may be determined by the specificity of oestrus cycle stages as well as various hormones and their receptors activity during these stages. In relation to the endometrium, there is a term of different receptivity of uterus to embryo attachment during implantation. A similar mechanism may determine the oocytes receptivity to each hormone or growth factor that may have various effects on oocytes maturation. Additionally different molecules (in most cases there are polypeptides) may extent different effects on signal metabolic pathways that lead to reach a full developmental competence by oocytes (Rodrigues and Rodrigues 2003).

The role of selected media supplements in canine oocytes maturation *in vitro*

The effect of estrous stage on *in vitro* canine oocytes meiotic competence was investigated in many studies eg. Nakao et al. 1985, Weilenmann et al. 1993). They used oocytes collected from donors at different physiological stages of estrous, such as: follicular (proestrus and oestrus), luteal (dioestrus) or anoestrus, with recognized pregnancy and oocytes collected from pathological conditions of pyometra. After collection the evaluation of a possible ability of oocytes to maturation *in vitro* has been performed. Canine oocytes were cultured in TCM 199 media supplemented with 0.5 mg/ml FSH and 0.03 IU/ml hCG (Santos et al. 2006). Studies reveled that *in vitro* nuclear maturation was not influenced by the reproductive status of donor females and that the hormonal environment of *in vitro* culture was not a reliable marker of oocytes maturation competence. It may be suggested that the quality of oocytes recovered from the bitches is a more important indicator of following stages of proper IVM than the addition of gonadotropine hormones into the culture media (De Los Reyes et al. 2005). Similar results were obtained by Luvoni et al. (2001), who compared different *in vitro* meiotic competence and reaching MII stage by canine oocytes isolated from donors at various oestrus stages. In this experiment, they used bitches at anoestrus or late pro-oestrus as the experimental model. In addition, they focused on the presence of gap junction communication pathways as the indicator of maturation ability of oocytes, after microinjecttion of a 3% (w/v) Lucifer dye yellow solution into female gametes.

These experiments showed that in oocytes recovered at anoestrus there were no gap junction connections between the cumulus cells and oocytes. Moreover these COC's (cumulus oocyte complex) had a low ability to reach MII stage and to go through proper IVM stages, since no communication between these somatic cells and oocytes exist and no transport of any molecule is possible. However, visible gap junction connections were detectable in COC's collected from bitches at proestrus. Thus, these cells have increased the ability to reach the MII stage. In the same experiment, the authors cultured the canine COC's in media supplemented with 0.3% (w/v) BSA, FSH (0.5 IU/ml) and LH (0.5 IU/ml). However, no significant relationships was found between the oocytes isolated from anoestrus and proestrus donors. It may be concluded that the application of media supplements, such as gonadotropins in the assessment of IVM efficiency should be considered in future investigations. Moreover, the possible use of combination of several other hormones with the addition of gonadotropins must be included (Rodrigues et al. 2009). Similar, however in some parts contrary, results were first presented by Bolamba et al. (1998). They showed that oocytes isolated form preantral and early antral follicles and then cultured *in vitro* in media supplemented with FSH (1 μg/ml), hCG (10 μ/ml) and E2 (1 μg/ml) may successfully reach the MII stage and they are full meioticaly competent to resume meiosis. Bolamba et al. (2006) investigated the role of EGF and additional hormones (LH, FSH, and estradiol-17beta) in granulosa expansion and meiotic competence of canine oocytes. In the experiment, they used in vitro culture system (F-12/DME medium with 20% fetal bovine serum (FBS), 2 mM glutamine and 1% antibiotic-antimycotic) with combination of (I) EGF (5 ng/ml), (II) FSH (0.5 mg/ml) + LH (5 mg/ml) and (III) FSH + LH + estradiol-17beta (E2, 1 mg/ml). The study revealed that EGF supplementation led to a higher granulosa expansion as compared to that found in other experiments groups. When using the EGF in combination with other hormones, the number of oocytes reaching the GVBD-MII (germinal vesicle break down-metaphase II) stage was increased. These results, contrary to the previous ones, suggest that the combination of EGF with LH, FSH and E2 may support the efficiency of IVC system with special relation to the number of oocytes reaching the MII stage. However, no significant differences were found between the ability of oocytes to reach the meiotic competence and the type of follicles form which the COC's were recovered. In other experiments, Vannucchi et al. (2009) investigated the role of E2 and human somatotropin (hST) on canine oocytes IVM efficiency and meiotic progression. They cultured the bitch COC's in maturation medium (TCM-199) supplemented with different concentrations and combinations of these hormones; (I) E2 (1 mg/ml) E2 and/or (II) E2 (20 mg/ml) E2 + hST (1 mg/ml). After IVM the oocytes were *in vitro* fertilized and then cultured in synthetic oviduct fluid (SOF) medium with BSA 4 mg/ml. They showed 10.1% embryos that undergo developing to the early stages. Moreover, the cleavage rate was similar among the oocytes recovered from the groups of donors (at the follicular, anestrus, and luteal stages), although the pronuclear development was significantly increased in the oocytes collected from bitches at the follicular phase (Vannucchi et al. 2009). Similar to previous results they found that the supplementation of IVC media with hST (1 mg/ml) and E2 (20 mg/ml) led to increasing the number of canine oocytes that reached the meiotic competence. The number of oocytes that reach MII stage was significantly higher when the media supplements were applied, although the cleavage rate did not differ statistically among the groups investigated. In other research, Alhaider and Watson (2009) determined the developmental competence of canine oocytes after maturation with media supplemented with hCG and growth factors. In this experiment, the canine COC's were cultured in a basic tissue culture medium supplemented with 0.3% BSA and P4 (7 mg/ml). Moreover, they cultured the COC's with the supplementation of hCG and selected growth factors, such as: GH, IGF-1, TGF-α and FGF. They showed an increased number of oocytes reaching the MII stage and decreased the degeneration rate after maturation of COC's in media supplemented with hCG. Furthermore, the supplementation of media with growth factors (mentioned above) improved the number of oocytes that resume meiosis and reduce significantly the degeneration rate. They also found that the strong effect of growth factors may be raised by the addition of hCG into the culture medium. These impressive results first described the strong effect of growth factors on the meiotic competence of canine oocytes (49% of total oocytes resume meiosis) (Alhaider and Watson 2009).

The effect of EGF on meiotic competence of canine oocytes as well as the expression of maturation related genes, such as: EGF receptor (EGFr), luteinizing hormone receptor (LHr) and gap junction protein h 5 (GJA5) was investigated by Song et al. (2010). As the culture system they used the standard medium (TCM-199) with supplements: 10% FBS, LH (10 μg/ml) and FSH, and different concentrations of EGF (0, 10 and 30 ng/ml). The study showed that the number of oocytes that reached the MII stage was significantly higher in medium supplemented with 10 ng/ml of EGF compared to that observed in media

supplemented with 0 and 30 ng/ml. They did not find the expression of EGFr in both cumulus cells and oocytes, the level of LHr expression was decreased, although the expression of GJA5 was significantly increased. These results suggested that the supplementation of medium with EGF in concentration 10 ng/ml led to the higher ability of canine oocytes that reached the meiotic competence. Moreover, the GJA5 may be used as a marker of this ability. However, the EGFr and LHr expression is not associated with the maturation ability of canine oocytes. Kim et al. (2010) described the effect of gonadotropine hormones (hCG, equine chorion gonadotropine- eCG) on canine oocytes maturation *in vitro*. In the first experiment, they treated the *in vitro* cultured cells with hCG and eCG separately. In the second experiment the combination of both of these hormones, was used. The investigations reveled indicated that there was no significant differences between the number of oocytes that reached the MII stage between the hCG and eCG groups. However, the authors observed an increased number of cells that reached the full meiotic competence when a combination of both hCG and eCG was applied, as compared to that found in groups treated with each hormone separately.

Gonadotropine hormones, especially FSH and LH, play an important role in cumulus expansion and resuming of meiosis in oocytes of several species of mammals. Lee et al. (2007) researched the role of these hormones in canine oocytes maturation and then their influence on meiotic competence. They cultured the COC's in medium supplemented with FSH or LH at different concentration (0.5, 5 or 50 mg/ml) for 72 h. Similar to the previous results they found that the cumulus expansion was increased in the group of cells treated with 5 mg/ml of FSH as compared to that observed with the other two concentrations of this hormone. However, they did not find differences among the LH treated group (used in the same concentrations). Similarly, the number of cells that reached the MII stage was higher in the group of 5 mg/ml of FSH as compared to others, however there was no differences among the LH groups.

The role of oestrus cycle (follicular, luteal or anestrous stages) and the influence of E2 or P4 supplementation on *in vitro* meiotic competence was investigated by Kim et al. (2005). They cultured the canine COC's in different concentration of E2 (0, 0.1, 1.0 or 2.0 mg/ml) and/or P4 (0, 0.5, 1.0 or 2.0 mg/ml) for 72 h. Additionally, they treated the oocytes with the combination of both of these hormones 2 mg/ml E2 and various concentrations of P4 (0, 0.5, 1.0 or 2.0 mg/ml). The study revealed the increased number of MII oocytes isolated from donors at the follicular stage of oestrus with the addition of 2 mg/ml of E2. The MII rate was also higher in oocytes supplemented with 1.0 and/or 2.0 mg/ml of P4 as compared to that found in other groups. Moreover, the combination of E2 and P4, used in the concentration 2.0 mg/ml, support the number of oocytes with full meiotic competence as compared to that observed with E2 alone. However, the addition of 2 mg/ml E2 + 0.5 mg/ml P4 decreased the maturation rate. These results suggested that supplementation of E2 or P4 alone significantly increased the number of oocytes that reached the MII stage and the use of both E2 and P4 in a combination led to the low IVM efficiency.

Taking into account all the results mentioned before, it may be suggested that in canine species there is a special requirement for oocytes culture conditions, which may be a species specific phenomenon. It may be a result of specificity of the oestrus cycle in this species and associated with this a different hormonal activity of gonadotropins and progesterone. It may be concluded that the higher MII rate is achieved when a combination of several hormones and the addition of growth factors in canine oocytes *in vitro* culture are applied. However, not always the combination of several hormones leads to the higher rate of oocytes reaching the MII stage. This is especially related to the use of combined E2 and P4 in a combination. Using both of these hormones in a single culture system suggested that the IVM efficiency is in the progesterone specific concentration dependent manner.

Selected aspects of canine oocytes maturation with relation to the molecular and morphological changes

In connection with differences in specific of oocyte maturation there are many problems with effective culture of these gametes (Suzukamo et al. 2009). Until now there have been many studies performed on maturation and related with rearrangements within the oocyte and cumulus cells using *in vitro* model. Suzukamo et al. (2009), using mitogen, activated kinase (MAPK) and p34 kinase, typical markers of development demonstrated that the optimal oocyte culture time is 72 h, because at this time MAPK and p34 reveled the highest activity. In contrast to these results, culture performed with the combination of chemical and biological agents allowed to obtain oocytes in meiosis II after 48 h (Hanna et al. 2008).

Chebrout et al. (2009). and Otoi et al. (2007) obtained the same 72 h optimal culture time in studies focused on the influence of separation of cumulus cells from oocytes. Additionally, they have shown, using confocal and electron microscopy, that denuded oocytes have the same degradation levels and both

irregular and seldom patterns of organelles distribution with smooth endoplasmatic reticulum aggregates in the cortical zone as compared to the control (functional COCs). Cells that were completely denuded showed a better maturation level described as the resumption of meiosis. Studies on the influence of cumulus cells in COCs performed by Rodrigues et al. (2009) have shown that in spite of the morphological quality of cumulus cells that can give some valuable information about oocyte maturation, the influence of P4 dose *in vivo* does not give such information. The localization of organelles was studied also by De los Reys et al. (2011) where patterns of mitochondria distribution were analyzed. Using fluorescence microscopy images of three different patters that refer to three groups of mitochondria (germinal vesicle oocyte, ovulated oocyte and cultured after 72 h) were obtained. Results suggested that most of oocytes cultured *in vitro* refer to the pattern characteristic to ovulated oocytes. Additionally, the authors concluded that the pattern of distribution can give information about a degree of nuclear development but is not associated with the ability of resumption of meiosis.

On the other hand, De los Reyes et al. (2009) have shown that the condition of *zona pellucida* also refers to the development of oocyte. Using scanning electron microscopy they compared immature oocytes (obtained by ovariohysterectomy) with *in vitro* cultured gametes and showed irregularities that indicate the influence of culturing on oocyte maturation. Additionally, the phase of the estrous cycle also gives information about the quality of gametes as previously shown by Lee et al. (2008) and Otoi et al. (2001).

In spite of many relationships between factors that are important for appropriate oocytes maturation it has to be pinpointed that there is a possibility to obtain morphologically approval oocytes from bitches at various age and under pathological conditions, such as pyometra (Hshinuma et al. 2004) by using a morphological system based on physical factors proposed by Diagone et al. (2008). On the other hand, the quality assessment of oocytes should be based not only on the core maturation but also on the cytoplasmatic maturation because they are not related to each other (Nickson et al. 1993, Viaris de Lesegno et al. 2008).

The influence of induction and synchronization of the estrous in canine species on quality of oocytes for IVM procedure

There are several reports indicating the role of media *in vitro* supplements on the meiotic maturation competence of canine oocytes (Otoi T et al. 1999, Rodrigues and Rodrigues 2003, Cui et al. 2006). However, the *in vitro* maturation procedures still bring low efficiency and only 15-20% of bitch oocytes reach the MII stage. Therefore, exogenous treatment, such as estrus induction may provide new results in research related to canine COC's *in vivo* maturation. The estrus induction belongs to good models for research related to canine reproductive disorders, study of pregnancy and teaching specific features of canine reproduction. The stages between early to late anoestrus is associated with increased pulse of GnRH, as well as increased sensitivity of pituitary to GnRH and ovarian response to higher concentration of LH and FSH (Jeffcoate 1993, Van Haaften B et al. 1994, Tani et al. 1996). The FSH serum concentration is increased during anoestrus although LH is low and increased only at the end of this phase (Concannon 1993). It was proved that serum FSH and LH induced ovarian folliculogenesis, stimulated the expression of LH receptors in granulosa cells and improved the COC's ovarian maturation (Monniaux et al. 1997, Kooistra et al. 1999, Onclin et al. 2001). Since secretion of pituitary FSH and LH stimulate *in vivo* canine COC's maturation, many of *in vitro* experiments include these hormones as the basic media supplements in IVM procedures. However, in this article we presented data which showed that FSH or LH may have no stimulatory effect on IVM of canine COC's. Moreover, several other supplements used alone or in combinations may have a beneficial effect on bitches COC's IVM. Therefore, to focus on canine IVM "enigma", the *in vivo* maturation condition have to be mimic.

Perspectives

Despite many efforts devoted to developing high efficiency *in vitro* culture systems for canine species, the number of oocytes available for reproduction is still low and there is no evidence for successful production of canine offspring using IVP. It is clear that there is still a need to improve and develop new systems for effective culture of canine oocytes. However, cell culture studies may be supported in this field by molecular analysis of oocyte developmental potential. These analyses could precisely link the stage of estrous cycle and effect of culture supplementation with condition of oocytes. Vast genome research in combination with completely new methods of real time imaging like microfluidics lab-on-chip analysis may bring last piece of puzzle which have been thought to be missing for 60 years.

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