BIOTECHNOLOGY IN CANINE REPRODUCTION: AN UPDATE

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Abstract : *Canine biotechnology studies are far less developed than in other species. Canine reproduction and gametes have unique characteristics compared to other mammals which makes adaptation of knowledge from other species difficults. Culture media for oocytes with or without serum, hormonal or protein supplementation, and oviductal cells have been used for* in vitro *maturation. Age and phase of the estrous cycle of the donor, oocyte size and nuclear and cumulus morphology influence* in vitro *maturation rates. Canid oocytes can be fertilized and developed* in vitro*, but at a reduced rate and to a limited stage of embryo development. Embryo transfer has shown to be possible but with low success in both dogs and foxes. Additional refinement of freezing regimens, improvement of donor recipient synchronization, handling of embryos and transfer techniques are still necessary. Canine spermatozoa can be capacitated and undergo acrosome reaction* in vitro*. Various procedures to cryopreserve canine spermatozoa have been described and differences in cooling and freezing sensitivity have been observed among* Canidae*. Intravaginal or uterine artificial inseminations can be successfully performed in both dogs and foxes achieving high whelping rates. During the last five years research on canine biotechnology has substantially increased and most of the reproductive mysteries of these species will probably be unveiled in the near future.*

Key words: Canidae- biotechnology- reproduction- dog

BIOTECNOLOGÍA EN LA REPRODUCCIÓN CANINA: UNA ACTUALIZACIÓN

Resumen: *Los estudios en biotecnología canina están menos desarrollados que en otras especies. Las gametas y la reproducción canina en general tienen características únicas comparadas con las de otros mamíferos lo que hace que la extrapolación de otras especies sea dificultosa. Para la maduración* in vitro *de oocitos se han utilizado medios de cultivo con o sin suero, suplementación hormonal o proteica, y células oviductales. La edad y la fase del ciclo estral de la donante, el tamaño del oocito y la morfología del cúmulus y núcleo influencian la tasa de maduración* in vitro*. Los oocitos de Cánidos pueden ser fertilizados y desarrollados* in vitro*, pero en una tasa reducida y a una etapa limitada del desarrollo embrionario. La transferencia embrionaria ha sido posible pero con bajo éxito en perras y zorras. Es necesaria todavía, la puesta a punto de regímenes de congelación, el mejoramiento de la sincronización de celos entre donantes y receptoras, el manejo de embriones y de las técnicas de transferencia. Los espermatozoides caninos pueden ser capacitados y experimentar reacción acrosómica* in vitro*. Se han descrito varios procedimientos de congelación de espermatozoides caninos y se ha observado diferencias en la sensibilidad al enfriado y congelado entre cánidos. La inseminación artificial intravaginal o uterina se puede realizar con éxito en perras y los zorras alcanzando altas tasas de preñez. Durante los últimos cinco años la investigación sobre biotecnología canina ha aumentado sustancialmente y la mayoría de los misterios reproductivos de estas especies serán revelados probablemente en un futuro cercano.*

Palabras clave: cánidos- biotecnología- reproducción- perro

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INTRODUCTION

In general all techniques to manipulate gametes outside the body i.e. *in vitro* are called reproductive biotechnology. Assisted reproductive technology takes on many forms, from simple assisting a natural mating under controlled settings to cloning of adult animals (1). Reproductive biotechnologies such as artificial insemination (AI), *in vitro* maturation (IVM) and fertilization (IVF), *in vitro* embryo production (IVP), embryo transfer (ET) and gamete cryopreservation are essential to improve reproductive performance, to preserve biodiversity and to develop basic research. Research in this area will permit the use of assisted reproductive methods in commercially or affectively valuable animals. *In vitro* fertilization, IVP and ET are powerful tools to study fertilization and to preserve genetic material.

Canine biotechnology is much less developed than in other animal species, this may be due to a number of reasons being the lack of commercial interest probably the most important. Furthermore, canine female gametes have unique characteristics compared to oocytes of many other domestic mammals. The main differences are represented both by the follicular environment and the oocyte meiotic stage at ovulation. These characteristics have complicated the adaptation of biochemical knowledge gained from other species. In practice, assisted reproductive techniques have been mainly limited to AI and semen cryopreservation in these species and the more advanced technologies have been limited to research laboratories.

Presently, the domestic dog is not only considered a pet but a useful experimental model for the study of other canine species. Interestingly, foxes have also been useful models for canine gamete and embryologic research. Nine wild canids, including the Mexican wolf *(C. lupus baileyi*), South America Sabannah Dog *(Speothos venaticus)* and the maned wolf *(Chrysaocon brachyarus)* are considered endangered by the Convention of the International Trade in Endangered Species (2). Therefore, the need for strategies enhancing conservation of these species is increasing. The aim of this article was to review the present situation of assisted reproductive technologies in canids with special emphasis on the domestic dog.

Canine reproductive physiology

Canine reproductive physiology has unique characteristics. Canids are monoestrous, with a seasonality nearly lost through domestication in the dog but present in wild species i.e. wolves and foxes (3, 5). Canine estrous cycle is characterized by the slow motion of its phases (6) and a long obligate anestrus, which can be normally as long as 11 months in certain breeds (5).

Although neither canine ovary nor pituitary is quiescent during anestrus, it has been less described than the other phases of the estrous cycle. Anestrus is characterized by a slow decline of plasma progesterone concentrations to basal values (7). Gonadotrophin secretion during the transition from early to late anestrus is also controversial in this species (7, 8, 9). Recently, it has been shown that progression from early to late anestrus is associated with an increase in plasma follicle stimulating hormone (FSH) without a concomitant rise in luteinizing hormone (LH) (10, 11). Finally, prolactin concentrations slowly decrease throughout anestrus to reach basal values around the beginning of the new estrous cycle (12).

Preovulatory luteinization of follicles occurs in bitches and vixens, exposing oocytes to high concentrations of progesterone, as opposed to the situation in many mammalians, where estrogens dominate the periovulatory follicular environment. Ovulation occurs 1-2 days after LH surge in both dogs and foxes (13).

Oocytes are ovulated spontaneously as primary oocytes, at the beginning of the first meiotic division and the germinal vesicle is broken shortly after ovulation**.** Subsequent stages of meiotic maturation are resumed in the oviduct and take 2 or 3 days to be completed (14).

Fox and dog oocytes appear morphologically similar (14, 15, 16). The ooplasm is very dark and uniform due to the high lipid content and the cumulus cell mass around the oocyte is tight and multilayered and remains attached to the gamete after fertilization (3, 17). Cumulus expansion is observed as oocytes mature but the innermost layer remains attached to the ovum until the morula stage in dogs (17), while complete cumulus expansion including the corona radiata, is observed at the completion of oocyte nuclear maturation *in vivo* 2-3 days after LH peak in foxes (16).

The presence of primary oocytes in the oviducts increases the chance of the oocyte meeting spermatozoa before or during maturation, so that primary oocytes can be fertilized and a male pronucleus can be formed despite of the stage of oocyte maturation (18). Canine embryos require a long time passaging of the oviduct and enter the

uterus approximately 7-9 days after ovulation as an embryo of 16 cells or more (14). In the oviducts oocytes complete maturation, undergo fertilization and develop up to the morula – blastocyst stage.

Oocyte maturation

A prerequisite for fertilization and embryo development is full nuclear (metaphase II stage) and cytoplasmic maturation of the oocyte. *In vitro* oocyte maturation is a complex process in which it is attempted to mimic the dynamic changes occurring in the preovulatory ovarian follicle and in the oviduct.

It has been shown that canine oocytes resume meiosis *in vitro*, although at a much lower rate than oocytes of other species. Full nuclear maturation is achieved in about 20 % of cultured oocytes. The low maturation rate could be due to either the low meiotic competence of the oocytes or the suboptimal culture conditions for this species.

Age of the donor, oocyte size and nuclear and cumulus morphology do influence success of IVM rates (19, 20, 21). A difference in maturation rates has been found for oocytes with diameters < 100 mm (22). Similarly, it has been shown that oocytes < 100 mm are meiotically incompetent in foxes (23). Furthermore, oocytes are smaller and have less complete cumulus layers and therefore mature less frequently in prepubertal bitches(19).

In the bitch, the stage of estrous cycle influences the functional status of communications between cumulus cells and oocyte (24). Cumulus oocytes complexes isolated from the ovary during anestrus are unable to complete meiosis and communications and gap junctions are absent (24). Conversely, communications between cumulus cells and oocyte were present in complexes isolated during late proestrus and these cumulus oocytes complexes were able to resume meiosis at a higher rate (24).

Culture requirements for canine oocytes have not been fully elucidated. Tissue Culture Medium (TCM) 199, modified Krebs Ringer Bicarbonate or Ham F-10 with or without serum, hormonal or protein supplementation, and oviductal cells have been used for IVM (25, 26).

Hormone supplementation (LH and FSH) of the culture medium had an effect on the proportion of oocyte maturing (27). Some research groups have not been able to find beneficial effects of adding hormones (19, 28). The maturation rates of oocytes cultured in recombinant FSH treatments was statistically different from control treatment in a recent study (29). Relative amounts of different hormones, their source and interaction may be important.

It was shown that the use of an environment similar to that of the composition to the oviductal fluid with high concentrations of proteins and the presence of oviductal cells improved maturation rates after a prolonged maturation time (96 h) (30). Higher meiotic resumption rate was found in canine oocytes cultured with cells from either the infundibulum or the ampulla of the estrous bitch oviduct compared with culture without oviduct cells (59 % and 60 % vs. 40 %, respectively), and more oocytes progressed to metaphase II in the co-culture systems (28). A synthetic oviduct fluid medium could support nuclear maturation of a small proportion of bitch´s oocytes *in vitro* in one study (31). It was recently found that oocyte culture in isolated ligated oviduct was better (31.9% metaphase I/II) and with fewer degenerate oocytes than culture in open oviduct or drop culture after 30 h (32). It has also been reported that canine oocytes may complete nuclear maturation in protein free media at a very low rate (33).

In foxes, the IVM of ovarian oocytes have resulted in maturation rates similar to those of the bitch (34).

In vitro fertilization and embryo production

Canine embryos have been produced after fertilization *in vitro* or *in vivo* (17, 18). *In vitro*, oocytes matured up to the stage of eight cells (35). IVF was reported to have a cleavage rate of 5- 20 % and pronuclear formation in 20-37 % of oocytes in dogs and foxes (18, 28).

It has been reported the development of one ? embryo of two cell obtained during a study of canine oocyte penetration (35). One blastocyst out of 217 inseminated oocytes was obtained in another study (36). Furthermore, it was reported a 22 day pregnancy by IVF (37), and another one from nuclear transfer/ ET, although no pregnancy went to term (38).

Finally, it was recently obtained a small portion of early canine embryos after culture of *in vitro* produced zygotes in a protein–free medium (33). Fertilized *in vivo* matured fox oocytes, cleaved from the 2 to the 16 cells stages in foxes (39). No pro-

duction of puppies after IVF of neither *in vitro* nor *in vivo* matured oocytes exists in the literature for dogs and foxes.

Sperm testing, capacitation and cryopreservation

Functional tests for dog sperm include techniques for sperm binding assay and sperm penetration assays using entire, hemi-zonae or intact either fresh or cooled oocytes (4). Semen may be collected from fresh or cooled epididymis up to 8 days (41) and be able to bind to homologous zonae in a time dependent manner.

Fresh semen is commonly used for *in vitro* insemination, with the use of special media for capacitation. It has been demonstrated that ejaculated sperm capacitation occurs *in vitro* after 7 hours and that Ca^{++} is essential for this process (42). *In vitro* capacitation may be achieved in Canine Capacitation Medium (CCM) (42) or in a modified Tyrode´s (43). When CCM was used, removal of proteins was detrimental to sperm motility and glucose withdrawal reduced the percentage of acrosome reacted sperm (42). Calcium ionophore A23187 can promote capacitation and acrosome reaction in a similar manner as Ca++ acts *in vitro* (22). Bitch follicular fluid may also induce capacitation of dog sperm (28).

During cryopreservation the spermatozoa have to survive changes in temperature to which they are exposed during the freezing (down to temperature of liquid nitrogen -197 °C) and thawing process. The spermatozoa damage due to these processes depends on the species, the cooling rate and temperature intervals and changes (44). The spermatozoa can be damaged both when they are frozen too slowly or too rapidly. Thawing methods should also depend on the freezing protocol (45, 46). To minimize injuries from the freezing-thawing process, spermatozoa must be diluted in special extenders and cryoprotectants like glycerol (47). Various procedures to cryopreserve canine spermatozoa have been described (13, 15, 47, 48). The most common diluent to cryopreserve canine spermatozoa is Tris fructose- citric supplemented with egg yolk and glycerol (15, 50, 51, 52). Detergents such as Orvus ES paste and sodium dodecyl sulfate added to freezing media have been found beneficial (53, 54). Modifications of the commonly used TRIS-egg yolk extender by the addition of 0.5 % Equex STM paste produced overall pregnancy rate of 84 % after vaginal or intrauterine AI (55).

Procedures used to cryopreserve spermatozoa from non- domestic canids have been modified from those of the domestic dogs (56, 57). Differences in spermatozoa cooling and freezing sensitivity have been observed among *Canidae* (13).

Artificial insemination

Intrauterine AI with frozen semen has mostly proven to yield higher whelping rates than intravaginal. Results from frozen-thawed semen AI have been reported to be up to 80-85 % whelping rate (58, 59). Success rate depends on the quality of the semen, insemination timing and extending, freezing, storing and thawing techniques. Recent studies on dogs have also shown that, results by intrauterine AI are significantly better than those obtained by vaginal AI (58, 59) either for fresh or chilled semen (60, 61, 62).

Intrauterine AI in the bitch can be carried out with a specially designed metal intrauterine catheter (Scandinavian catheter) (62) which is passed through the cervical canal during abdominal fixation of the cervix (63), or by a flexible plastic tube entering with endoscopic visualization (64, 65). Intrauterine AI can also be carried out by laparoscopy, or abdominal surgery, althought ethically objected.

AI is performed with the intrauterine metal catheter mentioned above in foxes (3). Frozen silver fox semen has resulted in 80 % pregnancy rates, while blue fox spermatozoa seem more sensitive to cooling and freezing/thawing. Thus lower pregnancy rates have been reported in this species (3). Differences have been found in the fatty acid composition of the plasma membrane sperm between these two species (66).

Embryo transfer

Embryo transfer from one female that has produced embryos through in vivo fertilization to a recipient has shown to be success but with low success rates in both dogs and foxes (67, 69). In one study in the dog, 8 morulae were transferred to a recipient bitch which gave birth to 2 puppies (70). In another study, 28 embryos were collected on days 14 and 15 from 5 donors and transferred into another five recipients (67). Although embryo transfer has been carried out in foxes, birth of live young from canine embryos has not been reported (68).

Embryo transfer to the lower part of the oviduct, but not to the upper, was carried out with greater success than uterine transfer, since 50 %

of recipient dams became pregnant (71). Transfer to only one side seemed enough, since transuterine migrations occured in dogs (72). Recently, the transfer of *in vitro* fertilized dog oocytes was also reported (73).

Furthermore, the use of ET in canids may increase when *in vitro* produced embryos become available for transfer, or when cryopreserved canid embryos can be stored and transferred to naturally synchronized females. An exception is the report of the successful ET in the silver fox (74).

Embryo transfer requires synchronization of cycles between donor and recipient females, so that the recipient's uterus provide an endocrinological environment similar to the donor´s (71). In farm foxes, due to the seasonality of their oestrus cycle, natural synchronization made embryo transfer possible (74).

Estrous synchronization is a major problem in non seasonal monoestrus species (11). Estrus induction in the bitch has not gained a widespread use because the lack of reliability of most of the protocols proposed (11). In this species estrus induction protocols have been difficult to devise because of the lack of understanding of the hormonal events necessary for folliculogenesis. Moreover, the natural ending cause of the long obligatory anestrus and onset of a new cycle is not clearly understood in this species (18).

The stage of the estrus cycle influences the effectiveness of estrus induction protocols. To provide the best results, protocols should begin during anestrus, estrus induction during diestrus has poor or no results. Even the stage of anestrus has an influence on the response to treatment. As a general rule, treatments instituted in early anestrus are less effective than those initiated in late anestrus (11, 74, 75).

Estrus induction has been traditionally achieved by the administration of gonadotrophic hormones (FSH, equine chorionic gonadotropin [eCG], LH (76); human chorionic gonadotropin [hCG] and human menopausal gonadotropin [hMG]) (77). Efficacy and safety of hormonal protocols may be diminished by ovarian hyperstimulation, ovulation failure, premature luteolysis and antibody formation. Moreover, there may be problems in availability, quality, and consistency of hormone preparations. Dopaminergic agonists are considered reliable compounds for this purpose (75,78). They have been recently used during 12 to 15 days to synchronize oestrus in bitches for ET (49).

Intracytoplasmic sperm injection and cloning

Intracytoplasmic sperm injection (ICSI) involves injecting a spermatozoon into the cytoplasm of a mature ovum. Fertilization can be successful as long as the sperm DNA is intact and in its stable condensed form. This procedure can be useful not only in cases of immotile sperm, but also for epididymal sperm of species threatened by extinction.

Only one experiment of ICSI has been published in the dog with chilled semen achieving the formation of male pronucleous in 7.8 % of oocytes, although no offspring was produced (79).

CONCLUSIONS

The development of assisted reproductive technologies in canids has been very slow. Dissimilarities of reproductive physiology of the dog compared to other species and the lack of information on the oviduct environment make *in vitro* systems applied for other species, unsuitable for canine oocytes (25).

The immature stage of ovulation and the persistence of cumulus cells during the transport and maturation could explain the low efficiency of IVM in these species. Maturation rates have shown limited success, ranging from 0 to 58 % and usually around 20 % in different culture systems and media (3, 13).

Although, much information has been gained during the last 5 years on the factors influencing canine oocyte maturation (3, 49, 80), it still seems to be the limiting step for success with *in vitro* production of embryos, as well as, ICSI or cloning by nuclear transfer.

Reliable systems for *in vitro* production of embryos, embryo cryopreservation and ET are yet to be developed in dogs. Canid oocytes can be fertilized and developed *in vitro*, but at a reduced rate and to a limited stage of embryo development. Embryo transfer would be viable in cases of female gestational infertility but, from what has been described above about the difficulties in canine IVM, it is deduced that the results of IVF and embryo development are still limited in this species.

Some control over the estrous cycle is necessary when any assisted reproductive technologies must be used. Although ET requires synchro-

nization of donors and recipient females, canine estrus induction still represents a challenge for researchers. Currently, procedures to induce estrus or superovulate bitches are relative ineffective.

Additional refinement of freezing regimens, improvement of donor recipient synchronization, *in vitro* handling of embryos and transfer techniques may also render both a cryobanking of embryos and ET feasible in foxes.

Canine semen can be successfully cryopreserved and intravaginal or uterine AIs can be performed in both dogs and foxes. It is today possible to achieve high whelping rates using AI in the dog. Differences in cooling and freezing sensitivity of their spermatozoa have been observed among *Canidae* [13] and more basic research on membrane function during exposure to cooling and freezing regimens and media is still necessary. Canine semen can be capacitated and undergoes acrosome reaction *in vitro* and spermatozoa are able to fertilize homologous oocytes in *in vitro* culture conditions.

Although canine biotechnologies are being developed at a much lower rate than in other species, research in the last five years has substantially increased and most of the reproductive mysteries of this species will probably be unveiled in the near future.

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