

Original article

Current progress on assisted reproduction in dogs and cats: in vitro embryo production

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Abstract — The objective of the development of assisted reproduction techniques in dogs and cats is their application to non-domestic canine and feline species, most of which are considered threatened or endangered. Among these techniques, an entirely in vitro system for embryo production is effectively an important tool for conservation of wildlife. In the last decade, progress has been made in embryo production in carnivores. It has been shown that canine oocytes can resume meiosis in vitro and that these oocytes can be fertilized and developed in vitro, although at a much lower rate than most other domestic animal oocytes. The reason lies in the dissimilarities of reproductive physiology of the dog compared to other species and the lack of precise information concerning the oviductal environment, in which oocyte maturation, fertilization and early embryonic development take place. Successful in vitro embryo production in the domestic cat has been attained with oocytes matured in vitro, and kittens were born after transfer of IVM/IVF derived embryos. On the basis of these results the in vitro fertilization of oocytes has also been applied in several non-domestic feline species. The effectiveness of such protocols in the preservation of genetic material of rare species can be improved by developing better techniques for long-term storage of gametes. In dogs and cats sperm cells have been successfully frozen and the cryopreservation of oocytes would greatly increase their availability for a range of reproductive technologies. Cryopreserved cat oocytes can be fertilized successfully and their development in vitro after fertilization is enhanced when mature oocytes are frozen. Thus refined techniques of oocyte maturation and fertilization in vitro coupled with oocyte cryopreservation could allow for an easy establishment of genetic combinations when male and female gametes in the desired combination are not simultaneously available, and the propagation of endangered carnivores would be facilitated.

canine / feline / in vitro embryo production

Résumé — Progrès récents de la reproduction assistée des chiens et des chats : production in vitro d'embryons. L'objectif du développement des techniques de reproduction assistée chez le chien et le chat ont leur application à des espèces canines et félines non domestiques, la plupart d'entre elles menacées ou en danger. En effet un système de production d'embryons entièrement

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in vitro est un outil important pour la préservation de la faune sauvage. Pendant les dix dernières années, des progrès ont été effectués dans les techniques de reproduction assistée des carnivores. Il a été montré que les ovocytes canins peuvent achever leur maturation méiotique *in vitro* et que ces ovocytes peuvent être fécondés et se développer *in vitro*, cependant avec beaucoup moins de succès que dans la plupart des autres espèces domestiques. La cause tient dans la différence de physiologie de la reproduction entre le chien et les autres espèces et l'absence d'information précise sur le milieu tubaire dans lequel la maturation ovocytaire, la fécondation et le début de développement embryonnaire se produisent. La production d'embryons de chat a été obtenue avec des ovocytes matures *in vitro* et des chatons sont nés après transfert *in vivo* des embryons cultivés. À la suite de ces résultats, la fécondation *in vitro* a été néanmoins appliquée à plusieurs espèces félines non domestiques. L'efficacité de tels protocoles de préservation de matériel génétique d'espèces rares peut être améliorée en développant de meilleures techniques de préservation à long terme de gamètes. Les spermatozoïdes de chiens et de chats ont été congelés avec succès, mais la cryopréservation d'ovocytes augmenterait beaucoup leur disponibilité pour les technologies de reproduction. Des ovocytes cryopréservés de chat peuvent être fécondés et le développement *in vitro* des embryons obtenus est meilleur quand ce sont des ovocytes mûrs qui sont congelés. Ainsi, des techniques perfectionnées de maturation et de fécondation *in vitro* couplées à la congélation des ovocytes pourraient permettre d'obtenir aisément des combinaisons génétiques même si les gamètes mâles et femelles de la combinaison désirée ne sont pas disponibles simultanément, et la préservation d'espèces carnivores menacées en serait facilitée.

canin / félin / production d'embryon *in vitro*

1. INTRODUCTION

The improvement of reproductive performances is the ultimate goal of assisted reproductive technologies (ART) and the main impulse to these studies in carnivores started when the scientific community began to be interested in the conservation of biodiversity. In fact, carnivores have been considered not only as pets, but also as comparative models for studies aimed at non-domestic canine and feline species.

In the last 200 years more than 50 animal species disappeared and more than 200 are considered endangered by the Convention of International Trade in Endangered Species [5]. There are 37 feline species and all of them, besides the domestic cat, are endangered, as well as 9 wild canids.

Placing animals in captivity, even under intense management, does not always resolve the loss and variety of species or ensure that a viable population will be established. Certain species thrive in captivity

and others experience subfertility or infertility [40].

Assisted reproductive technologies as artificial insemination (AI), *in vitro* fertilization (IVF) and embryo production (IVP), embryo transfer (ET) and gamete cryopreservation, are important biotechnologies not only for preserving biodiversity but also for basic research, and improvements of reproductive performances. Among these, IVF-IVP and ET potentially are the most powerful reproductive tools currently available for studying fertilization and preserving genetic material of rare species.

Current progress in assisted reproduction techniques concerns mainly *in vitro* embryo production and the scope of this paper is to provide a review of this aspect of ART in canids and felids.

Successful *in vitro* techniques depend on the possibility to mimic *in vivo* conditions. For this reason knowledge on reproductive physiology is necessary to define an efficient entirely *in vitro* system to produce embryos.

2. IN VITRO EMBRYO PRODUCTION IN DOGS

In canine species the female gamete has 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. In fact, in most mammalian species, estrogen dominates the preovulatory follicular environment and the meiotic division is resumed shortly before ovulation as a consequence of the preovulatory LH-surge. Ovulated oocytes are in metaphase II of the meiotic division and are ready for fertilization.

In dogs, foxes and other canids, ovarian follicles luteinize prior to ovulation, exposing oocytes to high concentrations of progesterone. After the LH-surge, the oocytes are ovulated spontaneously as primary oocytes, at the beginning of the first meiotic division. Subsequent stages of the meiotic maturation are resumed in the oviduct and take 2–3 days to complete [14]. Moreover, the canine oocyte is very rich in lipids and the cumulus cell mass around the oocyte is tight and multilayered and remains attached to the gamete longer after fertilization [37].

The presence of primary oocytes in the oviducts increases the chance of the oocyte meeting spermatozoa before or during maturation and it has been shown in the fox that primary oocyte can be fertilized and a male pronucleus can be formed irrespective of the stage of oocyte maturation [7].

The canine embryos require long time for the passage of the oviduct and enter the uterus, 7–9 days after ovulation, as an embryo of 16 cells or more [14]. Thus, the oviduct supports long term survival of oocytes that in this tract complete maturation, undergo fertilization, and develop up to the morula-blastocyst stage.

These interesting dissimilarities of reproductive physiology of the dog compared to other species and the lack of precise

information on the oviductal environment, make the defined in vitro system routinely applied for oocytes of other species, unsuitable for canine oocytes.

In the last few years, research has been directed to the identification of adequate cultural conditions for canine oocytes, but still relatively limited success in terms of embryo development has been achieved.

2.1. In vitro maturation

Collection of follicular oocytes from ex situ ovaries obtained post mortem or at ovariectomy could provide a large source of potential embryos. A prerequisite for fertilization and embryo development is oocyte full nuclear (metaphase II stage) and cytoplasmic maturation.

In vitro oocyte maturation is a complex process in which the attempt is made to mimic the dynamic changes occurring in the preovulatory ovarian follicle and in the oviduct. A successful maturation system supports all components of the cumulus-oocyte complex, including the cumulus cells, nuclear material and cytoplasm.

In vitro maturation of canid oocytes is, at present, characterized by low and greatly variable success rates. It has been shown that canine oocytes resume meiosis in vitro, although at a much lower rate than oocytes of other species. In fact, full nuclear maturation is achieved in about 20% of cultured oocytes [6].

Low maturation rate could be due either to low meiotic competence of the oocytes or to suboptimal culture conditions.

In vitro studies allowed a better understanding and the definition of some parameters indicative of maturation competence. Morphological appearance of the cumulus-oocyte complex and the diameter of the oocyte, have been already identified as factors influencing in vitro maturation rates. In fact, oocytes collected from ovarian follicles with dark and homogeneous

cytoplasm, completely surrounded by two or more layers of compacted cumulus cells and with a diameter of more than 110 μm , have better chances to mature in vitro [12].

Moreover, a technique for culturing oocytes in intact follicles dissected from the ovaries has been employed [3]. The results show that dog oocytes cultured within advanced preantral and early antral follicles in vitro are competent to resume meiosis to the metaphase II stage.

The immature stage of oocytes at ovulation and the persistence of cumulus cells during the transport and maturation period within the oviduct suggest that the investigation of the relationship between cumulus cells and oocyte could have contributed to clarifying the reasons behind the low efficiency of in vitro maturation of canine oocytes. It is well known, in fact, that in mammalian ovaries, communications through gap junctions between the somatic compartment of the follicle and the oocyte are involved in the regulation of its meiotic differentiation and maturation leading to the acquisition of meiotic and developmental competence [30]. In fox oocytes, gap junctions are present within the ovary and all junctional contacts between cumulus cell projections and the oocyte are disrupted 2–3 days after the LH-peak when the metaphase I stage is reached [15]. Viable and differentiated cumulus cells are needed to control the resumption of meiosis in vitro of fox oocytes [19]. These authors concluded that the role of cumulus cells in these species seems to be even more essential than in other animals.

In the bitch, the stage of the oestrous cycle influences the functional status of communications between cumulus cells and oocyte [26]. The results of this study indicated that dog cumulus-oocyte complexes isolated from the ovary during anoestrous are unable to complete meiosis and communications between the germinal and the somatic compartment through gap junctions are absent, thus suggesting a relationship

between presence of communications and meiotic competence. This was consistent with the observation that communications between cumulus cells and oocyte were present in cumulus-oocyte complexes isolated during late prooestrous, complexes which were capable to complete meiosis at a higher rate.

This finding seems particularly relevant to the understanding of the mechanisms which limit the efficiency of IVM in these species.

A similar effect of the phase of the oestrous cycle on meiotic competence of bitch oocytes has been previously described by Yamada et al. [45]. These authors reported that 32% of preovulatory oocytes collected from superovulated bitches reached metaphase II after 72 hours of culture, while oocytes from anoestrous bitches showed no tendency for increased maturation on culturing for up to 144 hours.

Research is in progress for studying culture requirements for dog oocytes. Culture media such as Tissue Culture Medium 199, modified Krebs Ringer Bicarbonate or Ham F-10 with or without serum or hormonal supplementation [4, 27, 28, 44, 45] have been used for IVM, but the requirements of canine oocytes in culture have not yet been elucidated. Recently, Hewitt and England [13] investigated the effect of using an environment different from that normally used in studies of mammalian oocyte maturation in vitro and more similar in composition to oviductal fluid. These authors demonstrated that synthetic oviduct fluid (SOF, [39]) with high concentrations of proteins (Bovine Serum Albumin) and in the presence of oviductal cells improved maturation rates, but only after a prolonged maturation time (96 h).

All these studies confirm that the morphological appearance and diameter of the oocytes, the developmental stage of the follicles, and the stage of the cycle, coupled with culture conditions are important factors to take into account for the successful IVM of dog oocytes.

2.2. In vitro fertilization and embryo development

From what has been mentioned above concerning the difficulties encountered in canine oocyte IVM, it follows that results of in vitro fertilization and subsequent embryo development are still limited in this species.

In 1992, Yamada et al. [44] reported 2% of inseminated oocytes that reached the 8-cell stage and recently Otoi et al. [29] obtained one blastocyst out of 217 inseminated oocytes.

New microinjection techniques such as intracytoplasmic sperm injection (ICSI) have been reported in the dog [8] and male pronucleus formation was observed in 7.8% of oocytes, but no further cleavage occurred.

3. IN VITRO EMBRYO PRODUCTION IN CATS

Reproductive physiology of the domestic cat is characterized by induced ovulation. Ovulation requires the release of LH from copulation, even if recent studies have demonstrated that ovulation can also occur in group-housed females in the absence of mating [20].

The ovulated cat oocyte is in metaphase II of meiosis and it is very dark in appearance because of a high intracellular concentration of lipid [11]. Moreover the cat zona pellucida is bilayered and the inner zona layer appears to function as a partial barrier to sperm penetration affecting the number and kind of sperm entering the oocyte [1].

In the last decade considerable progress has been made in in vitro embryo production in domestic cats and important goals have been achieved applying these techniques to non-domestic feline species [31].

3.1. In vitro maturation

Selection of oocytes and cultural conditions, as has been already stressed for dog oocytes, are the most important factors that affect in vitro maturation results.

Morphological criteria used to select cat oocytes do not differ from those used for canine oocytes and immature oocytes with dark and homogeneous cytoplasm, surrounded by compacted cumulus cells are destined to maturation in vitro.

The important role played by cumulus cells during maturation has been confirmed in cat oocytes by different authors [32, 42] that obtained the highest percentages of maturation and fertilization in vitro by selecting oocytes with intact cumulus cells.

Cultural conditions have also been investigated and gonadotropins were found to have a positive effect, but bovine serum [18, 22, 43] and estrous cat serum [10] had a negative effect on maturation compared to bovine serum albumin. Moreover it has been shown that antioxidant components, such as cysteine, significantly improve maturation rates that reach 70% [23].

3.2. In vitro fertilization and embryo development

Although cultural conditions for in vitro development of cat embryos are not yet completely defined, results are encouraging even though they are still lower than those obtained in other mammalian species such as bovine.

Cleavage rates in vitro range from 30 and 50% [17, 21, 38], and 20–30% of inseminated oocytes reach the blastocyst stage [32, 42]. Recently, in vitro development of embryos produced from IVM/IVF oocytes was improved (40% to > 50% blastocysts) by adding cysteine during IVM and culturing in a reduced oxygen atmosphere [34].

3.3. Embryo transfer of in vitro derived embryos

The ultimate goal of the in vitro procedure is the transfer of produced embryos and the subsequent pregnancy in the recipient animal and in 1997 kittens were born after transfer of IVM/IVF derived embryos [32].

Recently, new techniques for producing embryos in vitro have been applied in cats. Microinjection of sperm cells in the cytoplasm of the in vitro matured oocytes (ICSI) resulted in cleavage rates ranging from 40–60% [2, 9, 35, 36] and in developmental rates to the blastocyst stage of around 25% [35].

Up to now, kittens were born after transfer of the embryos obtained by ICSI only when in vivo matured oocytes had been used [33], but recently one pregnancy after transfer of the embryos produced by ICSI of in vitro matured oocytes has been reported [9].

3.4. Cryopreservation of oocytes

To maximize the practical effectiveness of in vitro protocols in the preservation of genetic material of feline species, improved techniques for long-term storage of gametes and embryos would be very useful.

Sperm cells and in vivo- or in vitro-derived cat embryos have been successfully frozen [31], but cryopreservation of oocytes is still considered experimental because adequate rates of survival, fertilization and embryo development of frozen oocytes have been reached mainly with murine oocytes.

The cryopreservation of oocytes would greatly increase their availability to a range of reproductive technologies.

Wolfe and Wildt [41] observed that cat oocytes collected from antral follicles can be stored at +4 °C for 24 h without losing their ability to mature and develop in vitro after fertilization and small preantral follicles seem to be more sensitive to prolonged cold

storage than larger preantral follicles. Recently, Jewgenow et al. [16] reported that preantral follicles from the domestic cat ovaries survive cryopreservation and remain structurally intact and physiologically active after thawing.

Moreover, it has been shown, by evaluating their subsequent development following IVM, that immature cat oocytes are able to survive after cryopreservation and results indicated that dimethylsulphoxide (DMSO) and ethylene glycol (EG) were both suitable cryoprotectants for slow freezing of immature cat oocytes. The ultrarapid procedure never resulted in resumption of meiosis in vitro, despite post-thaw intact morphology of the oocytes [24]. This demonstrates that morphology of oocytes after freezing and thawing is not predictive for ability to resume meiosis and it underlines the importance of freezing damage to the cytoplasmic system, which is not revealed by a morphological evaluation, and which affects maturation.

Recently, it has been demonstrated [25] that cryopreserved cat oocytes can be fertilized successfully and that their development in vitro after fertilization is enhanced when mature oocytes (metaphase II stage) are frozen with a slow procedure and EG. In fact, the cleavage rate obtained after IVF of mature oocytes cryopreserved with EG was greater than the rate obtained by freezing oocytes at the immature stage (38.7% vs. 6.8% $P < 0.001$). Moreover only those embryos derived from metaphase II oocytes developed beyond the 8 cell stage (11.3%), thus demonstrating a higher developmental competence than that of germinal vesicle stage (immature).

In order to facilitate the design of cryopreservation protocols which optimize the in vitro embryo development after thawing, further studies on intrinsic characteristics of cat oocytes are needed. In fact the permeability of the membrane or the presence of the intracellular lipid droplets can be responsible for uneven intracellular ice

formation which could affect the freezing-thawing process.

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