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Embryo biotechnology in dog: a review

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Abstract

Canine embryos are scarce biological material, due to the inefficiency of superovulation and cycle induction/synchronisation protocols. Difficulties encountered in collecting in vivo produced embryos and the impossibility to date to produce canine embryo sin vitro are other limiting factors. In vivo produced embryo transfer procedure is not under control, with only six attempts reported in the literature, leading to the birth of 45 puppies. In vitro, the fertilization rate is particularly low (about 10 %) and the incidence of polyspermy particularly high. So far, no puppy has been obtained from an in vitro produced embryo. In contrast, cloning of somatic cells is successfully used since 4 years with the birth of 41 puppies, with an efficiency not so lower to that obtained in other mammalian species. In the same period, canine embryonic stem sells and transgenic cloned dogs have been obtained. Last generation reproductive technologies are thus in advance over in vitro embryo production. The lack of fundamental studies on the specific features of reproductive physiology and developmental biology in the canine species is regrettable in view of the increasing role of dogs in our society and of the current need for new biological models in biomedical technology.

Introduction

In a number of domestic species, advances in the development of reproductive biotechnology have involved oocyte and embryo manipulation, with in vitro production of embryos, embryo transfer, cloning and transgenesis. In the canine species, if sperm technologies are as efficient as in other mammalian species, only a few laboratories in the world have made any progress with oocyte/embryo manipulation. This gap reflects peculiarities of reproductive physiology in the dog but also socio-economic considerations. The potential gain associated with the development of embryo biotechnology in the dog is indeed far lower than that expected in cattle or horses. It may sound strange to try and develop advanced techniques of reproduction in the dog while most countries suffer, if anything, from an overpopulation of dogs and while natural reproduction is regarded world-wide as sufficient or excessive (Zawitovsky *et al*, 1998; McNeil and Constandy , 2006; Purswell and Closter, 2006). Lastly, the biological material required for experimental purposes is rather scanty for dogs whereas it is plentiful for meat producing species since large quantities of ovaries can be collected in slaughterhouses.

In view of those difficulties, it is hardly surprising that relatively few research teams have become involved in the development of reproductive biotechnology in the dog. On the other hand, the growing importance of pets in urban societies and the current concern for preservation of endangered species have given a new impetus to the development of dogs as biological models.

Dog as a relevant model for human diseases and therapeutics

In many cases, the dog reveals to be a more relevant model of human diseases than mouse can be (Schneider *et al*., 2008), due to its size, its life time, its way of life, a closer physiology and a more similar reactivity to drugs and irradiation. But above all, dog appears as a good genetic model Of the nearly 400 known hereditary diseases described in the dog, more than half (224) have an equivalent in the human species, such as cardiomyopathies, muscular dystrophy and prostate cancer (http://omnia.angis.org.au). Recent sequencing of the dog genome (Kirkness *et al*., 2003; Lindblad-Toh *et al.*, 2005) positions the dog as a model to study the genetic basis of diseases. This species presents also three assets for genetic studies: the possibility to gain access to large families (much larger than in humans), to decide informative crossings between genetically characterized males and females and a non homogeneous gene background (at the opposite to inbred mouse lines). Moreover, dog is submitted to the same environmental factors as we are, that is of great importance since numerous common inherited human diseases (asthma, diabetes, epilepsy, cancers) involve complex interactions between genes and environment. The dog having to be considered not only a pet, but also as the modern mouse, a need in embryo biotechnologies is becoming more obvious.

Embryo development in the dog

Oocyte and embryo biology in the dog is quite different from that in other mammals and still largely unknown. In the bitch, 6 to 12 oocytes are delivered at each cycle (Tsutsui *et al*., 1975; Lee *et al*., 2005; Reynaud *et al*, 2006), with ovulations being spread over 24, even 36 hours (Boyd *et al*., 1993; Marseloo *et al*., 2004). This lack of synchronization may account in part for the diversity of embryonic stages observed within a single embryo cohort. (Bysted *et al*., 2001; Kim *et al*., 2002; Reynaud *et al*., 2005). While in most mammalian females, ovulation delivers haploid oocytes that can be readily fertilized, the oocytes delivered in the bitch are still blocked at stage I of meiosis prophase. Following ovulation, oocytes require a maturation of 54-60 hours in the oviduct to reach the metaphase II stage and become fertilizable (Tsutsui, 1989; Reynaud *et al*., 2005).

Thereafter, fertilization occurs in the oviduct 48 to 83 hours post ovulation. Some investigators have suggested that sperm penetration into the canine oocyte could be obtained at immature stages of meiosis (Van der Stricht, 1923; Farstad *et al*., 1993). While such an atypical fertilization can be obtained in vitro with oocytes collected during anoestrus (Saint-Dizier *et al*., 2001), examination by confocal microscopy of oocytes collected in vivo has demonstrated that this phenomenon occurred exceptionally in vivo. Out of 112 immature oocytes collected in vivo issued from 30 inseminated bitches, only three from a single bitch were found to be fertilized (Reynaud *et al*., 2005). In vivo, sperm penetration did not take place unless the canine oocyte had reached the metaphase II stage as in other mammals.

Embryos at the 2 pronuclei stage are found 72 to 124 hours (3 to 5 days) post ovulation. The two-cell stage is observed 96 to 168 hours (4 to 7 days) post ovulation (Reynaud *et al*., 2006). The activation of the embryonic genome, which corresponds to the initiation of the transcription of the embryonic genome, seems to take place at the 8-cell stage, occurring between 122 and 288 hours (4.5 to 12 days) post ovulation (Bysted *et al*., 2001; Fig. 1). Until that stage, embryo development takes place in the oviduct. Towards 8.5 to 10 days post ovulation, the embryos reach the morula stage and start to slip into the uterus (Reynaud *et al*., 2006). Thus, compared to other mammals, dog embryos spend a long time in the oviduct: roughly 9 days in a pregnancy lasting 63 days altogether, whereas in the cow for example, the time in the oviduct amounts to 4 days out of a total of 280 days (Guillomot, 2001).

The blastocysts, which appear around D10-12 post ovulation, hatch between D16 and D20, measuring around 2.5 mm at that time (figure 1). The implantation takes place shortly thereafter, between D 18 and D 21 post ovulation (Holst and Phemister, 1971; Concannon *et al*., 2001; Reynaud *et al*., 2005) again later than in other species studied. In the cow, embryo implantation takes place between D16 and D19 of a pregnancy lasting 280 days (Guillomot, 2001).

While in the bitch, passage into the uterus and implantation occur relatively late, compared to other mammalian females, embryo development itself is by no means slow. This common misconception relates to the 48- to 72- hour delay between ovulation and fertilization. Once embryonic development is computed starting with fertilization, its kinetics are largely comparable to those in other mammalian species.

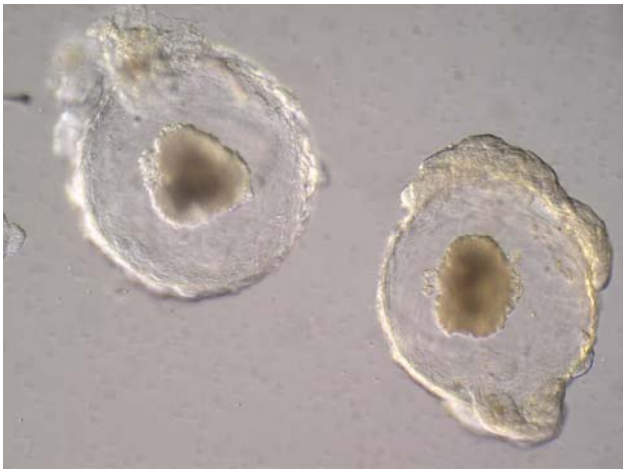

Figure 1. Canine blastocysts 13 days after fertilization. Inner cell masses are clearly visible.

In vivo production of embryos

In vivo production of embryos consists in collecting embryos by flushing the genital tract of a female after (super) ovulation and insemination. Thus, in this case, fertilization takes place in vivo. The collected embryos are then transferred into recipient females whose cycle is synchronous with that of the donor(s).

Several difficulties are encountered when this technique, commonly used in humans and cattle, is applied to dogs. Whereas superovulation in the donor female, intended to increase the number of embryos to be collected, can be routinely obtained by appropriate treatment in other species, the female dog does not respond adequately to common combinations used to induce superovulation (eCG / hCG) (Archbald *et al*., 1990; Yamada *et al*., 1992). Following natural oestrus, without superovulation, an average of 6 to 8 well-formed embryos can be collected from each female. Judging from the number of corpora lutea on the ovaries, the number of embryos that can be collected varies with the size of the dog., from 5.5±0.3 in small breed females to 7.8±0.7 in middle sized breeds (between 10 and 20 kg) and 10.1±1.4 in larger breeds.

The technique used for embryo collection has to be adapted. Until D9 post ovulation, the collection of embryos requires flushing of the oviducts and thus calls for surgery, an invasive procedure. For embryos at a later stage, one could imagine washing the uterine horns without surgery, through a catheter inserted through the cervical canal. Nevertheless, even under surgical approach, with a one-way liquid movement from the apex of one horn towards the uterine body, these procedures collect only 30-40 % of the embryos expected from the number of corpora lutea (Archbald *et al*., 1990; Tsutsui *et al*., 2001b). This poor recovery rate may result from the huge endometrial hypertrophy associated with oestrus in the bitch, embryos remaining trapped in the deep folds of the uterine mucosa. The rate can be definitely improved by flushing the oviducts and the uterus ex vivo after surgical ablation but with little practical interest (Tsutsui *et al*., 1989, 2001a, b).

For the small number of embryos collected, 80 to 90 % of the embryos collected are viable (Tsutsui, 1975; Tsutsui and Ejima,1988, Shimizu *et al*., 1990; Tsutsui *et al*., 2006).

Short of increasing the number of embryos by way of superovulation, which cannot be achieved at this time, one might obtain more embryos by shortening the interval between heat periods which is particularly long in the bitch (approximately six months). Attempts to use protocols similar to those that work in other species (including progestagens, oestrogens and prostaglandins F2alpha) have proved unsuccessful in dogs. Other attempts have been made using eCG and hCG with dopaminergic agents. Yet, the most promising compounds are GnRH agonists, deslorelin primarily (Kutzler, 2005; Fontaine, personal communication). Such protocols once developed could be used for cycle control in recipient females as well. Indeed, the recruitment of recipient females remains problematic as long as no treatment protocol is available to assure synchronization. Synchronization based on natural cycles requires keeping a very large number of females with the hope that one or more will ovulate at the same time as the donor female. No more than one or two days should elapse between ovulation in donor and recipient females to obtain a pregnancy (Tsutsui *et al*., 2001a, b).

Deep-freezing of embryos might represent an alternative to oestrus cycle control .In cattle and swine, the high amount of lipids in the cytoplasm of embryos is known to interfere with the ability of embryos to survive deep-freezing (Nagashima *et al.*, 1995; Diez *et al*., 2001; Fig. 2). Since the cytoplasm of canine embryos is particularly lipid-rich, chances of successful deep-freezing appear rather meagre. A single attempt is reported in the literature with no birth obtained after transfer of 8 frozen blastocysts (collected at D 13 after coitus; Kim e*t al.*, 2002).

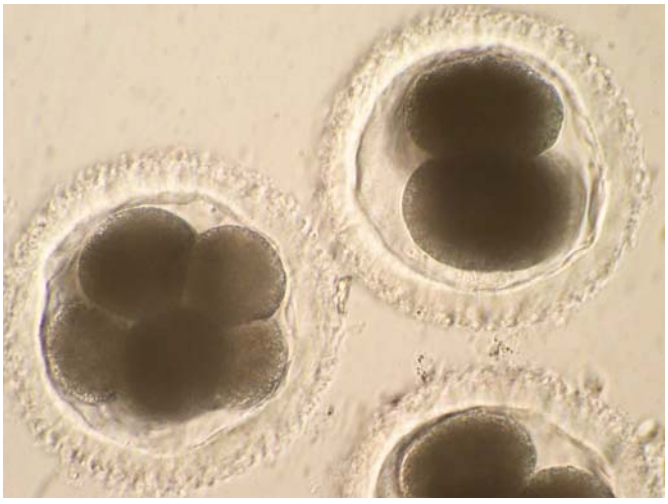

Figure 2: The opacity of the embryo cytoplasm is due to a high lipid content.

Embryo transfer in the dog is still inefficient, with low rates and surgery required for both embryo collection and transfer. A review of the international literature on this subject found no more than five reports of attempts with fresh embryos (transfers of embryos obtained by nuclear transfer excluded; see below). Altogether, the reported transfers involved 57 recipient females with no more than 45 births (Kinney *et al.* 1979, Tsutsui *et al.* 1989, 2001a, b, 2006; Kim *et al*., 2002). This figure compares with some 100 000 bovine embryos transferred

per year in Europe alone (www.aete.eu). Because of the poor recovery rates of embryo collections by uterine washings and because of the difficulties associated with transfer of the embryos into the oviducts, Tsutsui *et al*. (2001a) have attempted to transfer 52 embryos at tubal stages (zygote to 8-cell) into the uterus of 13 recipient females. Four pregnancies were obtained, giving a total of 6 puppies. This approach of intra-uterine transfer of embryos collected from the oviduct is a common and efficient practice in women.

The development of in vivo produced embryo transfer would make it possible to increase the progeny of genetically valuable females and from females unfit for conducting a full pregnancy. Embryo transfer would be applied to females undergoing embryo/fetal mortality, especially around term due to obstetrical complications: for example, embryos could be collected from English Bulldog females in which caesarean sections are often needed and transferred into Beagle bitches with easy whelping conditions. This situation would also provide an opportunity to evaluate epigenetic effects of foetus exposure to maternal environment during pregnancy and nursing.

Embryo transfer would also contribute to the eradication of genetic defects after pre-implantation diagnosis. In dogs, mutations responsible for numerous hereditary defects are already identified, such as blindness, deafness, and various neurological conditions (Quenet *et al*., 2003). Only embryos free from those unwanted genes would eventually be implanted into a recipient female. Finally, if procedures for deep-freezing of canine embryos can be developed (exchanges and conservation of genetic material) could become feasible.

In vitro production of embryos

Collection and in vitro maturation of canine oocytes is described elsewhere in the proceedings of the 18th congress CBRA. Once metaphase II oocytes obtained, in vitro fertilization is obtained by placing sperm in contact with them.

As precised above, the rate of in vivo fertilization is excellent and polyspermy has not been described. By contrast, the rate of in vitro fertilization is quite low in the bitch, rarely exceeding 10 to 20% (Mahi and Yanagimashi, 1976; Saint-Dizier *et al*., 2001) while it commonly reaches 80 to 90 % in cattle. Besides, in vitro, a remarkably high rate of polyspermy is observed: 47 % of fertilized oocytes were found with 2 to 12 sperm cells per oocyte, with an average of 3.3 sperm cells per oocyte (Saint-Dizier *et al*., 2001).

Intra-Cytoplasmic Sperm Injection (ICSI) may provide a solution to the dual problem of low fertilization rate and high rate of polyspermy. In dogs, a single study on the use of ICSI is available (Fulton *et al*., 1988): two pronuclei formed in no more than 8 % of the microinjected oocytes, but it is noteworthy that only 38 oocytes that were furthermore at the prophase I stage were included in this study.

In vitro produced embryos still remain thus exceptional. Only few morulas and blastocysts are described (Otoi *et al*., 2000; Hong *et al*., 2009; Jang *et al*., 2008).

Cloning

Alternatively to fertilization, embryos can be obtained in vitro after nuclear transfer (cloning): a donor cell, issued from a genetically interesting animal, is fused with a cytoplast obtained by the extraction of the metaphase II plate from a mature oocyte. This procedure is made easier in numerous mammalian species by the large number of metaphase II oocytes that can be obtained after in vitro maturation. Another obstacle in the bitch is the impossibility to date to culture or freeze reconstructed embryos together with the non availability of synchronisation process for recipients (see above). Nuclear transfer has been made successful in the canine species in 2005 with the birth of a male Afghan puppy, named Snuppy, from adult fibroblasts (Lee *et al*., 2005). From this birth onwards, canine clones have been obtained from a variety of donor cells: male and female, adult and fetal fibroblasts, young and aged donor dog, from small and large breeds, and even genetically-modified cells: the first six transgenic puppies have been born recently after nuclear transfer the with fetal canine fibroblasts transfected with Red Fluorescent Protein gene (Hong *et al*., 2009).

A total of 37 cloned puppies is reported in the scientific literature, but recently, four “commercial” puppies have been born from family dog (www.bestfriendsagain). The latest, named “Lancelot Encore”, has been sold in South Florida for 120 000 US dollars.

Compared to the protocols followed in other mammals, dog cloning is currently performed with in vivo produced oocytes. Reconstructed embryos are transferred after a very short time in culture (less than 4 hours after activation) into spontaneously synchronous recipients. Both oocyte collection and embryo transfer is surgically performed. Large kennels and specific skills are thus required. The optimal number of embryos to be transferred seems to be in the 11-25 range (Hossein *et al*., 2008; Jang *et al*., 2007, 2008). The reconstruction process is well controlled (with high fusion rates, around 80%), but the global efficiency of the process (number of puppies born / number of transferred embryos) between 0.4 and 4% is lower than in other mammals (Lagutina *et al*., 2007). The incidence of the *“Abnormal Offspring Syndrome”* (formerly called *“Large Offspring Syndrom”* Young *et al*., 1998) seems to be very limited, if any. No defect, such as placental hyperdevelopment, excessive fetal growth, anasarc, abnormalities of the circulatory system, immunity disorders are reported. This

may be due to the good quality of the recipient oocytes (collected in vivo and not in vitro produced as in other species) and to the immediate transfer of the embryos into surrogate females without culture, suspected to induce gene dysregulation. Some species may also be more robust in this concern than the bovine, Abnormal Offspring Syndrom being neither observed in horses and pigs. Nevertheless, late abortion and death of the clone in the neonatal period and until weaning, that are also part of the syndrome, are not so rare after somatic cloning in the dog: 23% abortion and 13% of puppies died during the 2 months after birth, compared to 33-43% abortions and 35% death before 2 months, 48% before adulthood. (Heyman et al, 2002; Cibelli et al, 2002 ; Chavatte-Palmer et al., 2004). Since mortality until adulthood might be increased, further follow-up of already born cloned puppies is a great importance.

Since it turned out to be so difficult to obtain canine oocytes at the metaphase II stage, heterospecific nuclear transfer was attempted. Cells from adult dogs were transferred into the caryoplasm from bovine oocytes. These represent an easily accessible (from the slaughterhouse) and plentiful biological material whose in vitro maturation is well under control. With such interspecific transfers, clivage rates are excellent (74 to 81 %) and few morula (1.3 %) and blastocysts (0.4 %) are obtained with no development to term after transfer (Westhusin *et al.*, 2001, 2003; Murakami *et al*., 2005). Similar results were obtained with donor cells from other species, (pigs, sheep, macaques) transferred into bovine recipient oocytes, without any full term development (Dominko *et al*., 1999). Nevertheless, with more closely related species between donor and recipient cells, offspring was obtained (Tecirlioglu *et al*., 2006). Since wolf oocytes are even scarcer than dog oocytes, interspecies somatic cell nuclear transfer was attempted with grey wolf fibroblasts transferred into canine recipient oocytes. Even with donor cells collected post-portem, the global efficiency was similar to that of canine cell transfer with the birth of 6 wolf puppies (Kim *et al*., 2007; Oh *et al*., 2008).

The major application of cloning in Canidae is the preservation of genotypes of interest. One could obtain by cloning the birth of a living replica of an animal, prior to its death and even post-mortem. This could apply to endangered canid species (as done for the grey wolf), to avoid genetic loss; to mountain rescue dogs, police dogs dedicated for explosive detection, guide dogs, all being neutered very early in life; breeders are interested in cloning beauty champions, as are some private owners for cloning of their own pet. Cloning could also be used to produce groups of genetically identical dogs for biomedical research, for instance as models for human diseases. Nevertheless, applicants for canine cloning expecting a perfect phenotypic copy should be made aware of the diversity of phenotypes that may occur in animals obtained from donor cells with the same genotype. Differences in coat characteristics, behaviour, performances have been reported among animals obtained from the same cellular source in cattle, horse, cat and pigs for example (see Chavatte-Palmer and Heyman, 2006 for review).

Embryonic stem cells

Controlling canine embryo biology and related techniques (production, culture and transfer) would allow to gain access to embryonic stem cells technology, first step towards cell therapy, one of the major therapeutic challenges in human species for the coming years. For example, a patient suffering from genetic muscular dystrophy would be injected with pluripotent cells, conducted in this case to differentiate in non-defective myocytes. Such pluripotent cells, called ES cells, for “Embryonic Stem cells”, issued from inner cell masses dissected from blastocysts. Under appropriate conditions, such cells can proliferate indefinitely in vitro while maintaining their pluripotency. They can be transfected and driven to differentiate in numerous cell types. To date, five characterizations of canine ES cells are available, the first being published in 2005 (Hatoya *et al*., 2005; Schneider *et al*., 2007; Hayes *et al*., 2008; Vaags *et al*., 2008; Wilcox *et al*., 2009). In comparison, murine ES cells were obtained in 1981 and in 1998 in human. Blastocysts collected 8-9 days post fertilization seem to be the ideal developmental stage for ES cell production. Such canine ES cells were successfully differenciated in various cell types, such as neurons, epithelial cells, fibroblasts, myocardic cells and haematopoetic progenitors. Nevertheless, the ability of the canine embryo-derived cell lines to differentiate in vivo remains to be demonstrated, together with the evaluation of their risk of tumorisation. ES cells can also be combined with nuclear transfer: fibroblasts from the patient could be used as donor cells to produce blastocysts, from which patient-specific embryonic stem cells would be derived; after transfection or not, such ES cells would be reinjected to the host, conducted to differenciate in the appropriate cell type without any risk of Graft Versus Host Disease.

ES cells are also a potent tool to obtain transgenic animals: thanks to canine genome sequencing, such cells could be genetically modified by homologous recombination before reinjection, for example after insertion of a normal dystrophin gene for the correction of Duchenne’s muscular dystrophy (Sampaolesi *et al*., 2005). Transgenic ES cells microinjected in a morula or a blastocyst, allowing the formation of chimeras, with in some cases germinal expression.

Conclusion

The specificities of dog reproduction require numerous adaptations of the efficient procedures used in other species. Even among carnivorous mammals, techniques efficient in cats could not be applied to dogs. Dog embryo biotechnologies are dramatically developing since the 5 past years, jumping directly to the most advanced ones, somatic cell nuclear transfer, transgenesis and ES-cells. Despite a lack of fundamental knowledge, they hugely progress in synergy with parallel advances in other fields, such as genetic, cell therapy and in situ wild life conservation.

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