CURRENT STATE OF IN VITRO TECHNIQUES IN CANID REPRODUCTION

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Introduction

In most domestic animal reproductive biotechnology, assisted reproductive techniques (ARTs) are aimed at manipulating the gametes outside the body of the animal, such a normal and assisted fertilization of the oocyte and culture *in vitro*, long- or short-term conservation and subsequent transfer of resulting embryos to synchronized recipients as in most domestic animal scenarios. Furthermore, the collection, processing and handling of semen followed by artificial insemination or assisted fertilization techniques are well developed for most domestic animals including the dog and cat. However, in the dog, reliable systems for in vitro production of embryos, embryo cryopreservation and embryo transfer are yet to be developed. Oocyte maturation and subsequent embryo culture in vitro are currently the rate-limiting steps to success in canine biotechnology. In vivo, the microenvironments provided by the follicle and the oviduct protect oocytes from oxidative damage to which they are exposed in vitro and allow an efficient exchange of regulatory molecules, which promote both nuclear and cytoplasmic maturation of the oocytes. Modifications of the cultural environment during maturation, either by addition of substances that stimulate endogenous systems of the egg cell and modulate the intracellular levels of regulatory molecules, or by the use of sequential culture systems adapted to the various demands of the oocyte and embryo during development in vitro, may be a feasible approach to solving the problem. In this paper a review of the state of the art in canine in vitro techniques is given.

Semen processing and assisted fertilization

Semen processing for short-term conservation in the fresh state for immediate or within 24-48 hour- artificial inseminations involves the use of extenders for canine semen (Farstad, 1998). Also, fresh semen is commonly used for in vitro insemination, necessitating the use of special canine capacitating media (CCM, Mahi and Yanagimachi, 1978) or media adapted from use in other species (sperm TALP). Sperm TALP contains albumin, which stimulates sperm capacitation, and even the acrosome reaction, in a variety of species (Hewitt & England, 1999a; Andrews et al, 1992, Sirivaidyapong et al., 2000).
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Furthermore, in vitro techniques for testing dog sperm function include sperm binding assay and sperm penetration assays using entire or hemi-zonae or intact oocytes (Hewitt and England, 1998). In this system both fresh, cooled ands salt-stored oocytes are used. However, salt-storing may cause damage to the oocytes, whereas up to 48h cooling of oocytes within the follicle does not seem to affect sperm-egg interaction and penetration rates compared with fresh oocytes (Mastromonaco et al., 2002). Semen may be collected from fresh or cooled epididymides and successfully be able to bind to homologous zonae in a time-dependent manner. Sperm that are cooled at 4 °C for up to 192 hours (8 days) within the epididymides may still be motile and bind to zonae, although the overall motility and number of sperm with intact acrosomes are significantly reduced when storage exceeds 48 hours (Yu and Leibo, 2002). The oviduct has a modulating effect on sperm selection and capacitation in

vivo. Coculture with porcine oviduct cells in vitro showed that these cells influenced canine sperm in a similar way as the canine oviduct in vivo, i.e., producing similar alterations in sperm head tyrosine phosphorylation and delaying capacitation in sperm bound to oviduct cells. Porcine coculture systems may be a possible way to assess canine sperm capacitation in vitro (Petrunkina et al, 2003).

Intracytoplasmic sperm injection (ICSI) involves injecting a spermatozoon into the cytoplasm of a mature ovum. This procedure enables even non-motile sperm to take part in fertilization, and the sperm tail no longer plays an essential role in fertilization. On the contrary, fertilization may be successful as long as the sperm DNA is intact and remains in its stable condensed form. This not only renders the use of sperm from dogs that have immotile sperm possible, but theoretically, epididymal sperm from extinct species obtained as freeze-dried specimens in museums of natural history, or specimens found enclosed in ice blocks in the permafrost state may be sufficiently well preserved to develop after injection into oocytes of closely related species as suggested by Seidel (1991). Only one limited experiment of ICSI has been published in the dog, but no live offspring were produced (Fulton et al., 1998). This is in contrast to the situation in cats, in which the birth of live kittens is reported after ICSI (Gomez et al, 2000).

Maturation of oocytes in vivo vs in vitro

In canines, the oocyte is ovulated at the beginning of the first meiotic division with the extrusion of the first polar body, and subsequent stages of oocyte maturation occur in the oviduct. At ovulation the cumulus mass around the oocyte is tight, as opposed to other mammals such as the cow, in which oocyte maturation is completed and the cumulus mass is expanded. During the process of oocyte maturation in canine oocytes, a similar cumulus expansion is seen as the bitch oocyte matures, and complete nuclear maturation with the extrusion of the first polar body was only observed in dog oocytes matured in vitro with at least two layers of cumulus (Nickson et al., 1993).

Penetration of the oocyte during maturation is possible in both dog and fox oocytes despite their immature state. Sperm penetration induces oocyte maturation (Mahi and Yanagimachi, 1976; Farstad et al., 1993a; Saint-Dizier et al., 2001). The canine oocyte seems to possess the ability to elicit the cortical reaction to prevent polyspermy, and a parallel synchronization between male and female pronucleus development was found to occur in both fox ova (Farstad et al, 1993a) and dog ova (Saint-Dizier et al., 2001). Fox and dog oocytes appear morphologically similar (Holst and Phemister, 1971; Farstad et al., 1989; Hyttel et al., 1990). Dog and fox oocytes require 1-3 days of maturation in the oviduct to resume and complete first meiotic division to allow fertilization (Farstad et al., 1989; Hyttel et al., 1990). In vitro studies of dog oocytes indicate that all oocytes capable of maturation had extruded the first polar body after 24h of in vitro culture (Nickson et al, 1993). After in vitro fertilization of in vitro matured dog oocytes with fresh dog spermatozoa, these authors found that no penetration of the zona pellucida occurred in oocytes cultured less than 24h, whereas oocytes matured for 48h or 72h zona penetration occurred within 1h after addition of spermatozoa. This indicated that 48h of maturation is needed in vitro before fertilization is possible, and that nuclear maturation alone judged by polar body extrusion, is not sufficient to allow fertilization. Thus, the in vitro studies confirm observations on oocytes obtained in vivo that the bitch oocyte needs at least 24-48h of maturation before developmentally competent embryos can be obtained after fertilization.

In vitro maturation of canid ovarian oocytes collected in anoestrus have resulted in poor maturation success (Krogenæs et al., 1993; Wen et al., 1994). During anoestrus many oocytes are still in their growing phase, and it has been stated by several authors that an oocyte diameter of >100-110um is necessary before the oocyte acquires meiotic competence (Otoi et al., 2002). Difficulty in maturing canid oocytes may also be related to these oocytes normally being matured in the oviduct, or the need for cumulus granulosa cells to stimulate resumption of meiosis. Mitogen-activated protein (MAP) kinase activation and Raf-1 synthesis and phosphorylation are controlled by cumulus cells (CCM) in foxes, and FSH receptor-dependent pathways may be present in granulosa cells capable of controlling oocyte maturation (Kalab et al., 1997). Luvoni et al. (2001) showed that estrus cycle influenced cumulus-acolyte communications in canine oocytes cultured in vitro. In anestrous oocytes there were no permeable gap junctions between oocytes and their surrounding cumulus cells as opposed to those in late proestrus, suggesting that CCM control oocyte maturation through gap junctions in the dog as well. From the above, it is now well established that canine oocytes resume meiosis in vitro (10-40%), although at a much lower rate than in vivo (for review also see, Farstad 2000).

The most common medium used for canine oocyte maturation is TCM 199, with or without serum supplementation (bovine serum albumin, canine serum from anestrus or metestrus bitches, or estrous bitch serum), hormones (FSH/LH, progesterone, estradiol), epidermal growth factor (EGF), somatotropin (human[1], bovine[2] or porcine[3]ST, Rodrigues and Rodrigues, 2003[1]; Srsen et al., 1998[2]; Willingham-Rocky et al., 2002[3]) or proteins, and somatic supporter cells, such as oviductal cells (Bogliolo et al., 2002). The influence of supplementing serum, using co-culture, growth promoting substances and hormones to induce nuclear maturation is widely discussed, and results and conclusions differ somewhat between studies. Hewitt and England (1999b) did not find any beneficial effect of co-culture with anestrous oviduct cells (location of sample site unspecified) on oocyte maturation. Contrary to their findings, significantly higher meiotic resumption rate was found in canine oocytes cultured with cells from either the infundibulum (59%) or the ampulla (60%) of the estrous bitch oviduct compared with culture without oviduct cells (40%), and more oocytes progressed to MII in the co-culture systems (Bogliolo et al, 2002). Luvoni and coworkers (2003) found that oocyte culture in isolated ligated oviduct was significantly better and with fewer degenerate oocytes than culture in open oviduct or drop culture within 30h of culture. Oviduct cell interaction with oocytes therefore seem beneficial to in vitro systems of oocyte maturation in the dog. Recently it has been shown that canine oocytes may also complete nuclear maturation in protein free media, although at a very low rate (1/135oocytes) (Songsasen et al, 2002). As of now, oocyte maturation seems to be the rate-limiting step for success with in vitro production of canine embryos, as well as for other forms of manipulation of canine oocytes, such as ICSI or cloning by nuclear transfer.

Fertilization of oocytes in vitro, embryo development and embryo culture

Valtonen (1984a,b) and Valtonen et al. (1984) described the developmental stages of early blue fox (*Alopex lagopus*) embryos in relationship to the day of mating. At 2-4 days after mating oviductal embryos were 1-cell, at 4-6 days after mating 1-4 cells, and at 6-8 days morulae were found in uterus, whereas expanded and hatched blastocysts were present on days 13-15 after mating. Red foxes (*Vulpes vulpes*) may experience a more rapid oocyte maturation after ovulation and a faster oviductal passage of embryos than blue foxes and dogs because embryos enter the uterus at the 14-16-cell stage 4-6 days after mating. Morulae are found on days 6-7, expanded blastocysts on days 9-10 and hatching blastocysts on days 11-12. Implantation occurs 16-18 days after mating in both species (Jalkanen, 1992a,b). Farstad et al (1993a) suggested that

the fox embryonic genome was switched on at the 6-8-cell stage. In the dog, embryos survive in the oviduct for 6-8days. Holst and Phemister (1971) recovered free-floating blastocysts from day 8-20 after mating. The major transcriptional activity occurs in the dog at the end of the 4th post-fertilization cell cycle, i.e., at the 10-12-cell embryo (Væver Bysted, 2001). This implicates that the particular sensitive period for suboptimal culture conditions may be later in the developmental program in dog embryos than in fox embryos (6-8-cell stage).

The first successful report of a dog ovum developing in vitro from a 1-cell zygote to the morula stage was reported in 1991 (Renton et al, 1991). Yamada et al (1992) showed that dog oocytes from preovulatory follicles could be matured, fertilized and cultured to the 8-cell stage in vitro. Songsasen and coworkers (2002) obtained a small portion of early canine embryos after in vitro culture of IVP zygotes in a protein–free medium. One canine pregnancy following in vitro fertilization of bitch oocytes has been reported, although the pregnancy did not go to term (England et al, 2001), and one pregnancy from nuclear transfer/ET was established but was unfortunately lost before term (Westhusin et al, 2001).

In vitro fertilization of in vivo-matured as well as in vitro-matured fox oocytes have been successfully carried out in the blue fox (Farstad, 1993b,c, Farstad et al, 2001). Farstad et al (1993c) fertilized immature fox oocytes in vitro, and were able to culture an embryo to the morula stage. In vitro culture of in vivo-produced silver fox embryos and subsequent surgical embryo transfer has been carried out with the birth of two live pups (Lindeberg et al, 1992; Jalkanen, 1993; Jalkanen and Lindeberg, 1998). All procedures involved the use of TCM 199 as culture medium with serum sources from bovine or fox, and in the case of the fox experiments, fox oviduct cell cultures were used to make conditioned media for embryo co-culture.

Progress is slow in this area because of the difficulty in obtaining oocytes and embryos, and the difficulties in maturing oocytes in vitro when they are collected from anestrous females. Although some experiments have been carried out on gamete and embryo culture conditions, there is still a lack of knowledge concerning maturation competence and hormonal stimulation regimes, and inexperience in optimum culture conditions and flushing methods for embryos. Modifications of the cultural environment during maturation, either by addition of substances that stimulate endogenous systems of cell defense and modulate intracellular levels of regulatory molecules, or by the use of sequential culture systems adapted to the various demands of the oocyte and embryo during development in vitro, may be a feasible approach to solving the problem.

Embryo transfer and recipient management

Transfer of embryos from one female to another requires synchronization of cycles between donor and recipients, i.e., the recipient's uterus should provide an endocrinological and cellular environment, which is as similar as possible in temporal development to the uterine environment of the donor female (±1 day) in day of ovulation relative to the donor bitch, Tsutsui et al, 2001). In foxes, natural synchronization is achieved in a farm situation due to the seasonal occurrence of estrus and the effect of surrounding females on the induction of estrus in neighboring vixens. This has enabled successful embryo transfer in the silver fox (Jalkanen and Lindeberg, 1998). Similar mechanisms, i.e., sexual pheromones, exist for natural synchronization in groups of bitches housed together in kennels or in private homes.

Artificial induction of estrus may be done using either gonadotropins or lately, dopaminergic compounds, such as the antiprolactin cabergoline, which affects the interestrus interval (for review, see Gobello, 2002 and Rota et al, 2003). However, no attempt has so far been published to use these substances for synchronization of estrus in bitches for embryo transfer. Despite these difficulties, successful surgical transfer of dog embryos leading to the birth of live pups, has been published both after intrauterine (Kinney et al, 1979; Tutsi et al, 1989), and intratubal transfer to the lower part of the oviduct. Transfer of embryos to the lower part of the oviduct was carried out with greater success than uterine transfer, since 50% of recipient dams became pregnant. None of the females which had embryo transferred to the upper part of the oviduct became pregnant (Tsutsui et al, 2001). Transfer to only one uterine horn (or oviduct) should be sufficient, since transuterine migrations has been found to occur in dogs (Tsutsui et al, 2002). 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 animals or to females in spontaneously occurring or induced estrus.

Cryopreservation

The ability to cryopreserve gametes and embryos is of considerable importance to future application of ARTs in canids. Whereas freezing protocols for semen are available for some canid species such as the domestic dog and red fox, and to a certain extent the arctic fox, spermatozoa from other canid species may be further away from being successfully cryopreserved (Farstad et al, 1992; Goodrowe et al, 2000; Zindl et al, 2002). Freezing of embryos from foxes has been attempted by both conventional freezing methods and by Open pulled straw (OPS) vitrification procedures (H. Lindeberg et al, cited in Farstad, 2000), but to date no live offspring has been born from frozen canid embryos.

Cloning of dogs by nuclear transfer

Biotechnology and the use of assisted reproductive techniques (ART) have reached much further in cats than in dogs. The refinement of invasive techniques with gametes and embryos in cats has now proven to be a successful tool. Nuclear transfer techniques have recently resulted in birth of the first live cloned cat, “CopyCat,” in 2002 (Shin et al, 2002). The company that finances the scientists who created Copycat (the Genetics Savings and Clone Company) claimed in 2000 that they expected to see canine clones of “Missy”(named after the female pet dog, Missy, the Missiplicity project (Nature Biotechnology, 1998, 16: 892) appear at the end of 2000 (Grisham, 2000). However, at the end of 2003 no cloned canine has yet been born.

References

Andrews, JC, Howard, JG, Bavister, BD, Wildt, DE. 1992. Sperm capacitation in the domestic cat (*Felis catus*) and leopard (*Felis bengalensis*) as studied with salt stored zona pellucida penetration assay. Mol Reprod Dev, 31, 200-207.

Bogliolo, L, Zedda, MT, Ledda, S, Leoni, G, Naitana, S, Pau, S. 2002. Influence of co-culture with oviduct epithelial cells on in vitro maturation of canine oocytes. Reprod Nutr Dev, 42, 265-273.

England, GCW, Verstegen JP, Hewitt DA. 2001. Pregnancy following in vitro fertilisation of canine oocytes. Vet Rec, 148, 20-2.

Farstad, W, Mondain-Monval, M, Hyttel, P, Smith, AJ, Markeng, D**.** 1989**.** Periovulatory endocrinology and oocyte maturation in unmated, mature blue fox vixens (*Alopex lagopus*). Acta Vet Scand 30, 313-319.

Farstad, W, Fougner JA, Andersen Berg K. 1992. Species differences in fertility after artificial insemination with frozen semen in fox pure breeding. N J Agric Sci 9 (Suppl), 115-121.

Farstad, W, Hyttel, P, Grøndahl, C, Mondain-Monval, M, Smith, AJ. 1993a. Fertilization and early embryonic development in the blue fox (*Alopex lagopus*). Mol Reprod Dev 36, 331-7.

Farstad, W, Krogenæs, A, Nagyova, E, Hafne, AL, Hyttel, P. 1993b. In vitro techniques in fox reproduction. Livestock Prod Sci 36, 23-7.

Farstad, W, Hyttel, P, Grøndahl, C, Krogenæs, A., Mondain-Monval,M, Hafne, A.L. 1993c. Fertilization in vitro of oocytes matured in vivo in the blue fox (*Alopex lagopus*). J Reprod Fertil 47 (Suppl.), 219-26.

Farstad W. 1996.Semen cryopreservation in dogs and foxes. Anim Reprod Sci, 42, 251-260.

Farstad, W.1998. Mating and artificial insemination in the dog. In: Simpson, GM, England , GCW and Harvey, M (Eds.). Manual of small animal reproduction and neonatalogy. British Small Animal Veterinary Association, Cheltenham, UK, Ch 10, pp 95-103.

Farstad, W.2000. Assisted reproductive technology in canid species. Theriogenology, 53, 175-86.

Farstad,W, Hyttel P, J Hafne A-L, Nielsen, J. 2001. Maturation and fertilization of blue fox (*Alopex lagopus*) oocytes in vitro. J Reprod Fertil 57(Suppl), 161-5.

Fulton, RM, Keskintepe, L, Durrant BS, Fayrer-Hosken RA.1998. Intracytoplasmic sperm injection for the treatment of canine infertility. Theriogenology 48, 366.

Gobello,C. 2002. Questions concerning estrus induction in the bitch and queen. 3rd EVSSAR Congress, Liége, Belgium. Proceedings, pp 43-4.

Goodrowe, KL, Walker, S, Ryckman, D,
Mastromonaco, G, Hay, MA, Bateman, H, Waddell WT. 2000. Piecing together the puzzle of carnivore reproduction. Anim Reprod Sci, 60-61, 389-403.

Gomez, MC, Pope, CE, Harris, R, Mikota, S, Dresser, BL. 2000. Birth of kittens produced by intracytoplasmic sperm injection of domestic cat oocytes matured in vitro. Reprod Fertil Dev, 12, 423-33.

Grisham, J. 2000. Beyond nine lives. Nature Biotechnology 18, 366.

Hewitt, DA, England GCW.1998. The canine oocyte penetration assay: its use as an indicator of dog spermatozoal performance in vitro. Anim Reprod Sci, 50, 123-39.

Hewitt, DA, England, CGW. 1999a.Culture conditions required to induce capacitation and the acrosome reaction of canine sperm in vitro. Vet Rec, 144, 22-3.

Hewitt, DA, England, CGW. 1999b. Synthetic oviduct fluid and oviductal cell coculture for canine oocyte maturation in vitro. Anim Reprod Sci, 55, 63-75.

Holst, PA, Phemister, RD. 1971**.** The prenatal development of the dog: Preimplantation events. Biol Reprod, 5, 194-206.

Hyttel, P, Farstad, W, Mondain-Monval, M, Bakke Lajord, K, Smith, AJ. 1990. Structural aspects of oocyte maturation in the blue fox (*Alopex lagopus*). Anat Embryol, 181, 325-31.

Jalkanen, L**.** 1992a. The female silver fox compared to the blue fox.
In: Tauson, A.H., Valtonen, M. (Editors), Reproduction in carnivorous fur bearing animals. Jordbruksforlaget, Copenhagen, pp 135-138.

Jalkanen, L. 1992b. Embryonal development and embryo losses during the preimplantation period in the silver fox. N J Agric Sci, 9 (Suppl.): 108-14.

Jalkanen, L. 1993. Prospects of embryo technology in fox research. Scientifur17, 287-91.

Jalkanen, L, Lindeberg, H. 1998. Successful embryo transfer in the silver fox (*Vulpes vulpes*). Anim Reprod Sci, 54,139-47.

Kalab, P, Srsen, V, Farstad, W, Krogenæs, A, Motlik, J, Hafne, AL. 1997.
MAP kinase activation and RAF-1 synthesis in blue fox oocytes is controlled by cumulus granulosa cells. Theriogenology 47, 400.

Kinney, GM, Pennycook, JW, Schriver, MD, Templeton, JW, Kraemer, DC. 1979. Surgical collection and transfer of canine embryos. Biol Reprod 20 (suppl 1), 96A (abstr).

Krogenæs, A, Nagyova, E, Farstad, W, Hafne, A-L. 1993. In vitro maturation of blue fox oocytes and cAMP production in oocyte-cumulus cell complexes. Theriogenology 39, 250 (abstr.).

Lindeberg, H, Jalkanen, L, Savolainen, R. 1992**.** Experiences of in vitro culture of silver fox embryos. The Nordic A.I. Virtanen Inst. Symp On In Vitro Culture of Domestic Animal Embryos, University of Kuopio, pp 42-3.

Luvoni, GC, Luciano, AM, Modina, S, Gandolfi, F. 2001. Influence of different stages of the oestrus cycle on cumulus-oocyte communications in canine oocytes: effects on the efficiency of in vitro maturation. J Reprod Fertil, 57(Suppl): 141-146.

Luvoni. GC, Chigoni, S, Allievi, E, Macis. D. 2003. In vitro maturation of canine oocytes in isolated oviduct. Reprod Dom Anim, 38, 410-414.

Mahi CA, Yanagimachi, R. 1976. Maturation and sperm penetration of canine ovarian oocytes in vitro. J Exp Zool 196:189-96.

Mahi, CA, Yanagimachi, R. 1978. Capacitation, acrosome reaction and egg penetration by canine spermatozoa in a simple defined medium. Gamete Research 1, 101-9.

Mastromonaco, GF, Hay, MA, Goodrowe, KL. 2002. The effects of oocyte storage and cumulus cell presence on canine zona penetration by domestic dog spermatozoa. Theriogenology 57, 1123-34.

Nickson, DA, Boyd, JS, Eckersall, PD, Ferguson, JM, Harvey, MJA, Renton, JP. 1993. Molecular biological methods for monitoring oocyte maturation and in vitro fertilization in bitches. J Reprod Fertil 47(Suppl.), 231-40.

Otoi, T, Fujii, M, Tanaka, M, Ooka, A, Suzuki, T. 2000. Oocyte diameter in relation to meiotic competence and sperm penetration. Theriogenology 54, 535-42.

Petrunkina AM, Simon,K, Gunzel Apel, AR, Töpfer- Petesen , E. 2003. Regulation of capacitation of canine spermatozoa during co-culture with heterologous oviductal epithelial cells. Reprod Dom Anim, 38, 455-463.

Rota, A, Mollo, A, Marinelli, L, Gabai, G, Vincenti, L. 2003. Evaluation of cabergoline and buseriline efficacy for oestrus induction in the bitch. Reprod Dom Anim, 38, 440-443.

Renton, JP, Eckersall, PD, Ferguson, JM, Harvey, MJA, Mullaney, J, Perry**,** B. 1991. Ovulation, fertilization and early embryonic devlopment in the bitch (*Canis familiaris*). J Reprod Fertil 93, 221-31.

Rodrigues, BA , Rodrigues JL. 2003. Meiotic response of in vitro matured canine oocytes under different proteins and heterologous hormone supplementation. Reprod Dom Anim, 38, 58-62.

Saint-Dizier, M, Renard, J-P, Chastant-Maillard, S. 2001. Induction of final maturation by sperm penetration in canine oocytes. Reproduction 121, 97-105.

Seidel,GE. 1991. Embryo transfer: the next 100 years. Theriogenology 35, 171-80.

Shin, T, Kraemer, D, Pryor, J, Liu, L, Rugila, J, Hower, L, Buck, S, Murphy, K, Lyons, L, Westhusin, M. 2002. Nature 415, 859.

Sirivaidyapong, S, Cheng, FP, Marks, A, Voorhout, WF, Bevers, MM, Colenbrander, B. 2000. Effects of sperm diluents on the acrosome reaction in canine sperm. Theriogenology 53,798-802.

Songsasen, N, Yu, I, Leibo, SP. 2002. Nuclear maturation of canine oocytes cultured in protein–free media. Mol Reprod Dev 62, 407-15.

Srsen,V, Kalous, J, Nagyova, E, Sutovsky, P, King WA, Motlik J. 1998. Effects of follicle stimulating hormone, bovine somatotropin and okadaic acid on cumulus expansion and nuclear maturation of blue fox (*Alopex lagopus*) oocytes in vitro. Zygote 6, 299-309.

Tsutsui, T, Shimada, K, Nishi M, Kubo, N, Muraop, I, Shimizu, T, Ogasa, A. 1989. An experimental trial on embryo transfer in the dog. Jap J Vet Sci 51, 797-800.

Tsutsui,T, Hori, T, Kawakami, E. 2001. Intratubal transplantation of early canine embryos. J Reprod Fertil 57 (Suppl), 309-14.

Tsutsui, T, Shimizu, T, Hori, T, Kawakami, E. 2002. Transuterine migration of canine embryos. 3rd EVSSAR Congress, Liége, Belgium. Proceedings, pp 183-4.

Valtonen, M. 1984a. Från äggcell till blårevsvalp I. Fosterutveckling. (From egg cell to blue fox cub I. Embryonic development). Finsk Pälstidsskrift 18: 253-5.

Valtonen, M. 1984b. Från äggcell till blårevsvalp II. Fosterdödlighet (From egg cell to blue fox cub II. Embryonic death). Finsk Pälstidsskrift 18: 296-8.

Valtonen, M, King, WA, Gustavsson, I, Mäkinen, A. 1984. Embryonic development in the blue fox. 3e Congr. Int. Sci. Prod. Anim. Fourrure, Versailles, Com. 29, 1p.

Valtonen, M. 1992. Comparative aspects of reproductive physiology in domestic animals. The female reproductive system. In: Tauson, A.H., Valtonen, M. (Editors), Reproduction in carnivorous fur bearing animals. Jordbruksforlaget, Copenhagen, pp 1-15.

Vaever-Bysted , Greve,T, Hyttel, P. 2001. Activation of the embryonic genome in the dog. In: Periovulatory Events and Early Embryonic Development in the Dog. PhD Thesis, Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Waterhouse, KE, Miller, Jr. RR, Cornett, CL, Haugan, T , Fougner" JA, Farstad, W. 2002. Comparison of membrane fatty acid composition of sperm from silver fox and blue fox. 9th-International Symposium on Spermatology, Cape Town, South Africa, 72 (abstr).

Wen, XH, Feng, HL, Sun, QY. 1994. In vitro maturation of follicular oocytes of the silver fox (*Vulpes fulva desm*). Theriogenology 41: 333.

Westhusin, ME, Burghardt, RC, Rugila, JN, Willingham, LA, Liu, L, Shin, T, Howe, LM, Kraemer, DC. 2001. Potential for cloning dogs. J Reprod Fert il 57 (Suppl): 287-93.

Willingham-Rocky, LA, Hanna, CBM, Westhusin, ME, Kraemer, DC. 2002.
Effects of porcine somatotropin and epidermal growth factor on the resumption of meiosis of canine oocytes matured in vitro. Theriogenology 57:742.

Yamada, S, Shimazu, Y, Kawaji, H, Nakazawa, M, Naito, K, Toyoda,Y. 1992. Maturation, fertilization and development of dog oocytes in vitro. Biol Reprod 46: 853-8.

Yu, I, Leibo, S.2002. Recovery of motile, membrane intact spermatozoa from canine epididymides stored for 8 days at 4 °C. Theriogenology 57: 1179-90.

Zindl, C, Günzel-Apel, A-R, Asa, CS. 2002.
Semen freezing in the Mexican wolf (*Canislupus baileyi*). Effects of different cooling rates and addition of Equex on sperm post-thaw viability and longevity. 3rd EVSAR Congress, Liége, Belgium, Proceedings, pp 189-90.