The Domestic Dog Embryo: *in vitro* fertilization, culture, and transfer

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i. Summary/Abstract

Advances in embryo technologies in the domestic dog have made significant strides in the past decade. This progress has been spurred by interests in taking advantage of the dog as a biomedical research model for human and companion animal medicine, developing assisted reproductive technologies to manage genetic diversity in endangered canids maintained ex situ, and improving breeding in rare or working breeds of dogs. Here we focus on recent advancements and techniques for collection of *in vivo*-matured oocytes, *in vitro* fertilization (IVF), *in vitro* culture of early (≤ 8-cell) and advanced stage (≥ 16-cell) embryos, and embryo transfer.

ii. Key Words

Domestic dog, *in vitro* fertilization, oocyte, embryo culture, embryo cleavage kinetics

1. Introduction

Understanding of reproductive biology in the domestic dog is surprisingly limited. This includes the regulators of ovarian follicle development, oocyte maturation, anestrus termination, and embryo development. In turn, this lack of understanding has restricted our ability to develop assisted reproductive technologies (ART) in canids and advance the domestic dog as a research model. Over 410 genetic diseases have been identified in domestic dogs with homologies to human conditions [1,2], making it an attractive model for human biomedical research. Toward these ends, transgenic puppies have been produced via somatic cell nuclear transfer [3-5], and CRISPR/Cas9 [6]. Still, rates of successful transgenesis remain low, and the process is dependent on the use of zygotes, which limits broad utilization. The domestic dog is also the ideal model to develop ARTs for the conservation of its endangered cousins. The International Union for Conservation of Nature has designated 5/35 extant wild canid species as either endangered or critically endangered, and two have already gone extinct [7]. As result, there is growing need for the creation of *ex situ* insurance populations. However, maintenance of genetic diversity within a small group of animals over many generations is a critical challenge.

Natural breeding requires the animals to be at the same geographic location, which means that achieving specific matches involves the often-stressful and expensive transport of valuable animals to distant locations. Improved ARTs such as *in vitro* fertilization introduces the ability to transfer gametes or embryos to distant locations, rather than the animals themselves, and still produce genetically valuable, live offspring.

Unfortunately, ARTs have been historically challenging to develop in the domestic dog due to their unique reproductive biology. Domestic dogs experience a reproductive cycle consisting of roughly one week periods of proestrus and estrus, followed by a 2 month obligatory diestrus [8]. Diestrus is followed by a variably long (2-10 month) anestrus [9], resulting in ovulation only 1-2 times annually. The variable duration and final termination of anestrus are not well understood, despite decades of work investigating the traditional reproductive hormones (gonadotropins, estradiol, progesterone) that drive estrus. Many attempts have been made to develop estrus induction protocols for the dog, with varying results (See Review: [10]). For example, supplementation of exogenous follicle stimulating hormone (FSH) has been used to stimulate follicle growth [11]. Dopamine agonists and gonadotropin-releasing hormone agonists have both been utilized to induce estrus by stimulating gonadotropin release [12,13]. The latter two treatment types have successfully induced estrus in most studies, but effectiveness is highly dependent on the stage of reproductive cycle at the time of hormone treatment [14,15] and, in the case of GnRH agonists, long-term treatment resulted in estrus suppression [16], and contraception [17]. Thus, while many different estrus induction techniques have been attempted in the dog, few have yielded consistent results.

After the onset of estrous behavior and two days following the surge of LH, a germinal vesicle stage oocyte is ovulated into the bitch's oviduct, which is embedded in the fatty bursa encompassing the ovary. Dog oocytes require an additional 48-72 hours in the oviduct to reach metaphase II (MII) and become fertilizable [18]. The evolutionary advantage for this timing,

which differs between canids and all other mammals, is unclear. In addition, the mechanism underlying this delayed oocyte maturation is not yet understood, though it has been postulated to involve the prolonged close association of cumulus cells with ovulated oocytes [18]. Nevertheless, attempts to recapitulate this maturation process *in vitro* have generally yielded low rates of MII development. Several excellent reviews exist on dog *in vitro* oocyte maturation (IVM) efforts [19-21], but the overall conclusion is that attempts to mature eggs *in vitro* have generally yielded low rates of MII development. Additionally, a comprehensive review of domestic dog embryo biotechnologies, from *in vivo* embryo development to cloning is also already available [22]. Therefore, in this chapter, we concentrate on recent advancements in *in vitro* embryo production with an emphasis on culture medium composition and early embryo cleavage kinetics *in vitro,* followed by detailed techniques for the collection of *in vivo*-matured oocytes, *in vitro* fertilization (IVF), *in vitro* culture of early and late stage embryos, and embryo transfer.

Techniques for collection of *in vivo*-matured oocytes

As result of the unique characteristics of the domestic dog's reproductive biology described above, obtaining mature oocytes is a particular challenge in this species. Ideally, the vast stores of immature oocytes present in the ovary at any stage of the reproductive cycle could be utilized for ART. Detailed methods exist on oocyte collection from ovaries [23]; however, the success of various *in vitro* oocyte maturation protocols have proved variable [19-21]. Thus far, the only reliable source of MII dog oocytes is via collection of *in vivo* matured ova. This strategy requires precise timing of the luteinizing hormone (LH) surge and/or ovulation. This is best accomplished via serum progesterone [24,25] and/or identifying the LH surge [26-28], with monitoring of changes in vaginal cytology being used in a supportive, confirmatory fashion [29]. Recovery of MII oocytes from the oviduct can be accomplished *in situ,* via inserting a catheter into the distal oviduct (isthmus) then retrograde flushing toward a needle or another catheter inserted into the infundibulum [30-32]. This *in situ* technique has been successfully applied to collect mature

oocytes for use in somatic cell nuclear transfer, and zygotes for autologous transfer after modification via CRISPR-Cas9 [33]. In a less technically challenging method, *in vivo* matured oocytes can also be flushed from isolated oviducts following specifically-timed ovario-hysterectomy. Day 6 post-LH surge (i. e., *in vivo*-matured) dog oocytes collected in this manner have recently produced live young following IVF, demonstrating their fertilization competence on this day of collection [28].

***In vitro* fertilization and embryo culture in the dog**

While the production of IVF puppies was primarily attributed to use of *in vivo* matured oocytes and modifications in sperm capacitation medium [28], the extent to which fertilization and embryo culture media contributed to the success is unclear. *In vivo*-matured ova recovered on Day 6 post-LH surge had previously been used in IVF in the dog [34]. Hori and Tsutsui timed ovulation based on plasma progesterone levels over 2 ng/ml, based on previous work in their laboratory correlating progesterone with LH and ultrasonographic imaging of the ovary [27]. They collected oocytes and co-incubated them in a modified Krebs-Ringer bicarbonate solution (See TYH-BSA and mTYH-BSA in **Table 1**) with sperm capacitated in TYH for 20 hours [34]. Two-cell embryos were observed 48-108 hours following initiation of co-culture, with cleavage to the 4-cell stage after an additional 12-24 hours. Across all treatment groups, 10 oocytes cleaved out of 94 oocytes fertilized (**Table 2**). The authors attributed the low rate of cleavage to suboptimal fertilization and embryo culture medium.

In our study of IVF that yielded live births, *in vivo* matured oocytes were collected on Day 6 following the LH surge, which was determined based on serum progesterone levels of 1.5 - 2.5 ng/ml and a positive LH snap test. Serum progesterone levels of ~4.5 ng/ml confirmed the day of ovulation, two days after the LH surge [28]. Ova were co-incubated with sperm for 14 hours in a ‘complete’ NCSU-23 medium (cNCSU, see **Table 1**), with both essential and nonessential amino acids, as well as 6 µg/ml progesterone. Sperm had been collected and pre-incubated for 2.5 - 3.5 hours under capacitating conditions in modified Canine Capacitation Medium

(containing 25 mM HEPES and 1.0 mM $MgCl_2$). No cleavage was observed 24 hours post initiation of co-incubation, but 115 oocytes cleaved out of 146 fertilized (**Table 2**), with the majority developing to the 4-cell stage by 48 hours post co-incubation.

In the same study, Day 5 oocytes were similarly collected and fertilized, yielding 11 embryos of 37 oocytes (**Table 2**), although 6 of these did not cleave until 72 hours post co-incubation. Our laboratory has also previously evaluated Tissue Culture Medium 199 (M199) + 20% fetal bovine serum (FBS) for IVF, followed by culture in a modified synthetic oviductal fluid (mSOF) or NCSU-23 (mNCSU). Both mSOF and mNCSU lack the full complement of amino acids used in cNCSU (See **Table 1**). This strategy produced four embryos from five Day 6 oocytes (2/3 and 2/2 embryo development in mSOF and mNCSU, respectively, **Table 2**), three of which developed to the 8-cells stage in culture.

It should be noted that at least part of the difference in embryo production rates between the Nagashima et al., and Hori and Tsutsui studies may be due to a difference in the designation of Day 0. Although it is difficult to compare assays performed using different methods in different laboratories, one can make the observation that serum progesterone values in our study on the designated day of ovulation were typically ~ 4.5 ng/ml [28], whereas plasma progesterone values higher than 2 ng/ml were used to identify ovulation in the Hori and Tsutsui study [34].

When assessing these studies in the context of the broader literature on embryo production and development, it is apparent that the presence of phosphate and/or glucose does not hinder early embryo (< 8-cell) development in the dog, as in the hamster [35]. Also, although essential and non-essential amino acids were both present in the media producing live young, it cannot be said conclusively that their presence benefited embryo production and early development, as in the cow [36], because the relevant comparisons were not made. Fertilization was accomplished in the Hori and Tsutsui study (as well as those of others [37-39]) in the absence of essential and non-essential amino acids. In our laboratory, IVF embryos cultured in mSOF developed to the 8-cell stage as readily as those cultured in the presence of the full complement

of amino acids. Additional investigation is needed to determine the influence of amino acid supplementation on developmental competence of canine embryos generated by IVF.

Kinetics of dog embryo cleavage events and *in vitro* culture

Time-lapse imaging has advanced our ability to understand the relationship between embryo cleavage kinetics and developmental capacity *in vitro.* In human IVF, shorter times of cleavage to the 8-cell stage are associated with increased development to the blastocyst stage, improved blastocyst quality [40], and better implantation rate [41,42]. Alterations in ovarian stimulation protocols [43] and culture medium [44] do not appear to significantly influence cleavage kinetics of human embryos. However, developmentally delayed embryos are typically aneuploid [45], which may explain the deficiencies observed at the blastocyst and implantation stages.

To our knowledge, time-lapse imaging of domestic dog embryo development has not been completed. Still, it is likely that cleavage rates which align to *in vivo* development rates are indicative of better embryo quality. Early embryo development *in vivo* has been well described in the dog [22]. Briefly, two-cell stage embryos are typically observed 144-216 hours post-LH surge, 4-cell between 160-192 hours, and 8-cell stage embryos 160-336 hours after the LH surge [18,46] – **Figure 1**. The high temporal variability is likely due in part to asynchrony of ovulation after the LH surge (which can range up to 36 - 50 hours [46]), in time from ovulation to complete nuclear maturation (48 – 60 hours [18,47]), and in fertilization time point following the former two events.

Of the oocytes which developed into embryos in our IVF study, >92% did so within 48 hours of co-incubation with sperm (~192 hours or Day 8 post-LH surge) [28]. At this time point, the vast majority (> 81%) of embryos were at the 4-cell stage. No cleavage was observed at the 24-hour post co-incubation in our study; therefore, the first two cleavage events both took place between hours 24 and 48 following the onset of co-incubation in the fertilization droplet. By the subsequent check (72 hours post co-incubation), embryos were typically at the 6- or 8-cell

stage. Despite using different sources of oocytes, embryo cleavage kinetics reported from dog IVF studies utilizing *in vitro*-matured ova previously reached the same consensus, with initial embryo cleavage occurring between 24-48 hours post co-incubation with sperm, and development to 4- to 8- cell stage by the 72 hour mark [39,37,38]. Therefore, it appears that the kinetics of embryo development in the dog are fairly conserved across gamete sources, media preparations, and protocol variations.

In spite of this apparent consistency, we also noted that a subset of embryos (5.4% of oocytes co-incubated with sperm) experienced 'delayed' cleavage, wherein development to the 2-cell stage did not occur until sometime around 72-120 hours post co-incubation, or 216-264 hours (Day 9-11) post-LH surge [28]. Frequency of delayed cleaving embryos was higher in oocytes collected on Day 5 post LH-surge, and with one of the individual males used as a sperm donor. A similar phenomenon was described by Hori and Tsutsui, with cleavage to the two-cell stage occurring up to 108 hours after initial co-culture. As a comprehensive evaluation of the developmental competence of these 'delayed' embryos has not been completed, it is still uncertain if delay is indicative of poor embryo quality, as in the human. Domestic dog ova are known to have a uniquely long period of fertility, with live young having been produced after intrauterine artificial insemination up to 10 days post-LH surge [48]. Therefore, it is also possible that these 'delayed' embryos could produce normal pregnancies.

Embryonic genome activation and advanced stage embryo culture

In the Nagashima et al IVF study, 15 of the 21 embryos cultured for 96 hours (i. e. to 240 hours or Day 10 post-LH surge) developed to ≥ 8-cell stage but development halted between the 8- and 16-cell stage *in vitro.* This block coincides with the time of genome activation in the canid embryo [49]. Results suggest that, although culture conditions described are sufficient for short term culture of early stage domestic dog embryos, they are not sufficient to overcome this developmental block during long-term *in vitro* culture*.* Nevertheless, embryo development *in vitro* to the 16-cell stage and beyond has been observed, albeit infrequently, following *in vitro*

oocyte maturation and IVF in the dog. Otoi et al. produced four 16-cell stage or beyond from 30 embryos produced using an M199-based medium [50]. IVF and culture of *in vitro* matured dog oocytes in M199-based media have produced morulae and blastocyst stage embryos in other studies as well [51,52]. The formulation of M199 is much more complex compared to even the modified NCSU used for the fertilization and early embryo culture of the IVF-derived embryos that produced live young, and so may provide embryos with better support during embryonic genome activation. Further studies are needed to identify component(s) that may be responsible for this effect.

In vivo-derived wild canid embryos have also been cultured to advanced stages *in vitro.* Post-embryonic genome activation-stage silver fox embryos have been maintained *in vitro* using M199 [53]. Renton et al. reported the development of a one-cell stage embryo collected five days after ovulation to the morula stage after 120 hours *in vitro* [54]. The embryo was also cultured in M199 supplemented with 20% FBS. Further, our laboratory has had some success in culturing compact morula-stage embryos in a similar medium to the blastocyst stage (**Figure 2**), indicating this base medium is supportive to the development of advanced stage embryos.

Interestingly, in the latter two experiments, as well as in the elegant work describing preimplantation development of later stage dog embryos by Abe et al, *in vivo*-derived dog blastocysts were observed to initially shrink within their zona pellucidae around the time of collection [54-56]. In all cases, the blastocoel cavity recovered after a short period of *in vitro* culture. Renton et al noted that recovery could be accomplished quickly utilizing a 1:1 Ham's F10:distilled water medium, suggesting the rapid change in cavity volume is likely related to a change in the osmolarity of their environment. In our work, blastocoel re-expansion was typically achieved after ~2 hours incubation in M199 with 20% FBS. Further, we have previously physically collapsed cavities of expanded blastocysts via microinjection (n=8), then transferred to a recipient uterus and produced two live births (Data not shown). Human blastocyst studies have deliberately collapsed this cavity to reduce opportunity for ice crystal formation during

cryopreservation [57,58], and also produced successful pregnancies. Although not ideal, these data indicate blastocoel cavity collapse *in vitro* does not prohibit successful birth in humans or dogs following transfer.

Embryo Transfer

Currently, there are three key methods to transfer embryos which have been documented as resulting in live births. Choice of method depends in part on the stage of embryos being transferred, as transfer location must be physiologically appropriate for the embryo (Details of embryo stages/locations have been well described in the dog [55,59]). The first two are surgical transfers, to either the oviduct or the uterine horn. The oviduct is preferred for early stage embryos (< 8-cell), whereas transfer into the uterine horn is technically more straightforward but appears better suited for later stage embryos (≥ 8-cell stage). Both strategies are currently performed via laparotomy, but have the potential for completion via laparoscopy to reduce invasiveness. The third method, non-surgical embryo transfer, is a minimally-invasive standing procedure, involving passage of a cystoscope-equipped catheter through the cervix into the uterine body and horns [59]. As deposition of embryos is into the uterine horn, this again likely necessitates use of ≥ 8-cell stage embryos.

Future Directions

Although we have focused on the recent advances in canine embryo technologies, we would be remiss not to also highlight the areas in need of additional study. The development of *in vitro* fertilization in this species opens the door to re-evaluate *in vitro* oocyte maturation protocols with a proven-method end point. Establishment of protocols that will result in the production of *in vitro* matured oocytes will, in turn, allow for more studies to understand the mechanisms regulating embryo development and embryo genome activation. Therefore, developing a robust IVM protocol for oocytes collected from diestrus and anestrus dogs is the highest priority. Following this, high rates of success in terms of live birth after oviductal embryo transfer should quickly facilitate translation of these advances to broader applications, such as

producing viable biomedical research models for human medicine [33], or applying IVF to the conservation of endangered canid species.

2. Materials

All media should be prepared using ultrapure water (18 mΩ with low VOC (ie. milliQ)) and cell culture grade reagents. Unless otherwise indicated, store all reagents at room temperature. All plastic materials in contact with gametes (i. e. culture tubes, petri dishes) should be polystyrene.

2.1 Oocyte collection

1. PB1 [60]: 136.87 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 0.49 mM MgCl2*6H2O, 8.09 Na_2HPO_4, 0.33 Na pyruvate, 5.56 mM glucose, 3.00 g/l BSA, 100 U/ml Penicillin and Streptomycin sulfate, and 0.90 mM $CaCl_2$*$2H_20$ (**Note 1**) Adjust pH to 7.55-7.6, then make up to 1 L with ultrapure water. Store at 4°C up to 2 weeks.
2. Warming pad
3. #10 blades and scalpel blade holder
4. Sterile forceps
5. Sterile scissors
6. Leur-lock syringe (5 – 20 cc)
7. Sterile curved tip fine forceps
8. 23 G winged infusion set (**Note 2**), or straight needle
9. 100 x 15 mm Petri dishes
10. 60 x 15 mm Petri dishes
11. 35 x 10 mm Petri dishes

2.2 IVF and early embryo culture

1. mCCM [61,28]: 83.29 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$ (**Note 1**), 1.19 KH_2PO_4, 0.25 Na pyruvate, 2.78 mM glucose, 100 IU/ml Penicillin G and streptomycin sulphate, and 1.0 mM $MgCl_2$*$6H_20$. In ultrapure water. Store at 4°C for up to 2 weeks. Osmolality should be around 300 mOsm

i. Add 37.61 mM $NaHCO_3$, 21.66 mM Na lactate 60% syrup (**Note 3**), and 2 g/ml bovine serum albumin (BSA) on the day of IVF. Adjust pH to 7.8. Warm in incubator at 38.5°C and 5% CO_2

2. cNCSU: **See Table 2**. Prepare cNCSU with ultrapure water, pH to 7.55 – 7.6 and store at 4°C. Added 1% BME and 1% Minimum Essential Medium (MEM) Nonessential Amino Acids solutions (also stored at 4°C) on the day of IVF
3. Polyvinyl alcohol-coated tubes [62] (**Note 4**)
4. Mineral oil, cell culture grade
5. 35 x 10 mm non-tissue culture treated Petri dishes
6. Mouth pipette
7. Hypoxia chamber
8. 5% CO_2, 5% O_2, 90% N_2 gas
9. 10 mM progesterone in DMSO (optional)

2.3 Late stage embryo culture

1. Blastocyst culture medium: M199, 10% v/v FBS, and 100 U/ml penicillin and streptomycin sulfate (pH 7.5, Osm 340)
2. Mineral oil, cell culture grade
3 Hypoxia chamber
4 5% CO_2, 5% O_2, 90% N_2 gas

2.4 Oviductal Embryo Transfer (Surgical)

1. Transfer medium: M199, 20% w/v FBS (**Note 5**)
2. Recipient bitch at synchronized stage of estrus cycle
3. Sterile forceps
4. Sterile surgical scissors
5. 3.5 FR, 4.5” (11.4 cm) tomcat catheter
6. Leur-lock syringe (1-3 cc)

7. Dissecting scope with warming plate

2.5 Uterine Horn Embryo Transfer (surgical)

1. Transfer medium (See 2.4.1)
2. Recipient bitch at synchronized stage of estrus cycle
3. #11 scalpel blade and holder, sterile
4. Autoclaved wide-orifice 200 ul pipette tips
5. 200 ul pipette
6. Dissecting scope with warming plate
7. Suture (optional)

3. Methods

3.1 In Vivo Matured Oocyte Retrieval

1. Monitor onset of donor bitch's estrous cycle via behavioral and physical signs of proestrus. Use vaginal cytology, serum progesterone [24,25], and/or LH surge, to determine the date of the LH surge (counted as Day 0). Specifically time spay for Day 6 post-LH surge.
2. Warm 500 ml PB1 overnight in incubator at 38.5°C prior to collection
3. Transfer excised tract from Day 6 post LH surge bitch immediately after removal during spay procedure into at least 300 ml warmed PB1 in a large beaker. Transport to laboratory.
4. In tissue culture hood using forceps and scissors, remove entire bursal compartment around an ovary, including a small portion of the uterine horn just distal to the uterotubal junction. Transfer to 60 x 100 mm petri dish on warming pad or warmed surface, keeping moist with additional PB1 (approx. 5 ml, but will depend on the size/breed of the dog)
5. Identify bursal window then create an opening in this region large enough to invert the bursal sac without cutting into the oviduct running through (**Figure 3A, B, Note 6**)

i. Record # of corpora lutea on each ovary

6. Isolate oviduct from bursa using #10 scalpel blade (**Figure 3C**, **Note 7**)
7. Make a transverse section of each oviduct with a scalpel blade (**Figure 3D, E**, **Note 8**) and transfer each isolated oviduct section to an individual 60 mm petri dish with ~3 ml fresh warmed PB1.
8. For each oviduct half, cannulate from the end cut in Step 3.1.7 with 23 g needle infusion set attached to syringe filled with warmed PB1. This can be done using curved fine tip forceps to sheath oviduct over the needle.
9. Flush (**Figure 3F**) oviduct by holding cannulated oviduct firmly over needle end with forceps to prevent backflow while slowly depressing syringe with PB1. Flush each side with at least 3 ml of PB1, into the petri dish, then transfer oviductal tissue to a fresh dish with PB1 and set aside on a warming plate.
10. Observe dish with flushed medium under a dissecting microscope. Oocytes will typically settle to the base of the dish. Using either a mouth pipette or pipette set to small volume (< 10 µl), collect oocytes (**Note 9**) into a new 35 mm petri dish with warmed cNCSU to wash. Maintain dish in incubator at 38.5°C and 5% CO_2 until all oocytes have been collected.
    i. Repeat flushing of oviducts set aside in Step 3.1.9 if the number of oocytes recovered is significantly less than the number of CLs on the corresponding ovary noted in Step 3.1.5.i.
11. Maintain room lights on low or off once oocytes have been recovered (**Note 10**)

3.2 In vitro fertilization

1. Sperm Preparation (**Note 11**)
    i. Prepare PVA-coated tubes
        a) Prepare 2 g polyvinylalcohol in 50 ml ultrapure water (4% w/v) by stirring while heating up to 98°C.

b) Slowly add 50 ml isopropanol, continue to stir and heat until solution becomes clear.

c) Cool to room temperature, then fill ultra-clear sterile polystyrene tubes with the solution and let sit for 15 minutes

d) Aspirate the solution (will leave a clear film on the tube walls), then leave the tubes open to dry overnight at room temperature

e) Rinse tubes with distilled water then fill with distilled water and allow to stand for 1-2 hours at room temperature. Rinse once more with distilled water, tap to remove excess liquid, then leave to dry.

ii. If necessary, prepare temperature-controlled centrifuge to 37°C

iii. Prepare CCM with BSA, bicarbonate and sodium lactate, pH to 7.8, sterile filter and maintain at 38.5°C and 5% CO_2

iv. Collect first and second fraction of semen via manual stimulation into sterile tube or collection bag (**Note 12**)

v. Transfer semen to PVA-coated tube

vi. Centrifuge at 100g for 1 min to remove debris (primarily epithelial cells).

vii. Transfer supernatant to fresh PVA-coated tube diluted 1:1 with warmed mCCM

viii. Centrifuge at 400g x 5 min to pellet the sperm. Remove supernatant and discard, minimally disrupting pellet.

ix. Resuspend sperm pellet in 1 ml mCCM

x. Using a hemocytometer and a small sample of the washed sperm in water (typically 1:200 dilution), assess sperm concentration

xi. Incubate washed sperm at a concentration of 7.5 x 10^6 sperm/ml in mCCM in PVA-coated tubes at 38.5° C and 5% CO_2 for at least 2 hours to capacitate (**Note 13**)

2. Fertilization

i. Prepare 100 µl droplets of cNCSU (Step 2.2.2) overlaid with mineral oil by first pipetting 5 µl onto the base of a NON tissue-culture treated 35 mm petri dish, then overlaying with ~4.5 ml cell culture grade mineral oil, then pipetting the remaining 95 µl cNCSU into the 5 µl droplet under the oil (**Note 14**). Equilibrate for at least 1 hour

ii. After 2 hr capacitation, count sperm again (**Note 15**), then adjust volume of cNCSU droplet prepared in Step 3.2.2.i to add capacitated sperm for a final concentration in a 100 µl droplet of 1.0 x 10^6 sperm/ml (**Note 16**)

iii. Transfer mature oocytes (From Step 3.2.10, if applicable) by mouth pipette (minimal medium) into the cNCSU fertilization droplet with capacitated sperm.

iv. Co-incubate gametes for 14 hr at 38.5°C and 5% CO_2, 5%O_2 and 90% N_2 in a humidified hypoxia chamber. (**Note 17**)

v. Prepare individual culture dishes for each oocyte collected, with a 50 □l droplet of cNCSU under mineral oil (As in Step 3.2.2.i)

vi. After 14 hrs co-incubation (**Note 18**), transfer presumptive zygotes into a dish with 5 ml warmed cNCSU and wash by gentle pipetting to remove loosely attached sperm and/or cumulus cells

vii. Transfer each presumptive zygote in minimal medium to a pre-equilibrated culture dish prepared in Step 3.2.2.v then return all dishes to the hypoxia chamber for culture at 38°C and 5% CO_2, 5%O_2 and 90% N_2 (**Note 19**)

viii. Evaluate embryo cleavage at 48 hr post-IVF

3.3 Embryo culture

1. Early embryos (1 – 8-cell stage)
    i. Prepare 50 □l droplets of cNCSU overlayed with mineral oil, as in Step 3.2.2.i. Allow the medium to equilibrate for at least one hour at 38.5°C and 5% CO_2, 5% O_2 and 90% N_2 in a humidified hypoxia chamber.
    ii. Culture each embryo individually in the pre-equilibrated dishes at 38.5°C and 5% CO_2, 5%O_2 and 90% N_2 in a humidified hypoxia chamber.
    iii. Exchange half the medium (25 µl) with fresh every 48 hours.
2. Advanced Embryos (Compact morulae to blastocysts) (**Note 20**)
    i. Prepare 25 □l droplets of Culture Medium overlaid with mineral oil, as in Step 3.2.2.i
    ii. Transfer blastocysts using wide-orifice, 200 µl or smaller pipette tips (**Note 21**) and culture each embryo individually in the pre-equilibrated dishes at 38.5°C and 5% CO_2, 5%O_2 and 90% N_2 in a humidified hypoxia chamber
    iii. Exchange half the medium (12.5µl) with fresh every 24 hours.

3.4 Oviductal Embryo Transfer

1. Monitor estrus as in Step 3.1.1 to time synchronization of recipient bitch with stage of embryos
2. Exteriorize both left and right ovarian bursa of recipient via laparotomy (**Note 22**), manually dissect the suspensory ligament as far from the ovarian bursa as possible while minimizing manipulation of the area.

3. Using forceps and scissors, make a slit through the bursal window wide enough to access the infundibulum. This will vary depending on the size of the animal and maneuverability possible based on field exteriorized via laparotomy.
4. Perform a 'dry run' cannulation of the infundibulum with a 3.5 FR, 4.5" tomcat catheter to locate lumen
5. Draw up embryos with ~20 µl medium into the tomcat catheter connected to a 1 cc syringe (**Note 23**). First, draw up air, then embryos in medium, then additional medium with no embryos in the distal tip of the catheter.
6. Advance the catheter approximately 2.5 cm into the oviduct via the previously identified (Step 3.4.4) ostium
7. Slowly dispense embryos, withdrawing catheter slightly as syringe plunger is depressed. Last portion dispenses air to 'push' embryos the remaining distance before removing catheter from infundibulum
8. Check to ensure that there was no backflow from oviduct
    i. If so – attempt to pipette off and check under microscope, re- transfer if needed
9. Check tom cat catheter under a microscope to confirm all embryos dispensed, may see presence of ciliated cells on outside (brushed off from oviductal lumen)
10. Fold bursa closed back over the ovary

3.5 *Uterine horn embryo transfer*

1. Monitor estrus as in Method 3.1 to time synchronization of recipient bitch with stage of embryos
2. Exteriorize both uterine horns of recipient via laparotomy (**Note 22**)
3. Using an #11 blade scalpel, make a small (size) stab incision (avoiding blood vessels, as possible) into the cranial portion of the uterine horn

4. Draw up embryos in minimal medium (M199 + 20% FBS, 15–30 µl) using a large-orifice 200 µl pipette tip (**Note 23**).
5. Insert pipette tip, angled caudally, as far as possible into the uterine lumen from the stab incision. Slowly but firmly depress plunger to expel embryos, as you will likely encounter back-pressure.
    i. When plunger is fully depressed, leave pipette tip embedded in the lumen but remove pipette. Release plunger then re-attach pipette to tip. Expel air while slowly backing tip out of the lumen and holding the sides of the incision closed to prevent backflow of embryos
    ii. Check pipette tip to make sure no embryos adhered to the side of the tip.
6. Press sides of stab incision site closed for an additional minute following transfer to prevent backflow of embryos and blood entering lumen of the uterine horn.
7. If desired, close incision site with suture.

4. Notes

1. Add $CaCl_2$ last or dissolve first in 1 ml water and slowly add to media, to avoid forming calcium phosphate precipitate.
2. Depending on the size of the dog (and therefore oviduct), a different gauge needle may be required. 23 g was utilized for work with beagles (~9 to 14 kg). Winged infusion sets, as in Figure 3F, are easier to maneuver than straight needles for flushing.
3. Sodium lactate syrup may suddenly ‘go bad’, which will result in all sperm capacitating in CCM made with it to die fairly quickly into the incubation period.
4. Use of PVA coated tubes are preferred for the sperm incubation to reduce sperm adherence to the tube walls during capacitation. Uncoated sterile tubes can also be used if needed.

5. Embryo transfer medium might be adjustable – this high protein version was utilized in [28] as embryos were post-thaw. The critical portion is to avoid adding amino acids, as this might cause inflammation at ET site. This is also the rationale for minimal medium volume used during the transfer.
6. Some bursae are thicker / more fatty than others, making it difficult to follow where the oviduct is running. Rather than attempting to isolate the oviduct solely with tools, grasp the bursa through the opening created through the window with thumb and forefinger (with sterile glove) and gently roll the tissue between them to feel for the oviduct. With this position, once a shallow slice is made into the bursa, you can then also use your thumb to pull away the excess tissue to expose the oviduct (**Figure 3C**)
7. It is helpful to reduce the amount of excess tissue/fat left on the oviduct while you are isolating it, as this will make it easier to cannulate for flushing later and reduce the amount of lipid droplets in the flushing dish later. However, this should also be balanced out with time, to get the oocytes collected quickly post-spay.
8. Flushing from the center out toward the infundibulum or utero-tubule junction is preferred because cannulation of the lumen from a clean cut is straightforward. Oocytes are unlikely to be in this region anyway, as typically by 48 hrs post-ovulation all oocytes have passed through the middle of the oviduct [63]. It is recommended to leave the most proximal portion of the uterine horn on the isthmic end of the oviduct as oocytes tend to gather near the UTJ. It will require more pressure to flush, as the UTJ is restrictive. Flush 2-3 times with gentle but steady pressure, as too much pressure runs the risk of exerting negative shear forces on any oocytes trapped in this region (It should also be noted that abnormally shaped / elongated oocytes, likely the result of too-intensive flushing, still cleaved to 8+ cell stages in our IVF system).

9. As previously noted [18], dog ova and even early stage embryos maintain layer(s) of cumulus cells. While this obscures observations, it does not preclude successful fertilization or development (See **Figure 1** images)
10. Once the oviducts have been flushed, most work should be done in a dim room to reduce the potential of light damage to the oocytes/embryos.
11. If performing IVF with freshly collected semen and *in vivo* matured oocytes, it is best to collect sperm and start the capacitation at least one hour prior to spay, or about 1.5 hours prior to obtaining the reproductive tract. This provides time to collect oocytes within a 3 hour capacitation window.
12. Males were collected 3 days prior to the sperm capacitation for IVF, to improve the quality of sperm in the ejaculate.
13. Concentrations as low as 5×10^6 sperm/ml are sufficient for capacitation, but domestic dog sperm are very sensitive to both high and low concentrations. If working with low sperm count samples, reduce volume capacitated rather than incubating at a concentration below 5×10^6 sperm/ml.
14. All the published IVF-derived embryos producing puppies were fertilized and cultured in cNCSU supplemented with 10 mM progesterone in DMSO, for a final concentration of 6 µg/ml [28], but no significant difference in embryo production rates were observed in the presence/absence of progesterone. As no non-supplemented embryos were transferred into recipient oviducts, it is not known if progesterone had an impact on final pregnancy or birth outcome, but based on the embryo results progesterone supplementation is considered ‘optional’ to the method.
15. This concentration should be very close to the 7.5×10^6 sperm/ml, which results in 13 □l being added to 87 ul cNCSU; however, sperm tend to clump together and may be either difficult to count, or the clumps fall quickly to the base of the incubation tube

even after gentle mixing. As result, the density of sperm counted after 2 hrs of incubation was typically lower, so 15-20 µl capacitated sperm were more common

16. The lowest sperm concentration successfully producing embryos was 200,000 sperm/ml in the 100 µl fertilization droplet, wherein five of six oocytes developed into embryos.
17. It is possible to reduce the time of co-incubation of sperm and eggs for fertilization. More recently, we have produced embryos from the same IVF protocol, but using a 12 hour fertilization incubation.
18. Some loosely attached sperm and outer cumulus cells will detach during this gentle washing, but the majority will remain closely adhered. If the presumptive zygote is readily denuded at this time, it may have become degenerate.
19. At the 14 hr mark, embryos are typically spinning/rotating in the dish due to motility of attached sperm, as has also been noted in *in vivo*- derived zygotes [54].
20. Described culture conditions based on short-term (42 hours) culture in which *in vivo*-derived morulae developed into blastocysts (**Figure 2**)
21. Although blastocoel cavity shrinkage within the zona is typically attributed to changes in osmolarity, we have also noted fluctuations that appear to be due to mechanical pressure on blastocysts. Cavities will still re-expand after re-equilibration incubation.
22. Avoid use of non-steroidal anti-inflammatory drugs for pain management of recipient bitch during and after embryo transfer, to reduce risk of miscarriage/pregnancy complications if similar to humans [64]
23. It is useful to have embryos closely accessible to surgical suite for ET. Set up cart with microscope and warming stage for embryos in room adjacent to surgery, but wait until last moment to pipette up embryos. Hold pipette / catheter horizontally to prevent loss of embryos out the end while moving over to transfer site. We have typically utilized three individuals for a transfer – a surgeon preparing the recipient

bitch (completely sterile), an assistant drawing up embryos into the pipette or catheter (clean, but not sterile – unless access to surgical-suite level sterile microscope / setup is available), and an embryo transfer technician (half sterile – hand which receives and pipettes embryos from assistant becomes non-sterile, but able to stand over surgical site to pipette or depress plunger to transfer embryos.

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Figure Captions:

Figure 1: Kinetics of early embryo development *in vivo* (top, from Reynaud et al 2006) and *in vitro* (bottom, from Nagashima et al 2015).

Figure 2: Advanced stage embryo development. Images of (A) a freshly collected morula and (B) compact morulae. Panels B_8 hrs, 16 hrs and 30 hrs show the compact morulae from (B) cultured for the referenced time, with both initially developing to the early blastocyst stage after 8 hours, but only one expanding further after 30 hours and the other degenerating. (C and D) show expanding blastocysts collected Days 14 and 15 post-LH surge, respectively.

Figure 3: Isolation and flushing of the dog oviduct. A) Excised bursa-encompassed ovary and cranial uterine horn, with arrow indicating bursal window. B) Opened and inverted bursa with a white asterisk (*) indicating infundibulum. C and D) Isolation of oviduct within bursal fat pad. E) Dissected oviduct for ease of cannulation (F) and flushing to collect oocytes or early embryos.

Table Captions:

Table 1: Details of media preparations used in IVF studies with *in vivo* matured oocytes

Table 2: Comparison of oocyte collection day, and IVF and embryo culture medium *in vivo* matured oocyte IVF studies

Ingredient (mM)	TYH-BSA	mTYH-BSA	mSOF	mNCSU	cNCSU
NaCl	119.37	119.37	107.70	108.73	108.73
KCl	4.78	4.78	7.16	4.78	4.78
$CaCl_2*2H_2O$	1.71	1.71	1.71	1.67	1.67
KH_2PO_4	1.19	-	1.19	1.19	1.19
$MgSO_4*7H_2O$	1.19	1.19	-	-	-
$MgCl_2$	-	-	0.49	1.19	1.19
$NaHCO_3$	25.07	25.07	25.07	25.07	25.07
Glucose	5.56	-	1.50	0.55	0.55
BSA	4 mg/ml	4 mg/ml	32 mg/ml	4 mg/L	4 mg/L
Penicillin G	75 µg/ml	75 µg/ml	100 U/ml	100 U/ml	100 U/ml
Streptomycin sulfate	50 µg/ml	50 µg/ml	50 ug/ml	100 U/ml	100 U/ml
Methyl-β-cyclodextrin	0.74	0.74	-	-	-
Polyvinylalcohol	22.70	22.70	-	-	-
Minimum essential medium (100x)	-	-	-	-	100 µl/ml
Basal medium eagle (50x)	-	-	-	-	20 µl/ml
Na Pyruvate	1.0	1.0	0.33	0.33	0.33
Sodium Lactate	-	-	3.3		
Taurine	-	-	-	7.00	7.00
Hypotaurine	-	-	-	5.00	5.00
Glutamine				1.00	1.00

Study	Day of Oocyte Collection	IVF Medium	Culture Medium	# Oocytes Fertilized	# Embryos Produced (%)
Hori and Tsutui 2003	6	TYH-BSA	TYH-BSA	43	5 (11.6)
	6	mTYH-BSA	mTYH-BSA	38	5 (21.1)
	6	TYH-BSA	TYH-FCS	13	0 (0)
Nagashima et al 2015 and unpublished data	5	M199	mSOF	13	0 (0)
	5	M199	mNCSU	15	0 (0)
	5	cNCSU	cNCSU	37	11 (29.9)
	6	M199	mSOF	3	2 (67)
	6	M199	mNCSU	2	2 (100)
	6	cNCSU	cNCSU	146	115 (78.8)