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Assisted reproductive techniques for canines: preservation of genetic material in domestic dogs

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Abstract. Assisted reproductive techniques (ARTs), such as artificial insemination, *in vitro* fertilization, and cryopreservation of gametes/zygotes, have been developed to improve breeding and reproduction of livestock, and for the treatment of human infertility. Their widespread use has contributed to improvements in human health and welfare. However, in dogs, only artificial insemination using frozen semen is readily available as an ART to improve breeding and control genetic diversity. A recent priority in sperm cryopreservation is the development of alternatives to egg yolk, which is widely used as a component of the sperm extender. Egg yolk can vary in composition among batches and is prone to contamination by animal pathogens. The latter can be a problem for international exchange of cryopreserved semen. Low-density lipoprotein and skim milk are promising candidates for use as extenders, to ensure fertility after artificial insemination. Although not tested for its effects on fertility following artificial insemination, polyvinyl alcohol may also be a useful alternative to egg yolk as an extender. The development of cryopreservation techniques for canine embryos lags behind that for other mammals, including humans. However, given the success of non-surgical embryo transfer in 2011, studies have sought to refine this approach for practical use. Research on sperm cryopreservation has yielded satisfactory results. However, investigation of other approaches, such as cryopreservation of oocytes and gonadal tissues, remains insufficient. Techniques for the efficient induction of estrus may aid in the development of successful canine ARTs.

Key words: Assisted reproductive technique, Cryopreservation, Dog, Embryo, Sexing, Spermatozoa

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Introduction

Assisted reproductive techniques (ARTs) in dogs, which may contribute to the control of genetic diversity and efficient breeding, have strictly limited utility compared to those in other mammals, including humans. Although successful somatic cell cloning has been reported, artificial insemination using frozen semen is the only readily available ART for improved breeding of dogs. The development of canine ARTs has a long history, beginning with reports of artificial insemination by Spallanzani in 1780 [1]. However, the level of their development is poor compared to that of ARTs available for other mammals. This is because the reproductive physiology of dogs is unique and poorly understood. Furthermore, the anatomical characteristics of the female dog reproductive tract complicate the development of ARTs. In addition, considerable research effort is required to study a sufficient number of animals. This may have been a roadblock in the development of canine ARTs. A research group has successfully managed ART in 500 dogs, to enable synchronous ovulation in multiple bitches (personal communication). However, this scale is extremely difficult for a research institution to achieve, by taking into consideration the animal maintenance requirements that must be fulfilled to ensure animal welfare.

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Domestic bitches are non-seasonal monoestrus animals that spontaneously ovulate only once or twice a year. Diestrus occurs after ovulation, with obligatory diestrus periods of 9-10 weeks. Following regression of the corpus luteum at the end of diestrus, the bitch enters a phase hormone quiescence period termed anestrus for three or more months [2]. The interestrus interval averages 31 weeks, ranging from 16 to 56 weeks [3]. Bitches with longer interestrus intervals have fewer opportunities to become pregnant, and thus have reduced the chance of their use in the development of ARTs.

There is a great demand for ARTs in dogs, for the efficient breeding of companion animals and to ensure the diversity of genetic resources of working dogs and endangered canidae. Application of embryo cryopreservation and embryo transfer technology for artificial insemination could also contribute to breeding management in companion dogs, service dogs (including guide dogs), and working dogs (including drug-detecting and quarantine dogs). This technology would also facilitate the transport and storage of genetic materials and aid in the elimination of vertically transmitted diseases in dogs.

With these goals in mind, this paper reviews recent research trends in the development of canine ARTs, especially in the conservation of gene resources.

Frozen Spermatozoa for Artificial Insemination

To successfully complete artificial insemination, it is important to determine the optimal time for insemination. To determine the ovulation time, it is necessary to consider that the cells and environment of the vagina is altered due to changes in estrogen levels. In addition, changes in the levels of blood luteinizing hormone (LH) and progesterone have also been observed. Practically, it is possible

to estimate ovulation date and optimize the time of insemination by identifying the day when the blood progesterone concentration exceeds 2 ng/ml as the day when the LH surge occurs [4].

Four types of insemination of bitches have been described: vaginal insemination, blind transcervical insemination (Norwegian catheter method), endoscopy-assisted transcervical insemination (TCI), and surgical insemination [2]. Vaginal insemination is commonly used for fresh or chilled semen and is not recommended for frozen spermatozoa [2]. This is because vaginal insemination has a requirement for sufficient sperm motility and maintenance of that motility for a period of time, in order for the inseminated sperm to pass through the cervix and reach the oviduct. The Norwegian catheter was first developed as a non-surgical intrauterine device in 1975 [5]. The operator transabdominally palpates the cervix of the bitch while passing the catheter into the vagina with the opposite hand. While holding the cervix steady with the thumb and index finger, the stylet with a blunted, rounded distal tip is passed into the cervical os and through the cervix of the bitch [2]. The procedure is quick and simple, but is difficult for large-breed bitches, such as Labrador retrievers. TCI was first described in 1993 [6]. The endoscope is initially passed into the vagina and advanced cranially past the dorsomedial folds to the cervix. The TCI catheter is then advanced through the endoscope and cervical os. Unlike the Norwegian method, as the TCI visualizes the cervix, it is always guaranteed that the catheter is intrauterine. Surgical insemination is the insemination of semen into the uterus of a bitch under general anesthesia *via* laparotomy. Surgical insemination has recently been prohibited in the United Kingdom [7]. It is anticipated that similar legislation or guidelines will be enacted globally. Fortunately, the fertility obtained by means of TCI is the same or better than that obtained by means of surgical insemination, especially when cryopreserved spermatozoa are used [8, 9]. TCI is usually completed within 10 min [2], while avoiding risks associated with anesthesia and surgery, and is typically performed without the need for sedation. Thus, it is clear that TCI is preferable from the viewpoint of animal welfare.

Cryopreservation of spermatozoa is essential for artificial insemination, the most widely used ART in Canidae. The main goal of sperm cryopreservation is to conserve the fertility of animals with high genetic value or to preserve endangered species. In addition, cryopreservation of spermatozoa for artificial insemination can prevent sexually transmitted diseases, such as brucellosis and herpes virus infections. Artificial insemination using cryopreserved spermatozoa provides a number of potential advantages, including avoidance of transport-related stress, breeding problems caused by copulation failure due to behavioral issues (including female aggressiveness and male indifference), and the quarantine placed on live animals.

Extender for Canine Spermatozoa

Cryopreservation of animal semen has escalated with the discovery of the addition of the cryoprotectant glycerol to semen extenders, and subsequently, the benefit of adding chicken egg yolk to the extenders. Since the first success of artificial insemination with frozen semen in 1969 [10], many further improvements in freezing procedures and extenders have been made (Tables 1–3). Tris-egg yolk-glycerol buffer has been widely used as an extender to freeze canine semen. Chicken egg yolk is often the cryoprotectant substance used as an extender, despite its disadvantages. A major concern in the application of ARTs is the risk of bacterial or mycoplasma contamination, which can be a source of endotoxins that can reduce the fertility of spermatozoa and transmit diseases [11]. There have been increasing arguments against the use of egg yolks. The main drawbacks of the same are the variability in composition among batches and contamination by animal pathogens. The latter can be problematic for international exchange of cryopreserved semen. In particular, chicken egg yolk is a biologically hazardous compound due to the recent spread of endemic zoonotic diseases in birds, such as avian influenza [12]. Consequently, studies on alternative extenders that can replace egg yolk are necessary and very important. Table 1 summarizes the reports on improvements in the development of extenders for the cryopreservation of canine spermatozoa. Many attempts have been made to add sugars and antioxidants to known or reported extenders or to replace the constituents of existing extenders with other chemicals. However, few studies have attempted artificial insemination of spermatozoa that have been cryopreserved using these modified extenders. Lack of knowledge reflects the major bottleneck in ART studies in dogs. When a paper related to artificial insemination is submitted to a journal, the reviewers always request information from both the experimental and control groups. This requires a large number of animals. The care and fate of the large number of weaned offspring that would be obtained from both the experimental and control groups owing to the artificial insemination experiments is an important issue that needs to be considered. To avoid a large number of offspring, a plausible solution is to use the litter size obtained from natural breeding of the relevant breed as control data.

Sperm motility is a key parameter that determines the quality of frozen-thawed samples for artificial insemination [13]. This motility is positively correlated with viability, as well as, plasma membrane/mitochondrial integrity [14]. Owing to the known correlation of fertility with the total number of progressively motile spermatozoa in thawed canine semen [15], Table 1 provides comparative values (%) of motility and progressive motility in the test groups, as compared to those in the control group after thawing, in each published report. For canine spermatozoa, glycerol has been used as a penetrating intracellular cryoprotectant to preserve fertility after cryopreservation [16]. Other penetrating intracellular cryoprotectants include ethylene glycol [17–20], propylene glycol [20], dimethyl sulfoxide [16], methyl-formamide [20, 21], dimethyl-formamide [20, 21], methyl-acetamide [20], and N-N-dimethylformamide [22]. Glycerol is best at preserving the motility of cryopreserved spermatozoa after thawing.

As described above, egg yolk has long been used as an extracellular cryoprotectant for the cryopreservation of canine spermatozoa, with a lack of standardization and a risk of contamination. Consequently, studies on alternative extenders that can replace egg yolk are of great importance. As shown in Table 1, several materials have been examined as candidates for cryoprotectants that can replace egg yolk [11, 12, 23–35]. Among them, only low-density lipoprotein (LDL) [25, 27] and skim milk [12, 35, 36] have been assessed as alternative components of extenders, for their benefits in improving fertility by means of artificial insemination. LDL can protect spermatozoa against cold shock by preventing the efflux of phospholipids and cholesterol from the sperm cell membrane and by promoting the addition of phospholipids and cholesterol to the cell membrane [26]. Considering sugar as a partner of skim milk, raffinose or trehalose is superior to glucose in supporting motility and average path velocity of canine spermatozoa after cryopreservation (Table 2; Asano *et al*., unpublished). Skim milk in combination with raffinose resulted in a sufficient litter size of 5.3 (Asano *et al*., unpublished data) and 6.0 [36] after artificial insemination (Table 1). Milk [24], egg yolk plasma [31, 37–39], lecithin [11, 32–34, 40], lipoprotein [41], and liposomes [38] appear to have comparable or better effects on sperm

Table 1. Attempts to improve the components of the extender for frozen canine spermatozoa

Extender	Supplement or replacement	Comparison with control group after thawing (%)		Results of artificial insemination	References
		Total motility	Progressive motility		
Tris-egg yolk	Glycerol	229	–	Pregnancy rate: 25%–67%; litter size: obscure	Olar *et al.*, 1989 [16]
		126	–	ND	Gharajelar *et al.*, 2016 [24]
	Egg yolk to milk	151	–	ND	Gharajelar *et al.*, 2016 [24]
Tris-egg yolk-glycerol	Dimethyl-sulfoxide	33	–	ND	Olar *et al.*, 1989 [16]
	Glycerol to ethylene glycol	89	85	ND	Martins-Bessa *et al.*, 2006 [17]
		88 [c]	100 [c]		
		47	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to propylene glycol	38	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to methyl-formamide	86	82	ND	Futino *et al.*, 2010 [21]
	Glycerol to dimethyl-formamide	64	61	ND	Futino *et al.*, 2010 [21]
	Glycerol to methyl-acetamide	28	–	ND	Cheema *et al.*, 2021 [20]
	Egg yolk to LDL	196 [a]	–	Delivery rate: 67%; litter size: obscure	Bencharif *et al.*, 2008 [25]
		127	–	ND	Varela-Junior *et al.*, 2009 [26]
		180 [a]	–	Delivery rate: 100%; litter size: 4.0	Bencharif *et al.*, 2010a [27]
		150 [a]	–	ND	Bencharif *et al.*, 2010b [28]
		180 [a]	–	ND	Bencharif *et al.*, 2012 [29]
		109	110	ND	Neves *et al.*, 2014 [30]
	Egg yolk to egg yolk plasma	136	–	ND	Corcini *et al.*, 2016 [31]
	Egg yolk to soybean lecithin	78	–	ND	Beccaglia *et al.*, 2009 [32]
		55 [c]	54 [c]	ND	Axnér & Lagerson, 2016 [33]
		124	134	ND	Sánchez-Calabuig *et al.*, 2017 [11]
		107	–	ND	Dalmazzo *et al.*, 2018 [34]
	Tris-egg yolk to skim milk-glucose	103	101	Delivery rate: 100%; litter size: 3.0	Abe *et al.*, 2008 [12]
	Tris-egg yolk to skim milk-trehalose	138	108	ND	Asano *et al.*, unpublished (Table 2)
	Tris-egg yolk to skim milk-raffinose	132	80	Delivery rate: 100%; litter size: 5.3	Asano *et al.*, unpublished (Table 2)
	Vitamin C	103	122	ND	Michael *et al.*, 2007 [56]
		116	107	ND	Monteiro *et al.*, 2009 [57]
	Vitamin E	114	157	ND	Michael *et al.*, 2007 [56]
	Vitamin B16	63	57	ND	Michael *et al.*, 2007 [56]
	Butylated hydroxytoluene	151	157	ND	Sun *et al.*, 2020 [61]
	Curcumin	98	95	ND	Aparnak & Saberivand, 2019 [62]
	Resveratrol	128	125	ND	Bang *et al.*, 2021 [64]
	Catalase	128	187	ND	Michael *et al.*, 2007 [56]
	Glutathione	102	117	ND	Monteiro *et al.*, 2009 [57]
		–	153	Delivery rate: 50%; litter size: 2.5	Ogata *et al.*, 2015 [66]
		–	375 [c]		
	Astaxanthin	111	102	ND	Qamar *et al.*, 2020 [65]
	N-acetyl-L- cysteine	118	165	ND	Michael *et al.*, 2007 [56]
	Equex STM (SDS)	435 [c]	–	ND	Rota *et al.*, 1997 [42]
		–	–	Pregnancy rate: 100%; implantation rate: 63%#	Nöthling *et al.*, 1997 [43]
		114	–	Pregnancy rate: 100%; mean no. conceptus: 3.2##	Rota *et al.*, 1999 [44]
		228 [c]	272 [c]	ND	Peña & Linde-Forsberg, 2000 [45]
		353 [c]	700 [c]	ND	Peña *et al.*, 2003a [46]
		112	–	ND	Peña *et al.*, 2003b [47]
		272 [d]	–		
		–	164	ND	Schäfer-Somi *et al.*, 2006 [48]
		173 [a]	–	ND	Bencharif *et al.*, 2010a [27]
		173 [a]	–	ND	Bencharif *et al.*, 2012 [29]

Table 1. Continued.

Extender	Supplement or replacement	Comparison with control group after thawing (%)		Results of artificial insemination	References
		Total motility	Progressive motility		
	Orvus ES paste (SDS)	159	–	ND	Tsutsui *et al.*, 2000a [49]
		433 [c]	–	ND	Tsutsui *et al.*, 2000b [50]
		93	–	Delivery rate: 80%; litter size: 3.6	Tsutsui *et al.*, 2000c [51]
		131 [c]	111	ND	Nizański *et al.*, 2001 [52]
		126	–	ND	Hori *et al.*, 2006 [53]
	SDS	117	–	ND	Hori *et al.*, 2006 [53]
	Taurine	113	150	ND	Michael *et al.*, 2007 [56]
	Monosaccharide	94–130	–	ND	Yildiz *et al.*, 2000 [76]
	Disaccharide	76–117	–	ND	Yildiz *et al.*, 2000 [76]
	Trisaccharide	116	–	ND	Yildiz *et al.*, 2000 [76]
	Spermine	103	–	ND	Setyawan *et al.*, 2016 [77]
	Kinetin	117	96	ND	Qamar *et al.*, 2020a [78]
	Cholesterol-loaded cyclodextrin	168	–	ND	Khan *et al.*, 2017 [79]
	Iodixanol	115	–	ND	Abdillah *et al.*, 2019 [80]
	Metformin	139	158	ND	Gradhaye *et al.*, 2020 [81]
	Mesenchymal stem cell	129	–	ND	Qamar *et al.*, 2020b [82]
	Mesenchymal stem cell-conditioned medium	129	115	ND	Mahiddine *et al.*, 2020 [83]
	3,4-dihydroxyphenyl glycol	157	–	ND	Shakouri *et al.*, 2021 [84]
	Tris to lactose	63	–	ND	Olar *et al.*, 1989 [16]
Tris-egg yolk-glycerol-Equex STM	Vitamin E and C	115	115	ND	Lecewicz *et al.*, 2018 [58]
	Butylated hydroxytoluene	126	–	ND	Neagu *et al.*, 2010 [59]
		116	–	ND	Sahashi *et al.*, 2011 [60]
	Prostatic fluid	–	94	ND	Nöthling *et al.*, 2007 [67]
	IGF-I	–	225	ND	Shin *et al.*, 2014 [85]
	Glycerol to ethylene glycol	166	206	ND	Rota *et al.*, 2006 [18]
		102	97	ND	Marins-Bessa *et al.*, 2006 [17]
		50 [c]	43 [c]		
		120	–	Pregnancy rate: 100%; mean no. conceptus: 3.8[#]	Rota *et al.*, 2010 [19]
	Egg yolk to egg yolk plasma	85	82	ND	Schäfer-Somi *et al.*, 2021 [37]
	Egg yolk to soybean lecithin	0	–	ND	Beccaglia *et al.*, 2009 [32]
		39–46	–	ND	Hermansson *et al.*, 2021 [40]
	Egg yolk-Equex to soybean lecithin	72	–	ND	Beccaglia *et al.*, 2009 [32]
Tris-egg yolk-glycerol-Orvus ES paste	Egg yolk to lipoprotein from ostrich egg yolk	79	80	ND	Strzezek *et al.*, 2012 [41]
	Glycerol to N-N-dimethylformamide	–	45	ND	Hernández-Avilés *et al.*, 2020 [22]
Tris-egg yolk-glycerol-SDS	Tris to skim milk	117 [a]	119 [a]	ND	Rota *et al.*, 2001 [23]
		122 [b]	109 [b]		
		350 [c]	400 [c]		
Tris-egg yolk plasma-glycerol	Glycerol to dimethyl-formamide	96	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to methyl-acetamide	34	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to ethylene glycol	45	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to propylene glycol	15	–	ND	Cheema *et al.*, 2021 [20]
Tris-glycerol	Polyvinyl alcohol	–	1200	ND	Nabeel *et al.*, 2019 [55]
		–	167 [b]		
Tris-PVA-glycerol	Essential amino acid (EAA)		486	ND	Talha *et al.*, 2021 [75]
	Non-essential amino acid (NEAA)		700	ND	Talha *et al.*, 2021 [75]
	EAA+NEAA		514	ND	Talha *et al.*, 2021 [75]
Tris-LDL-glycerol	Glutamine	107 [a]	–	ND	Bencharif *et al.*, 2010b [28]
		110 [a]	–	ND	Bencharif *et al.*, 2012 [29]
	LDL to egg yolk plasma	95	92	ND	Belala *et al.*, 2016 [38]
		96 [a]	96 [a]		
		95 [a]	103 [a]	ND	Belala *et al.*, 2019 [39]

Table 1. Continued.

Extender	Supplement or replacement	Comparison with control group after thawing (%)		Results of artificial insemination	References
		Total motility	Progressive motility		
	LDL to liposomes	91	88	ND	Belala *et al.*, 2016 [38]
		93 [a]	89 [a]		
	Glycerol to dimethyl-foramide	85	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to methyl-acetamide	31	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to ethylene glycol	102	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to propylene glycol	26	–	ND	Cheema *et al.*, 2021 [20]
Tris-coconut water-glycerol	Glycerol to dimethyl-foramide	126	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to methyl-acetamide	87	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to ethylene glycol	108	–	ND	Cheema *et al.*, 2021 [20]
	Glycerol to propylene glycol	100	–	ND	Cheema *et al.*, 2021 [20]
Lactose-egg yolk	Dimethyl-sulfoxide	81	–	ND	Olar *et al.*, 1989 [16]
Skim milk-raffinose-glycerol		–	–	Delivery rate: 83%; litter size: 4.2	Abe *et al.*, 2018 [35]
		–	–	Delivery rate: 80%; litter size: 6.0	Abe *et al.*, 2020 [36]
Skim milk-glucose-glycerol	Quercetin	197 [c]	242 [c]	Delivery rate: 100%; litter size: 6.0	Kawasaki *et al.*, 2020 [63]

a: 10 min after thawing, b: 20 min after thawing, c: 2 h after thawing, d: 5 h after thawing, e: 6 h after thawing. # All females underwent ovariohysterectomy during pregnancy. ##Abortion was induced at 28 days after pregnancy. ND, not determined; LDL, low-density lipoprotein; IGF-1, insulin-like growth factor; SDS, sodium dodecyl sulfate.

Table 2. Results of the quantitative assessment of the sperm quality of frozen-thawed canine spermatozoa with skim milk-sugar based extender

Parameter	Extender			
	Tris-egg yolk	Skim milk with 0.3 M glucose	Skim milk with 0.2 M trehalose	Skim milk with 0.2 M raffinose
Motile (%)	53.7 ± 9.3 [a]	59.6 ± 8.6 [a]	74.3 ± 7.0 [b]	70.9 ± 10.2 [b]
Progressive (%)	7.6 ± 5.1	7.8 ± 7.1	8.2 ± 4.5	6.1 ± 2.1
VAP (µm/sec)	29.8 ± 8.3 [a]	28.0 ± 13.1 [a]	34.6 ± 14.4 [b]	34.1 ± 13.2 [b]
VSL (µm/sec)	21.4 ± 3.7	19.3 ± 5.6	22.6 ± 6.1	22.2 ± 5.0
VCL (µm/sec)	54.4 ± 18.1	49.8 ± 27.1	59.6 ± 29.6	58.8 ± 27.2
ALH (µm)	4.1 ± 2.7	4.5 ± 2.7	4.5 ± 2.8	4.9 ± 2.8
BCF (Hz)	21.7 ± 9.6	22.2 ± 7.1	21.8 ± 7.9	21.5 ± 6.6
STR (%)	35.2 ± 20.4	38.2 ± 19.9	37.3 ± 18.7	37.5 ± 19.2
LIN (%)	22.3 ± 7.1	22.6 ± 9.0	22.3 ± 8.9	22.6 ± 8.9

[a, b] Values in the same row indicated using different superscripts differ significantly ($P < 0.05$, n = 5 replicates). VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness of average path; LIN, linearity of curvilinear path. For a skim milk-based extender, 30 mg/ml of skim milk and glucose, trehalose, or raffinose were dissolved in water for embryo transfer at 60°C, following which the solution was centrifuged at 10,000 × *g* for 15 min at room temperature. The supernatants were filtered and used as skimmed milk-based extender. A Tris-egg yolk extender composed of 20% (v/v) egg yolk, 24 mg/ml tris(hydroxymethyl)aminomethane, 14 mg/ml citric acid monohydrate, 0.8 mg/ml glucose, 0.65 mg/ml penicillin G potassium, and 1 mg/ml streptomycin sulphate was prepared.

motility after thawing, as alternatives to egg yolk. However, the results varied between the studies. In particular, various sources of soybean lecithin differ in their composition and may differ in their suitability as cryoprotectants.

The significant effects of replacing Tris with skim milk in Tris-egg yolk-glycerol-SDS extender on the *in vitro* motility of frozen-thawed spermatozoa have been documented [23]. However, artificial insemination has not yet been attempted with the same.

The addition of detergents such as Equex STM paste, Orvus ES paste, and sodium dodecyl sulfate (SDS) to egg yolk-supplemented extenders appears to have beneficial effects on spermatozoa motility after thawing [27, 42–53]. Although SDS is believed to have effects on membrane fluidity upon interaction with the egg yolk [54], there are relatively few litters following artificial insemination using the same (Table 1).

Polyvinyl alcohol (PVA) is an attractive alternative to egg yolk. Although no comparative experiment has been conducted with the extender containing egg yolk, approximately 35% of progressive motility has been reported in cryopreserved spermatozoa that used an extender with PVA [55]. Further studies are needed to confirm the fertility of these spermatozoa upon artificial insemination.

After natural mating or artificial insemination, reactive oxygen species may damage the spermatozoa in the reproductive tract [54]. After dilution of ejaculate, the quantity of seminal fluid components,

including antioxidants, may be less than optimal for potential fertility of spermatozoa. Accordingly, several antioxidant substances have been examined, alone or in combination, for their role in protection of spermatozoa from oxidative damage. These include vitamins [56–58], phenols [59–61], polyphenols [62–64], carotenoids [65], enzymes [56, 57, 66], and others [56]. Among the latter, catalase [56], glutathione [66], and quercetin [63] markedly improve the motility of spermatozoa after thawing. In particular, pups are frequently obtained after artificial insemination of cryopreserved spermatozoa to which quercetin is added [63]. The effect of adding homologous prostatic fluid to the sperm extender on the motility of cryopreserved canine sperm after thawing has not yet been examined [67]. However, as shown in Table 3, addition of prostatic fluid after thawing may be appropriate to improve sperm motility [67–69]. In addition, sperm motility is reportedly improved by post-thawing addition of pentoxifylline [70–72], epigallocatechin-3-gallate [73], caffeine [71, 72], and 2'-deoxyadenosine [71, 72]. However, the fertility of treated spermatozoa after artificial insemination has not yet been assessed.

As a consequence of low-temperature exposure, some plants or animals accumulate amino acids and are capable of surviving at temperatures below zero [74]. The effects of amino acids on semen characteristics during cryopreservation have been examined [56, 75]. The addition of essential/non-essential amino acids, alone or in combination, significantly improves the motility of spermatozoa after thawing, although the fertility of these spermatozoa has not been examined [75]. Current knowledge of the mechanisms of action of amino acids on spermatozoa exposed to cold environments is relatively limited. The mechanism of cryoprotection by amino acids remains to be fully elucidated.

More suitable energy sources for frozen-thawed sperm have also been examined [16, 76]. In addition, the effects of spermine [77], kinetin [78], cholesterol-loaded cyclodextrin [79], iodixanol [80], metformin [81], stem cells [82], stem cell-conditioned medium [83], norepinephrine metabolite [84], and insulin-like growth factor-1 [85] have been examined. The effects of each treatment on the *in vitro* motility of frozen-thawed spermatozoa have been documented. However, artificial insemination using these compounds has not yet been attempted.

In the development of sperm extenders for cryopreservation, components of the diluent modulate the post-insemination inflammatory reaction in the uterus, which could be detrimental to the fertilizing ability of the spermatozoa.

Sexing Spermatozoa

The specific production of male or female offspring by separation of X- or Y-chromosome-bearing spermatozoa, followed by artificial insemination, is a long-standing goal in the breeding of domestic and other animals. For dogs, higher percentages of female puppies are desired for the breeding of guide dogs. Most blind people prefer to use a female guide dog, rather than a male, because they are easier to manage. Male dogs are stronger and require two evacuation bags, while female dogs require only one evacuation bag. Thus, for equal numbers of male and female trained guide dogs, there is a mismatch between the supply and demand. Generally, when sexing is performed using spermatozoa, X- or Y-chromosome-bearing sperm are fractionated using flow cytometry, and either of the sperm population is then subjected to artificial insemination or *in vitro* fertilization. To assess the sorting accuracy of canine spermatozoa using flow cytometry, direct visualization of sex chromosomes in a single sperm by means of dual [86] and triple color [87] fluorescence *in situ* hybridization has been developed. The purities of flow-sorted sex chromosomes in the spermatozoa of the dogs were 91% for the X-chromosome fraction and 90% for the Y-chromosome fraction [86]. An accuracy of 90% is ideal because a certain percentage of male offspring is necessary to maintain the breeding colony. Sorted frozen-thawed spermatozoa after surgical artificial insemination reportedly yielded pups with a significantly biased sex ratio [88]. In

Table 3. Supplements added after thawing, to improve the motility of the cryopreserved canine spermatozoa

Extender	Supplement	Comparison with control group after thawing (%)		Results of artificial insemination	References
		Total motility	Progressive motility		
Tris-egg yolk-glycerol	Pentoxifylline	–	140 [c]	ND	Koutsarova *et al.*, 1997 [70]
Tris-egg yolk-glycerol-Equex STM	Prostatic fluid	–	–	Pregnancy rate: 100%; implantation rate: 57%#	Nöthling & Volkmann 1993 [68]
		–	153 [b]	Pregnancy rate: 67%; implantation rate: 58%#	Nöthling *et al.*, 2005 [69]
		–	357 [a]	ND	Nöthling *et al.*, 2007 [67]
	Epigallocatechin-3-gallate	110 [d]	97 [d]	ND	Bucci *et al.*, 2019 [73]
	Caffeine	107 [a]	106 [a]	ND	Milani *et al.*, 2010 [71]
		245 [c]	282 [c]		
	Pentoxifylline	108 [a]	117 [a]	ND	Milani *et al.*, 2010 [71]
		374 [c]	482 [c]		
	2'-deoxyadenosine	109 [a]	90 [a]	ND	Milani *et al.*, 2010 [71]
		281 [c]	373 [c]		
Tris-egg yolk-glycerol-Orvus ES paste	Caffeine	132 [c]	130 [c]	ND	Lecewicz *et al.*, 2019 [72]
	Pentoxifylline	127 [c]	140 [c]	ND	Lecewicz *et al.*, 2019 [72]
	2'-deoxyadenosine	118 [c]	140 [c]	ND	Lecewicz *et al.*, 2019 [72]

a: 1 h after thawing, b: 90 min after thawing, c: 2 h after thawing, d: 6 h after thawing. # All females underwent ovariohysterectomy during the pregnancy.

general, freezing and thawing procedures and cell sorting adversely affect sperm motility. Since surgical artificial insemination of dogs is soon expected to be restricted worldwide, it is necessary to examine whether sorted and cryopreserved sperm can be fertilized using TCI.

Freeze-dried Spermatozoa

Freeze-drying is a preservation method in which frozen materials are dried by sublimation of ice. The material can then no longer support biological growth or chemical reactions. The first successful offspring derived from freeze-dried spermatozoa were obtained by means of intracytoplasmic sperm injection (ICSI) using mouse spermatozoa in 1998 [89]. Freeze-drying of spermatozoa is expected to replace cryopreservation as a method for storing genetic resources. A further advantage over storage in liquid nitrogen is that freeze-dried sperm can be temporarily stored and transported at room temperature. It is possible to transport freeze-dried mouse sperm overseas at ambient temperature without the need for a special container [90]. The production of live offspring derived from freeze-dried spermatozoa in dogs has not yet been reported. However, the formation of pronucleus in freeze-dried canine spermatozoa following the injection of freeze-dried sperm into mouse oocytes has been described [91, 92].

Cryopreservation of Gonadal Tissues

When spermatozoa are not preserved, testicular tissues or cell suspensions can be cryopreserved instead. However, testicular tissue cryopreservation has not yet been reported in dogs. The preservation of the female gonadal tissue could allow the storage of a large pool of follicles for subsequent oocyte retrieval. Cryopreservation of female gametes is very important for preserving the fertility of female cancer patients who may lose ovarian function due to chemotherapy or radiation therapy [93]. In dogs, successful cryopreservation of ovarian tissue began with xenografting in immunodeficient mice [93]. One of the most recognized obstacles after ovarian tissue transplantation is ischemic injury [94–96]. Delayed revascularization and ischemia appear to be responsible for follicular loss and apoptosis [96–98]. Administration of desialylated erythropoietin [94, 99, 100], erythropoietin [97], or hyperoxygen therapy [101] is effective in suppressing follicular loss after ovarian transplantation in dogs. Vitrification is more suitable than slow freezing for cryopreserving canine ovarian tissues [93–96, 99, 100, 102–105]. Future challenges include the induction of growth from primary and secondary follicles to antral follicles in the transplanted ovarian tissue, followed by oocyte maturation and ICSI or *in vitro* fertilization.

Cryopreservation of Oocytes and Embryos

Due to the lack of an efficient *in vitro* maturation method [106–109], there is limited possibility of embryo development after immature oocyte cryopreservation. However, the possibility of preservation of the viability of stored oocytes has been examined [110, 111], in addition to oocyte ultrastructure, meiosis resumption, and gene expression in oocytes [112], primarily as preliminary studies on the cryopreservation of embryos [110, 111]. Canine oocytes are similar to porcine oocytes in terms of their diameter and high cytoplasmic lipid content [110, 111]. Thus, the method used for cryopreservation of porcine oocytes may be suitable for canine oocytes as well. When dog cumulus oocyte complexes were collected from ovaries and vitrified in dimethylsulfoxide-based medium (DAP213) in a cryotube or an ethylene glycol-based (E30S) medium on a cryotop sheet, no significant morphological differences were evident following warming (59%–62%) [110]. Immature canine oocytes could be successfully vitrified with 44% plasma membrane integrity [111], along with resumption of meiosis and development to the metaphase II stage [112].

Embryo cryopreservation and subsequent embryo transfer have greatly contributed to efficient production in livestock animals and infertility treatment in humans. However, there have been few studies on the cryopreservation of canine embryos [113–115], and their practical application remains elusive. Surprisingly, the generation of the first canine offspring from frozen embryos was reported in 2011 [113], while that from *in vitro* fertilization was reported in 2015 [114]. In contrast, the first human "test tube baby" was born more than 40 years ago. When canine zygotes and embryos were vitrified in E30S, over 80% of the 1-cell to morula-stage embryos exhibited normal morphology. However, most cryopreserved blastocysts showed abnormal morphology after warming. The viability of morphologically normal embryos at the 1–16-cell, morula, and blastocyst stages was 90%–100%, 50%, and 40%, respectively [113]. Differentiation of canine embryos during pre-implantation may influence their susceptibility to vitrification. In addition, the sensitivity of canine oocytes/embryos to cryopreservation may be related to their high lipid content. If so, tolerance to cryopreservation might increase if lipid content is reduced. In the first report on the generation of puppies from embryos preserved in liquid nitrogen, when 77 vitrified embryos at the 4-cell to morula stage were transferred into the uteri of nine recipient bitches 8–11 days after LH surge, five of the recipients became pregnant. Of these, four delivered a total of seven pups (9.1%) [113]. The conditions for embryo transfer that contributed most to the development of offspring were developmental embryonic stage from 8- to 16-cell, in a recipient at the stage of 8–9 days after LH surge. The developmental rate of offspring when embryos were transferred under these conditions was 15.9% (7/44). Although no offspring were derived from the morula, this may actually reflect a difficulty in evaluation, because of the small number of transferred embryos at the morula stage. In another report, when 35 vitrified embryos at the 8-cell to early blastocyst stage were surgically transferred into the uteri of five recipients, two became pregnant and delivered two pups (5.7%), presumably derived from the morula or early blastocyst [115]. The first canine "test tube baby" was derived from a frozen embryo. When 19 vitrified embryos at the 4-cell stage were surgically transferred into the oviduct of a recipient bitch, the recipient delivered seven pups (36.8%) [114]. Paternity testing based on microsatellite genotypes has been introduced to guarantee experimental methods and results in embryo transfer [113]. The testing demonstrated that the delivered puppies were derived from donor embryos, and not from recipients [113–115]. Most offspring derived from cryopreserved embryos are obtained by surgical embryo transfer into the uterus or oviduct. The development of offspring by non-surgical transfer has been reported in only one study [113]. This non-surgical embryo transfer system was originally developed for the artificial insemination of large breeds in dogs [6]. The surgical method might be more commonly applied because of the difficulty of non-surgical embryo transfer owing to the unique morphology of the vagina and cervix in dogs. However, non-surgical embryo transfer techniques can be applied in clinical and field situations. In addition, as mentioned above, surgical artificial insemination has been banned in the UK, on the grounds of animal welfare [7]. Since it forces recipient animals to conceive and give birth to genetically different bitch offspring, it may be desirable to provide a non-invasive transcervical embryo transfer from the viewpoint of animal welfare. However, if early

embryos prior to the 8-cell stage are to be transferred, they may need to be surgically injected into the oviduct. In future studies, it will be necessary to improve the developmental rate of embryos after transfer by further improving cryopreservation methods and examining appropriate embryonic stages for vitrification, as well as, a suitable timing after the LH surge of transferred embryos and recipients.

To develop methods for cryopreservation and transfer of embryos, it is essential to understand *in vivo* early embryonic development in all mammals. However, owing to singular reproductive features, the actual situation and mechanisms of early development, such as oocyte maturation, fertilization, and embryogenesis, have not been fully elucidated and controlled in dogs, as compared to those in many other domestic mammalian species. The pre-implantation development of Labrador retrievers has been clarified through studies on embryo cryopreservation [113, 116, 117]. A total of 620 embryos were collected from oviducts, isthmus, and uteri of 134 Labrador Retriever bitches inseminated with ejaculates, following which the developmental stages and localization of the collected embryos were examined [117]. As shown in Fig. 1, embryos at the 16-cell to morula stage migrated from the oviduct into the uterus on day 10 after LH surge and likely completed the migration within 24 h. By day 12 after LH surge, all of the developing embryos were localized in the uteri. Embryos developed to the morula by 11–12 d and to the blastocyst by 12–13 d after LH surge [117].

Postscript

To achieve efficient breeding or to apply ARTs to animals with long estrus intervals, it is necessary to develop an efficient method for estrus manipulation. Since 1939, many studies have assessed the possibility of estrus induction in dogs using hormones such as follicle-stimulating hormone, synthetic estrogen, gonadotropin-releasing hormone, dopamine agonists, human chorionic gonadotropin (hCG), and equine chorionic gonadotropin (eCG) [118]. However, the protocols used vary widely in terms of the results achieved, such as estrus-inducing efficacy, pregnancy rates following induced estrus, and success in ongoing pregnancies. In addition, some protocols are expensive and/or labor-intensive, and thus not suitable for clinical veterinary practice. Each method has advantages and disadvantages, and no standard method for inducing estrus in dogs has been established as of yet. However, the following reports are simple and efficient methods for inducing estrus. When bitches were treated with a single injection of eCG followed by hCG 7 days later, all bitches at late anestrus showed signs of estrus. Moreover, 80% of these animals became pregnant after natural mating or artificial insemination [119]. Recently, it was reported that inhibin anti-serum, in combination with eCG, induced estrus and promoted a considerable number of ovulations in anestrus dogs [120]. These findings and further studies may contribute to the development of canine ARTs.

One of the most effective applications of ARTs is in the improvement of the breeding system of guide dogs for the blind, as well as, that of other working dogs. Although guide dogs remarkably improve the quality of life of blind people, many countries suffer from an acute shortage of the same. This is partly because only a certain percentage of the available dogs meet the requisite aptitude standards. For example, only 30% of the dogs that are trained ultimately participate as guide dogs in Japan [93]. A further problem in breeding systems

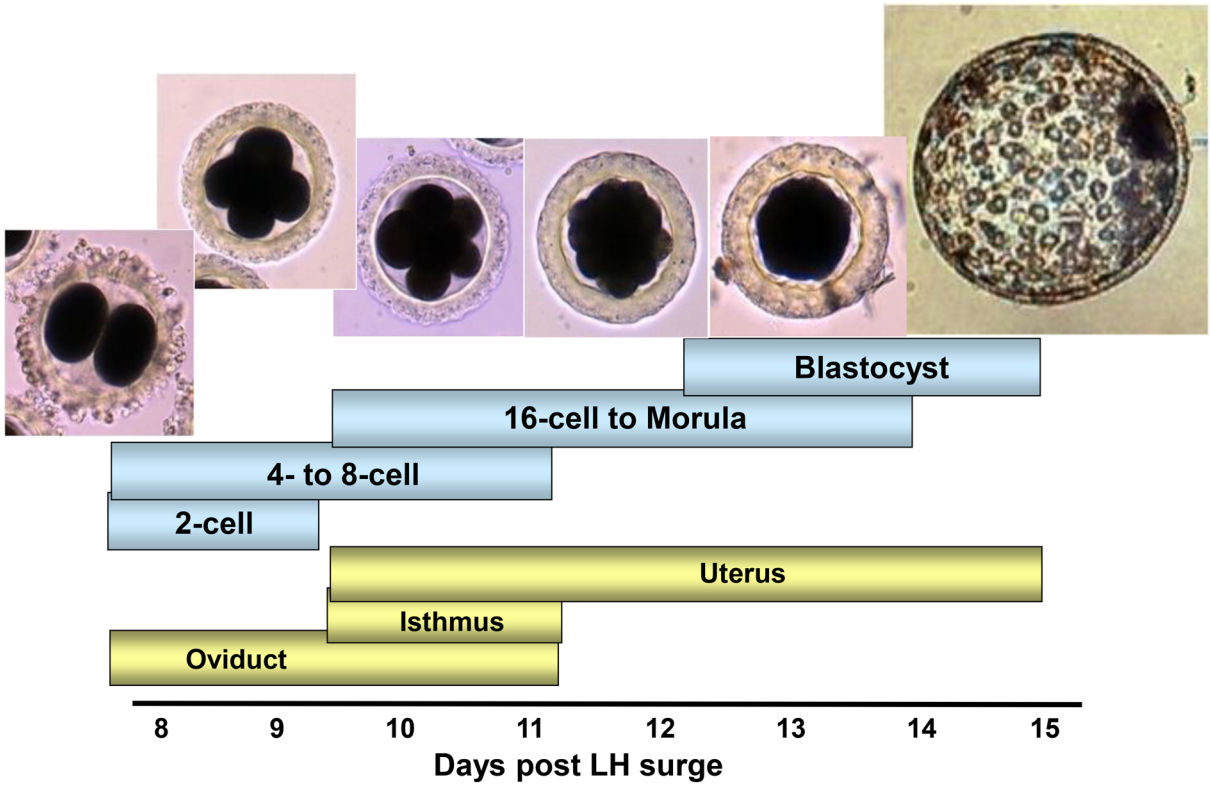

Fig. 1. Pre-implantation development and localization of embryos in Labrador retrievers. Embryos at the 16-cell to morula stage migrate from the oviduct into the uterus on day 10 after LH surge and likely complete the migration within 24 h. By day 12 after the LH surge, all of the developing embryos have localized in the uteri. Embryos develop to the morula by days 11–12 and to the blastocyst by day 12–13 after LH surge.

for guide dogs is that these guide dogs must be spayed before training starts, which means that the dogs who succeed in becoming guide dogs can then never be used for breeding. ARTs can overcome these difficulties, at least in part. Thus, canine ART development is a socially rewarding research.

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