

Individual fertility differences in the frozen-thawed spermatozoa among semen donors in the Labrador Retriever

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Abstract

Purpose We aimed to validate and determine the possible application of transcervical insemination of frozen semen for improved breeding in guide dogs for the blind in Japan.

Methods From February 2004 to March 2007, a total of 53 Labrador Retriever bitches, used for the breeding of guide dogs for the blind, were transcervically inseminated with frozen-thawed semen from 13 males by means of a cystoscope.

Results The overall whelping and pregnancy rate with the frozen semen was 42%. Pregnancy rates ranged widely from 0 to 100% depending on the semen donor male. Of 13 males, 6 males exhibited severely poor fertility (less than 20% pregnancy rate) and 3 males exhibited high fertility (over 70% pregnancy rate) on artificial insemination. However, the spermatozoa motility after thawing was not significantly different among these dogs. In addition, heterospermic insemination revealed the optimal timing for transcervical insemination with frozen-thawed semen to be by day 6 after the LH surge.

Conclusions Although transcervical insemination of frozen-thawed semen is effective for breeding of guide dogs for the blind, some modification of freeze-thawing procedures might be required to overcome individual fertility differences in the frozen-thawed spermatozoa among semen donor dogs. In addition, the motility of

spermatozoa after thawing might not be an appropriate indicator of the relative fertility of frozen-thawed spermatozoa in dogs.

Keywords Artificial insemination · Cryopreservation · Dog · Heterospermic insemination · Spermatozoa

Introduction

Guide dogs make a remarkable contribution to the quality of life of the blind people, but many countries suffer from an acute shortage of guide dogs such that not all those who need them are able to be supplied. Approximately 1,000 guide dogs for the blind are presently at work in Japan. However, it is estimated that the demand for such guide dogs is approximately 7,800, including latent demand. Although about 120 trained dogs are provided to the society for the blind annually, the actual number of increase is only 20–30 dogs, since 90–100 of them retire due to ageing every year. This is far from an adequate supply of these dogs for the blind in Japan. In addition, only about 30% of the dogs that are trained in fact work out as guide dogs [1]. One of the important challenges from the point of view of animal and veterinary science, as well as social welfare, is to establish a stable and effective breeding and reproduction system for guide dogs for the blind. However, only about 150 breeding dogs, counting both males and females, are currently available in Japan. In addition, it is known that the Labrador Retriever, which is extensively used as a guide dog for the blind in Japan, is a carrier of certain severe, high frequency genetic defects, including some conditions that are specifically problematic for guide dogs such as hip dysplasia, elbow joint dysplasia, cataracts and retinopathy.

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Thus, urgent countermeasures are required for a stable supply of guide dogs of high quality and in high quantity in Japan. Although the development and extension of assisted reproductive techniques in canines, such as artificial insemination, embryo transfer and in vitro fertilization has been remarkable, it is not as successful as in other mammalian species such as mice, pigs, cows and humans. However, successful artificial insemination with frozen canine semen has been well documented [2] since the first conception in 1969 [3]. Thus, in terms of the diffusion of the guide dogs for the blind, one of the solutions associated with these problems seems to be an application of artificial insemination by using frozen-thawed semen imported from developed countries such as the USA, UK and Australia. The transcervical insemination technique seems to be readily applicable to artificial insemination with frozen-thawed spermatozoa for large sized canines such as the Labrador Retriever, which is one of most popular breeds for the guiding dog [4]. Here we report successful delivery, with individual differences in fertility of frozen semen among the dogs, after transcervical artificial insemination with frozen-thawed spermatozoa of the Labrador Retriever.

Materials and methods

Animals and timing of artificial insemination

Both male and female Labrador Retrievers used as breeding dogs for guide dogs for the blind in the Hokkaido Guide Dog Association, Japan, were used in this study in the period from February 2004 to March 2007. Plasma progesterone concentrations of bitches were measured by enzyme linked fluorescent assay (SV-5010, SPOTCHEM VIDAS, Arkray, Kyoto, Japan) daily after the appearance of a blood-tinged vaginal discharge and vaginal swelling. The day when the plasma concentration of progesterone exceeded 2 ng/ml was estimated as the occurrence of the LH surge (Day 0) [5]. Bitches were inseminated with frozen-thawed semen 4–8 days after the estimated LH surge with some exceptions. A total of 13 semen donors with proven fertility in natural mating were used in this study. Unsuccessful pregnancy of the bitches after the experimental artificial insemination by frozen-thawed spermatozoa would considerably influence planning for the production and providing of the guide dogs in the guide dog association. Thus, in some cases, heterospermic insemination using both frozen-thawed and freshly ejaculated semen from different dogs was performed to avoid the failure of conception of the bitches. Paternity for the delivered pups was examined by using micro-satellite markers, as previously described [6].

Freezing and thawing of canine spermatozoa

The method of freezing and thawing of the spermatozoa was similar to that described by Christiansen and Schmidt [7] with diluent developed by Rota et al. [8]. Semen was collected by digital manipulation. The first fraction (seminal plasma) of the ejaculate was discarded, and 3–4 ml of the second (sperm-rich fraction) and third (seminal plasma) fractions mixture were pooled and then centrifuged at 700 g for 10 min. The sperm pellet was resuspended with recovered supernatant by gentle mixing at a concentration of 1×10^9 cells/ml. The sperm suspension was diluted with an egg yolk-Tris-citrate-glucose extender [8] at a concentration of 2×10^8 cells/ml at room temperature and then cooled to 4°C in a refrigerator for 4 h. The sample was further diluted with a second diluent, consisting of the first diluent supplemented with 16% (v/v) of glycerol [8], at a final concentration of 1×10^8 cells/ml. 0.5 ml of the diluted sperm suspension was loaded into a 0.5 ml plastic straw (Type 133, NFA101, Fujihira, Tokyo, Japan). Straws were placed in an atmosphere of liquid nitrogen vapor, i.e. placed horizontally 6 cm above the surface of the liquid nitrogen with a height of 8 cm in a styrene foam box (17.5 cm $\times$ 24.5 cm $\times$ 17.5 cm), and were kept there for 15 min, and then were plunged into the liquid nitrogen. For thawing, straws were put into a water bath at 38°C for 1 min. After examination of motility under light microscopy at a magnification of $\times 100$, the frozen-thawed spermatozoa were subjected to transcervical insemination [4]. The motility of spermatozoa was classified into the following grades: +++, progressively motile at a high speed; ++, progressively motile at a moderate speed; +, progressively motile at a low speed; $\pm$, motile without progression; –, immotile. The proportion of spermatozoa exhibiting a motility grade of +++ or ++ was used as the parameter for sperm motility.

Transcervical artificial insemination and diagnosis of pregnancy

Inseminations were performed on standing and non-sedated bitches. A catheter (8 Fr, Nippon Sherwood, Tokyo, Japan) equipped with a cystoscope for human use (Karl Storz, Germany) was inserted into the *corpus uteri* through the cervical canal [4]. And then 2×10^8 spermatozoa were inseminated through the catheter. Care was then taken to avoid backflow of the semen, so the catheter was withdrawn one minute after the insemination and the hind quarters of the bitch were kept elevated for 5 min. Conception was diagnosed by Doppler ultrasonography (VPU-011A, Toshiba, Tokyo, Japan).

The animals used in this study were treated and cared for under the Guiding Principles for the Care and Use of

Research Animals established by Obihiro University of Agriculture and Veterinary Medicine, Japan.

Results and discussion

As shown in Table 1, of the 53 bitches that were inseminated, 22 (42%) gave birth to a litter derived from frozen-thawed spermatozoa. All of the live pups exhibited normal appearance and growth. The motility of frozen-thawed spermatozoa from 13 dogs ranged from 55 to 75%. However, the delivery rates after insemination were considerably different between the dogs, ranging from 0 to 100%. Of 13 males, 6 males exhibited severely poor fertility (less than 20% pregnancy rate) and 3 males exhibited high fertility (over 70% pregnancy rate) on artificial insemination. Since the optimal time for insemination with frozen semen theoretically appeared to be around day 4 after the LH surge [9], and it has been suggested that artificial insemination with frozen semen is successful on days 4 and 6 [10], and days 5 and 7 [11, 12] after the LH surge in canine species, we thus performed transcervical insemination between days 4 and 8 after the estimated LH surge in the Labrador Retriever. However, as shown in Table 2, heterospermic insemination in the present study revealed the optimal timing for transcervical artificial insemination with

frozen-thawed semen to be by day 6 after the estimated LH surge. When frozen-thawed spermatozoa were inseminated into the bitches by day 6 after the LH surge in heterospermic insemination, only the frozen-thawed spermatozoa contributed to conception in many of the cases (mating no. 1, 2, 3, 4, 6 and 10 in Table 2). When frozen-thawed spermatozoa from RYU were inseminated into bitches on day 4 or 5, however, those spermatozoa did not participate in the pregnancy (mating no. 11 and 12 in Table 2). Although 3 of 11 inseminations from RYU did not even-tuate in pregnancy (Table 1), these failures of pregnancy included the above 2 cases (mating no. 11 and 12) and one abortion which was inseminated with frozen semen on LH 6 and 7. These results indicate that the ‘hot spot’ for the successful conception might be on day 6 after the LH surge. On the other hand, day 7 after the LH surge seems to be still fertile. Because when the frozen-thawed and freshly ejaculated semen were inseminated into the bitches on days 6 and 7, respectively, both frozen-thawed and freshly ejaculated semen successfully contributed to the conception, (mating no. 5, 7 and 13; Table 2). In mating no. 14, spermatozoa from NUR did not contribute to the conception in spite of insemination on day 6 after the estimated LH surge, but the cause of this fertilization or implantation failure appeared to be an individual difference rather than the timing of insemination. This is because the frozen-thawed spermatozoa from NUR never participated in pregnancy in any of the 7 trials in this study (Table 1).

It has been reported that whelping rates after transcervical insemination with frozen-thawed spermatozoa using a stainless steel catheter (the Norwegian method) in canines ranged from 60 to 90% in several breeds which were inseminated on the second and third day after estimated ovulation [13]. However, the Norwegian method seems to require considerable skill for successful insemination through the cervix in large breeds such as the Labrador Retriever. The catheter could not be inserted through the cervix in approximately 3% of any sized bitches by the Norwegian method [13]. And less than 10% of the bitches of both large and giant breeds required sedation to enable abdominal fixation of the cervix [13]. In the present study, the catheter was successfully inserted into the uterus through the cervix without any sedation required in any of the trials. Thus, transcervical insemination using a stereoscope with a long sheath as is used for human cystoscopy might be an effective technique for artificial insemination of large sized bitches such as the Labrador Retriever. This technique enables spermatozoa to be deposited non-surgically into the uterus in standing, non-sedated bitches. Since the number of fertile spermatozoa and the longevity of the spermatozoa seem to be reduced, it would be more effective to make the deposit closer to the site of fertilization rather than intra-vaginally. Since a fiber optic endoscope

Table 1 Results of transcervical artificial insemination with frozen-thawed spermatozoa in the Labrador Retriever guide dog

Semen donor	Motility of sperm after thawing (%)	No. of parturition/no. of bitches inseminated (%)	No. pups delivered	Litter size
ODY	65–75	4/5 (80)	13	3.3
RYU	70–75	8/11 (73)	28	3.7
BUZ	75	4/4 (100)	19	4.8
ERI	75	1/1 (100)	7	7.0
MAX	70	1/1 (100)	2	2.0
LEG	70	1/3 (33)	6	6.0
JAS	75	2/6 (33)	8	4.0
PEA	70	1/7 (14)	1	1.0
NUR	55–65	0/7 (0)	0	–
MAR	70	0/3 (0)	0	–
QUI	75	0/2 (0)	0	–
ATO	70	0/1 (0)	0	–
KEN	70	0/2 (0)	0	–
Total		22/53 (42)	84	3.8

Motility of spermatozoa was classified into the following grades: +++ progressively motile at a high speed, ++ progressively motile at a moderate speed, + progressively motile at a low speed; ± motile without progression, – immotile. The proportion of spermatozoa exhibiting a motility grade of +++ or ++ was used as the parameter for sperm motility

Table 2 Results of heterospermic insemination with frozen-thawed and freshly ejaculated spermatozoa in the Labrador Retriever guide dog

Mating no.	Bitch	Dogs	Days after LH surge at insemination					No. of live pups
			4	5	6	7	8	
1	ERZ	BUZ		FT	FT			2
		MAR				NM		0
2	SHI	BUZ		FT	FT			3
		MAR			NM			0
3	PAG	BUZ		FT	FT			5
		RYU				EJ		0
4	BEG	BUZ		FT	FT			7
		FUN					NM	0
5	HAN	RYU			FT	FT		4
		PEA				EJ		4
6	BEL	RYU		FT	FT			6
		PEA				EJ		0
7	IND	RYU			FT	FT		5
		MAR				EJ		2
8	GUC	RYU		FT	FT	FT		5
		PEA				EJ		0
9	DRE	RYU		FT	FT	FT		1
		KEI				NM		0
10	BEL	RYU		FT	FT			1
		PEA				EJ		0
11	ION	RYU	FT					0
		PEA		NM				4
12	KER	RYU		FT				0
		TOR		NM		NM		7
13	HAN	RYU			FT	FT		4
		PEA				EJ		4
14	BEL	ODY	FT					0
		NUR			FT			0
		PEA			EJ		EJ	7
15	CHR	LEG	FT	FT	FT			6
		PAL			EJ	EJ		1

FT frozen-thawed spermatozoa, *EJ* freshly ejaculated spermatozoa, *NM* natural mating

and expensive equipment is required to achieve transcervical insemination, the spread of intrauterine insemination for the breeding of guide dogs, as well as other working dogs, may still face certain difficulties. However, in a view of the urgent demand of the increasing numbers of handicapped people, it is necessary to develop and diffuse new assisted reproductive techniques, including artificial insemination, to improve the effective use of genetic resources in dogs. Although the cause of the lower conceptus number and litter size in pregnancy is still unclear in our present study, it might be influenced by the quality of the frozen-thawed semen, the age and gravidity of the bitches used, and/or the optimal timing of insemination. Further studies would be required to develop a novel

cryopreservation method which is available to male dogs proven fertile in natural matings.

The total of 15 homospermic and 38 heterospermic inseminations yielded 84 pups from frozen-thawed semen and 91 pups from freshly ejaculated semen, respectively. Heterospermic insemination in combination with cryopreserved spermatozoa and ejaculated semen appeared to be effective to avoid a shortage of production in a colony of guide dogs.

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