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Establishment of Normal Reference Data of Analysis in the Fresh and Cryopreserved Canine Spermatozoa

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Abstract

The cryopreservation has been extensively applied in many cells including spermatozoa (semen) during past several decades. Especially, the canine spermatozoa cryopreservation has contributed on generation of progeny of rare/genetically valuable dog breeds, genome resource banking and transportation of male germplasm at a distant place. However, severe and irreversible damages to the spermatozoa during cryopreservation procedures such as the thermal shock (cold shock), formation of intracellular ice crystals, osmotic shock, stress of cryoprotectants and generator of reactive oxygen species (ROS) have been addressed. According as a number of researches have been conducted to overcome these problems and to advance cryopreservation technique, several analytical methods have been employed to evaluate the quality of the fresh or cryopreserved canine spermatozoa in regards to the motility, morphology, integrity of membrane and DNA, mitochondrial activity, ROS generation, binding affinity to oocytes, in vitro fertilization potential and fertility potential by artificial insemination. Because the study designs with certain application of analytical methods are selective and varied depending on each experimental objective and laboratory condition, it is necessary to establish the normal reference data of the fresh or cryopreserved canine spermatozoa for each analytical method to monitor experimental procedure, to translate raw data and to discuss results. Here, we reviewed the recent articles to introduce various analytical methods for the canine spermatozoa as well as to establish the normal reference data for each analytical method in the fresh or cryopreserved canine spermatozoa, based on the results of the previous articles. We hope that this review contributes to the advancement of cryobiology in canine spermatozoa.

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INTRODUCTION

Cryopreservation indicates long-term storage in condition of subzero ℃ temperatures without the metabolic activity on the cellular systems [Stănescu and Bîrţoiu, 2010; Polge et al., 1949]. Since the introduction of glycerol and dimethyl sulfoxide (DMSO) as the cryoprotectants, cryopreservation techniques have been extensively applied in many cells including spermatozoa (semen); spermatozoa cryopreservation is a way for preserving genetic information and diversity in several species for an unlimited time theoretically and enables male germplasm to use in the future [Bainbridge and Jabbour, 1998; Farstad, 2000; Lovelock and Bishop, 1959; McLachlan, 1998; Polge et al., 1949; Rhemrev et al., 2001; Varesi et al., 2014].

Of particular, there are additional benefits of spermatozoa cryopreservation in canine. Canine spermatozoa cryopreservation may help to create progeny of rare dog breeds, genetically valuable dogs such as the guide dog, detection dog and disease model dog, and the dog whose the owner wants to breed; cryopreservation technology has contributed to the protocol of genome resource banking in canine as well [England, 1993; Farstad, 2000; Hay et al., 1997; Oettle, 1986]. In addition, transportation of cryopreserved spermatozoa can make female dogs fertilized at a distant place, which can reduce their stress and shipping cost [Hori et al., 2011; Umamageswari et al., 2012]. Furthermore, canine spermatozoa cryopreservation can be done even post mortem; if the male dog dies accidently or unexpectedly, the procedures with collection of spermatozoa from the epididymis and cryopreservation are feasible to produce his offspring in the future via the artificial insemination [Hori et al., 2009; Hori et al., 2015; Varesi et al., 2013].

Despite the aforementioned advantages of cryopreservation, its process may directly or indirectly affect severe and irreversible damages to the spermatozoa with respect to the thermal shock (cold shock), formation of intracellular ice crystals, osmotic shock, stress of cryoprotectants and generator of reactive oxygen species (ROS) [Henry, 1993; Holt, 2000; Oettle, 1986; Rhemrev et al., 2001; Watson, 2000]. Because the spermatozoa are highly differentiated, are very structural and are functionally motile, these kinds of damage results in the loss of viability, motility and fertility of spermatozoa [Henry et al., 1993; Stănescu and Bîrţoiu, 2012; Thomas et al., 1993]. For instance, the formation of intracellular ice crystals and osmotic imbalance can damage the integrity of spermatozoa, membrane structure and function [Henry et al., 1993; Oettle, 1986; Stănescu and Bîrţoiu, 2012; Thomas et al., 1993; Yoon et al., 2015; Watson, 2000]. Acrosome reaction during fertilization takes place in only intact acrosome of the spermatozoa, however, acrosome is the most commonly affected part during freezing [Oettle, 1986, Szász et al., 2000]. Since polyunsaturated fatty acids is highly rich in the spermatozoa membrane, they are particularly sensitive to the generation of ROS by the mitochondrial activity, resulting in interference with spermatozoa quality, capacitation and fertility; once the membrane components are damaged by oxidative stress, the spermatozoa is not able to resynthesize it [Aitken et al., 1989; Lucio et al., 2017; Neagu et al., 2011; Starkov, 2008; Wang et al., 1997].

In case of canine spermatozoa cryopreservation, a number of researches have been conducted to overcome the adverse effect of cryopreservation into canine spermatozoa as well as to advance this technique to facilitate in the veterinary clinics during past several decades [Martins et al., 2009; Stănescu and Bîrţoiu, 2012; Varesi et al., 2014]. The researchers have focused in terms of collection method of spermatozoa or semen, storage condition of fresh spermatozoa, types of extender, cooling rate/program and thawing method to improve protocol for the canine spermatozoa cryopreservation, however, the each experimental condition is too multi-factorial and diverse to consolidate a single protocol. In case of collection of canine spermatozoa or semen, several methods to get the canine semen such as massaging the penis and flushing/mincing the epididymis were compared; there were no considerable different in each method [Hori et al., 2011; Hori et al., 2015]. When the proper storage condition of fresh semen from isolated epididymis so as to investigate the time range without lowering quality of canine spermatozoa was studied, the cryopreserved spermatozoa from left epididymis at 20 ℃ for up to 6 hr or 4 ℃ for up to 24 hr showed normal qualities in viability, motility and acrosomal integrity [Hori et al., 2005a; Hori et al., 2009]. In the comparative studies of several types of extender and supplement, Bovimix® or egg yolk (EG) plasma or ACP-106c or 4% LDL supplemented extender showed improved quality of cryopreserved canine spermatozoa than TRIS or whole EG or TRIS or EG supplemented one, respectively [Corcini et al., 2016; Martins et al., 2012; Mota Filho et al., 2014; Prapaiwan et al., 2016]. In addition, supplementation of 10 mM glutathione in the extender resulted in acrosome protection and preservation of fertility of cryopreserved canine spermatozoa, but melatonin

did not [Lucio et al., 2016b; Varesi et al., 2014]. On the purpose of establishment of global protocol of canine spermatozoa cryopreservation, the application of commercial extenders (TRILADYL®, Norwegian, Dutch, Uppsala-Equex, CERREC and CERCA extender) or protocols (Conventional bull semen freezing protocol modified, self-made, Norwegian, Uppsala-Equex, CERREC, CERCA and Dutch canine semen freezing protocol) into canine spermatozoa was compared or reviewed [Oettle, 1986; Stănescu and Bîrţoiu, 2010; Stănescu and Bîrţoiu, 2012; Umamageswari et al., 2012]. In case of thawing the cryopreserved canine spermatozoa, slow thawing at 37 ℃ for 30 sec was recommended than fast thawing at 70 ℃ for 8 sec [Brito et al., 2017].

The quality of fresh or cryopreserved canine spermatozoa in the aforementioned articles was evaluated by several analytical methods with respect to concentration of spermatozoa in the semen, sperm morphology, sperm motility, computer-assisted sperm analysis, sperm membrane integrity, sperm mitochondrial activity, sperm DNA integrity (sperm DNA fragmentation and sperm chromatin structure), mitochondrial membrane potential, hyposmotic swelling test, susceptibility to ROS, sperm binding assay, status of capacitation in the spermatozoa, in vitro fertilization and artificial insemination. However, as mentioned above, the study designs of the previous researches are multi-factorial and diverse, depending on the experimental objective and laboratory condition. Such being the case, it is necessary to establish the normal reference data of the fresh or cryopreserved canine spermatozoa; if the normal reference data are unclear, monitoring the experiments, proper translation of raw data and discussion of result are not possible. Therefore, we aimed to review the recent articles to introduce the analytical methods for canine spermatozoa in detail as well as to establish the normal reference data in the fresh or cryopreserved canine spermatozoa, based on the results of each analytical method obtained from the previous articles. The analytical methods, applied in more than 2 articles, were only employed to calculate the mean value in this review; the values from results of each analytical method were selectively taken from the freshest (the most naive) spermatozoa group (e.g. 0 hr storage group in the article of Hori et al., 2005a) or the cryopreserved spermatozoa group with the best result between/among experimental groups (e.g. slow thawing group in the article of Brito et al., 2017) when the fresh (unfrozen) or cryopreserved groups were diverse, respectively. If a certain datum from the article was too distant from the average of relevant data from its analysis, it was intensively excluded.

The normal reference data in the analysis of the fresh or cryopreserved canine spermatozoa

The normal reference data for sperm morphology, sperm motility, progressively motile sperm, sperm viability, mean velocity, linear velocity, curvilinear velocity, amplitude of lateral head displacement, beat cross frequency, mean coefficient, linear coefficient, sperm membrane integrity, sperm acrosome integrity and sperm DNA integrity (sperm DNA fragmentation) were consolidated and were presented in Table 1.

The concentration of spermatozoa in the semen is defined as the number of spermatozoa/mL and is measured in sperm-rich fraction using the hemocytometer counting chamber [Brito et al., 2017; Hori et al., 2015].

In the sperm morphology (MOR) and viability (VIA) analysis, the spermatozoa should be stained with various kinds of staining methods such as Eosin-Nigrosin, Bengal Rose, Victoria Blue B and Williams. Thereafter, the total number of 100-500 stained spermatozoa is smeared and is observed under a light microscope at × 1000 magnification with emersion oil to record the percentage (%) of alive spermatozoa and defected spermatozoa in head, neck, midpiece and tail; abnormal spermatozoa can be recognized by pear-shaped head, narrow head, detached head, bent neck, cytoplasmic droplet at midpiece, bent tail, coiled tail and broken tail [Hori et al., 2004; Mota Filho et al., 2014; Prapaiwan et al., 2016; Rhemrev et al., 2001; Varesi et al., 2014]. Of particular, it is important for further study to secure more than 60% morphologically normal spermatozoa population to prevent reduction of fertility of canine spermatozoa [Oettle, 1986].

The sperm motility (MOT) is conducted under a light microscope and warm plate at 37-38 ℃ with a scale of 0-4 (0, absent; 1, weak; 2, definite; 3, good; 4, vigorous; a score of 3 or 4 indicates progressive motility) or with the percentage (%) of progressively motile (PMOT) sperm (+, active movements; ±, slow movement; -, motionless) [Hori et al., 2015; Mortimer, 1994]. It was suggested that 30-50% was acceptable in the cryopreserved canine semen [Umamageswari et al., 2012].

The development of computer-assisted sperm analysis (CASA) has been very useful for evaluating the parameter of spermatozoa

Table 1. Mean, average of mean values from results from each article; Max, the maximum value among the data; Min, the minimum value among the data; Remark; the total number of articles; MOR, sperm morphology; MOT, sperm motility; PMOT, progressively motile sperm; VIA, sperm viability; VAP, mean velocity; VSL, linear velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, mean coefficient; LIN, linear coefficient; SMI, sperm membrane integrity; SAI, sperm acrosome integrity; SDI (SDF), sperm DNA integrity (sperm DNA fragmentation)

Method	Fresh canine spermatozoa				Cryopreserved canine spermatozoa				Reference
	Mean	Max	Min	Remark	Mean	Max	Min	Remark	
MOR	80.3	96.5	52.2	n=10	42.5	69.3	21.3	n=14	Watson, 2000; Martins et al., 2009; Corcini et al., 2016; Brito et al., 2017; Merlo et al., 2015; Varesi et al., 2014; Mota Filho et al., 2014; Ortiz et al., 2017; Lucio et al., 2016b; Hori et al., 2004; Hori et al., 2011; Hori et al., 2009; Hori et al., 2015; Umamageswari et al., 2012; Silva et al., 2005; Prapaiwan et al., 2016; Hewitt et al., 2001
MOT	66.2	84.1	47.9	n=4	53.5	70.5	17.2	n=4	Corcini et al., 2016; Varesi et al., 2014; Mota Filho et al., 2014; Silva et al., 2005; Hewitt et al., 2001
PMOT	26.6	32.5	20.7	n=2	26.3	40.5	13.8	n=5	Martins et al., 2012; Martins et al., 2009; Brito et al., 2017; Mota Filho et al., 2014; Lucio et al., 2016b; Hewitt et al., 2001
VIA	84.7	91.4	76.2	n=4	54.6	60.6	43.0	n=5	Hori et al., 2004; Hori et al., 2011; Hori et al., 2009; Hori et al., 2015; Umamageswari et al., 2012
VAP	80.3	-	-	n=1	81.2	88.9	70.6	n=4	Martins et al., 2012; Martins et al., 2009; Brito et al., 2017; Mota Filho et al., 2014; Lucio et al., 2016b
VSL	74.8	-	-	n=1	69.1	77.9	60.9	n=4	Martins et al., 2012; Martins et al., 2009; Brito et al., 2017; Mota Filho et al., 2014; Lucio et al., 2016b
VCL	88.8	-	-	n=1	124.9	132.4	118.4	n=3	Martins et al., 2012; Brito et al., 2017; Mota Filho et al., 2014; Lucio et al., 2016b
ALH	3	-	-	n=1	5.1	6.9	2.8	n=3	Martins et al., 2012; Brito et al., 2017; Mota Filho et al., 2014; Lucio et al., 2016b
BCF	9.5	-	-	n=1	22.0	31.1	12.8	n=2	Martins et al., 2012; Brito et al., 2017; Mota Filho et al., 2014; Lucio et al., 2016b
STR	84.4	-	-	n=1	82.4	85.6	75.8	n=4	Martins et al., 2012; Brito et al., 2017; Mota Filho et al., 2014; Lucio et al., 2016b
LIN	65.3	-	-	n=1	58.4	66.3	47	n=4	Martins et al., 2012; Brito et al., 2017; Mota Filho et al., 2014; Lucio et al., 2016b
SMI	80.6	86.2	74.9	n=2	51.8	60.9	33.8	n=4	Martins et al., 2009; Corcini et al., 2016; Umamageswari et al., 2012; Prapaiwan et al., 2016
SAI	78.5	97.9	55.5	n=5	46.9	65.0	33.9	n=6	Corcini et al., 2016; Merlo et al., 2015; Varesi et al., 2014; Hori et al., 2015; Umamageswari et al., 2012; Prapaiwan et al., 2016
SDI (SDF)	3.3	-	-	n=1	2.8	3.6	1.9	n=2	Varesi et al., 2014; Lucio et al., 2016b

movement including total motility (%), progressive motility (%), mean velocity (VAP, μm/s; the mean trajectory of the spermatozoa per unit of time), linear velocity (VSL, μm/s; the straight trajectory of the spermatozoa per unit of time), curvilinear velocity (VCL, μm/s; the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time), amplitude of lateral head displacement (ALH, μm; mean width of sperm head oscillation), beat cross frequency or frequency of head displacement (BCF, Hz; the number of lateral oscillatory movements of the sperm head around the mean trajectory), mean coefficient (straightness, STR, %; (VSL/VAP) × 100) and linear coefficient (linearity, LIN, %; (VSL/VCL) × 100), wobble coefficient (WOB, %; the oscillation of the curvilinear trajectory upon the mean trajectory; (VAP/VCL) × 100), distance curved line (DCL, mm; the actual distance that the sperm cell moved during the analysis period), distance straight line (DSL, mm; the distance from the point in which the cell was first found to the last location in a straight line), distance average path (DAP, mm; the measured distance using a smooth line as a reference), and average orientation change (AOC, degrees; the

average number of degrees that the head of the sperm moved from left to right during the analysis) [Martins et al., 2009; Martins et al., 2012; Rhemrev et al., 2001]. Of particular, some of the aforementioned parameters can be applied to determine the fertilization ability of spermatozoa; high values of VAP, VSL and VCL of spermatozoa were correlated with their ability for oocyte fertilization, in addition, VCL and ALH, inversely proportional to LIN, indicated the hyperactivation of spermatozoa, implying successful penetration to the zona pellucida of oocytes [Schäfer-Somi and Aurich, 2007; Silva et al., 2006a; Verstegen et al., 2002].

The sperm membrane integrity (SMI) is a valuable factor to assess spermatozoa viability due to its importance for general cell function such as maintenance of alive cell, capacitation, acrosome reaction and attaching ability to oocytes. The percentage (%) of SMI of spermatozoa might be simply counted using Eosin/Nigrosin staining under epifluorescence microscope with WG filter; because the stains unable to penetrate an intact membrane, spermatozoa with green or red (pink) fluorescence indicate intact or damaged, respectively [Brito et al., 2017; Martins et al., 2009]. In the recent days, a commercialized kit, consisting of propidium iodide (PI) and SYBR, is available on this assay using a fluorescence microscope; viable spermatozoa can be seen with SYBR positive and PI negative, in contrast, PI positive indicates non-intact membranes or death of spermatozoa [Merlo et al., 2015; Rhemrev et al., 2001]. In addition, PI and Pisum sativum agglutinin conjugated with fluorescein isothiocyanate (FITC-PSA) is also applied to concurrently assess SMI and acrosome damage by flow cytometry; sperm membrane or acrosome damage was expressed by a red or green color, respectively [Lucio et al., 2016b].

The sperm acrosome integrity (SAI) is important to maintain the acrosomal enzymes, indicating fertility potential [Lucio et al., 2016b]. The acrosome reaction is initiated to fuse the sperm plasma of the sperm's head, exposing the contents of the acrosome membrane, with oocyte when the first sperm meet with oocyte [Yanagimachi and Usui, 1974]. However, SAI is easy to be lost by mechanical stress such as centrifugation, freezing and thawing processes, followed by loss of fertility [Hewitt et al., 2001; Hori et al., 2005b; Rijsselaere et al., 2002]. The assay of SAI (%) can be achieved by counting (100-200 cells) Fast Green/Rose Bengal stained spermatozoa under microscopic observation; the purple or darker stained acrosomal region than the post-acrosomal area indicates intact acrosome, in contrast, unstained or brighter acrosomal region than the post-acrosomal area does damaged one [Brito et al., 2017]. In another way, the presence or absence of a green acrosomal fluorescence was considered as indication for an intact or damaged acrosome, respectively, in a same manner with the aforementioned PI and FITC-PSA (or Peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC), FITC-PNA) [Lucio et al., 2016b; Merlo et al., 2015; Varesi et al., 2014].

The 3.3′ diaminobenzidine (DAB) staining is mainly applied in sperm mitochondrial activity (SMA) assay. The total number of 100-200 DAB-stained spermatozoa (%) is counted and is classified in four different classes: Class I with 100% of the mid-piece stained indicating full mitochondrial activity; Class II with more than 50% of the mid-piece stained indicating medium activity; Class III with less than 50% of the mid-piece stained indicating low activity; Class IV with the absence of staining in the mid-piece indicating no mitochondrial activity [Brito et al., 2017; Lucio et al., 2016b].

The sperm DNA integrity (SDI; sperm DNA fragmentation, SDF) is associated with fertility potential, because the paternal DNA plays a critical role in the functions of spermatozoa and embryo development [Andrabi, 2007; Silva et al., 2006b; Virro et al., 2004]. The evaluation of SDI is conducted in 300-500 spermatozoa (%) with the commercial kit (Sperm-Halomax®) under bright-field microscopy or epifluorescence microscope after additional staining with SYBR. The indexes of SDI consist of intact or fragmented or highly fragmented DNA with showing a small/compact halo around the spermatozoa head or a widespread/soft halo of chromatin dispersion or a halo of dispersion, respectively [De Ambrogi et al., 2006; Varesi et al., 2014]. The flow cytometer is also involved to evaluate SDF and sperm chromatin structure assay (SCSA) [Chohan et al., 2006; Lucio et al., 2016b; Ortiz et al., 2017]. In brief, snap-frozen spermatozoa are stained with acridine orange (AO), followed by evaluation of 10,000 cells via the flow cytometer equipped with Ar ion laser. Green fluorescence from AO bound double-strand DNA was detected at a 530/30 nm bandpass filter, whereas, red fluorescence from AO bound single-strand DNA was done at a more than 670 nm longpass filter; the ratio of single strand DNA is considered as SDF (%) [Lucio et al., 2016b; Ortiz et al., 2017].

The previous articles suggested that the sperm motility was dependent on the mitochondrial function, therefore, the assay for mitochondrial membrane potential (MMP) has been widely used to evaluate spermatozoa of human and rodents [Davis et al., 1991; Hirano et al., 2001; Rhemrev et al., 2001]. However, the assay of MMP is still limited in research for canine spermatozoa; the 5, 5`, 6, 6`, tetrachloro-1, 1`, 3, 3`-tetraethyl-benzimidazol carbocyanine iodide (JC-1) is added to 7 million spermatozoa, followed

by analysis with flow cytometry. The spermatozoa with high or low mitochondrial potential are detected by red fluorescence (complex form of JC-1) or green fluorescence (monomer form of JC-1), respectively [Lucio et al., 2016b].

The hyposmotic swelling test (HOST) is applied to evaluate the membrane functionality of spermatozoa. The sets of serial concentration of distilled water are exposed to spermatozoa, thereafter, spermatozoa (%) with functional membrane is counted when the sperm has a curled tail [Mota Filho et al., 2014].

The oxygen is essential to maintain the function of spermatozoa; high concentrations of oxygen can arise severe damage to cells via the formation of ROS and free radicals [Bennetts and Aitken, 2005; Lucio et al., 2017]. Whereas low concentration of ROS are necessary to achieve sperm capacitation and hyperactivation fertilizing capacity, the gap between formation and removal of ROS and free radicals (oxidative stress) results in an oxidant state, followed by decrease in motility, DNA damage and cellular death of spermatozoa [Lucio et al., 2016a; Lucio et al., 2017; Saleh and Agarwal, 2002]. In case of the assay for susceptibility of spermatozoa to oxidative stress, malondialdehyde (MDA) levels, a product of lipid peroxidation of the spermatozoa under ROS generating system, are indirectly measured by thiobarbituric acid reactive substances (TBARS) by quantification of a pink chromogen (reaction of MDA with TBARS) with a spectrophotometer at a wavelength of 532 nm; the obtained values are compared with result of standard curve of MDA, and the index of lipid-peroxidation was evaluated as ng of TBARS/mL [Lucio et al., 2016b; Lucio et al., 2017].

The sperm binding assay (SBA) is conducted as a functional sperm test with perivitelline membrane chicken EG in even canine spermatozoa; when segments of the perivitelline membrane of chicken EG are incubated with canine spermatozoa, the area (mm^2) of spermatozoa bound to the perivitelline membranes were measured under microscopic observation [Brito et al., 2017].

Status of capacitation in the spermatozoa can be detected by the dual fluorescent staining, chlortetracycline (CTC) and Hoechst 33258 [Fraser et al., 1995; Hewitt et al., 2001]. In the results, spermatozoa with bright blue staining were defined as dead and were excluded from further assessment for CTC-staining pattern. In contrast, blue violet fluorescent spermatozoa at 400-440 nm wave length are considered as live ones and are classified as uncapacitated and intact acrosome (F) or capacitated and intact acrosome intact (B) or capacitated and reacted acrosome reacted (AR) [Hewitt et al., 2001; Kay et al., 1994]. This technique allows the acrosomal status of sperm as well as capacitation status of spermatozoa.

In vitro fertilization (IVF) is regarded as a great method to evaluate the comprehensive function of canine spermatozoa with respect to viability, motility, capacitation, oocyte binding affinity and fertility; however, it is limitation that collection of canine oocytes is harder than that of other species such as bovine and swine, easily available to get the oocytes from slaughter house [Martins et al., 2009]. The canine oocytes commonly obtained from ovariohysterectomized ovaries or via uterine-flushing, thereafter, the ones that are approximately 100 mm in diameter with a dark ooplasm surrounded by several layers of cumulus cells are selectively collected. To conduct IVF, the oocytes are then incubated with spermatozoa in condition of humidified atmosphere containing 5% CO_2 at 38 ℃ for 4-18 hr. The fertilized oocytes are vortexed, are stained with Hoechst 33258 and are analyzed regarding the percentage (%) of spermatozoa that bound to zona pellucida or penetrated oocytes [Hewitt et al., 2001; Martins et al., 2009; Rhemrev et al., 2001].

The artificial insemination (AI) has great functional significance about whether the spermatozoa maintain the real fertility potential or not. It has been addressed that AI with fresh semen to female dogs has similar pregnancy rate with that of natural mating [Lucio et al., 2017]. However, the relationship between normal morphology and fertility in canine spermatozoa was previously investigated; the population of less than 60% morphologically normal spermatozoa, possible to occur in the cryopreservation, caused a significant reduction in fertility [Oettle, 1986]. In addition, cryopreservation attenuated the pregnancy rate as a reduction of 30% on pregnancy rate and litters than those from fresh spermatozoa [Linde-Forsberg et al., 1999; Yu et al., 2002]. Furthermore, the success of AI with fresh or cryopreserved canine spermatozoa is dependent on the AI technique, accuracy of ovulation detection, the time for AI and the number and concentration of spermatozoa [Eilts, 2005]. To detect the estrus cycle and ovulation of the female dogs, several methods are applicable. Comprehensively, the small volume of blood is daily or every other day aspirated post the vulval bleeding on clinical observation and/or presence of keratinized (superficial) cells in the vaginal cytology and/or observation of pale and primary folding with secondary wrinkling vaginal mucosa (crenulation) via the vaginoscopy [Hori et al., 2011; Lucio et al., 2016b]. The progesterone (P4) level in the serum is thereafter measured by

fluorescence enzyme immunoassay (fluorescence immunochemistry analyzer or enzyme immunoassay); the day on which the P4 level reached more than 2 ng/mL is expected as the ovulation day [Hase et al., 2000; Munro and Stabenfeldt, 1984]. Then AI is conducted at 3 to 4 days after estimated ovulation when P4 level is expected as 8.5-12 ng/mL [Hori et al., 2005b; Hori et al., 2011; Tsutsui et al., 2000]. As AI is performed, the number and concentration of spermatozoa are recommended as 1-4 × 10^8 spermatozoa in 150-200 μL extender to guarantee the fertility with oocytes, in accordance with previous articles [Hori et al., 2004; Hori et al., 2005a; Hori et al., 2011; Lucio et al., 2016b; Tsutsui et al., 2000]. The introduction of spermatozoa to female dogs can be done by surgical or non-surgical methods, which are the intrauterine insemination via insertion of catheter (e.g. venous catheter, glass capillary and Norwegian catheter) to the exposed uterine after midline incision of the linea alba or the transcervical insemination via catheter (e.g. Minitube® and Norwegian catheter) to each uterine, respectively [Hori et al., 2004; Linde-Forsberg et al., 1999; Lucio et al., 2016b]. Additionally, if possible, two inseminations at intervals of 24 hr are recommended for accuracy of the procedure of AI [Lucio et al., 2017]. The most common way to check the diagnosis is ultrasonography around 30 days after AI [Hori et al., 2005a; Hori et al., 2011; Lucio et al., 2016b]. Then the conception rate during the pregnancy and counting new born at delivery around 60 days after AI are acquired as the result of AI in fresh or cryopreserved canine spermatozoa [Hori et al., 2005a; Hori et al., 2011; Lucio et al., 2016b].

CONCLUSION

Taken together, diverse analytical methods to evaluate the fresh or cryopreserved canine spermatozoa has been introduced and applied in the relevant researches during past several decades. Because the cryopreserved spermatozoa would be exposed in the stressful circumstance than the fresh ones, it is unavoidable to be damaged during cryopreservation procedure. Therefore, the comparative studies between the fresh and cryopreserved canine spermatozoa should be conducted so as to understand the physiological and functional alteration of cryopreserved spermatozoa and to facilitate the cryopreservation of canine spermatozoa in the veterinary clinics with guaranteeing fertility potential. In the light of this objective, the establishment of normal reference data for each analytical method is necessary. Therefore, this review may contribute to the advancement of cryobiology in canine spermatozoa.

DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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