NOTE *Theriogenology*

Effects of Storage Temperature and Semen Extender on Stored Canine Semen

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(Received 12 June 2013/Accepted 17 September 2013/Published online in J-STAGE 1 October 2013)

ABSTRACT. The objective of the present study was to determine an optimum temperature and extender for short-term transport of canine ejaculated semen. There was no significant difference in the qualities of semen diluted with two kinds of extender, egg yolk Tris-citrate fructose (EYT-FC) or glucose (EYT-GC) extender, between the 2, 8 or 12 and the 4°C control groups during storage for up to 48 hr, while the 16–24°C groups showed decreased sperm motility during storage for 48 hr. However, the 2°C group showed slightly lower sperm motility and slightly higher sperm abnormality than the 4°C group. Therefore, we concluded that semen qualities can be maintained for up to 48 hr when canine semen samples are extended with EYT-FC or EYT-GC and stored at a temperature in the range of 4–12°C.

KEY WORDS: canine, semen, semen extender, storage temperature.

doi: 10.1292/jvms.13-0303; *J. Vet. Med. Sci.* 76(2): 259–263, 2014

To produce or maintain superior pedigrees, some breeders desire mating between dogs from remote locations. However, land and air transport of dogs for mating markedly places a large amount of stress on female dogs. Air transport during summer is difficult, particularly for short-nosed dog breeds. To solve these problems, frozen or chilled semen are routinely transported for artificial insemination.

Frozen semen can be stored semipermanently, facilitating their use at any time according to the female estrous cycle. However, the optimum timing of insemination should be carefully determined, because damage of sperm during freezing and thawing resulted in low sperm motility and a short lifespan after thawing and negative effects on fertilization due to accelerated capacitation [20]. In addition, some problems, such as risks of general anesthesia and invasive surgery, occur, because conception with frozen semen in bitches requires surgical intrauterine insemination and not intravaginal insemination [18]. Recently, intrauterine sperm injection has been performed using transvaginal, nonsurgical procedures with a small veterinary endoscope to avoid invasive surgery [7, 11]. However, this method is difficult for small-sized dogs and requires expensive equipment, precluding its clinical application. In addition, there are disadvantages, such as the need for special equipment to prepare frozen semen, maintenance cost for long-term semen storage (e.g., replenishment of liquid nitrogen) and transport cost due to the use of a dry shipper containing liquid nitrogen for semen transport [3, 12]. Thus, the use of frozen canine semen is impeded by these problems, although it is beneficial.

In contrast, chilled semen should be clinically applied, because it requires no special expensive equipment and maintains high sperm motility over the short term, providing a high conception rate for intravaginal insemination [16, 19]. Tsutsui *et al.* [19] extended canine semen with an egg yolk Tris-fructose citrate solution (EYT-FC) and stored it at 4°C for 48 hr. As a result, the mean sperm motility after storage for 48 hr was maintained at as high as 84.3 ± 4.3 (SE), although the rate of abnormal sperm slightly increased with the storage time. In addition, the conception rate with intravaginal artificial insemination using the semen was as high as 83.3%. Thus, they demonstrated that canine chilled semen was very useful. Specifically, if the female dog is in an area in which the semen sample can be transported from a male dog within 48 hr after semen collection, artificial insemination by chilled semen can be performed.

However, it is difficult to transport semen samples in a Styrofoam carrier box containing a refrigerant with the temperature maintained constantly at 4°C for 48 hr. Temperature rise in the carrier box is inevitable. We previously measured the temperature in a Styrofoam carrier box containing a refrigerant for the transport of semen samples and noted that the temperature rose to 12–15°C at 48 hr [unpublished data]. However, the semen samples retained their good qualities even after 48 hr. Thus, we suggested that canine semen samples could retain their qualities even after storage above 4–5°C.

Semen samples from various animals have been investigated at a storage temperature of 4–5°C [1, 8, 17, 20]. Although boar semen can be stored at 5–7°C for as long as five days, sperm viability markedly declines at the start of storage. Boar semen maintain their good qualities when stored for two or three days at around 15°C [4, 10, 21]. In addition, equine semen showed no significant difference in qualities when stored at 15°C for one day or 4°C using a extender, INRA96, based on modified Hanks' salts [2]. Canine sperm has as low resistance to cooling as sperm from swine [13]. Storage at a comparatively high temperature during a short transport period is unlikely to damage sperm, particularly the sperm acrosome [5, 6, 13]. Therefore, we assume that the optimum temperature for transportation of canine semen samples is different from those for many other animals.

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However, no report has been published on the storage of canine semen samples above 4–5°C.

In addition, sugars contained in semen extenders mitigate damage caused by cold shock or freezing [14]. Besides EYT-FC, egg yolk Tris-glucose citrate solution (EYT-GC) is routinely used as an extender for canine semen. As reported by Ponglowhapan *et al.* [15], a comparison of two extenders containing glucose or fructose used for canine semen storage at 5°C for 23 days demonstrated higher sperm motility with fructose than glucose, although there was no significant difference. However, the effects of storage temperatures, except for 4–5°C, on the qualities of canine semen samples diluted with these extenders have not been examined.

The objective of the present study was to examine the effects of a storage temperature of 2–24°C on the qualities of canine semen samples extended with EYT-FC or EYT-GC in order to determine the optimum temperature for their storage and an extender suitable for low-temperature transport.

Animals: The animals were 7 male beagles bred at our colony and were 1 to 7 years of age (mean ± SE: 3.3 ± 1.1 years). These experimental dogs were kept in 160 × 75 × 65 cm cages with 2 dogs in each cage. Commercial dog food (Health Nutrition, Royal Canin Japon, Inc., Tokyo, Japan) was given once daily, and drinking water was given three times daily (morning, afternoon and evening).

This study was conducted in conformity with the animal study guidelines of Nippon Veterinary and Life Science University.

Semen collection and storage: The semen samples, collected by digital manipulation, were divided into three fractions. We used the second (sperm-rich) fraction. In order to perform examinations at various temperatures under the same conditions, semen from two male dogs was mixed and used in one experiment after confirming that semen parameters from each dog showed no difference.

Storage methods were performed as previously reported [19]. The semen samples were first centrifuged at 650 × *g* for 5 min to remove seminal plasma, and the number of sperm was adjusted to 1×10^8/m*l* with two extenders, EYT-FC or EYT-GC. To prepare EYT-GC, glucose, instead of fructose, was added to EYT-FC at a concentration of 56 mM [19]. The experiment was divided into two parts in consideration of equipment for storage. In Experiment 1, semen samples extended with the two extenders were aliquoted into four plastic tubes, and the samples were stored in an e-Thermo-Bucket (TAITEC, Saitama, Japan) at 4, 8, 12 or 16°C for 72 hr. In Experiment 2, semen samples were extended in the same manner as in Experiment 1 and were stored at 4, 20 or 24°C in an e-ThermoBucket or at 2°C in a programmed low-temperature bath (UH-JF, Chino Co., Ltd., Japan) for 72 hr. In both experiments, semen stored at 4°C was set as the control. In these experiments, to prevent a rapid temperature drop, the temperature was gradually lowered to the target. In addition, to reduce the effects of ambient temperature, the experiments were conducted in the room with a temperature controlled to 20°C using an air conditioner. The difference in the storage temperatures in these experiments was less than ± 1°C. Each experiment was conducted five times.

Semen quality test: The tests of semen quality, sperm motility, viability, abnormality and sperm acrosome status were performed immediately after semen collection and every other 24 hr until 72 hr of storage by methods described previously [19].

Sperm motility was assessed as the percentage of progressively motile sperm using a sperm motility examination plate (Fujihira Industry Co., Ltd., Tokyo, Japan) and a warmplate at 37˚C. The percentages of viable sperm and morphologically abnormal sperm were assessed by eosin-nigrosin staining. The percentage of spermatozoa with an intact acrosome was evaluated in a smear after staining with Spermac® stain (FertiPro NV, Beernem, Belgium), and the spermatozoa were classified into normal or abnormal groups. Spermatozoa with normally appearing acrosomes were considered normal, and spermatozoa with vesiculated, swollen, lost or ruptured acrosomes were considered acrosome reacted or abnormal.

Statistical analysis: Differences in sperm parameters between the groups were analyzed using one-way ANOVA (extender) or two-way ANOVA (storage time × storage temperature), and post hoc multiple comparisons between groups were made by Tukey-Kramer test using Statview 5.0 (Abacus Concepts Inc., Berkeley, CA, U.S.A.). A significance level lower than 5% was defined as significant.

In all sperm parameters for each storage time and storage temperature, no significant difference was observed between the two extenders, EYT-FC and EYT-GC.

Sperm motilities after storage for various time periods in Experiments 1 and 2 are shown in Figs. 1 and 2, respectively. In the EYT-FC group in Experiment 1, in the EYT-FC group, the 16°C group showed significantly lower sperm motility than the 4 and 8°C groups after storage for 24 hr, and the 4°C group showed significantly lower sperm motility after storage for 48 and 72 hr ($P<0.05$). In contrast, in the EYT-GC group, there was no difference due to the storage temperature for the time periods. In Experiment 2, the 24°C group showed significantly lower sperm motility than the 4°C group with both extenders after storage for 24 hr (EYT-FC, $P<0.01$; EYT-GC, $P<0.05$). In the EYT-FC group, the 24°C group showed significantly lower sperm motility than the other groups after storage for 48 and 72 hr ($P<0.05$, $P<0.01$). In the EYT-GC group, there was a significant difference only between the 4 and 24°C groups for 24 and 48 hr, and the 24°C group showed significantly lower sperm motility than the other groups after storage for 72 hr ($P<0.05$, $P<0.01$). The 2°C group showed lower sperm motility than the 4°C group after storage for 24–72 hr, although there was no significant difference between these groups ($P=0.08$–0.13).

As compared with storage for 0 hr, the 12 and 16°C groups showed lower ($P<0.05$) sperm motility with both extenders after storage for 24 hr. The 2°C group of the EYT-FC group after storage for 48 hr and that of the EYT-GC group after storage for 24 hr showed significant differences ($P<0.05$, $P<0.01$). All groups in both experiments showed significantly lower sperm motility with both extenders after storage for 72 hr than after storage for 0 and 24 hr ($P<0.05$, $P<0.01$).

Although sperm viability decreased slightly with the

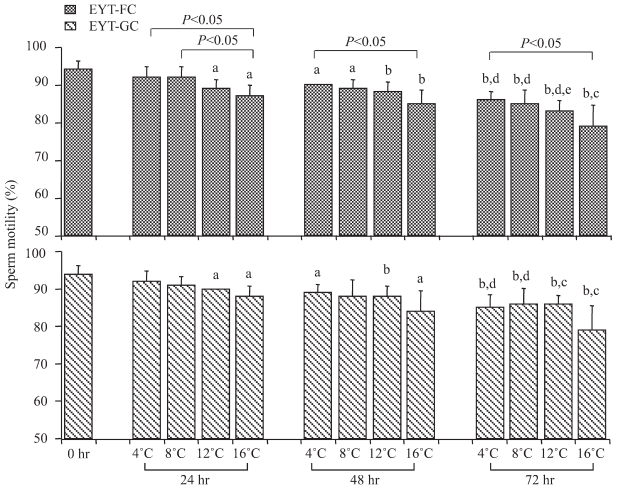

Fig. 1. Time-course changes in sperm motility after storage for various time periods in each group in Experiment 1. Significantly different from that of the 0 hr group at $P<0.05$ (a) and $P<0.01$ (b). Significantly different from that of the 24 hr group at $P<0.05$ (c) and $P<0.01$ (d). Significantly different from that of the 48 hr group at $P<0.05$ (e).

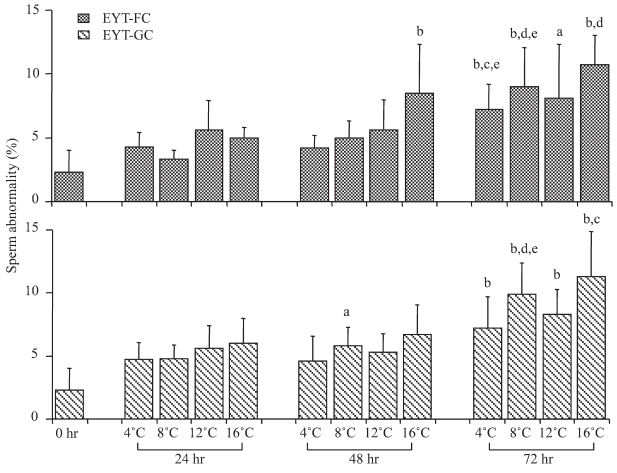

Fig. 3. Time-course changes in sperm abnormality after storage for various time periods in each group in Experiment 1. Significantly different from that of the 0 hr group at $P<0.05$ (a) and $P<0.01$ (b). Significantly different from that of the 24 hr group at $P<0.01$ (d). Significantly different from that of the 48 hr group at $P<0.05$ (e).

storage time, there was no significant difference in sperm viability due to the storage time and temperature in either experimental group, except that the 16°C group of the EYT-FC group showed significant differences after storage for 72 hr (86.6 ± 2.7%) compared with 0 hr (95.4 ± 1.4%) ($P<0.05$).

Sperm abnormalities in Experiments 1 and 2 are shown in Figs. 3 and 4, respectively. There were no significant differences among all groups in the two experiments after storage for 24 hr. However, sperm abnormality increased over time at 48 hr and later. In Experiment 1, there was no significant difference between storage temperatures. In

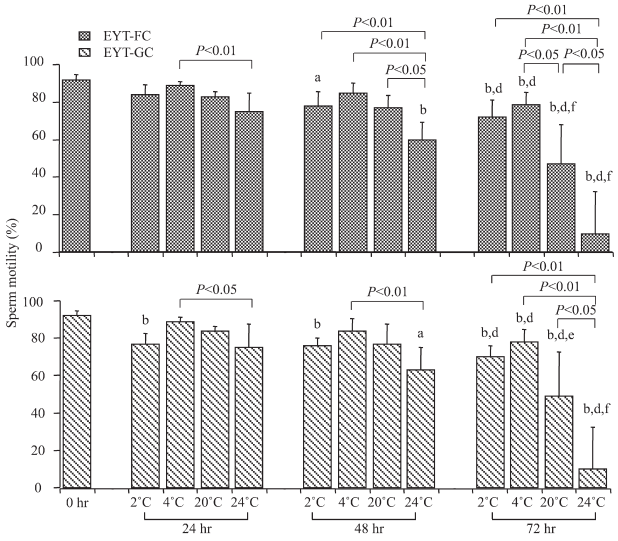

Fig. 2. Time-course changes in sperm motility after storage for various time periods in each group in Experiment 2. Significantly different from that of the 0 hr group at $P<0.05$ (a) and $P<0.01$ (b). Significantly different from that of the 24 hr group at $P<0.01$ (d). Significantly different from that of the 48 hr group at $P<0.05$ (e) and $P<0.01$ (f).

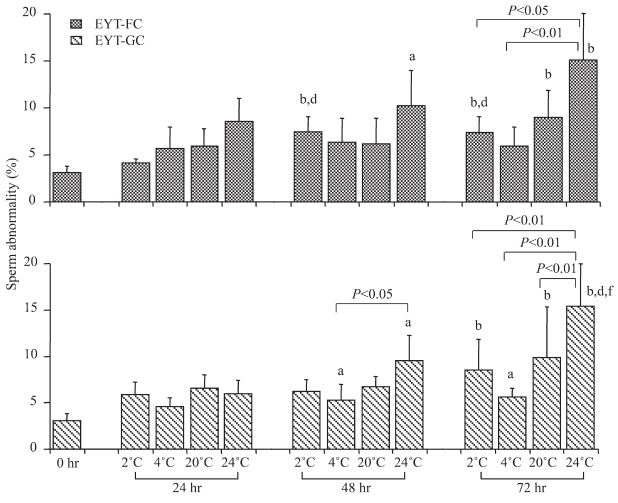

Fig. 4. Time-course changes in sperm abnormality after storage for various time periods in each group in Experiment 2. Significantly different from that of the 0 hr group at $P<0.05$ (a) and $P<0.01$ (b). Significantly different from that of the 24 hr group at $P<0.01$ (d). Significantly different from that of the 48 hr group at $P<0.01$ (f).

Experiment 2, there was a significant difference between the 4 and 24°C groups of the EYT-GC group after storage for 48 hr ($P<0.05$). In the EYT-FC group, the 24°C group showed significantly higher sperm abnormality than the 2 and 4°C groups after storage for 72 hr ($P<0.05$, $P<0.01$). In the EYT-GC group, the 24°C group showed significantly higher sperm abnormality than the other groups after storage for 72 hr ($P<0.01$).

As compared with storage for 0 hr, the 16°C group of

the EYT-FC group ($P<0.01$) and 8°C group of the EYT-GC group ($P<0.05$) showed significantly higher sperm abnormality after storage for 48 hr. All groups showed significantly higher sperm abnormality with both extenders after storage for 72 hr than after storage for 0 hr ($P<0.05$, $P<0.01$). The 2 and 24°C groups of the EYT-FC group ($P<0.05$, $P<0.01$) and 4 and 24°C groups of the EYT-GC group ($P<0.05$) showed significantly higher sperm abnormality after storage for 48 hr than after storage for 0 hr. All of the EYT-FC group ($P<0.05$, $P<0.01$), except for the 4°C group, and all of the EYT-GC group ($P<0.01$) showed significantly higher sperm abnormality after storage for 72 hr than after storage for 0 hr.

The percentages of spermatozoa with an intact acrosome in Experiments 1 and 2 are shown in Figs. 5 and 6, respectively. In Experiment 1, the percentage of spermatozoa with an intact acrosome decreased with the storage time, although there was no significant difference due to the storage time and temperature. After storage for 48 and 72 hr, the 16°C group of the EYT-FC group and the 2 and 4°C groups of the EYT-GC group showed significant differences compared with after storage for 0 hr. Similarly, in Experiment 2, the percentage of spermatozoa with an intact acrosome decreased with the storage time. The 24°C group of the EYT-FC group showed a significantly lower percentage of spermatozoa with an intact acrosome than the 2 and 4°C groups after storage for 72 hr ($P<0.05$). The 2°C group showed a significantly lower percentage of spermatozoa with an intact acrosome with both extenders after storage for 24–72 hr than after storage for 0 hr ($P<0.05$, $P<0.01$). All of the EYT-FC group, except for the 4°C group, and all of the EYT-GC group showed significantly lower percentages of spermatozoa with an intact acrosome after storage for 72 hr than after storage for 0 hr ($P<0.05$, $P<0.01$). The 2°C group showed an increase in swollen sperm acrosomes, and the 16°C or higher group showed an increase in sperm acrosome loss.

In the present study, canine semen was stored at 2–24°C to determine an optimum temperature for short-term storage based on semen qualities. The results showed that there was no significant difference in semen qualities during storage at 2–12°C for up to 48 hr, demonstrating that the semen qualities could be maintained. Although there was no significant difference between the 2 and 4°C groups, the percentage of motile spermatozoa was slightly low and sperm abnormality was slightly high in the 2°C group. In addition, acrosomal defects in the 2°C group of the EYT-GC group were slightly increased after 24–72 hr storage, but this was not observed in the 4°C group. The temperature error during semen storage was ± 1°C. Thus, the temperature may fall below 2°C even if stored at 2°C. Specifically, sperm could be irreversibly damaged by cold shock, causing an abnormal sperm morphology, particularly an abnormal tail and acrosome, and slightly reducing sperm motility. Thus, semen qualities may deteriorate when semen samples are exposed to temperatures below 4°C during storage at a low temperature. In addition, the 16°C or higher groups showed decreased sperm motility and markedly increased sperm abnormality. Furthermore, the percentage of spermatozoa with an intact acrosome sharply declined in the 20°C or higher groups. Sperm motility and

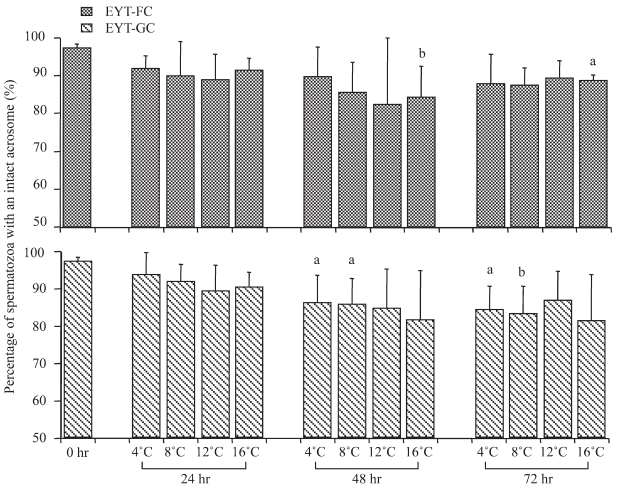

Fig. 5. Time-course changes in the percentage of spermatozoa with an intact acrosome after storage for various time periods in each group in Experiment 1. Significantly different from that of the 0 hr group at $P<0.05$ (a) and $P<0.01$ (b).

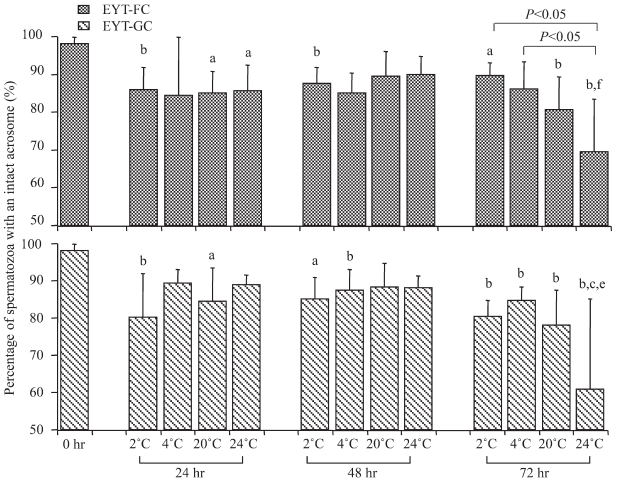

Fig. 6. Time-course changes in the percentage of spermatozoa with an intact acrosome after storage for various time periods in each group in Experiment 2. Significantly different from that of the 0 hr group at $P<0.05$ (a) and $P<0.01$ (b). Significantly different from that of the 24 hr group at $P<0.01$ (c). Significantly different from that of the 48 hr group at $P<0.05$ (e) and $P<0.01$ (f).

metabolism increase as the temperature rises. Thus, sperm motility cannot be completely suppressed at these temperatures. Metabolites decrease the pH of semen. Oxidative stress caused by active oxygen damages the cell membrane containing unsaturated fatty acids and impairs the enzyme activity and cellular functions of sperm, resulting in reduced sperm motility and an abnormal morphology, particularly an abnormal tail [19]. In addition, freezing or cooling sperm alters the plasma membrane or intracellular functions, such as capacitation [20]. Thus, evaluating the percentage of sperm with an intact sperm acrosome is important for indirectly evaluating sperm fertility. Our results suggest that the rate

of swollen sperm acrosomes increased in the 2°C group. In addition, the rate of sperm acrosome loss increased in the 16°C or higher group. Therefore, these storage temperatures were unsuitable.

In our previous study [unpublished data], the temperature of a Styrofoam carrier box (Canine Transport Box, Minitube, Tiefenbach, Germany) containing two ice packs frozen at −20°C declined to 4°C. Subsequently, the temperature rose to 12–15°C after 48 hr with the average temperature being 13.5°C. However, the sperm motility of canine semen samples stored in this carrier box was as high as 85–90% even after 48 hr with the average being 86.7 ± 2.5%. In the present report, it is impossible to discuss the effects of temperature changes, because semen samples were stored at a constant temperature. However, the effects of temperature changes on sperm may be negligible as long as sperm motility is suppressed at a low temperature. Our results demonstrated that there is no effect on sperm at a temperature of 4–12°C, supporting the results of preliminary experiments. Semen qualities were not rapidly deteriorated during storage at a constant temperature of 4°C for 72 hr, but were rapidly deteriorated by the rise in temperature in the carrier box during storage for 72 hr. Thus, it was important to transport semen samples within 48 hr. However, it was suggested that a new carrier box should be developed to maintain a transport temperature below 12°C in order to allow transport for 48 hr or longer.

No significant difference was observed between the extenders containing glucose or fructose. As reported by Ponglowhapan *et al.* [15], comparison of the two extenders during storage at 5°C for 23 days demonstrated higher sperm motility with fructose than glucose, although there was no significant difference. As reported by Iguer-ouada *et al.* [9], an egg yolk Tris-glucose solution was more suitable for long-term storage than an egg yolk Tris-fructose solution, although there was no significant difference between these extenders until about 72 hr. Our results demonstrated that there was no significant difference between these extenders during short-term low-temperature storage for up to 72 hr above 4°C. Hence, semen qualities can be maintained with both EYT-FC and EYT-GC semen extenders during short-term transport at a low temperature.

In summary, canine semen qualities can be maintained for up to 48 hr when semen samples are extended with EYT-GC or EYT-FC and stored at a temperature in the range of 4–12°C.

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