**NOTE** *Theriogenology*

# IGF-I Improves Mitochondrial Membrane Potential during Hypothermic Storage of Canine Spermatozoa

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ABSTRACT. The aim of study was to evaluate the effects of insulin-like growth factor I (IGF-I) on canine sperm function during cooled and freeze-thaw storage. Extenders supplemented with different IGF-I concentrations (0, 100 and 200 *n*g/m*l*) were added to canine spermatozoa, and the sperm samples were stored at 4°C for 48 hr or freeze-thawed. Sperm motility, morphology, plasma-membrane integrity (PMI) and mitochondrial membrane potential (MMP) were evaluated. IGF-I had no effect on PMI or morphology during cooling and freeze-thawing. However, IGF-I alleviated the reduction in progressive motility and MMP caused by cooled storage and led to an improvement in MMP after freeze-thawing. In conclusion, IGF-I can be helpful to maintain progressive motility of canine spermatozoa during hypothermic storage via increased MMP.

KEY WORDS: canine spermatozoa, hypothermic storage, insulin-like growth factor I (IGF-I), mitochondrial membrane potential (MMP).

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Dogs are not only companions but also valuable animals capable of representing human disease, because of their pathological similarities with humans [9]. Thus, strategies for long-term storage of canine spermatozoa are promising for the future. Various additives have been used to preserve functional spermatozoa longer [5]. Growth factors are potential candidates to maintain sperm function as an energy source for spermatozoa. In particular, insulin-like growth factor I (IGF-I) improves the quality of mammalian spermatozoa [1, 5, 12, 14]. However, no study has investigated the effect of IGF-I on canine spermatozoa. Therefore, the goal of this study was to determine whether IGF-I plays a beneficial role in canine spermatozoa during hypothermic storage.

Twelve ejaculates were collected from six beagles, and spermatozoa were diluted with an extender (20% [v/v] egg yolk, 5% [v/v] glycerol and 0.5% [v/v] Equex STM paste in a Tris diluent) containing different IGF-1 concentrations (0, 100 or 200 *n*g/m*l*). The samples were cooled for 0, 12, 24, 36 and 48 hr or freeze-thawed using a standard cryopreservation protocol [10]. Progressive motility [11], morphology [7], plasma membrane integrity (PMI) [4] and mitochondrial membrane potential (MMP) [3] were evaluated. In addition, PMI and MMP of fresh spermatozoa were evaluated after subjecting the samples to different IGF-I concentrations. Sperm PMI and MMP were analyzed using a FACScalibur flow cytometer (Becton Dickinson, San José, CA, U.S.A.) and Cell Quest Pro software (Becton Dickinson) after 6-CFDA/propidium iodide (PI) and JC-1 staining, respectively. CFDA+/PI− and JC-1 aggregate-forming spermatozoa were considered to have intact plasma membranes and a high MMP, respectively.

Statistical analysis was performed using SPSS software (SPSS, Inc., Chicago, IL, U.S.A.). One-way repeated-measures analysis of variance or Friedman test was used according to the normality of the distribution. Statistical significance was set at $P<0.05$, and all data were presented as mean ± standard error.

IGF-I had no effect on PMI or MMP of fresh spermatozoa (Fig. 1). Progressive motility of spermatozoa decreased in the IGF-I-free condition at 48 hr of cooled storage ($P<0.05$) (Fig. 2A). However, progressive motility of spermatozoa under IGF-I treatment was not different following 48 hr of cooled storage (Fig. 2A), indicating that progressive motility of spermatozoa can be maintained by IGF-I for long periods of cooled storage. Moreover, IGF-I treatment resulted in increased progressive motility of cooled spermatozoa compared to 0 *n*g/m*l* IGF-I at 36 and 48 hr for 100 *n*g/m*l* IGF-I and at 36 hr for 200 *n*g/m*l* IGF-I ($P<0.05$) (Fig. 2A). Cooling for 48 hr did not affect sperm morphology or PMI, and IGF-I did not influence morphology or the PMI of cooled spermatozoa (Fig. 2B and 2C).

Sperm MMP decreased gradually beginning at 12 hr during cooled storage in the IGF-I-free condition ($P<0.05$) (Fig. 2D). However, MMP of sperm treated with 100 and 200 *n*g/m*l* IGF-I increased for 12 hr and 24 hr of cooled storage compared to that at 0 hr, respectively ($P<0.05$) and was maintained by 36 hr of cooled storage (Fig. 2D). Although sperm MMP decreased at 48 hr of cooled storage compared to that at 0 hr despite IGF-I treatment ($P<0.05$), the reduction in sperm MMP at 48 hr of cooled storage was mitigated

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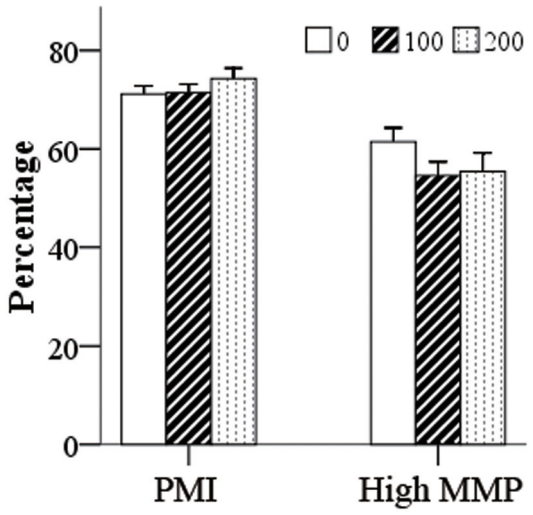


Fig. 1. Effect of insulin-like growth factor-I (IGF-I) on fresh spermatozoa. Fresh spermatozoa were treated with different IGF-I concentrations (0, 100 and 200 *n*g/m*l*), and plasma-membrane integrity (PMI) and mitochondrial membrane potential (MMP) of fresh samples were evaluated (n=12).

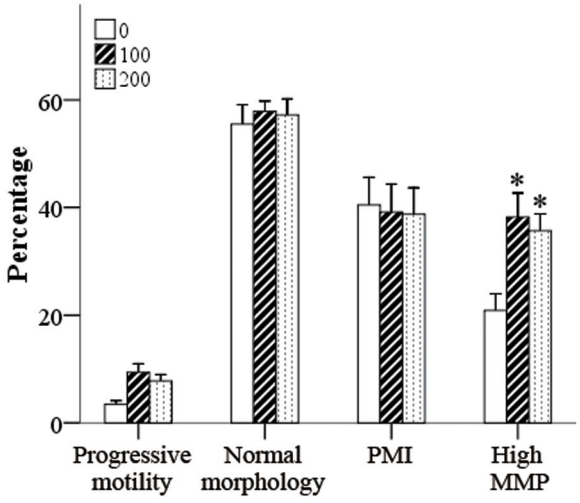


Fig. 3. Effect of IGF-I on freeze-thawed spermatozoa. Fresh spermatozoa were frozen after treated with different IGF-I concentrations, and the functions of thawed spermatozoa were evaluated. **P*<0.005 vs. 0 *n*g/m*l* IGF-I (n=5 for progressive motility and morphology, n=12 for PMI and MMP).

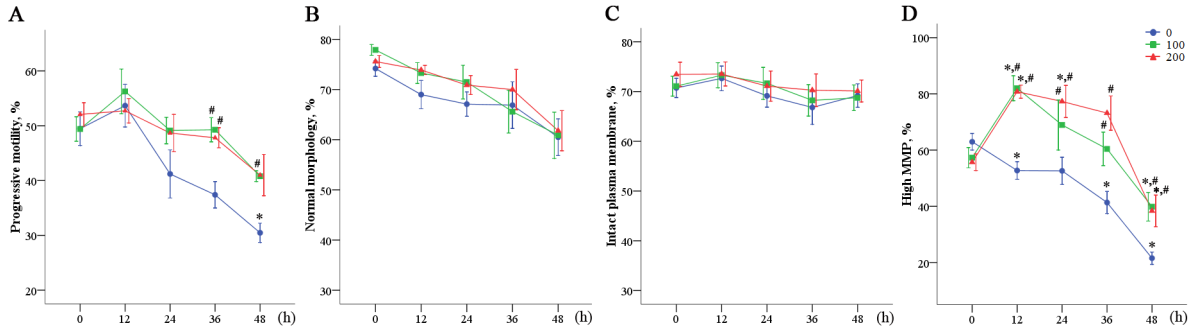


Fig. 2. Effect of IGF-I following cooled storage time. Spermatozoa were stored at 4°C for 48 hr after treated with different IGF-I concentrations (0, 100 and 200 *n*g/m*l*). Progressive motility (A), morphology (B), PMI (C) and MMP (D) of the cooled samples were evaluated (n=5, 5, 10 and 8 for each evaluation). **P*<0.05 vs. 0 hr within the same IGF-I concentration, $^{\#}P$<0.05 vs. 0 *n*g/m*l* IGF-I within the same storage time.

by IGF-I, showing increase in high MMP in IGF-I treated samples compared to that in IGF-I-free samples at 48 hr of cooled storage ($P$<0.05) (Fig. 2D).

Progressive motility, morphology and PMI were not different among freeze-thawed spermatozoa in different IGF-I concentrations, whereas the percentage of freeze-thawed spermatozoa with a high MMP increased following IGF-I treatment ($P$<0.005) (Fig. 3).

This study is the first to investigate the effect of IGF-I on canine sperm function during hypothermic storage. IGF-I alleviated sperm damage caused by cooling and freezing by maintaining progressive motility of spermatozoa and preventing a reduction in sperm MMP during hypothermic storage. The IGF-I receptor (IGF-IR) signaling pathway mediated by IGF-I may be involved in enhanced canine sperm function. Specific IGF-IRs have been demonstrated in human [8] and bovine [1] spermatozoa, suggesting a possible role of IGF-I as a regulator of sperm function [5]. As the presence of IGF-IR and IGF-I in sperm and semen and the ability of IGF-I to stimulate sperm motility have been identified [1], a relationship between the IGF system and fertilization has been suggested.

Although no reports have identified IGF-IR in canine spermatozoa, our study indirectly shows the presence of an IGF-IR in canine spermatozoa via the IGF-I effect. IGF-I stimulated MMP and motility of hypothermically stored canine spermatozoa in our study. The possible mechanism of how IGF-I maintains motility and MMP is assumed to be through energy metabolism [1], antioxidant effects [13] and high intracellular calcium level by increased ion transport

[2]. In contrast, activation of cellular metabolism by IGF-I may also be related to the generation of free radicals [6]. In our study, the 100 and 200 *n*g/m*l* IGF-I concentrations were optimal level and had a positive effect without toxicity to canine spermatozoa. We cannot clearly state the role of IGF-1 in membrane stability [5] of canine spermatozoa, because of less damage to canine sperm PMI during cooling. Overall, the IGF-IR signaling cascade may be a clue to identify molecular mechanisms regulating motility and membrane integrity of canine spermatozoa.

Our results suggest that IGF-I is an effective supplement to improve canine sperm quality for longer periods of cooling and freeze-thawing. IGF-I may enhance canine sperm fertilizing ability by maintaining motility and MMP and preventing a decrease in sperm longevity during hypothermic storage.

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