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# Effect of seminal plasma vesicular structures in canine frozen-thawed semen

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ABSTRACT

Membrane vesicles (MVs) in the ejaculate have been identified in various species and are considered to affect membrane fluidity due to their characteristic molecular composition. Addition of MV to human frozen semen has been shown to improve post-thaw motility. Similarly, a beneficial effect has been suggested for frozen equine semen. As post-thaw canine semen quality varies widely between dogs, the aim of our study was to test for the effect of addition of canine MV on post-thaw semen quality in dogs. Semen samples from 10 male dogs were purified from MV and prepared for freezing. In experiment 1, three groups were compared: sperm frozen (1) with MV (S1); (2) without MV, but MV added immediately after thawing (S2); and (3) without MV (C). Semen analysis included computer-assisted sperm analysis of motility parameters immediately after thawing (t0), after 10 (t10) and 30 minutes (t30), % living sperm, % membrane intact, % morphologically normal sperm (all t0 and t30). Computer-assisted sperm analysis motility distance and velocity parameters (all $P < 0.05$) and % living sperm ($P < 0.001$) were significantly affected by treatment with a temporary increase of distance and velocity parameters at t0 to t10, but a significant decrease of the aforementioned parameters at t30 in samples with MV. In experiment 2, different MV protein concentrations added after thawing were compared: 0.05 mg, 0.1 mg, and 0.2 mg/mL. Computer-assisted sperm motility analysis was performed at t0, t10, and t30. No differences between MV concentrations were identified, only a significant interaction between effect of treatment and time for progressive motility ($P < 0.01$). Our study identified a short-term beneficial effect of canine MV on post-thaw distance and velocity parameters, whereas at t30 progressive motility, motility parameters and % living sperm were reduced in samples with MV compared to C. The results point to species-specific differences regarding the MV effect on frozen semen and indicate the need for further studies using different semen and MV purification protocols and more frequent analyses. At the moment, addition of MV is not an option to improve post-thaw semen quality in dogs.

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## 1. Introduction

Although sperm are the major component of the ejaculate, the seminal plasma containing various amorphous proteins and membrane vesicles (MVs)—at least in man, stallion, rabbit, boar, bull, ram, dog, and laboratory rodents such as mouse, rat, and hamster—has also been focus of

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research. MV in seminal plasma may be derived from various organs within the male reproductive tract—with species-specific differences, but they predominantly originate from the prostate in man [1], stallion [2], and rabbit [3]. This is the reason why often the term “prostasomes” is used synonymously for these MVs. Membrane vesicles have been extensively investigated especially in humans (for reviews, see [4–6]), and meanwhile, they are known for many interesting properties. They can enhance progressive motility (PM) and induce hyperactivation [7], influence capacitation, and acrosome reaction [8,9]; they induce complement inhibitory activity and are immunosuppressive (thereby protecting sperm against immune-mediated recognition and/or destruction in the female genital tract) [10–12], antioxidative [13,14], and antibacterial [15]. Their molecular characteristics as e.g., the unusually high concentrations of cholesterol and sphingomyelin are responsible for some of the aforementioned properties [16–18]. It has been shown that prostasomes can fuse with sperm cells [18,19] resulting in a decreased fluidity of the sperm membrane [18]. As several membrane-bound systems, such as enzymes and receptors, depend on membrane fluidity for their activity, it is considered one of the most important parameters in membrane biology [18]. Changes in membrane fluidity have been shown to affect survival of ram spermatozoa during storage at 5 °C and motility [20], and it has been hypothesized that addition of MV might improve sperm survival after cryopreservation in the stallion [21], but not proven.

Semen freezing has become a popular tool in canine reproduction [22], and due to the fact that dogs and stallions, different to bulls, are not selected for their fertility and semen freezability but for their competition and/or show success, semen quality is often not optimum before freezing. Furthermore, as also “good” and “bad” freezers exist, post-thaw results are disappointing [22,23] in some dogs with acceptable semen quality before freezing. Various extenders and freezing protocols have been tested to overcome this problem, but the reason for different freezability is largely unknown. As addition of MV—recently identified and partly characterized in canine seminal plasma [24–26]—has been shown to affect human post-thaw motility beneficially and has been hypothesized to improve freezing results in stallions, the aim of our study was to investigate the effect of addition of canine MV on canine post-thaw semen quality in dogs.

## 2. Materials and methods

The study consisted of two different experiments: The aim of experiment 1 was to test for the influence of MV on canine frozen-thawed semen quality with a special focus on motility parameters. To study this, MVs were removed from the second fraction of ejaculates to obtain pure sperm. These sperm were frozen in straws. To some samples, MVs were added before freezing (S1), whereas the remaining samples were frozen without MV (S2 and control, C). Membrane vesicles were added to some of the straws frozen without MV (S2) to see whether addition of MV after freeze-thaw has an impact on semen quality. Only MVs from the same dog obtained from the third fraction of the same ejaculate were added to the semen sample (no foreign MV from other dogs). Due to the very limited yield of canine MV in this fraction and the variable protein concentrations, the concentration chosen for addition to samples (0.04-mg protein/mL) was relatively low compared to previous human studies, but could be obtained from all dogs. To test for the influence of MV concentration on semen quality, experiment 2 was performed. In this experiment, only pure sperm were frozen, and MVs were added after thawing to the frozen sperm comparing three protein concentrations (0.05-/0.1-/0.2-mg protein/mL) of MV.

### 2.1. Animals, semen collection, and evaluation

Ejaculates were collected by manual masturbation from 10 clinically healthy intact male dogs of seven breeds (Beagle, Bearded Collie, Rottweiler, German Shepherd, Welsh Corgi Pembroke: n = 1 each; Labrador Retriever: n = 2; Golden Retriever: n = 3) after sexual/mating abstinence for at least a week in the presence of a teaser bitch in

**Table 1**
Technical settings of the CASA system SpermVision for motility analysis.

| Parameter | Setting |
|---|---|
| Field-of-view depth = depth of sample chamber | 20 μm |
| Light adjustment | 80–110 |
| Total number of cells evaluated or number of fields | 4000 spermatozoa or 8 fields |
| Sperm recognition area | 20–60 $\mu m^2$ |
| Frame rate | 60 frames/s |
| Points assessed for sperm motility | 11 |
| Total motility | Progressive motility + local motility |
| Immotile sperm | AOC $< 9.5°$ |
| Local motility | DSL $< 6.0$ μm |
| Progressive motility | Every cell that is not “immotile” or “local motile” |
| Hyperactive sperm | VCL $> 118$ μm/s, ALH $> 6.5$ μm, and LIN $< 0.5$ |
| Linear sperm | STR $> 0.9$ and LIN $> 0.5$ |
| Nonlinear sperm | STR $\leq 0.9$ and LIN $\leq 0.5$ |
| Curvilinear sperm | DAP/radius $\geq 3$ and LIN $< 0.5$ |

Abbreviations: ALH, amplitude of lateral head displacement (μm); AOC, average orientation change; CASA, computer-assisted sperm analysis; DAP, distance average path (μm); DSL, distance straight line (μm); LIN, linearity (VSL/VCL, %); STR, straightness (VSL/VAP, %); VCL, velocity curved line (μm/s).

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