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Morphological characterisation of vesicular structures in the canine ejaculate☆

Goericke-Pesch S.[a,b,*,1], Hauck S.[a,1], Bergmann M.[c], Wehrend A.[a]

[a] Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Justus-Liebig-University Giessen, Frankfurter Str. 106, 35392 Giessen, Germany
[b] Department of Large Animal Sciences, Section for Veterinary Reproduction and Obstetrics, Faculty of Health and Medical Sciences, University of Copenhagen, Dyrlægevej 68, 1870 Frederiksberg C, Denmark
[c] Institute of Veterinary Anatomy, Histology and Embryology, Justus-Liebig-University Giessen, Frankfurter Str. 94, 35392 Giessen, Germany

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ABSTRACT

Membrane vesicles (MV) have been identified in seminal plasma from various species and they are thought to have a significant impact on semen quality and fertilisation. Although recently presence of MV has been also described in the canine ejaculate, detailed knowledge on their morphology is missing by now. This is, however, needed to provide a basis for detailed biochemical and functional studies as it is generally assumed that different MV populations are responsible for distinct tasks. MV were prepared for light (LM) and transmission electron microscopy (TEM) analysis using samples from normospermic dogs ($n = 15$), hypokinozoospermic dogs ($n = 2$, h) and one castrated azoospermic dog (a). For TEM, a new preparation protocol was used resulting in a higher MV retrieval rate. Using fractionated semen samples, most MV were identified in the second (sperm-rich) fraction in LM. Using pooled ejaculates, three different MV types could be identified in LM: (1) large MV with a marginal accumulation of opaque, granulated material, (2) medium- to small size MV with dense, opaque content and (3) small MV with no further defined contents. No direct contact between sperm and MV could be visualised. In TEM, 11 different MV types were identified based on diameter, structure, contents and electron density of contents as well as presence, number and size of smaller MV inside the MV itself. In normospermic males, secondary vesicles (type i, H, K1/2) included smaller vesicles and had a weighted mean diameter of 409.46 nm; hereof types i, H and K1 were smaller (mean: 287.55 nm, range: 51.25–994.86 nm) and type K2 was larger (mean: 1746.43 nm, range: 1003.66–3289.34 nm). Primary vesicles (mean diameter: 135.29 nm) – without vesicles inside – were differentiated into larger MV (A, B, C1/2) with a mean diameter of 219.63 nm (range: 39.08–1300.13 nm) and small primary MV (F, G) with a mean diameter of 66.12 nm (range: 24.62–99.84 nm). Whereas all mentioned MV were round to oval and mostly double-, rarely multiple-membrane surrounded, one longish primary MV type (L) was identified. In general, small primary vesicles were most common independent of semen quality, but distribution frequency of vesicle types differed between normospermic, pathospermic dogs and the castrated male. Mean weighted diameter of MV was 195.14 nm (range: 24.62–3289.34 nm) in normospermic males with the maximum diameter being smaller in the other dogs (h: 2096.78 nm; a: 1314.06 nm). Our results provide new information about ultrastructure and distribution frequency of canine MV in normospermic males and point to possible differences in MVs depending on semen quality. They provide the basis for further detailed functional analysis of MV subpopulations. Furthermore, the presence of MV in the castrated azoospermic male confirms an at least partly prostatic origin of canine MV.

☆ Address where the work was conducted: Clinic for Obstetrics, Gynecology and Andrology of Large and Small Animals, Justus-Liebig-University Giessen, Frankfurter Str. 106, 35392 Giessen, Germany.

* Corresponding author. Present address: Department of Large Animal Sciences, Section for Veterinary Reproduction and Obstetrics, Dyrlægevej 68, 1870 Frederiksberg C, Denmark. Tel.: +45 35 33 29 85.
E-mail addresses: sgp@sund.ku.dk (S. Goericke-Pesch), sabine.hauck@gmail.com (S. Hauck), Martin.Bergmann@vetmed.uni-giessen.de (M. Bergmann), Axel.Wehrend@vetmed.uni-giessen.de (A. Wehrend).

[1] Both authors contributed equally.

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1. Introduction

Membrane vesicles (MV) have been identified in the seminal plasma from various species, like man, stallion, rabbit, boar, bull, ram, but also laboratory rodents like mouse, rat and hamster; and extensive research has been performed about their function. Although Frenette et al. (1985, 1986) already mentioned secretory granules from the prostate with a similar arginine esterase activity as measured in the seminal plasma, to the best of our knowledge no research has been performed about MV in canine semen for 28 years. While our experiments were running, presence and some ultrastructural (Zelli et al., 2013) and enzymatic properties (Ronquist et al., 2013; Zelli et al., 2013) of these MV in canine seminal plasma had been described for the first time.

In general, MV in seminal plasma may originate from various organs within the reproductive tract – with species-specific differences: In man (Carlsson et al., 2003), stallion (Minelli et al., 1999) and rabbit (Castellini et al., 2012), the prostate is considered the major source of small vesicles. Different to these species, the origin of porcine MV is not clear, ram MV seem to derive mainly or totally from the epididymis, "epididymosomes", (Gatti et al., 2005) and bovine MV obviously seem not to derive from the prostate, but from the vesicular gland, "vesiculosomes", (Agrawal and Vanha-Perttula, 1987) and from the epididymis, "epididymosomes" (Frenette and Sullivan, 2001).

MV play an important role in reproductive processes and various different functions are proposed or even meanwhile proven – mainly based on research about human MV (for reviews see Ronquist, 2012; Sullivan and Saez, 2013; Aalberts et al., 2014). They are known to have a positive impact on sperm progressive motility (Stegmayr and Ronquist, 1982a,b; Fabiani et al., 1994b; Carlsson et al., 1997; Arienti et al., 1999) and hyperactivation (Fabiani et al., 1994a), and to influence capacitation and acrosome reaction (Palmerini et al., 2003; Pons-Rejraji et al., 2011). Furthermore, they have an immunosuppressive and complement inhibitory activity protecting the spermatozoa in the potentially hostile environment of the female genital tract (Kelly et al., 1991; Kelly, 1995); they protect spermatozoa like antioxidants against reactive oxygen species (Saez et al., 1998, 2000; Goyal et al., 2006) and have antibacterial properties (Carlsson et al., 2000). Although it seems possible that a single type of prostasome is able to function in this large variety of unrelated processes (Aalberts et al., 2012), several authors consider it more likely that distinct MV populations carry out different tasks (Poliakov et al., 2009; Aalberts et al., 2012; Zelli et al., 2013) indicating the need for detailed morphological and functional description of the MV subpopulations. In dogs, description is by now restricted to "dark" and "light" MV (Zelli et al., 2013). Consequently, we aimed to provide this knowledge about the ultrastructure of canine MV using light and transmission electron microscopy (TEM). Our detailed characterisation can provide the basis for follow-up studies on functional analysis of the described prostasome-subpopulations and their origin. Furthermore, due the impression obtained by clinical work that only semen samples from fertile dogs contain MV, we also included ejaculates from two dogs with known subfertility due to semen deviations from reference values and one castrated male dog that was available for ejaculate collection.

2. Materials and methods

2.1. Animals and semen collection

Ejaculates were collected by manual masturbation from 17 clinically healthy intact male dogs of 11 breeds after sexual/mating abstinence for at least a week in the presence of a teaser bitch in estrus as previously described (Pesch et al., 2007; Goericke-Pesch et al., 2012; Goericke-Pesch and Failing, 2013). Additionally, a sample was collected in the same way from a clinically healthy male dog, castrated more than 2 years ago. Mean age of all 18 dogs was 4.8 ± 2.4 years (range 1.25–11 years) and mean body weight was 30.3 ± 20.3 kg (range: 8.5–80 kg). The three different fractions were collected separately into pre-warmed sterile glasses. Additionally, two healthy intact male Beagle dogs were collected repeatedly on following days for the pre-trials.

2.2. Semen analysis

All three fractions were light microscopically checked for presence of spermatozoa and – if present – for their motility. Additionally, presence of other cells (erythrocytes, leucocytes, round cells, MV) was checked and semi quantitatively recorded (range: – to +++). In randomly selected animals, images from MV in different fractions were taken (Leitz DMRM with integrated digital camera Leica DC300, Leica Microsystems, Wetzlar, Germany) and individual largest and smallest MV measured using a measuring tool (Leica Image Manager IM1000, version 1.20, Leica Microsystems, Wetzlar, Germany). A detailed examination of the second, sperm-rich fraction was performed immediately after collection as previously described (Pesch et al., 2007; Goericke-Pesch and Failing, 2013). It included volume, pH using pH indicator paper (Merck, Darmstadt, Germany), estimation of the percentage of spermatozoa with progressive motility (37 °C, phase contrast microscope, 400-fold magnification), evaluation of the percentage of unstained (membrane intact, live) and stained (membrane defect, dead) spermatozoa and morphologically normal and abnormal spermatozoa using eosin-stained sperm smears (evaluation of 200 spermatozoa each, bright-field, 400-fold magnification). In the following text, unstained spermatozoa in the eosin smear – considered as spermatozoa with intact plasma membrane – were referred to as living sperm. Sperm concentration was assessed using a Neubauer haemocytometer counting chamber to calculate the total sperm count (TSC) according to the equation: TSC = volume (second fraction in ml) × sperm concentration/ml.

2.3. Preparation of vesicles

Only one sample from each animal was used for electron microscopic examination. Following a brief light-microscopical check of all fractions for vesicles (400-fold magnification, phase contrast, range: – to +++) and detailed semen analysis of the second fraction (see Section 2.2), all fractions were pooled for further preparation of vesicles. As the published protocols were found to be not suitable for preparation of pure vesicles, three methods were compared in respect to their suitability for transmission electron microscopic (TEM) evaluation in a pre-trial: (1) 1.5 ml of pooled ejaculate, one centrifugation at 13,000 × *g* (Biofuge fresco, Heraeus, Kendro Laboratory Products GmbH, Hanau, Germany) for 10 min; (2) 1.5 ml pooled ejaculate, centrifugation at 100 × *g* (Rotina 38, Rotor 1794, Andreas Hettich GmbH & Co., KG, Tuttlingen, Germany) for 10 min, repetition of the centrifugation with the supernatant at 100 × *g* for 10 min, ultracentrifugation for 1 h at 4 °C and 100,000 × *g* (Optima L-70, Rotor TI-50, Beckman Coulter GmbH, Krefeld, Germany). Method 3 was most time-consuming and work-intensive: 1.5 ml of pooled ejaculate was centrifuged for 10 min at 100 × *g* (Rotina 38, Rotor 1794 – Andreas Hettich GmbH & Co., KG, Tuttlingen, Germany); the supernatant was pipetted into a new tube and centrifuged for another 5 min at 200 × *g*. The resulting supernatant was centrifuged for 15 min 30 s at 500 × *g* followed by an ultracentrifugation for 1 h at 4 °C and 100,000 × *g* (Optima-L 70, Rotor TI-50, Beckman Coulter GmbH, Krefeld, Germany). The pellet was resuspended in 250 μl TRIS-NaCl-Puffer (30 mM TRIS, 130 mM NaCl, pH