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Equilibration in freezing extender alters *in vitro* sperm–oviduct binding in the domestic cat (*Felis catus*)

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ABSTRACT

For the preservation of endangered felid species, epididymal sperm may be received from valuable individuals after castration or death and they need to be cryopreserved for storage. However, pregnancy rates with epididymal or cryopreserved sperm are lower than with ejaculated and non-frozen semen even if insemination is surgically performed into the oviduct.

To investigate whether equilibration, the first step of the cryopreservation procedure, has an impact on sperm-oviduct binding, we generated oviduct epithelial cell vesicles from isthmus segments of pre-ovulatory domestic cats. Binding assays were performed with epididymal sperm in a cell culture medium (M199) without supplements, or after cooling to 15 °C in a freezing extender (TestG), supplemented with glycerol and the water-soluble fraction of hen's egg yolk mainly comprising LDL. The sperm-oviduct binding was assessed both quantitatively and qualitatively (head or tail binding of sperm with active or inactive mitochondria).

Most of the bound sperm prepared in M199 had active mitochondria and were attached to the vesicles by their heads. In equilibrated samples, the proportion of bound sperm with active mitochondria and the proportion of head-bound spermatozoa were reduced. The total motility of the sperm after 1 h of incubation in the absence or presence of vesicles were also affected by the preparation (higher in equilibrated) and the incubation (lower in co-incubated), while mitochondrial activity was influenced just by the preparation. Obviously, LDL has a beneficial effect on sperm motility, but we suggest that it interferes with the molecular sperm-oviduct crosstalk and causes a reduced binding of "good" sperm.

1. Introduction

During natural mating, felid sperm are deposited in the anterior part of the vagina and must overcome the natural barriers such as vagina, cervix, uterus and the utero-tubal junction to reach the oviduct [1,2]. The oviduct in cats like in many mammals acts as a sperm reservoir [3] and the place of both fertilization and early embryo development. Most felids are induced ovulators [4] and sperm need to be stored prior to ovulation which occurs 25–32 h after mating [5]. In addition to storage, sperm need to become conditioned in the oviduct for fertilization. This process of capacitation is well described in some domestic mammalian species and happens upon sperm interaction with oviduct epithelial cells [6].

25 of the 38 wild felid species are classified as threatened or near threatened on the IUCN Red List of Threatened Species [7]. Therefore, the use of assisted reproductive techniques (ARTs) is of increasing importance to preserve the species and their genetic diversity. Cryopreservation of semen and artificial insemination are currently the most promising ARTs and enable long-term storage and distribution of male gametes. In many endangered felids, only epididymal sperm are available from valuable individuals collected after castration or death and these are mostly cryopreserved and stored in wildlife cryobanks [8]. However, in felid species, artificial insemination with cryopreserved semen or epididymal sperm requires surgical techniques to deposit sperm deep in the female genital tract [9–11]. However, even with these techniques, pregnancy rates are much lower with frozen/thawed semen or sperm than those with fresh semen or sperm after laparoscopic insemination into uterus or oviduct [12].

Several reasons may account for this deficiency. The lower quality and longevity of cryopreserved sperm are probably the

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main factor which hampers successful sperm transit, particularly in species where the natural semen deposition occurs in the vagina and penetration of the cervix by insemination instruments is difficult, as for example in domestic dog and sheep [13,14]. Besides the loss of viability upon cryopreservation, further modifications of sperm may interfere with their passage through the female genital tract. Before freezing, sperm are commonly exposed to extender media which contain cryoprotectants, lipids and proteins. The latter, which are derived from egg yolk, milk or plant supplements, adhere to the sperm surface [15,16] and may disturb the natural communication of male gametes with the components of the female genital tract even if a standard sperm quality criterion such as motility seems promising.

Considering the low success rate of frozen-thawed sperm compared to non-frozen felid sperm, even after surgical deposition into the oviduct, we assume that functional sperm binding to the oviduct is altered in cryopreserved sperm when compared with the performance of non-frozen gametes. In the current study, we examined whether the equilibration of epididymal sperm in a freezing extender, the first step of a common freezing procedure, has an impact on *in vitro* sperm-oviduct binding in the domestic cat, which serves as a model for wild felids. For this purpose, epididymal sperm were diluted in extender TestG and subjected to slow cooling over 40 min to 15 °C. To mimic the situation in the oviduct, viable explant vesicles were generated during short term culture for 16 h from isthmus oviduct epithelial cells of preovulatory female cats (FOECi). Those FOECi-vesicles, also sometimes referred to as explants in other studies, were successfully applied in sperm binding assays for several species [17]. Comparing quantity and quality of bound populations of equilibrated and non-equilibrated epididymal sperm we tested the hypothesis that the contact of sperm to the freezing extender during equilibration might change sperm-oviduct binding patterns.

2. Material and methods

2.1. Reagents and media

The cell culture medium DMEM/Ham's F12 (1:1, FG 415), FBS (S 0115, Lot 1030B) and amphotericin B were obtained from Merck Millipore (Darmstadt, Germany), other chemicals, media and supplements were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless otherwise stated.

Media used in culture procedures were modifications of previously described media for mouse tracheal cell culture [18] as well as for air-liquid interface culture of mouse and swine oviduct epithelial cells [19]. The basic medium (FOECbasic) for culture of feline oviduct epithelial cells (FOEC) consisted of DMEM/Ham's F12 supplemented with 15 mmol/lHEPES (H0887), 100 U/ml penicillin/100 μg/ml streptomycin (P4333) and 0.25 μg/ml amphotericin B (171375, Merck, Darmstadt, Germany). For the proliferation medium (FOECprol), FOECbasic was supplemented with 5 μg/ml insulin (I6634), 5 μg/ml transferrin (T8158), 0.025 μg/ml cholera toxin (C8052), 5 ng/ml epidermal growth factor (E4127), 30 μg/ml bovine pituitary extract (P1476), 0.05 μM retinoic acid (R2625) and 5% (v/v) FBS.

The transport medium for ovary-oviduct complexes was HEPES-MEM (M7278) supplemented with 1% (v/v) Antibiotic Antimycotic Solution (A 5955) and 0.3% (w/v) BSA (126579). The freezing extender TestG consisting of buffer (210.7 mol/l N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonicacid (9090.3, Carl Roth, Karlsruhe, Germany), 95.75 mmol/l tris(hydroxymethyl)-aminomethane) (T5691) and 11.1 mmol/l fructose (F0127) (supplemented with 15% (v/v) of the water-soluble fraction of hen's egg yolk (containing low-density lipoproteins (LDL)) and with 7% (v/v) glycerol (A3092, AmpliChem, Darmstadt, Germany) [20].

2.2. Animals

Tissue samples from nine male and nine female domestic cats (*Felis catus*) were obtained from the rescue shelter or veterinary clinics in Berlin, Germany, after regular gonadectomy. The experimental protocols were approved by the Ethics Committee of the Leibniz Institute for Zoo and Wildlife Research (2013-05-05).

2.3. Experimental design

We investigated whether the contact of feline sperm to the freezing extender during equilibration, the first phase of a common cryopreservation procedure, affects sperm-oviduct binding. The detailed time schedule is shown in Fig. 1.

2.4. Preparation of feline isthmus oviduct epithelial cell (FOECi) vesicles

The protocols of isolation of FOECi vesicles and sperm binding test were adapted according to former studies [21–24]. Ovaries with oviducts were transported to the laboratory in transport medium at 4 °C and were processed within 5 h. Only oviducts from preovulatory cats (follicles with more than 2 mm in diameter on ovaries) were used. After removal of ovary and surrounding vessels the isolated oviducts were washed in PBS (D8537) supplemented with 100 U/ml/100 μg/ml penicillin/streptomycin, 0.05 mg/ml gentamicin (G1272) and 0.25 μg/ml amphotericin B. The oviducts were injected with 1 mg/ml collagenase in PBS (D8537) and incubated for 30 min at 38 °C. Epithelial cells from isthmus were squeezed out with the outer edge of a scissor onto a glass slide and flushed into 600 μl FOECbasic in a 4-well dish and cultured for 16 h at 38.5 °C, 5% CO_2.

During this period, explanted cell associations form three-dimensional vesicles (Fig. 2). After culture, FOECi-vesicles mainly consisting of viable cells were separated from vesicles containing many dead cells as follows. The FOECi-vesicle suspension was pipetted up and down (~50 times). Viable cells are firmly connected and form stable FOECi-vesicles while vesicles containing dead cells collapse and disaggregate by mechanical shear stress. Vesicles consisting of viable cells sink fast to the bottom. The supernatant containing the disaggregated vesicles and dead single cells was removed and PBS was added to the pellet to repeat the separation procedure two times. To quantify the amount of FOECi-vesicles, vesicles were transferred to a 250 μl capillary which was sealed with 2% agarose. Within 30 min of vertical incubation (38.5 °C, 5% CO_2), the vesicles sediment to the bottom. One mm pellet height in the capillary with a diameter of 1.6 mm equals a vesicle volume of 2 mm^3. Pellet height was measured and FOECi-vesicles were flushed out by applying 400 μl FOECprol per 2 mm^3 vesicles. To visualize remaining dead cells in isolated FOECi-vesicles during the experiment, samples were stained with propidium iodide (PI, Invitrogen, Molecular Probes, Eugene, OR, USA) to a final concentration of 0.00187 mmol/l. To remove the excessive dye, the supernatant was removed and the sample was rinsed three times with 500 μl FOECprol. For the sperm binding assay, 2 mm^3 FOECi-vesicles were finally re-suspended in 400 μl FOECprol in 4-well dishes.

2.5. Preparation of sperm

After castration, testes and epididymides were transported without medium to the laboratory and stored for ~24 h at 4 °C until usage. Sperm were collected as previously described [20]. Briefly,

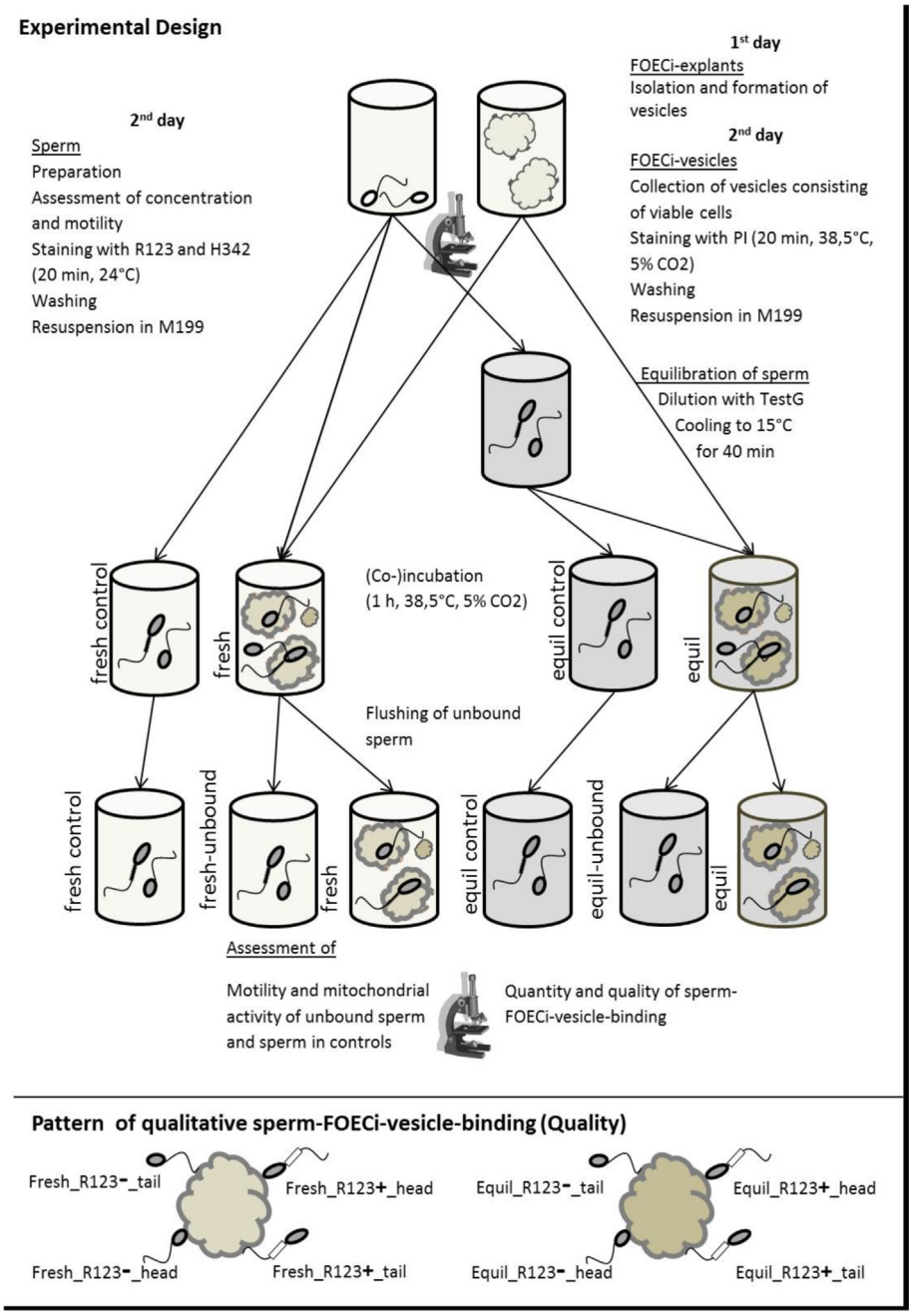

Fig. 1. Experimental design. Vesicles formed from feline isthmus oviduct epithelial cell (FOECi) explants during overnight culture were used for sperm binding assays with fresh and equilibrated sperm prepared from epididymides of domestic cats. Control sperm (without co-incubation) and unbound sperm from co-incubated samples were analyzed for motility and mitochondrial activity. The number and mitochondrial activity of bound sperm and the type of binding (by head or tail) was also determined. Before co-incubation, vesicles consisting of viable cells were selected and stained by PI, sperm were stained by R123 and H342. Equilibration was performed in a freezing extender mainly supplemented by the water soluble fraction from hen's egg yolk and glycerol.

each cauda epididymis and the proximal ductus deferens were sliced in 1 ml culture medium M199 (M7528, HEPES modification) at room temperature. Sperm were flushed through a 30 μm filter (Partec, Goerlitz, Germany) to remove remaining tissues. Sperm concentration was determined in a counting chamber and motility was measured by CASA (see below). Two aliquots with 8×10^6 motile sperm each were centrifuged at 800 X g for 5 min. Supernatants were removed and sperm were kept in the soft pellets for 30 min at room temperature while FOECi-vesicles were isolated. To ensure that each sample is processed under the same conditions, pellets were re-suspended consecutively. After a sperm aliquot was re-suspended with 100 μl M199, sperm were stained with Rhodamine 123 (R123, Invitrogen, Molecular Probes, Eugene, OR, USA) and Hoechst 33342 (H342, Cfm Oskar Tropitzsch GmbH, Marktredwitz, Deutschland) to a final concentration of 0.021 mmol/l and 0.285 mmol/l, respectively. After an incubation of 20 min at room temperature (24 °C) the stained sperm suspension was centrifuged at 800 X g for 5 min. Supernatants were removed and pellets re-suspended in either 300 μl or 100 μl M199 to obtain a concentration of ~27×10^6 or ~80×10^6 motile sperm per ml in the fresh subsample and the subsample designated to equilibration, respectively. About 1×10^6 motile sperm of the fresh sample were

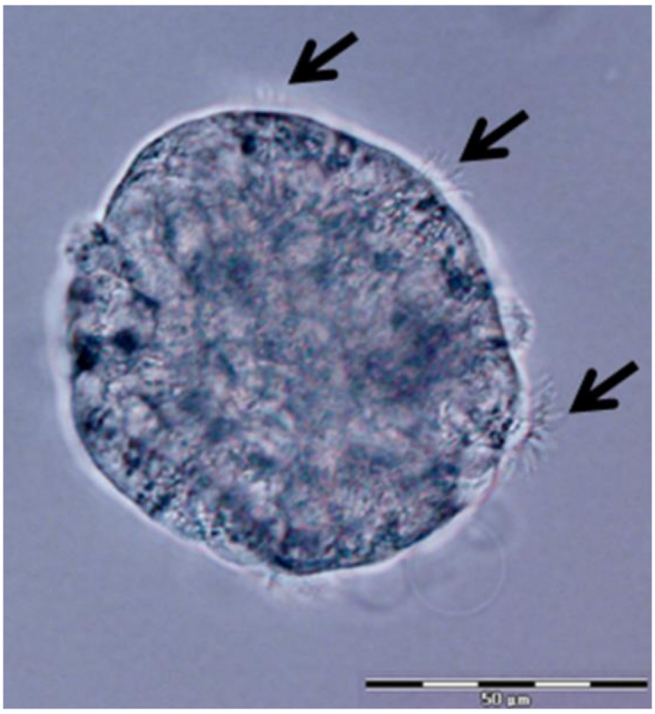

Fig. 2. FOECi-vesicle in transmitted light with cilia (arrows) orientated to the outer vesicle side. Bar represents 50 µm.

co-incubated with 2 mm^3 of vesicles or incubated without vesicles. For equilibration, the respective sperm subsample was diluted with TestG 1:3 (v/v) to obtain a final sperm concentration of ~27×10^6 motile sperm/ml, and transferred to a 2 ml cryovial. According to our protocol which was successfully developed to shorten the equilibration period [20] the cryovial was placed in a foam floater that was positioned in a double water bath, tempered at 21 °C. For equilibration, the water bath was placed in a fridge (4 °C) for 40 min to reach a temperature of 15 °C. Subsequently, the equilibrated sperm subsample was rewarmed to room temperature and 1×10^6 sperm were co-incubated in the presence of 2 mm^3 of vesicles or absence of FOECi-vesicles in 4-well dishes. All variants were incubated for 1 h (38.5 °C, 5% CO_2).

2.6. Evaluation of sperm-oviduct binding and quality of unbound sperm

After co-incubation of sperm and FOECi-vesicles, the vesicles were thoroughly flushed with 500 µl FOECprol through a 30 µm cell strainer (Partec, Goerlitz, Germany). Unbound sperm were collected in a 1.5 ml tube to assess motility and mitochondrial activity (see below). FOECi-vesicles were flushed back from the strainer and 50 µl vesicle suspension was placed between two coverslips for examination. An inverted wide-field fluorescence microscope (Olympus IX81, Hamburg, Germany) equipped with a triple band filter set suited for H342, R123 and PI (excitation 455 nm) and a color camera DP72 (Olympus, Hamburg, Germany) was used to record pictures. Nine randomly chosen vesicle areas were photographed one to nine times at different z-levels in fluorescence and transmitted light mode (xcellence rt, Olympus). PI was used as marker for dead oviduct cells in FOECi-vesicles. Vesicles containing dead oviduct cells after co-incubation were excluded from the assessment. R123 was used as marker for active mitochondria in sperm. H342 was applied to detect all sperm, in particular those which did not accumulate R123. In some cases, not

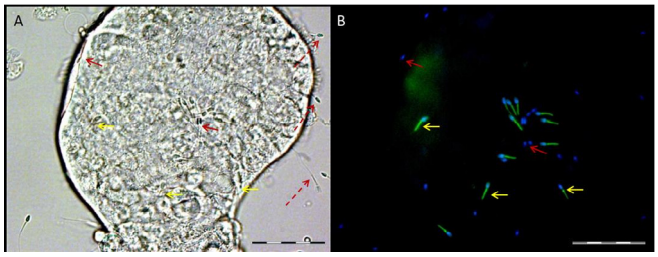

Fig. 3. Sperm binding to FOECi-vesicles *in vitro*. Fresh sperm with active (R123+, H342+) and inactive (R123-, H342+) mitochondria attached to the vesicles (yellow and red arrows, respectively) are shown in transmitted (A) and fluorescence light mode (B). Tail-bound sperm marked by the dashed line arrows (A), not all are visible in the z-level of the presented fluorescence picture (B). An assessment of the binding type for sperm on the surface of the vesicle is performed by focusing through the different z-levels. Bar represents 50 µm.

only sperm but also oviduct cells incorporated R123 (visible in Fig. 3) or H342 because there are still dye molecules remaining in sperm suspensions after washing. Similarly, also the co-incubated dead sperm may incorporate PI which stems from staining of FOECi-vesicles.

Pictures recorded in transmitted light mode were used to measure the vesicle area. Fluorescence pictures were applied to determine the sperm binding. Binding pattern was distinguished according to active (R123+) or inactive (R123-) sperm mitochondria as well as to the type of sperm attachment by head or by tail. Sperm cells stained by R123 (R123+) were considered to be viable. The latter was possible to assess in most cases if sperm were observed in different z-levels. Considering the fresh (fresh) or equilibrated (equil) state of sperm preparation, each sperm was assigned to one of the occurring four sperm classes shown in Fig. 1.

In the fraction of unbound or not co-incubated (control) sperm, motility was analyzed in a Makler chamber (Sefi-Medical Instruments, Haifa, Israel) using a CASA system (AndroVision, Minitube, Tiefenbach, Germany) with a phase-contrast microscope (Olympus CX41) equipped with a 20 X objective and a 0.5 X camera adapter. Videos were recoded for 0.5 s at a frame rate of 100/s. To analyze sperm mitochondrial activity 10 µl of sperm solution were pipetted on a slide and pictures were recorded using the AndroVision-software. According to the fluorescence signals, the percentage of sperm with active mitochondria (R123+, H342+) was determined.

2.7. Statistical analysis

All statistical analyses were performed with R version 3.5.3. All statistical models were fitted using the package lme4 [25]. We tested the effect of preparation (fresh or equilibrated sperm) on four variables: the proportion of viable (characterized by active mitochondria (R123+)), bound spermthe proportion of head bound sperm, the proportion of motile sperm, and the proportion of sperm with active mitochondria (R123+). Proportions of viable bound sperm and head bound sperm were calculated with respect to all observed bound sperm. All four variables were analyzed with generalized linear mixed-effects models with a binomial family (logit link), with preparation (fresh or equilibrated) included as an independent variable in both models.

Analysis of the proportion of motile sperm and the proportion of sperm with active mitochondria (R123+) included an independent variable to account for incubation procedure (control or co-incubated). To analyze the proportion of viable bound sperm, we included binding type (head or tail) as an additional independent variable in the model. To analyze the proportion of head bound

sperm, we included sperm viability of bound sperm (viable (R123+) or non-viable (R123-)) as additional independent variable in the model.

Multiple observations were taken from the same experimental replication (cells from the same male and female), which leads to a lack of independence. Experimental ID was included as a random intercept term in all models to account for this problem. The random intercept term is assumed to be drawn from a normal distribution with mean 0 and variance σ^2. The experiment was repeated nine times.

3. Results

The influence of equilibration in TestG freezing extender on binding of epididymal cat sperm to FOECi-vesicles was studied. Four sperm classes were distinguished according to sperm mitochondrial activity and type of sperm binding. In Fig. 4, proportions of fresh and equilibrated sperm classified into the different binding categories are shown in relation to the respective total number of sperm attached to FOECi-vesicles. The highest proportions of attached sperm were recorded for fresh and head-bound sperm with active mitochondria. In both, fresh and equilibrated samples, tail-bound sperm with active mitochondria were the least represented.

In Table 1 the results from the generalized linear mixed-effects models are summarized. Sperm preparation as well as the binding type has a significant effect on binding of sperm with active mitochondria to FOECi-vesicles. There was a higher proportion of viable bound sperm (with active mitochondria) attached to FOECi-vesicles in fresh trials than equilibrated trials. There was also a higher proportion of viable bound sperm among sperm that bound by the head than those that bound by the tail.

Sperm preparation also has an effect on the proportion of head-bound sperm on FOECi-vesicles. There was a higher proportion of head bound sperm in fresh than equilibrated trials. As expected from model 1, there was a relationship between bound sperm viability and binding type, such that viable sperm were more likely to bind by the head.

Sperm preparation as well as incubation to FOECi-vesicles has an effect on sperm motility (see Fig. 5A). It was more likely, that the total motility was higher in equilibrated than in fresh trials. There was also a higher proportion of motile sperm when sperm were incubated in absence of FOECi-vesicles (control). Sperm preparation has an effect on mitochondrial activity of sperm (see Fig. 5B). There was a higher proportion of sperm with active mitochondria in equilibrated than in fresh trials. The co-incubation had no influence on the proportion of sperm with active mitochondria.

4. Discussion

For the investigation of sperm-oviduct binding *in vitro*, either short-term fresh oviduct cell explants or long-term primary cell culture systems have been used in several species [23,26–28]. In our previous study, we used explants of cat oviduct epithelial cells immediately after isolation to test the influence of seminal fluid on feline sperm binding to oviduct [24]. To extend the time period for the usage of oviduct explants in the present study, fresh explants had been incubated until they formed vesicles where both, ciliated and secretory cells are maintained. Whereas organoids of human fallopian tube cells turn the apical side of cells to the organoid lumen [29], explants from bovine oviducts form vesicles with the apical side of cells directed to the outer vesicle side [26,30]. Similar FOECi-vesicles were generated from the domestic cat oviduct explants in our short-term culture system (Fig. 2). This has already been observed by Lengwinat and Blottner (1994) who used FOEC-vesicles in co-culture experiments to improve the outcome of IVF [21]. However, several epithelial cells die during cell isolation and vesicle formation. Lengwinat and Blottner (1994) observed only

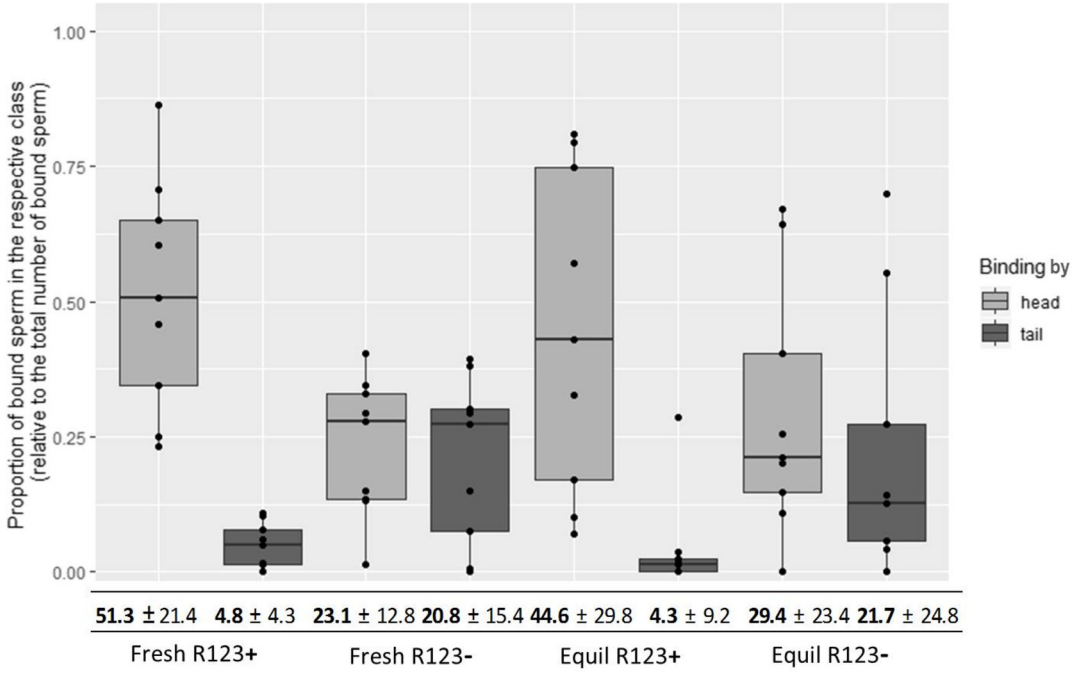

Fig. 4. Binding pattern of fresh (Fresh) and equilibrated (Equil) sperm in relation to the respective total number of sperm attached to FOECi-vesicles (N = 9) after 1 h of co-incubation at 38.5 °C under 5% CO_2. Sperm classes were distinguished according to active (R123+) or inactive (R123-) sperm mitochondria and sperm binding by head (head) or by tail (tail). Data are presented by vertical box plots ((R 3.5.3). Medians, the 25 and 75th percentiles are shown as vertical boxes and whiskers, outliers are visualized by circles. The numbers below give the mean ± SD of sperm in the respective binding class.

Table 1
Main effects between 1. preparation (Fresh and Equil), binding (head and tail) on proportion of bound R123 + sperm/all bound sperm, 2. preparation (Fresh and Equil), mitochondrial activity (R123 + and R123-) on proportion of head bound sperm/all bound sperm, 3&4 preparation (Fresh and Equil), incubation (co-incubated and control) on total motility & on percentage of R123 + sperm in a generalized linear mixed-effects models (glmer) in the lme4 package, R 3.5. (N = 9).

	Reference level	Estimate	Std. Error	z value	*P* value	
1. Proportion of R123 + sperm/all bound sperm ~ Preparation + Binding						
(Intercept)		0.338	0.347	0.976	0.329	
Preparation	Equil	0.389	0.137	2.849	0.004	**
Binding	head	−2.334	0.186	−12.552	<2e-16	***
2. Proportion of head bound/all bound sperm ~ Preparation + Mitoch. activity						
(Intercept)		0.958	0.428	2.237	0.025	*
Preparation	Equil	−0.537	0.153	−3.499	4.68e-4	***
Mitoch. activity	R123-	2.322	0.184	12.63	<2e-16	***
3. Proportion of motile sperm/all flushed sperm ~ Preparation + Incubation						
(Intercept)		−0.958	0.129	−7.404	1.32E-13	***
Preparation	Equil	−0.223	0.033	−6.821	9.05E-12	***
Incubation	Co-incub	0.234	0.033	7.207	5.73E-13	***
4. Proportion of R123 + sperm/all flushed sperm ~ Preparation + Incubation						
(Intercept)		−0.544	0.183	−2.968	0.003	**
Preparation	Equil	−0.198	0.047	−4.211	2.54E-05	***
Incubation	Co-incub	0.017	0.047	0.363	0.717	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

about 16% of viable cells after 48 h of cultivation [21]. Since many sperm of all categories and samples strongly bind to dead epithelial cells (not shown) it was necessary to select vesicles consisting of viable cells for the presented binding experiments.

Artificial insemination significantly differs from natural mating, especially when frozen/thawed epididymal sperm are surgically deposited into the oviduct where mammalian sperm are stored and primed for fertilization [12]. Fresh and cryopreserved feline epididymal sperm are able to perform *in vitro* fertilization [8,21] and the basic ability of fresh epididymal feline sperm to bind to the oviduct has been proven by an *in vitro* binding assay [24]. Even acceptable conception rates could be achieved if cryopreserved epididymal semen had been inseminated into the oviduct [31]. However, compared to fresh semen the fertilization competence is diminished after sperm cryopreservation [12,32], and it is unknown whether this is caused by the freezing/thawing or already due to the equilibration, which comprises cooling of sperm to 15 °C in our protocol and the dilution of sperm in an artificial medium.

Sperm from different species differ in their sensitivity to cooling. While boar sperm undergo membrane injuries already at 15 °C [33,34], cat sperm are more resistant to cold shock [35]. Cooling predominantly causes capacitation-like changes [36], which can be prevented by supplementation of egg yolk to the medium [37,38]. Several studies failed to find changes in membrane and/or acrosome integrity after cooling to 4 °C when cat sperm were stored in egg yolk extender [35,39,40]. Toyonaga et al. (2011) also showed

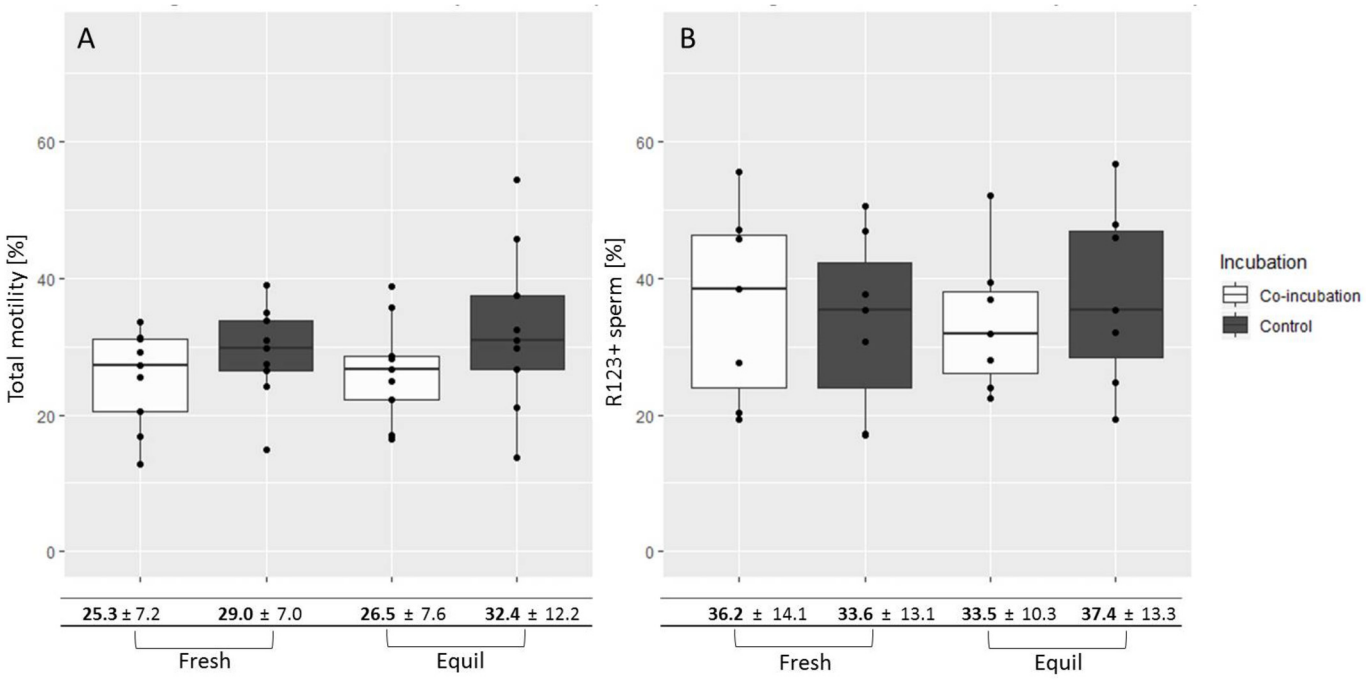

Fig. 5. Total motility (A) (N = 9) and percentage of sperm with active mitochondria (R123+) (B) (N = 7) of control sperm and unbound sperm flushed after 1 h of co-incubation with FOECi-vesicles at 38.5 °C under 5% CO_2. Sperm were distinguished according to fresh (Fresh) or equilibrated (Equil) stage of sperm preparation. Data are presented by vertical box plots (medians, the 25 and 75th percentiles are shown as vertical boxes and whiskers, outliers are visualized by circles. The numbers below give the mean ± SD of sperm in the respective binding class.

there were no differences in the conception rates when epididymides were stored at 4 °C for 24 h before freezing compared to not coldstored sperm in tissue before artificial insemination [31].

In the current study we demonstrated that the equilibration of epididymal sperm from domestic cats in a freezing extender influences sperm-oviduct binding *in vitro*. The freezing extender (TestG) which was used for the study contains buffers, ions, fructose, glycerol and only the water-soluble fraction of hen's egg yolk. This fraction mainly comprises LDL [41] which are considered responsible for sperm protection during cryopreservation [15,42,43]. Bergeron and Manjunath (2006) reviewed several proposed protective mechanisms mediated by LDL studied on ram and bull sperm:

- sperm membrane stabilization,
- formation of a protective film on the sperm surface,
- replacement of sperm membrane phospholipids that are degraded or lost during cryopreservation,
- replacement of detrimental seminal plasma peptides attached to the sperm membrane,
- prevention of Binder of sperm (BSP) protein mediated lipid efflux via LDL-BSP protein interaction [15].

All the protective actions of LDL target the sperm membrane and have the potential to affect binding sites on sperm and/or oviduct cells. In the light of a LDL-BSP protein interaction, Bergeron and Manjunath (2006) argued that the presence of egg yolk in a freezing extender might be a reason for the poorer fertilization rate after artificial insemination with cryopreserved compared to fresh semen [15]. BSP proteins as well as spermadhesins are mainly provided by the seminal vesicles but are already partly secreted in the epididymis. Both types of proteins attach to the surface of the rostral sperm head region and BPS proteins also bind to the principal piece of the tail in mice, rats and rabbits [44,45]. BSP proteins are later involved in the formation of the oviductal sperm reservoir as part of the capacitation process [16,43,44]. Therefore, they are candidate molecules being masked or blocked by LDL or other egg yolk components. Genes for BSP proteins and spermadhesins have been described in many species, mostly in ungulates, but so far not in felids. However, very recently the proteome of cat spermatozoa and seminal plasma had been described, and proteins with sperm binding activity were identified [46]. The epididymal sperm binding protein (ELSPBP1) was found and even if its function is unknown yet, its sequence is similar to the bovine BSP [47]. Inactive copies of spermadhesin genes were also discovered in the genomes of domestic dogs [48]. It remains unknown whether the mentioned homologous sperm binding proteins are relevant for sperm-oviduct binding in felids and were blocked or altered in our experiments.

Reports on sperm binding in several species, including domestic dog, described that the male gametes bind to the oviduct or uterus epithelium by their heads [49–51]. In the cat, we observed that most epididymal sperm attached by their heads to the oviductal explants, but some sperm were also captured by their tails [26]. Therefore, we distinguished the type of binding for the present study and investigated the mitochondrial activity of sperm to assess their viability. Whereas other authors used the mitochondrial dye JC-1 to visualize bound sperm in sperm-oviduct binding assays [52–54], we applied R123 to distinguish active and inactive sperm mitochondria in the head- or tail-bound cat sperm visualized by H342. Schulze et al. (2013) showed that there is a positive correlation between mitochondrial activity determined by R123 staining and pregnancy rate in pigs [55]. Only sperm with active mitochondria are competent to move through the female genital tract, undergo capacitation culminating in hyperactivation and oocyte penetration [56].

Within the attached population of fresh prepared epididymal sperm, head-bound sperm with active mitochondria achieved the highest numbers per FOECi surface area. In both, fresh and equilibrated samples, tail-bound sperm with active mitochondria were the least represented (<5%). Proportions of about 20% of sperm contained inactive mitochondria and were attached with their tail in the fresh and equilibrated samples. This leads us to the assumption that tail-binding might be an initial event preceding phagocytosis of dying and dead sperm by oviduct epithelium, originally described in domestic cats by Murakami et al. (1985) [57]. In contrast, head binding seems to be more selective for viable sperm. About half of the fresh and a somewhat smaller fraction of the equilibrated sperm were head-bound and showed positive mitochondrial activity. We suggest that LDL interferes with the natural sperm-oviduct molecular interaction and causes a reduced binding of "good" sperm (head bound_R123+).

Following our standard freezing protocol we commonly prepare epididymal sperm in a cell culture medium M199 without stimulatory supplements to restrict metabolism and motility before freezing. However, when sperm were equilibrated in TestG, and samples were analyzed after 1 h of incubation, the total motility was significantly higher in the equilibrated samples than in samples solely diluted in medium M199 without equilibration. This is in accordance with our previously published results [20] and is at least partly due to the above-mentioned beneficial roles of LDL in the extender.

5. Conclusion

In conclusion, we showed that FOECi-vesicles are suitable to study sperm-isthmus interaction in the domestic cat *in vitro*. Right-side out vesicles form of fresh oviduct explants in a short term overnight culture, but manual isolation of FOECi-vesicles consisting of viable oviduct cells is required before binding experiments. Staining the vesicles by PI and sperm by H342 and R123 enables to monitor vesicle viability, visualize bound sperm and distinguish sperm with active or inactive mitochondria.

We suggest that the main sperm population attached by head to FOECi-vesicles and possessing active mitochondria is the competent sperm population which would be primed and subsequently released for fertilization *in vivo*. The binding capacity of feline epididymal sperm was reduced after dilution in freezing extender TestG and equilibration to 15 °C, but there were still head-bound sperm with active mitochondria observed. Our assay will be helpful to optimize extender ingredients for their minimum interference with the binding efficiency of sperm and potential releasing mechanisms. The supplementation of equilibrated/cryopreserved sperm by recombinant seminal plasma proteins to restore their binding capacity is one possible option to be tested in future to improve artificial insemination in endangered felids.

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Author contribution statement

SE, KM primarily designed the study and drafted the manuscript. SE performed the experiments and conceived the experiments. SE, LDB primarily analyzed the data. LDB edited the English language.

Declaration of competing interest

There is no conflict of interest that could be perceived as

prejudicing the impartiality of the research reported.

CRediT authorship contribution statement

Susanne Eder: Writing - original draft, Writing - review & editing, Visualization, Methodology, Investigation, Formal analysis. **Liam D. Bailey:** Writing - review & editing, Visualization, Formal analysis. **Karin Müller:** Writing - original draft, Writing - review & editing, Methodology.

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