Characterization of sperm-oviduct interactions in felids using the domestic cat (*Felis catus*) as model species

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CONTENT

SUMMARY

The family Felidae includes 38 non-domestic species and the domestic cat (*Felis catus*). Twenty-four of the non-domestic species are listed in the Red List of the IUCN as near-threatened or threatened. In order to counteract species extinction and further loss of genetic diversity, breeding programs and effective application of assisted reproductive technologies (ARTs) are poised to make a substantial contribution to ensure that they will not go extinct. Important ARTs used in *ex situ* breeding programs are sperm cryo-preservation and artificial insemination (AI). It is possible to obtain offspring from a single artificial insemination in felids, but the success of fertilization depends, among other things, strongly on the type of sperm. Both epididymal and ejaculate sperm are cryo-preserved in banks for future AI and further ARTs. Ejaculate sperm have a higher degree of maturity and contact with seminal plasma and therefore a better fertilization competence than epididymal sperm. Fertilization success is also higher when fresh and not cryo-preserved sperm are used for AI and for *in vitro* fertilization. Depending on the method of AI (intra-vaginal, trans-cervical, intra-uterine, intra-tubal) the sperm has to overcome different barriers to reach the oviduct. A functional sperm reservoir is formed in the isthmus of the oviduct during the period when the oocytes mature; they are fertilized in the isthmus- ampulla junction. Early embryo development also takes place in the oviduct.

Especially the sperm storage in the isthmus, where the final sperm maturation, capacitation, occurs, is decisive for sperm to obtain fertilization competence and to prevent polyspermy. However, it is unknown whether the lower proportions of successful fertilization following AI using epididymal and / or cryo-preserved sperm in felids are due to the fact that the sperm reservoir cannot be formed because epididymal sperm lack contact with seminal plasma (SP) proteins that mediate sperm-oviduct binding or the sperm membrane is damaged by the freezing process and can therefore no longer bind to the oviduct epithelial cells (OEC).

We therefore investigated two important aspects which may play a role in the interaction between sperm and oviduct. Using the domestic cat as model species, we wanted to develop suggestions to optimize preservation and insemination protocols to improve the efficiency of ARTs in endangered felid species.

First, we investigated the influence of the seminal plasma on sperm-oviduct binding (**chapter I,** also called SP-study) and aimed to find out whether (1) the SP has an influence on epididymal sperm quality, (2) epididymal sperm are able to bind to oviduct epithelial cells *in vitro*, and (3) whether the SP has an influence on *in vitro* binding efficiency. Epididymal spermatozoa (with or without SP) were co-incubated with freshly isolated feline OEC (FOEC) so-called explants. The SP-study revealed that epididymal spermatozoa of the domestic cat are able to bind to FOEC-explants and that the SP plays a supporting role in the sperm binding process.

In the second study (**chapter II**, also referred to as equilibration-study) we examine whether the contact of sperm to the freezing extender and cooling to 15°C during equilibration might change sperm-oviduct binding patterns in quantity and quality (bound by sperm head or tail, sperm with active or inactive mitochondria). Epididymal sperm (with or without equilibration) were co-incubated with isolated FOEC-vesicles. FOEC-vesicles formed from FOEC-explants during the short-term suspension culture. The use of the vesicles had the advantage that they survived longer and retained their sperm binding capacity longer than explants. The equilibration-study showed that the binding capacity of epididymal sperm to FOEC-vesicles was reduced when sperm were diluted in freezing extender and equilibrated to 15°C. We suggest that the main sperm population bound by head to FOEC-vesicles and possessing

active mitochondria is the competent sperm population that would be primed and subsequently released for fertilization *in vivo*.

Explants and vesicles which were used in the first and second study, respectively, have the disadvantage that their viability and differentiation state is limited to a short time. The polarized-monolayer culture of OEC in a compartmentalized system is well characterized and established in laboratory mouse, domestic pig, domestic cattle and human OEC where OEC have a phenotype similar to *in vivo* tissue and are well differentiated. In the third study (**chapter III**) we took the first steps to establish a polarized monolayer long-term culture of FOEC of the domestic cat to establish a reproducible *in vitro* model for use in basic reproductive research and assisted reproduction. The experiments of the third study showed that the long-term culture of FOEC in a compartmentalized culture system is possible in principle. However, the desired result – a differentiated, monolayered, highly prismatic epithelium with secretory as well as ciliated cells – could not yet be reliably (and reproducibly) achieved. In future studies, progenitor cells should be identified and isolated from the epithelium of the feline oviduct in order to use them as starting material for culture.

With this work, we have shown for the first time that a highly differentiated monolayer culture of FOEC in a compartmentalized system is possible in principle. In addition, we also elucidated two important aspects of sperm-oviduct interaction that may help to optimize protocols for sperm cryo-preservation and sperm handling prior to artificial insemination.

ZUSAMMENFASSUNG

Die Familie der Katzen (Felidae) umfasst 38 nicht-domestizierte Arten und die Hauskatze (*Felis catus*). Vierundzwanzig der nicht-domestizierten Arten sind in der Roten Liste der IUCN als nahezu bedroht oder gefährdet aufgeführt. Um dem Aussterben der Arten und dem weiteren Verlust der genetischen Vielfalt entgegenzuwirken, leisten Zuchtprogramme und die wirksame Anwendung von assistierten Reproduktionstechnologien (ART) einen wichtigen Beitrag. Wichtige ART, die in *ex-situ*-Zuchtprogrammen eingesetzt werden, sind die kryo-Konservierung von Spermien und die künstliche Besamung (KB). Es ist möglich in Feliden Nachkommen aus einer einmaligen künstlichen Besamung zu erhalten, jedoch sind die Fertilisationserfolge u.a. stark von der Art der Spermien abhängig. Sowohl epididymale als auch ejakulierte Spermien werden kryo-konserviert. Aufgrund des höheren Reifegrads und des Kontakts mit dem Seminalplasma (SP) haben Ejakulatspermien eine bessere Befruchtungskompetenz als Nebenhodenspermien. Außerdem werden bessere Befruchtungserfolge erzielt, wenn frische im Vergleich zu kryo-konservierten Spermien für die *In-vitro*-Fertilisation sowie für die KB verwendet werden. Je nach Methode der KB (intravaginal, transzervikal, intrauterin, intra- tubal) müssen die Spermien unterschiedliche Barrieren überwinden, um den Eileiter zu erreichen. Im Isthmus des Eileiters wird ein Spermienreservoir gebildet, die Eizellen werden in der Ampulla bzw. im Übergang vom Isthmus zur Ampulla befruchtet. Auch die frühe Embryonalentwicklung findet im Eileiter statt. Insbesondere die Spermienlagerung im Isthmus ist entscheidend für die Erlangung der Befruchtungskompetenz der Spermien. Es ist jedoch nicht bekannt, ob die niedrigeren Befruchtungsratennach KB mit Nebenhodenspermien bei Feliden darauf zurückzuführen sind, dass das Spermienreservoir nicht gebildet werden kann, weil den epididymalen Spermien der Kontakt mit den Seminalplasma-Proteinen fehlt, die die Bindung zwischen Spermien und Eileiter vermitteln, und/oder ob die Spermien durch den Gefrierprozess geschädigt sind und daher nicht mehr an die Epithelzellen des Eileiters binden können.

Daher haben wir zwei wichtige Aspekte untersucht, die bei der Interaktion zwischen Spermien und Eileiter eine Rolle spielen könnten. Mit der Hauskatze als Modellspezies wollen wir zur Optimierung von Konservierungs- und Besamungsprotokollen beitragen, um die Effizienz von ART bei gefährdeten Katzenarten zu verbessern.

Zunächst untersuchten wir den Einfluss von SP (**Kapitel I**) und wollten herausfinden, ob (1) SP einen Einfluss auf die epididymale Spermienqualität hat, (2) epididymale Spermien in der Lage sind, *in vitro* an Epithelzellen des Eileiters (feline oviduct epithelial cells, FOEC) zu binden, und (3) ob SP einen Einfluss auf die *in vitro*-Bindungskapazität hat. Epididymale Spermien (mit oder ohne SP) wurden mit frisch isolierten FOEC, sogenannten Explants (Kapitel I), co-inkubiert. Die SP-Studie ergab, dass epididymale Spermien der Hauskatze in der Lage sind, an FOEC-Explants zu binden und dass SP darüber hinaus eine unterstützende Rolle bei der Spermienbindung spielt.

In der zweiten Studie (**Kapitel II**) wurde untersucht, ob der Kontakt der Spermien mit einem Gefrier-Extender und die Abkühlung auf 15°C während der Equilibrierung die Spermien-Oviduk-Bindungsmuster in Quantität und Qualität (Bindung mit Spermienkopf oder Schwanz, Spermien mit aktiven oder inaktiven Mitochondrien) verändern. Dazu wurden epididymale Spermien (mit oder ohne Equilibrierung) mit FOEC-Vesikeln co-inkubiert. Die FOEC-Vesikel formierten sich während kurzzeitiger Suspensionskultur aus FOEC-Explants. Die Verwendung der Vesikel hatte den Vorteil, dass sie länger überlebten und länger ihre Bindungskapazität für Spermien behielten als Explants. Die Equilibrierungsstudie zeigte, dass die Bindungskapazität von epididymalen Spermien an FOEC-Vesikel reduziert war, wenn die Spermien in Gefrier-Extender verdünnt und auf 15°C equilibriert wurden. Wir

vermuten, dass die Hauptpopulation der Spermien, die mit dem Kopf an die FOEC-Vesikel binden und aktive Mitochondrien besitzen, die Spermienpopulation ist, die Befruchtungskompetenz besitzt.

Explants und Vesikel, die in der ersten bzw. zweiten Studie verwendet wurden, haben den Nachteil, dass ihre Lebensfähigkeit und ihr Differenzierungszustand auf kurze Zeit begrenzt sind. Eine polarisierte Monolayerkultur von OEC, kultiviert in einem kompartimentierten System, ist von Mäusen, Schweinen, Rindern und Menschen gut charakterisiert und etabliert. Die Kulturen ähneln im Phänotyp dem *in vivo*-Gewebe und sind gut differenziert. In der dritten Studie (**Kapitel III**) haben wir erste Schritte zur Etablierung einer polarisierte-Monolayer-Langzeitkultur von felinen OEC der Hauskatze unternommen, um ein reproduzierbares *in-vitro*-Modell für den Einsatz in der Grundlagenforschung der Reproduktionsbiologie und der assistierten Reproduktion zu schaffen. Die Experimente der dritten Studie haben gezeigt, dass eine Langzeitkultur von FOEC in einem kompartimentierten Kultursystem prinzipiell möglich ist. Das gewünschte Ergebnis - ein differenziertes, einschichtiges, hochprismatisches Epithel mit sekretorischen sowie Zilien-tragenden Zellen - konnte jedoch noch nicht reproduzierbar erreicht werden. In zukünftigen Studien sollten Progenitorzellen aus dem Epithel des felinen Eileiters identifiziert und isoliert werden, um sie als definiertes Ausgangsmaterial für die Kultur zu verwenden.

Mit dieser Arbeit haben wir zum ersten Mal gezeigt, dass eine hochdifferenzierte epitheliale Eileiter-Zellkultur bei der Hauskatze prinzipiell möglich ist. Außerdem haben wir zwei wichtige Aspekte der Spermien-Eileiter-Interaktion aufgeklärt, die dabei helfen können, Protokolle für die kryo-Konservierung von Spermien sowie das Spermien-Handling vor der künstlichen Besamung zu optimieren.

ABBREVIATIONS

AEA	N-arachidonoylethanolamide or anandamide
AI	artificial insemination
AL	air-liquid
ARTs	assisted reproductive technologies
AV	artificial vagina
BSP	binder of sperm protein
CB1	cannabinoid receptor type 1
CL	corpus luteum
EE	electro-ejaculation
ETCIUI	endoscopic transcervical intrauterine insemination
FOEC	feline oviduct epithelial cells
GnRH	gonadotropin-releasing hormone
HE	hematoxylin-eosin
HDL	high density lipoprotein
IUCN	International Union for Conservation of Nature
IVI	intravaginal insemination
KLF4	Krupple-like factor 4
LDL	low-density lipoprotein
LITI	laparoscopic intratubal insemination
LIUI	laparoscopic intrauterine insemination
LH	luteinizing hormone
LL	liquid-liquid
NANOG	named after the fountain of eternal youth *Tír na nÓg*
NB	natural breeding
OEC	oviduct epithelial cells
OEP	orvus es paste
OVGP1	oviduct-specific glycoprotein
PAX8	paired box gene 8
PI	propidium iodide
RSPO1	R-spondin-1
R123	Rhodamine 123
ROS	reactive oxygen species
SP	seminal plasma
SLS	sodium lauryl sulfate
SSEA3	stage-specific embryonic antigen 3
SSEA4	stage-specific embryonic antigen 4
TCIUI	transcervical intrauterine insemination
UIUI	unilateral intrauterine horn insemination
TVICI	transvaginal intracervical insemination
TRPV1	transient receptor potential vanilloid 1
UrCa	urethral catheterization
UTJ	utero-tubal junction

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EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, die Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze keinen entsprechenden Doktorgrad. Icherkläre, dass ich die Dissertation oder Teile davon nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht habe und dass sie dort weder angenommen, noch abgelehnt wurde.

1. General introduction

1.1. The importance of rescue programs and assisted reproduction techniques (ARTs) for endangered feline species and challenges

The family Felidae includes 38 non-domestic species and the domestic cat (*Felis catus*). Twenty-four of the non-domestic species are listed in the Red List of the IUCN as near-threatened or threatened. Residential and commercial development, agriculture and aquaculture as well as energy production and mining are increasing and contribute to habitat loss, habitat fragmentation and climate change. In the remaining areas, excessive tourism and/or hunting/trapping can reduce wild felid populations. Invasive species and diseases bring additional problems [IUCN, 2022]. Populations that are small in number and fragmented often have a reduced resistance against diseases because of limited genetic diversity [King and Lively, 2012; Spielman et al., 2004]. And even if there is an improvement in terms of habitat availability achieved by current conservation efforts, some populations and species are already so severely reduced in numbers and genetic diversity that special breeding programs and an effective application of ARTs are necessary to prevent them from extinction [Fernandez-Gonzalez et al., 2019]. Unfortunately, the success of ARTs in feline species is relatively low compared to their application in livestock or humans [Kochan et al., 2019]. ART protocols developed for livestock cannot be transferred one-to-one to felids. This is due to species-specific differences in estrus cycle, anatomy of the genital tract and mating behavior. In the following chapters, the estrus cycle and the morphology and anatomy of the reproductive tract of felids will be described, especially considering the location of fertilization, the oviduct. Cell culture models can be useful tools to answer basic questions of reproduction biology including the oviduct as a central organ of interest. Different cell culture methods are discussed which are used for the development of ARTs. Finally, sperm preservation and artificial insemination, as the most important and most widely used methods in breeding programs, are described. Possible reasons for the relatively low proportion of conception success after artificial insemination in felids are introduced as the rationale for the studies conducted as part of this dissertation.

1.2. The estrous cycle of felids

Most feline species are polyestrous and, depending on the species and its geographical range, seasonal or non-seasonal breeders [Brown, 2011; Göritz et al., 2009]. Ovulation may be induced by mating or occurs spontaneously [Brown, 2011]. This is not only species-specific but may also vary between individuals within the same species [Binder et al., 2019; Brown, 2011]. The estrous cycle in felids starts with a proestrus, lasting less than 24 h [Andrews et al., 2019]. It is characterized by the increase of estradiol concentrations and the presence of small follicles. At this stage mating does not occur. When estradiol concentrations increase further, follicles grow, oocytes mature and females show the typical behavior. A female in estrus rolls over the ground, calls or meows to attract the attention of males, crouches on the ground with her forelegs pressed down, her back in a lordosis position, and bends her tail to the side to present her vulva. The female is also restless and has little appetite. Felids have promiscuous mating behavior. The mating induces ovulation. Estrus lasts three to ten days [Brown, 2011]. In induced ovulators mating stimulates the release of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) [Brown, 2011]. After ovulation the corpus luteum (CL) develops from the ovulated follicle. In spontaneously ovulating female CL formation also occurs by ovulation or by

luteinization of follicles without previous ovulation [Binder et al., 2019]. The length of the luteal phase (also called diestrus) depends on the lifespan of the corpus luteum. The anestrus and the interestrus are characterized by basal levels of estradiol production and absence of follicles. The duration of anestrus is determined by the length of the day. The phase occurs when the daylight period does not exceed eight hours. To be distinguished from this is the lactation anestrus, which is the phase in which the female nurses the kittens - after parturition before a new cycle starts. In contrast, interestrus is a variable period between follicular phase (estrus) and a new cycle (proestrus) when no mating event, no ovulation, no conception or a miscarriage has occurred [Andrews et al., 2019]. Gestation differs among non-domestic felid species between 56 and 96 days in small cat species (*Felinae*) and between 90 and 110 days in the panther lineage (*Pantherinae*) [Macdonald, 2004]. In the domestic cat, gestation lasts between 58 and 65 days [Brown, 2011]. A generalized reproductive cycle of the domestic cat is shown in figure 1. ARTs also comprise the hormonal stimulation of estrus and ovulation. They are not discussed in this thesis in detail. However, if a female does not develop natural estrus, hormonal stimulation is a prerequisite for successful artificial insemination [Pelican et al., 2006]. There are several hormonal regimes tested to stimulate estrus and ovulation of a female, but to date no universal protocol has been established for felids [Thongphakdee et al., 2018].

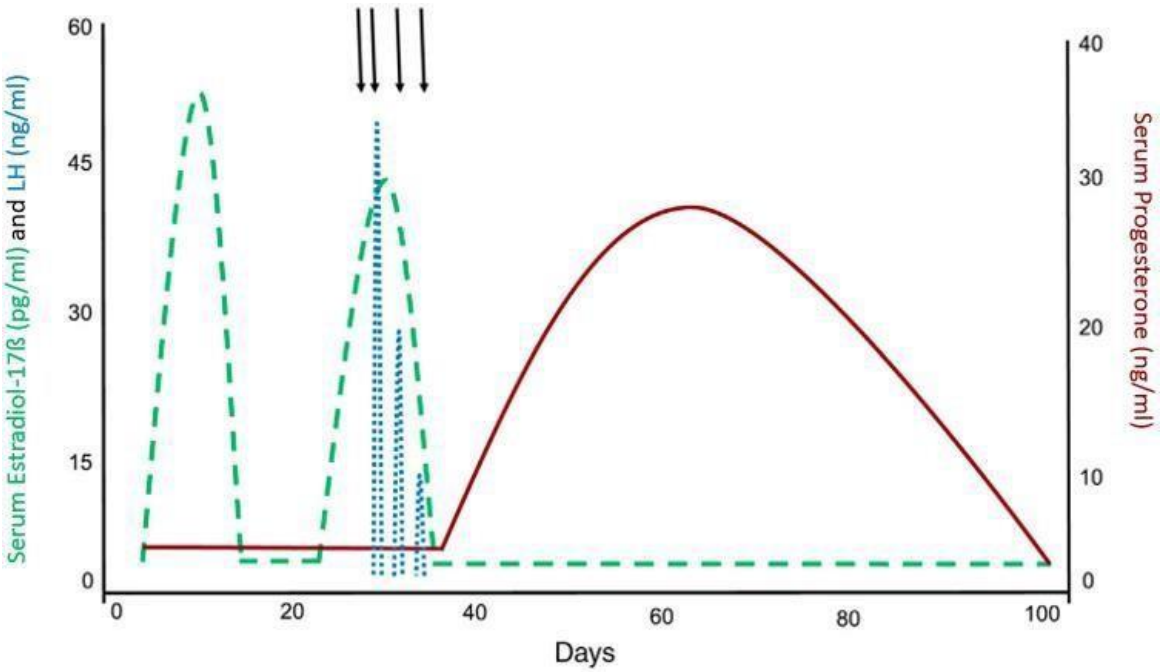

Fig. 1. Schematic diagram of circulating estradiol-17b (dashed line), LH (dotted line) and progesterone (solid line) concentrations during the estrous cycle and post-ovulation in domestic cats. Arrows designate the time window for mating [Brown and Comizzoli, 2018]. Reuse of the figure by permission of Elsevier (license number 5277690971716).

1.3. Transit of sperm through the male and female reproductive tract

New life starts with the fertilization of an oocyte by a sperm. Male and female gametes develop and mature in mammals separately in different individuals. In males, sperm development and maturation occur during the process called spermatogenesis. This development takes place in the testes and

epididymis and its duration is species-specific. After spermatogenesis, testicular sperm cells are not yet able to fertilize an oocyte. During transit through the epididymis further sperm maturation takes place, a necessary step [Gatti et al., 2004]. Here, sperm come in contact with epididymal fluid and undergo membrane modifications that result in changes in lipid and protein composition [Girouard et al., 2011; Yanagimachi, 1994]. During ejaculation mammalian sperm are diluted with the secretion products of the accessory sex glands – the ampullary glands, seminal vesicles, prostate gland, bulbourethral glands and urethral glands [Kent, 2021; Britannica, 2018]. In felids only the prostate gland and bulbourethral glands are present [Bragulla, 2019]. During copulation, ejaculated sperm diluted in seminal plasma are deposited in species-specifically distinct sites inside the female genital tract. In domestic horses and many rodents, sperm are placed directly into the uterus [Coy et al., 2012]. In the domestic pig, semen is deposited deep into the cervix close to the uterine cavity [Levis, 1914]. In humans and domestic cattle, sperm are ejaculated into the vagina, in domestic dogs as well as in domestic cats even into the anterior vagina [Coy et al., 2012]. Sperm deposited vaginally (fig. 2) as in felids must overcome many natural barriers such as vagina, cervix, uterus and the utero-tubal junction (UTJ) to reach the place of fertilization, the oviduct [Chatdarong et al., 2004]. During the passage through the female genital tract processes of sperm selection operate. Of several million sperm, only a few hundred in the domestic cat and the domestic pig [Chatdarong et al., 2004; Viring, 1980] and some thousands in golden hamster [Smith et al., 1987] arrive in the oviduct, where they accumulate in a functional sperm reservoir. The sperm attach to the oviduct epithelial cells (OEC) where binding extends their fertile lifespan [Suarez, 2008]. This has been observed in several mammalian species such as human, domestic cattle, domestic pig and domestic horse [Dobrinski, 1996; Murray and Smith, 1997; Petrunkina et al., 2001; Talevi and Gualtieri, 2010].

How the prolongation of sperm lifespan is achieved has not yet been fully elucidated. One mechanism is discussed for equine and porcine sperm. Possibly, vitality is maintained by preventing premature capacitation and the concomitant rise in cytoplasmic Ca^{2+} [Dobrinski, 1996; A Petrunkina et al., 2001] In parallel, catalase, which has been detected in high amounts before ovulation in the bovine oviduct, is suggested to protect sperm membranes against oxidative damage [Lapointe et al., 1998]. After some time of sperm binding, capacitation – comprising a destabilization process of the plasma membrane – is triggered, results in hyperactivation and finally in sperm release from the OEC [Gervasi et al., 2016; Suarez, 2008]. This process and the subsequent sperm release from the oviductal reservoir are poorly understood. Sperm release from OEC is induced by ovulation associated signals [Suarez, 2008]. In domestic cattle it is proposed that AEA (N-arachidonoylethanolamide or anandamide) activates CB1 (cannabinoid receptor type 1) and TRPV1 (transient receptor potential vanilloid 1) receptors, which boost Ca^{2+} influx and flagellar motion resulting in sperm release from the OEC [Gervasi et al., 2016]. Also sulfated glycoconjugates secreted by bovine OEC during estrus seem to play an important role in sperm release [Talevi and Gualtieri, 2001]. Furthermore, Talevi et al. [2007] demonstrated that disulfide-reductants are able to release sperm bound to the oviduct. Machadoet al. [2019] demonstrated in the domestic pig that release of sperm from oviduct cells is promoted by progesterone which binds to the non-genomic receptor α/β hydrolase domain-containing protein 2 (ABHD2) that leads to Ca^{2+} influx via CatSper channel proteins [Machado et al., 2019]. In the domestic dog, no specific molecule has been identified so far. Freeman and England [2013] provided evidence in the domestic dog that motility changes and release of sperm can be induced by flushed post-ovulation oviduct fluid, which contains components of follicular fluid as well as by solubilized zona pellucida. In domestic cats, no molecule has yet been identified that mediates the release of sperm from the oviduct.

Finally, the released sperm undergo an acrosome reaction, the final maturation step prior to oocyte penetration. As outlined in this chapter, decisive processes take place in the oviduct, which is introduced in the next section in more detail.

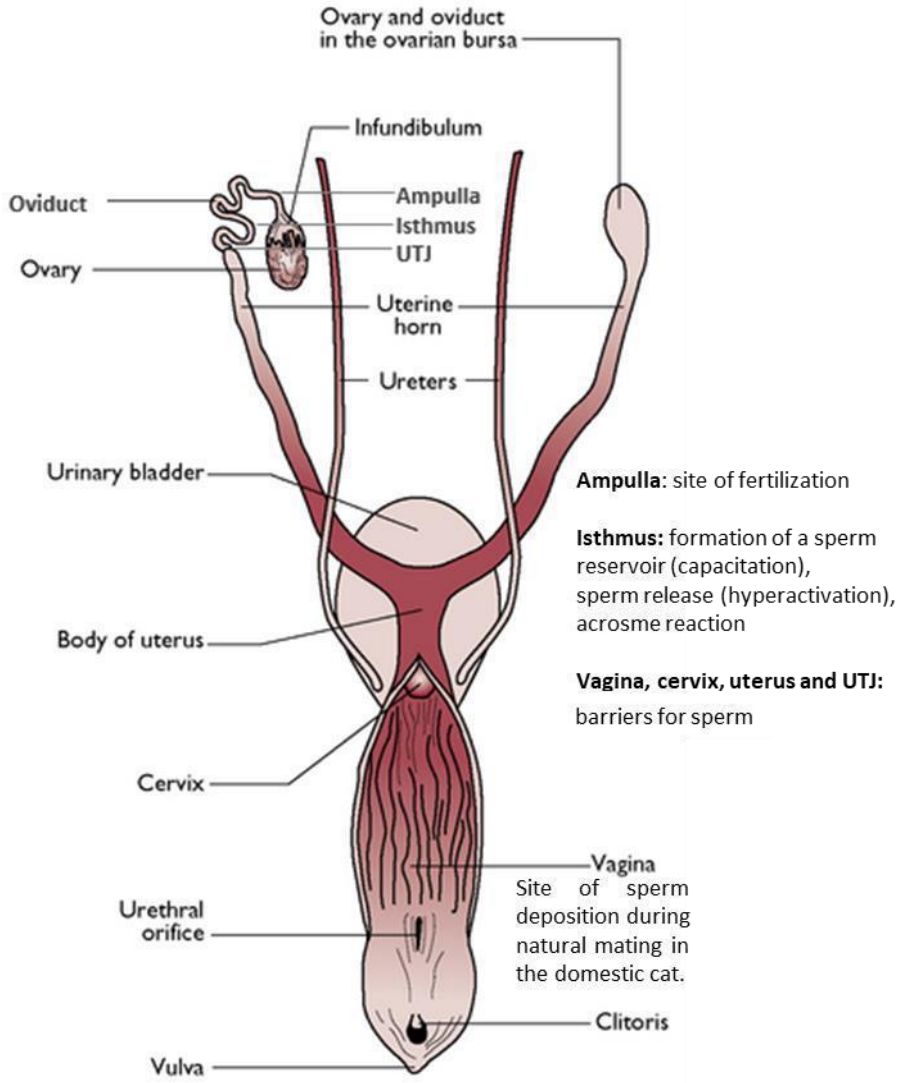

Fig. 2. Dorsal view of the female reproductive system of a carnivore. Events from the perspective of sperm were assigned to the parts of the female genital tract. [Modified from Colville, and Bassert, 2015]. Reuse of the figure by permission of Elsevier (license number 220329-010778).

1.4. The oviduct

The oviduct is a part of the female reproductive tract and plays an important role in reproductive processes. It is a paired organ and consists of the utero-tubal junction (UTJ), isthmus, ampulla and infundibulum. The isthmus is connected to the uterus via the UTJ. On the distal site, the infundibulum loosely envelops the ovary. These parts of the oviduct have different functions and, therefore, also differ in morphology. The UTJ and isthmus are known as functional sperm reservoirs. The isthmic-ampullary junction or the ampulla is the site of fertilization. Early embryo development occurs during the transit through the oviduct towards the uterus [Ellington, 1991; Hunter, 2012]. The cross-sectional structure of the oviduct consists of serosa, longitudinal muscle with embedded veins and arteries, circular muscle as well as mucosal folds (fig. 3).

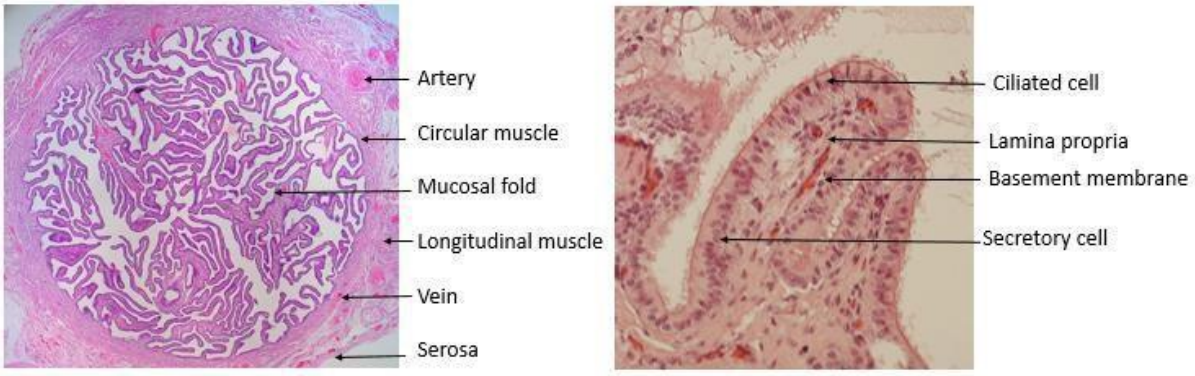

Fig. 3. left: Cross section through a human oviduct. Hematoxylin -eosin stain [modified from https://de.wikibrief.org/wiki/Ampulla_of_Fallopian_tube, accessed: 29.3.22, permission CC-BY-SA 2.0], right: Cross section through mucosalfolds of an isthmus from domestic cat. Hematoxylin-eosin stain.

The mucosal folds are composed of connective tissue (the so called lamina propria), the extracellular basement membrane and epithelial tissue. The oviduct epithelial tissue is a monolayer of highly prismatic cells. The two main cell types are ciliated and secretory cells. The oviduct epithelium is described as secretory [Leese, 1988]. A smaller number of cells are immune cells such as lymphocytes, monocytes, mast cells and granulocytes. These are mainly located in the lamina propria (fig. 3) [Eriksen et al., 1994; Givan et al., 1997; Valle et al., 2009]. As in all epithelia, the cells are polarized. The basal cell side is directed to the basement membrane and the apical cell side to the oviduct lumen. The epithelium receives nutrients via diffusion from the blood vessels of the subepithelial connective tissue [Leese, 1988].

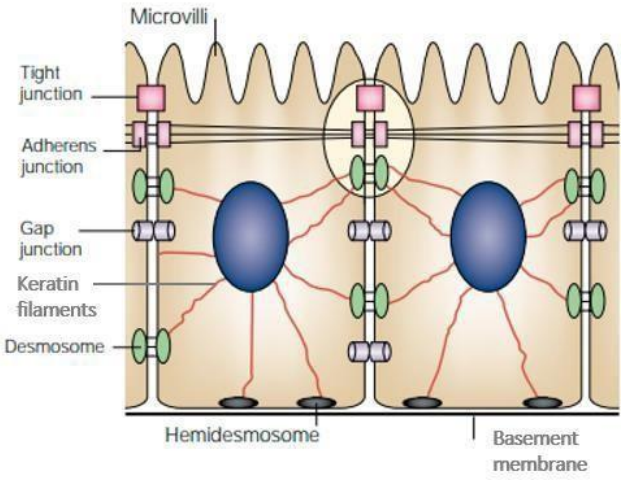

Fig. 4. Junctional complex and tight junctions. Schematic drawing of epithelial cells. Modified from Tsukita et al., 2002. Reuse of the figure by permission of Springer Nature (license number 5277710397049)

Epithelial tissue can be characterized by intermediate keratin filaments. The OEC are in close contact via different junction types and, therefore, the intercellular space is small. In this way, the epithelium forms a barrier that ensures that a specific milieu can be induced and maintained in the oviduct lumen depending on the cycle stage. Communication and adhesion between cells or between cells and matrix are enabled via tight junctions, adherens junctions, gap junctions, desmosomes and hemidesmosomes (fig. 4). Hemidesmosomes form the connection between basal membrane and the epithelial cells on the basal site. The other four connection types are positioned between epithelial cells and connect them.

The tight junctions are the most important junctions to ensure a barrier between oviduct lumen and the basal membrane and matrix. The formation of the barrier between the OEC is an important quality parameter in an OEC culture and can be demonstrated by measuring the transepithelial electrical resistance (TEER) [Chen et al., 2015].

The ratio of ciliated to secretory cells, cellular height and the volume of the oviduct fluid change during the estrous cycle. During the follicular phase, the isthmus contains a higher number of secretory cells, whereas more ciliated than secretorycells are present in the ampulla and in the infundibulum [Binelli et al., 2018; Verhage and Brenner, 1975]. Cell height and oviduct fluid production are higher in estrus than anestrus [Leese, 1988].
Whereas in ciliated cells the nucleus is more apically located, the nucleus of secretory cells is positioned in the basal region. Ciliated cells have cilia on their apical side. These cilia are characterized by a microtubule skeleton consisting of tubulin. Secretory cells can be identified by microvilli. They secrete oviductal fluid which plays an important role in oocyte transport and maturation, sperm transport and maturation (storage, capacitation, release), fertilization as well as embryo transport and early development [Leese, 1988]. One protein which is synthesized and exclusively released by secretory, non-ciliated OEC is oviductin (oviduct-specific glycoprotein, OVGP1). Functional studies in domestic goat, domestic pig and domestic cattle indicate that oviductin affects oocyte-sperm interactions, fertilization and embryonic development as well as sperm capacitation, motility and viability [Abe et al., 1995; McCauley et al., 2003; Pradeep et al., 2011]. There is no evidence to date that oviductin affects sperm-oviduct binding. As mentioned earlier, sperm binding is mediated by binding molecules located in the sperm plasma membrane as well as in the apical plasma membrane of the OEC [Talevi and Gualtieri, 2010]. The binding molecules located in the sperm plasma membrane are discussed in more detail in the next section. Several studies suggest that sperm binding is mediated by species- specific glycoproteins and / or carbohydrate ligands. In golden hamster, fetuin and its terminal sugar sialic acid are involved, in domestic horse galactose, in domestic pig mannose, galactose, annexin 2, sperm binding glycoprotein (SBG) and galactose-beta 1-3 N-acetylgalactosamine (Galb1-3GalNAc), and in domestic cattle fucose as well as annexin 1, 2, 4, 5 participate in or inhibit binding of sperm to oviduct epithelium [Ball et al., 1997; DeMott, 1995; Ekhlasi-Hundrieser et al., 2005; Ignotz et al., 2001; Talevi and Gualtieri, 2010]. In felids, no molecule has been identified so far.

1.5. Seminal plasma

Seminal plasma (SP), also referred to as seminal fluid, contains secretions of the epididymis and the accessory sex glands (see above). Its components have decisive effects on sperm function and survival [Rodriguez-Martinez et al., 2021]. SP provides metabolic substrates, supports sperm transit through the female genital tract and the formation of a functional sperm reservoir in the oviduct [Caballero et al., 2012]. Via interaction with sperm, its components play key roles in capacitation, acrosome reaction and sperm-egg fusion [Caballero et al., 2012]. Furthermore, SP has also an impact on the female reproductive tract and on embryo development. SP induces inflammatory processes such as phagocytic clearance of superfluous sperm and microorganisms in the vagina, cervix and uterus [Robertson, 2005]. In addition to dead and morphologically abnormal sperm [Tomlinson et al., 1992], vital and morphologically intact sperm are also phagocytosed [Robertson, 2005]. Selection appears to be based on antigenic parameters in addition to fertilization competence [Taylor, 1982; Schimenti, 2000]. In the uterus, SP prepares the endometrium to receive and tolerate the embryo and promotes blastocyst development. Finally, SP contributes to the induction of ovulation and corpus luteum formation in the ovary [Schjenken and Robertson, 2015].

SP in mammals comprises fructose, the major metabolic substrate, inorganic substances such as sodium bicarbonate as a buffer, antioxidants, a variety of proteins and lipids which are mostly organized in extracellular vesicles [Mogielnicka-Brzozowska and Kordan, 2011; Rodriguez-Martinez et al., 2021].

Proteins related to sperm-oviduct binding are for instance spermadhesins and proteins containing fibronectin type-II domains. These proteins are described in several mammalian species such as humans, domestic pig, domestic horse, domestic cattle, domestic sheep, laboratory mouse, laboratory rat and domestic rabbit [Ekhlasi-Hundrieser et al., 2005; Manjunath et al., 2007; Nixon et al., 2008; Töpfer-Petersenetal., 1998]. *In vitro* studies indicate that in the domestic pig the spermadhesin AQN1 and in domestic cattle the BSP protein BSP-A1/A2 play a supportive role in the formation of the oviductal sperm reservoir [Ekhlasi-Hundrieser et al., 2005; Gwathmey et al., 2003]. Spermadhesins and proteins with fibronectin type II domains are associated with the rostral surface of sperm head or the mid-piece [Ekhlasi-Hundrieser et al., 2005; Nixon et al., 2008; Suarez, 2016]. So far, no specific protein that mediates sperm-oviduct binding has been identified in seminal plasma of the domestic cat. Only a few studies were performed in the domestic cat. Ekhlasi-Hundrieser et al. [2007] and Zambelli et al. [2010] investigated the composition of SP. Rowlison et al. [2020] characterized the proteomic profile of epididymal extracellular vesicles and Mogielnicka-Brozozowska et al. [2020] studied the proteomic profile of semen obtained after urethral catherization. One feline SP candidate protein for the interaction with sperm membranes is the epididymal sperm binding protein (ELSPBP1) that contains four fibronectin type-II domains arranged in tandems, although its function is still unknown. Whereas D`Amours et al. [2012] stated that ELSPBP1 specifically targets dead sperm cells to presumably initiate their degradation and removal from the maturing population, Ekhlasi-Hundrieser et al., [2007] found its sequence is similar to binder of sperm proteins (BSPs) in bulls, suggesting a possible involvement in the formation of the sperm reservoir in the oviduct.

1.6. Cell culture of oviduct epithelial cells (OEC)

Cell culture is a useful tool to get a basic understanding of cellular and molecular mechanisms, interactions and pathways of normal physiology and pathology, to test drugs and toxic substances on cells and tissues and to develop drugs, vaccines and therapeutic proteins. Other specific applications comprise stem cell research and the development of *in vitro* and *in vivo* protocols for ARTs.

Oviduct cells are cultivated using different methods: in short-term culture as a 2D monolayer or as vesicles in suspension as well as in long-term culture as a polarized monolayer in simple compartmentalized, in microfluidic, or in 3D-printed chambers or as organoids [Ferraz et al., 2017]. The cell culture should mimic the physiologic conditions as best as possible. This concerns the culture medium that provides the molecules required for nutrition, proliferation and differentiation as well as relevant physical and biochemical parameters such as appropriate temperature, pH and osmolality. The substrate on which the OEC adhere or the matrix in which they are cultivated is also important for successful proliferation and differentiation, so that a supply of nutrients is ensured via the basal cell side. This section provides an overview on culture methods of feline epithelial oviduct cells (FOEC) and OEC of other species in relation to the respective purpose.

In a **2D monolayer culture,** cells attach to the (coated) bottom of a culture dish or flask. Cells attach by their basal site and are covered on their apical site by medium where they are supplied with nutrients. This is not physiological for epithelial cells, because they normally receive nutrients via diffusion from the blood vessels of the subepithelial connective tissue beneath the basal cell side. As a consequence,

fast dedifferentiation (after several days) and cell death can be observed. This was shown several times for bovine OEC [Hishinuma et al., 1989; Joshi, 1988; Walter, 1995]. The loss of cilia was observed when the monolayer reaches confluence [Reischl et al., 1999; Thibodeaux et al., 1992; Walter, 1995]. Still, this type of culture was widely used to study oviduct physiology, specifically interactions of oviduct cells with gametes and embryos [Thibodeaux et al., 1992].

A **suspension culture** of OEC vesicles was also often used to cultivate OEC. Under this condition, isolated cells form floating aggregates within hours. These vesicles are also referred to as spheroids, explants and clusters and do not attach to the bottom of a culture dish or flask. The method is simple and vesicles can be used immediately after their formation because there is no need to wait until a confluent and re-differentiated monolayer has formed. Vesicles survive for a few days with their morphological and functional properties. Walter [1995] described that cilia were still present on bovine OEC vesicles after 10 days. Kaffenberger [2010] demonstrated the expression of the characteristic OEC protein oviductin for 19 days. An advantage is that the apical side faces outwards. This facilitates sperm-oviduct assays. After a period of several days, cells lose their functionality [Kaffenberger, 2010; Rottmayer et al., 2006]. The restricted supply of nutrients via the inward-facing basal side of the cells resembles the non-physiological situation in 2D monolayers. Nevertheless, this method was used to study sperm-oviduct interaction in domestic cattle [De Pauw et al., 2002; Saraf et al., 2017] and to improve the fertilization success and embryo development after IVF in the domestic cat [Lengwinat et al., 1993].

Polarized monolayers are cultured in compartmentalized culture systems for three weeks or longer. In this system isolated single cells and small clusters are seeded on the apical side of the transwell membrane. The culture begins with a proliferative phase in which medium is present in the baso-lateral as well as in the apical compartment. The period of liquid-liquid (LL) interface is followed by an air- liquid (AL) condition in which the medium is removed from the apical compartment and is solely available from the baso-lateral side. This compartmentalized device (fig. 5.), where cells are cultured in an AL interface, mimics the *in vivo* situation and the metabolism switches from anaerobic to aerobic. As a result, the cells proliferate and differentiate, including re-ciliation, after confluence [Johnson et al., 1993]. It seems that proliferation and differentiation of the OEC in a compartmentalized culture approach depends on the presence of stem cell-like progenitor cells [Chen et al., 2018] as this was shown for normal human bronchial epithelial cells [Prytherch et al., 2011] and bovine bronchial epithelial cells [Cozens et al., 2018]. OEC polarized monolayer cultures are established and well characterized in several mammalian species [Chen et al., 2017, 2013; Levanon et al., 2010; Palma-Vera et al., 2014; Rajagopal et al., 2006]. Polarized monolayer cultures were first established for epidermal and airways epithelial cells [Pruniéras et al., 1983; Whitcutt et al., 1988]. Later this method was adopted for ear [Portier et al., 2005], gastric [Tabuchi, 2001], cornea [Sygitowicz et al., 2011] and female reproductive tract epithelia [Chen and Schoen, 2019]. This culture method has been used to study the

regulation of estrus-dependent changes of OEC-properties as well as the interactions of gametes and embryos with the oviduct [Chen et al., 2013; 2017].

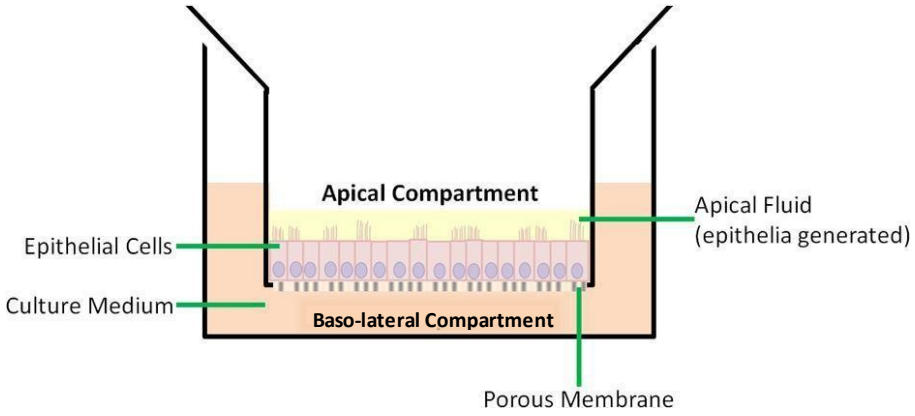

Fig. 5. Schematic cross-sectional representation of an air-liquid interface culture with epithelial cells (mucociliary phenotype). The culture system consists of an apical and a baso-lateral compartment, which are separated by a porous membrane support and the confluent epithelial tissue. Upon differentiation, epithelial cells of mucociliary phenotype secrete mucus fluid into the apical compartment. [Modified from Chen and Schoen, 2019] Reuse of the figure by permission of John Wiley and Sons (license number 5277690045384)

Organoids are derived from adult stem or progenitor cells. These cells are isolated from a primary cell culture and usually embedded in extracellular matrix components. They proliferate continuously and form a spheroid structure. In contrast to primary culture vesicles in suspension culture (mentioned above), the apical cell side is directed to the organoid lumen. The basal side of the cell is directed outwards and allows the continuous supply of nutrients from the surrounding medium to the cells. Oviduct epithelial organoid culture was reported for humans, laboratory mouse and domestic cattle [Bourdon et al., 2021; Chang et al., 2020; Kessler et al., 2015; Xie et al., 2018]. OEC organoids can reproduce the characteristic folds of the *in vivo* oviduct epithelium and can be maintained in culture for at least 16 months [Kessler et al., 2015]. The organoids grow continuously to a size of 2.5 mm in diameter, show an *in vivo* phenotype and differentiate similar to native tissue with expression of oviductin, paired box gene 8 (PAX8) and acetylated tubulin [Kessler et al, 2015; Xie et al, 2018]. The method was established in humans and laboratory mouse to investigate ovarian cancer [Kessler et al, 2015; Xie et al, 2018]. However, due to the specific cell orientation, an investigation of interactions with gametes or embryos is difficult.

Microfluidic cell culture allows a controlled fluid flow in the nanoliter scale. These chambers are in well-defined geometries and allow simultaneous manipulation and analysis from single cells up to tissues [Mehling and Tay, 2014]. It is used to characterize sperm migration, as well as oocyte and embryo development [Ferraz et al, 2017]. Ferraz et al. [2017, 2018] published the first oviduct on a chip model with microfluidics that function to simulate hormonal stimulation.

The recently developed option of 3D printing of cell culture substrates is likely to provide the best condition to mimic an organ-specific architecture. Ferraz et al. [2017] were the first who used an oviduct-on-a-chip model combining 3D printed substrates and microfluidics to mimic the bovine oviduct. A chamber was designed that includes a convex shaped transwell membrane as well as an independent double perfusion system, one to flush apical and another to flush the baso-lateral compartment. The system has been developed to investigate interactions of gametes and embryos with

the oviduct but also to examine the toxicity of different polymers commonly used in organ-on-chip devices [Ferrazet al., 2018; Ferrazet al., 2017].

1.7. Preservation of sperm

Sperm can be stored in the female reproductive tract for different time periods. In the domestic dog this period may last up to eleven days but some turtles can store sperm for four years and the Javan wart snake (*Acrochordus javanicus)* can maintain sperm for up to seven years [Holt, 2011]. Insects such as some bees and ants can store sperm for several years, which means almost their entire life [Orr and Zuk, 2012]. In terms of banking of genetic resource as carried out in the Felid Gamete Rescue Project [Fernandez Gonzalez et al, 2019] it is necessary to preserve semen and sperm *ex situ*. Besides sperm, oocytes, embryos and reproductive tissue are also cryopreserved in the Felid Gamete Rescue Project for future use in breeding programs or basic research. Assisted reproduction technologies (ARTs) such as artificial insemination mainly rely on frozen stored sperm, particularly in species where sperm cells have a high tolerance to cooling and freezing. Sperm of domestic cattle and domestic cat have a higher resistance against cold shock [Axnér et al., 2004.] than for instance domestic boar sperm [Green and Watson, 2001; Pursel et al., 1972]. Nevertheless, they cannot be cooled and frozen without protective agents since cooling, freezing as well as thawing cause damage to unprotected sperm cells.

Such damage affects the sperm membranes, which can be altered or damaged, but also sperm metabolism and mitochondrial energetic processes and DNA integrity [Hezavehei et al., 2018]. The damage is caused for instance by ice crystal formation, by dehydration and the resulting changes of cytosolic ion concentration and by irreversible temperature-related phase separations of membrane lipids [Hammerstedt et al., 1990; Öztürk et al., 2019]. Another issue is the release of reactive oxygen species (ROS) from dead and damaged sperm that cause oxidative stress [Khan et al., 2021]. The use of an extender should minimize osmotic, mechanical, biochemical and electrochemical stress [Ötztürk et al., 2019]. Therefore, the medium contains a buffer (typically TRIS buffer); one or more sugars as energy substrate or osmoticum (glucose, fructose, lactose, raffinose, saccharose, or trehalose); further solutes to adjust pH and osmolality (sodium citrate, citric acid), antioxidants to neutralize ROS (e.g. vitamin E analogues and L-carnitine) and cryo-protectants. Cryo-protectants are divided into membrane permeable and non-permeable substances. The permeable cryo-protectants include glycerol, DMSO and ethylenglycol [Hezaveheiet al., 2018; Layek et al., 2016].

Addition of cryo-protectants first increases extracellular osmolality, resulting in rapid dehydration of sperm. Much more slowly, an osmotic balance between intracellular and extracellular fluid becomes established as the cryo-protectants penetrate into the cells, accompanied by an influx of water. For this reason, freezing is preceded by a phase in which the cells are (simultaneously to the treatment with cryo-protectants) cooled to slow down metabolic processes. This phase in which sperm remain in contact with the cryo-protectant and cool down before freezing is defined as equilibration. During freezing, the extracellular water freezes earlier than the cytosol. Due to the lower extracellular vapor pressure, sperm are further deprived of water, which lowers the intracellular freezing point [Müller et al., 2009]. The glycerol which is now inside the sperm disturbs the hydrogen bonds between the water molecules and reduces the ice crystal formation. Thawing reverses the entire process.

Besides permeable cryo-protectants, which exert their action on the water structure inside the cells, non-permeable cryo-protectants including sugars as well as proteins and lipids exert their protective effect outside the cell. They promote dehydration of cells and have more direct effects on the sperm membranes. Commonly used proteins mainly derive from milk and egg yolk. Within the group of non-

permeable cryo-protectants, the low-density lipoprotein (LDL) is of special importance. LDL is an extract of mostly domestic chicken egg yolk and is composed of 83 % - 89 % lipids (69 % triglycerides, 26 % phospholipids and 5 % cholesterol) and 11 - 17 % proteins [Anton, 2013].
The precise mechanisms whereby LDL prevents sperm cells from cooling, freezing and thawing injuries are not fully understood. Different processes are discussed such as sperm membrane stabilization [Watson, 1975; Foulkes, 1977; MacDonald and Foulkes, 1981]. Foulkes et al. [1980] as well as Graham and Foote (1987) assume that phospholipids from LDL replace sperm membrane phospholipids which were degraded or lost during cryopreservation. Another theory suggests that LDL protects sperm not only against cold shock but also against the "toxic" action of seminal plasma constituents [Shannon and Curson, 1987]. These "toxins" are antibacterial cationic peptides that are released over time from large disaggregating proteins and cause severe membrane damage [Shannon and Curson, 1987]. It is possible that the cationic lipoproteins from the water-soluble extract of egg yolk compete with the detrimental cationic peptides of seminal plasma for binding to sites on the sperm plasma membrane [Vishwanath et al., 1992]. LDL can form complexes with BSP proteins, which prevent an early cholesterol and phospholipid efflux from the sperm membrane that would physiologically be mediated by BSP proteins to trigger the sperm capacitation [Bergeron et al., 2004; Bergeronand Manjunath, 2006; Manjunath et al., 2002].

1.8. Artificial insemination

During artificial insemination (AI), sperm can be deposited with the help of a catheter before, into or behind the cervix (intravaginal, intracervical, transcervical, intrauterine) or laparoscopically into uterus or oviduct (intrauterine, intratubal). The first methods are less invasive. However, the penetration of the cervix is only possible to a limited extent in felids, namely in the context of natural or ideally stimulated estrus [Chatdarong etal., 2001; Zambelli et al., 2004]. Fertilization success after AI varies. Since felids have a promiscuous mating behavior, the question arises whether a single intravaginal insemination may not be sufficient for successful fertilization. This does not seem to be the case, Sojka et al, [1970], Tanaka et al., [2000] and Callealta et al., [2019] showed that after a single AI with fresh semen, offspring could be produced in domestic cat and in the African lion (*Panthera leo*). It is reasonable to assume that other factors play a role.

Some scientists investigated the influence of the number of inseminated sperm cells on the success of fertilization [Lambo et al., 2012; Platz et al., 1978; Tanaka et al., 2000; Tsutsui et al., 2004, 2000]. In addition, the success of fertilization was examined in relation to sperm storage (fresh or cryopreserved) [Lambo et al., 2012]. Table 1 summarizes the studies where AI was performed in the domestic cat and in non-domestic felids, listed by method of AI. Mostly fresh or frozen/thawed ejaculated sperm were inseminated [Tsutsui, 2006]. Only two attempt were performed with cryopreserved epididymal sperm [Tsutsui et al., 2003; Toyonaga et al., 2011]. The closer the sperm are placed to the site of fertilization, the higher was the proportion of successful fertilizations. Fresh ejaculated sperm fertilized better than frozen/thawed semen. Until now, there was no direct comparison between insemination with fresh and cryopreserved epididymal sperm. *In vitro* studies indicate proportions of successful cleavage between 46 % and 68 % if cryopreserved ejaculated sperm were used for IVF [Herrick et al., 2010; Swanson, 2006; Zambelli et al., 2006] and proportions of successful cleavage between 25 % and 33 % for cryopreserved epididymal sperm [Eriani et al., 2008; Kunkitti, 2016; Lengwinat and Blottner, 1994]. Deficiencies in the functional sperm interaction of sperm with components of the female genital tract, including the oocytes, might be responsible for the limited success of frozen/thawed sperm compared to fresh semen and epididymal sperm compared to ejaculated sperm in AI and IVF.

We hypothesize that the missing contact of epididymal sperm with seminal plasma results in a decreased sperm-oviduct binding due to lack of sperm-associated seminal plasma proteins that mediate the specific oviduct-binding. Without building the functional sperm reservoir, these sperm cannot undergo the final maturation steps, namely capacitation, and do not attain the ability for fertilization. Another aspect concerns the handling of sperm during the cryopreservation process that affects both epididymal and ejaculate sperm. We hypothesize that, on the one hand, the temperature changes and, on the other hand, the extender components alter the sperm membrane in such a way that sperm-oviduct binding occurs only to a limited extent. Components of the freezing medium could block binding sites on the sperm membrane or change the composition of the membrane, as has been described in other animal species.

Tab. 1. Summary of the AI-trials in felids. OEP= orvus es paste, SLS = sodium lauryl sulfate¸ AV= artificial vagina, IVI = intravaginal insemination, UIUI = unilateral intrauterine horn insemination, TCIUI = transcervical intrauterine insemination, LIUI = laparoscopic intrauterine insemination, TVICI = transvaginal intracervical insemination, ETCIUI = endoscopic transcervical intrauterine insemination, EE = electro-ejaculation, UrCa = urethral catheterization, LITI = laparoscopic intratubal insemination, NB = natural breeding

Reference	**AI method**	**Species**	**Sperm collection**	**Sperm preservation**	**Numbers of inseminated sperm**	**Conception success** [% (n/n_{total})]
Sojka et al., 1970	IVI	Domestic cat	AV	fresh	1x with 5 – 25 *10^6 total 2x with 5 – 25 *10^6 total	50 % 75 %
Tanaka et al. 2000	IVI	Domestic cat	AV	fresh	20*10^6 total 80*10^6 total	7 % (1/16) 33 % (6/18)
Tsutsuiet al. 2004	UIUI	Domestic cat	AV	fresh	2*10^6 total 4*10^6 total 8*10^6 total	9 % (1/11) 18 % (2/11) 71 % (5/7)
Chatdarong et al., 2007	IVI vs TCIUI	Domestic cat	EE	fresh frozen/thawed	6.8-22*10^6 total 4.4-22*10^6 total 20*10^6 total 20*10^6 total	IVI at time of hCG Fresh 29 % (2/7) IVI 28 h later hCG Fresh 38 % (3/8) IVI at time of hCG Frozen 0 % (0/12) IUI at time of hCG Frozen 42 % (5/12)
Villaverde et al., 2009	IVI vs. LIUI	Domestic cat	AV	frozen/thawed	100*10^6 total	IVI 75 % (6/8) LIUI 0 % (0/8)
Goeritz et al., 2012	TVICI	African lion (*Pantheraleo*)	EE	fresh	72*10^6 total	67 % (2/3)
Lueders et al., 2014	TCIUI IVI	Asiatic golden cat (*Catopuma temmincki*)	UrCa EE	fresh	Not stated	100 % (1/1)
Callealta et al., 2019	TCIUI IVI	African lion (*Panthera leo*)	UrCa EE	fresh	1.4 - 1215.2*10^6	33 % (4 /12)
Zambelli et al., 2015	ETCIUI	Domestic cat	UrCa	fresh	2x with 8 – 53.3*10^6 total	100 % (3/3)
Howard et al., 1992a	LIUI	Domestic cat	EE	fresh	5.5*10^6 motile 6.8*10^6 motile	pre-ovulated 14 % (2/14) post-ovulated 50 % (9/18)

Tsutsui 2000	LUIUI	Domestic cat	AV	fresh	$2*10^6$ total $4*10^6$ total $8*10^6$ total	13 % (2/16) 31 % (5/16) 80 % (8/10)
Barone et al., 1994	LIUI	Puma (*Felis concolor*)	EE	fresh	$5 – 50*10^6$ motile	11 % (1/9)
Howard et al., 1997	LIUI	Cheetah (*Acinonyx jubatus*) Clouded leopard (*Neofelis nebulosa*)	EE	fresh	$1.4 – 28.6*10^6$ motile $2.5 – 43.2*10^6$ motile	32 % (6/19) 0 % (0/18)
Howard et al., 1996	LIUI	Clouded leopard (*Neofelis nebulosa*)	EE	fresh	$40 – 70*10^6$ motile	20 % (1/5)
Roth et al., 1993	NB LIUI	Domestic cat	EE	fresh	$2\text{-}14*10^6$ motile	100 % (24/24) 100 % (6/6)
Howard et al., 1992b	LIUI	Cheetah (*Acinonyx jubatus*)	EE	fresh	$1.5 – 13.6*10^6$ motile	17 % (1/6)
Tajima et al., 2017	LIUI	Amur leopard cat (*Pionailurus bengalensis eutilura*)	EE	fresh	$9.6*10^6$ - $21*10^6$ totalper cat	100 % (2/2)
Jordan et al., 1996	LIUI	Domestic cat	EE	fresh frozen/thawed	$2\text{-}90*10^6$ total	50 % (3/6) 0 % (0/6)
Tsutsuiet al. 2000	LUIUI	Domestic cat	AV	frozen/thawed	$50 *10^6$ total	57 % (8/14)
Tsutsuiet al. 2003	LIUI	Domestic cat	epididymal sperm	frozen/thawed	$50*10^6$ total	27 % (3/11)
Tsutsuiet al., 2011	LIUI	Domestic cat	AV	frozen/thawed	$40*10^6$ total	OEP 70 % (7/10) SLS 30 % (3/10)
Baran, 2010	LIUI	Domestic cat	EE	frozen/thawed	$50*10^6$ total	EY-TFC 43 % (3/7) EY-SMGT 33 % (2/6)
	LIUI	Domestic cat	AV	fresh	$1*10^6$ motile per AI side	18 % (2/11)

Conforti et al., 2013	LITI LIUI & LITI					46 % (5/11) 36 % (4/11)
Toyonaga et al., 2011	LIUI LITI	Domestic cat	epididymal sperm	frozen/thawed	$40*10^6$ total $10*10^6$ total	0h stored before cryo-preservation 29 % (2/7) 24h stored before cryo-preservation 29 % (2/7) 24h after hCG injection 80 % (4/5) 30h after hCG injection 20 % (1/5)
Lambo et al. 2012	LITI	Domestic cat	AV	fresh frozen/thawed	$2*10^6$ motile $4*10^6$ motile	100 % (7/7) 43 % (3/7)
Chansaenroj et al., 2011	LITI	Domestic cat	E	frozen/thawed	$5*10^6$	20 % (1/5)
Tipkantha et al., 2017	LITI	Clouded leopard (*Neofelis nebulosa*)	EE	fresh	$10.7*10^6$ motile	100 % (1/1)

2. Aims of the thesis

In endangered felid species, ARTs and especially AI are a tool to maintain and expand genetic diversity. Because of the peculiarities in feline reproduction it is necessary to first improve the knowledge about the physiological processes in the female reproductive tract, especially in the oviduct. The overall aim of this work was to optimize AI in felids. It is known that fertilization success is lower using epididymal and/or cryopreserved sperm than with ejaculated and fresh sperm. But it is not clear whether the lack of seminal plasma and/or the contact with the freezing extender during cooling and freezing are responsible for the reduced conception success. Therefore, we looked at these two important aspects which may play a role in sperm-oviduct interaction. Using the domestic cat as model species, we wanted to develop suggestions to design more physiological preservation and insemination protocols to improve the efficiency of ARTs in endangered felids. In addition, we took initial steps to establish a long-term culture of OECs of the domestic cat to accomplish a reproducible *in vitro* model for application in basic reproductive research as well as in assisted reproduction.

First, we investigated the impact of seminal plasma (chapter I, also referred to as SP-study) and second, the influence of sperm dilution in a freezing extender and cooling to 15°C (chapter II, also referred to as equilibration-study) on the sperm-oviduct binding capacity in the domestic cat. The aim of the first study was to investigate, whether (1) SP has an effect on epididymal sperm quality, (2) epididymal sperm are able to bind to oviduct epithelial cells *in vitro*, and (3) whether SP has an effect on the *in vitro* binding efficiency. Therefore, epididymal sperm (with or without SP) were co-incubated with freshly isolated OECs referred to as explants (chapter I).

In the second study we examined whether the contact of sperm to the freezing extender during equilibration might change sperm-oviduct binding patterns in quantity and quality. For this purpose, epididymal sperm (with or without equilibration) were co-incubated with isolated OECs after short-term suspension culture, referred to as feline OEC (FOEC) vesicles (chapter II).

Explants and vesicles which were used in the first and second study, respectively, have the disadvantage that their viability and differentiation state is limited to a short time. The polarized OEC cultured in a compartmentalized system is well established and characterized in the laboratory mouse, domestic pig, domestic cattle and humans and provides a stable model for at least three weeks. These OEC have a phenotype similar to *in vivo* tissue and are well differentiated. The aim of the third study (chapter III) was to test whether (1) the compartmentalized culture approach using media optimized for laboratory mouse and domestic pig OEC is generally applicable to FOEC, (2) it is possible to generate long-term culture cells from the ampulla and isthmus region of the feline oviduct separately, and (3) highly differentiated FOEC cultures can be obtained from the heterogeneous basic material available after routine castrations of domestic cats.

3. References

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4. Research articles

4.1. Chapter I - 1st Publication

Seminal fluid promotes *in vitro* sperm–oviduct binding in the domestic cat (*Felis catus*)

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Contribution of each co-author to the manuscript:

FH, SE, KR, JS, AF, KM designed the study.
FH, SE conceived and performed the experiments.
FH, SE primarily analyzed the data.
GW performed electron microscopy.
FH, SE, KM drafted the manuscript.
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Seminal fluid promotes *in vitro* sperm–oviduct binding in the domestic cat (*Felis catus*)

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ABSTRACT

From many endangered or threatened species which are expected to profit from assisted reproduction techniques, mainly epididymal sperm of dead or freshly castrated males are available. These sperm had contact to epididymal secretion products but not to seminal fluid components. Notably, products of accessory sex glands have been shown in domestic animals to condition sperm for fertilization, in particular by mediating sperm–oviduct interaction. We report for the first time that motile epididymal sperm from domestic cats are able to bind to fresh oviduct epithelial cell explants from preovulatory females (median [min, max] of 10 [8, 16] and 10 [8, 17] sperm per 0.01 mm^2 explant surface from both isthmic and ampullar regions, respectively). More sperm attach to the explants when epididymal sperm were preincubated for 30 minutes with seminal fluid separated from electroejaculates of mature tomcats (median [min, max] of 17 [13, 25] and 16 [12, 21] sperm per 0.01 mm^2 explant surface from isthmus and ampulla, respectively). The proportion of bound sperm increased from a median of 54% to 62% by seminal fluid treatment. Sperm–oviduct binding could be facilitated by the decelerated sperm motion which was observed in seminal fluid–treated samples or supported by seminal fluid proteins newly attached to the sperm surface. Seminal fluid had no effect on the proportion of sperm with active mitochondria. Extent and pattern of sperm interaction *in vitro* were independent of explant origin from isthmus or ampulla. Sperm were attached to both cilia and microvilli of the main epithelial cell types present in all explants. In contrast to published sperm-binding studies with porcine and bovine oviduct explants where predominantly the anterior head region of sperm was attached to ciliated cells, the tails of some cat sperm were firmly stuck to the oviduct cell surfaces, whereas the heads were wobbling. Whether this response is a preliminary step toward phagocytosis or a precondition to capacitation and fertilization remains to be determined. In conclusion, treatment of epididymal sperm with seminal fluid or particular protein components should be considered in future investigations for its potential to improve the outcome of artificial insemination in felids.

1. Introduction

Most felid species are listed on the International Union for Conservation of Nature's red list of threatened or endangered species. Therefore, optimized technologies for

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assisted reproduction are of increasing importance to support conservation strategies. Testes and epididymides of dead or euthanized individuals often are the only source of male gametes available for artificial insemination. However, those sperm have not been in contact with seminal fluid which is known to mediate the interaction of sperm with epithelial cells in the female reproductive tract [1] particularly in the oviduct [2].

Previous work in other mammalian species has revealed that sperm are caught by epithelial cells in the uterotubal junction and caudal isthmus creating a sperm reservoir (for review see [3–5]). Hunter [6] was the first to propose the term "functional sperm reservoir" for the pig oviduct as contact to the oviduct epithelium maintains sperm viability until ovulation and controls sperm transport, capacitation, and fertilization. In the cow and pig, it was shown that seminal fluid proteins are involved in sperm–oviduct binding [7,8].

To investigate reproductive physiology in felid species and to develop assisted reproduction techniques in endangered nondomestic felids, the domestic cat (*Felis catus*) serves as a valuable comparative model. In 1993, Roth et al. [9] observed that washed electroejaculated cat sperm, after swim-up, bind to oviductal epithelial cells generated from a primary cat oviduct cell culture to support fertilization *in vitro*. Chatdarong et al. [10] reported that the cervix and the uterotubal junction serve as barriers for sperm transport in the domestic cat genital tract, a species in which ovulation is induced by mating and occurs 25 to 32 hours later. Although the uterine crypts and the uterotubal junction act as sperm reservoirs before ovulation, the oviduct, especially the isthmus, is the primary sperm reservoir around the time of ovulation.

In both cats [11] and dogs [12], intrauterine insemination with frozen ejaculated semen has been more successful than with frozen epididymal sperm [13,14]. Thus, seminal fluid components may enhance the outcome of artificial insemination in domestic carnivores. When epididymal sperm are deposited directly into the preovulatory oviduct (24 hours after hCG treatment), pregnancy rates were higher than those obtained after insemination at 30 hours after hCG administration [11]. Because it is known that cat sperm also undergo capacitation in the female genital tract [15], we suggest that a functional sperm–oviduct interaction must occur to prime epididymal sperm for the fertilization process. Cat epididymal sperm are phagocytized by luminal macrophages and nonciliated epithelial cells of the oviduct [16], but it is not known whether or not epididymal cat sperm can bind to uterine or oviductal cells. In pigs and cows, epididymal sperm interact with oviduct cell explants *in vitro*, and seminal fluid has a positive effect on the quantity of bound sperm [17,18]; however, sperm binding to the isthmic part of the oviduct is not absolutely necessary for *in vivo* fertilization to occur [19].

The aim of the present study was to investigate, in the domestic cat, whether (1) seminal fluid has an effect on sperm quality *in vitro*, (2) epididymal sperm are able to bind to oviduct epithelial cells *in vitro*, and (3) whether seminal fluid has an effect on binding efficiency. To accomplish our goals, an *in vitro*–binding assay was established with fresh oviduct epithelial cell explants and sperm from cauda epididymis and proximal ductus deferens. Seminal fluid was obtained from electroejaculates of mature male cats. We anticipate that results of the present study will give suggestions to design more physiological preservation and insemination protocols for improving the efficiency of assisted reproduction techniques in endangered species.

2. Materials and methods

Unless otherwise stated, chemicals were obtained from Sigma–Aldrich Chemical Company (St Louis, MO, USA or Steinheim, Germany). The experimental protocols were approved by the Ethics Committee of the National Veterinary School of Alfort and the Leibniz Institute for Zoo and Wildlife Research.

2.1. Experimental design

To analyze sperm–oviduct binding, epididymal sperm of one mature male and an oviduct from one follicular stage female were obtained on the same day. Two aliquots of epithelial cells were prepared each from the ampulla and caudal isthmus of the oviduct. The epididymal sperm suspension was split and incubated for 30 minutes with culture medium or seminal fluid before it was added to the oviduct epithelial cells for 60 minutes. Seminal fluid of mature males was prepared from samples obtained by electroejaculation and stored frozen (see in the following). To avoid repeated thawing and freezing, two or three aliquots from different individuals were thawed immediately before the binding experiment and pooled to give a volume of 80 μL and a protein concentration of about 10 mg/mL. Sperm quality was evaluated after incubation with culture medium or seminal fluid. Sperm–oviduct cell binding was assessed by two independent methods. First, after coincubation and flushing, unbound sperm were counted and subtracted from the initial sperm number to subsequently calculate the percentage of bound sperm. Second, bound sperm were counted directly on the oviduct epithelial cell surface after flushing, and the number was extrapolated to a standardized surface area (binding index). Seven independent experiments were performed.

2.2. Collection of semen by electroejaculation

Semen samples were collected by an electroejaculation procedure at the veterinary college of Alfort and at the rescue shelter, Society for Protection of Animals in Paris, France, from privately owned or stray cats that were aged between 7 months and 4 years. The electroejaculation procedure was done under the supervision of a veterinarian with appropriate training on the use of equipment and an understanding of and ability to recognize and manage potential adverse outcomes. The equipment and procedures used were appropriate for cats. Electroejaculation was performed during anesthesia induced by ketamine HCl (intramuscular, 5 mg/kg of body weight) in combination with medetomidine (80 μg/kg of body weight, Domitor veterinary). An electroejaculator (PT Electronics, OR, USA)

with a rectal probe gives electrical stimuli to the hypogastric and the pudendal nerves which are involved in penile erection, stimulation of the accessory glands, and in ejaculation. The rectal probe had three longitudinal electrodes directed ventrally during electrical stimulation. The cat was placed in lateral recumbency, and the probe was lubricated and inserted in the rectum to a depth of 6 cm. To induce electroejaculation, each cat was first given a series of 40 stimuli from 2 to 4 V, according to the protocol derived from Howard et al. [20]. If an electroejaculate was obtained, a second series of 40 stimuli from 3 to 5 V was given after 5 minutes of rest followed by a final series of 40 stimuli from 4 to 6 V. Because of ejaculate variability, each ejaculate was evaluated as an individual sample. Because the volume of most seminal fluid samples was not sufficient for a binding experiment, individual samples were pooled immediately before sperm treatment (described previously).

2.3. Evaluation of electroejaculates and separation of seminal fluid

Immediately after collection, ejaculate volume was measured with a variable micropipette (10–100 μL) and 20 μL of the ejaculate was diluted with 40-μL M199 (HEPES modification, Sigma–Aldrich M7528) for evaluation of motility and sperm concentration. Then, the sample was centrifuged at 1000 × *g* for 10 minutes, and the resulting supernatant was centrifuged a second time at 12000 × *g* for 1 minute to remove sperm. The seminal fluid was transferred into a 1.5-mL tube; its volume was determined with a micropipette and stored at −20 °C until it was used for the experiments. A 2.5-μL aliquot of diluted semen was loaded into a prewarmed (38 °C) Leja chamber (20 μm depth), and sperm motility was evaluated under a phase-contrast microscope (magnification: × 20, objective) with the SpermVision software (Minitüb, Germany). Sperm concentration was determined in a Thoma chamber under a phase-contrast microscope. The protein concentration (mg/mL) in seminal fluid was determined in 2-μL samples by spectrophotometry at 280 nm. In total, 16 seminal fluid samples of 10 to 80 μL from 12 males were used for the binding experiments (see in the following). The mean sperm motility in the respective ejaculates was 48 ± 23%, the mean sperm number was 11 ± 8 × 10^6, and the mean protein concentration in seminal fluid samples was 10 ± 6 mg/mL.

2.4. Collection of sperm from cauda epididymis

After castration of male cats in the rescue shelter or by veterinarians in Berlin, Germany, testes and epididymides were recovered and transported to the laboratory at 4 °C. Sperm of one male per experiment were collected by slicing the cauda epididymis and the proximal ductus deferens together in 1-mL culture medium (M199 Sigma M4530, supplemented with fetal calf serum, 10%; cysteine, 0.01%; HEPES, 5.67 mM; sodium pyruvate, 2.2 mM; L-glutamine, 2 mM; gentamicin, 53 μg/mL) at 38 °C. The sperm were flushed through a 20-μm filter (Partec, Germany). Sperm concentration was adjusted to ~13 × 10^6 cells/mL.

2.5. Incubation of epididymal sperm with culture medium or seminal fluid

The sperm suspension was split, transferred into two 1.5-mL tubes, each containing ~6.5 × 10^6 sperm, and sperm were washed by centrifugation at 800 × *g* for 8 minutes. The pellet was resuspended with 80 μL of culture medium or with 80 μL of seminal fluid. After incubation (30 minutes, 38 °C, 5% CO_2, high humidity), sperm were added to oviduct explants without further washing. An aliquot of each sample was used to determine the effect of seminal fluid on sperm viability and motility.

2.6. Evaluation of epididymal sperm after incubation (culture medium or seminal fluid)

Motility and sperm concentration were analyzed in epididymal sperm samples after incubation by the aforementioned methods for samples obtained by electroejaculation. The percentages of motile and progressively motile sperm, the mean velocity (curvilinear velocity) of progressively motile sperm, and the percentage of high-speed sperm (curvilinear velocity > 120 μm/s) were determined. Also, sperm viability was evaluated [21] using rhodamine 123 (R123; Invitrogen, Molecular Probes, Eugene, OR, USA) and propidium iodide (PI; Invitrogen, Molecular probes). One hundred microliters of sperm suspended (about 1 × 10^6 sperm) in either culture medium or seminal fluid was stained with 20-μL R123 (5 mg/100 mL stock in aqua bidest. diluted 1:50 with PBS [Sigma D8662]; final concentration, 20 ng per 10^6 sperm) and 5-μL PI (1 mg/mL stock in aqua bidest diluted 1:5 with PBS; final concentration, 1 μg per 10^6 sperm). Stained sperm were incubated in the dark for 20 minutes at 38 °C in a humidified atmosphere of 5% CO_2 in air. Twenty-five microliters of stained sperm suspension (~0.2 × 10^6 sperm) were diluted in 2-mL PBS at 38 °C for measurement in a flowcytometer (CyFlow space and FloMax software; Partec) equipped with a 50-mW solid-state laser (Ex 488 nm), a 515- to 560-nm band-pass for R123 (green), and a 620-nm long-pass filter for PI (red). The system was triggered on the forward light scatter, and 15,000 cells per sample were characterized for their fluorescence at a flow rate of about 150 cells/s. Sperm with active mitochondria accumulate R123 and show bright green fluorescence, whereas dead sperm allow PI to enter the nucleus and appear red. Rhodamine 123–positive sperm were considered to be viable.

2.7. Preparation of oviduct epithelial cell explants (OEC)

During the spring season, oviducts of mature female domestic cats were collected after castration in the rescue shelter or by veterinarians in Berlin, Germany. The stage of oviducts was assessed by examination of ovarian follicle sizes. Only oviducts from cats with follicles greater than 2 mm were used for the binding experiments. The surrounding tissues were removed, and the oviducts were flushed with PBS (Sigma D8662). The ampulla and isthmus were separated and gently squeezed between two toothpicks to expell their contents onto glass slides. The OEC were recovered in 500-μL culture medium (mentioned

previously) and washed two times by centrifugation steps at 300 × *g* for 3 minutes, After measuring biomass in VoluPAC tubes (VWR, Germany) by centrifugation at 13,000 × *g* for 1 minute, the OEC concentration was adjusted so that all experiments were performed under similar conditions. The viability of OEC was assessed microscopically by evaluating their ciliary activity at × 200 magnification.

2.8. Coincubation of sperm with OEC and binding assays

In a 1.5-mL tube, 20-μL sperm suspended (~1.6×10^6 sperm) in either culture medium or seminal fluid was added to 40-μL OEC. After coincubation for 1 hour (38 °C, humidified atmosphere of 5% CO_2 in air), OEC were thoroughly washed with 500-μL culture medium through a 20-μm filter (Partec). First, the unbound sperm were recovered in a 1.5-mL tube, and the concentration was determined. The percentage of bound sperm was calculated for either culture medium or seminal fluid in each region of the oviduct. Second, after flushing, some of the OECs were pipetted between two coverslips to be observed under a wide-field inverted microscope (Olympus IX81) using differential interference-contrast optics at × 400 magnification. The OECs were photographed and videotaped within 10 minutes. In five consecutive photographs, the complete area of OEC was screened for bound sperm, and the binding index (number of bound sperm per 0.01 mm^2) was determined.

Some OEC were fixed in Karnovsky's solution (Serva, Heidelberg, Germany) for 12 hours at 4 °C. The samples were washed in PBS three times for 5 minutes each. Subsequently, dehydration was performed through a graded ethanol series (30%, 50%, 75%, 95%, 100%), and samples were submerged in hexamethyldisilazane for 10 minutes. After allowing to air-dry, specimens were sputter coated with gold–palladium. Examination was performed by scanning electron microscopy (Zeiss Supra 40VP) at different magnifications.

All binding experiments were performed within 4 hours after OEC preparation.

2.9. Statistical analysis

The determination of sperm concentration was performed (three times) in the flushing medium before and after coincubation with OEC. The determination of bound sperm was performed in five photographs with a total OEC area of 0.01 to 0.03 mm^2 per sample. Data are presented by means of boxplots (R 2.15). Medians, the 25th and 75th percentiles are shown as vertical boxes and whiskers. Outliers are denoted by circles. Means are included by squares. Comparisons of paired data sets were done using nonparametric Wilcoxon signed-rank test (PASW Statistics 18, SPSS Inc.). The criterion for statistical significance was an error probability of 0.05.

3. Results

Epididymal sperm were prepared and incubated for 30 minutes in either seminal fluid or culture medium. The influence of seminal fluid on epididymal sperm quality was analyzed before coincubation of treated sperm with oviduct epithelial cell explants. As shown in Fig. 1, the number of viable sperm in seminal fluid (median of 53%) determined by flowcytometry after staining with rhodamine 123 and PI was not different compared with incubation in medium (54%). Sperm motility was analyzed in parallel by a computer assisted sperm analysis system. The percentages of motile and progressively motile sperm were significantly lower in seminal fluid (median of 48% and 30%, respectively) than those in medium (64% and 49%, respectively). Also, the proportion of high-speed sperm and accordingly, the mean velocity of progressively motile sperm were significantly reduced in seminal fluid.

Using standardized sperm and oviduct cell concentrations in our binding experiment, 53.5% and 54.2% (medians) of epididymal sperm attached to fresh oviduct epithelial cell explants from isthmus and ampulla, respectively (Fig. 2A). This corresponds to 10.4 and 10.3 sperm per 0.01 mm^2 explant surface (Fig. 2B). Both binding parameters were similar for the different oviduct regions.

When epididymal sperm were preincubated in seminal fluid before the binding experiment, the percentage of bound sperm and the number of bound sperm per explant surface area were significantly higher in both oviductal compartments (Fig. 2), medians of 61.8% and 61.2%, in the isthmus and ampulla, respectively, than those found when sperm were not preincubated. With two exceptions, the ratio between the percentage of seminal fluid– and medium-treated bound sperm (isthmus: 1.07–1.36, ampulla: 0.97–1.41) and the ratio of corresponding data pairs for the binding index (isthmus: 1.07–3.11, ampulla: 1.00–2.18) were greater than 1.

Observation of explants from each oviduct by wide-field microscopy revealed that, independently of sperm pretreatment and oviduct region, nearly all bound sperm were

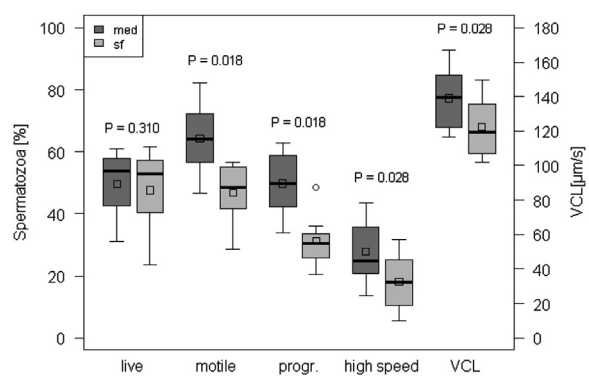

Fig. 1. Viability and motility parameters of epididymal cat sperm after incubation in medium (med) or seminal fluid (sf) for 30 minutes at 38 °C under 5% CO_2: proportion of vital sperm after rhodamine 123/propidium iodide staining as determined by flowcytometry. Proportions of motile, progressively motile, and high-speed sperm, as well as curvilinear velocity (VCL) of progressively motile sperm are measured using SpermVision (Minitüb) software. Data are displayed by means of boxplots (R 2.15). Medians, the 25th and 75th percentiles are shown as vertical boxes and whiskers. Outliers are denoted by circles. Means are included by squares. P values (Wilcoxon signed-rank test) of significant differences between med and sf are given (N = 7).

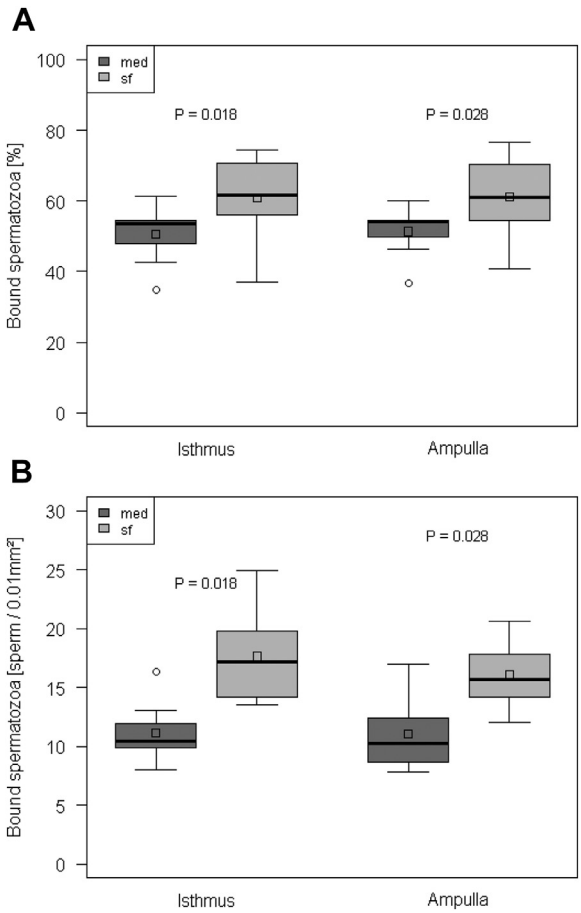

Fig. 2. Sperm–oviduct binding of cat epididymal sperm to oviduct epithelial cell explants. Epididymal sperm were preincubated with medium (med) or seminal fluid (sf). The percentage of bound sperm was calculated after counting unbound sperm in the flushing medium (A), the number of bound sperm per area was counted directly on the explants (B). Data are displayed by means of boxplots (R 2.15). Medians, the 25th and 75th percentiles are shown as vertical boxes and whiskers. Outliers are denoted by circles. Means are included by squares. P values (Wilcoxon signed-rank test) of significant differences between med and sf are given (N = 7).

motile (see Supplementary Video files). Two types of sperm binding were observed: Most sperm heads were attached to the oviductal cells with the tails freely moving. On the other hand, some sperm tails were tightly attached to the oviductal cells, and head displacement was observed.

Scanning electron microscopy analysis confirmed that both interaction types of epididymal sperm (head and tail binding) occur with both types of oviduct explant cells (ciliated and secretory cells), and this is independent of (1) preincubation in seminal fluid and (2) the origin of explants (isthmus or ampulla; Fig. 3).

4. Discussion

Sperm–oviduct interaction is a physiological part of the reproductive process. The oviduct as a functional sperm reservoir has been extensively characterized in domestic animals [3–5]. Although *in vitro* systems lack the regulatory function of the ovaries on oviductal epithelium and the physical barriers of oviductal mucus [22], fresh oviduct epithelial cell explants (OEC) provide a simple approach to study basic principles of sperm–oviduct interaction [17,23].

Previous studies in the pig and bovine showed that epididymal sperm are able to bind to OEC *in vitro*, although to a lower extent than ejaculated sperm [7,17,18]. Almost all bound sperm show flagellar motion. Proteins known to participate in the interaction of sperm with the carbohydrate moieties of oviduct epithelial cells are derived from secretions of epididymal cells and male accessory sex glands (for review see [24]). They belong to the groups of fibronectin type II proteins (such as PDC-109 in bovine) and of spermadhesins (such as AWN or AQN in pigs). Sperm may acquire the proteins during epididymal transit, but their predominant presence is in seminal fluid and, thus, acquisition by sperm occurs mostly at ejaculation [25,26]. This difference in proteins between the epididymal and ejaculated sperm could explain the improved OEC binding capability of sperm obtained by ejaculation. In our study, we show for the first time that motile epididymal cat sperm bind to feline preovulatory OEC and that the portion of bound sperm is increased after preincubation in seminal fluid. Our results suggest the mechanism(s) for sperm–oviduct binding in cats is similar to that of mammals studied previously. A variety of unidentified proteins in the range from 3.5 to 200 kDa is present in domestic cat seminal fluid (a mean protein concentration of 3.1 mg/mL) [27]. In our study, seminal fluid separated from ejaculates of mature males had a mean protein concentration of 10 ± 6 mg/mL. The sperm concentration during incubation in seminal fluid was adjusted to ~30% to 50% of the values reported by Zambelli et al. [27] or that of the electro-ejaculates used in our study.

Incubation in seminal fluid reduced sperm motility, whereas sperm viability remained unchanged. However, the determination of R123-positive cells in the control (incubation in culture medium) revealed a lower amount of vital than motile sperm. Because the proportion of vital sperm was at the level of progressively moving cells, we suggest that the population of nonprogressively motile sperm is comparatively susceptible to the staining procedure. This is normally not the case in ejaculated sperm but may be indicative of a high sensitivity in a subpopulation of cat epididymal sperm to the staining procedure.

The lower sperm velocity in the presence of seminal fluid could be related to the decapacitation effects of seminal fluid. A number of proteins found in seminal fluid of domestic animals have been identified as regulators of *in vitro* capacitation [28]. Baas et al. [29] reported that bull seminal fluid not only contains low-molecular motility-stimulating components but also high-molecular weight factors which inactivate sperm with time. This is in accordance with a study of Thuwanut and Chatdarong [30] where incubation of postthaw epididymal cat sperm with seminal fluid resulted in reduced percentages of motile sperm, lower scores of progressive motility, and less sperm with intact plasma membrane. When ejaculated and epididymal cat sperm were applied to cooling, inconsistent sperm properties were described regarding the presence of seminal fluid. Although ejaculated and

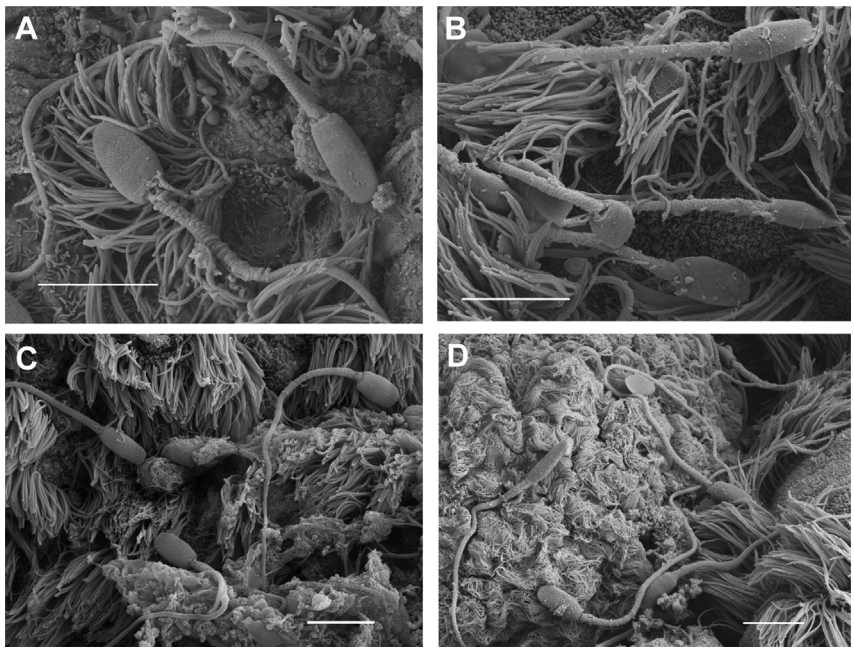

Fig. 3. Illustration of sperm binding to oviduct epithelial cell explants from isthmus (A, B) and ampulla (C, D) region by scanning electron microscopy. Epididymal sperm were preincubated with medium (A, C) or seminal fluid (B, D) for 30 minutes at 38 °C under 5% CO_2. Each bar corresponds to 5 μm.

epididymal cat spermatozoa were not statistically compared in the study of Harris et al. [31], mean motility was higher in epididymal sperm than that in ejaculated sperm up to 23 days of cooled storage in TEST yolk buffer. Epididymal sperm motility was better preserved in the presence of egg yolk than in a glucose–BSA solution, whereas ejaculated sperm had similar motility in both media. Hermansson and Axnér [32] reported that both motility and plasma membrane integrity were lower in cooled epididymal sperm than those in ejaculated sperm. The presence of egg yolk was beneficial for both sperm types. Tebet et al. [33] found no differences between the electroejaculated and epididymal fresh or frozen-thawed sperm of the same individuals for overall and progressive motility as well as membrane integrity. An interpretation of these results is difficult because interactions between seminal fluid components in ejaculates, cryoprotective agents, and cooling are still unknown and may interfere with the processes designated to occur *in vivo*.

However, the decapacitating ability of cat seminal fluid on rabbit fertilization *in vivo* has been observed [34]. Uncapacitated sperm have lower velocity than do capacitated sperm, and binding assays in pigs [35] and cows [36] showed a superior attachment of uncapacitated sperm. Sperm–oviduct interaction is initiated by binding of uncapacitated sperm and is continued by the induction of capacitation [35]. Induction of capacitation and initiation of the hyperactivated flagellar beating pattern appear to coincide with the state of sperm when released from the oviductal reservoir. Within the transilluminated oviducts of naturally mated mice, hyperactivated sperm were seen swimming freely, whereas nonhyperactivated sperm appeared to be tightly bound to the epithelium [37].

Sperm binding for storage and proper timing of capacitation and sperm release with respect to ovulation are of crucial importance for successful fertilization. We hypothesize that the presence of seminal fluid regulatory factors supports epididymal sperm binding as a preparative step for fertilization also in the cat oviduct. We do not know if the increased number of bound seminal fluid–treated sperm is solely a result of their reduced velocity or motility or is, additionally, mediated by sperm-associated seminal fluid proteins with specific oviduct-binding sites.

The oviduct is lined with a columnar epithelium, which undergoes morphologic and biochemical changes because of steroid hormone oscillations during the estrous cycle [38,39]. Oviductal epithelial cells extracted during the follicular stage typically consist of two types, ciliated cells and secretory cells exhibiting numerous microvilli. In species studied so far, sperm bind primarily to ciliated cells, but binding also occurs to nonciliated cells [5]. The binding is limited mostly to sperm heads. From studies applying OEC or cultured monolayers, it is well known that the sperm tails perform an undulating movement. Roth et al. [9] noted that electroejaculated cat sperm interacted in a similar manner with an oviductal cell monolayer generated from primary cultures after ovariohysterectomy. The first report on sperm–oviduct interaction in cats [16], however, only described phagocytotic events by nonciliated cells which were fixed three hours after surgical injection of epididymal sperm into the oviduct of a female at an unknown stage of the estrous cycle. Previously, such events were observed in the lower isthmic region of the oviduct in mice [40] and bats [41] and are believed to remove surplus male gametes from the female genital tract after fertilization [16].

Our binding studies revealed that ciliated and non-ciliated cells of OEC from preovulatory follicular phase oviducts interact with vital sperm and prevent them from moving freely in the surrounding medium. After natural mating, cat sperm were observed to attach firmly to all epithelia of the female genital tract [10]. Although we found that most sperm were attached to OEC by their heads, some sperm were tightly stuck to the OEC by their tails. Sperm attached at the tail showed vivid flagellar activity (head movement) indicating that they were still vital. Whether particular sperm–oviduct interaction is a mechanism to store functional sperm, or a selective step to disable individual sperm from participating in fertilization, remains open.

Cat sperm are normally ejaculated into the anterior vagina [5]. During the basically passive passage through the cervix and transit through the uterotubal junction, the sperm selection process occurs, and the isthmus is the site for sperm storage during the periovulatory period [10]. Fewer sperm were attached to the epithelium of the ampullar region. In our study, no differences in sperm binding between the isthmic and ampullar part of the oviduct were observed. This was also described in a sperm-binding study using OEC from pigs [17]. However, because of unphysiological culture conditions and the loss of the local fine regulation by estrogen and progesterone, oviduct epithelium properties are rapidly lost in epithelial explant culture systems currently used [42,43]. The development of new cell culture techniques that would enable the successful establishment of highly differentiated and polarized primary long-term cultures of oviduct epithelial cells would be a useful tool for investigating local aspects of epithelial function and physiology of sperm [44].

In the present study, we showed that motile epididymal cat sperm are selected to interact with oviduct epithelial cells and more sperm attach after preincubation with seminal fluid. Sperm–oviduct interaction may prime sperm for fertilization by storing them until ovulation, mediating their capacitation, and gradually releasing them to avoid polyspermia. Cat seminal fluid has been reported to be able to reduce sperm motility during preservation [30], and it interferes with fertilization by acting as an decapacitating agent when added to the oviduct [34]. Nonetheless, we contend that preincubation of epididymal sperm with seminal fluid or particular protein components may improve the outcome of artificial insemination by supporting more physiological sperm behavior in the oviduct. Epididymal semen is often the only source of gametes from valuable males after castration or *postmortem* in conservation breeding programs.

4.1. Conclusions

In conclusion, sperm–oviduct binding in cats seems to be selective for motile sperm. Although epididymal sperm are able to attach to oviduct epithelial cell explants, our results suggest that seminal fluid components may play a supportive role in the binding process. The oviductal explant binding assay represents a useful but limited approach for studying sperm–oviduct interaction. The nature of sperm interactions with cilia and microvilli in the oviduct remains to be determined, and the future identification of mechanisms and molecules involved in sperm–oviduct interaction may advance our understanding of sperm physiology within the female reproductive tract.

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Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2015.01.031.

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4.2. Chapter II – 2nd Publication

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

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Contribution of each co-author to the manuscript:

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

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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,

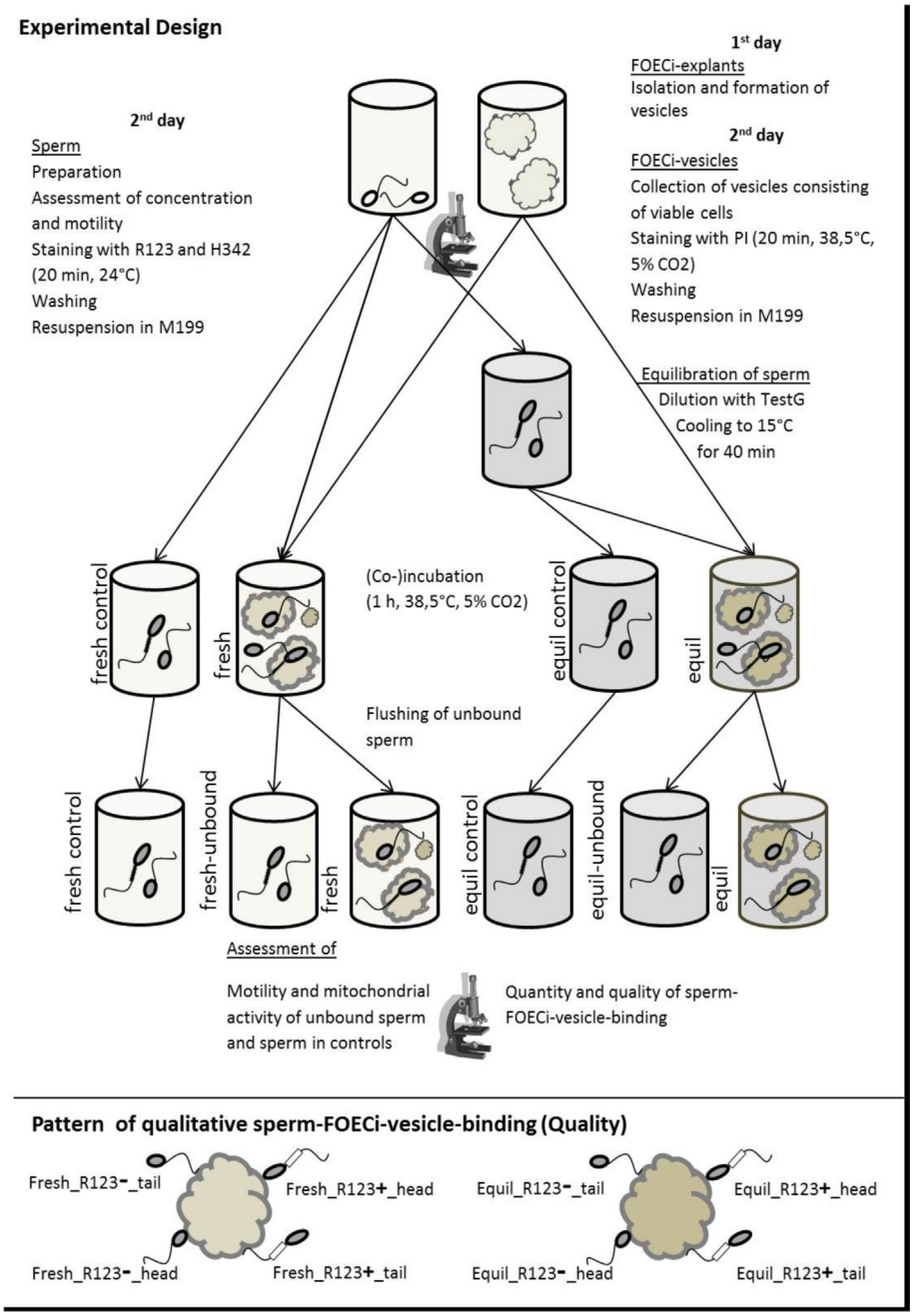

Fig. 1. Experimental design. Vesicles formed from feline isthmus oviduct epithelial cell (FOECi) explants during overnight culture were used for sperm binding assays with fresh and equilibrated sperm prepared from epididymides of domestic cats. Control sperm (without co-incubation) and unbound sperm 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

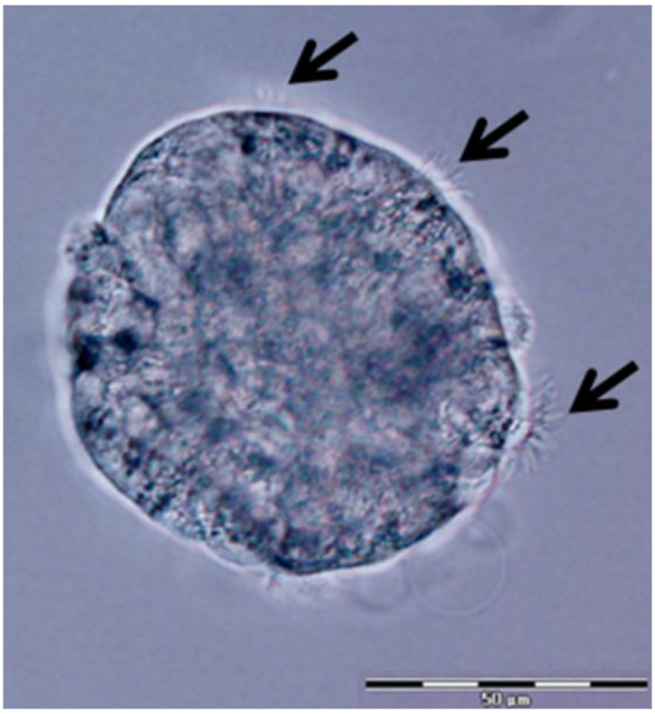

Fig. 2. FOECi-vesicle in transmitted light with cilia (arrows) orientated to the outer vesicle side. Bar represents 50 µm.

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

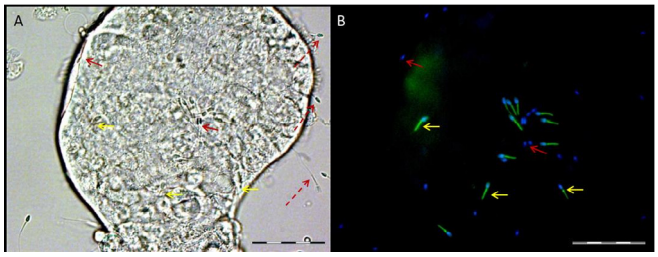

Fig. 3. Sperm binding to FOECi-vesicles *in vitro*. Fresh sperm with active (R123+, H342+) and inactive (R123-, H342+) mitochondria attached to the vesicles (yellow and red arrows, respectively) are shown in transmitted (A) and fluorescence light mode (B). Tail-bound sperm marked by the dashed line arrows (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

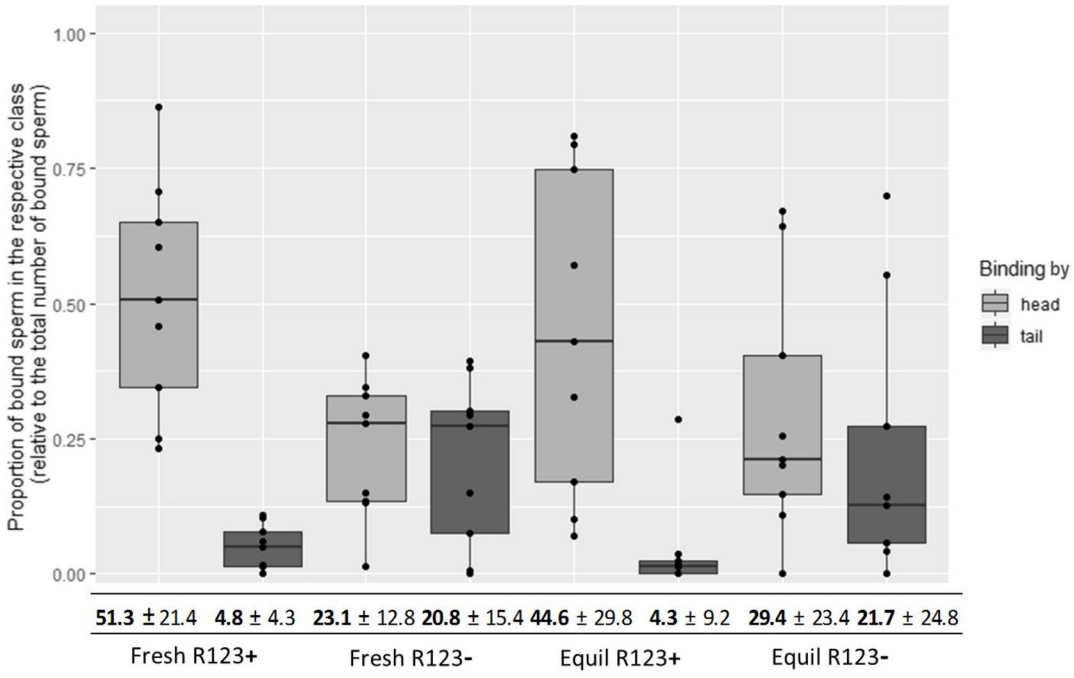

Fig. 4. Binding pattern of fresh (Fresh) and equilibrated (Equil) sperm in relation to the respective total number of sperm attached to FOECi-vesicles (N = 9) after 1 h of co-incubation at 38.5 °C under 5% CO_2. Sperm classes were distinguished according to active (R123+) or inactive (R123-) sperm mitochondria 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

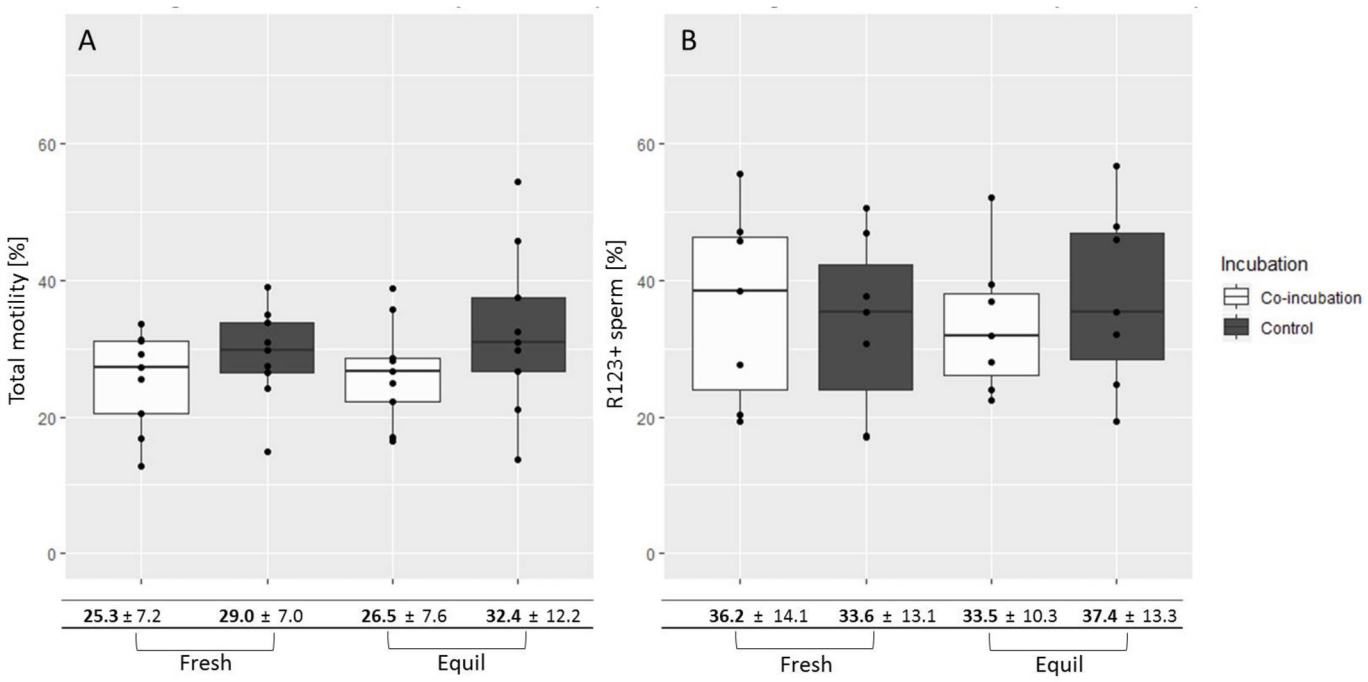

Fig. 5. Total motility (A) (N = 9) and percentage of sperm with active mitochondria (R123+) (B) (N = 7) of control sperm and unbound sperm flushed after 1 h of co-incubation with FOECi-vesicles at 38.5 °C under 5% CO_2. Sperm were distinguished according to fresh (Fresh) or equilibrated (Equil) stage 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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4.3. Chapter III – Submitted manuscript

Long-term culture of feline oviduct epithelial cells on porous filter supports

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Contribution of each co-author to the manuscript:

SE, SC, KM, JS designed the study.
SE, SC conceived and performed the experiments.
SE primarily analyzed the data.
SE drafted the manuscript.
All authors revised and approved the final manuscript.

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Long-term culture of feline oviduct epithelial cells on porous filter supports

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Abstract

Basic knowledge about cellular and molecular mechanisms underlying feline reproduction is required to improve reproductive biotechnologies in endangered felids. Commonly, the domestic cat (*Felis catus*) is used as a model species, but many of the fine-tuned, dynamic reproductive processes can hardly be observed *in vivo*. This necessitates the development of *in vitro* models. The oviduct is a central reproductive organ hosting fertilization in the ampulla and early embryonic development in the isthmus part, which also functions as a sperm reservoir before fertilization. In other species, culturing oviduct epithelial cells in compartmentalized culture systems has proven useful to maintain oviduct epithelium polarization and functionality. Therefore, we made the first attempt to establish a compartmentalized long-term culture system of feline oviduct epithelial cells from both ampulla and isthmus. Cells were isolated from tissue samples (n = 33 animals) after routine gonadectomy, seeded on porous filter supports and cultured at the liquid-liquid or air-liquid interface. Cultures were harvested after 21d and microscopically evaluated for epithelia l differentiation (monolayer formation with basal-apical polarization) and protein expression of marker genes (oviductal glycoprotein 1, acetylated tubulin). Due to the heterogeneous and undefined native tissue material available for this study, the applied cell culture approach was only successful in a limited number of cases (five differentiated cultures). Even though the protocol needs optimization, we proved that the compartmentalized culture approach is suitable to maintain differentiated epithelial cells from both isthmus and ampulla of the feline oviduct.

Keywords

Oviduct epithelial cells, Long-term culture, Domestic cat

Statements and Declarations

All authors declare that there are no financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

Introduction

Many felid species are classified as near threatened or threatened on the IUCN Red List of Threatened Species (The IUCN Red List of Threatened Species). To preserve these species and their genetic diversity, the continuous improvement of assisted reproductive techniques (ARTs) based on a better understanding of feline reproduction is urgently needed.

The oviduct and especially the epithelium lining the oviduct lumen create the optimal milieu for central reproduction events such as final gamete maturation, fertilization, and early embryo development. However, to date there is only little knowledge about feline oviduct epithelium physiology. As *in vivo* experiments are technically challenging and ethically questionable, *in vitro* models are established in accordance to the 3R principle (replacement, refinement, reduction of animal experiments). So far, mainly suspension cultures or in one case, 2D adherent submerged cultures have been used for studying feline oviduct epithelium physiology. The two main applications of feline oviduct epithelial cell (FOEC) cultures were the characterization of sperm-oviduct interactions (Henry et al. 2015; Ferraz et al. 2019; Eder et al. 2020) and the improvement of fertilization and developmental competence of early cat embryos (Lengwinat et al. 1992; Roth et al. 1993; Swanson et al. 1996). However, even though the suspension and 2D adherent submerged systems are easy to use, cells quickly dedifferentiate under these conditions as shown for other mammalian species (Reischl et al. 1999; Rottmayer et al. 2006; Danesh Mesgaran et al. 2016; Chen and Schoen 2019). Therefore, they are not applicable for long-term functional studies.

Our group successfully optimized compartmentalized culture systems using the air-liquid interface (ALI) approach for long-term culture of oviduct epithelial cells (OEC) of different species including pig, cattle, and mouse (Miessen et al. 2011; Chen et al. 2013b; Chen et al. 2017). In these species, the ALI-OEC system leads to the formation of functional epithelia l tissues *in vitro* (Chen et al. 2017). In the compartmentalized system, OEC grow on permeable membrane supports and provision of nutrients is ensured (as *in vivo*) via the basolateral cell side, which triggers basal-apical polarization and thereby differentiation. The approach enables co-culture with embryos/gametes or application of embryonic signals in the apical cell compartment and provision of maternal effectors (e.g. metabolic or hormonal stimuli) in the basolateral compartment. Therefore, such compartmentalized *in vitro* systems are powerful tools to investigate embryo-maternal interactions and the dynamic composition of the early embryonic environment (Chen and Schoen 2019). Aim of the current study was to apply the compartmentalized culture approach for the establishment of differentiated long- term cultures of FOEC separately isolated from both, the ampulla and isthmus region.

The domestic cat (*Felis catus*) is frequently used as a model species for felids. To prevent undesired pregnancies and control stray populations, cats are routinely neutered in veterinary clinics and animal shelters. Although these cats are highly diverse with respect to age, reproductive and general health status compared to farm and laboratory animals, it is possible to obtain and examine oviduct samples for their use *in vitro*. We tested whether (i) the compartmentalized culture approach using media optimized for murine and porcine OEC is generally applicable for FOEC, (ii) it is possible to long-term culture cells from the ampulla and isthmus region of the feline oviduct separately and (iii) highly differentiated FOEC

cultures can be obtained from the heterogeneous basic material available after routine castrations.

Material and methods

Reagents and media

Cell culture media used in culture procedures (Table 1) were modifications of previously described media for mouse tracheal cell culture (You et al. 2002) as well as for ALI culture of murine and porcine pig OEC (Chen et al. 2017). 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), while other chemicals, media and supplements were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless otherwise stated.

Table 1 Media compositions used for compartmentalized cell culture

Media / Reagents	**FOEC-BASIC**	**FOEC-PROL**	**FOEC-DIFF**	**Transport medium**	**Washing medium**
Basic medium	DMEM/Ham's F12 (1:1, FG 415)	FOEC-Basic	FOEC-Basic	HEPES-MEM (M7278)	PBS (D8537)
HEPES (H0887)	15 mM				
Penicillin, Streptomycin (P4333)	100 U/ml, 100 µg/ml				100 U/ml, 100 µg/ml
Amphotericin B (171375)	0.25 µg/ml				0.25 µg/ml
Gentamicin					0.05 mg/ml
Antibiotic Antimycotic Solution (A5955)				1% (v/v)	
Insulin (I6634)		5 µg/ml	2.5 µg/ml		
Transferrin (T8158)		5 µg/ml	5 µg/ml		
Cholera toxin (C8052)		0.025 µg/ml	6.26 ng/ml		
Epidermal growth factor (E4127)		5 ng/ml	1 ng/ml		
Bovine pituitary extract (P1476)		30 µg/ml	30 µg/ml		
Retinoic acid (R2625)		0.05 µM	0.05 µM		
FBS (S 0115, Lot 1030B)		5%			
BSA (126579)			1 mg/ml	3 mg/ml	

Animals

Oviduct tissue samples of 33 domestic cats (*Felis catus*) were obtained from the animal rescue shelter or veterinarians in Berlin, Germany. They were collected by the local veterinarians after routine gonadectomy. The experimental protocols were approved by the Ethics Committee of the Leibniz Institute for Zoo and Wildlife Research (2013-05-05).

Tissue and sample preparation

Ovaries with oviducts were transported to the laboratory in transport medium at 4°C and were processed within 5 h. However, the storage time between gonadectomy and transport to the laboratory varied between 1 and 6 h. The organs markedly differed in size and ovarian status. Oviducts from both, late follicular phase ovaries (follicles larger than 2 mm in diameter visible) and inactive ovaries (neither follicles nor *corpora lutea* visible) were used.

Isolation of FOEC was conducted according to a previously published protocol (Eder et al. 2020). Briefly, after removal of the ovary and surrounding tissues, oviducts were washed in washing medium. The oviducts were injected with 1 mg/ml collagenase 1A (C5894) in PBS (D8537) and incubated for 30 min at 38°C. Epithelial cells from isthmus and ampulla were squeezed out separately with the outer edge of a scissor onto a glass slide, flushed into 600 µl FOEC-BASIC in a 4-well dish and pre-cultured for 16 h at 38.5°C, 5% CO_2. If both oviducts of one cat were available, the two samples of each region were pooled.

During the pre-culture, extracted cell associations formed three-dimensional vesicles. After pre-culture, FOEC-vesicles mainly consisting of viable cells were separated from vesicles comprising many dead cells (Eder et al. 2020). They were resuspended in 10×Trypsin/EDTA (59418C) solution for subsequent single cell isolation. After 10 min digestion at 38°C in a water bath, the reaction was stopped by adding 750 µl FBS. Single cell isolation was completed by gentle pipetting through a wide pipet tip and filtering through a cell strainer of 40 µm pore size. Cell concentrations were determined in a counting chamber. FOEC solutions were centrifuged for 5 min at 200 × g, pellets were resuspended with proliferation medium (FOEC-PROL) and adjusted to a cell concentration of 1×10^6/ml.

Long-term culture of FOEC

For the long-term culture, 24-well hanging inserts (0.4 µm PET, non-transparent, Millipore, Switzerland) were used. Inserts were coated with human placenta collagen (C5533) according to the manufacturer's instructions.

In dependence on the number of isolated viable cells, $1 - 2 \times 10^5$ FOEC were seeded onto the apical side of each insert. Each well (basal side of insert) was filled with 1 ml FOEC-PROL. All samples were maintained submerged during the proliferation phase for three to five days. Once a confluent cell monolayer was established, cells were either grown at the ALI with 1 ml FOEC-DIFF in the basolateral compartment and without addition of medium on the apical side, or with 1 ml FOEC-DIFF in the basolateral and 50 µl FOEC-DIFF in the apical compartment of the inserts (liquid- liquid interface, LLI). Medium was changed twice a week

in all compartments containing liquid. During a culture period of 3 weeks the FOEC were incubated in humidified atmosphere with 5% CO_2 at 38.5°C.

Histology and morphological evaluation

Histological processing was performed as previously described by our group (Chen et al. 2013a, b). Briefly, the membranes were fixed in Bouin`s solution, stabilized in agarose and post-fixed in 4% formaldehyde. After dehydration in an ascending ethanol series, the samples were embedded in paraffin. Samples were cut into 3 µm sections, stained with haematoxylin/eosin (HE) and microscopically evaluated for epithelial differentiation using the criteria depicted in Fig.1.

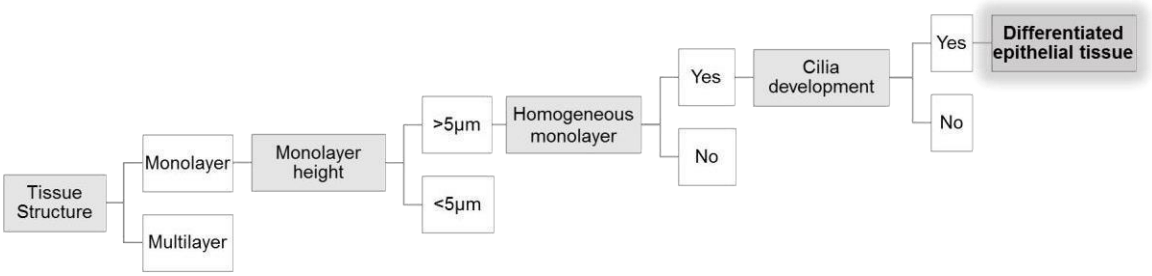

Fig. 1 Criteria applied for the evaluation of FOEC cultures after three weeks of culture.

Immunohistochemistry

Immunolocalization of marker proteins for cilia development (acetylated tubulin) and oviduct specific secretory activity (oviductal glycoprotein 1, OVGP1) was conducted in morphologically differentiated cultures. Antigen retrieval was performed either enzymatically (acetylated tubulin) by incubation with 0.06% trypsin, pH 7.8 or via heat-induced antigen retrieval (OVGP1) using sodium citrate buffer (10 mM sodium citrate, 0.05% tween 20, pH 6.0). Unspecific binding sites were blocked with 5% BSA plus 10% goat serum in PBS (30 min, room temperature). Slides were incubated with the primary antibodies anti-tubulin (Sigma T7451; 1:1000 in PBS with 1% BSA) or anti-OVGP1 (Abcam ab118590; 1:500 in PBS with 1% BSA), respectively (overnight, 4°C). The corresponding secondary antibodies were goat anti-mouse Alexa 568 (Invitrogen A-11031; 1:40 in PBS with 1% BSA) and goat anti-rabbit IgG, Alexa 647 (Invitrogen A-21245; 1:200 in PBS with 1% BSA), respectively, and were applied for 1 h at room temperature. Negative controls were performed by omitting the primary antibody. SYBR Green I (Mobitec, Berkheim) was used for nuclei counterstaining. Pictures were captured using a Zeiss LSM800 equipped with fluorescence optics and ZEN software.

Results and discussion

The compartmentalized culture approach was originally developed for human epidermal and rodent respiratory epithelial cells to better mimic *in vivo* epithelia (Pruniéras et al. 1983; Whitcutt et al. 1988). Later, this method was adopted for gastric (Tabuchi 2001), ear (Portier et al. 2005), cornea (Sygitowicz et al. 2011) and female reproductive tract epithelia (Chen and

Schoen 2019). In cats, only trachea epithelial cells had been cultured by a compartmentalized approach (Nelli et al. 2016).

The oviduct samples that could be obtained from the animal shelter and private veterinary clinic s were expectedly very heterogeneous. Since cats of private owners are often neutered before they come into heat for the first time more samples were available from cats with inactive ovaries. In total, 25 inactive and 8 cats in late follicular phase of estrous were sampled. The cell numbers isolated per oviduct were highly variable (inactive isthmus: $2.1 \pm 1.4 \times 10^5$, inactive ampulla $4.6 \pm 3.8 \times 10^5$, active isthmus: $2.4 \pm 1.9 \times 10^5$, active ampulla: $6.7 \pm 4.1 \times 10^5$). The reproductive tract of the young and inactive females is still very delicate. Therefore, a minimum of 1×10^5 cells of at least one of the oviduct segments (ampulla or isthmus) could only be collected from 18 individuals with inactive ovaries. This was the case for all eight individuals in late follicular phase of estrous. Because of the unbalanced cell numbers per ovary stage, individual and oviduct region, we assigned the original cells in mostly non-paired approaches to the culture treatments. Only five and three "full" sets of sample allocation were possible in the groups with inactive or estrous ovaries, respectively. Therefore, we did not perform statistic evaluation and only consider the information on potentially successful culture strategies important for the progress of feline oviduct long-term culture.

Table 2 Success of compartmentalized long-term culture of feline oviduct epithelial cells in air- liquid (ALI) or liquid- liquid (LLI) approaches

Ovary stage	**Inactive**				**Estrous (late follicular phase)**				**∑**
Oviduct segment	**Isthmus**		**Ampulla**		**Isthmus**		**Ampulla**		
Number of segments with ≥10^5 cells	15		18		6		8		
Culture method	**ALI**	**LLI**	**ALI**	**LLI**	**ALI**	**LLI**	**ALI**	**LLI**	
Number of cultures with 1-2× 10^5 seeded cells	9	9	17	15	5	4	8	4	**71**
Number of cultures with polarized epithelial monolayers	2	1	0	2	0	0	0	0	**5**

In total, only five cultured samples (all derived from the more numerous females with inactive ovaries) reached a differentiated status (Tab. 2). Three of these samples were obtained from the same donor (isthmus ALI and isthmus LLI, ampulla LLI), further two samples stem from two other females. Differentiated samples formed a homogeneous epithelial monolayer consisting of polarized cells (Fig. 2A) as shown for pig, cattle and mouse by the same approach (Miessen et al. 2011; Chen et al. 2013b; Chen et al. 2017). OVGP1, a marker for functional oviduct epithelia was expressed in all of the differentiated cultures as exemplary shown for one ampulla LLI sample (Fig. 2B). The presence of cilia was verified by staining of

acetylated tubulin. However, cilia formation was only rarely observed as shown for the ampulla LLI sample (Fig. 2C).

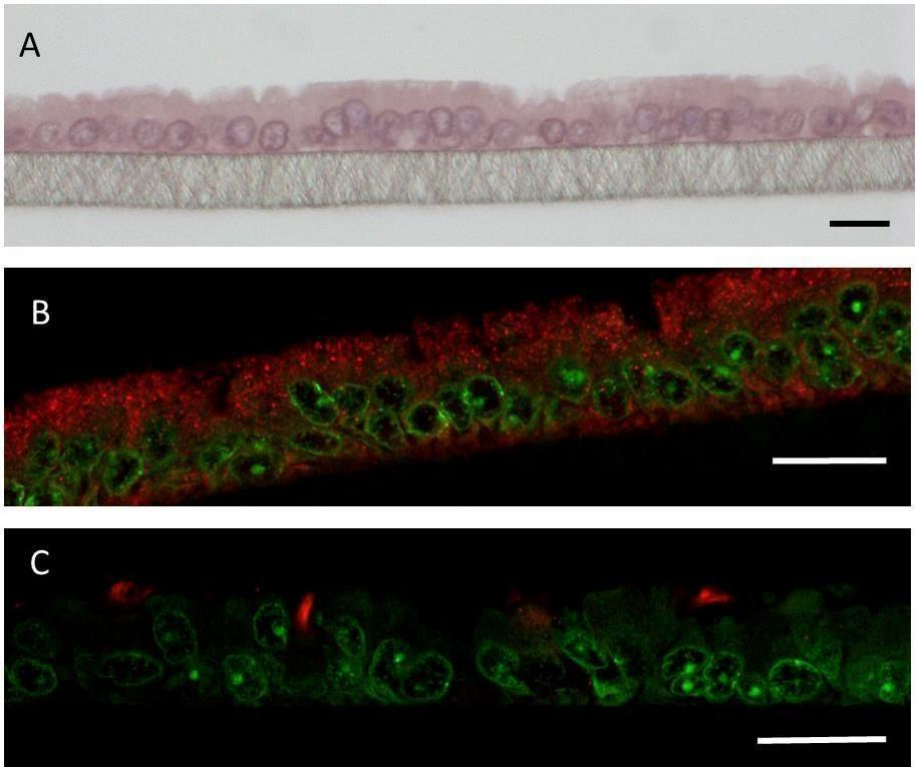

Fig. 2 Long-term primary cell culture of feline oviduct epithelial cells after three weeks in a compartmentalized liquid- liquid (LLI) approach. Cells from inactive ampulla were HE stained (A) or labeled for oviductal glycoprotein 1 (B, red) as well as acetylated tubulin to visualize cilia (C, red). Nuclei were stained green by SYBR Green I. The bars represent 10 µm.

With this report we demonstrate that a long-term differentiated culture of FOEC is possible although the pronounced variability of the native material from routine castrations and the limited cell yield are basic handicaps. To select viable cells, a pre-culture of FOEC- vesicles was applied. However, we suspect that not only the number of viable cells but especially proliferative progenitor- like cells within the isolated cell population is crucial for the success of the compartmentalized culture approach. Previous reports (Kessler et al. 2015) describe the formation of fully differentiated human oviduct epithelium organoids from clonal cells by experimental control of their stemness and, therefore, their proliferative potential prior to induction of differentiation. In species with limited sample availability and heterogeneous sample quality such as human, mouse, cat or wildlife, protocols which increase the proliferative capacity of the primary cell/tissue material, such as organoid pre-culture to increase the number of progenitor-like cells, are required to accomplish stable, reproducible long-term cultures for application in reproductive research and assisted reproduction.

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Declarations

Author's contribution SE and SC: Investigation; SE: Writing – original draft; KM and JS: Conceptualization, Supervision; JS, KM and SC: Writing – review & editing. All authors read and approved the final manuscript.

Compliance with ethical standards The experimental protocols were approved by the Ethics Committee of the Leibniz Institute for Zoo and Wildlife Research (2013-05-05).

Conflict of interest The authors declare no conflict of interest.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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5. General discussion

5.1. Summary of the research articles

In this study we addressed two problems which arise upon use of ARTs in felids. The necessity to use epididymal sperm to preserve the genetic potential of valuable males, mostly in combination with cryopreservation, results in a supply of male gametes for ARTs, particularly AI, that do not correspond to the physiological state. We suggested that the lack of seminal fluid in epididymal sperm as well as the modification of sperm during cryopreservation may have an impact on the sperm-oviduct interaction. Without the formation of the functional sperm reservoirs, feline sperm do not undergo the final developmental steps that enable them to fertilize oocytes. Moreover, we performed the first steps to establish a compartmentalized long-term FOEC-culture system to enable further investigations of sperm-oviduct interaction *in vitro*. The domestic cat was used as model for non-domestic feline species.

First, we investigated the effect of seminal plasma on sperm-oviduct binding (**chapter I**) by using FOEC-explants and epididymal sperm. It was the first time that an *in vitro* sperm-oviduct binding assay was performed in a feline species. We could show that epididymal sperm are able to bind to oviduct epithelial cell explants. Furthermore, our results suggest that seminal fluid components may play a supportive role in the binding process.

Second, we examined whether equilibration, the first step of the cryopreservation procedure, has an impact on sperm-oviduct binding *in vitro* (**chapter II**). Equilibration comprises dilution in freezing extender and equilibration to 15°C. We optimized the protocol to generate FOEC-vesicles from fresh isolated explants, because the usage of fresh explants was restricted to four hours after preparation. For this study, cell material obtained from the isthmus region was used and vesicle formation occurred within a twelve-hour culture period. The binding assay with FOEC-vesicles and epididymal sperm revealed that the binding capacity of sperm to vital FOEC was significantly reduced after equilibration compared to non-equilibrated sperm. Furthermore, we showed in both studies, that the sperm-binding pattern in the domestic cat differs from those in livestock. Epididymal sperm attach not solely by the head but also by their tail to FOEC, possibly preparing for a selection step of phagocytosis of dying and dead sperm. Epithelial cell explants and vesicles represent useful but limited tools for studying sperm-oviduct interaction.

Third, we tested the first steps to establish a compartmentalized FOEC-culture system for long-term culture (**chapter III**). We demonstrated for the first time that a long-term differentiated culture of FOEC is possible in principle. However, repeatability was still poor as compared for instance to the established porcine system.

Since the SP and the equilibration-study have some themes and methods in common, they are compared and discussed together concerning (i) the importance of sperm viability for oviduct binding, (ii) the impact of seminal plasma and equilibration on feline epididymal sperm binding to OEC, and (iii) the consequences for artificial insemination. Difficulties in establishing a long-term FOEC-culture in a compartmentalized approach and options of further optimization are subsequently discussed. Finally, the general conclusions follow.

5.2. Comparison of FOEC-explants and FOEC-vesicles for their use in sperm-oviduct binding assays

Both sperm-oviduct binding studies were performed with domestic cat epididymal sperm. Cell explants were used in the SP-study and FOEC-vesicles in the equilibration-study (fig. 6). They differ in terms of the culture period before the binding assays were performed. While explants are freshly isolated planar cell layers, the three-dimensional vesicles are formed from explanted cell associations during twelve hours of pre-culture. The suspension of explants contained a mixture of primarily cell associations as well as single vital and dead cells. During the experiments, care was taken to evaluate only explants containing vital cells. Vitality was assessed according to cilia movement of ciliated cells.

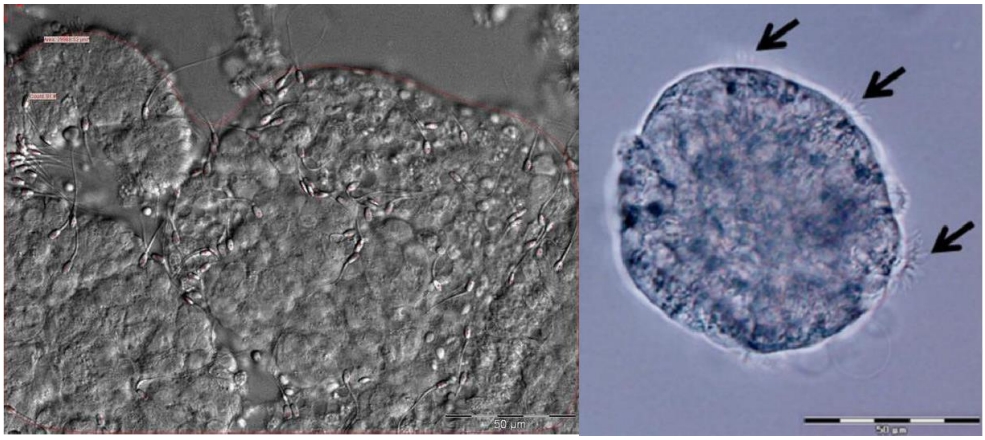

Fig. 6. FOEC-explant with attached sperm (left) and FOEC-vesicle with cilia (arrows) orientated to the outer vesicle side (right) (modified from Eder et al., 2020) in transmitted light. Bar represents 50 µm.

In the equilibration-study, FOEC-vesicles were used in the sperm-binding assay. These vesicles comprised viable cells, since dead cells did not associate in stable vesicles. We isolated these stable vesicles containing viable cells from the overnight cultured suspension. The selection of vesicles is an important step to ensure conditions that are as close as possible to physiological conditions. To monitor the viability of FOEC-vesicles during the sperm-oviduct binding assay, the contained cells were evaluated by fluorescent dyes. The dye propidium iodide (PI) was applied to label dead cells, Hoechst dye 33342 stains the nuclei of all cells. Even after the selection of FOEC-vesicles, a small number of FOEC-vesicles containing PI-positive cells were found in the samples. We suggest that these PI-positive FOEC died as a result of the handling (isolation procedure, adjusting the sperm to vesicle ratio) and culture conditions.

During the assessment of sperm binding to FOEC-vesicles, it was observed that a large number of epididymal sperm bind also to the contained dying or dead cells. This is not considered to be the physiological situation even though it is unknown whether and to what extent dead OEC are present in the oviduct *in vivo*. However, we did not want to leave this interesting observation unnoticed. So, in addition to sperm bound to vesicles consisting of viable FOEC, the sperm attached to FOEC-vesicles consisting of many dying/dead cells were also counted but not included in the manuscript data. FOEC-vesicles which contained PI-positive cells comprised about 10 % of the total vesicle population. In figure 7, the numbers of sperm bound to FOEC- vesicles consisting of viable or of dying/dead cells are shown. The median of the number of fresh or equilibrated viable sperm per mm^2 of vesicles consisting of dying/dead FOEC was 630 and 1684, respectively. In comparison, medians of 472 and 338 for fresh and equilibrated viable sperm were counted per mm^2 of vesicles consisting of viable FOEC (fig. 7). It seems that in particular the equilibrated sperm lose their ability to select viable OEC or are preferentially attracted by dead OEC. The reason why sperm attach to dying/dead cells is not known. We assume that the loss of the barrier function of membranes of dead FOEC causes a leakage of intracellular components. These components may include for instance proteins, peptides and

enzymes synthesized and secreted by OEC to create a fine-tuned micro-environment which supports gamete function, fertilization as well as early embryo development [Killian, 2004]. One of these proteins is oviductin. Its presence in the oviduct of the domestic cat was demonstrated by Hachen et al., [2012]. Oviductin has not yet been described to mediate sperm-oviduct binding but does affect, amongst other processes, oocyte-sperm interactions [Buhi, 2002]. There is evidence in the literature that various molecules, including oviductin, are secreted by the secretory OEC and released into the lumen of the oviduct, where they associate with sperm and/or oocytes to mediate sperm-oocyte binding [Ghersevich et al., 2015]. An excessive presence of these molecules on the cell surface caused by uncontrolled leakage could result in an increased unspecific binding of sperm to the FOEC that does not reflect the physiological situation. It is therefore important to work with cell material that contains as few dead cells as possible.

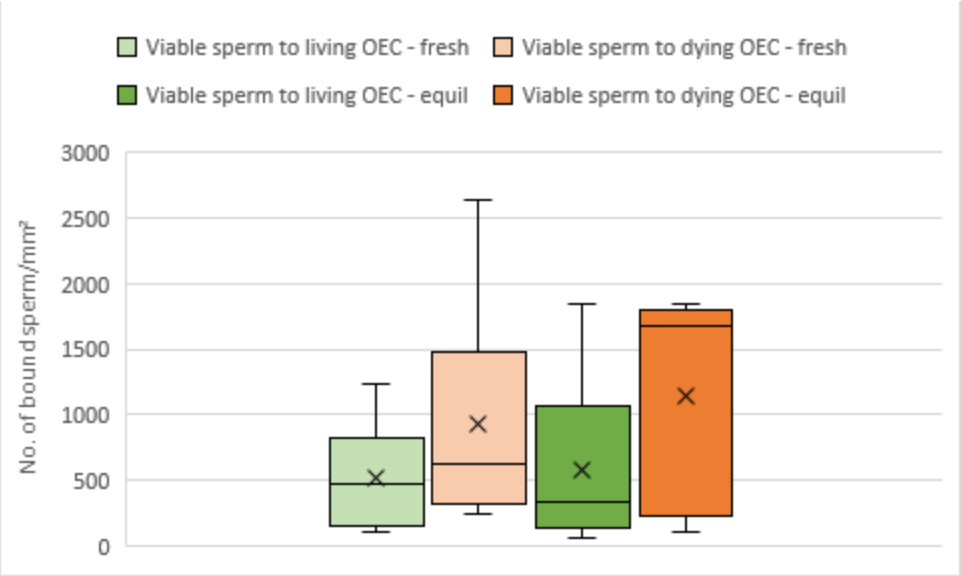

Fig. 7. Number of viable fresh (bright green and orange) and equilibrated (dark green and orange) sperm per mm² attached to vesicles consisting of viable (green) or dying/dead (orange) FOEC. Data are presented by vertical box plots. Medians, the 25 and 75th percentiles are shown as vertical boxes and whiskers. Means are indicated by crosses (N = 9 experiments). Between seven and eleven vesicles containing mainly viable and between one and three vesicles containing mainly dying/dead FOEC were evaluated in the experiments, in three experiments in the control (fresh) and in four experiment s in the treatment (equil) group, no vesicles with dying/dead FOEC were observed.

In preliminary experiments for the SP-study, we had observed that FOEC-explants exhibited a reduced viability and lost their sperm binding capacity when pre-cultured overnight in medium M199 (also referred to as TCM-199). Therefore, we performed the sperm-oviduct binding assays on FOEC-explants within four hours after FOEC preparation. The reason for the loss of binding capacity is unknown. One possibility is that progressive de-differentiation of the FOEC could be responsible. One factor influencing the survival and progressive de-differentiation of OEC appears to be the culture medium and additives. De Pauw et al. [2002] and Leemans et al. [2014] tested the viability of bovine and equine OEC-vesicles incubated in different media before using them in the sperm-oviduct binding assay. The pre-culture of bovine OEC overnight for vesicle formation was performed in TCM-199 medium supplemented with 10 % FCS and resulted in good quality FOEC-vesicles [De Pauw et al., 2002]. Leemans et al. [2014] tested the viability of the OEC vesicles over 24 hours in capacitation and non-capacitation medium (based on Whitten medium) and found no decrease in cilia activity or membrane integrity. The most common methods to test viability before and during the sperm- oviduct binding assay are the assessment of cilia activity and/or live/dead fluorescence staining. Unfortunately, the viability of a cell does not correspond to its stage of differentiation. A common marker for OEC differentiation is oviductin, which is exclusively synthesized and secreted by differentiated secretory OEC. It has been shown in the domestic cat that the mRNA expression of oviductin decreased during a FOEC-vesicle culture period of 19 days but remained detectable [Kaffenberger, 2010]. Kaffenberger [2010] cultured the

FOEC-vesicles in a M199 medium. It is important to realize that there are several variants of the medium M199. Even though we used M199 for the preparation and incubation of FOEC-explants in our SP-study, preliminary tests had revealed that they lose their sperm-binding capacity overnight.

For the equilibration study, we changed the medium for the pre-culture of FOEC to DMEM/F12. This resulted in the formation of FOEC-vesicles with rapidly beating cilia, and binding capacity for sperm was maintained for at least 12 h. Besides oviductin as a marker for the differentiation of FOEC, proteins that mediate sperm- oviduct binding should be identified in future as a characteristic of the sperm-binding capacity which is a particular aspect of FOEC differentiation. Interesting candidates are oviduct-specific proteins such as mucin 1(MUC1), phosphatidylethanolamine binding protein 4 (PEBP4), and tachykinin 3 (TAC3) [Acuña et al., 2017]. Acuna et al. [2017] demonstrated their presence in the porcine oviduct, and more specifically that they were synthesized in the oviduct and also secreted into the lumen. Acuna et al. [2017] also showed that the coding genes were differentially expressed at different estrous stages.

The numbers of bound fresh epididymal sperm per mm^2 FOEC (median (min – max) were 1040 (800 – 1630) in the SP study and 472 (105 – 1298) in the equilibration experiments. In sperm-oviduct binding studies in other species, values in the three- to five-digit range were determined [De Pauw et al., 2002; Leemans et al., 2014; Thomas et al., 1994; Waberski et al., 20064]. Therefore, the order of magnitude of our values corresponds to published data for other mammalian species. Detailed comparisons are not possible since the experimental factors differ, such as sperm maturation level (epididymal or ejaculated), sperm preservation stage (fresh, liquid or cryo-preserved), sperm concentration, explant and vesicle geometry, co-incubation time, media and additives.

Another factor that can affect sperm-binding to OEC vesicles appeared to be the vesicle size [De Pauw et al., 2002; Saraf et al., 2017]. On OEC of dairy cow and of Murrah buffalo (*Bubalus bubalis*), significantly more sperm bound if OEC-vesicle size was smaller than 20,000 and 40,000 μm^2, respectively, compared to large vesicles [De Pauw et al., 2002; Saraf et al., 201]. De Pauw et al. [2002] suggest that the distribution of cell types probably differs with vesicle size, i.e. small vesicles contain more ciliated cells that bind sperm in cattle [Lefebvre et al., 1995]. In the SP and equilibration-study, the evaluated FOEC-explant or vesicle surfaces were smaller than 30,000 and 36,000 μm^2, respectively. In order to obtain more reliable results, it could be tested whether there is a more suitable vesicle-size in the FOEC-vesicle model.

The influence of media on sperm-oviduct binding is particularly difficult to test because media can affect cell-physiological properties of sperm and/or OEC. With regard to media, the conditions within the SP and equilibration-study are not directly comparable. An M199 medium was used for explant culture and co-culture in the SP study, DMEM/F12 was used for vesicle formation and mixed with sperm diluted in M199 or freezing media TestG (~12:1) for co-culture in the equilibration-study.

We do not know whether media components may directly influence sperm binding or whether solely the different handling of the FOEC before the binding assay resulted in different cell-physiological and sperm binding properties of the formed vesicles. Nevertheless, we achieved the aim of prolonging FOEC-viability and delaying de-differentiation with regard to sperm binding. This means that the FOEC-vesicle model can be used longer and more flexibly than the explant model. However, an established long-term polarized culture would offer even more flexible options and produce more reliable results.

In conclusion, FOEC-explants and vesicles are suitable for sperm-oviduct binding experiments. However, they are limited in time and to obtain reliable and consistent results several points must be considered. To reflect a physiological state, it is important to co-incubate sperm with viable OEC and reduce the number of dead OEC to a minimum. Marking the dead cells during the experiment is not sufficient, because a large part of the sperm gets bound to them, which could change the results. Another important point is the choice of the culture and incubation media used in a sperm-oviduct binding assay. It must be considered which impact they may have

on the physiological state of OEC or sperm and their interaction capacity. Therefore, it is necessary to identify the molecules which mediate the sperm-oviduct binding in the domestic cat.

5.3. Importance of sperm viability for oviduct binding

The sperm-oviduct binding studies differed not only with regard to the oviduct cell preparation but also with regard to sperm characterization. In the SP-study all bound sperm were counted. Although no staining was performed to evaluate sperm quality, it was observed that most of the bound sperm were viable as assessed by the movement of their tails or heads. It is important to note that different from observations in domestic cattle or domestic pig [Ekhlasi-Hundrieser et al., 2005; Lefebvre et al., 1995], domestic cat sperm may be attached to the OEC not only by the anterior part of the head but also by their tails (see below).

In the equilibration-study we differentiated between viable and non-viable sperm to investigate not only the quantity but also the quality of bound sperm. This distinction was made by using a triple staining protocol with the fluorescent dyes Hoechst 33342, PI and Rhodamine 123 (R123). Hoechst 33342 enters all cells independent of their vitality and incorporates into the nuclei. PI binds to nucleic acids but is not membrane-permeable, therefore it is used to differentiate between dead (PI-positive) and viable cells (PI-negative) based on membrane integrity. R123 is accumulated in the mitochondria membrane in dependent of the membrane potential. R123-positive cells are considered to be viable.

As already mentioned before, we observed in the SP-study that most sperm were attached to FOEC-explants by their heads and some sperm were tightly stuck to the FOEC by their tails. This coincides with the observations of the equilibration-study where we quantified the type of sperm-oviduct binding in combination with the triple staining procedure. Among the attached population of freshly preparedepididymal sperm, head- bound sperm with active mitochondria achieved the highest numbers per FOEC-surface area. In both, fresh and equilibrated samples, tail bound sperm with active mitochondria were the least represented (<5 %). About 20 % of the fresh and equilibrated sperm contained inactive mitochondria and were attached with their tail in both samples. This leads us to the suggestion that the sperm-oviduct interaction via sperm heads is a mechanism to store functional sperm and that tail binding might be an initial event preceding phagocytosis of dying and dead sperm by the oviduct epithelium, originally described in domestic cats by Murakami et al., [1985]. In Llama (*Lama glama*) it was also found that sperm bind by their head or tail to OEC-vesicles [Apichela et al., 2009] but to our knowledge, there is no observation of phagocytosis in the oviduct of camelids. In the mouse, phagocytosis of surplus sperm, namely sperm which will not participate in fertilization, by isthmic OEC was observed [Chakraborty and Nelson, 1975]. Light and electron micrograph pictures show that sperm with and also without signs of degeneration were phagocytosed [Chakraborty and Nelson, 1975]. Austin described already in 1959 the phenomenon that sperm heads had been observed in the epithelial layer of the oviduct in the laboratory rat, the domestic rabbit, the West European hedgehog (*Erinaceus europaeus*), the European mole (*Talpa europaea*) and the stoat (*Mustela erminea*). Sperm has been found in phagocytotic vacuoles in the OEC layer [Chakraborty and Nelson, 1975].

Taken together, there are differences in the sperm-oviduct interaction *in vitro* between mammalian species. While in the domestic pig almost exclusively viable sperm bind by their heads to the oviduct epithelium, in the domestic cat viable and non-viable sperm attach to FOEC-vesicles. Furthermore, the binding behavior of viable sperm seems to be different than the binding of non-viable sperm. Possibly different molecules are involved when sperm build a functional sperm reservoir or when they are selected for phagocytosis. As already stated in the previous paragraph, further investigations are necessary to identify these molecules. In general, the triple staining with the fluorescence dyes Hoechst 33342, PI and R 123 is helpful to visualize differences in sperm binding pattern.

5.4. Impact of seminal plasma and equilibration on feline epididymal sperm-binding to OEC and consequences for artificial insemination

The SP-study indicated that seminal plasma components may play a supportive role in the binding process of sperm to OEC in the domestic cat. This was also observed in the domestic cattle and the domestic pig, where BSP proteins and spermadhesins are involved. Gwathmey et al. [2003] observed that the addition of the BSP protein PDC-109 to bovine epididymal sperm increased the number of sperm per OEC explant area to the level observed for ejaculated sperm. The porcine spermadhesin AQN1 was identified to initiate sperm-binding to the oviduct epithelium [Ekhlasi-Hundrieser et al., 2005]. In felids, less is known about seminal plasma proteins. Zambelli et al. [2010] compared the seminal plasma protein profile (by SDS-PAGE) from ejaculates obtained using urethral catheterization or electro-ejaculation. The profiles differed and a few proteins were identified. While some similarities between domestic cat SP-proteins and SP-proteins in other species were found, there was no evidence for spermadhesins in the domestic cat. One candidate to mediate sperm-oviduct binding in domestic cats might be the epididymal sperm binding protein 1 (ELSPBP1) that contains four fibronectin type 2 (Fn2) domains arranged in tandems [Rowlison et al., 2020]. This protein is produced and secreted by the epididymal duct epithelium and binds to sperm [Saalmann et al., 2001]. Its function in the domestic cat is currently unknown. D`Amours et al. [2012] described that ELSPBP1 in male domestic cattle specifically targets dead sperm cells in the epididymis to presumably initiate their degradation and removal from the maturing population. Ekhlasi-Hundrieser et al. [2007] found its sequence similar to the BSPs in male domestic cattle. Further investigations are necessary to identify SP-proteins as well as proteins from sources such as epididymal extracellular vesicles that mediate sperm-oviduct binding.

The equilibration-study indicates that dilution of feline epididymal sperm in freezing extender containing LDL and equilibration to 15°C reduces their binding capacity to vital OEC compared to the non-chilled sperm diluted in culture medium without LDL. To our knowledge, there is no other study that has examined the influence of the equilibration process on sperm-oviduct binding *in vitro*. In this study we could not clarify whether reduced binding competence is caused by cooling of sperm to 15°C and/or the dilution of sperm in a freezing extender. Sperm from different species differ in their sensitivity to cooling. While domestic boar sperm undergo membrane damage already at 15°C [Green and Watson, 2001; Pursel et al., 1972], domestic cat sperm are more resistant to cold shock [Axnér et al., 2004; Müller et al., 2020]. The different sensitivities might be caused by differences in the lipid composition of the sperm membrane. The lipid composition defines membrane fluidity and permeability. Eder et al. [2016] investigated the physico-chemical membrane properties and incorporated spin-labelled lipid analogs into the sperm cell membranes to record their electron spin resonance (ESR) spectra at varying temperatures. The less pronounced loss of fluidity upon cooling in domestic cat sperm was suggested to be related to their better equilibration tolerance whereas the freezing process seems to be more critical for domestic cat sperm [Müller et al., 2020]. Therefore, we assume that the cooling process and related membrane changes have only a minor effect on domestic cat sperm binding properties. The freezing extender (TestG) which was used for the study contains the water-soluble fraction of domestic chicken egg yolk that mainly comprises LDL [Oloyede and Ikuelogbon, 2004] which are considered responsible for sperm protection during cryo-preservation [Bergeron and Manjunath, 2006; Pace and Graham, 1974; Watson, 1976]. From studies in domestic cattle it is known that LDL interacts with BSP proteins. These LDL-BSP complexes prevent a lipid efflux from the sperm membrane of ejaculated sperm and its destabilization [Bergeron and Manjunath, 2006]. However, since very little is known about seminal plasma proteins, we can only speculate about such interactions or the blocking of binding receptors by the freezing medium. In this context, it would be important to know whether ELSPBP1 is functionally analogous to bovine PDC-109. In this case, rapid sperm dilution with an LDL-extender after sperm collection (from the epididymis as well as after ejaculation) would be important to prevent premature membrane destabilization during the preservation process but would also have the potential to disturb the mediating role of the protein for sperm- oviduct binding.

Which consequences can be drawn for the adjustment of freezing protocols and subsequent handling of sperm samples before artificial insemination? Both, ejaculated and epididymal sperm are frozen preserved in gamete banks for later artificial insemination. During the freezing and thawing process, seminal plasma is highly diluted or completely washed out. It can be speculated that LDL also interacts with sperm membrane proteins in felids. Since we have shown that SP has a positive effect on sperm-oviduct binding and the freezing extender has obviously a negative effect on sperm-oviduct binding in the domestic cat *in vitro*, the question arises whether it is useful to wash out the freezing extender after thawing and to dilute the sperm with seminal plasma? It should be tested whether the effect of the freezing extender can be reversed by removing the extender after thawing and before artificial insemination. Chatdarong et al. [2010] recommended post-thaw dilution with Tris-buffer when an egg yolk Tris-extender containing Equex is used as a freezing medium to enhance immediate post-thaw semen quality.

Regarding the addition of seminal plasma to feline sperm after freezing/thawing, Thuwanut and Chatdarong [2009] indicated that seminal plasma did not have beneficial effects on quality parameters (motility, progressive motility, membrane and acrosome integrity) over a Tris-buffer when used as a diluent for post- thaw epididymal cat spermatozoa. They did not assess the impact on the oviduct binding or fertilizing capacity. In the domestic goat (race Cabra Blanca de Rasquera), the addition of SP did not improve sperm quality after thawing *in vitro* [Ohaneje et al., 2021]. Again, oviduct binding capacity or the ability to fertilize were not assessed. The impact of SP in our experiments was not very strong. So far, there is no study published yet that examines the impact of SP added post-thawing on the fertilization ability of feline sperm *in vitro* or *in vivo*. Sperm-oviduct binding assays *in vitro* seem to be a useful tool to predict fertility in domestic pig and domestic cattle [Waberski et al., 2006]. RamírezVasquez et al. [2019] conclude that seminal plasma can reduce the damage generated by the freezing/thawing procedure of sperm of the domestic sheep and this effect is increased when the extender was removed, particularly the egg yolk. In alpaca (*Vicugna pacos*), sperm obtained from the vas deferens were positively influenced by addition of SP post-thawing with regard to sperm motility, plasma membrane functionality and morphology [Aisen et al., 2021].

In conclusion, it should be investigated in a further study whether there is a positive effect on sperm-oviduct binding if the freezing extender is washed out after thawing and seminal plasma is subsequently added to the sperm.

5.5. Establishment of a compartmentalized long-term culture system for primary FOEC

In principle, FOEC-explants and vesicles are suitable to study sperm-oviduct interactions. However, they have some disadvantages, such as time limitation, viability, progressive de-differentiation, and they are not standardized in terms of cell type composition, size and surface area. A more reliable model is a polarized monolayer long-term culture. We took the first steps in the third study (chapter III) to develop such a long-term culture of FOEC in a compartmentalized culture system. Compared to the established equivalents for porcine, bovine and murine OEC, the success of our FOEC compartmentalized long-term culture approach was low. Seventy-one samples from 33 individuals were cultured. Five samples degenerated during the culture period of three weeks due to technical problems. After the three-week culture, hematoxylin-eosin (HE) sections were prepared from 66 samples and epithelia differentiation was evaluated according to morphological criteria. This evaluation showed that only five of 66 samples reached a differentiated status with a homogeneous epithelial monolayer (>5 µm height) consisting of polarized ciliated and non-ciliated cells.

In addition to the data in chapter III, further observations were made during the culture period of three weeks. From the beginning of the second week a so-called dome formation was observed in a few samples: starting from domes, tubule-like structures developed. After two weeks most samples showed these structures.

Interestingly, these structures and their direct surroundings were primarily lined with ciliated cells. Dome and tubule-like structures were described before in laboratory rat mammary gland and canine kidney epithelial cell cultures [Chen et al., 2019; Zucchi et al., 2002]. In mammary gland cell culture, cells located in domes are highly differentiated cells which express an early stage of lactogenic differentiation [Zucchi et al., 2002]. Chen et al. [2019] showed that the dome- and tubule-like structure forming canine kidney epithelial cells have stem cell characteristics. The formation of tubule-like structures in the canine kidney epithelial cell culture took 10 to 14 days, which is similar to our FOEC-cultures [Chen et a. 2019]. In case of our FOEC-cultures it has not yet been investigated whether the dome and tubule-like structures were caused by an uncontrolled growth or, similar to the mammary gland and kidney cell culture, by differentiating cells. It is also possible that the cell monolayer spontaneously develops folds, as it is the physiological situation in the oviduct. The phenomenon of spontaneous folding has been observed by Kessler et al. (2015) in the organoid culture of human OEC that derived from adult stem/progenitor cells [Kessler et al., 2015]. Further experiments are necessary to clarify whether the dome-like structures in our FOEC long-term cultures are physiological structures based on a sufficient number of isolated oviduct epithelial progenitor/stem cells before culture. Cross-sections of the respective regions in future experiments are suitable to visualize whether the monolayer is grown in folds or degenerated to irregular multi-layer structures. In parallel, appropriate markers are required to detect progenitor or stem cells. In human and mouse LGR5 (Leucin rich motif G-protein coupled receptor-5) [Van der Flier et al., 2007] as well as SSEA3 and SSEA4 (stage-specific embryonic antigen 3 and 4) [Chang et al., 2020] were identified as stem cell markers within those structures. In the porcine air-liquid (AL) interface OEC model, two stemcell markers, KLF4 (Krupple-like factor 4) and NANOG (Homeobox protein NANOG) were detected [Chen et al., 2018], which have been reported before in other epithelial tissues [Choobineh et al., 2016; Piazzolla et al., 2014]. These markers might be tested for their applicability in the domestic cat in future approaches.

Media used in culture procedures were modifications of previously described media for mouse tracheal cell culture [You et al., 2002] as well as for AL interface culture of murine and porcine OEC [Chen et al., 2017]. The proliferation medium consisted of DMEM/Ham's F12 supplemented with HEPES, antibiotics and anti- mycotic agents, FBS and various growth factors such as insulin, transferrin, cholera toxin, epidermal growth factor, bovine pituitary extract and retinoic acid. The differentiation medium was serum free but contains BSA and differs in the concentrations of the growth factors. In the studies on mouse tracheal cells as well as murine and porcine OEC a cell differentiation was achieved and the cells could be maintained under AL interface conditions for at least two and three weeks, respectively [You et al. 2002; Chen et al. 2017]. According to Kessler et al [2015], successful cultivation of human OEC requires supplementation with suitable growth factors that support paracrine signaling pathways (Wnt and Notch). These growth factors are Wnt3a and RSPO1 (R-Spondin-1) and help maintain the stem cell niche [Kessler et al., 2015]. They should be tested for long-term FOEC culture.

Originally, the compartmentalized culture system in which epithelial cells grow under AL interface conditions was developed for keratinocytes and tracheal epithelial cells [Pruniéras et al., 1983; Whitcutt et al., 1988*]. In vivo*, these epithelial cells are supplied with nutrients from the basal cell side. The apical cell side is covered just with a thin film of liquid. The compartmentalized culture system mimics the *in vivo* situation by basal feeding and availability of oxygen on the apical cell side [Prunieras et al., 1983, Whitcutt et al., 1988]. Johnson et al. [1993] demonstrated that a shift from an anaerobic to an aerobic metabolic pattern leads to differentiation under AL interface conditions.

In contrast to keratinocytes and airway epithelial cells, the oviduct is filled with oviduct fluid. On the one hand, the oviduct fluid is synthesized and secreted by oviduct cells themselves. On the other hand, there are also small contributions from follicular cells, peritoneal cells and the seminal plasma and by reflux of uterine fluid [Leese, 1988]. Against the background that the oviduct fluid is not solely secreted by the OEC, we tested a liquid-liquid (LL) interface beside the classical AL interface condition. According to Whittcutt et al. [1988],

polarization and differentiation of the epithelial cells is efficiently promoted by the basal supply of nutrients and does not only depend on the AL interface.

The question whether one of the two culture conditions is preferred for long-term FOEC-culture has not yet been answered. In our study, samples cultured under both AL and LL conditions were among the differentiated samples. Kreft et al. [2015a; 2015b] compared the AL and LL culture conditions for the Calu-3 cell line that demonstrates many characteristics of bronchial epithelium as well as for the RPMI 2650 cell line, a nasal epithelium model evaluated with immune-labeling of junctional proteins, ultrastructural analysis by electron microscopy, transepithelial electrical resistance (TEER) measurements, permeation studies, and determination of drug transporter genes. Calu-3 cells form tight epithelia at both culture conditions and the AL interface promotes appropriate cell differentiation and increases the drug transporter expression, whereas the LL interface results in a higher TEER [Kreft et al., 2015a]. The TEER measurement can be used as an indicator of the barrier function of a cell layer grown on a porous membrane which depends on cell integrity and permeability. During the determination of electrical resistance, a continuous current flows through the cells in both the paracellular as well as transcellular pathways [Powell, 1981]. The higher TEER values confirmed the tighter barrier function of Calu-3 cells under LL conditions against penetration of a high molecular weight dextran [Kreft et al., 2015a]. The study that compared RPMI 2650 cells under AL and LL interface demonstrated that RPMI 2650 cells are able to form tight cell barriers under both conditions. The models formed a multi-layered epithelium at both interfaces, however, with more layers under the AL compared under LL interface conditions. However, under the LL interface RPMI 2650 cells developed more cell junctions as well as microvilli, suggesting the LL interface being more suitable for RPMI 2650 differentiation than the AL interface. However, the mean TEER was higher in RPMI 2650 cells cultured at the AL than at the LL interface, which indicates a tighter barrier function of RPIM 2650 cells cultured under AL condition, which was also confirmed by the permeation study. The higher TEER value could result from a higher number of cell layers [Višnjar and Kreft, 2013].

These examples show that various quality parameters can be differentially affected by the culture conditions and a clear decision for either AL or LL condition is difficult. Further experiments are necessary to find out whether FOEC can be cultured more successful and reproducible under AL or LL conditions. Besides histology and immunohistology, TEER measurement and sperm binding assay should also be used for evaluation. Beside media supplementation and interface conditions, novel approaches are necessary to test further effective factors to establish a repeatable protocol for the long-term culture of FOEC. It is worth considering whether, in addition to the hanging insert, a compartmentalized microfluidic system should be tested. It is superior to the ordinary compartmentalized system because oviductal fluid flow can be simulated. Alternatively, we could try to culture FOEC in a suspension Matrigel® culture as described by Kessler et al. [2015]. An established FOEC long-term culture system provides an important tool for basic reproductive research and to optimize ARTs in the domestic cat as well as in threatened feline species. The domestic cat is therefore an important model species for the non-domestic feline species.

5.6. Conclusions

The first two studies showed that both, FOEC-explants and -vesicles are suitable for sperm-oviduct binding experiments. The SP-study revealed that epididymal sperm of the domestic cat are able to bind to FOEC-explants and, furthermore, SP has a supportive role in the sperm binding process. The equilibration-study disclosed that the binding capacity of epididymal sperm to FOEC-vesicles was reduced when sperm were diluted in freezing extender and equilibrated to 15°C. We suggest that the sperm population bound by head to FOEC-vesicles and possessing active mitochondria is the competent sperm population which would be primed and subsequently released for fertilization *in vivo*. These findings from both studies can contribute to the optimization of artificial insemination in felids. Further experiments are needed to optimize the cryopreservation procedure, including thawing and handling of sperm prior to insemination. This potentially comprises the removal of freezing extender and supplementation with SP. The third study showed that the long-term culture of FOEC in a compartmentalized system is basically possible. However, the desired result, a differentiated, monolayer, prismatic epithelium with secretory as well as ciliated cells, could not yet be reliably achieved. Therefore, future investigations should aim at the identification and isolation of progenitor cells from the epithelium of the feline oviduct in order to use them as defined starting material for the culture. With a reliable FOEC-culture system, a non-animal model would be available to test fundamentals of reproductive processes in the oviduct (as for instance the contribution of individual molecules in sperm-binding and release) and to conduct experiments to optimize ARTs including artificial insemination in felids.

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