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Original article

Reaction of fresh and frozen bull spermatozoa incubated with fresh and frozen bovine oviduct epithelial cells

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Abstract – The hypothesis was tested that frozen bovine oviduct epithelial cells (BOEC) could provide a physiologic and standardized system for studying motility and acrosomal changes of fresh and frozen-thawed sperm. The BOEC were collected by lavage from cows and monolayers prepared from both fresh and frozen-thawed cells. Fresh and frozen semen processed from five bulls was extended in a modified Tyrode's medium and coincubated with both types of monolayers at 39 °C in 5 % CO_2 and 95 % humidified air. After 5, 10 and 24 h of incubation, percentages of motile, progressively motile and acrosome-reacted unattached sperm were measured. The percentage of motile fresh sperm exceeded the motility of frozen sperm, and more fresh sperm attached to BOEC. More frozen sperm than fresh sperm were acrosome reacted. Fresh and frozen epithelial cells produced similar effects, except sperm motility was higher after 5 h of incubation with fresh BOEC. Thus, the convenient frozen-thawed coincubation protocol provides a practical in vitro system for studying capacitation and the acrosome reaction. © Inra/Elsevier, Paris

fresh bull spermatozoa / frozen bull spermatozoa / fresh oviduct cells / frozen oviduct cells

Résumé – Réaction acrosomique de spermatozoïdes frais ou décongelés de bovins, incubés en présence de cellules epithéliales fraîches ou congelées d'oviducte. Cette expérience vise à déterminer si des cellules épithéliales congelées d'oviductes bovins peuvent être utilisées comme système physiologique standard pour déterminer la capacité de la semence fraîche ou congelée à subir la réaction de l'acrosome. Des cellules épithéliales d'oviductes de vaches ont été recueillies par lavage et des monocouches ont été préparées à partir de cellules fraîches ou décongelées. La semence fraîche et congelée de cinq taureaux a été diluée dans du milieu Tyrode modifié et co-incubée avec les deux types de monocouches à 39 °C, 5 % de CO_2 et 95 % d'humidité. Après 5, 10 et 24 h d'incubation, les pourcentages de spermatozoïdes motiles, progressivement motiles

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et de spermatozoïdes non liés ayant subi la réaction acrosomique ont été déterminés. Le pourcentage de motilité des spermatozoïdes frais était supérieur à celui des spermatozoïdes congelés. Plus de spermatozoïdes provenant de la semence fraîche se sont attachés aux cellules épithéliales d'oviductes. Un nombre plus élevé de spermatozoïdes congelés a subi la réaction acrosomique comparativement aux spermatozoïdes frais. Les cellules épithéliales fraîches ou décongelées ont donné des résultats similaires. La seule exception est que le pourcentage de spermatozoïdes motiles était plus élevé aprés 5 h d'incubation avec des cellules épithéliales fraîches. L'incubation de spermatozoïdes avec des cellules épithéliales décongelées représente donc une méthode pratique pour l'étude de la capacitation et de la réaction acrosomique. © Inra/Elsevier, Paris

spermatozoïdes frais de bovin / spermatozoïdes congelés de bovin / cellules fraîches d'oviducte / cellules congelées d'oviducte

1. INTRODUCTION

The major events in the final stages of oocyte and sperm maturation, sexual union and early embryo development occur in the oviduct. Despite numerous studies [2, 3, 19, 42] these events are poorly understood. Following matings in cattle only a few hundred of the billions of sperm deposited in the anterior vagina migrate through the cervical barrier, uterus, tubo-uterine junction and reach the oviduct [17, 19, 21, 41]. In artificial insemination the cervical barrier usually is bypassed, but again only a few sperm out of millions deposited reach the oviducts [7, 17].

Although some sperm may traverse the tubular structures in a few minutes [40], this is controversial [39] and sperm likely participating in fertilization arrive later at the site of fertilization. Fertilizing sperm are thought to come from a pool established near the tubo-uterine junction and caudal isthmus [19–21, 36]. A local effect of oviduct tubal fluids secreted at estrus on capacitation and the acrosome reaction have been reported [1, 9, 18, 24, 33].

These studies have provided considerable information on sperm changes in the female reproductive system. However, sperm recovery from the oviduct in vivo is low and variable [17, 29, 36], causing uncertainty that the sperm assessed in samples obtained are representive of the population of sperm poised to fertilize the oocyte. Another approach to studing sperm interactions with the oviductal epithelial cells has been the use of sperm-oviductal epithelial cell coincubation systems [5, 6, 8, 10–12, 15, 16, 22, 23, 26, 28 34]. In some studies fresh sperm have been used and in other studies frozen-thawed sperm were used. Usually BOEC monolayers have been prepared from fresh oviductal tissue, but it is difficult to always have these available when needed, particularly if tissue from animals in estrus are used. Furthermore, use of frozen stocks of BOEC offers the potential of comparing test results on material originating from the same cow or duplicated from a series of cows.

No reports were found studying the possible interaction of fresh versus frozen sperm coincubated with the fresh versus frozen-thawed epithelial cells. While fresh semen was used for many years in artificial insemination of cattle, most current commercial artificial insemination is with frozen sperm [15]. It is often inferred that storage and survival time of frozen-thawed bull sperm is shorter than for fresh sperm in the female reproductive tract. Therefore, this in vitro study was designed to compare changes in fresh and frozen-thawed bull sperm from the same bulls coincubated with monolayers prepared from fresh and frozen-thawed oviductal epithelial cells from the same cows.

2. MATERIALS AND METHODS

2.1. Semen

Semen was obtained from five bulls randomly chosen at the Eastern Artificial Insemination, Coop., Inc. Bulls were on a regular semen collection schedule. After collection, the semen was held in a 35 °C water bath while subsamples were used to determine the sperm concentration and the percentage of motile sperm.

The semen to be frozen was extended with whole milk (WM) at 35 °C. The WM had been prepared by heating to 95 °C for 10 min in a double boiler, cooled and filtered. Two fractions of WM were prepared, one without glycerol for cooling the sperm to 5 °C and a fraction with glycerol so that sperm, when frozen, were in WM containing 7 % glycerol, vol/vol. The extended semen was packaged in 0.5 mL French straws, containing 20×10^6 sperm, and frozen by the standard procedure used at the insemination center about 4 h after semen collection. These straws of frozen semen were used in the ensuing days when fresh bull sperm were obtained for comparison. Straws were thawed in warm water at 35 °C. The newly collected fresh semen from the same bulls used to provide the frozen sperm was also initially evaluated for sperm concentration and the percentage of motile sperm. The ejaculates within the same bulls differed by less than 5 %. Thus, similar quality semen from the same bulls could be used simultaneously to test the two kinds of BOEC.

2.2. Preparation of oviduct cell monolayers

Oviducts were obtained from cows on the day of standing estrus, programmed by treatment with prostaglandin $F_2\alpha$. BOEC were recovered by luminal lavage and gentle massage of the oviducts using 3 mL of PBS plus 1 % polyvinyl alcohol and 2 % of an antibiotic mixture. Then detached cells were washed and resuspended. Then 0.5 mL portions of the clumps of detached cells were placed in 4-well tissue culture plates and cultured for 4 days at 39 °C in 5 % CO_2: 95 % air in 0.5 mL of incubation medium consisting of Ham's F10 + 10 % fetal bovine serum and growth factors [10, 11] until monolayers formed. Then cells from several wells were stripped by freeing the cells with trypsin-EDTA in calcium-free medium, and frozen in 50 % bovine serum containing 10 % DMSO. As needed, frozen cells were thawed, washed and replated in incubation medium for 4 days to form new monolayers before use. Whenever fresh or frozen-thawed BOEC were replated the trypsin-EDTA medium was used. Wells containing at least 80 % monolayer formation from fresh or frozen-thawed BOEC from the same cow were selected to compare the effects on each sample of semen. Before adding the sperm preparations, the selected cultures of BOEC were washed with sperm-TALP, modified from Parrish et al. [32] to contain 25 mM potassium and no glucose [31].

2.3. Experimental preparation of semen

Seven straws of frozen-thawed semen (20×10^6 sperm per straw) were diluted in 6 mL of sperm-TALP and centrifuged for 15 min on a 45–90 % Percoll gradient. This removed the milk from the frozen-thawed sperm and seminal plasma from all samples to facilitate capacitation and the acrosome reaction [4]. The sperm pellet was resuspended in 1 mL of sperm-TALP. Sperm in the sperm-TALP were counted with a Coulter counter to determine the volume of sperm in sperm-TALP needed to give 25×10^6 sperm/mL with 0.5 mL/well. Also 1 mL of fresh semen from these bulls was obtained, counted with the Coulter counter, and sufficient sperm added to sperm-TALP to provide 25×10^6 sperm/mL.

2.4. Sperm incubation and evaluation

Fresh sperm-TALP (0.5 mL) was added to each well containing BOEC for co-incubation with sperm. Sperm were incubated at 39 °C in 5 % CO_2: 95 % air, and were assayed at 5, 10 and 24 h. The sample in each well was agitated by pipetting all of the supernatant ten times with protein-coated pipettes to prevent sperm from sticking. Than a 100 μL subsample from the well-mixed fluid in each well was sampled for analysis. Sperm motility was assessed using the HTM Hamilton-Thorn 2030 (Hamilton Thorne Research, Beverly, MA) sperm analyzer [13, 38]. The sperm concentration of 25×10^6/mL was suitable for direct assessment by CASA of the motility characteristics of sperm.

Two chambers and eight fields per chamber were examined. Although many motion characteristics were measured, the ones of most interest are the percentages of motile sperm (PM) and the percentages of progressively motile sperm (PPM) presented here.

Sperm also were examined for changes in the acrosomal status. The percentage of AR sperm was determined by using the Giemsa stain, as described by Kovács and Foote [25]. Two slides were prepared for each subclass of sperm and 100 sperm per slide were evaluated for the acrosome reaction.

Attachment of the different types of sperm preparations to BOEC prepared from fresh and frozen-thawed BOEC was examined at 5 h by removing a small aliquot of unattached sperm from a well-mixed supernatant and counting them with a Coulter counter. The counting fluid usually reduced clumped sperm. This count was subtracted from the total sperm placed in the well to obtain the attached cell count. Enough clumping of sperm occurred at longer intervals of incubation to prevent obtaining accurate counts at 10 and 24 h.

2.5. Experimental design and statistical analysis

The design consisted of a factorial arrangement with two types of BOEC monolayers (fresh versus frozen-thawed) and two types of semen from the same bull (fresh versus frozen-thawed). Observations were made after 5, 10 and 24 h of incubation. The experiment was replicated with fresh and frozen semen from the same five randomly chosen bulls tested with each fresh and frozen BOEC monolayer prepared from the same cow. A mixed model general linear analysis of variance was used. Data were analyzed by blocking on bulls and the experimental model consisted of bulls as a random variable, and the fixed effects of type of BOEC, type of sperm, interactions, and sampling variation associated with replication. Data for each time of incubation were analyzed separately. Means were compared using Duncan's multiple range test, and values differing at $P \leq 0.01$ were considered to be statistically significant.

3. RESULTS

The mean percentages of motile sperm (PM), progressively motile sperm (PPM), and acrosome-reacted sperm (AR) are summarized in *table I*. There was no difference ($P > 0.05$) between sperm incubated with fresh versus frozen-thawed BOEC for PM or PPM, except at 5 h of incubation. In contrast there was a major difference between fresh and frozen-thawed sperm. The latter sperm were consis-

Table I. Motility and acrosome reaction of cocultured sperm.

Treatment	Incubation (h)								
	5			10			24		
(%)	PM	PPM	AR	PM	PPM	AR	PM	PPM	AR
Fresh sperm/fresh BOEC	68	62	16	42	37	35	15	13	62
Fresh sperm/frozen BOEC	60	53	17	47	43	29	14	11	55
Frozen sperm/fresh BOEC	32	28	54	21	18	63	6	4	68
Frozen sperm/frozen BOEC	26	23	55	20	17	66	7	5	74
Means for fresh BOEC	49^a	44^a	35^a	31^a	27^a	49^a	11^a	9^a	65^a
Means for frozen BOEC	42^b	37^b	36^a	33^a	30^a	47^a	11^a	8^a	64^a
Means for fresh sperm	64^a	58^a	16^a	45^a	40^a	32^a	15^a	12^a	58^a
Means for frozen sperm	29^b	25^b	54^b	20^b	18^b	64^b	6^b	5^b	71^b

* PM = % of total motile sperm; PPM = % progressively motile sperm; AR = % of acrosome-reacted sperm; ^{a,b} values for paired main treatment effects with different superscripts differ, $P < 0.01$

tently less motile throughout the incubation period and a higher proportion of the frozen-thawed sperm were acrosome-reacted ($P < 0.01$). This effect was consistent regardless of whether either type of sperm were incubated with fresh or frozen-thawed BOEC. The interaction between the two was not significant, reflecting, in part, the fact that the fresh and the frozen-thawed BOEC had the same effect on sperm. The same relationship was observed for sperm velocity estimated by CASA, but for simplicity these data are not reported.

It was noted visually that the heads of fresh sperm attached rapidly to both fresh and frozen BOEC, with the tails exhibiting rapid flagellar movement. No major difference in appearance was apparent, except more fresh sperm attached than frozen-thawed sperm. At 5 h there was an average of $5.0 \pm 0.8 \times 10^6$ fresh sperm and $2.8 \pm 0.6 \times 10^6$ frozen-thawed sperm attached to both types of BOEC ($P < 0.05$). Attachment of both types of sperm to fresh and frozen BOEC was $3.5 \pm 0.8 \times 10^6$ and $4.3 \pm 0.8 \times 10^6$, respectively ($P > 0.05$).

4. DISCUSSION AND CONCLUSIONS

The present studies extend previous research. Ellington et al. [10, 11] and Pollard et al. [34] described a BOEC culture system which could be used to examine several effects on fresh bull sperm in vitro. Sperm attached tightly to the BOEC monolayers and more sperm underwent the acrosome reaction (AR) when they were incubated with the monolayers than when incubated in monolayer-conditioned medium. Chian and Sirard [5], Chian et al. [6] and Lefebvre et al. [26, 28] reported that freshly prepared BOEC monolayers promoted capacitation, but that fewer capacitated sperm bound to the epithelial cells [28]. Dobrinski et al. [8] reported that freezing and thawing equine sperm also decreased their attachment to oviductal epithelial cells.

For uniformity all BOEC monolayers used in our studies were prepared from oviducts of cows in 'standing' estrus, although BOEC obtained from oviducts at different stages of the estrous cycle gave similar results [27]. Monolayers were either used directly or frozen and new monolayers prepared from the frozen-thawed cells. The monolayers from both sources were similar in appearance, and the monolayers from fresh cells have been described [11, 37, 38]. Monolayers prepared from both sources have numerous microvilli that make fibril-like attachments to the sperm.

The studies reported here provided a comparison between the effects of fresh and frozen-thawed BOEC prepared as monolayers from the same cows for coincubating fresh and frozen-thawed sperm. No previous reports of these direct comparisons were found in the literature. When sperm were coincubated with monolayers from fresh or frozen BOEC, only at 5 h was any difference found in the proportion of motile sperm and no difference in the percentage of AR sperm on fresh versus frozen BOEC was found at any time interval (*table I*).

Attachment of sperm heads to both types of monolayer was similar and the appearance has been described by Ellington et al. [11], Suzuki and Foote [37] and Suzuki et al. [38], as viewed in detail by electron microscopy. Also, the number of sperm attached to the two sources of monolayers was not significantly different. Thus, the estimates for the proportion of motile and AR sperm are not biased by any difference in the proportion of sperm attached to the two kinds of monolayers.

Fresh and frozen-thawed sperm attach rapidly to BOEC monolayers, reaching a peak in 3 h [37]. The attachment at 5 h likely represents this initial attachment

with gradual subsequent release after 4–5 h [11]. More fresh sperm than frozen-thawed sperm were attached to the BOEC at 5 h. The difference in attachment between fresh and frozen sperm would likely be less after longer exposure [11], but clumping of sperm in the present experiment prevented accurate counting at the longer time intervals. Suzuki and Foote [37] reported that the concentration of sperm placed on the BOEC also influenced the number of sperm bound.

The PM and PPM of fresh sperm exceeded that of frozen sperm throughout the incubation period (*table I*). Subjective visual checking of the frozen-thawed sperm immediately after thawing indicated that the proportion of motile sperm had been decreased by freezing.

The proportion of AR fresh sperm was considerably less than for frozen sperm. The increase in AR over time presumably represents capacitation and some death. The extent that this is due to physiological changes in the acrosome and those accompanying senescence of the sperm cannot be determined in the present experiments, as the sperm were not sub-classified as live or dead cells. However, Ellington et al. [11] reported that most of the AR sperm after 4 h of incubation were dead sperm. This is consistent with the hypothesis that the AR sperm found up to 5 h of incubation represent sperm killed by freezing, and supports the commercial practice of using more total frozen-thawed sperm than fresh sperm for insemination. However, with 10×10^6 or 100×10^6 sperm inseminated Nadir et al. [30] found no difference between fresh and frozen semen in accessory sperm attached per egg.

Samper et al. [35] reported that survival of fresh equine sperm coincubated with BOEC was correlated with fertility. There were similarly large differences in sperm survival among semen samples from the five bulls used in the present study. Replication was not sufficient to characterize bulls and further work is needed to pursue this promising lead of the relationship between survival of sperm coincubated with BOEC and fertility. This is supported by the finding [14] that most of the variance in CASA characteristics among semen collected from bulls under the same conditions, as reported here, is due to differences among bulls, with less than 10 % due to variability of samples within bulls.

In conclusion, quality of the fresh sperm coincubated with BOEC was superior to frozen-thawed sperm. More fresh sperm adhered to BOEC monolayers, indicating that freeze thawing caused a change in the surface membranes of the sperm which participate in attachment. The BOEC stored frozen and then thawed provided essentially equivalent monolayer conditions for coincubating either fresh or frozen-thawed bull sperm. Large batches of BOEC can be prepared and frozen with the convenience that subsets are available for replicated use through a series of experiments, thus, also avoiding the usual confounding of different sources of BOEC.

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