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Bovine oviductal monolayers cultured under three-dimension conditions secrete factors able to release spermatozoa adhering to the tubal reservoir *in vitro*

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ABSTRACT

Different *in vitro* models have been developed to understand the interaction of gametes and embryos with the maternal reproductive tract. We recently showed that bovine oviductal monolayers three-dimensionally cultured in Gray's medium on collagen-coated microporous polycarbonate inserts under liquid-air interface conditions are well polarized, develop cilia, remain viable for at least 3 weeks postconfluence, and mantain the viability of bound spermatozoa significantly better than bidimensionally cultured monolayers. Herein, we used these culture conditions to understand whether: (1) spermatozoa adhering to three-dimensionally cultured oviductal monolayers can be released by heparin or penicillamine as previously shown with bidimensionally cultured oviductal monolayers and explants; and (2) media conditioned by three-dimensionally cultured oviductal monolayers were able to release spermatozoa adhering to oviductal explants. Findings demonstrated that (1) spermatozoa adhering to three-dimensionally cultured oviductal monolayers are readily released by heparin and penicillamine, (2) media conditioned by three-dimensionally cultured oviductal monolayers are able to release spermatozoa bound to oviductal explants, (3) do not depress sperm motility and viability, (4) they improve sperm kinetics, and (5) promote binding to the zona pellucida. In conclusion, *in vitro* data suggest that the release of spermatozoa adhering to the oviductal reservoir *in vivo* can be triggered by factors secreted by the oviduct itself that induce sperm capacitation.

1. Introduction

In mammals, sperm ascension within the female reproductive tract involves a transient adhesion to the caudal isthmus of the oviduct [1]. Sperm adhesion to this specialized region, which is termed the "oviductal reservoir" [2], extends the sperm fertile life by delaying capacitation until, at approximately the time of ovulation, stored spermatozoa are released for fertilization [3]. Little is known about the nature and the source of signals that allow the release of spermatozoa from the oviductal reservoir in different species. In cattle, molecules responsible for release of spermatozoa from the oviductal reservoir have been searched among those described in the oviductal fluid and that have increased concentration at estrus [3]. As a result of these studies we provided for the first time direct evidence, among all species examined thus far, that sulfated glycoconjugates [4] and disulfide-reductants (SS-reductants) [5,6] are powerful inducers of the release of spermatozoa adhering to *in vitro* cultured oviductal epithelium. These two different classes of molecules have been suggested to represent physiologic signals for sperm release as they are similar to heparin-like glycosaminoglycans and reduced glutathione, respectively, both of which are present in the bovine oviductal fluid and increase in concentration at estrus [7–9]. Both

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inducers were shown to act on spermatozoa, and release was accompanied by the reduction of surface protein disulfides to sulfhydryls and capacitation [4–6,10]. As concerns the source of releasing signals, the oviduct itself under the influence of hormones could be able to secrete them. Alternatively, or in synergy with oviductal secretions, the cumulus oocyte complex and/or the follicular fluid could contain the specific signals able to release the spermatozoa stored in the oviductal reservoir. Previous experiments in our lab failed to demonstrate an effect of media conditioned by bidimensionally cultured oviductal monolayers (OMs) on sperm release. This could have been because of a dedifferentiation of the oviductal epithelium cultured under those conditions. We recently showed that bovine OMs three-dimensionally cultured in Gray's medium (GM) on microporous inserts under air-liquid interface conditions (3D OMs), adapting a 3-D cell culture method applied on monkey oviductal epithelium [11], are better differentiated than bidimensional OMs [12]. In particular, under these conditions epithelial cells become polarized, develop cilia, bind spermatozoa, and maintain their viability better over time, and express these features at least for 3 weeks after confluence [12].

In this study, we used these modified culture conditions to understand whether spermatozoa adhering to 3D OMs can be released by heparin or penicillamine as previously shown with bidimensional OMs and oviductal explants, and the effects of media conditioned by 3D OMs on the release of spermatozoa adhering to oviductal explants, and on sperm motility, kinetics, viability, and binding to the zona pellucida (ZP).

2. Materials and methods

2.1. Chemicals

Medium 199 (M4530), BSA (fraction V), penicillin, streptomycin, gentamycin, amphotericin B, fetal calf serum (FCS), human placental collagen, acetic acid, paraformaldehyde, hydrocortisone, insulin, transferrin, epinephrine, triiodothyronine, epidermal growth factor, all trans-retinoic acid, bovine pituitary extract, sucrose, heparin sodium salt, and penicillamine were purchased from Sigma (Milan, Italy); Dulbecco Minimum Essential Medium (DMEM), Ham's F12 and bronchial epithelial cell basal medium were purchased from Lonza (Milan, Italy); glutaraldehyde was purchased from SIC (Rome, Italy). Reagents and water for preparation of salines and culture media were all cell culture tested.

2.2. Preparation of oviductal epithelial cells

Oviducts were collected at the time of slaughter and transported to the laboratory in Dulbecco's phosphate buffered saline supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin at 4 °C. Laminae of epithelial cells were recovered from oviducts of single animals by squeezing (3 experiments with 2 individuals each) and cultured at 39 °C, 5% CO_2 in air, 95% humidity, in M199 supplemented with 50 μg/mL gentamycin, 1 μg/mL amphotericine B, and 10% FCS. After 24 hours of culture, bovine oviductal epithelial cells rearranged to form swimming-everted vesicles, referred to as explants. Explants were either used within 24 hours of culture for sperm-oviduct adhesion assays with conditioned media (see below) or for preparation of 3D OMs. In particular, within each experiment, explants collected from a single individual were cultured to form confluent OMs in a semidefined culture medium according to Gray et al. [13] (GM), on collagen-coated polycarbonate nembrane 12-well Transwell permeable supports, with 0.4 μm pores (Corning, Milan, Italy) under air-liquid interface conditions. In particular, explants cultured in 10-cm Petri dishes (Falcon; Becton Dickinson, Milan, Italy) for 24 hours, were seeded at 0.5×10^6 cells per cm^2 onto collagen-coated polycarbonate inserts. Collagen coating was done with 0.05% human placental collagen according to Coleman et al. [14]. Cells were initially cultured in an immersed condition with 1.5 mL of medium in the well and 0.5 mL in the insert. Culture was performed 24 hours in 1:1 DMEM/Ham's F12 supplemented with 5% fetal bovine serum and 50 μg/mL gentamycin and then in GM until cell confluence that was achieved in 3 to 5 days. Confluence was assessed through labeling of cells with Hoechst 33342 10 μg/mL for 10 minutes, excision of the membrane and evaluation was done at the fluorescence microscope. After cell confluence, the medium on inserts was removed and 3D OMs were cultured with 1.5 mL of GM in the well (basolateral side of the cells) changing the medium every 48 hours. Gray's medium is a 1:1 mixture of DMEM and bronchial epithelial cell basal medium supplemented with 1.4 μM hydrocortisone, 5 μg/mL insulin, 10 μg/mL transferrin, 2.7 μM epinephrine, 9.7 nM triiodothyronine, 0.5 ng/mL epidermal growth factor, 50 nM all trans-retinoic acid, 2% bovine pituitary extract (14 mg protein per mL), 1.5 μg/mL BSA, 100 μg/mL gentamycin, and 2.5 μg/mL amphotericin B. All components were freshly purchased and prepared for each experiment. 3D OMs and explants derived from six individuals were used for preparation of conditioned media and for experiments on sperm-oviduct binding and release.

2.3. Preparation of conditioned and control media

Previous studies [12] showed that 3D OMs, cultured as described above, require 2 to 3 weeks of postconfluence culture to achieve a differentiated condition as shown by cell height and ciliation. Therefore, in the present study, conditioned media (CM) were prepared adding 500 μL of GM per insert to the apical surface of 3D OMs at 3 weeks of postconfluence culture. Control media (bottom well media [BWM]) were prepared adding 500 μL of GM in the bottom of wells containing the inserts of the same samples mentioned above. After 48 hours of incubation, CM and BWM were separately collected, pooled, centrifuged at 600× *g* for 5minutes and the supernatants were stored at −20 °C until use. To ascertain that media recovered by the inserts were really conditioned by secretions of the apical side of the cultured epithelium, the integrity of each 3D OM used to produce CM was evaluated through labeling of cells with Hoechst 33342 10 μg/mL for 10 minutes, excision of the membrane, and observation with the fluorescence