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A phosphodiesterase type-5 inhibitor, sildenafil, induces sperm capacitation and penetration into porcine oocytes in a chemically defined medium

Sumire Ioki [a], Qing-Shan Wu [a], Osamu Takayama [b], Hideyuki H. Motohashi [b], Takuya Wakai [a], Hiroaki Funahashi [a,b,*]

[a] Department of Animal Science, Okayama University, Okayama, Japan
[b] Assisted Reproductive Technology Center, Okayama University, Okayama, Japan

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ABSTRACT

The present study was undertaken to determine the effect of a phosphodiesterase (PDE) type-5 (cyclic guanosine monophosphate-specific) inhibitor, sildenafil, on capacitation and penetration of boar spermatozoa in a basic chemically defined medium (adenosine- and theophylline-free PGM-tac4). When ejaculated spermatozoa were cultured for 90 minutes in the absence or presence of sildenafil at 2.5 mM, the inhibitor significantly increased the percentage of capacitated/acrosome-reacted spermatozoa, as a result of the chlortetracycline assay. When fresh spermatozoa were co-cultured with oocytes in the presence of sildenafil at a different concentration (0, 2.5, 25, or 250 μM), higher sildenafil concentrations (25 and 250 μM) significantly resulted in higher sperm penetration rates. When oocytes matured *in vitro* were co-cultured with spermatozoa in the presence of 25 μM sildenafil or 25 mM caffeine benzoate for 8 hours, the incidence of penetrated oocytes did not differ between two groups, whereas the incidence of monospermic oocytes in penetrated one was significantly higher in the presence of sildenafil. Immunocytochemical analysis reported the presence of PDE type-5 on the acrosome region of boar spermatozoa. These results report that regulation of cyclic guanosine monophosphate-specific PDE type-5 by sildenafil somehow can increase the penetrability of boar spermatozoa *in vitro*.

1. Introduction

An increased intracellular cyclic adenosine monophosphate (cAMP) level is required to trigger the sequence of mammalian sperm capacitation, including a change of intracellular pH, activation of protein kinase A activity, and induction of phosphorylation of protein tyrosine [1]. *In vitro* capacitation of spermatozoa in domestic animals, including pigs, has been induced for decades by commonly using caffeine, a general nonspecific phosphodiesterase (PDE) inhibitor [2–4], whereas the presence of serum albumin or methyl-beta-cyclodextrin (as inducer of cholesterol efflux) [5,6], bicarbonate (as an initiator of early event in the capacitation) [7,8], and calcium (as a messenger of late calcium-dependent event of capacitation) are also essential in the medium. Recently, a chemically defined medium, PGM-tac4, has been developed for *in vitro* fertilization of porcine oocytes [9] and supported blastocyst development and piglet production well [10]. This medium contains a nonspecific PDE inhibitor, theophylline (2.5 mM), and an accelerator of adenylyl-cyclase, adenosine (1 μM) [9]. Nonspecific PDE inhibitors, such as caffeine and theophylline, may allow increase in not only the intracellular level of cAMP but also that of cyclic guanosine monophosphate (cGMP) by inhibition of the reductions into AMP and GMP. Recently, it has been reported that nitric oxide, which

* Corresponding author. Tel./fax: +81-86-251-8329.
E-mail address: hirofun@okayama-u.ac.jp (H. Funahashi).

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interacts with both cAMP and cGMP signal pathways [11], is including the induction of sperm capacitation [12,13]. It has been also reported that a cGMP-specific PDE inhibitor stimulates motility and capacitation of human spermatozoa [14]. However, it appears to be still unclear whether the cGMP-specific PDE inhibitor can regulate successfully to induce capacitation of spermatozoa and to increase the penetration into IVM oocytes *in vitro* in domestic animals.

So far, three cAMP-specific PDEs (type-4, type-7, and type-8), 3 cGMP-specific PDEs (type-5, type-6, and type-9), and five nonspecific PDEs (type-1, type-2, type-3, type-10, and type-11) PDEs have been identified [15,16]. Expression of PDEs types-1, -3, and -4 has been found in human spermatozoa [17,18]. Whereas a type of antibodies for cGMP-specific PDE type-5 did not give a distinct signal in mouse spermatozoa [19], effect of PDE type-5 inhibitor on capacitation and/or acrosome reaction of human spermatozoa is debatable with positive [20,21] and negative results [14,22]. Furthermore, effect of PDE type-5 on capacitation and penetrability of boar spermatozoa is not known well.

In the present study, therefore, we investigated effects of PDE type-5 inhibitor, sildenafil, in a chemically defined medium on the induction of capacitation/acrosome reaction *in vitro* and then examined the penetrability of boar spermatozoa in the presence of sildenafil, in the comparison with a common nonspecific PDE inhibitor, caffeine. In the previous experiment, we determined whether PDE type-5 exists on boar spermatozoa.

2. Materials and methods

2.1. Chemicals and culture media

Potassium chloride, KH_2PO_4, $MgCl_2 \bullet 6H_2O$, $CaCl_2 \bullet 2H_2O$, sodium citrate, and citric acid were purchased from Ishizu Pharmaceutical Co., Ltd (Osaka, Japan). Sodium chloride and paraffin liquid were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Equine chorionic gonadotropin (eCG ; Serotropin) and human chorionic gonadotropin (hCG; gonadotropin) were purchased from ASKA Pharmaceutical Co., Ltd. (Tokyo, Japan). Unless specified, other chemicals were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan). A basic diluent solution for transportation and keeping boar spermatozoa was modified Modena solution [23] composed of 152.61 mM glucose, 23.46 mM sodium citrate $2H_2O$, 11.9 mM $NaHCO_3$, 6.99 mM EDTA-2Na$\bullet 2H_2O$, 46.66 mM TRIS, 15.10 mM citric acid, and 10 mg/mL gentamicin. Egg yolk–based extender (20% [v:v] hen's egg yolk in mMS) was used as the cooling extender. The medium used for washing and manipulating spermatozoa and cumulus–oocyte complexes was modified TL-HEPES-PVA medium composed of 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM KH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2 \bullet 6H_2O$, 2 mM $CaCl_2 \bullet 2H_2O$, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w:v) polyvinyl alcohol, 25 μg/mL gentamicin, and 65 μg/mL potassium penicillin G [24]. The basic IVM medium used was a BSA-free chemically defined medium, porcine oocyte medium (POM; Research Institute for the Functional Peptides, Yamagata, Japan) [9] modified with 50 μM beta-mercaptoethanol (mPOM) [10]. This IVM medium supports blastocyst development well after IVF if gonadotropins are added during IVM [9,10]. The chemically defined medium for sperm treatment and IVF was adenosine- and theophylline-free PGM-tac4 (Research Institute for the Functional Peptides, Yamagata, Japan). All media (except for modified TL-HEPES-PVA) were equilibrated at 39 °C in an atmosphere of 5% CO_2 in air overnight before use (only adenosine- and theophylline-free PGM-tac4 was under paraffin liquid).

2.2. Preparation of fresh boar spermatozoa

Semen-rich fractions (about 50 mL) were collected from totally four Birkshire boars by glove-hand method at a local experimental station and were diluted five times with modified Modena solution [23]. The diluted semen samples were transported to the laboratory at 26 °C to 32 °C within 2 hours after collection. Spermatozoa were diluted at a concentration of 1×10^8 cells/mL with modified Modena solution containing 5-mM cysteine and 20% (v:v) boar seminal plasma. Ten milliliters of the resuspended sperm cells were washed three times by centrifugation at 750 *g* for 3 minutes with modified TL-HEPES-PVA solution and then used for experiments.

2.3. Preparation and culture of cumulus-oocyte complexes

Ovaries were collected from slaughtered prepubertal gilts at a local abattoir and transported to the laboratory in 0.9% (w:v) NaCl solution containing 75 μg/mL potassium penicillin G and 50 μg/mL streptomycin sulfate at 27 °C to 33 °C. Using an 18-gauge needle and a disposable 10-mL syringe, cumulus-oocyte complexes were aspirated from antral middle follicles with 3 to 6 mm in diameter on the surface of ovaries and washed three times with modified TL-HEPES-PVA medium at room temperature (25 °C) [24]. The complexes with uniform ooplasm and a compact cumulus cell mass were selected and washed three times with mPOM. Forty to fifty of these complexes were subsequently cultured in 500 μL of mPOM added 1 mM dibutyryl cAMP, 10 IU/mL eCG, and 10 IU/mL hCG for 20 hours, and then in 500 μL of mPOM without these additives for a further 24-hour period, at 39 °C in an atmosphere of 5% CO_2 in air with 100% humidity [24]. After IVM culture, oocytes were denuded from cumulus cells by pipetting with 0.1% (w:v) hyaluronidase.

2.4. Chlortetracycline fluorescence assessment of spermatozoa

To examine the functional status of spermatozoa, chlortetracycline (CTC) fluorescence assay was done as described previously [4,25]. Treated sperm cells were assessed immediately under a phase-contrast microscope, equipped with epifluorescent optics. Each cell was first observed under ultraviolet illumination to determine the live and/or dead status; the sperm cells showing bright blue staining of the nucleus (bisbenzimide Hoechst 33258-positive cells) were considered as dead and not counted. Two hundred live spermatozoa were then examined under blue–violet illumination and classified according to CTC staining patterns. The three fluorescent staining patterns