Zygote **14** (November), pp. 275–285. © 2006 Cambridge University Press
doi:10.1017/S0967199406003819 Printed in the United Kingdom

Inhibition of boar sperm hyaluronidase activity by tannic acid reduces polyspermy during *in vitro* fertilization of porcine oocytes

Hideki Tatemoto[1], *Isao Tokeshi*[1], *Satoshi Nakamura*[2], *Norio Muto*[3] *and Tadashi Nakada*[1]

Department of Bioproduction, Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan, Laboratory of Swine, Okinawa Prefectural Livestock Experiment Station, Okinawa, Japan and Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Japan

Date submitted: 25.11.05. Date accepted: 30.01.06

Summary

The present study was conducted to examine the effects of three polyphenols (tannic acid, apigenin and quercetin) on hyaluronidase activity and *in vitro* fertilization (IVF) parameters. Among them, tannic acid showed by far the strongest potency for blocking hyaluronidase activity extracted from preincubated boar sperm, causing a dose-dependent inhibition over the range of 2–10 μg/ml. When cumulus-intact and cumulus-free oocytes were inseminated in IVF medium containing tannic acid, the penetration and the polyspermy rates were significantly decreased in the presence of 10 μg/ml tannic acid compared with those in the absence of tannic acid, and the addition of 5 μg/ml tannic acid significantly reduced the polyspermy rate ($p < 0.05$) compared with that of the control while maintaining the high penetration rate. However, apigenin and quercetin had no effect on the rate of polyspermy. Interestingly, the incidence of polyspermy was significantly reduced in oocytes inseminated with sperm pretreated with 5 μg/ml tannic acid ($p < 0.05$), although the pretreatment of oocytes had no effect against the polyspermy after insemination with untreated sperm. Treatment with tannic acid caused neither a protective proteolytic modification of the zona pellucida matrix before fertilization, nor a reduction of the proteolytic activity of acrosomal contents or the number of zona-bound spermatozoa. These data suggest that an appropriate concentration of tannic acid prevents polyspermy through the inhibition of sperm hyaluronidase activity during IVF of porcine oocytes.

Keywords: Boar spermatozoa, Hyaluronidase, *In vitro* fertilization, Polyphenols, Polyspermy

Introduction

Recent progress in *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) technologies has promoted the use of porcine embryos for the study of early zygotic development (Tatemoto *et al.*, 2001, 2004; Brad *et al.*, 2003; Marchal *et al.*, 2003). However, in porcine IVF, the abnormally high incidence of polyspermy, which often exceeds 50% (Nagai *et al.*, 1984; Wang *et al.*, 1994; Asano & Niwa, 2004), remains a major impediment.

Various improvements have been applied to the method of porcine IVF to decrease the polyspermy rate while maintaining a high sperm penetration rate. The addition of oviductal epithelial cells (Nagai & Moor, 1990; Kano *et al.*, 1994), oviductal fluid (Kim *et al.*, 1996), or porcine oviduct-specific glycoprotein (Kouba *et al.*, 2000; McCauley *et al.*, 2003) to IVF medium has been used to reduce the incidence of polyspermy in porcine oocytes, suggesting that factor(s) secreted from the oviduct may be associated with the functional block of polyspermy during IVF. Our recent study clearly demonstrated that chondroitin sulfate A-derived oligosaccharide (ChSAO) strictly inhibited the hyaluronidase activity extracted from boar

All correspondence to: H. Tatemoto, Department of Bioproduction, Faculty of Agriculture, University of the Ryukyus, Nishihara-cho, Okinawa 903-0213, Japan. Fax: +81 98 895 8757. e-mail: hidettmt@agr.u-ryukyu.ac.jp
[1]Department of Bioproduction, Faculty of Agriculture, University of the Ryukyus, Nishihara-cho, Okinawa 903-0213, Japan.
[2]Laboratory of Swine, Okinawa Prefectural Livestock Experiment Station, Nakijin-son, Okinawa 905-0426, Japan.
[3]Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023, Japan.

spermatozoa, and the incidence of polyspermy was effectively reduced to 30% by the addition of 10 μg/ml ChSAO to IVF medium in cumulus-free oocytes while maintaining high sperm penetrability (Tatemoto *et al*., 2005). From these results, it is expected that the high incidence of polyspermy will be repressed by blocking sperm hyaluronidase activity during IVF. However, since the addition of anti-hyaluronidase ChSAO to IVF medium significantly decreased the number of sperm bound to the zona pellucida (ZP) in a concentration-dependent manner, it is not clear whether the prevention of polyspermy by ChSAO during IVF results from inhibiting sperm hyaluronidase activity or blocking primary sperm-ZP binding related to carbohydrate-mediated events.

In general, the hyaluronidase in sperm plasma membrane is thought to hydrolyse hyaluronic acid in the extracellular matrix of the expanded cumulus cells to allow sperm to penetrate the cumulus layer and proceed to fertilize the egg (McRorie & Williams, 1974; Yanagimachi, 1994; Thaler & Cardullo, 1995). Although the various mammalian hyaluronidases are derived from different genes, they share certain structural and biological characteristics with one another and with other glycosidases. Sperm hyaluronidase activity is provided by the PH-20 molecule, which is a glycosyl phosphatidylinositol-anchored membrane protein (Phelps *et al*., 1988). PH-20 homologues have been identified in guinea pigs (Primakoff & Myles, 1983; Primakoff *et al*., 1985), mice (Lin *et al*., 1994; Baba *et al*., 2002), monkeys (Cherr *et al*., 1996, 1999), horses (Meyers, 2001) and humans (Gmachl *et al*., 1993). The 64 kDa membrane-bound isoform has a broad optimal pH range, with the strongest activity at neutral pH (pH 7.0), while the 53 kDa soluble form exhibits activity only at acidic pH (pH 4.0) (Cherr *et al*., 1996, 2001; Li *et al*., 1997*a*, 2002). According to the findings reported by Fléchon *et al*. (2003), when porcine oocytes were prevented from accumulating hyaluronidase-sensitive extracellular matrix in the perivitelline space and the ZP surface during IVM, although there was no effect on meiotic division, the incidence of polyspermy was increased during subsequent *in vivo* fertilization. In short, these data are the first experimental evidence that the extracellular matrix synthesized on both sides of the ZP during oocyte maturation may constitute a favourable factor for appropriate sperm penetration. However, to our knowledge there have been no reports directly demonstrating interactions between hyaluronidase activity and sperm penetration during IVF of porcine oocytes.

Interestingly, a previous investigation showed that some polyphenols are potent inhibitors of commercial bull testis hyaluronidase (Kuppusamy *et al*., 1990). Tannic acid, apigenin and quercetin have also been shown to inhibit the hyaluronidase activity of guinea pig sperm PH-20 (Hunnicutt *et al*., 1996) and cynomolgus monkey sperm PH-20 (Li *et al*., 1997*b*; Meyers *et al*., 1997), as well as the recombinant form of cynomolgus monkey sperm PH-20 (Lin *et al*., 1994). Thus, it appeared possible that the addition of anti-hyaluronidase polyphenols to IVF medium might decrease the incidence of polyspermy in porcine oocytes. To test this possibility the following experiments were undertaken: (1) an examination of the inhibitory effects of three polyphenols – tannic acid, apigenin and quercetin – on the hyaluronidase activity extracted from boar sperm; (2) an evaluation of the effects of the addition of polyphenols to IVF medium on fertilization responses, including the penetration rate and incidence of polyspermy in porcine oocytes matured *in vitro*; (3) an examination of the effects of polyphenols on zona solubility, proteolytic activity of acrosomal contents and sperm binding to ZP.

Materials and methods

All chemicals used in the study were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated.

Culture media

The culture medium used for oocyte maturation was North Carolina State University (NCSU) 37 medium (Petters & Wells, 1993) supplemented with 0.57 mM cysteine, 200 μM ascorbic acid 2-*O*-α-D-glucoside (Hayashibara Biochemical Laboratory, Okayama, Japan), 0.04 units/ml ovine FSH, 0.02 units/ml ovine LH and 10% (v/v) porcine follicular fluid. Porcine follicular fluid was aspirated from follicles 2–6 mm in diameter, centrifuged at 10 000 *g* for 15 min at 4 °C to remove cellular debris, and stored at −30 °C until use. The basic medium used for IVF was essentially the same as the modified Tris-buffered medium (mTBM) used by Abeydeera & Day (1997). This mTBM was designated as IVF medium when supplemented with 2 mM caffeine sodium benzoate and 0.1% (w/v) bovine serum albumin (BSA), and as sperm preincubation medium when supplemented with 2 mM caffeine sodium benzoate, 0.1% (w/v) BSA and 0.5% (v/v) porcine follicular fluid.

Extraction of sperm hyaluronidase

Hyaluronidase was extracted from boar sperm according to the method of Li *et al*. (1997*b*). For sperm preparation, frozen-ejaculated Landrace spermatozoa (three pellets of volume 100 μl and containing about 5×10^7 sperm/pellet) were thawed (39 °C) and washed twice by centrifugation at 400 *g* for 4 min in 4 ml of Dulbecco's phosphate-buffered saline (PBS; Invitrogen, Carisbad, CA) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA) at pH 7.2. The sperm pellet

https://doi.org/10.1017/S0967199406003819 Published online by Cambridge University Press

was resuspended at 4×10^8 sperm/ml in the sperm preincubation medium, and then incubated for 90 min at 39 °C in an atmosphere of 5% (v/v) CO_2 in air to induce capacitation. After preincubation, sperm suspensions were washed by centrifugation at 400 *g* for 10 min in HEPES-buffered saline (HBS; 5 mM HEPES, 150 mM NaCl, pH 7.0). The sperm pellet was resuspended in 3 ml of HBS, layered onto 3 ml of 40% Percoll (Amersham Pharmacia Biotech), then centrifuged at 300 *g* for 15 min to remove BSA. After washing three times by resuspension in 10 ml of HBS and centrifugation, the sperm pellet was resuspended at 2×10^8 sperm/ml in an appropriate volume of cold HBS containing protease inhibitors (20 mM EDTA, 1 mM (*p*-hydroxymercuro)benzoate, 5 mM *N*-ethymaleimide and 1 mM benzamidine). After addition of Triton X-100 to a final concentration of 1% (v/v), the sperm suspension was vortexed for 5 min and then centrifuged at 10 000 *g* for 15 min at 4 °C. The supernatant was stored at −80 °C until assayed for hyaluronidase activity. The concentration of protein in sperm extract solution was determined using a Bradford protein assay reagent kit (Bio-Rad Corp., Hercules, CA).

Measurement of hyaluronidase activity

The polyphenols were dissolved in dimethylsulfoxide (DMSO) with the exception of tannic acid, which was dissolved in absolute ethanol. Aliquots (25 µl) of the polyphenol solution in the final concentration range of 0–10 µg/ml were mixed with 25 µl of sperm extract solution and preincubated for 1 h at room temperature with shaking. Control assays that contained the sperm extract and the solvent (DMSO or ethanol) without polyphenols were carried out simultaneously. The final concentration of the solvents was less than 0.1% (v/v). Hyaluronidase activity was measured by the colorimetric method using Alcian Blue 8 GX, as described previously (Baba *et al*., 2002). Human umbilical cord hyaluronic acid was dissolved at a concentration of 90 µg/ml in 50 mM sodium acetate buffer (pH 7.0) containing 50 mM $MgCl_2$. The reaction was conducted in a mixture of 450 µl of hyaluronic acid solution and 50 µl of the sperm extract–polyphenol solution. Following incubation at 37 °C for 100 min, the reaction mixture was mixed with 700 µl of 0.02% (w/v) Alcian blue solution, and centrifuged at 10 000 *g* for 5 min. Absorbance of the supernatant solution at 603 nm was measured using a spectrophotometer (Shimadzu Co.). Blanks containing only the buffer and polyphenol or solvent were assayed in parallel. The units of hyaluronidase activity were determined based on the standard curve of hyaluronidase activity of bull testicular hyaluronidase with a specific activity of 500 units/mg (Sigma, H-3506).

Substrate gel assay for hyaluronidase activity

To detect proteins exhibiting the hyaluronidase activity, SDS-PAGE in the presence of hyaluronic acid was carried out under non-reducing conditions as described previously (Cherr *et al*., 1996). Non-reducing SDS-sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, and 0.5% bromphenol blue, final concentrations) was added to sperm extract solution at 4 °C. Then, the samples were subjected to SDS-PAGE on 7.5% polyacrylamide gels copolymerized with 200 µg/ml human umbilical cord hyaluronic acid (Biozyme Laboratories). Following electrophoresis, gels were washed with Tris-buffered saline, pH 7.0, containing 3% (v/v) Triton X-100 at room temperature for 2 h to remove SDS and then incubated in 100 mM sodium acetate buffer with or without supplementation with 10 µg/ml polyphenol at either pH 4.0 or pH 7.0 at 37 °C for 30 h. To visualize regions of digestion of hyaluronic acid in the gels, the gels were stained with 0.5% (w/v) Alcian blue 8 GX in 3% (v/v) acetic acid for 3 h, and then destained in 7% (v/v) acetic acid. The presence of activity was revealed by lack of colouration in the gel. The relative enzyme activities within a given lane were determined using an Image PC (Scion).

In vitro maturation

Ovaries were collected from maturing gilts at a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl containing 100 mg/l kanamycin sulfate (Meiji Seika) at 30 °C. Within 2 h of slaughter, the follicular contents were recovered by excising the visible small antral follicles (about 2–6 mm in diameter) on the ovarian surface using a razor, and by scraping the inner surface of the follicle walls with a disposable surgical blade. Only cumulus–oocyte complexes (COCs) with uniform ooplasm and a compact cumulus cell mass were collected and washed three times with HEPES-buffered Tyrode medium containing 0.01% (w/v) PVA (H-TL-PVA). After washing in IVM medium, groups of 20 COCs were transferred into 100 µl droplets of IVM medium that had previously been covered with mineral oil and equilibrated in a 5% CO_2 incubator. After 20 h of maturation culture, the oocytes were washed and transferred to 100 µl droplets of IVM medium without hormonal supplementation for an additional 24 h of culture. After a total 44 h of maturation culture, a proportion of the COCs were freed from their cumulus cells by treatment with H-TL-PVA containing 0.1% (w/v) hyaluronidase, followed by repeated passage through a narrow-bore pipette.

Effect on IVF of different concentrations of tannic acid

Cumulus-intact and cumulus-free oocytes were washed three times with IVF medium, and 25–30

https://doi.org/10.1017/S0967199406003819 Published online by Cambridge University Press

oocytes were transferred to 50 µl droplets of IVF medium containing 0, 0.5, 1, 2, 5 or 10 µg/ml tannic acid, which had been covered with warm mineral oil. The droplets containing oocytes were kept in an incubator for 1 h until spermatozoa were added for fertilization. After thawing and washing frozen-ejaculated spermatozoa as described above, the sperm pellets were resuspended at 4×10^8 cells/ml in sperm preincubation medium and then incubated for 90 min at 39 °C in an atmosphere of 5% CO_2 in air. After sperm preincubation, 50 µl of diluted sperm suspension in IVF medium containing 0, 0.5, 1, 2, 5 or 10 µg/ml tannic acid was added to a droplet containing oocytes at a final sperm concentration of 1×10^6 cells/ml. Oocytes were co-incubated with spermatozoa for 7 h at 39 °C in an atmosphere of 5% (v/v) CO_2 in air. After insemination, oocytes were removed from the fertilization drops, washed three times and cultured in 50 µl drops of NCSU 37 medium at 39 °C in an atmosphere of 5% CO_2 in air.

Effect on IVF of different polyphenols at a fixed concentration

Cumulus-free oocytes were washed three times with IVF medium, and 25–30 oocytes were transferred to 50 µl droplets of IVF medium containing 5 µg/ml of tannic acid, apigenin or quercetin, which had been covered with warm mineral oil. The concentration of polyphenols was determined at 5 µg/ml based on the data from the treatment with tannic acid in order to examine whether the reduction of the polyspermy rate during IVF was attributable to an anti-hyaluronidase action of polyphenols. The following procedures were the same as described above, except that 50 µl of diluted sperm suspension in IVF medium containing 5 µg/ml of tannic acid, apigenin or quercetin was used.

Examination of oocytes

At 10 h post-insemination, groups of 30–40 oocytes were mounted, fixed in acetic acid–ethanol (1:3, v/v) for 72 h, stained with 1% (w/v) lacmoid in 45% (v/v) acetic acid, and examined for fertilization parameters under a phase-contrast microscope at ×400 magnification. Oocytes were designated as penetrated when one or more sperm heads and/or male pronuclei and corresponding sperm tails were present. The rates of polyspermy, male pronucleus formation and mean number of sperm per oocyte were determined from the oocytes that had been penetrated.

Effect on IVF of pretreatment of oocytes and/or spermatozoa with 5 µg/ml tannic acid

Cumulus-free oocytes matured *in vitro* were treated in the presence or absence of 5 µg/ml tannic acid in pre-equilibrated IVF medium for 90 min, washed three times in tannic-acid-free IVF medium, and transferred to droplets of tannic acid-free IVF medium. Washed spermatozoa were resuspended at 4×10^8 cells/ml in sperm preincubation medium with or without 5 µg/ml tannic acid, and incubated for 90 min at 39 °C in an atmosphere of 5% (v/v) CO_2 in air. Preincubated spermatozoa were washed once by centrifugation at 400 *g* for 4 min in tannic acid-free IVF medium, and diluted in the same medium. Several combinations of pretreated oocytes and spermatozoa were co-incubated at a final sperm concentration of 1×10^6 cells/ml for 7 h at 39 °C in an atmosphere of 5% (v/v) CO_2 in air. After insemination, oocytes were removed from the fertilization drops, washed three times and cultured in 50 µl drops of NCSU 37 medium at 39 °C in an atmosphere of 5% CO_2 in air.

Assessment of ZP solubility

Cumulus-free oocytes matured *in vitro* were cultured for 4 h in IVF medium with or without 5 µg/ml polyphenols, and then washed three times in H-TL-PVA. Fifteen or 20 oocytes were transferred into 100 µl of H-TL-PVA containing 0.1% (w/v) protease and were continuously observed for dissolution of the ZP at room temperature under an inverted microscope at ×200 magnification. The time required for dissolution of the ZP in a given oocyte was recorded as the ZP dissolution time.

Assessment of proteolytic activity of acrosomal contents

The proteolytic activity of the acrosomal contents was assessed by the gelatin substrate slide method, as described previously (Maeda *et al*., 1990; Thundathil *et al*., 2001). Slides were cleaned with alcohol and cooled by keeping them in a moist chamber at 4 °C for 2 h. A 3% (w/v) gelatin suspension was prepared by dissolving gelatin in distilled water at 50 °C. A volume of 40 µl of gelatin suspension supplemented or not with 5 µg/ml polyphenol and 5 µg/ml trypsin inhibitor ovomucoid, as a negative control, was placed across on one clean slide and smeared uniformly with another slide by rubbing the two slides together. The gelatin-film-covered slides were placed horizontally in a moist chamber, and stored at 4 °C for 24 h. The slides were then fixed for 10 min by immersion in 0.05% (v/v) glutaraldehyde solution, and stored vertically in a moist chamber at 4 °C until use. Before use for digestion by spermatozoa, the gelatin slides were warmed at room temperature for 5 min.

After 90 min of preincubation, 20 µl of sperm suspension was placed on one end of a slide and smeared with a cover glass taking care not to disrupt the gelatin film. After air-drying, the smears were covered

with a cover glass (24 × 50 mm), which was held in place with nail polish at the four corners. The slides covered with the gelatin membrane were incubated in a moist chamber at 37 °C for 2 h. Halos produced around acrosomes of spermatozoa on the gelatin film were observed under a phase-contrast microscope. The horizontal diameter of individual halos around 40–50 sperm heads was measured with a micrometer, and regarded as an indication of the proteolytic activity of the acrosomal contents.

Sperm–ZP binding assay

After IVM culture, cumulus-free oocytes were transferred to IVF medium and co-incubated with capacitated spermatozoa for 2 h in the presence or absence of 5 μg/ml polyphenol as described above. After the co-incubation, the oocytes and bound sperm were gently pipetted 10 times with a wide-bore pipette to remove loosely bound sperm in H-TL-PVA, and fixed at room temperature for 40 min with 2% formaldehyde. The oocytes were then placed in 50 μl drops of H-TL-PVA containing 10 μg/ml of *bis*-benzimide Hoechst 33342 and incubated for 10 min. The oocytes were washed in H-TL-PVA, mounted, and the sperm tightly bound to ZP were counted under a fluorescent microscope (Olympus, Tokyo, Japan).

Statistical analysis

Values are presented as the mean of four independent experimental replicates. Variation between experiments is illustrated using SEM. For evaluation of the differences between groups, data on the percentage of fertilization parameters were checked for homogeneity, pooled, and then subjected to contingency table analysis followed by the Tukey test for non-parametric multiple comparisons (Ryan, 1960). Statistical analyses of data on hyaluronidase activity, mean number of spermatozoa per penetrated oocyte, the ZP dissolution time, the halo diameter and the number of sperm binding to ZPs were carried out by the Shapiro–Wilk normality test and one-way or two-way ANOVA followed by the Tukey–Kramer test. All statistical analyses were carried out with the Statistical Analysis System R software package (Cary). A probability of $p < 0.05$ was considered to be statistically significant.

Results

Effect of polyphenols on hyaluronidase activity

The data in Fig. 1 show the effect of three polyphenols – tannic acid, apigenin and quercetin – on the hyaluronidase activity of preincubated boar spermatozoa extracts. Among them, tannic acid markedly inhibited

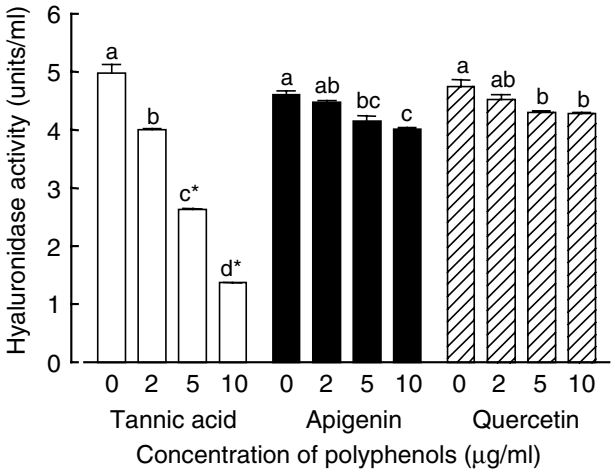

Figure 1 Inhibitory effect of polyphenols on the hyaluronidase activity of preincubated boar sperm extract. Values are expressed as the mean ± SEM. [a–d]Values with different superscripts within each polyphenol are significantly different ($p < 0.05$). *Values are significantly different compared with those of other polyphenols at a given concentration ($p < 0.05$).

hyaluronidase activity in a concentration-dependent manner over the range of 2–10 μg/ml ($p < 0.05$). A significant decrease in hyaluronidase activity (2.63 ± 0.02 units/ml and 1.37 ± 0.01 units/ml) was found at the concentrations of 5 and 10 μg/ml, respectively, compared with that in the absence of tannic acid (4.97 ± 0.16 units/ml) ($p < 0.05$). In contrast, both apigenin and quercetin exerted weak inhibition in a concentration-dependent fashion, but substantial hyaluronidase activities (4.02 ± 0.03 units/ml and 4.28 ± 0.03 units/ml, respectively) were still observed when these agents were added at a concentration of 10 μg/ml.

To detect the isoenzymes of hyaluronidase in the extracts of preincubated boar spermatozoa, hyaluronic acid substrate gel analyses were performed. As shown in Fig. 2, the hyaluronidase activity at pH 7.0 was associated with the 89, 73, 64 and 59 kDa proteins (lane 1). However, this activity was reduced to less than 12% at pH 4.0, and the hyaluronidase which corresponds to the 53 kDa protein activity was not detected (lane 2). Moreover, tannic acid (10 μg/ml) inhibited the hyaluronidase activity, as indicated by the relative digested regions of the gels at pH 7.0 (less than 6%, lane 3), but apigenin and quercetin showed no such inhibition (data not shown).

Effect on IVF of different concentrations of tannic acid

In both cumulus-intact (Table 1) and cumulus-free oocytes (Table 2), penetration rates were significantly reduced to 41.7% and 57.1%, respectively ($p < 0.05$), in

https://doi.org/10.1017/S0967199406003819 Published online by Cambridge University Press

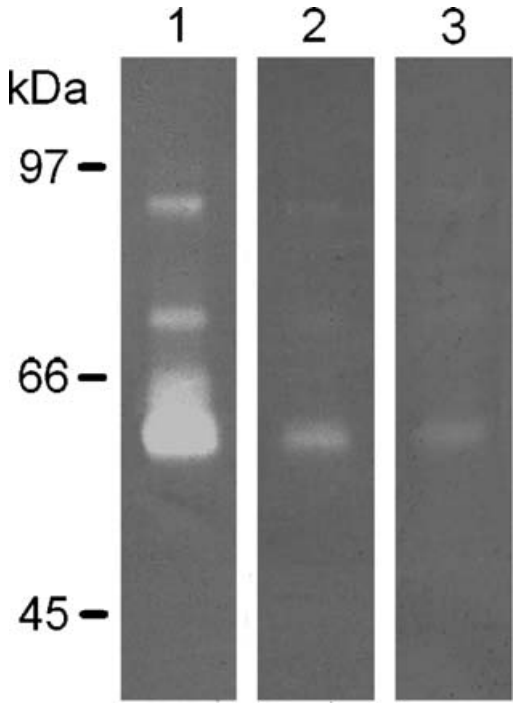

Figure 2 Hyaluronic acid substrate SDS-PAGE of preincubated boar sperm extracts. The samples (approximately 5.6 μg protein/lane) were subjected to non-reducing SDS-PAGE, and the gels were incubated at 37 °C for 30 h at pH 7.0 (lanes 1 and 3) and pH 4.0 (lane 2). Tannic acid (10 μg/ml) was added to the incubation buffer (lane 3). Positions of molecular weight standards (kDa) are shown on the left. The analysis was repeated four times; the figure shown is from a representative experiment.

the presence of 10 μg/ml tannic acid compared with when no tannic acid was added (63.2% and 75.8%, respectively). However, in the presence of 5 μg/ml tannic acid, no decrease in penetration rate was observed but the incidence of polyspermy and mean number of sperm per penetrated oocyte were significantly reduced ($p < 0.05$) compared with in the absence of tannic acid. Male pronucleus formation was not affected by the presence of tannic acid and its concentration in either cumulus-intact or cumulus-free oocytes. Consequently, the addition of 5 μg/ml polyphenols to the IVF medium in cumulus-free oocytes was selected for the subsequent experiments to examine whether there was a direct correlation between the inhibition of hyaluronidase activity and polyspermic fertilization.

Effect on IVF of different polyphenols at a fixed concentration

No difference was found in the incidences of penetration and male pronucleus formation irrespective of the addition of polyphenols to IVF medium (Table 3), but the rate of polyspermy in oocytes treated with tannic acid was significantly lower than that in oocytes treated with apigenin or quercetin or without polyphenols ($p < 0.05$). In addition, prevention of polyspermy was not detected in the oocytes fertilized in the presence of apigenin and quercetin.

Pretreatment of oocytes and spermatozoa prior to IVF

The optimal concentration of tannic acid (5 μg/ml, as determined above) was used in this experiment. Pretreatment of oocytes did not decrease the incidence of penetration (68.2%) or polyspermy (51.1%), but pretreatment of spermatozoa significantly reduced the polyspermy rate (34.1%) compared with the control (52.9%), without affecting the rate of penetration ($p < 0.05$) (Fig. 3). No synergistic effect was observed when both spermatozoa and oocytes were pretreated with tannic acid.

Effect of polyphenols on ZP solubility

No effect was observed on the ZP dissolution time of the oocytes incubated for 4 h in the presence of tannic

Table 1 Effect of tannic acid on fertilization parameters of cumulus-intact porcine oocytes matured and fertilized *in vitro*

Concentration of tannic acid (μg/ml)	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
		Penetrated	Polyspermic[a]	Male pronucleus[a]	
0	125	63.2 ± 4.3^{b}	48.1 ± 5.6^{b}	70.1 ± 5.1	1.61 ± 0.07^{b}
0.5	127	55.1 ± 4.4^{bc}	35.7 ± 5.7^{b}	72.9 ± 5.3	1.40 ± 0.08^{bcd}
1	135	56.3 ± 4.3^{bc}	38.2 ± 5.6^{b}	65.8 ± 5.4	1.51 ± 0.05^{bc}
2	120	53.3 ± 4.6^{bc}	32.8 ± 5.9^{bc}	65.6 ± 5.9	1.43 ± 0.04^{bcd}
5	127	53.5 ± 4.4^{bc}	25.0 ± 5.3^{c}	67.7 ± 5.7	1.31 ± 0.03^{cd}
10	139	41.7 ± 4.2^{c}	22.4 ± 5.5^{c}	51.7 ± 6.6	1.26 ± 0.06^{d}

[a]Percentage of oocytes that were penetrated.
[b–d]Values with different superscripts within a column are significantly different ($p < 0.05$).

https://doi.org/10.1017/S0967199406003819 Published online by Cambridge University Press

Table 2 Effect of tannic acid on fertilization parameters of cumulus-free porcine oocytes matured and fertilized *in vitro*

Concentration of tannic acid (μg/ml)	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
		Penetrated	Polyspermic[a]	Male pronucleus[a]	
0	132	75.8 ± 3.7[b]	58.0 ± 4.9[b]	68.0 ± 4.7	1.85 ± 0.09[b]
0.5	122	71.3 ± 4.1[bc]	52.9 ± 5.4[b]	66.7 ± 5.1	1.80 ± 0.12[b]
1	121	71.9 ± 4.1[bc]	48.3 ± 5.4[b]	67.8 ± 5.0	1.66 ± 0.08[b]
2	126	70.6 ± 4.1[bc]	39.3 ± 5.2[bc]	64.0 ± 5.1	1.50 ± 0.06[bc]
5	115	70.4 ± 4.3[bc]	25.9 ± 4.9[c]	69.1 ± 5.1	1.31 ± 0.10[cd]
10	119	57.1 ± 4.5[c]	25.0 ± 5.3[c]	66.2 ± 5.7	1.25 ± 0.04[d]

[a]Percentage of oocytes that were penetrated.
[b–d]Values with different superscripts within a column are significantly different ($p < 0.05$).

Table 3 Effect of polyphenols on fertilization parameters of cumulus-free porcine oocytes matured and fertilized *in vitro*[a]

Treatment during IVF	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
		Penetrated	Polyspermic[b]	Male pronucleus[b]	
None	129	74.4 ± 3.8	54.2 ± 5.1[c]	60.4 ± 5.0	1.73 ± 0.06[c]
Tannic acid	137	75.2 ± 3.7	29.1 ± 4.5[d]	65.1 ± 4.7	1.39 ± 0.06[d]
Apigenin	129	72.1 ± 4.0	51.6 ± 5.2[c]	61.3 ± 5.1	1.63 ± 0.07[cd]
Quercetin	124	72.6 ± 4.0	50.0 ± 5.3[c]	61.1 ± 5.1	1.66 ± 0.08[cd]

[a]Concentrations of all polyphenols were 5 μg/ml.
[b]Percentage of oocytes that were penetrated.
[c, d]Values with different superscripts within a column are significantly different ($p < 0.05$).

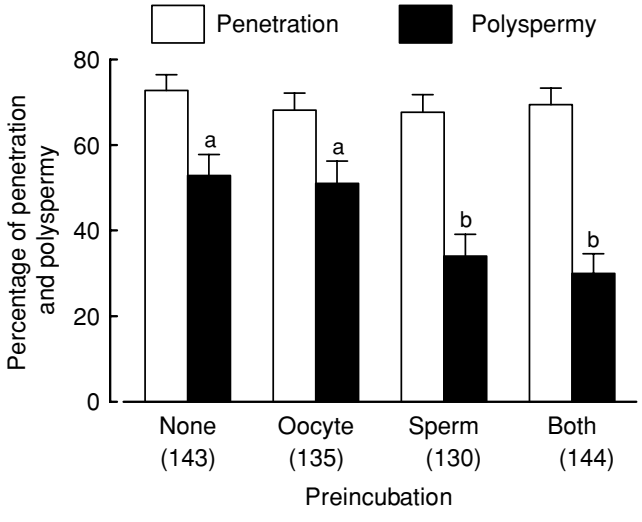

Figure 3 Effects of pretreatment of oocytes and/or spermatozoa with tannic acid on the penetration and polyspermy rates. *In vitro* matured oocytes and/or spermatozoa were incubated in the presence or absence of 5 μg/ml tannic acid for 90 min immediately before insemination. Values are expressed as the mean ± SEM. The numbers of oocytes examined are indicated in parentheses. Within the same category, values with different superscripts are significantly different ($p < 0.05$).

acid, apigenin or quercetin (5.0 ± 0.1 min, 5.0 ± 0.1 min and 5.1 ± 0.1 min, respectively), compared with that in the absence of polyphenols (5.1 ± 0.2 min).

Effect of polyphenols on proteolytic activity of acrosomal contents

As shown in Fig. 4, preincubated spermatozoa produced halos on the gelatin substrate slide as a result of gelatin digestion by the acrosomal contents from spermatozoa. Treatment with the trypsin inhibitor ovomucoid significantly decreased the halo diameter (14.8 ± 0.6 μm) compared with the control (40.9 ± 0.9 μm) ($p < 0.05$), but no significant difference in the halo diameter was observed with any treatments with polyphenols (Table 4).

Effect of polyphenols on sperm binding to ZP

No difference was observed in the number of sperm bound to the ZP regardless of treatment with tannic acid, apigenin or quercetin (114.5 ± 9.1, 116.5 ± 9.6 or 115.5 ± 6.7 sperm/oocyte, respectively), compared with the control (104.5 ± 6.8 sperm/oocyte).

https://doi.org/10.1017/S0967199406003819 Published online by Cambridge University Press

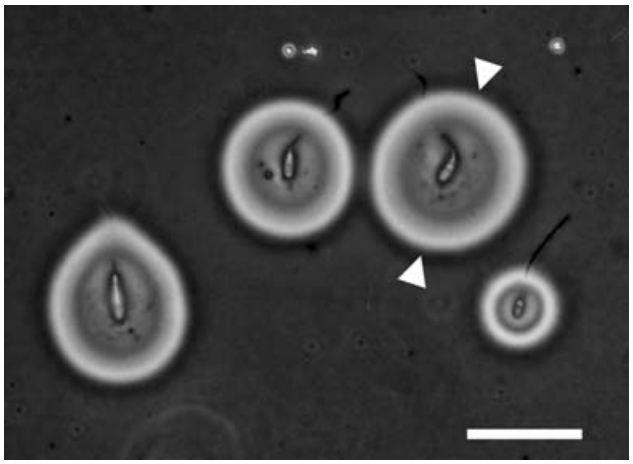

Figure 4 Halos made by the digestion with acrosomal contents from preincubated boar spermatozoa on a gelatin substrate slide in the presence of 5 μg/ml tannic acid. Halo diameter is the distance between arrowheads. Scale bar represents 50 μm.

Table 4 Effect of polyphenols on the halo diameter as an indication of the proteolytic activity of acrosomal contents in preincubated boar spermatozoa[a]

Treatment	No. of sperm examined	Halo diameter (μm; mean ± SEM)
None	200	40.9 ± 0.9^{b}
Tannic acid	197	43.3 ± 0.9^{b}
Apigenin	196	41.5 ± 0.9^{b}
Quercetin	199	41.6 ± 1.0^{b}
Ovomucoid	203	14.8 ± 0.6^{c}

[a]Concentration of all reagents was 5 μg/ml.
[b, c]Values with different superscripts within a column are significantly different ($p < 0.05$).

Discussion

Polyphenols such as tannic acid, apigenin and quercetin are known to be competitive hyaluronidase inhibitors (Kuppusamy *et al*., 1990). In the present study, tannic acid inhibited the hyaluronidase activity associated with 89, 73, 64 and 59 kDa proteins in the extracts from preincubated boar spermatozoa more effectively than apigenin and quercetin (Figs. 1, 2). In agreement with these results, it has been reported that tannic acid more potently blocks the activity of commercial bull testis hyaluronidase than luteolin, apigenin, kaempferol and quercetin (Kuppusamy *et al*., 1990).

When cumulus-intact and cumulus-free oocytes were inseminated in IVF medium containing 0–10 μg/ml tannic acid, the penetration and the polyspermic fertilization rates were significantly decreased in a concentration-dependent manner, and the incidence of polyspermy was significantly reduced to 25.0% and 25.9%, respectively, by tannic acid at a concentration of 5 μg/ml without an effect on the penetration rate (Tables 1, 2). However, the presence of apigenin or quercetin at the same concentrations as tannic acid could not prevent polyspermy (Table 3). Inhibitory effects of anti-hyaluronidase polyphenols on sperm penetration into cumulus-intact oocytes were observed in monkeys (Li *et al*., 1997*b*). It is interesting that mouse sperm lacking PH-20 show delayed cumulus dispersion and a significantly lower rate of fertilization than wild-type sperm (Baba *et al*., 2002). This suggests that fertilization still occurs despite the absence of sperm PH-20, presumably because of the presence of other hyaluronidase(s) within the acrosome of mouse sperm. The data in the present study indicate that the effective decrease in the incidence of polyspermy is well correlated with the potency of hyaluronidase inhibition by polyphenols. However, to our knowledge there is no report describing the possible role of hyaluronidase in sperm penetration of the ZP.

According to the findings reported by Honda *et al*. (2002), analysis of acrosin-deficient mouse spermatozoa demonstrates that acrosin is not essential for sperm–ZP interactions, including sperm penetration of the ZP, and these authors suggested that acrosin is presumably involved in the limited proteolysis and/or processing of other proteins in the acrosome and on the membranes during the acrosome reaction. Furthermore, anti-acrosin IgG had no effect on sperm penetration of the macaque ZP *in vitro*, while anti-PH-20 IgG was capable of blocking sperm penetration of the ZP by interference with secondary sperm–ZP binding with no effect on primary sperm–ZP binding or the zona-induced acrosome reaction (Yudin *et al*., 1999). As reported by Cherr *et al*. (1996), the neutral-active (pH 7.0) form of PH-20 is present on the plasma and inner acrosomal membranes and gives rise to the soluble acid-active (pH 4.0) form at the time of the acrosome reaction, and the soluble form of PH-20 may be generated as a result of protease, glycosidase and phospholipase activities that can alter the neutral-active form of PH-20. The recent report by Fléchon *et al*. (2003) made it clear that the extracellular matrix and its hyaluronan moiety are accumulated in the perivitelline space and the ZP surface during the maturation of porcine oocytes, and can be partially extracted with hyaluronidase but not with neuraminidase. Those authors also found that in oocytes matured in the presence of tunicamycin, an inhibitor of glycoprotein synthesis, meiotic division was not prevented, although the amount of glycoproteins in the perivitelline space was significantly decreased, and the

https://doi.org/10.1017/S0967199406003819 Published online by Cambridge University Press

incidence of polyspermic penetration was relatively increased subsequently during *in vivo* fertilization. From these results, it seems that the interaction between the sperm hyaluronidase activity and the glycoproteins accumulated on both sides of the ZP during oocyte maturation may play a crucial role in preventing polyspermic fertilization in pigs. The present study confirmed that the penetration and the polyspermy rates were reduced by treatment with tannic acid, which did not disturb the proteolytic activity of acrosomal contents of preincubated spermatozoa (Table 4). In addition, we are now establishing that there is no toxic effect of treatment with 5 μg/ml tannic acid during IVF on embryo development to the blastocyst stage (20.7%) and the nucleus number in blastocysts (49.4 ± 2.4) as compared with no treatment (15.0% and 48.9 ± 2.6, respectively) (unpublished data). It is therefore speculated that treatment with tannic acid at an appropriate concentration during porcine IVF prevents polyspermy through the inhibition of hyaluronidase activity, which may be responsible for sperm penetration of the ZP.

Although treatment with tannic acid reduced the incidence of polyspermy, it is still uncertain whether the effect of tannic acid was limited to inhibition of the hyaluronidase activity or extended to blocking other events of polyspermic fertilization. To address this question, we examined the resistance of the ZP to dissolution by protease and the number of zona-bound spermatozoa. In mammalian species, spermatozoa activate oocytes by triggering calcium oscillations that persist for more than 1 h after sperm penetration (Miyazaki, 1990; Kline & Kline, 1992; Miyazaki *et al*., 1992; Wu *et al*., 2001; Gordo *et al*., 2002). The increase in cytoplasmic free-Ca^{2+} is recognized as the primary intracellular signal responsible for initiating the activation process, which evokes exocytosis of cortical granules, followed by modification of ZP glycoproteins. This ZP modification is mandatory for an effective block to polyspermy (Wolf & Hamada, 1977). We previously reported that the complete ZP modification of porcine oocytes was seen after 3.5 h of oocyte activation, accompanied by delayed exocytosis of cortical granules (Tatemoto & Terada, 1999*a*, *b*). This delayed ZP modification in porcine oocytes may account for the notable increase in the frequency of polyspermy during IVF. This notion was experimentally supported by the finding that polyspermic fertilization after insemination with spermatozoa at a concentration of 1×10^6 cells/ml was inhibited in porcine oocytes that were pretreated with an appropriate concentration (6.25 μM) of calcium ionophore A23187 to induce moderate exocytosis of cortical granules in advance (Asano & Niwa, 2004). In the present study, pretreatment of spermatozoa with tannic acid prior to insemination reduced the polyspermy rate, whereas pretreatment of oocytes with tannic acid showed no effect on the incidence of polyspermy after insemination with untreated spermatozoa (Fig. 3). In addition, the treatment of oocytes with tannic acid had no effect on the protease digestion of ZP. These findings clearly imply that the reduction in the rate of polyspermy by treatment with tannic acid during porcine IVF does not result from effects on physiological functions related to ZP modification.

In the present study, the number of zona-bound spermatozoa was not decreased by the addition of three polyphenols to IVF medium, indicating that tannic acid and the other two polyphenols do not form a physical barrier to sperm binding or modify sperm binding sites on ZP. In contrast, treatment with porcine oviduct-specific glycoprotein strongly reduced the incidence of polyspermy in porcine oocytes through a reduction of the number of zona-bound spermatozoa (Kouba *et al*., 2000; McCauley *et al*., 2003), and the same phenomenon was detected with oocytes fertilized in the presence of ChSAO (Tatemoto *et al*., 2005). Therefore, the present study is the first to show directly that inhibition of the sperm hyaluronidase activity can protect porcine oocytes against polyspermic fertilization during IVF without affecting carbohydrate-mediated events during primary sperm–ZP binding.

In summary, the data reported here demonstrate that tannic acid potently blocked the hyaluronidase activity extracted from boar spermatozoa, resulting in an effective reduction in the polyspermy rate in *in vitro* matured oocytes during IVF. This lower incidence of polyspermy elicited by tannic acid was not due to protective proteolytic modification of the ZP matrix before fertilization, nor to reduction of the proteolytic activity of acrosomal contents or the number of zona-bound spermatozoa. It is concluded that the anti-hyaluronidase action of tannic acid can promote the normal fertilization process by preventing polyspermy during porcine IVF. Further studies will be required to investigate the detailed mechanism of hyaluronidase functions during sperm penetration of the ZP in porcine oocytes.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (C) (no. 147580247 to H.T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Research Project for Utilizing Advanced Technologies in Agriculture, Forestry and Fisheries of Japan. We are grateful to the staff of the Meat Inspection Office of the city of Oosato, Japan, for supplying the porcine ovaries.

References

Abeydeera, L.R. & Day, B.N. (1997). Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified Tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biol. Reprod.* **57**, 729–34.

Asano, A. & Niwa, K. (2004). Activation and penetration *in vitro* of pig oocytes treated with calcium ionophore. *J. Reprod. Dev.* **50**, 77–85.

Baba, D., Kashiwabara, S., Honda, A., Yamagata, K., Wu, Q., Ikawa, M., Okabe, M. & Baba, T. (2002). Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. *J. Biol. Chem.* **277**, 30310–14.

Brad, A.M., Bormann, C.L., Swain, J.E., Durkin, R.E., Johnson, A.E., Clifford, A.L. & Krisher, R.L. (2003). Glutathione and adenosine triphosphate content of *in vivo* and *in vitro* matured porcine oocytes. *Mol. Reprod. Dev.* **64**, 492–8.

Cherr, G.N., Meyers, S.A., Yudun, A.I., VandeVoort, C.A., Myles, D.G., Primakoff, P. & Overstreet, J.W. (1996). The PH-20 protein in cynomolgus macaque spermatozoa: identification of two different forms exhibiting hyaluronidase activity. *Dev. Biol.* **175**, 142–53.

Cherr, G.N., Yudin, A.I., Li, M.-W., Vines, C.A. & Overstreet, J.W. (1999). Hyaluronic acid and the cumulus extracellular matrix induce increases in intracellular calcium in macaque sperm via the plasma membrane protein PH-20. *Zygote* **7**, 211–22.

Cherr, G.N., Yudin, A.I. & Overstreet, J.W. (2001). The dual functions of GPI-anchored PH-20: hyaluronidase and intracellular signaling. *Matrix Biol.* **20**, 515–25.

Fléchon, J.E., Degrouard, J., Kopečný, V., Pivko, J., Pavlok, A. & Motlik, J. (2003). The extracellular matrix of porcine mature oocytes: origin, composition and presumptive roles. *Reprod. Biol. Endocrinol.* **1**, 124–36.

Gmachl, M., Sagan, S., Ketter, S. & Kreil, G. (1993). The human sperm protein PH-20 has hyaluronidase activity. *FEBS Lett.* **336**, 545–8.

Gordo, A.C., Roodrigues, P., Kurokawa, M., Jellerette, T., Exley, G.E., Warner, C. & Fissore, R. (2002). Intracellular calcium oscillations signal apoptosis rather than activation in *in vitro* aged mouse eggs. *Biol. Reprod.* **66**, 1828–37.

Honda, A., Siruntawineti, J. & Baba, T. (2002). Role of acrosomal matrix proteases in sperm–zona pellucida interactions. *Hum. Reprod. Update* **8**, 405–12.

Hunnicutt, G.R., Primakoff, P. & Myles, D.G. (1996). Sperm surface protein PH-20 is bifunctional: one activity is a hyaluronidase and a second, distinct activity is required in secondary sperm-zona binding. *Biol. Reprod.* **55**, 80–6.

Kano, K., Miyano, T. & Kato, S. (1994). Effect of oviductal epithelial cells on fertilization of pig oocytes *in vitro*. *Theriogenology* **42**, 1061–8.

Kim, N.-H., Funahashi, H., Abeydeera, L.R., Moon, S.J., Prather, R.S. & Day, B.N. (1996). Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during fertilization of pig oocytes *in vitro*. *J. Reprod. Fertil.* **107**, 79–86.

Kline, D. & Kline, J. (1992). Repetitive calcium transient and the role of calcium in exocytosis and cell cycle activation in the mouse. *Dev. Biol.* **149**, 80–9.

Kouba, A.J., Abeydeera, L.R., Alvarez, I.M., Day, B.N. & Buhi, W.C. (2000). Effects of the porcine oviduct-specific glycoprotein on fertilization, polyspermy, and embryonic development *in vitro*. *Biol. Reprod.* **63**, 242–50.

Kuppusamy, U.R., Khoo, H.E. & Das, N.P. (1990). Structure–activity studies of flavonoids as inhibitors of hyaluronidase. *Biochem. Pharmacol.* **40**, 397–401.

Li, M.-W., Cherr, G.N., Yudin, A.I. & Overstreet, J.W. (1997*a*). Biochemical characterization of the PH-20 protein on the plasma membrane and inner acrosomal membrane of cynomolgus macaque spermatozoa. *Mol. Reprod. Dev.* **48**, 356–66.

Li, M.-W., Yudin, A.I., VandeVoort, C.A., Sabeur, K., Primakoff, P. & Overstreet, J.W. (1997*b*). Inhibition of monkey sperm hyaluronidase activity and heterologous cumulus penetration by flavonoids. *Biol. Reprod.* **56**, 1383–9.

Li, M.-W., Yudin, A.I., Robertson, K.R., Cherr, G.N. & Overstreet, J.W. (2002). Importance of glycosylation and disulfide bonds in hyaluronidase activity of macaque sperm surface PH-20. *J. Androl.* **23**, 211–19.

Lin, Y., Mahan, K., Lathrop, W.F., Myles, D.G. & Primakoff, P. (1994). A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *J. Cell Biol.* **125**, 1157–63.

Maeda, T., Tatemoto, H., Terada, T. & Tsutsumi, Y. (1990). A convenient method for evaluation of frozen-thawed damage to acrosomes of fowl spermatozoa using a gelatin substrate slide. *Jpn. Poult. Sci.* **27**, 66–71.

Marchal, R., Caillaud, M., Martoriati, A., Gerard, N., Mermillod, P. & Goudet, G. (2003). Effect of growth hormone (GH) on *in vitro* nuclear and cytoplasmic oocyte maturation, cumulus expansion, hyaluronan syntheses, and connexins 32 and 43 expression, and GH receptor messenger RNA expression in equine and porcine species. *Biol. Reprod.* **69**, 1013–22.

McCauley, T.C., Buhi, W.C., Wu, G.M., Mao, J., Caamano, J.N., Didion, B.A. & Day, B.N. (2003). Oviduct-specific glycoprotein modulates sperm-zona binding and improves efficiency of porcine fertilization *in vitro*. *Biol. Reprod.* **69**, 828–34.

McRorie, R.A. & Williams, W.L. (1974). Biochemistry of mammalian fertilization. *Annu. Rev. Biochem.* **43**, 777–803.

Meyers, S.A., Yudin, A.I., Cherr, G.N., VandeVoort, C.A., Myles, D.G., Primakoff, P. & Overstreet, J.W. (1997). Hyaluronidase activity of *in vitro* capacitated macaque sperm assessed by an *in vitro* cumulus penetration assay. *Mol. Reprod. Dev.* **46**, 392–400.

Meyers, S.A. (2001). Equine sperm–oocyte interaction: the role of sperm surface hyaluronidase. *Anim. Reprod. Sci.* **68**, 291–303.

Miyazaki, S. (1990). Cell signaling at fertilization of hamster eggs. *J. Reprod. Fertil. Suppl.* **42**, 163–75.

Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakada, S. & Mikoshiba, K. (1992). Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1,4,5-triphosphate receptor in fertilized hamster eggs. *Science* **257**, 251–5.

Nagai, T. & Moor, R.M. (1990). Effect of oviductal cells on the incidence of polyspermy in pig eggs fertilized *in vitro*. *Mol. Reprod. Dev.* **26**, 377–82.

Nagai, T., Niwa, K. & Iritani, A. (1984). Effect of sperm concentration at preincubation in chemically defined medium on fertilization *in vitro* of pig follicular oocytes. *J. Reprod. Fertil.* **70**, 271–5.

Petters, R.M. & Wells, K.D. (1993). Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **48**, 61–73.

Phelps, B.M., Primakoff, P., Koppel, D.E., Low, M.G. & Myles, D.G. (1988). Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein. *Science* **240**, 1780–82.

Primakoff, P. & Myles, D.G. (1983). A map of the guinea pig sperm surface constructed with monoclonal antibodies. *Dev. Biol.* **98**, 417–28.

Primakoff, P., Hyatt, H. & Myles, D.G. (1985). A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida. *J. Cell Biol.* **101**, 2239–44.

Ryan, T.A. (1960). Significance tests for multiple comparison of proportions, variances, and other statistics. *Psychol. Bull.* **57**, 318–28.

Tatemoto, H. & Terada, T. (1999*a*). Analysis of zona pellucida modifications due to cortical granule exocytosis in single porcine oocytes, using enhance chemiluminescence. *Theriogenology* **52**, 629–40.

Tatemoto, T. & Terada, T. (1999*b*). Time course analyses of cortical granule exocytosis and zona pellucida modifications after artificial activation in *in vitro* matured porcine oocytes. *J. Mamm. Ova Res.* **16**, 110–17.

Tatemoto, H., Ootaki, K., Shigeta, K. & Muto, N. (2001). Enhancement of developmental competence after *in vitro* fertilization of porcine oocytes by treatment with ascorbic acid 2-*O*-α-glucoside during *in vitro* maturation. *Biol. Reprod.* **65**, 1800–6.

Tatemoto, H., Muto, N., Sunagawa, I., Shinjo, A. & Nakada, T. (2004). Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. *Biol. Reprod.* **71**, 1150–7.

Tatemoto, H., Muto, N., Yim, S.-D. & Nakada, T. (2005). Anti-hyaluronidase oligosaccharide derived from chondroitin sulfate A effectively reduces polyspermy during *in vitro* fertilization of porcine oocytes. *Biol. Reprod.* **72**, 127–34.

Thaler, C.D. & Cardullo, R.A. (1995). Biochemical characterization of a glycosylphosphatidylinositol-linked hyaluronidase on mouse sperm. *Biochemistry* **34**, 7788–95.

Thundathil, J., Palomino, J., Barth, A., Mapletoft, R. & Barros, C. (2001). Fertilizing characteristics of bovine sperm with flattened or indented acrosomes. *Anim. Reprod. Sci.* **67**, 231–43.

Wang, W.H., Abeydeera, L.R., Okuda, K. & Niwa, K. (1994). Penetration of porcine oocytes during maturation *in vitro* by cryopreserved, ejaculated spermatozoa. *Biol. Reprod.* **50**, 510–15.

Wolf, D.P. & Hamada, M. (1977). Induction of zonal and oolemmal blocks to sperm penetration in mouse eggs with cortical granule exudates. *Biol. Reprod.* **17**, 350–4.

Wu, H., Smyth, J., Luzzi, V., Fukami, K., Takenawa, T., Black, S.L., Allbritton, N.L. & Fissore, R.A. (2001). Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide pathway. *Biol. Reprod.* **64**, 1338–49.

Yanagimachi, R. (1994). Mammalian fertilization. In *The Physiology of Reproduction*, 2nd edn (ed. E. Knobil & J.D. Neill), pp. 135–85. New York: Raven Press.

Yudin, A.I., Vandevoort, C.A., Li, M.-W, & Overstreet, J.W. (1999). PH-20 but not acrosin is involved in sperm penetration of the macaque zona pellucida. *Mol. Reprod. Dev.* **53**, 350–62.