Protamine dissociation before decondensation of sperm nuclei during *in vitro* fertilization of pig oocytes

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The correlation between morphological changes and the dynamics of protamine in boar sperm chromatin during *in vitro* fertilization of pig oocytes matured *in vitro* was assessed. For this purpose, protamine was purified from boar sperm nuclei and an antiserum against protamine was developed. After affinity purification, the antiserum reacted exclusively with boar protamine during western blotting, showing no crossreactivity with core histones. Immunohistochemical evaluation revealed that only fully developed spermatid nuclei in boar testes stained strongly with the antiserum. When pig oocytes matured *in vitro* were fertilized *in vitro*, sperm penetration was observed in 37% of oocytes at 2 h after insemination and the penetration rate increased to 99% by 5 h after insemination, accompanied by an increase in polyspermic penetration. Paraffin wax sections of the inseminated oocytes were examined by immunohistochemical analysis with the antiserum. The proportion of condensed sperm nuclei that reacted with the antiserum was 87% of the sperm nuclei that penetrated by 2 h after insemination, and this decreased to 20 and 13% at 3 and 5 h after insemination, respectively. However, none of the decondensing sperm nuclei or male pronuclei reacted with the antiserum during the entire insemination period. These results indicate that a specific antiserum against boar protamine can be raised and, using this serum, it has been demonstrated that protamine is dissociated from boar sperm nuclei before decondensation during *in vitro* fertilization.

Introduction

Highly condensed nuclei of mature spermatozoa from most animals contain a specific set of strongly basic DNA-binding proteins, which are termed protamines or sperm-specific nuclear proteins, depending on their biochemical properties. Of such proteins, small arginine-rich proteins are categorized as protamines. Protamines directly replace histones in most species, with the exception of mammals. In mammals, nucleosomal histones are replaced transiently by transition proteins, and finally by protamines (Poccia, 1986). Mammalian sperm protamines differ from those of other species, such as fish, amphibians and fowl, with respect to the presence of cysteine (reviewed by Poccia, 1986; Zirkin *et al.*, 1989). The fundamental structure of sperm chromatin is not understood completely. However, it has been shown that mammalian sperm protamines have many disulphide (S-S) bonds. During epididymal maturation of spermatozoa, the thiol (SH) groups of the cysteine residues are progressively oxidized and S-S bonds are formed (Calvin *et al.*, 1973; Marushige and Marushige, 1975; Akama *et al.*, 1989). The

formation of the disulphide linkages makes sperm nuclei tight, or compact, and resistant to detergents (Calvin and Bedford, 1971; Bedford and Calvin, 1974), trypsin (Meistrich *et al.*, 1976) and micrococcal nuclease (Tobita *et al.*, 1984). Mature spermatozoa are transcriptionally inactive (Katagiri and Ohsumi, 1994).

After penetration of mature spermatozoa into oocytes, the chromatin of the sperm nuclei is remodelled into a typical somatic state, participating in subsequent chromosomal activities such as decondensation of sperm nuclei and formation of male pronuclei for the completion of fertilization. Investigation of the events of chromosomal remodelling in penetrating sperm nuclei would help to elucidate the molecular changes that occur at this early stage of fertilization and may contribute to the success of technologies for assisted reproduction in mammals, such as intracytoplasmic sperm injection. Yanagimachi (1994) suggested that chromosomal remodelling requires two steps: firstly, reduction of S-S to S-H and, secondly, replacement of protamines by histones. The ability for S-S reduction in ooplasm may depend on the concentration of free thiol glutathione (Zirkin *et al.*, 1989) as a result of cytoplasmic maturation (Yoshida, 1993; Yoshida *et al.*, 1993). However, the mechanism of the replacement of protamines by histones is still unclear. Perreault (1992) concluded that, in mammalian

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fertilized oocytes, removal of protamine during fertilization occurs as the sperm nucleus decondenses. However, in mice, Nonchev and Tsanev (1990) reported that protamine loss occurs as histones appear in the well-developed male pronucleus. Thus, it remains unclear exactly when protamine dissociation occurs during fertilization.

Experiments to detect protamine in penetrating sperm nuclei have been carried out using radiolabelled protamine (Ecklund and Levine, 1975; Kopečný and Pavlok, 1975) or antibodies raised against sperm basic nuclear protein (Rodman *et al.*, 1981) or protamine (Nonchev and Tsanev, 1990). An immunohistochemical approach with a well-characterized antibody seems to be useful for analysing the dynamics of proteins during fertilization. In some mammals, protamine has been detected during spermatogenesis by using immunofluorescence or immunoperoxidase techniques (Rodman *et al.*, 1979; Roux *et al.*, 1988); however, studies during fertilization have been performed only in mice (Rodman *et al.*, 1981; Nonchev and Tsanev, 1990). Although the amino acid sequence of protamines is conserved among mammals (Tobita *et al.*, 1983), the specificity of the antibody against protamine must be demonstrated (Stanker *et al.*, 1993). Thus, protamine-specific immunological studies provide a new approach that could be useful for investigating the remodelling of sperm chromatin during fertilization.

In the present study, protamine was purified from boar sperm nuclei and an antiserum against protamine was developed. This antiserum was used to investigate the correlation between the morphological changes and the dynamics of protamine in boar sperm chromatin during *in vitro* fertilization (IVF) of pig oocytes matured *in vitro*.

Materials and Methods

Purification of boar reduced protamine

Sperm nuclei. Ejaculated semen from a crossbred boar was filtered with cotton gauze and stored at -20°C until used for purification. Isolation of sperm nuclei was performed at 4°C as described by Tobita *et al.* (1982), with slight modifications. After thawing, semen (300 ml) was centrifuged at 4000 g for 20 min. The sediment was washed three times with 125 ml of a buffer solution containing 0.05 mol Tris-HCl l^{-1} (pH 8.3) and 0.14 mol NaCl l^{-1} . The washed sediment was suspended in 150 ml of a buffer containing 0.05 mol Tris-HCl l^{-1} (pH 8.0) and 0.02 mol 2-mercaptoethanol l^{-1} , and was incubated at 25°C for 30 min. After incubation, the suspension was centrifuged at 4000 g for 20 min at 25°C . The resulting sediment was washed four times with 75 ml 0.05 mol Tris-HCl l^{-1} (pH 7.0) supplemented with 1% (w/v) cetyltrimethylammonium bromide and washed three times with 75 ml 1% (w/v) citric acid. The washed sediment containing sperm nuclei was dispersed in 100 ml buffer containing 1.62 mol sucrose l^{-1} and 0.01 mol Tris-HCl l^{-1} (pH 7.4), and was recovered by centrifugation at 4000 g for 40 min. This process was repeated several times until the supernatant was reasonably clear and pure nuclei were obtained.

Purification of protamine. The purified sperm nuclei were suspended in 200 ml buffer containing 2 mol NaCl l^{-1} , 4 mol urea l^{-1} , 0.02 mol EDTA l^{-1} and 0.5 mol Tris-HCl l^{-1} (pH 8.5). Dithiothreitol (1.65 g) was added to the suspension with stirring under a nitrogen barrier. After incubation for 3 h at 37°C , two volumes of cold ethanol were added to the mixture to precipitate DNA. The mixture was allowed to settle on ice for 1 h and was centrifuged at 12 000 g for 15 min at 4°C . A four-thirds volume of ice-cold ethanol was added to the supernatant with gentle stirring and the mixture was kept at -20°C overnight, after which it was centrifuged at 12 000 g for 17 min at 4°C . The resulting precipitate was dried under vacuum. The precipitated sperm proteins were dissolved in 200 ml buffer containing 0.2 mol NaCl l^{-1} , 0.01 mol 2-mercaptoethanol l^{-1} , 4 mol urea l^{-1} and 0.05 mol sodium acetate l^{-1} (pH 5.3), and diluted with a buffer containing 0.01 mol 2-mercaptoethanol l^{-1} and 0.01 mol sodium acetate l^{-1} (pH 5.3). The mixture was applied to a CM-32 column (1.2 cm $\times$ 33.0 cm; Whatman, Maidstone) that had been equilibrated with a buffer containing 0.2 mol NaCl l^{-1} , 0.01 mol 2-mercaptoethanol l^{-1} , 4 mol urea l^{-1} and 0.05 mol sodium acetate l^{-1} (pH 5.3). The proteins were eluted with a linear gradient of 0.2–1.0 mol NaCl l^{-1} in a buffer containing 0.01 mol 2-mercaptoethanol l^{-1} , 4 mol urea l^{-1} and 0.05 mol sodium acetate l^{-1} (pH 5.3). The eluted proteins were precipitated by addition of 80% (w/v) trichloroacetic acid to a final concentration of 20% (w/v). The precipitated proteins were dissolved in a buffer containing 0.7 mol 2-mercaptoethanol l^{-1} , 7 mol guanidine-HCl l^{-1} , 0.01 mol EDTA l^{-1} and 0.05 mol Tris-HCl l^{-1} (pH 8.5). The pH of the solution was adjusted to 2.0 with 10% (v/v) trifluoroacetic acid and the solution was incubated at 37°C for 15 min. The proteins were purified by reverse-phase high-performance liquid chromatography on a Nucleosil 3007018 column (Macherey-Nagel, Düren) equilibrated in 0.1% (v/v) trifluoroacetic acid. The proteins were eluted with a gradient of 0–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml min^{-1} .

Preparation of antiserum against boar sperm protamine

Immunization and purification. Antiserum against boar protamine was raised in New Zealand White rabbits. Purified protamine was dissolved in Dulbecco's modified phosphate buffered saline (DPBS; Nissui Pharmaceutical Co Ltd, Tokyo) to a final concentration of 4 mg ml^{-1} . The protamine solution was emulsified in an equal volume of Freund's complete adjuvant (Yatron, Tokyo). The animals were injected with 1 ml of the emulsion intracutaneously at 2 week intervals and blood was collected through an ear vein 1 week after each injection. Serum was obtained and characterized with respect to titre using dot blotting as described by Hawkes *et al.* (1982).

The antiserum against protamine was affinity-purified using an *N*-hydroxysuccinimide-activated HiTrap column (Amersham Pharmacia Biotech, Uppsala) coupled with 2 mg purified protamine ml^{-1} . Saturated ammonium sulphate (2.7 ml) and DPBS (2 ml) were added to 2 ml antiserum. After incubation for 1 h at 4°C , the mixture was centrifuged at

10000 g for 30 min at 4°C. The precipitate was dissolved in Tris-buffered saline (TBS; 0.05 mol Tris-HCl l⁻¹ (pH 7.3) and 0.15 mol NaCl l⁻¹) containing 0.05% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (Chaps; Sigma Chemical Co, St Louis, MO) and applied to the protamine-coupled column. The column was incubated at room temperature for 1 h, followed by three washes with TBS containing 0.05% (w/v) Chaps. The antiserum against protamine was eluted from the column with 0.1 mol glycine-HCl l⁻¹ (pH 2.0). The eluate was neutralized by adding 150 µl of 2 mol Tris-HCl l⁻¹ (pH 8.5) to each fraction (1.5 ml). The affinity-purified antiserum was stored at -80°C until required.

Immunoblotting. The purified protamine (1 µg) and histones (4 µg; Sigma Chemical Co) were separated by electrophoresis in acid-urea gel as described by Panyim and Chalkley (1969). Gels of 15% (w/v) acrylamide in 5.4 mol urea l⁻¹ and 0.9 mol acetic acid l⁻¹ were made and pre-run at 200 V for 4 h using 0.9 mol acetic acid l⁻¹ as a running buffer. The proteins were dissolved in 10 µl of 7 mol urea l⁻¹ and 0.9 mol acetic acid l⁻¹, applied to the gel and electrophoresis was performed. The gel was soaked in the transfer buffer (7% (v/v) acetic acid) for 10 min, placed against a polyvinylidene difluoride membrane (Immobilon; Millipore Corporation, Bedford, MA) and the proteins were transferred using a transblot apparatus (MilliBlot; Millipore) operated according to the manufacturer's instructions. After transfer, the membrane was blocked in TBS with 0.5% (w/v) casein (casein TBS) at room temperature for 0.5 h. The membrane was incubated with the affinity-purified antiserum against protamine (1:100) overnight at 4°C. After washing with TBS containing 0.05% (v/v) Tween 20, the membrane was incubated with peroxidase-labelled anti-rabbit immunoglobulin (Cappel Research Product, Durham, NC). A peroxidase immunostaining kit (Wako Pure Chemical Industries Ltd, Osaka) was used to visualize the immunoreaction.

Immunohistochemistry. Immunohistochemical analysis was performed as described by Noguchi *et al.* (1997), with slight modifications. Testes from an adult Landrace boar were fixed with Methacarn solution (methanol-chloroform-acetic acid, 6:3:1, by volume) and embedded in paraffin wax. Sections were cut at 4 µm thickness. These sections were deparaffinized, autoclaved at 121°C for 5–15 min in 0.1 mol citric acid l⁻¹ (pH 6.0) for generation of antigen and incubated in 0.5% (w/v) periodic acid to block endogenous peroxidase. After blocking with casein TBS, the sections were incubated overnight at 4°C with the affinity-purified antiserum against protamine at a dilution of 1:80 in casein TBS. The Elite ABC kit (Vector Laboratories Inc, Burlingame, CA) with diaminobenzidine was used to visualize the reaction. All the experiments were repeated at least twice.

The specificity of the antiserum was examined by testing whether immunopositive reactions were diminished by the use of immunoabsorbed antiserum, which was prepared as follows: the affinity-purified antiserum (1:40 dilution in TBS) was mixed with protamine to a final concentration of 2.5–25.0 µg ml⁻¹. After incubation at 37°C for 1 h and subsequently at 4°C overnight, the mixture was centrifuged at

10000 g for 1 h at 4°C. The supernatant was used for immunohistochemical staining at the same dilution as untreated antiserum.

Detection of protamine in fertilized pig oocytes

In vitro maturation and IVF of pig follicular oocytes. Pig ovaries were obtained from prepubertal gilts at a local abattoir and were transported to the laboratory at 35°C. Cumulus-oocyte complexes (COCs) were collected from follicles 3–5 mm in diameter as described by Kikuchi *et al.* (1993, 1995). The COCs were transferred to a maturation medium (NCSU-37 solution; Petters and Wells, 1993) containing 10% (v/v) pig follicular fluid, 200 µmol cysteamine l⁻¹, 2.5 µg FSH ml⁻¹ (Sigma Chemical Co), 100 iu penicillin G potassium ml⁻¹ (Sigma) and 0.1 mg streptomycin sulphate ml⁻¹ (Sigma). About 40 COCs were cultured for 46–48 h in 500 µl maturation medium at 39°C under 5% CO₂ in air. The COCs were transferred to PBS containing 150 iu hyaluronidase ml⁻¹ (Sigma) for 5 min and freed from cumulus cells mechanically using a fine bore pipette. Denuded oocytes that emitted the first polar body were selected as matured oocytes. IVF was carried out as described by Kikuchi *et al.* (1995). Epididymal spermatozoa from a Landrace boar were frozen (Kikuchi *et al.*, 1998). After thawing, spermatozoa (5 × 10⁸ cells ml⁻¹) were preincubated at 37°C for 30 min in TCM-199 (with Earl's salts; Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics after adjusting the pH to 7.8 (Nagai *et al.*, 1988). Preincubated spermatozoa were diluted once with Bracket and Oliphant solution (Bracket and Oliphant, 1975) supplemented with 5 mmol caffeine l⁻¹ (Sigma) and 100 mg casein phosphopeptide ml⁻¹ (Meiji Seika Kaisha Ltd, Tokyo), and a portion of preincubated spermatozoa (10 µl) was transferred to 90 µl fertilization medium containing ten matured oocytes. The final sperm concentration was 2 × 10⁵ cells ml⁻¹. Co-incubation of oocytes with spermatozoa was carried out for 3.5 h, and the oocytes were then transferred to BMOC-II solution (Brinster, 1965) containing 4 mg BSA ml⁻¹. The oocytes were subsequently cultured for 1.5 h. At 1, 2, 3 and 5 h after insemination the oocytes were freed from spermatozoa attached to the zona pellucida. Some oocytes were whole-mounted on a glass slide, fixed in acetic alcohol (1:3), stained with 1% (w/v) aceto-orcein solution and examined under a phase contrast microscope. The other oocytes were fixed in 3% (w/v) paraformaldehyde and were processed for histological evaluation.

Immunohistochemical evaluation of protamine in penetrated sperm nuclei. The fixed *in vitro*-matured and *in vitro*-fertilized oocytes were embedded in 4% (w/v) agar (Difco Laboratories, Detroit, MI); the agar was dehydrated and embedded in paraffin. Serial sections were cut at 3 µm thickness. After deparaffinization, oocytes fixed at 1–5 h after insemination were processed for immunohistochemical analysis. The procedure for immunohistochemical analysis of oocytes was basically the same as described above, except that the oocytes were autoclaved for 5 min and blocked

with 1.5% normal goat serum (Vector Laboratories). After immunostaining, the sections were counterstained with haematoxylin. Morphological changes of penetrating sperm nuclei and immunoreactivity with anti-protamine antiserum were evaluated.

Artificially induced decondensing sperm nuclei were evaluated following the same procedure as described for the fertilized oocytes to assess the immunoreactivity of decondensing sperm nuclei with anti-protamine antiserum. Frozen-thawed epididymal spermatozoa were incubated in DPBS containing 5 mmol dithiothreitol l^{-1} for 30 min at 25°C (1×10^7 cells ml^{-1}), fixed with paraformaldehyde, embedded in agar and immunohistochemical analysis was performed as described previously. Condensed sperm nuclei that had not undergone dithiothreitol treatment were also evaluated. Some of the sections reacted without the first antibody (anti-protamine antiserum) during immunohistochemical analysis. All sections were counterstained with haematoxylin after the immunoreaction.

Sequential changes of protamine dissociation during fertilization. The correlation between sperm nuclear changes in whole mount preparations and the status of protamine in serial sections were analysed to understand the sequential changes of protamine dissociation in fertilized oocytes. Firstly, the reliability of the data obtained by sectioning was determined by comparing morphological changes of penetrating sperm nuclei with those obtained by the whole mount method. Some sections containing oocytes at 5 h after insemination were incubated for 15 min in PBS containing 10 μg Hoechst-33342 ml^{-1} (Hoechst; Calbiochem, CA) and the nuclear configuration of the penetrating sperm (condensation, decondensation and male pronuclei formation) was determined under a fluorescent microscope. The sperm nuclear status was recorded and reconstructed for each whole oocyte, and the results were compared with those for oocytes in the whole mount preparation. After this confirmation of data reliability, sequential changes in protamine dissociation were analysed.

Statistical analysis

Data were subjected to ANOVA using general linear model procedures (SAS/STAT, 1988). Morphological changes of penetrated sperm nuclei and immunohistochemical analysis of condensed sperm nuclei during IVF were analysed by Duncan's multiple range test after transformation using arcsin of percentages (Snedecor and Cochran, 1989).

Results

Specificity of the antiserum against protamine

The specificity of the antiserum for protamine was examined by acid-urea gel electrophoresis followed by western blotting and by immunohistochemical analysis (Figs 1 and 2, respectively). After transfer onto nitrocellulose membrane and staining with Coomassie brilliant blue, the

protamine band was relatively weak compared with the bands for core histones (Fig. 1, lanes 1 and 2). When electrophoresis was followed by immunoblotting with the antiserum, a specific band for protamine was detected, but bands for somatic histones were not detected (Fig. 1, lanes 3 and 4). Immunohistochemical evaluation of boar testis showed specific staining in nuclei of fully elongated spermatids, but no reaction in other cells, such as round spermatids, spermatocytes and spermatogonias (Fig. 2a). The immunohistochemical analysis was repeated using antiserum preincubated with purified protamine (immunoabsorbed antiserum) to confirm the specificity of the antiserum for protamine. No immunoreaction was observed after immunohistochemical analysis with the preincubated antiserum (Fig. 2b).

Sequential changes of penetrating sperm nuclei

Sequential changes of penetrating sperm nuclei were observed in whole mount preparations of inseminated oocytes. The rates of sperm penetration, polyspermy and the number of spermatozoa per oocyte are shown (Fig. 3). Sperm penetration was observed in >30% of oocytes at 2 h after insemination. At 3–5 h after insemination, sperm penetration was observed in almost all oocytes. The incidence of polyspermic penetration and the mean number of penetrated spermatozoa per oocyte increased with increasing duration of insemination. Penetrating sperm nuclei were classified as condensed sperm nuclei,

Fig. 1. Detection of core histones and protamine. Core histones (lanes 1 and 3) and purified protamine (lanes 2 and 4) were run on an acid-urea gel, transferred to nitrocellulose membrane and stained with Coomassie brilliant blue (lanes 1 and 2) or immunostained with the anti-protamine antiserum (lanes 3 and 4). Both core histones and purified protamine stained with Coomassie brilliant blue, whereas purified protamine but not core histone was detected by immunostaining.

Fig. 2. Boar testis stained using (a) purified anti-protamine antiserum and (b) immunoabsorbed antiserum. Note that nuclei of fully elongated spermatids were positive, but not other cells such as round spermatids, spermatocytes and spermatogonia. The positive reaction in the fully elongated spermatids disappeared when the sections were treated with absorbed antiserum. Scale bar represents 10 µm.

Fig. 3. Rate of sperm penetration (□), rate of polyspermy (◇) and sperm number per oocyte (■) of pig oocytes fertilized *in vitro*. Oocytes matured *in vitro* were inseminated and fixed in whole mount preparation at 1, 2, 3 and 5 h after insemination. The oocytes were stained with 1% (w/v) aceto-orcein and examined under a phase-contrast microscope.

Fig. 4. Morphological changes of penetrating boar sperm nuclei in whole mount preparations. (a) A condensed sperm nucleus at 2 h after insemination, (b) a decondensing sperm nucleus 5 h after insemination and (c) a male pronucleus 5 h after insemination. Arrows indicate sperm tails associated with sperm nuclei. Scale bar represents 10 µm.

decondensing sperm nuclei and male pronuclei (Fig. 4a,b,c). Sequential changes in sperm nuclei after insemination are shown (Table 1). At 2 h after insemination, all sperm nuclei were in a condensed state. Decondensing spermatozoa were first observed at 3 h after insemination, and male pronuclei were first observed at 5 h after insemination. The condensed sperm nuclei changed to decondensing nuclei and then to male pronuclei as the duration of the insemination period was increased.

Protamine status in penetrated sperm nuclei

Examination of sectioned oocytes under light microscopy enabled all condensed sperm nuclei and some of the decondensing sperm nuclei to be observed. Immuno-

Table 1. Morphological changes in penetrating sperm nuclei during *in vitro* fertilization of pig oocytes

Culture period (h after insemination)	Total number of sperm nuclei examined	Number (and %) of penetrating sperm nuclei		
		Condensed	Decondensing	Male pronuclei
2	31	31 (100 ± 0.0) ^a	0 (0 ± 0.0) ^a	0 (0 ± 0.0) ^a
3	161	104 (65 ± 4.8) ^b	57 (35 ± 4.0) ^b	0 (0 ± 0.0) ^a
5	258	37 (14 ± 3.6) ^c	95 (37 ± 3.6) ^b	126 (49 ± 4.6) ^b

Three replicate trials were performed.

Values are mean number (percentage ± SEM).

Inseminated oocytes were examined in whole mount preparations.

^{abc}Different superscripts within a column indicate significant differences between percentages by Duncan's multiple-range test ($P < 0.05$).

histochemical reactivity in the condensed sperm nuclei after penetration is shown (Fig. 5). Three types of sperm nuclei with or without immunoreactivity were detected (Fig. 5a,b). The three types of sperm nuclei were: (i) protamine associated with nucleus; the diaminobenzidine signal was observed all over the sperm nuclei and haematoxylin staining was not visible; (ii) protamine partially dissociated from the nucleus; part of the area of sperm nuclei was stained

with diaminobenzidine and the remaining area was stained with haematoxylin; and (iii) protamine fully dissociated from the nucleus; no diaminobenzidine signal was observed and sperm nuclei were stained with haematoxylin only. Sequential changes of immunoreactivity in condensed sperm nuclei during fertilization are shown (Table 2). At 2 h after insemination, >90% of condensed sperm nuclei were classified as 'protamine associated with nucleus', and no sperm nuclei were classified as 'protamine fully dissociated from the nucleus'. At 3 h after insemination, the incidence of sperm nuclei classified as 'protamine associated with nucleus' compared with the condensed spermatozoa decreased significantly ($P < 0.05$) and about half of the sperm nuclei were determined as 'protamine fully dissociated from the nucleus'. At 5 h after insemination, the incidence of sperm nuclei classified as 'protamine partially dissociated from the nucleus' and as 'protamine fully dissociated from the nucleus' decreased significantly ($P < 0.05$) and additional sperm penetration (an increase in the incidence of sperm nuclei classified as 'protamine associated with nucleus' compared with at 3 h after insemination) was observed. Decondensing sperm nuclei showed no staining with diaminobenzidine, but stained to varying degrees with haematoxylin (data not shown).

As decondensing sperm nuclei were not stained with diaminobenzidine, the question arose as to whether antibody binding to retained protamine was not observed because decondensing chromatin was very dispersed. Sperm nuclei

Fig. 5. Immunohistochemical analysis of penetrating boar sperm nuclei in oocytes, using anti-protamine antiserum. (a, b and c) Paraffin wax sections of oocytes at 3 h after insemination were examined by immunohistochemistry and counterstained with haematoxylin. Penetrated sperm nuclei were classified into three groups according to immunoreactivity: (i) protamine associated with nucleus (PA): the diaminobenzidine signal was observed all over the sperm nuclei and haematoxylin staining was not visible; (ii) protamine partially dissociated from nucleus (PPD): part of the area of sperm nuclei was stained with diaminobenzidine and the remaining area was stained with haematoxylin; and (iii) protamine fully dissociated from nucleus (PD): no diaminobenzidine signal was observed and sperm nuclei were stained with haematoxylin only. Sperm head (SH) before penetration located in the zona pellucida (ZP) was also immunoreactive. Scale bar represents 10 μ m.

Table 2. Protamine immunohistochemical analysis in condensed sperm nuclei during *in vitro* fertilization of pig oocytes

Culture period (h after insemination)	Total number of condensed sperm nuclei examined	Number of condensed sperm nuclei		
		Protamine associated (%)	Protamine partially dissociated (%)	Protamine fully dissociated (%)
2	51	47 (87 ± 19.4) ^a	7 (13 ± 5.9) ^{ab}	0 (0 ± 0.0) ^a
3	199	60 (30 ± 4.8) ^b	44 (22 ± 1.9) ^b	95 (48 ± 7.1) ^b
5	69	61 (88 ± 11.3) ^a	4 (6 ± 1.5) ^a	4 (6 ± 1.5) ^a

Values are mean number (percentage ± SEM).

Condensed sperm nuclei: serial sections of fertilized oocytes were examined immunohistochemically using anti-protamine antiserum reactivity of condensed sperm nuclei in the oocytes.

Protamine associated: the immunoreaction with anti-protamine antiserum was observed all over the sperm nuclei and haematoxylin staining was not visible.

Protamine partially dissociated: part of the area of sperm nuclei was immunoreacted and the remaining area was stained with haematoxylin.

Protamine fully dissociated: no immunoreaction was observed and sperm nuclei stained only with haematoxylin.

^{abc}Different superscripts within a column indicate significant differences between percentages by Duncan's multiple-range test ($P < 0.05$).

were made to decondense *in vitro* and were evaluated with the same procedure as for fertilized oocytes to eliminate this possibility. After incubation for 30 min with dithiothreitol, sperm nuclei decondensed randomly. *In vitro* decondensed sperm nuclei (Fig. 6c) and condensed sperm nuclei without dithiothreitol treatment (Fig. 6a) could be stained with anti-protamine antiserum. Both condensed and decondensed sperm nuclei were not stained with diaminobenzidine when the immunoreaction was performed without the antibody (Fig. 6b,d). These results also show that staining of decondensed sperm nuclei with haematoxylin was lower than that of condensed sperm nuclei. These results indicate clearly that the lack of staining with diaminobenzidine in decondensing sperm nuclei during fertilization is not caused by dispersion of chromatin.

Protamine dissociation from sperm nuclei during fertilization

It was difficult to detect all decondensing sperm nuclei and male pronuclei by haematoxylin staining during immunohistochemical analysis because of their low staining. The data for morphological changes obtained by the whole mounted method should be used to confirm the relationship between morphological changes and the dynamics of protamine in penetrated sperm nuclei. The reliability of the sectioning method was checked earlier by staining with Hoechst and comparing the results with those obtained by the whole mounted method. Hoechst staining enables decondensing sperm nuclei and male pronuclei to be visualized, as well as condensed sperm nuclei (Fig. 7). The incidence of each of these categories in sectioned oocytes stained with Hoechst was not different from those in whole mounted samples (Table 3). Thus, we are able to compare the results from immunohistochemical analysis (Table 2) with those from the whole mount method (Table 1), showing the relationship between morphological changes and the

dynamics of protamine in penetrated sperm nuclei (Fig. 8). This analysis shows clearly that the incidence of sperm nuclei classified as 'protamine associated with nucleus' decreased as the duration of the insemination period was increased.

Discussion

In the present study, the relationship between protamine dissociation and morphological changes of sperm nuclei during fertilization was investigated. For this purpose, boar protamine was purified by high-performance liquid chromatography, polyclonal antiserum against boar protamine was raised and further affinity-purified with purified protamine. The antiserum reacted exclusively with boar protamine on western blotting and there was no crossreactivity with core histones. Furthermore, indirect immunohistochemical examination of testes using the antiserum revealed that only fully developed spermatid heads stained strongly. This specificity has been observed not only in pigs but also in other mammalian species (rats and mice; J. Noguchi, A. Shimada, K. Kikuchi and H. Kaneko, unpublished). Rodman *et al.* (1981) and Nonchev and Tsanev (1990) reported that antibodies against sperm-specific protein or protamine of mice also reacted with proteins of the female pronucleus in fertilized mouse oocytes. In contrast, no component other than condensed boar sperm nuclei was apparently stained with anti-protamine antibody in the present study (data not shown). These results indicate that the antiserum developed in the present study is quite specific and, thus, should be a powerful tool for investigating the dynamics of protamine during fertilization.

The present results, based on immunohistochemical analysis, indicate that protamine dissociates from sperm nuclei before decondensation. These results also indicate that there are condensed sperm nuclei lacking protamine in pig oocytes

Table 3. Comparison of pig oocyte examination methods

Oocyte examination	Total number of oocytes examined	Total number of sperm that penetrated	Number (%) of penetrated sperm nuclei		
			Condensed (%)	Decondensing (%)	Pronucleus formed* (%)
Mounted whole	186	220	86 (39)	90 (41)	44 (20)
Sectioned	34	82	29 (35)	37 (45)	16 (20)

All oocytes were collected and fixed at 5 h after insemination.

Sectioned: stained with Hoechst 33342 and examined under a fluorescent microscope.

*One of the pronuclei was not included in the data analysis as it was a female pronucleus.

Fig. 6. Immunoreactivity of artificially induced decondensing boar sperm nuclei with anti-protamine antiserum. (a and b) Condensed sperm nuclei were evaluated without dithiothreitol treatment. (c and d) Frozen-thawed epididymal spermatozoa were treated with dithiothreitol for 30 min, fixed with paraformaldehyde, embedded in agar and evaluated by immunohistochemical analysis following the same protocol as for fertilized oocytes. Some of the sections reacted without the first antibody (anti-protamine antiserum) during immunohistochemistry (b and d). All sections were counterstained with haematoxylin after the immunoreaction. Arrows indicate sperm tails associated with sperm nuclei. Scale bar represents 10 μ m.

penetrated by a spermatozoon or spermatozoa. In toads, sperm nuclei lose protamines within 1 min after penetration, accompanied by nuclear decondensation (Ohsumi and Katagiri, 1991), indicating that removal of protamines occurs simultaneously with decondensation of nuclei during pronucleus formation (Katagiri and Ohsumi, 1994). However, hamster sperm nuclei microinjected into oocytes required 45–60 min for decondensation (Perreault *et al.*, 1987). Taking our results into consideration, it is possible that removal of protamine is not sufficient for the decondensation of mammalian sperm nuclei, and that an unknown factor (or factors) other than protamine may participate in maintenance of the condensed state in the sperm nuclei. Rodman *et al.* (1981) reported that, in mice, sperm basic nuclear proteins are retained in decondensed sperm nuclei. Nonchev and Tsanev (1990) reported that protamine is retained in the male pronucleus before the onset of DNA replication. The reasons

for this discrepancy in the timing of protamine disappearance among species are unclear. Spermatozoa from many species, including boars, contain only a single type of protamine, called protamine 1 (Balhorn, 1989). However, there are two different protamines, protamine 1 and protamine 2, in mice (Bellve *et al.*, 1988). This variety of protamines may mean that, in mice, protamines are remodelled through a pathway different from that in pigs or other mammals.

Perreault *et al.* (1988) and Zirkin *et al.*, (1989) suggested that reduction of protamine S-S is required for decondensation of sperm nuclei *in vivo*, and Katagiri and Ohsumi (1994) suggested that protamine is reduced by glutathione. Furthermore, pronuclear eggs lack sufficient reducing power to effect sperm nuclear decondensation (Perreault *et al.*, 1984), and mature oocytes contain significantly more glutathione than do pronuclear eggs (Perreault *et al.*, 1988). At 2–3 h after insemination the incidence of protamine-dissociated nuclei

Fig. 7. Morphological changes in penetrating boar sperm nuclei after histological preparation and staining with Hoechst 33342 dye. (a, b and c) Examples of oocytes fixed at 5 h after insemination. Condensed (Cn) and decondensing (Dc) sperm nuclei and pronuclei (PN) are visible. Sperm heads (SH) before penetration, attached to the zona pellucida, are also visible. Scale bar represents 10 μ m.

(both partially and fully dissociated) was increased. However, at 5 h after insemination, almost all the condensed sperm nuclei, which were derived from delayed polyspermic penetration, were found in association with protamine. Thus, at 3–5 h after insemination, protamine is rarely dissociated from the penetrating sperm nuclei. This inability to bring about dissociation of protamine is probably caused by a low concentration of glutathione in the polyspermic oocyte at 5 h after insemination. In fact, the glutathione concentration decreases in fertilized pig oocytes by 6 h after insemination (Funahashi *et al.*, 1995). It is possible that reduction of protamine by cytoplasmic glutathione is a prerequisite for protamine dissociation from sperm condensed nuclei.

In mammals, little is known about the oocyte factors that are responsible for protamine removal and nuclear

Fig. 8. Protamine dissociation from boar sperm nuclei before decondensation. ▨: Pronuclei. ▮: Decondensing sperm nuclei. □: Protamine fully dissociated from the nucleus. ▤: Protamine partially dissociated from the nucleus. ■: Protamine associated with the nucleus. These last three categories all represent condensed nuclei.

decondensation during fertilization (Zirkin *et al.*, 1989). Recent studies have revealed that nucleoplasmin plays a major role in the removal of sperm-specific protein in amphibians (Ohsumi and Katagiri, 1991; Philpot *et al.*, 1991; Philpot and Leno, 1992). Although the protamine releasing factors have not been identified in mammals, human spermatozoa treated with dithiothreitol are decondensed in *Xenopus* egg extracts (Brown *et al.*, 1987; Ohsumi *et al.*, 1988). Furthermore, nucleoplasmin purified from *Bufo* egg extract selectively removes protamines from human sperm nuclei *in vitro* (Itoh *et al.*, 1993). These studies indicate that the mammalian egg cytoplasm contain factors, similar to amphibian nucleoplasmin, that participates in the removal of protamine. Further studies are needed to elucidate the mechanisms by which protamine is removed from sperm nuclei in the cytoplasm of mammalian eggs.

In conclusion, a specific antiserum was raised against boar protamine and, by using this antiserum, it was found that protamine dissociates from sperm nuclei before decondensation during fertilization of pig oocytes.

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