Replacement of nuclear protein by histone in pig sperm nuclei during *in vitro* fertilization

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Sperm-specific nuclear protamines are dissociated before decondensation of sperm nuclei during fertilization in pigs. In the present study, replacement of nuclear protein by histone in boar spermatozoa during *in vitro* fertilization was evaluated by immunohistochemistry using anti-histone antibody. First, the specificity of the antibody used in this study was examined. Immunohistochemistry of the testes and epididymides indicated that somatic nuclei, but not elongated spermatids or maturing spermatozoa, were immunoreactive. Furthermore, immunoreaction was diminished after the antibody had been preincubated with unfractionated histone, indicating that the antibody was specific for the somatic nuclear histone. Immunohistochemistry of serial sections of oocytes, which were

matured and co-cultured with boar spermatozoa for 2 to 6 h indicated that, at 2 to 3 h after insemination, penetrating sperm nuclei in the condensed state were not immunoreactive. At 4 to 5 h after insemination, some of the condensed sperm nuclei were immunoreactive in part or over the whole area of the nucleus, and all of the decondensing nuclei and male pronuclei were immunoreactive. At 6 h after insemination, the decondensing sperm nuclei and well-developed male pronuclei were immunoreactive. These results imply that, in pigs, remodelling of sperm nuclear protein from protamine to histone is initiated at the time of sperm penetration, before onset of decondensation and male pronuclear formation.

Introduction

Fertilization is the first step for embryonic development. Before fertilization, sperm nuclear DNA is packed tightly with strongly basic DNA-binding proteins termed protamines or sperm-specific nuclear proteins. For completion of fertilization, penetrating sperm heads undergo cytological and biochemical changes different from those of oocyte chromatin. In summary, the cytological changes are: fusion of the plasma membrane of acrosome-reacted spermatozoa with the oocyte membrane; decondensation and then recondensation into a mass of chromatin, but not back to the original shape (oval spatulate nuclei); and decondensation of the nuclei again, resulting in male pronucleus formation. During these events, biochemical changes occur to the sperm nuclei. One of the most important events is chromatin remodelling from sperm-specific protamine to somatic nuclear histone. Yanagimachi (1994) suggested that the remodelling requires two steps: first, reduction of S–S to S–H in protamines, and second, replacement of

protamines by histones. The ability for S–S reduction in ooplasm correlates with the concentration of free thiol glutathione (Perreault *et al.*, 1988; Zirkin *et al.*, 1989). Biochemical analysis of the replacement process of sperm nuclear protein has been carried out using amphibian egg extracts (Lohka and Masui, 1983; Katagiri and Ohsumi, 1994) because these physiological events terminate quickly in the fertilized oocytes of organisms such as sea urchins. However, these events progress slowly in mammalian fertilized oocytes, which might be an advantage for analysis. Shimada *et al.* (2000) demonstrated that protamine dissociation occurs in fertilized pig oocytes just after sperm penetration but before sperm nuclear decondensation. This observation indicates that replacement by histones is likely to be initiated during the period just after sperm penetration and completed during sperm chromatin decondensation before DNA synthesis in a male pronucleus; this contention differs slightly from conclusions drawn by Nonchev and Tsanev (1990) in mice.

Understanding of the mechanism of sperm nuclear chromatin remodelling during fertilization may be advantageous not only for studies in developmental biology but also for the improvement of the successful rates of using the developing reproductive techniques of intracytoplasmic sperm injection (ICSI) or cloning by nuclear transfer. The

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injected sperm heads should complete their chromatin remodelling in the same way as they do in sperm penetration; however, the relationship between this phenomenon and successful development of sperm-injected oocytes has not yet been determined. In transplanted nuclei (Adenot *et al.*, 2000), interactions may occur between somatic histones brought by the nuclei and oocyte-specific linker histones originating from oocytes, as has been reported in sea urchins (Mandl *et al.*, 1997), frogs (Ohsumi and Katagiri, 1991a) and mice (Tanaka *et al.*, 2001).

In the present study, the correlation between the morphological changes of penetrated sperm nuclei and the dynamics of histone transfer onto sperm nuclei was evaluated by immunohistochemical methods during *in vitro* fertilization of pig oocytes. Chromatin remodelling during the early period of pig fertilization is discussed in the light of results from this and a previous study (Shimada *et al.*, 2000).

Materials and Methods

In vitro maturation and fertilization

Pig ovaries were obtained from prepubertal cross-bred gilts (Landrace, Large White and Duroc breeds) at a local abattoir and transported to the laboratory at 35°C. Cumulus-oocyte complexes (COCs) were collected from follicles 2–5 mm in diameter in TCM-199 with Hanks' salts (Gibco, Life Technologies Inc., Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Gibco), 20 mmol Hepes l⁻¹ (Dojindo Laboratories, Kumamoto), 100 iu penicillin G potassium ml⁻¹ (Sigma Chemical Co., St Louis, MO), and 0.1 mg streptomycin sulphate ml⁻¹ (Sigma; Kikuchi *et al.*, 1993). *In vitro* maturation (IVM) of oocytes was carried out as described by Kikuchi *et al.* (1999, 2002). In brief, about 50 COCs were cultured in each 500 µl of maturation medium, a modified NCSU-37 solution (Petters and Wells, 1993) containing 10% (v/v) pig follicular fluid, 0.6 mmol cysteine l⁻¹, 0.05 mmol β-mercaptoethanol l⁻¹, 1 mmol dibutyryl cAMP l⁻¹ (dbcAMP; Sigma), 10 iu eCG ml⁻¹ (PMS 1000 iu; Nihon Zenyaku Kogyo, Koriyama), and 10 iu hCG ml⁻¹ (Puberogen 500 unit; Sankyo, Tokyo), in four-well dishes (Nunc Multidishes, Nalge Nunc International, Roskilde) for 20 h. COCs were subsequently cultured in the maturation medium without dbcAMP and hormones for 24 h. The maturation culture was carried out under 5% CO₂ in air at 39°C. After maturation culture, oocytes with expanded cumulus cells were treated briefly with 150 iu hyaluronidase ml⁻¹ and denuded of cumulus cells by gentle pipetting. The denuded oocytes with the first polar body were harvested under a stereomicroscope and served as matured oocytes for *in vitro* fertilization (IVF). IVF was carried out as described by Kikuchi *et al.* (1995). Epididymal spermatozoa from a Landrace boar were frozen (Kikuchi *et al.*, 1998; Ikeda *et al.*, 2002). After thawing and pre-incubation for 1 h at 37°C in TCM-199 (with Earle's salts; Gibco) adjusted to pH 7.8 (Nagai *et al.*, 1988; Ikeda *et al.*, 2002), the 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 the diluted spermatozoa (10 µl) was transferred to 90 µl of fertilization medium containing ten matured oocytes. The final sperm concentration was 1 × 10⁵ cells ml⁻¹.

Examination of fertilization status

After co-incubation of gametes for 2, 3, 4, 5, 6, 8 or 10 h, some oocytes were freed from the attached spermatozoa, whole-mounted on to a glass slide, fixed in acetic alcohol (1:3), stained with 1% (w/v) aceto-orcein solution, and examined under a phase-contrast microscope.

Evaluation of specificity of anti-histone antibody

Before examination in samples from fertilized oocytes, the specificity of anti-histone antibody (monoclonal anti-histone H1; Leinco Technologies, Inc., St Louis, MO) used for immunohistochemistry was evaluated. Immunohistochemical approaches were conducted essentially as described by Noguchi *et al.* (1997). Tissue samples of testis and epididymis from adult Landrace boar were fixed with Methacarn solution (methanol–chloroform–acetic acid, 6:3:1 by volume), and dehydrated with a series of alcohols, and embedded in paraffin wax. Sections were cut at 4 µm, deparaffinized, treated with 8 mol urea l⁻¹ for 30 min for generation of antigen, and then all sections were incubated in 0.5% (w/v) periodic acid to block endogenous peroxidase. After blocking with 0.5% (w/v) casein Tris-buffered saline (TBS), the sections were incubated overnight at 4°C with the anti-histone antibody at a dilution of 1:5 in casein TBS. The Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) with diaminobenzidine was used to visualize the reaction.

The specificity of the antibody was also examined by testing whether immunopositive reactions were diminished by the use of immunoabsorbed antiserum, which was prepared as follows. The antibody at a dilution of 1:40 in TBS was mixed with unfractionated whole histone (Type II-A from calf thymus; Sigma) to a final concentration of 0–2560 µg ml⁻¹. After incubation at 37°C for 1 h and then at 4°C overnight, the mixture was centrifuged at 10 000 *g* for 1 h at 4°C. The supernatant was used for immunohistochemical staining at the same dilution as untreated antiserum.

Detection ability was also determined in oocytes. Maturing oocytes cultured for 24 h, matured oocytes cultured for 44 h and fertilized oocytes at 2 h after insemination were fixed in 3% (w/v) paraformaldehyde and processed for immunohistochemical evaluation as described by Shimada *et al.* (2000). In brief, the fixed oocytes were embedded in 4% agar (Difco Laboratories, Detroit, MI), and then the agar containing oocytes was dehydrated and embedded in paraffin wax. After deparaffinization, serial sections (3 µm in thickness) were processed for immunohistochemistry. The procedure for immunohistochemistry

Fig. 1. (a) Immunohistochemistry with anti-histone H1 antibody of adult boar testis. (b) Immunohistochemistry followed by counterstaining with haematoxylin. Nuclei of elongated spermatids in the seminiferous epithelium showed staining with haematoxylin but not with diaminobenzidine, indicating dissociation of histone H1, whereas spermatogonia, spermatocytes and round spermatids associated with histone H1 showed staining with diaminobenzidine. (c) Immunohistochemistry of boar testis with anti-histone antibody preincubated with unfractionated histones ($2560 \mu\text{g ml}^{-1}$). Most of the positive reactions in nuclei of germ cells such as spermatogonia, spermatocytes and round spermatids, and those of the interstitial cells are no longer present. (d–f) Immunohistochemistry applied in pig oocytes. (d) Maturing (unfertilized) oocyte at metaphase I. (e) Matured (unfertilized) oocyte at metaphase-II, and (f) fertilized oocytes at anaphase II emitting the second polar body. Oocyte chromatin at metaphase (M) or anaphase (A) stages and both first (1PB; out of focus) and second (2PB) polar bodies were immunoreactive. Scale bars represent $10 \mu\text{m}$.

of oocytes was the same as described above, except for autoclaving at 121°C for 5–15 min in $0.1 \text{ mol citric acid l}^{-1}$ (pH 6.0) and blocking with 1.5% normal goat serum (Vector).

Immunohistochemical evaluation of IVF oocytes

The IVF oocytes at 2, 3, 4, 5, 6, 8 or 10 h after insemination were fixed and processed for immunohistochemistry as described above. After immunohistochemical staining, serial sections were counterstained with haematoxylin. Morphological changes of penetrating sperm nuclei and immunoreactivity with anti-histone antibody were evaluated.

Results

Specificity of the anti-histone antibody in pig tissues

Immunohistochemical staining of boar testis showed specific reaction in nuclei of interstitial cells or those of germ cells such as spermatogonia, spermatocytes and round spermatids in seminiferous epithelium but not in nuclei of elongated spermatids (Fig. 1a). When immunostaining was followed by staining with haematoxylin, nuclei of elongated

spermatids located in the luminal area were stained with haematoxylin alone, whereas the other cells in the seminiferous epithelium were dark brown, indicating staining by both diaminobenzidine and haematoxylin (Fig. 1b). No positive reaction was detected in the matured epididymal spermatozoa (data not shown). An immunoabsorbance test with the antibody preincubated with unfractionated histone was conducted to confirm the specificity of the staining. Nuclei of germ cells such as spermatogonia, spermatocytes and round spermatids were positive without immunoabsorbance (Fig. 1a); however, most of the positive reactions diminished after preincubation with histone, as the histone concentration increased (Fig. 1c). The ability of the antibody to detect histone in maturing, matured and activated oocytes was also examined. Metaphase I and II and anaphase II chromosomes (Fig. 1d–f) and nuclei of first and second polar bodies (Fig. 1e,f) were detected by immunohistochemistry with this antibody.

Sequential nuclear changes of inseminated oocytes and penetrating spermatozoa

Sequential changes of penetrating sperm nuclei were observed in whole-mount preparations of inseminated

Fig. 2. Rate of sperm penetration (○), rate of polyspermy (●) and number of spermatozoa per oocyte (histogram) of pig oocytes fertilized *in vitro*. Oocytes matured *in vitro* were inseminated and fixed in whole mount preparation at 2, 3, 4, 5, 6, 8 or 10 h after insemination. The oocytes were stained with 1% (w/v) acetoorcein and examined by phase-contrast microscopy. Means $\pm$ SEM are presented. At least three replicated trials were performed and a total of 90–153 oocytes were examined for each category.

oocytes. The rates of sperm penetration, polyspermy and the number of penetrated spermatozoa per oocyte are shown (Fig. 2). Sperm penetration was observed in > 10% of the oocytes at 2 h after insemination. At 4–10 h, about 80% of inseminated oocytes were penetrated, and most of them showed polyspermic fertilization, indicating increased numbers of penetrating spermatozoa. Inseminated oocytes were activated to be at anaphase II at 2 h, proceeded to telophase II at about 4 h, and began to form a female pronucleus from 4 h after insemination (Fig. 3a). Whole-mount preparations of inseminated oocytes also showed the detail of morphological changes of sperm nuclei (Fig. 3b). Sperm entry was first observed at 2 h after insemination, when sperm nuclei were in a condensed state. Decondensation of the nuclei started from 3 h and continued to 10 h after insemination. A male pronucleus was first observed to form at 4 h after insemination, but was small. Well-developed pronuclei with a nucleolus appeared from 6 h after insemination. This development almost matches female pronuclear development.

Histone status in penetrated sperm nuclei

Examination of sectioned oocytes under light microscopy enabled visualization of (i) condensed sperm nuclei of the original shape, (ii) decondensing (including recondensing) sperm nuclei, and (iii) male and female pronuclei. Immunoreactivity in the sperm nuclei after penetration is shown (Fig. 4). Three types of condensed sperm nuclei were identified by the pattern of staining (Fig. 4a–c): (i) histone

Fig. 3. (a) Oocyte nuclear status and (b) morphological changes of penetrating sperm nuclei of pig oocytes fertilized *in vitro*. (a) Sperm penetration was initiated at 2 h after insemination; however, oocyte chromatin was arrested at metaphase II (○). Oocytes were activated and their nuclear status proceeded to anaphase II (●) at 3 h. After reaching telophase II (□), the female pronucleus began to develop from 4 h. Female pronucleus formation with second polar body (■) occurred in more than 70% of the oocytes. (b) Sperm penetration was first observed at 2 h after insemination, and at this stage all of the spermatozoa were condensed (○). Decondensing nuclei (●) began to be observed after 3 h and male pronuclei (■) after 4 h. Male pronuclear formation occurred in about 60% of the inseminated oocytes. At least three replicated trials were performed and a total of 90–164 oocytes were examined for each category.

not associated with the sperm nucleus: no diaminobenzidine signal observed and sperm nuclei stained with haematoxylin only; (ii) histone associated with part of the nucleus: part of the area of sperm nuclei stained with diaminobenzidine and the remaining area stained with haematoxylin; and (iii) histone associated with the entire nucleus: diamino-

Fig. 4. Immunohistochemistry with anti-histone H1 antibody followed by staining with haematoxylin allowing histone H1-associated sperm nuclei from non-associated nuclei to be distinguished in fertilized pig oocytes. Immunoreaction resulted in dark-brown staining with both diaminobenzidine and haematoxylin, whereas chromatin without histone H1 association resulted in blue staining with haematoxylin. (a) A condensed sperm nucleus at 3 h after insemination showed staining with haematoxylin alone (NH), indicating no association of histone H1 with the sperm nucleus. (b) A part of a condensed sperm nucleus at 4 h after insemination showed staining with haematoxylin and the remaining area showed staining with diaminobenzidine (PH). This finding indicates that some of the condensed sperm nuclei had associated with histone H1. (c) A condensed sperm nucleus showed staining with diaminobenzidine (H) at 4 h after insemination. (d) A sperm nucleus in an oocyte showing heavy polyspermy. Sperm nuclei were highly decondensed (DS), resulting in the broad staining with diaminobenzidine at 5 h after insemination. (e) Male and female pronuclei (PN) showed staining with diaminobenzidine at 6 h after insemination. They were not distinguishable from each other. The first polar body (1PB) was immunoreactive. Arrows indicate sperm tails. Scale bars represent 10 µm.

Fig. 5. Relationship between morphological changes of penetrating pig sperm nuclei and their immunoreactivity with anti-histone H1 antibody. At 2–3 h after insemination, all of the penetrated sperm nuclei were condensed and showed staining only with haematoxylin. At 4–5 h, some condensed sperm nuclei were immunoreactive in part of (haematoxylin and diaminobenzidine) or over the whole area (diaminobenzidine) of the nuclei. Up to 6 h, all of the decondensing sperm nuclei and male pronuclei were

benzidine signal observed all over the sperm nuclei and haematoxylin staining not visible. All the decondensing sperm nuclei (Fig. 4d) and both male and female pronuclei (Fig. 4e) were stained with diaminobenzidine and the signal from haematoxylin only was not detected. Because distinguishing the male from the female pronucleus was impossible in the sections, one was subtracted as a female pronucleus in the case of multiple pronuclear formation. In the case of polyspermy, all the decondensing sperm nuclei and pronuclei were equally labelled with diaminobenzidine.

The sequential changes of penetrated spermatozoa according to their morphological status and their immunoreactions are summarized (Fig. 5). Before 4 h after insemination, all penetrated sperm nuclei were condensed, and immunoreactivity showed that histone not associated with the sperm nucleus. At 4–5 h after insemination, about 80% of penetrated sperm nuclei were condensed, but all three types of immunohistochemical reactivity ('histone not associated with the sperm nucleus', 'histone associated with

immunoreactive (diaminobenzidine). ■: Pronucleus, diaminobenzidine; ▨: decondensing, diaminobenzidine; ▩: condensed, diaminobenzidine; ▤: condensed, haematoxylin and diaminobenzidine; and □: condensed, haematoxylin. Numbers on the bars represent the total number of sperm nuclei used for analysis.

Fig. 6. Schematic diagram of remodelling of boar sperm nuclear protein from protamine to histone in relation to changes in sperm nuclear morphology. Protamine dissociation occurs at 2–3 h and histone association occurs until 4 h after insemination, before decondensation or male pronucleus (MPN) formation.

part of the nucleus' and 'histone associated with the entire nucleus') were observed. Decondensing sperm nuclei and pronuclei, which were judged to have developed from sperm nuclei, were also immunoreactive and considered to show 'histone associated with the entire nucleus'. At 6 h after insemination, most of the penetrated sperm nuclei were decondensing and developing into male pronuclei; these were considered to show 'histone associated with the entire nucleus'. A small percentage of condensed sperm nuclei without immunoreaction was also observed.

Discussion

During fertilization, protamines dissociate from penetrating boar sperm nuclei that are still condensed at 2–3 h after insemination (Shimada *et al.*, 2000), at which time the spermatozoa begin to penetrate the oocytes. The results of the present study clearly indicate that some of the condensed sperm nuclei at 4–5 h after insemination are immunoreactive and that all of the decondensing nuclei and pronuclei are immunoreactive after 4 h. On the basis of these results, it is suggested that protamines dissociate from the boar nuclei just after penetration and, just after the protamine dissociation, histones began to associate in the nuclei before decondensation or formation of the pronucleus (Fig. 6). In mice, although the idea that protamine loss occurs as histones appear in the well-developed male pronucleus is accepted (Nonchev and Tsanev, 1990), it can be concluded that sperm basic proteins are retained in the decondensed nuclei (Rodman *et al.*, 1981). As the timing of the remodelling in mice (whether in condensed or decondensed sperm nuclei) is not yet known, the present study in

pigs is the first to have used immunohistochemistry to clarify the remodelling of penetrated sperm nuclei. In other mammalian species, further attention should be paid to the mechanism of remodelling of the nuclei after the sperm penetration.

After sperm penetration, protamine dissociation occurs and histone association and then somatic nucleosome reconstruction are observed (Yanagimachi, 1994). In frogs, remodelling of nuclear protein from protamine to histone occurs just after penetration (Ohsumi and Katagiri, 1991b). In mammals, especially in domestic animals such as pigs, the remodelling processes are not yet well understood. However, the observations in the previous (Shimada *et al.*, 2000) and present studies indicate that the dissociation of protamine and association of histone in penetrating sperm nuclei occur just after sperm penetration and before decondensation of nuclei, indicating that the nucleosome structure in sperm chromatin is reconstructed at the initial stage of fertilization and is similar to that of oocyte chromatin. The equality in the molecular structure between male and female chromatin appears to be important for the synchronization of decondensation and subsequent pronucleus formation between male and female chromatin, and may affect pronuclear functions such as DNA replication or transcriptional activity. In fact, synchronization has been detected between formation of male and female pronuclei in the present study, and in DNA replication within the pronuclei in fertilized mammalian oocytes (hamster: Naish *et al.*, 1987; pig: Laurincik *et al.*, 1995). Further functional analysis, for example examining transcriptional activity, of the pronuclei is expected to produce similar results in pigs as it has in mice (Aoki and Schultz, 1999).

In general, histones are well-conserved; however, many subtypes of linker histone (H1) are known (van Holde, 1988). Oocyte- or embryo-specific H1 subtypes, which can be distinguished by immunological approaches, have been reported, in particular, in sea urchins and frogs (Pehrson and Cohen, 1984; Smith *et al.*, 1988). Somatic H1 subtypes have not been detected in early embryos of mammals or non-mammalian animals. In mice, somatic H1 subtypes are not detected in germinal vesicles, the nuclei of polar bodies or the nuclei of one- or two-cell-stage embryos, but are detected in the nuclei of embryos beyond the four-cell stage (Clarke *et al.*, 1992). In cattle, somatic H1 assembly is similar to that found in mice (Smith *et al.*, 1995). Tanaka *et al.* (2001) reported evidence of a mouse oocyte- or embryo-specific linker histone subtype, named H1oo. H1oo has been detected in germinal vesicles, metaphase II chromosomes, the nuclei of polar bodies, pronuclei, and nuclei at the two-cell stage, and shows similarity to the oocyte-specific linker histone (B4/H1M) of frogs (Dworkin-Rastl *et al.*, 1994) and the cleavage stage-specific linker histone (cs-H1) of sea urchins (Poccia *et al.*, 1981). The antibody used in the present study detected somatic histone in pig testes and epididymides and its specificity was confirmed by an immunoabsorbance test. Penetrated sperm nuclei, oocyte chromatin in both metaphase to anaphase chromosomes, the nuclei of polar bodies and the female pronucleus were all shown to be immunoreactive with the antibody. This finding indicates that the anti-histone antibody used in the present study might detect both oocyte-specific H1 and somatic H1 subtypes in pigs; however, there is no evidence for a pig oocyte-specific H1 subtype. Further investigation is necessary to determine the H1 subtype involved in the remodelling during pig fertilization.

In mammals, little is known about the oocyte factors that are responsible for protamine–histone exchange and nuclear decondensation during fertilization. Cytoplasmic proteins called nuclear chaperones mediate protamine–histone exchange and decondensation of sperm nuclei in oocytes (for a review, see Philpott *et al.*, 2000). One of the well-studied nuclear chaperones is nucleoplasmin. In amphibians, nucleoplasmin removes protamine or sperm-specific basic proteins, while it deposits an H2A–H2B dimer (Ohsumi and Katagiri, 1991b; Philpott *et al.*, 1991; Philpott and Leno, 1992). Itoh *et al.* (1993) demonstrated that remodelling of human sperm chromatin is observed in amphibian egg extracts and is mediated by nucleoplasmin from egg extracts. Furthermore, there is a nuclear factor in interphase egg nucleus that affects sperm decondensation and is released into the ooplasm as oocytes mature (Maeda *et al.*, 1998). These results indicate the presence of nucleoplasmin-like substances in mammalian oocytes. Members of the nucleoplasmin family of nuclear chaperones have been isolated in mice (MacArthur and Shackleford, 1997) and humans (Shackleford *et al.*, 2001). However, there is no firm evidence as yet for nucleoplasmin-like chaperone molecules functioning in protamine–histone exchange of mammalian sperm. In addition, the mechanism

for transfer of histone H1 to sperm nuclei has not yet been identified even in amphibians. Further attempts to clarify the mechanism are needed.

In conclusion, the results of the present study indicate that somatic histone or its variant associates with boar sperm nuclei just after their penetration and after dissociation of protamine from the nuclei.

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