DNA stability and thiol-disulphide status of rat sperm nuclei during epididymal maturation and penetration of oocytes

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Summary

DNA stability and thiol-disulphide status of rat sperm nuclei was observed *in vivo* during maturation in the epididymis and penetration of oocytes. When spermatids and spermatozoa were stained with acridine orange after fixation with acetic alcohol, the red/green fluorescence ratio observed under a confocal microscope was not different between spermatids (3.81 ± 0.16) and testicular spermatozoa (4.03 ± 0.34), and then decreased sharply ($p < 0.01$) as the spermatozoa descended the epidymis to the caput epididymis (1.13 ± 0.03). However, the ratio was not different among corpus (0.69 ± 0.01), cauda epididymis (0.68 ± 0.11) and ejaculated spermatozoa (0.63 ± 0.01). On the other hand, when spermatozoa were labelled with monobromobimane (mBBr), the fluorescence intensities gradually decreased ($p < 0.01$) during passage of spermatozoa from testis (4.74 ± 0.16) through epididymis (caput, 2.72 ± 0.08; corpus, 1.07 ± 0.03; cauda, –0.05 ± 0.05; ejaculated, 0.08 ± 0.03). The acridine orange red/green fluorescence ratio increased ($p < 0.01$) during zona penetration (binding sperm, 0.52 ± 0.09; perivitelline sperm, 0.64 ± 0.16) and sperm decondensation (decondensed sperm, 0.69 ± 0.12). When spermatozoa in the perivitelline space were labelled with mBBr, the fluorescence was detected. These results demonstrate that DNA stability against acid appears to be ahead of the oxidation of protamine during sperm maturation in the epididymis and is an initial event of the unpackaging process in rat genome occurring during or just after zona penetration but before ooplasm penetration.

Keywords: Acridine orange, Fertilisation, Monobromobimane, Rat, Spermatozoa

Introduction

In order that sperm DNA can participate in embryonic development, it must be unpackaged in the oocyte early in the process of fertilisation. An initial event of the unpackaging process is a reduction in the protamine disulphide bonds that have been formed during epididymal maturation (Perreault *et al.*, 1988). Sperm nuclear decondensation of various mammalian species has been demonstrated *in vitro* by incubation of spermatozoa with disulphide-reducing agents (see Zirkin *et al.*, 1989). Since decondensation of microinjected sperm nuclei was blocked in the hamster by treating oocytes with sulphhydryl blocking agent or glutathione oxidant (Perreault *et al.*, 1984), it is believed that intracellular reducing activity, such as glutathione and other thiols, might play a role in sperm nuclear decondensation. In the mouse, the stability of sperm chromatin as assessed with toluidine blue staining changed only after penetration into the ooplasm (Krzanowska, 1982). Since the degradation of reduced glutathione (GSH) in the γ-glutamyl cycle occurs extracellularly (Meister, 1995), intracellular GSH could be transported outside the ooplasm. The perivitelline space, which is surrounded by zona pellucida, may provide a reducing condition. However, there is little evidence that the disulphide bonds of spermatozoa begin to be reduced before penetration into the ooplasm.

The dynamics of the thiol status of rat spermatozoa during epididymal maturation has been investigated using the fluorescent labelling agent monobromobimane (mBBr; Shalgi *et al.*, 1989), which reacts with cellular thiol groups under physiological conditions and results in fluorescent derivatives. Staining with acridine orange following fixation with acetic alcohol has

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also been used to assess thiol-disulphide status of the sperm nuclei in several species, including hamster, mouse and human (Kosower *et al.*, 1992). This assessment method is based on the stability of DNA against acid. Sperm DNA with disulphide poor protamines appears to be denatured following treatment with acid, whereas the DNA of disulphide-rich sperm nuclei is resistant to denaturation even after exposure to acid (Kosower *et al.*, 1992). Binding of acridine orange to denatured DNA emits red fluorescence, whereas binding to native DNA emits green fluorescence (Ichimura *et al.*, 1971; Kosower *et al.*, 1992). A previous observation using mBBr has demonstrated that the reactive thiols of rat spermatozoa decrease markedly between the corpus and the cauda of the epididymis (Shalgi *et al.*, 1989). Oxidation of sperm thiols as determined by acridine orange fluorescence after acid treatment occurred during passage through the caput epididymis (Kosower *et al.*, 1992). Recent progress in technology has made it possible to measure the fluorescence and to compare the values.

In the present study we investigated the thiol-disulphide status of rat sperm nuclei during epididymal maturation and the early events of fertilisation *in vivo*, by examining the intensities of mBBr fluorescence and the red/green fluorescence ratio of acridine orange after treatment with acid.

Materials and methods

Preparation of spermatids and spermatozoa

Spermatids and spermatozoa were collected from mature male Wistar rats (4–8 months old). To obtain spermatids and testicular spermatozoa, an excised testis was placed in Dulbecco's phosphate-buffered saline solution containing 5.6 mM glucose and 5.4 mM sodium lactate (GL-PBS: Ogura & Yanagimachi, 1993). After removal of the tunica albuginea with fine forceps, seminiferous tubules were allowed to spread in the medium and cut into small pieces with a pair of fine scissors. The spermatogenic cells and spermatozoa were dispersed throughout the medium by gentle aspiration in and out of a pipette. The cell suspension was filtered through a cell strainer 40 μm nylon mesh (Falcon, Becton Dickinson, NJ), and centrifuged at 700 *g* for 10 min. Sedimented cells were resuspended in a small quantity of fresh GL-PBS. To obtain a high concentration of spermatids and testicular spermatozoa, the resuspended cells were separated by Percoll density gradients. A gradient was made with 3 ml of 40% Percoll onto which was layered 3 ml of 30% Percoll, 3 ml of 20% Percoll and 2 ml sample in a 15 ml centrifuge tube, and centrifuged at 6000 *g* for 30 min. Highly concentrated spermatids and testicular spermtozoa collected in the 20% and 30% Percoll, respectively. Epididymal spermatozoa from the proximal caput, corpus and cauda epididymis were obtained by placing a small piece of the tract in a drop of GL-PBS and squeezing the tract gently using a fine forceps to release spermatozoa into the medium. Ejaculated spermatozoa were collected from the vagina of mature females the morning after mating.

Egg collection

Sexually mature female Wistar rats (2–3 months old) were maintained under controlled lighting conditions (14L : 10D; lights on at 06:00 hours). To obtain eggs, rats at pro-oestrus, which was identified by examination of vaginal smears, were naturally mated overnight with males of the same strain. On the following morning, rats were examined for the presence of a vaginal plug or spermatozoa in the vagina. To collect eggs with spermatozoa binding to the zona pellucida, eggs with penetrating spermatozoa in the perivitelline space and eggs with a decondensing sperm head, mated rats were killed by cervical dislocation at 06:00, 08:00, 10:00 and 15:00 hours. The oviducts were isolated and placed in a dish containing mKRB supplemented with 0.1% hyaluronidase. The cumulus–egg complexes were dissected out of the oviducts, placed in the medium, and freed from cumulus cells by repeated passage through a fine pipette. Under a dissecting microscope, eggs were selected in which one or more spermatozoa were binding to the zona pellucida, in which a spermatozoon was located in the perivitelline space, or in which the sperm head was not visible but a penetrating sperm tail was seen. When eggs with a sperm tail but no visible sperm head were examined under a phase-contrast microscope after fixation and staining, a decondensed sperm nucleus was observed in each of them ($n = 27$).

Staining with acridine orange

Samples of spermatids and spermatozoa were smeared on glass slides, air-dried, fixed for 2 h in acetic alcohol (1 part glacial acetic acid plus 3 parts 100% methanol) and air-dried again. Egg samples were placed between four spots of Vaseline on a glass slide, compressed gently with a coverslip, and fixed for 2 h in acetic alcohol. After fixation, eggs with sperm on the zona pellucida, in the perivitelline space or in the ooplasm were stained with acridine orange solution (at 1000× dilution with GL-PB) overnight. After staining, each slide was washed with distilled water and sealed with synthetic resin to prevent it from drying. Slides were examined at ×1200 magnification in a laser-scanning confocal microscope (BioRad MRC-1024). Fluorescence intensities of red and green emissions of the

sperm head were examined and scored separately using image analysis software (Laser Sharp, BioRad).

Labelling with mBBr

A stock solution of 50 mM mBBr (Sigma) in acetonitrile (Aldrich, Milwaukee, WI) was prepared. For sperm samples, stock mBBr was diluted to 2 mM in GL-PBS and added to the sperm suspension to give a final concentration of 1 mM. This solution was prepared before use. After 15 min of incubation in the dark at room temperature, sperm samples were washed two times by centrifugation, and smeared on glass slides, compressed gently with a coverslip. Specimens were examined for 450 nm emission at ×1000 magnification under an epifluorescence microfluorometer (Olympus OSD-SRF20). mBBr alkylates reactive thiols and the products (mB-SR) emit blue fluorescence with an emission maximum at 480–490 nm (Kosower *et al.*, 1992). Eggs with a spermatozoon in the perivitelline space were directly diluted in the stock mBBr (Sigma) to a final concentration of 1 mM. Labelling by mBBr was carried out in the dark for 15 min at room temperature. Eggs were then washed three times with GL-PBS, and observed under a dissecting fluorescence microscope.

Statistical analyses

Statistical analyses of samples from three or four replicated experiments for treatment comparisons were carried out by ANOVA and Fisher's protected least significant difference test using the STATVIEW program (Abacus Concepts, Berkeley, CA). When ANOVA revealed a significant treatment effect, the treatments were then compared with each other by Fisher's protected least significant difference test. Data were expressed as means ± SEM. A probability of $p < 0.05$ was considered to be statistically significant.

Results

When spermatids and spermatozoa were stained with acridine orange after fixation with acetic alcohol, the mean red/green fluorescence ratio was not different ($p = 0.22$) between spermatids (3.81 ± 0.16) and testicular spermatozoa (4.03 ± 0.34; Fig. 1). The red/green fluorescence ratio decreased ($p < 0.01$) during passing through the caput region of the epididymis (1.13 ± 0.03 in the caput, 0.69 ± 0.01 in the corpus; Fig. 1). Although the ratio continued to decrease until the corpus region, there were no differences ($p > 0.60$) in the ratio among spermatozoa in the corpus and cauda regions (0.68 ± 0.11) of the epididymis and ejaculated spermatozoa (0.63 ± 0.01; Fig. 1).

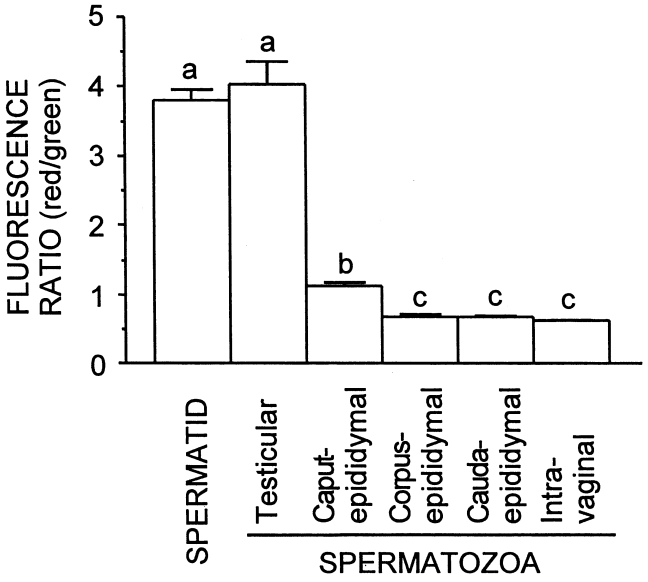

Figure 1 Changes in the acridine orange fluorescence of rat sperm nuclei during maturation. The red/green fluorescence ratio of the nucleus was measured by confocal fluorescence microscopy. Bars with different letters differ ($p < 0.05$).

As shown in Figs. 2 and 3, on the other hand, when spermatozoa were labelled with mBBr the fluorescence intensities gradually decreased ($p < 0.01$) during passage of spermatozoa from testis (4.74 ± 0.16) through the epididymis (2.72 ± 0.08 in the caput, 1.07 ± 0.03 in the corpus, –0.05 ± 0.05 in the cauda). The intensity of mBBr fluorescence in the heads of spermatozoa was very difficult to detect in the cauda epididymis and ejaculated spermatozoa (0.08 ± 0.03), and there was no difference between these two groups ($p = 0.27$).

As shown in Fig. 4, the mean red/green fluorescence ratio increased ($p < 0.01$) during zona penetration (0.52 ± 0.09 in spermatozoa binding to the zona pellucida, 0.64 ± 0.16 in spermatozoa in the perivitelline space) and sperm decondensation (0.69 ± 0.12 in decondensed spermatozoa). When spermatozoa in the perivitelline space after labelling with mBBr was examined under a fluorescence microscope with ×400 magnification, the fluorescence was detected (Fig. 5).

Discussion

In the present study we have demonstrated that the degree of DNA stability against an acid treatment can be evaluated by using confocal microscopy. The mean acridine orange red/green fluorescence ratio changed dramatically during passage of spermatozoa from testis to the corpus region of the epididymis, especially between the testis and caput epididymis. Kosower *et al.*

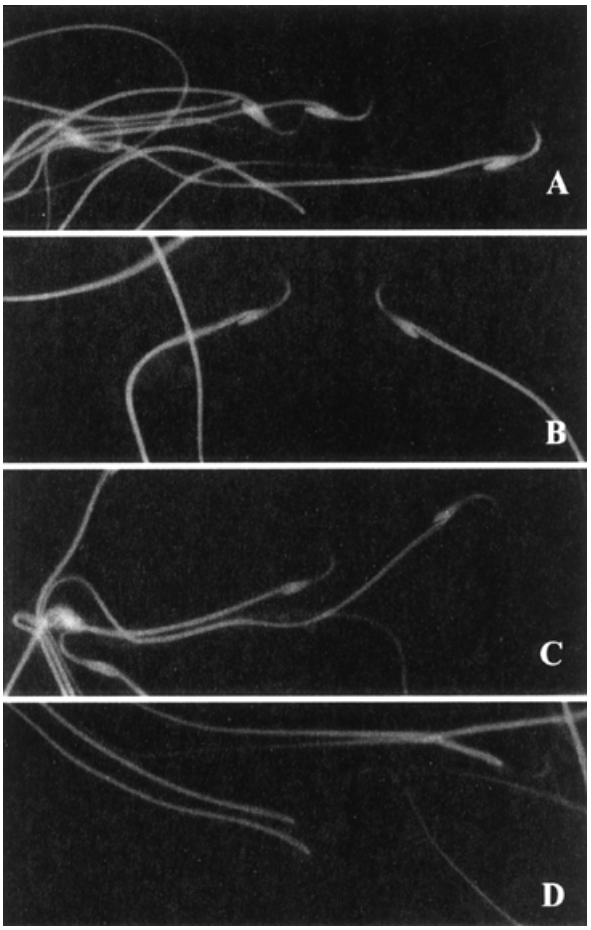

Figure 2 Monobromobimane (mBBr) fluorescence of rat sperm nuclei during epididymal maturation. (*A*) Testicular spermatozoa; (*B*) caput–epididymal spermatozoa; (*C*) corpus–epididymal spermatozoa; (*D*) cauda–epididymal spermatozoa.

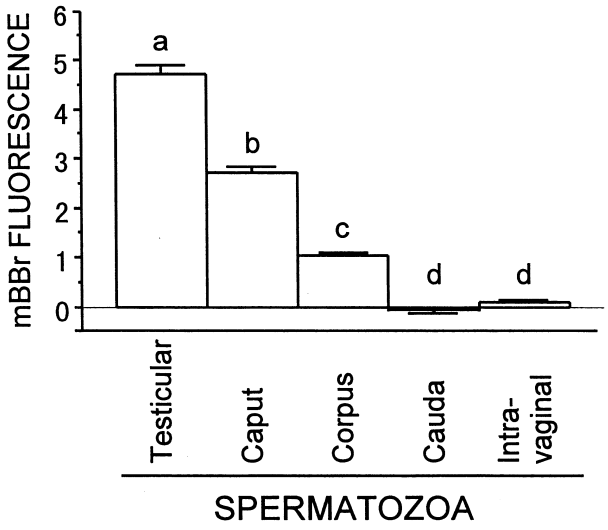

Figure 3 Changes in the monobromobimane (mBBr) fluorescence of rat sperm nuclei during epididymal maturation. The fluorescence intensity of sperm nucleus was measured using an epifluorescence microfluorometer. Bars with different letters differ ($p < 0.05$).

(1992) demonstrated using a fluorescence microscope that acridine orange fluorescence of acid-treated spermatozoa changed from red to green during passage from testis to caput epididymis in mouse and rabbit, but between the caput and corpus regions of epididymis in hamster. Our observation using confocal microscopy showed that the change in acridine orange fluorescence in rat spermatozoa was similar to the findings in mouse and rabbit. Acridine orange intercalates into double-stranded DNA as a monomer, whereas it binds to single-stranded DNA as an aggregate. Upon excitation at 470–490 nm, the monomeric acridine orange bound to double-stranded DNA fluoresces green with an emission maximum at 530 nm, and the aggregated acridine orange on single-stranded DNA fluoresces red with an emission maximum at about 640 nm (Ichimura *et al.*, 1971; Peacocke, 1973). Kosower *et al.* (1992) compared the colour of acridine orange fluorescence in sperm nuclei after acid treatment with the mBBr fluorescence reacted with sperm thiols, and concluded that acridine orange fluorescence after fixation with acetic alcohol is useful for assessing the status of disulphides in sperm protamines since the resistance of DNA against acid reflected the status of sperm disulphides. In the present study, although the fluorescence of sperm heads was very difficult to detect in cauda-epididymal and ejaculated spermatozoa, the fluorescence intensities from mBBr-labelled spermatozoa decreased gradually during passage from testis to cauda epididymis. Our observation using mBBr was basically consistent with a previous observation by Shalgi *et al.* (1989). In the rat, cysteine represents about 20% of the sperm protamine (Calvin, 1976; Betzalel *et al.*, 1986) and most of the cysteine groups in the sperm head are oxidised and form disulphides until reaching the cauda epididymis (Shalgi *et al.*, 1989). Therefore, our evidence demonstrates that colour assessment of acridine orange fluorescence after acid treatment does not reflect the thiol-disulphide status of rat spermatozoa in detail. In the rat, DNA stability against acid appears to be ahead of the oxidation of protamine.

Decondensation of mammalian spermatozoa has been demonstrated *in vitro* by exposure of spermatozoa to disulphide-reducing agents (see Zirkin *et al.*, 1989). Microinjected hamster spermatozoa were prevented from decondensing by treating oocytes with sulphhydryl blocking agent or glutathione oxidant (Perreault *et al.*, 1984). Glutathione and other thiols have been believed to play a role in sperm nuclear decondensation. In the mouse, the stability of sperm

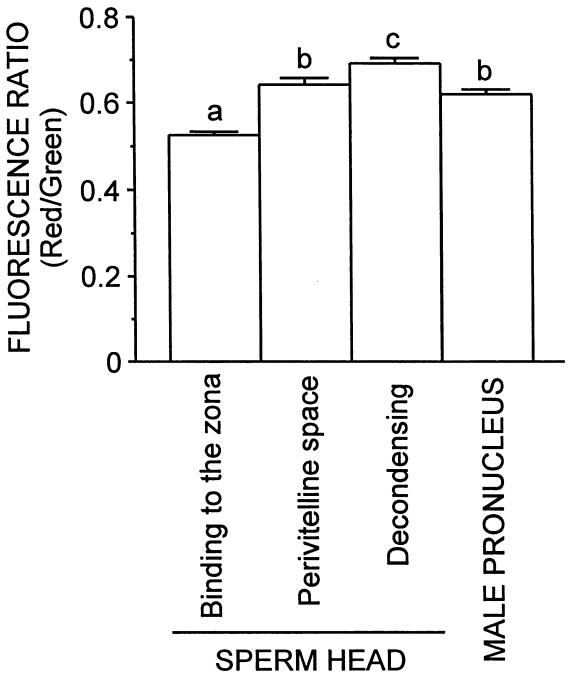

Figure 4 Changes in the acridine orange fluorescence of sperm nuclei during penetration of rat oocytes. The red/green fluorescence ratio of the nucleus was measured by confocal fluorescence microscopy. Bars with different letters differ ($p < 0.05$).

chromatin as assessed with toluidine blue staining changed only after penetration of the ooplasm (Krzanowska, 1982). Microinjected rat sperm nuclei, containing only protamine 1, appear to be more stable in Syrian hamster oocytes compared with mouse sperm nuclei containing protamines 1 (33%) and 2 (67%) (Perreault, 1990). In general, the higher proportion of protamine 2, the faster the nuclei decondensed (Perreault, 1990), because protamines 1 and 2 differ in the numbers and arrangements of disulphide bonds. In the present study, however, the red/green fluorescence ratio significantly increased in zona-penetrated spermatozoa compared with zona-binding ones, following treatment with acetic acid. This suggests that the stability of sperm DNA against acid decreases slightly in the perivitelline space. Furthermore, reduced thiols as determined by mBBr fluorescence were detected in zona-penetrated spermatozoa but not in zona-binding ones. This clearly indicates that the disulphide bonds of rat spermatozoa begin to be reduced during or just after zona penetration but before penetration of the ooplasm. Reduction of the sperm nuclei disulphide bonds may occur prior to fusion of the nuclei with ooplasm.

Although the red/green fluorescence ratio of acridine orange decreased dramatically from testicular spermatozoa to corpus epididymis spermatozoa during epididymal maturation, the ratio did not increase to the same level during fertilisation. These observations suggest that the stability of sperm DNA against acid is maintained at relatively high levels during and after penetration of the oocyte. Recently, Sakkas *et al.* (1995) also proposed that decondensation of mouse sperm chromatin did not appear to proceed as the reverse of the packaging procedure.

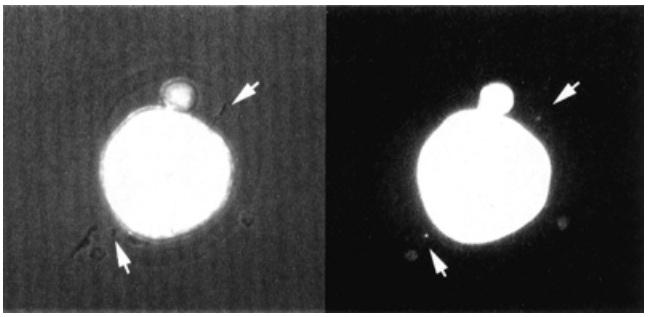

Figure 5 Monobromobimane (mBBr) fluorescence of rat sperm nuclei in the perivitelline space (arrows).

In conclusion, our observation demonstrates that DNA stability against acid appears to be ahead of the oxidation of protamine during sperm maturation in the epididymis and that an initial event of the unpackaging process occurs during or just after zona penetration but before penetration of the ooplasm.

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References

Betzalel, M., Shalgi, R. & Moav, B. (1986). Protamine in the rat, its fate *in vivo*, and its degradation *in vitro* by egg homogenate. *Gamate Res.* **14,** 293–306.

Calvin, H.I. (1976). Comparative analysis of the nuclear basic proteins in rat, human, guinea pig, mouse and rabbit spermatozoa. *Biochim. Biophys. Acta* **434,** 377–89.

Ichimura, S., Zama, M. & Fujita, H. (1971). Quantitative determination of single-stranded section in DNA using the fluorescent probe acridine orange. *Biochim. Biophys. Acta* **240,** 485–95.

Kosower, N.S., Katayose, H., & Yanagimachi, R. (1992). Thiol-disulfide status and acridine orange fluorescence of mammalian sperm nuclei. *J. Androl.* **13,** 342–48.

Krzanowska, H. (1982). Toluidine blue staining reveals change in chromatin stabilization of mouse spermatozoa during epididymal maturation and penetration of ova. *J. Reprod. Fertil.* **64,** 97–101.

Meister, A. (1995). Glutathione metabolism. In *Biothiols*, part A, *Monothiols and Dithiols, Protein Thiols, and Thiyl Radicals*, ed. L. Packer, pp. 3–7. San Diego: Academic Press.

Ogura, A. & Yanagimachi, R. (1993). Round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy. *Biol. Reprod.* **48,** 219–25.

Peacocke, A.R. (1973). The interaction of acridine with nucleic acids. In *Acridines*, ed. R.M. Acheson, pp. 723–54. New York: Interscience.

Perreault, S.D. (1990). Regulation of sperm nuclear reactivation during fertilization. In *Fertilization in Mammals*, ed. B.D. Bavister, J. Cummins & E.R.S. Roldan, pp. 285–96. Norwell, Massachusetts: Serono Symposia.

Perreault, S.D., Wolf, R.A. & Zirkin, B.R. (1984). The role of disulfide bond reduction during mammalian sperm nuclear decondensation *in vivo. Dev. Biol.* **101,** 160–7.

Perreault, S.D., Barbee, R.R. & Slott, V. (1988). Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing ability in maturing hamster oocytes. *Dev. Biol.* **125,** 181–6.

Sakkas, D., Manicardi, G., Bianchi, P.G., Bizzaro, D., Bianchi, U. (1995). Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilising mouse spermatozoa. *Biol. Reprod.* **52,** 1149–55.

Shalgi, R., Seligman, J. & Kosower, N.S. (1989). Dynamics of the thiol status of rat spermatozoa during maturation: analysis with the fluorescent labeling agent monobromobimane. *Biol. Reprod.* **40,** 1037–45.

Zirkin, B.R., Perreault, S.D. & Naish, S.J. (1989). Formation and function of the male pronucleus during mammalian fertilization. In *The Molecular Biology of Fertilization*, ed. H. Schatten & G. Schatten, pp. 91–114. San Diego: Academic Press.