Fertilizing capacity and ultrastructure of fowl and turkey spermatozoa before and after freezing

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Summary. The fertilizing capacity, motility and ultrastructure of fowl and turkey spermatozoa were examined at various stages of the freezing process. For both species, fertility and motility were depressed after equilibration with dimethylsulphoxide at 5°C. After freezing, motility was maintained at 55% for fowl spermatozoa and 40% for turkey spermatozoa; however, fertility was 55% for the fowl and 0% for the turkey. Qualitatively, the damage to the spermatozoa of both species was nearly identical, as revealed by scanning and transmission electron microscopy. The plasmalemma was the primary site of damage. ‘Bent’ spermatozoa, coiled tails and swollen mitochondria were also present. Damage to the acrosome was only observed in spermatozoa which had been frozen to –180° or –196°C. These changes were attributed to adverse osmotic conditions. Binding of cationic ferritin to the plasmalemma of spermatozoa from both species remained unaltered.

Introduction

Harris, Thurston & Cundall (1973) have shown that fowl spermatozoa are damaged ultrastructurally not only by freezing and thawing but also by the cryoprotectants used, e.g. glycerol and dimethylsulphoxide. Turkey spermatozoa are similarly affected by the presence of glycerol (Marquez & Ogasawara, 1977). The type of cryoprotective agent and the equilibration time needed are known to influence the fertilizing capacity of fowl spermatozoa before freezing (Sexton & Fewlass, 1976).

Limited fertility has been achieved with frozen–thawed fowl spermatozoa (see Lake, 1970; Sexton, 1976a) but similar treatments have been even less successful with turkey spermatozoa (Marquez & Ogasawara, 1973; Oderkirk & Buckland, 1976). Although much information has been published about the damage to fowl and turkey spermatozoa by freezing (Polge, 1951; Harris *et al.*, 1973; Westfall & Harris, 1975; Sexton, 1976a; Oderkirk & Buckland, 1976; Marquez & Ogasawara, 1977), the two species have not been studied simultaneously and it is difficult to assess whether the differences in response are specific or related to the experimental conditions. The present study is therefore of the changes in the fertilizing capacity, motility and ultrastructural characteristics of fowl and turkey spermatozoa before, during and after freezing and storage at –196°C when tested simultaneously under similar experimental conditions.

Materials and Methods

Treatment of spermatozoa

Semen samples were collected concurrently from 15 White Leghorn (*Gallus domesticus*) fowl cocks and 15 Large White (*Meleagris gallopavo*) turkey toms, of known fertility, by abdominal massage (Burrows & Quinn, 1937). The semen samples were immediately pooled

according to species, and Beltsville Poultry Semen Extender (BPSE) (Sexton, 1977) was added at room temperature at a rate of 1 part semen to 5 parts BPSE. The extended semen was then evaluated for sperm concentration and motility by conventional methods (Sexton, 1976b) before being placed at 5°C for 2 h. Dimethylsulphoxide (DMSO) was then added to give a concentration of 4% (v/v). After a further 2 h equilibration period at 5°C, the semen was packaged in glass ampoules (0.7 ml capacity) and cooled at 1.1°C/min to -20°C. The ampoules were then placed horizontally on a rack 7.5 cm above liquid nitrogen vapour for 1 min, which resulted in a semen temperature drop to -180°C, before being immersed in liquid nitrogen (-196°C). The ampoules were thawed by being placed directly in an ice bath.

Semen samples were removed for analyses after: (1) addition of BPSE, (2) cooling to 5°C, (3) equilibration at 5°C with 4% DMSO, (4) freezing to -20°C, (5) freezing to -180°C, (6) storage for 6 weeks at -196°C. Progressive sperm motility was evaluated microscopically (without knowledge of the treatment) at a magnification of $\times 430$ by examining several areas of a slide and estimating the percentage of spermatozoa moving in a forward motion. The fertilizing ability of the spermatozoa was tested by inseminating 10 fowl and 10 turkey hens for 5 weeks with about 1×10^8 spermatozoa from each treatment. Eggs were collected daily, held in a cool room, and incubated in weekly batches. On Day 7 of incubation the eggs were candled and fertility was expressed as the average percentage of viable embryos for the entire 5-week test period. The data were transformed to $\arcsin \sqrt{\%}$ and significance was determined at the 5% level of probability by analysis of variance.

Transmission and scanning electron microscopy

The spermatozoa were fixed at 5°C for 35 min by adding 3 volumes 2% glutaraldehyde in 0.15 M-cacodylate buffer or 1.15 M-sodium phosphate buffer, pH 7.3. Some glutaraldehyde-fixed spermatozoa were labelled with cationic ferritin as described by Bakst & Howarth (1977). This substance is electron-dense and binds to anionic sites on membranes at neutral pH (Dannon, Goldstein, Marikovsky & Skutelsky, 1972). For transmission electron microscopy fixed spermatozoa were pelleted by centrifugation and washed once with the respective buffer, to which 5% sucrose had been added, and left overnight at 5°C. The spermatozoa were post-fixed in 2% osmium tetroxide in 0.1 M sym-collidine buffer for 2 h at 5°C, dehydrated in ethanol and embedded in Spurr Low-Viscosity Embedding Medium (Polyscience, Inc., Warrington, Philadelphia). Thin sections were stained with saturated aqueous uranyl acetate for 15 min and then with lead citrate for 5 min before examination in a Philips 200 electron microscope. For scanning electron microscopy, a drop of glutaraldehyde-fixed spermatozoa was allowed to settle on a polylysine-coated coverslip for at least 2 h at 5°C (Mazia, Sale & Schatten, 1975). The coverslip with adhering spermatozoa was passed through an ascending series of ethanols, critical-point dried, mounted on an aluminium stub and coated with gold/palladium. A Hitachi HHS-2R scanning electron microscope was used.

The percentage of damaged spermatozoa within a treatment was based on a minimum count of 100 spermatozoa for each replicate. These counts were made directly from the microscope viewing screens or from photographic prints of randomly selected areas of the prepared treatment samples.

Results

Fertility and motility

The data are summarized in Table 1. Fertility and motility of fowl spermatozoa were not affected by dilution or cooling to 5°C, both decreased after equilibration with DMSO, and motility but not fertility decreased further when the semen was cooled to -20°C. Storage for 6

Table 1. Effect of various stages in the freezing process on the fertility and motility of fowl and turkey spermatozoa (mean $\pm$ s.e.m. of 5 replicates)

Stage of freezing process	% Fertility		% Motility	
	Fowl	Turkey	Fowl	Turkey
Diluted in BPSE	90 $\pm$ 3 ^a	90 $\pm$ 3 ^a	95 $\pm$ 2 ^a	95 $\pm$ 1 ^a
Cooled to 5°C	90 $\pm$ 2 ^a	80 $\pm$ 8 ^a	95 $\pm$ 4 ^a	95 $\pm$ 4 ^a
Equilibrated with DMSO at 5°C	75 $\pm$ 5 ^b	45 $\pm$ 10 ^{b*}	70 $\pm$ 9 ^b	60 $\pm$ 7 ^b
Frozen to -20°C	75 $\pm$ 4 ^b	40 $\pm$ 10 ^{b*}	55 $\pm$ 7 ^c	40 $\pm$ 9 ^c
Frozen to -180°C	55 $\pm$ 5 ^c	0 $\pm$ 0 ^{c*}	55 $\pm$ 7 ^c	40 $\pm$ 8 ^c
Stored at -196°C for 6 weeks	55 $\pm$ 6 ^c	0 $\pm$ 0 ^{c*}	55 $\pm$ 5 ^c	40 $\pm$ 9 ^c

Within columns, values with different superscripts are significantly different $P < 0.05$.

* Significantly ($P < 0.05$) lower than the equivalent value for fowl spermatozoa.

weeks led to no further loss. Turkey spermatozoa, which were more affected, completely lost fertility at -180 and -196°C.

Ultrastructure

The damage to the spermatozoa of both species was qualitatively identical and the following comments, therefore, refer to the plate-figures regardless of the species or treatment illustrated. The ultrastructure of freshly ejaculated fowl and turkey spermatozoa was not evaluated, but the changes seen were referenced to the descriptions of Lake, Smith & Young (1968), Bakst & Howarth (1975) and Marquez & Ogasawara (1975).

Labelling with cationic ferritin. Fowl and turkey spermatozoa showed identical binding patterns within all treatments, indicating that the anionic sites were not modified. Distribution of the cationic ferritin was uniform over the plasmalemma regardless of the internal structure of the spermatozoon (Pl. 1, Fig. 5). In spermatozoa with a discontinuous plasmalemma, the cationic ferritin was bound to the outer mitochondrial membrane and to the periphery of the material comprising the amorphous sheath of the principal piece.

Diluted with BPSE. About 40% of the fowl spermatozoa examined were enveloped by a distended plasmalemma, ranging from a balloon-like appearance to a slight lifting of the plasmalemma overlying the midpiece and nucleus (Pl. 1, Fig. 3). About 20% of the spermatozoa were 'bent' (Pl. 1, Figs 1 and 3; Pl. 2, Figs 6 and 9) and 5% had swollen mitochondria (Pl. 1, Figs 2-4). 'Bent' spermatozoa were those in which the head was reflexed at the neck, midpiece or distal aspect of the nucleus. The plasmalemma of such spermatozoa was distended around the realigned segments (Pl. 1, Fig. 3) or, less frequently, closely apposed to the segments (Pl. 1, Fig. 1).

Nearly 50% of the turkey spermatozoa showed anomalies similar to those of fowl spermatozoa. In addition, about 10% had coiled tails (Pl. 1, Fig. 4; Pl. 2, Fig. 6). 'Bent' spermatozoa and swollen mitochondria were apparent in 20% of the samples which had a distended plasmalemma.

Cooling to 5°C. About 40-50% of the fowl spermatozoa exhibited plasmalemma irregularities which ranged from low ruffling (Pl. 1, Figs 1 and 2) to considerable distension along the length of the spermatozoon. This condition was particularly evident at the nuclear and midpiece regions (Pl. 1, Fig. 3). Swollen mitochondria, 'bent' spermatozoa and coiled tails were observed within a distended plasmalemma in approximately 20% of the spermatozoa. The amorphous sheath, which is present around the axonemal complex of the principal-piece of normal fowl spermatozoa (Lake *et al.*, 1968), was missing in spermatozoa with coiled tails (Pl. 1, Fig. 4). Occasionally, the microtubular doublets comprising the axoneme were in disarray around the periphery of the distended plasmalemma. Organelle displacement, a condition

whereby severed sperm segments are arranged within the distended plasmalemma, was observed in less than 10% of the spermatozoa (Pl. 1, Fig. 4).

About 60–70% of the turkey spermatozoa exhibited plasmalemma irregularities and, as with the plasmalemma of the fowl, distension appeared to be accompanied by further damage. The acute bending of the turkey sperm head in 'bent' spermatozoa appeared at the neck region, along the distal aspect of the nucleus and the full length of the midpiece (Pl. 2, Fig. 6). Swollen mitochondria were evident in nearly all spermatozoa with a distended plasmalemma (Pl. 1, Fig. 3) and 'bent'. In spermatozoa of both species the plasmalemma remained tightly apposed to the acrosome.

Equilibration with DMSO. Although 40–50% of fowl spermatozoa appeared abnormal by scanning electron microscopy, 60–70% of those viewed by transmission electron microscopy had a distended plasmalemma. Of the cells with distended plasmalemma, 25% were 'bent' and 75% had swollen mitochondria. The integrity of the acrosome was not altered.

EXPLANATION OF PLATES

PLATE 1

Structural changes in fowl and turkey spermatozoa examined by transmission electron microscopy at various stages during the freezing process.

Fig. 1. A 'bent' turkey spermatozoon with a ruffled but continuous plasmalemma is observed after equilibration at 5°C in BPSE. $\times 11\,800$.

Fig. 2. This turkey spermatozoon, frozen at -20°C , has within its distended plasmalemma several free, swollen mitochondria (M). The anomalous ultrastructure of the acrosome (arrow) is evident. $\times 16\,500$.

Fig. 3. Equilibration of fowl spermatozoa with DMSO at 5°C results in distended plasmalemma (clear arrows) which are occupied by swollen and displaced mitochondria. Transverse sections of 'bent' spermatozoa illustrate the electron-dense nucleus reflexed upon the tail (curved arrows). $\times 8850$.

Fig. 4. Organelle displacement, nuclear decondensation, swollen mitochondria and a coiled tail that lacks the amorphous sheath are features of this turkey spermatozoon frozen at -180°C over liquid nitrogen vapour. The perforatorium of another spermatozoon (arrow) is evident in the absence of the acrosome. $\times 14\,850$.

Fig. 5. After the same treatment as in Fig. 4, cationic ferritin is uniformly distributed on the plasmalemma of this turkey spermatozoon, even though the underlying acrosome has been lost. The perforatorium (P) and remnants of the acrosomal membrane (arrow) are evident. $\times 39\,100$.

PLATE 2

Structural changes in fowl and turkey spermatozoa examined by scanning electron microscopy at various stages of the freezing process.

Fig. 6. A 'bent' turkey spermatozoon frozen at -20°C illustrates acute tail flexion distal to the midpiece. Displaced mitochondria (M) are located at the bend. The coiled tail (T) is apposed to the lightly swollen nucleus (N). $\times 6000$

Fig. 7. The swollen acrosomes of two fowl spermatozoa stored for 6 weeks at -196°C . $\times 13\,650$.

Fig. 8. The absence of the plasmalemma in this turkey spermatozoon after storage for 6 weeks at -196°C is suggested by the defined appearance of the acrosome (A), nucleus (N) and mitochondria (M). $\times 8925$.

Fig. 9. This 'bent' turkey spermatozoon, which was stored for 6 weeks at -196°C , has a swollen acrosome (A). The plasmalemma overlying the midpiece (MP) appears discontinuous. $\times 8075$.

Fig. 10. An ultrastructurally normal fowl spermatozoon is observed after storage for 6 weeks at -196°C . $\times 6400$.

PLATE I

(Facing p. 4)

PLATE 2

About 80% of the turkey spermatozoa exhibited plasmalemma distension or partial loss. The mitochondrial sheath occasionally showed a cobblestone appearance (Pl. 2, Fig. 8). 'Bending' and organelle displacement were evident in 20% of the spermatozoa with distended plasmalemma and mitochondria and microtubular doublets were randomly distributed within the distended plasmalemma.

Freezing at -20°C . Distension of the plasmalemma was apparent in 90% of the fowl spermatozoa and nearly all the mitochondria were swollen; several were free in the intact, swollen plasmalemma or between the spermatozoa. Organelle displacement was observed in 15% of the spermatozoa and 'bending' in about 30%. Acrosomal loss (Pl. 1, Figs 4 and 5) or disfiguration (Pl. 1, Fig. 2; Pl. 2, Figs 7 and 9) were observed in less than 10% of the spermatozoa.

About 90–95% of the turkey spermatozoa were damaged in the same way as were the fowl spermatozoa but the loss of mitochondria (Pl. 2, Fig. 6) was greater. In some spermatozoa nuclear decondensation was manifested as a slight swelling in the nuclear region (Pl. 2, Fig. 6); with other spermatozoa individual chromatin granules were widely separated, thus forming severely swollen nuclei (Pl. 1, Fig. 4). Acrosomal damage was similar to that observed for fowl spermatozoa.

Freezing at -180 and -196°C . There were no differences in the effects at these two temperatures. About 90–95% of fowl spermatozoa showed plasmalemma distension and of these 20–30% were bent or coiled. Organelle displacement (Pl. 1, Fig. 4) and plasmalemma breakage were apparent in 70–80% of the spermatozoa. Individual swollen mitochondria were observed between spermatozoa and there was nuclear decondensation in 20% of the spermatozoa. The acrosome appeared bulbous (Pl. 2, Figs 7 and 9) or was completely absent in 10% of the spermatozoa. Some structurally normal spermatozoa were observed only by scanning electron microscopy (Pl. 2, Fig. 10).

The midpieces and nuclei of turkey spermatozoa were drastically affected. Nuclear decondensation was evident and few midpieces were fully enveloped by a plasmalemma (Pl. 2, Fig. 8). Organelle displacement and acrosome anomalies were observed in about 80% and 15% respectively, of the spermatozoa examined.

Discussion

The present results indicate that the method followed for the cryopreservation of fowl spermatozoa (Sexton, 1976a) is not adequate for turkey spermatozoa. The sequential decrease in fertility and motility appears to be correlated with structural damage of the spermatozoa of both species. Qualitatively, the damage conferred to these gametes is identical; however, the degree of damage caused appears to be greater with turkey spermatozoa. Although nearly identical ultrastructurally fowl and turkey spermatozoa are metabolically different (Lake, 1971; Sexton, 1974, 1975) and such differences may be related to the increased sensitivity of turkey spermatozoa to the DMSO and cryogenic treatments used in this study.

Distension of the plasmalemma is a prelude to further internal damage such as coiling of the axoneme, bending, mitochondrial swelling and organelle displacement, all of which are probably osmotically induced, as concluded by Bakst & Howarth (1977) and Westfall & Howarth (1977) who studied the effects of addition of glycerol and its removal by dialysis from fowl spermatozoa. Marquez & Ogasawara (1977) also attributed the plasmalemma and midpiece irregularities of turkey spermatozoa treated with glycerol and then frozen and thawed to osmotic effects. The results of the present study indicate that DMSO may act in a similar manner.

In every treatment 10–30% of the fowl and turkey spermatozoa showed coiled tails. A similar phenomenon, which is a direct result of osmotic swelling, occurs when bull spermatozoa are placed in medium which is progressively made hypotonic (Drevius, 1972, 1973). Possibly the coiling of the tails observed by Drevius (1972, 1973) for bull and by us for fowl and turkey

spermatozoa is due to abolition of plasmalemma-associated restrictions upon the axoneme resulting from plasmalemma distension.

The occurrence of 'bent' spermatozoa is also known to be associated with adverse osmotic conditions (Yamane, 1972; Van Wambeke, 1977). Yamane (1972) suggested that bending occurs because of rupture of the plasmalemma, but our observations indicate that bending can occur without rupture. The swelling and displacement of the mitochondria are also the result of a hypotonic environment. Yamane (1972) suggests that damage to the midpiece precedes the bending of 'bent' spermatozoa, but our results show that mitochondrial disruption does not always occur before bending. The occurrence of 'bent' spermatozoa and acrosomal anomalies such as swelling and partial or complete detachment from the apical tip of the nucleus have been reported for fowl spermatozoa cold shocked for at least 15–30 min. Their incidence increases with prolonged cooling time (Tsukunaga, 1971).

The most drastic form of sperm disruption, organelle displacement, is a redistribution of cell organelles along the full length of the spermatozoon. This condition was observed in about 60% of the frozen-thawed spermatozoa of both species and is irreversible. However, none of the treatments altered binding of cationic ferritin to the plasmalemma. This is an indication that the macromolecules (glycoproteins) associated with the exposed surface of the plasmalemma, which are suggested as being involved in gamete recognition and attachment (Fawcett, 1975; Johnson, 1975; Baccetti & Afzelius, 1976), were unaffected by the cryogenic treatments.

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