THE ABSORPTION OF BOAR SEMINAL ZINC BY COOLED SPERMATOZOA

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Westmoreland, First & Hoekstra (1967) demonstrated absorption of seminal zinc by boar spermatozoa on cooling. Partial association of this zinc with the seminal proteins has been studied by Bournsneill, Briggs, Lavon & Butler (1973a, b). More recently, Bournsneill & Roberts (1974), Roberts, Bournsneill & Brown (1974) and Roberts, Bournsneill, Winsor & Mustill (1974) reported a seminal zinc-protein which was absorbed in part by spermatozoa from cooled semen.

This paper begins a study of the conditions which control the zinc and zinc-protein absorbancies. Small-scale methods were devised which speeded the analyses and permitted triplicate determinations in all cases. Routine weighings, adopted to ensure quantitative retrieval of the centrifuged spermatozoa, revealed unsuspected weight changes in the sperm pellet centrifuged from cold semen. The cause of this problem was sought.

The method of centrifuging (5 min, room temperature, 3000 *g*) spermatozoa from 0.6-ml samples of gel-free fresh whole semen from fertile boars employed a Beckman 152 microfuge (Beckman Instruments, Glenrothes, Fife) and capped polypropylene tubes (30/8, W. Sarstedt (UK), Leicester). The caps of these tubes were trimmed with stainless steel scissors so that the flanges of the body of the closed centrifuge tube slid into the centrifuge rotor slots.

Seminal plasma was removed by Pasteur pipette from the centrifuged sperm pellet in the pre-weighed centrifuge tubes; residual plasma was drained from the inverted centrifuge tube by cut pieces of filter paper (Whatman No. 1) which almost touched the damp pellet. The pellets were then dried *in situ* for 24 hr over fresh CaCl_2 *in vacuo*. Weights of the damp and dry pellets were determined by difference.

The dry pellets were quantitatively transferred in water by a Pasteur pipette to 5-ml vitreosil crucibles (A3/530/79, Jencons, Hemel Hempstead). The crucible contents were slowly dried on a hot-plate and ashed in a muffle furnace at 700°C. The ash was treated with 1 ml 0.5 M-HCl and the contents again dried. Acetic acid (3.0 ml M) was added to dissolve the ash; the zinc content of this solution was determined by atomic absorption (Bournsneill, Baronos, Briggs & Butler, 1972).

The results of a preliminary study justify the constancy of the values for the damp and dry weights of centrifuged sperm pellets obtained from ten aliquots of semen. The damp pellets were weighed immediately (25.93 ± 1.14 mg, Mean $\pm$ S.D.) and after 6 hr (10.27 ± 1.03 mg) and 23 hr (4.45 ± 0.35 mg)

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drying *in vacuo* over CaCl_2 . These were reweighed after 72 hr over fresh P_2O_5 *in vacuo* (4.58 ± 0.37 mg). The 23-hr drying over CaCl_2 *in vacuo* gave an over-all dry:damp pellet weight ratio of 0.172 ± 0.005 and was adopted in this work.

A comparison (Text-fig. 1) of sperm weight changes and absorption of seminal plasma zinc was made with aliquots of semen which were kept at 4°C for 20 min followed by warming to room temperature for various times (5 to 120 min); control aliquots were either kept at room temperature or not rewarmed after the treatments at 4°C .

TEXT-FIG. 1. Weights of damp (○) and CaCl_2 -dried (□) spermatozoa centrifuged from aliquots of boar semen after periods at room temperature subsequent to 20 min exposure to 4°C . Δ, Zinc in corresponding centrifuged spermatozoa. The points on the vertical scale are those of samples kept for 20 min at room temperature only. Solid symbols = means of re-warmed samples. Vertical bars indicate $\pm$ S.D.

Weights were determined (Text-fig. 2) of spermatozoa from aliquots of semen kept at 4°C for various times (5 to 180 min) with and without subsequent return to room temperature for 120 min. Control semen samples at room temperature were examined at times 0 and 120 min. A similar experiment with cooled semen showed a much greater increase in pellet weight up to 60 min (mean % increase at 60, 90 and 120 min, $96\% \pm 9$ S.D., of the uncooled aliquots). By contrast, spermatozoa from this sample washed with three changes of 0.6 ml sperm-Ringer phosphate (Mann, 1964) and cooled in this solution, showed only a $1.3\% \pm 0.2$ increase above the room temperature value.

In a study of the effect of repeated cold absorption of seminal plasma with homologous spermatozoa, eight aliquots of semen were dispensed into eight centrifuge tubes and kept at room temperature except as subsequently described.

After centrifugation of the first two tubes in the series, the seminal plasma from the first tube was transferred onto the spermatozoa in the second tube and mixed. This mixture was cooled to 4°C for 10 min and then centrifuged together with the next (third) tube in the series. The treated seminal plasma was transferred with mixing onto the centrifuged spermatozoa from the third sample and this mixture was cooled to 4°C for 10 min. These treatments were continued until the original seminal plasma had been absorbed seven times. Text-figure 3 gives zinc and weight determinations on the seven sperm samples

TEXT-FIG. 2. Mean weights (○) of damp and CaCl₂-dried sperm samples from aliquots of boar semen after cooling to 4°C for the times shown. ●, Mean weights of identically treated samples after a subsequent period of 2 hr at room temperature. Mean weights (■, damp; □, dry) of control samples after 0 or 2 hr at room temperature only. Vertical bars indicate $\pm$ S.D. Note the change of scale between the 'damp' and 'dry' values.

used for the cold absorptions and on the untreated spermatozoa from the first sample which served as a room temperature control. The experiment was performed in triplicate. Similar results were obtained with five other semen collections from four different boars. Titration tests (Roberts, Bournsnel, Winsor & Mustill, 1974) carried out against sheep and pig red cells on four other samples of absorbed seminal plasma showed no loss of haemagglutinin. In five experiments, determinations on the absorbed seminal plasma showed that 45% (± 16 S.D.) of the original zinc remained unabsorbed.

Zinc acetate in the presence of citrate was added to a concentration of 6 mM to eight samples of seminal plasma (adjusted to pH 8) fully cold-absorbed as described above. Nitrogen determinations (Bournsnel, Hartree & Briggs, 1970)

on the centrifuged zinc precipitate showed that 46% (± 15 S.D.) of all the zinc-precipitable protein remained.

The weight changes observed showed that damp pellets from cooled semen weighed about 13% more than the uncooled samples. The weight increase was abolished, but the zinc content of the spermatozoa was unaffected (Text-fig. 1) when cooled semen samples were rewarmed for 5 min or more before centrifuging; it did not occur in later sequential cold absorbtions with fresh spermatozoa, nor was it evident when spermatozoa were pre-washed with sperm-Ringer solution and then cooled in this fluid. Proportionate weight increases were not shown by the dried pellets.

TEXT-FIG. 3. The zinc contents (●) and corresponding damp (x) and dry (+) weights of spermatozoa samples used for successive cold absorptions (4°C, 10 min) of one sample of homologous boar seminal plasma. Vertical bars indicate $\pm$ S.D.

In some cases, the pellet weight lost on rewarming was greater than the entire weight of the non-dialysable proteins in the seminal plasma aliquot. A value for this (14.9 mg/0.6 ml) can be calculated from the mean total N (3.64 mg/ml) and the non-dialysable N ratio (0.887) given by Bournnell *et al.* (1972), combined with the approximate mean N content of the dried proteins (13%) derived from values given by Rottenberg & Bournnell (1966) and McIntosh & Bournnell (1966). More important still, the slight gain apparent in the dry weight of the corresponding pellet after cooling was far smaller than this value. The observed increase in the cold damp sperm pellet weight was probably caused by seminal plasma trapped in the cold-precipitated protein matrix which, with the spermatozoa, comprised the pellet. The progressive pellet weight increase shown when semen was cooled for up to 160 min is reminiscent of the slow zinc-induced opalescence increase with time shown by seminal plasma cooled for long periods (Roberts, Bournnell & Brown, 1974).

This work shows that some seminal plasma zinc is initially absorbed by spermatozoa at 4°C but that successive freshly applied spermatozoa absorbed

no more, although much zinc remains in the absorbed fluid. Zinc-precipitable protein is reduced in, but not eliminated from, seminal plasma even by absorptions beyond those which absorb zinc. Haemagglutinin is not absorbed at all under these conditions.

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