Electron microscopy

Sperm cells were pelleted, fixed (40 min, 0°C) in 3% paraformaldehyde, 0.1% glutaraldehyde in Sörensen buffer (SB, 0.1 M Na/K phosphate buffer, pH 7.3) and washed in SB (2 × 10 min). After centrifugation into 1% agarose, blocks were incubated in 0.02 M glycine in SB (20 min), washed in SB and dehydrated in ethanol. Ethanol was replaced in two steps by LR White resin and blocks were polymerized by UV light (20 h, 4°C). Approximately 80–nm sections were prepared and observed in a Philips CM 100 electron microscope.

SDS electrophoresis and Western blotting

Sperm samples were washed twice with TBS and sperm pellets were extracted by boiling in SDS buffer for 3 min. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 9% slab gels according to the method of Laemmli (1970), and Western blotting was performed as described by Towbin et al. (1979). Nonspecific sites were blocked with PBS-blocking solution. Visualization of bands was examined by the autoradiography method. Monoclonal antibodies Tu-30 and Tu-31 against γ-tubulin were used as supernatant. The molecular weights of separated proteins were estimated by comparison with Pharmacia low-molecular-weight protein standards run in parallel.

Antibodies A-2668 against actin, T9026 against α-tubulin and S-1515 against spectrin have been already examined by SDS-electrophoresis and Western blotting techniques in our previous research (Paleček et al., 1999).

Sperm motility and viability

Sperm motility was checked under a light microscope in the washed stock suspension and after the various treatments of spermatozoa in all samples. The percentage of viable sperm was evaluated in all samples: 20 μl of sperm suspension was fixed 1 min with 20 μl 5% glutaraldehyde in PBS and diluted with 250 μl PBS. Next, 5 μl of fixed sperm suspension was mixed with 5 μl 0.2% (v/v) Hoechst dye 33258 and immediately examined with a 40/1.25 objective. At least 100 spermatozoa were scored as either dead (stained) or live (unstained) (Kaláb et al., 1998).

Results

Examination of the acrosome reaction and viability of spermatozoa

The control of the acrosome reaction was made by immunolabeling analysis using specific intra-acrosomal monoclonal antibody (ACR.2) against acrosin (Pěknicová and Moos, 1990; Pěknicová et al., 1994; Paleček et al., 1999). For our experiments we used uncapacitated spermatozoa and spermatozoa after induction of the AR by calcium ionophore A23187. In control samples of fresh spermatozoa we found a weak spontaneous AR (14%), which means a high percentage of labeled acrosome by the antibody ACR.2. On the other hand, after induction of the AR, evaluation of the AR rose to 83% (Table 1). The same experiment was made with samples where specific inhibitors of cytoskeletal proteins such as cytochalasine B, vinblastine, colcemide and nocodazole were added. It has been demonstrated that combination of drugs such as vinblastine and colcemide provoked the AR (98%), while vinblastine and colcemide, added alone, were without effect on the AR (22% and 9%, respectively). The concentration of 100 μg of nocodazole and addition of cytochalasine B induced the acrosome reaction in high percentage (70% and 98%, respectively) (Table 1).

Table 1. Evaluation of the acrosome reaction (expressed as the percentage of spermatozoa without labeled acrosome in the imunmunofluorescence test (IF) using monoclonal antibody (ACR.2) against acrosin) and viability of spermatozoa (expressed as the percentage of spermatozoa labeled with Hoechst 33258) in IF

Treatment of spermatozoa	Acrosome reaction (%)	Viability of sperms (%)
control	14	89
ionophore	83	83
vinblastine	22	75
vinblastine + ionophore	78	72
colcemide	9	95
colcemide + ionophore	78	96
vinblastine + colcemide	98	94
vinblastine + colcemide + ionophore	100	82
nocodazole (100 μg)	70	70
nocodazole (wash)	28	68
nocodazole (wash) + ionophore	83	83
nocodazole (50 μg)	18	73
nocodazole (20 μg)	36	68
cytocholasine B	98	79
cytocholasine B + ionophore	100	73

Simultaneously, we also investigated the viability of tested spermatozoa after the treatment with different drugs. It was shown that the viability status of boar spermatozoa ranged from 68% to 96% (Table 1). Similar samples were prepared for testing the motility, which was generally around 80% in the examined samples of spermatozoa (data not shown).

Cytoskeletal protein inhibitors

For our experiments we used different kinds of specific inhibitors of cytoskeletal proteins, such as microtubule inhibitors and actin inhibitors.

We used microtubule inhibitor vinblastine, which causes blocking of tubulin polymerization and irreversible inhibition of formation of tubulin paracrystals, and colcemide, which also blocks tubulin polymerization and its influence leads to formation of irreversible complexes with the tubulin heterodimer (Dustin, 1984).

Simultaneous influence of vinblastine and colcemide on the cytoskeleton leads to stabilization of the complex of tubulin heterodimer and colcemide by vinblastine (Dustin, 1984). This stabilization is irreversible, very strong and leads to the AR (Fig. 1). Nocodazole is another microtubule inhibitor that causes reversible inhibition of tubulin monomer polymerization. For our experiments we used different concentrations (20, 50, 100 μg/ml). Only the high concentrations (50, 100 μg/ml) had positive effect on the AR. We also used cytochalasine B, actin inhibitor, which leads to the blocking of actin polymerization and to the collapse of actin structures (Table 2).

Actin

Fluorescent labeling was performed with two different samples, either with or without induction of the AR by calcium ionophore A23187. A bright fluorescence was seen in the outer acrosomal membrane, similarly as in the whole acrosomal and postacrosomal segment of control spermatozoa (Fig. 2A). A different situation was observed in samples with cytochalasine B and in both samples with added calcium ionophore. Detectable changes were observed in the outer acrosomal membrane; on the other hand, the post-acrosomal segment and the region of the acrosome did not differ significantly from the control (Fig. 2B, C, D). Nevertheless,

Table 2. Induction of the acrosome reaction by different drugs

Treatment of spermatozoa	Influence on the acrosome reaction
vinblastine	–
colcemide	–
vinblastine + colcemide	+
nocodazole	+*
cytochalasine B	+

*concentration 50, 100 μg/ml

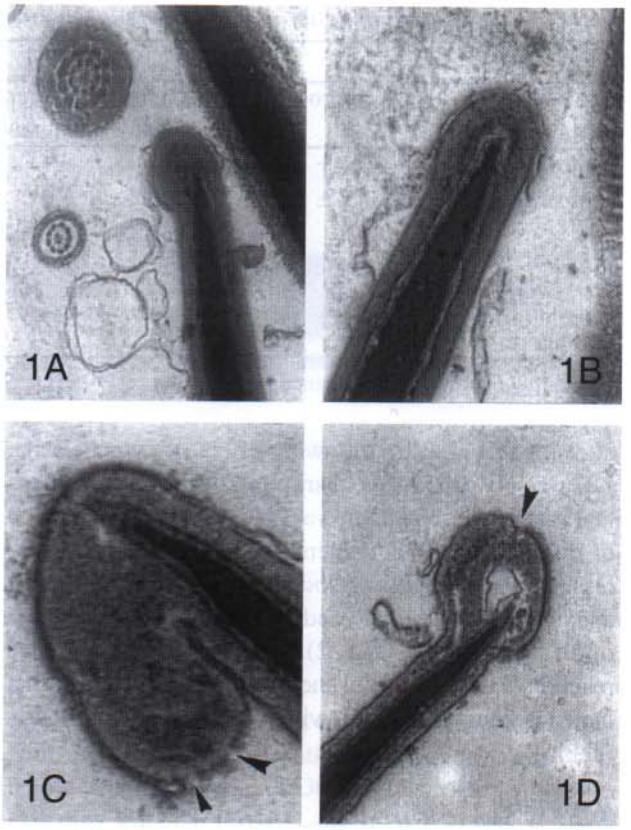

Fig. 1. Electron micrograph showing a detail of the acrosomal cap of a boar spermatozoon. A, B: control before the acrosome reaction, C, D: incubation with vinblastine, the arrows show perforation of the outer acrosomal membrane. Magnification 45000x. Arrows show penetration of the outer acrosomal membrane of the head of boar spermatozoa.

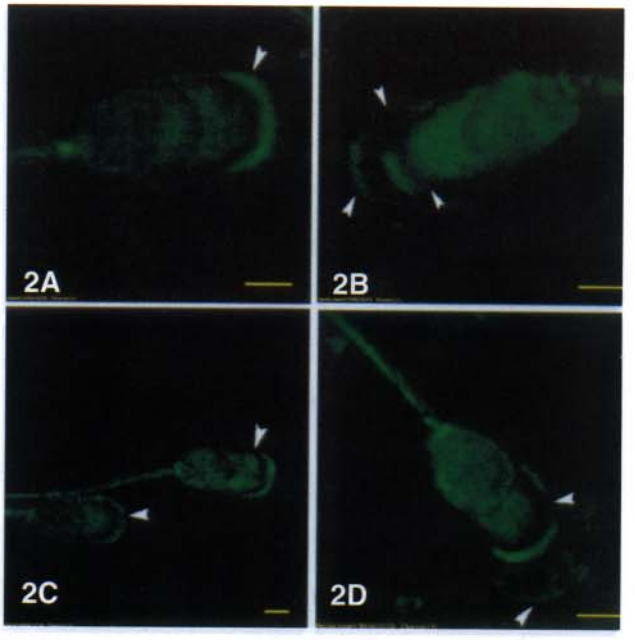

Fig. 2. Immunochemical localization of actin: A) control, B) calcium ionophore, C) cytochalasine B, D) cytochalasine B + calcium ionophore; confocal microscopy