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The effect of various capacitation active compounds and capacitation time on the *in vitro* fertility and protein tyrosine phosphorylation profiles of bovine sperm

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Summary

In this paper the effects of capacitation and fertilisation stimulating compounds (heparin, caffeine, glucose, D-penicillamine, bovine serum (BOS), bovine serum albumin (BSA), polyvinyl alcohol (PVA)) were analysed in several *in vitro* fertilisation protocols. Attention was paid to the rate of penetrated oocytes, kinetics of penetration and to polyspermic fertilisation. Cryopreserved bovine sperm and *in vitro* matured bovine oocytes were used throughout all the fertilisation experiments. As detected in the first 8 h fertilisation experiment with non-incubated sperm, the supplementation of medium with heparin, BOS and glucose supported the fertilisation rate most effectively (100%), including the kinetics of pronuclei formation (52.4%). The absence of BOS resulted in a decreased fertilisation rate (62.7%) as well as a delay in pronuclei formation (13.6%), similar to that after substitution of heparin with caffeine (73.0% and 25.4%, respectively). The penetration rate in the control medium with BOS (without heparin and caffeine) was surprisingly high, especially in medium without glucose (62.2%). The positive effect of glucose on sperm penetration was observed mainly in a chemically defined medium with PVA. High polyspermy rates were observed throughout all experiments in the media containing heparin or caffeine and BOS as the macromolecular component. D-Penicillamine was not shown to be a fertilisation-stimulating molecule. However, as detected in the second experiment in which oocytes were fertilised with 5 h incubated sperm, its positive effect on the prolongation of a fertile life span of cryopreserved spermatozoa was significant. The presence of either caffeine or heparin in the fertilisation medium (FM) with BOS during sperm incubation induced tyrosine phosphorylation of an approximately 90 kDa protein, detected after 5 h of sperm incubation. The absence of BOS reduced tyrosine phosphorylation of this protein in fertilisation medium with heparin. The percentage of motile spermatozoa and those with intact acrosomes were monitored throughout all experiments.

Key words: Bovine oocytes, Fertilisation, Sperm capacitation, Tyrosine phosphorylation

Introduction

Sperm capacitation in cattle, as well as in other mammalian species, is one of the important prerequisites of successful *in vitro* fertilisation. Although capacitation is at present a poorly understood process of sperm membrane alteration and enzymatic system activation, several compounds have been found to be capacitation stimulators – though with some differences in their effectiveness according to the mammalian species concerned (for review see Yanagimachi, 1994; Ward & Kopf, 1993; Brucker & Lipford 1995; Harrison, 1996; Visconti & Kopf, 1998). As documented in the early papers of Parrish *et al*. (1988), heparin was discovered to be a primary capacitating substance in bovine sperm. Apart from heparin there are several drugs with capacitation stimulating activity. Most of them are in some way involved with cAMP stimulation, either directly as cAMP analogues (db-cAMP,

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8-bromo-cAMP) (Galatino-Homer *et al*., 1997; Uguz *et al*., 1994) or indirectly as phosphodiesterase inhibitors such as caffeine or 3-isobutyl-1-methylxanthine (IBMX) (Niwa & Ohgoda, 1988; Galatino-Homer *et al*., 1997). Other compounds such as bicarbonate, calcium and BSA are also indispensable in supporting the capacitation stimulating effect of heparin or caffeine (for review see Visconti & Kopf, 1998; Visconti *et al*., 1999). Apart from the above-mentioned drugs, D-penicillamine has also been described as an effective capacitator of hamster (Andrews & Bavister, 1989) and bovine spermatozoa (Keskintepe & Brackett, 1996). In contrast, glucose was proved to have capacitation inhibiting activity in bull sperm (Parrish *et al*., 1989); however, there are also some controversial data concerning its role in capacitation of bull sperm (Tajik & Niwa, 1998).

Keskintepe & Brackett (1996) found that the capacitation effect of heparin and BSA can be substituted by D-penicillamine, caffeine and PVA. D-Penicillamine is known as a divalent cation chelator (Andrews & Bavister, 1989, Hottinger *et al*., 1997) and as a drug binding to cysteine of the cystine molecule, forming of drug–cysteine instead of cysteine–cysteine bonds (Wolf & Ruocco, 1997). However, its function in the process of sperm capacitation is not understood.

Several studies have described the detection of capacitation-related changes either in the morphological structure of the cytoplasmic membrane of the acrosomal region, mostly of its architecture (as reviewed by Cross, 1998), or in biochemical activities, mediated mainly by cAMP (reviewed by Harrison, 1996; Visconti & Kopf, 1998). Visconti *et al*. (1995*a*, *b*), Carrera *et al*. (1996), Leclerc *et al*. (1990, 1997) and Galantino-Homer *et al*. (1997) have demonstrated the tyrosine phosphorylation of several proteins during capacitation of mouse, human and bovine spermatozoa as a consequence of the elevated cAMP level. The only known downstream target for cAMP in sperm is protein kinase A (Visconti *et al*., 1997). When the intracellular concentration of cAMP increases, cAMP binds to a regulatory (R) subunit of protein kinase A causing the formation of the inactive (R-cAMP) complex and dissociation of the active catalytic (C) monomers. The latter can then phosphorylate substrate proteins, altering their functional properties (Visconti *et al*., 1997).

The objective of this study was to test not only the capacitation stimulating or inhibiting activity (as in the above-mentioned papers) but, more importantly, the fertile life span and fertilising activity of cryopreserved sperm, including the kinetics of pronuclei formation in medium with most of the known fertilisation stimulating compounds (BOS, BSA, PVA, glucose, heparin, caffeine or D-penicillamine) and combination thereof. The possible fertilisation stimulating or inhibiting effect of these substances was tested on *in vitro* matured oocytes. The analysis of sperm proteins phosphorylated on tyrosine was carried out after the same period of incubation and in the same combination of fertilisation activating compounds to see the effects on the fertilisation results. The acrosomal content of spermatozoa, indicating intact acrosomes, was tested by monoclonal antibody ACR.4 (Pěknicová & Moos, 1998) against a specific 28 kDa intra-acrosomal protein.

Materials and methods

Material

The following chemicals and their suppliers were used: crystalline bovine albumin (cat. no. 11920), silicone oil, sodium pyruvate and calcium lactate were from Serva (Heidelberg), tissue culture medium TCM 199 (10× concentrated stock), $NaHCO_3$ (7.5% (w/v) stock) and fluorescein isothiocyanate (FITC)-conjugated swine anti-mouse antibody were from Sevac (Prague), bovine serum (inactivated by heating for 30 min at 58 °C) was from Bioveta (Ivanovice na Hané), Suigonan PG-600 (FSH/LH gonadotropins 400/200 IU) was from Intervet, International B.V. (Boxmeer), Immobilon P was from Millipore (Bedford), horseradish peroxidase was from Jackson Labs. (Jackson), pY20 monoclonal antibody was from Transduction Labs. (Lexington), ECL chemiluminiscent kit was from Amersham (Little Chalfont). Polyvinyl alcohol (30–50 kDa), teleostean gelatine and all other reagents were from Sigma (St Louis). Deionised and Nanopure filtered water was used for all media. The plastic dishes for sperm capacitation, oocytes culture and fertilisation were from Nunclon (Roskilde).

Preparation of oocytes for culture and media composition

Ovaries were collected from slaughtered cows (Red-spotted Czech breed and their crosses with Holstein), transported in physiological saline at 20 °C to the laboratory, briefly washed in 70% (v/v) ethanol (for 20 s) and twice in physiological saline. Follicles of 2.5–8 mm diameter were dissected with fine scissors, placed in 90 mm Petri dishes with the basic culture medium (see below), punctured and pressed with a bent preparation needle to isolate the oocytes. For *in vitro* culture, only healthy-appearing cumulus–oocyte complexes (COCs) were selected (Pavlok *et al*., 1997), washed twice in the basic culture medium and transferred into 4-well Nunclon dishes with 0.5 ml medium under silicone oil.

For the manipulation of oocytes before their culture, the basic culture medium (BM) was composed of 9.4 ml TCM 199 (10× concentrated stock), 18.7 mM $NaHCO_3$ (2.1 ml 7.5% (w/v) stock), supplemented with 9.5 mM

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HEPES, 1.82 mM sodium pyruvate, 3 mg/ml PVA, 50 IU/ml penicillin K salt, 50 IU/ml streptomycin sulphate, 125 ng/ml amphotericin B and supplemented with H_2O to a final volume of 100 ml. For the *in vitro* maturation of oocytes the following medium was used: 8 ml TCM 199 (10× concentrated stock), 34.8 mM $NaHCO_3$ (3.9 ml 7.5% (w/v) stock), supplemented with 6.29 mM HEPES, 2.27 mM calcium lactate, 1.82 mM sodium pyruvate, 50 IU/ml penicillin K salt, 50 IU/ml streptomycin sulphate, 125 ng/ml amphotericin B and supplemented with H_2O to a final volume of 100 ml. Shortly before COC maturation the medium was supplemented with 10% (v/v) BOS and 10 IU/ml gonadotropins (PG 600). All cultures were prepared in a humidified atmosphere composed of 5% CO_2, 10% O_2 and 85% N_2 at 39 °C and incubated for 24 h.

Sperm preparation and fertilisation

Cryopreserved ejaculated bovine sperm of a bull of the Czech Red-spotted breed provided by Czech Breeders Cooperative Hradištko pod Medníkem, stored in sperm pellet form (used frequently for artificial insemination), were used throughout the fertilisation experiments. The sperm of another bull cryopreserved in straws, rather than pelleted sperm, was used in experiment 4 (see Results). Freshly ejaculated sperm, also provided by Czech Breeders Cooperative, and epididymal sperm from slaughtered bulls served as a control to cryopreserved sperm for analysis of proteins phosphorylated on tyrosine. For sperm preparation before capacitation or fertilisation the basic fertilisation medium (FM) (modified medium of Brackett & Oliphant, 1975) of the following composition was used: 115.7 mM NaCl, 5.4 mM KCl, 0.5 mM KH_2PO_4, 0.4 mM $MgSO_4$, 44.6 mM $NaHCO_3$, 2.3 mM calcium lactate, 1.82 mM sodium pyruvate, 6.29 mM HEPES, 50 IU/ml penicillin K salt, 50 IU/ml streptomycin sulphate, 125 ng/ml amphotericin B and supplemented with H_2O to a final volume of 100 ml. When this medium was modified by the addition of 13.9 mM glucose, the concentration of NaCl was reduced to 109.5 mM. The osmolality was adjusted by H_2O to 300 mosmol/l. The pH of medium equilibrated in the 5% CO_2 atmosphere was 7.7. The following 16 modifications of FM for fertilisation, or capacitation + fertilisation, were used in experiments 1 and 2, respectively:

A. FM + 10% (v/v) BOS and either (a) + 4 IU/ml heparin, (b) + 0.5 mg/ml D-penicillamine, (c) + 1 mg/ml caffeine or (d) + 0.5 mg/ml D-penicillamine with 1 mg/ml caffeine.
B. In all modifications listed in A, 13.9 mM glucose was added instead of the appropriate proportion of NaCl (to keep the final osmolality at 300 mosm as in the above-described media).
C. FM + 1 mg/ml PVA and either (a) + 4 IU/ml heparin, (b) + 0.5 mg/ml D-penicillamine, (c) + 1 mg/ml caffeine or (d) + 0.5 mg/ml D-penicillamine with 1 mg/ml caffeine.
D. Modifications identical to those in C, except that (as in B) isotonically adequate proportion of NaCl was substituted with 13.9 mM glucose.

All the modifications of FM are described schematically in Tables 1 and 3.

Four cryopreserved sperm pellets were thawed by embedding them in two tubes (two pellets in each) with 1.5 ml of FM with PVA and (according to medium composition) with or without glucose at 39 °C. After approximately 2 min the thawed semen was pooled into one 5 ml glass tube, centrifuged at 25 °C and 350 *g* for 8 min and washed twice in the same FM. After the second centrifugation, the sperm sediment was incubated in the same tube for 15 min, divided in two aliquots and then layered under 1 ml of FM with PVA (also either with or without glucose) in two 8 ml glass tubes and incubated for 15 min at 39 °C for the swim-up. The supernatants from both tubes with highly motile spermatozoa were pooled and centrifuged for 5 min at 25 °C and 300 *g*. The sperm sediment was diluted to a final concentration of 2×10^6/ml and placed into each well of 4-well Nunclon dishes with FM of appropriate modification.

In experiment 1, the sperm suspension at the above-mentioned concentration was used immediately for insemination of 24 h matured COCs – however, for an 8 h period only to test the kinetics of capacitation and subsequent fertilisation.

In experiment 2, the sperm samples treated by the same protocol (as for experiment 1) were first incubated also at a concentration of 2×10^6/ml for 5 h at 39 °C in 4-well Nunclon dishes but without COCs. After this time 24 h matured COC were added to each well containing incubated sperm for a 12 h period of fertilisation. This experiment was to test not only the kinetics of capacitation in relation to protein tyrosine phosphorylation, which should be a prerequisite of acrosome reaction and proper fertilisation, but also the fertile life span of differently treated and incubated spermatozoa.

In experiment 3 (Table 5), the samples of spermatozoa were incubated for 5 h in FM modified acccording to experiment 2 part D(a)–(d) and supplemented with 3 mg/ml BSA, 4 IU/ml heparin and with 24 h matured COC for 8 h fertilisation. This experiment was to test whether PVA-treated and preincubated spermatozoa with minimal fertilising capacity, as proved in experiment 2 (Tables 3, 4) still have, after a 5 h incubation, the capacity for improved fertilisation in better capacitation and fertilisation conditions, i.e. after treatment with BSA and heparin.

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Experiment 4 served as the control to show whether, in our hands, the 45 min swim-up of unwashed spermatozoa (as described by Keskintepe & Brackett, 1996) could stimulate the capacitation and fertilisation capacity of spermatozoa supplemented only with PVA, caffeine and D-penicillamine. One half of the oocytes ('a') were fertilised according to a slightly modified protocol of Keskintepe & Brackett (1996). Briefly, one straw containing frozen semen of one fertile bull (2×10^7 sperm in a 0.25 ml straw) was thawed in a 37 °C water bath for 30 s. The thawed semen was divided into two aliquots then layered under 1.5 ml of FM with 13.9 mM glucose supplemented with 0.1 mg/ml PVA, 1 mg/ml caffeine and 0.5 mg/ml D-penicillamine in two tissue culture tubes (14 × 90 mm) and maintained at a 45° angle for 45 min at 39 °C. The supernatant medium was centrifuged for 5 min at 300 *g* and part of the centrifuged sperm pellet was added to the 0.5 ml of FM (of identical composition as for swim-up) to a final sperm concentration of 2×10^6/ml. For fertilisation the matured COC were added. The second half of the COC control ('b') was fertilised according to above-described protocol as in experiment 1 (part B(a) in FM with glucose, BOS and heparin).

In all experiments, the capacitation or fertilisation temperature was 39 °C and the humidified atmosphere was composed of 5% CO_2, 10% O_2 and 85% N_2.

For fertilisation, 15–30 COCs were carefully washed four times in FM and placed in single wells with appropriate modification of FM with non-incubated (experiments 1 and 4) or 5 h incubated sperm (experiments 2 and 3). Following the appropriate time of fertilisation, the oocytes were fixed and stained to evaluate sperm penetration and pronuclei formation.

Fixation, staining of oocytes and their morphological analysis

At the end of culture, the residues of corona radiata surrounding the oocytes were removed by mechanically passing the oocytes through a manipulation pipette. The oocytes were mounted on microscope slides with Vaseline strips, covered with a cover slip and fixed in ethanol–acetic acid 3:1 for 24 h. Staining was performed with 2% (w/v) orcein, 1% (w/v) sodium citrate in 50%(v/v) aqueous acetic acid. Stained samples were differentiated by careful substitution of dye with 40% (v/v) aqueous acetic acid and observed in a phase-contrast NU Zeiss-Jena microscope.

SDS-PAGE and immunoblotting

Immunoblotting with antibodies against phosphotyrosine was carried out using a monoclonal pY20 antibody. The cryopreserved sperm samples were treated (washed and centrifuged) and incubated the same way as for experiments 2 and 3. The non-incubated cryopreserved ejaculated sperm of two other bulls, epididymal sperm of four slaughtered bulls and freshly ejaculated sperm of one bull were used as additional controls to non-incubated sperm of the previous experiments.

Directly after washing and/or after an appropriate time of incubation, the samaples of the final volume 1.0×10^6 were washed three times with 0.5 ml of TNEGTA buffer (buffer A, pH 7.15), centrifuged 2 min at 10 000 *g* and stored frozen at –80 °C until further use. Before analysis, the sperm pellets were supplemented with 20 µl of SDS sample buffer, boiled for 3 min and centrifuged for 2 min at 10 000 *g*. The proteins were then separated on 10% SDS-PAGE gels (Laemmli, 1970). Separated proteins were transblotted onto Immobilon-P membranes in a tank-buffer apparatus (200 mA, 1 h). Blots were incubated in 10% (v/v) teleostean gelatine, dissolved in 0.05% (v/v) Tween-20 in Tris-buffered saline, pH 7.4 (TTBS) 1 h prior to development with pY20 antibody (1:1000), followed by anti-mouse horseradish peroxidase linked Ig (1:5000), each 1 h at room temperature. After extensive washing (>1 h, >5 exchanges), blots were developed with ECL chemiluminiscent kit according to the manufacturer's instructions.

Monoclonal antibody against boar acrosomal antigen and indirect immunofluorescence

Acid extract of boar spermatozoa was used for immunisation of BALB/c mice (Breeding Unit at the Institute of Molecular Genetics, Prague). Hyperimmune spleen cells were fused with myeloma cells SP/20. Positive clones were selected by ELISA with boar and bull extracts and by indirect immunofluorescence with boar and bull spermatozoa. Details of the antibody production and characterisation have been described elsewhere (Pěknicová & Moos, 1998).

Aliquots of sperm suspension (approximately 0.5×10^6 sperm in 10 µl) was smeared onto a glass slide. Smears were dried and fixed with acetone for 10 min at room temperature (22 °C). Then they were rinsed with PBS and treated for 30 min with a monoclonal antibody (ACR.4) diluted in PBS to obtain an Ig concentration of 25 µg/ml. After washing with PBS, the smears were incubated with FITC-conjugated swine anti-mouse Ig, diluted 1:20 in PBS, for 60 min at 37 °C, washed with PBS and water, and mounted in 50% (v/v) glycerol in PBS, pH 9.0. Appropriate control smears were incubated with a non-specific antibody, with a supernatant of myeloma cells and the FITC conjugate only. The specimens were examined with a Nikon microscope equipped with a 50/1.00 fluorescence objective (Pěknicová & Moos, 1990).

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Viability assessment of spermatozoa

The viability of spermatozoa was assessed as described by Kaláb *et al*. (1998). Briefly, the frozen-thawed sperm washed or washed and incubated in FM (50 µl) were fixed with 50 µl of glutaraldehyde (1 ml 50% (v/v) glutaraldehyde + 24 ml PBS). After 1 min 250 µl of PBS was added. Fixed sperm suspension (50 µl) was mixed with 50 µl of Hoechst 33258 and incubated for 5 min in the dark (1 mg of Hoechst 33258 was diluted in 5 ml of citrate buffer pH 7.4; this stock was diluted 1:9 with citrate buffer for the staining). A small drop (5 µl) of this suspension was placed on the glass slide, covered with a cover slip and evaluated in a Nikon microscope as described above.

Statistical analyses

Instat software was used for statistical evaluation (GraphPAD Software). The effect of the composition of the fertilisation medium on the rate of fertilisation (proportion of oocytes penetrated or with two developing pronuclei) was evaluated by chi-squared analysis with Yates' (continuity) correction. Motility of free-swimming sperm, labelling by ACR.4 antibody and vitality staining were evaluated by one-way ANOVA.

Results

Assessment of fertilising capacity of spermatozoa in different media and after different capacitation times

The results of the experiment 1 are summarised in Tables 1 and 2. Among the different substances tested for capacitation (fertilisation) stimulating activity and their interaction, the most effective seem to be heparin and caffeine. As indicated by male and female pronuclei formation, which already appeared 8 h after COC fertilization, the sperm started the penetration and sperm swelling in the oocyte cytoplasm approximately 4–5 h after insemination. The highest proportion of zygotes already with both pronuclei was observed in

Table 1 Fertilising capacity of spermatozoa incubated with mature COCs for 8 h in FM with different capacitating supplements (n – 1276 in 8 replicates)

Glucose 13.9 mM	BOS/PVA		Supplements	No. of oocytes	Penetrated %	With both pronuclei %*	Mean no. of sperm per penetrated egg
–	+	–	Control	82	62.2^a	0.0	1.00
–	+	–	Heparin	77	93.5^b	36.4	1.22
–	+	–	D-p	73	53.4^a	1.4	1.02
–	+	–	Caffeine	75	89.3^b	17.3	1.06
–	+	–	Caffeine+ D-p	74	75.7^c	29.7	1.34
+	+	–	Control	59	30.5^a	0.0	1.00
+	+	–	Heparin	63	100.0^b	52.4	1.33
+	+	–	D-p	63	42.8^a	0.0	1.04
+	+	–	Caffeine	63	73.0^c	25.4	1.04
+	+	–	Caffeine+ D-p	59	69.8^c	16.9	1.12
–	–	+	Control	–	–	–	–
–	–	+	Heparin	73	27.4^a	0.0	1.00
–	–	+	D-p	74	2.7^b	0.0	1.00
–	–	+	Caffeine	73	21.9^a	0.0	1.08
–	–	+	Caffeine+ D-p	74	0.0^b	0.0	0.00
+	–	+	Control	60	0.0^a	0.0	0.00
+	–	+	Heparin	59	62.7^b	13.6	1.03
+	–	+	D-p	57	0.0	0.0^a	0.00
+	–	+	Caffeine	59	57.6^b	16.9	1.09
+	–	+	Caffeine+ D-p	59	3.4^a	0.0	1.00

Control, without capacitating supplements; Heparin, with 4 IU/ml heparin; D-p, with 0.5 mg/ml D-penicillamine; Caffeine, with 1 mg/ml caffeine; Caffeine+ D-p, 1mg/ml caffeine + 0.5 mg/ml D-penicillamine.

^{a,b,c}Data with different superscripts in separated blocks differ significantly ($p < 0.05$).

*The rest of the penetrated eggs are in the pre-pronuclear stage.

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Table 2 Statistical evaluation of the effect of the presence versus absence of glucose and the presence of BOS versus PVA in FM with different capacitating supplements on sperm penetrating capacity (as seen in the four separated blocks of results in Table 1)

FM	+glucose versus –glucose		BOS versus PVA	
	+ BOS *p* value	+ PVA *p* value	+ glucose *p* value	– glucose *p* value
Control	<0.001	–	<0.0001	–
Heparin	NS	<0.0001	<0.0001	<0.0001
D-p	NS	NS	<0.0001	<0.0001
Caffeine	<0.05	<0.0001	NS	<0.0001
Caffeine+ D-p	NS	NS	<0.0001	<0.0001

Capacitating supplements were added to FM as detailed in the notes to Table 1.

the medium with heparin, BOS and glucose. The absence of BOS resulted in a slight delay in pronuclei formation, as did the substitution of heparin with caffeine, indicating a delay in the start of penetration. The capacitation activity of D-penicillamine seems to be rather questionable, because it is effective only in combination with BOS. However, there are no significant differences in penetration rate between the control medium with BOS only and the medium supplemented with BOS and D-penicillamine. When compared with FM + PVA, FM + BOS supports fertilisation significantly better in nearly all tested combinations. The combination BOS + heparin, as well as BOS + caffeine and BOS + caffeine + D-penicillamine, induced a higher frequency of polyspermy. The combination of fertilisation medium with PVA + glucose resulted in a significantly higher proportion of fertilised oocytes than did a medium with PVA and no glucose.

The penetration capacity of spermatozoa after 5 h incubation (before insemination of oocytes) in a medium with different capacitation activating substances and subsequent 12 h fertilisation resulted in a decreased rate of penetrated oocytes in the medium with BOS + heparin or BOS + caffeine, in contrast to an increased penetration rate in the medium with BOS + D-penicillamine or BOS + D-penicillamine + caffeine, independently on the presence or absence of glucose. In this combination of FM supplements D-penicillamine was the main component that supported the fertile life span of spermatozoa during their time of incubation. the presence of glucose significantly stimulated the penetration activity of spermatozoa in protein-free media with heparin and caffeine (Tables 3, 4). Even in this experiment FM + BOS resulted in a significantly higher fertilisation rate and polyspermy than FM + PVA (particularly in media containing D-penicillamine).

The specific aim of the third experiment was to

Table 3 Penetrating capacity of spermatozoa incubated for 5 h in FM with different capacitating supplements and subsequent fertilisation of mature COCs for an additional 12 h (n = 1217 in 8 replicates)

Glucose 13.9 mM	BOS/ PVA		Supplements	No. of oocytes	Pene-trated %	Mean no. of sperm per pene-trated egg
–	+	–	Control	66	3.0[a]	1.00
–	+	–	Heparin	66	22.7[b]	1.00
–	+	–	D-p	67	80.6[c]	1.30
–	+	–	Caffeine	64	17.2[b]	1.00
–	+	–	Caffeine+ D-p	64	71.8[c]	1.33
+	+	–	Control	65	4.6[a]	1.00
+	+	–	Heparin	63	42.8[b]	1.04
+	+	–	D-p	61	88.5[c]	1.59
+	+	–	Caffeine	63	23.8[d]	1.13
+	+	–	Caffeine+ D-p	60	98.3[c]	1.98
–	–	+	Control	–	–	–
–	–	+	Heparin	69	7.2[a]	1.00
–	–	+	D-p	67	0.0[a]	0.00
–	–	+	Caffeine	69	0.0[a]	0.00
–	–	+	Caffeine+ D-p	69	0.0[a]	0.00
+	–	+	Control	60	0.0[a]	0.00
+	–	+	Heparin	60	18.3[b]	1.00
+	–	+	D-p	62	0.0[a]	0.00
+	–	+	Caffeine	61	11.5[b]	1.14
+	–	+	Caffeine+ D-p	61	8.2[ab]	1.00

Capacitating supplements were added to FM as detailed in the notes to Table 1.
[a,b,c]Data with different superscripts within separated blocks differ significantly ($p < 0.05$).

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Table 4 Statistical evaluation of the effect of the presence versus absence of glucose and the presence of BOS versus PVA in FM with different capacitating supplements on sperm penetrating capacity (as seen in the four separated blocks of results in Table 3)

	+ glucose versus − glucose		BOS versus PVA	
FM	+ BOS *p* value	+ PVA *p* value	+ glucose *p* value	− glucose *p* value
Control	NS	–	NS	–
Heparin	< 0.05	NS	<0.01	<0.05
D-p	NS	NS	<0.0001	<0.0001
Caffeine	NS	<0.05	NS	<0.01
Caffeine+D-p	<0.001	NS	<0.0001	<0.0001

Capacitating supplements were added to FM as detailed in the notes to Table 1.

Table 5 Penetrating capacity of spermatozoa incubated for 5 h in FM with glucose, PVA and with different capacitating supplements. Heparin (4 IU/ml), BSA (3 mg/ml) and mature oocytes were added to each well with incubated spermatozoa for subsequent 12 h of fertilisation (n = 422 in 4 replicates)

Capacitating supplements	Sperm motility %	No. of oocytes	Penetrated %	Mean no. of sperm per penetrated egg
Control	15–20	87	13.8[a]	1.00
Heparin	10–20	85	42.3[b]	1.00
D-p	50–60	83	86.7[c]	1.18
Caffeine	5–10	83	37.3[b]	1.00
Caffeine+D-p	20–30	84	3.6[a]	1.00

Capacitating supplements were added to FM as detailed in the notes to Table 1.
[a,b,c]Values in the fourth column with different superscripts differ significantly ($p < 0.05$).

Table 6 Penetrating capacity of spermatozoa not washed before 45 min of swim-up in FM with PVA, glucose, caffeine and D-penicillamine, and fertilised either in the identical medium 'a' or in medium 'b' (with heparin and BOS) (n = 129 in 2 replicates)

Fertilisation medium	No. of oocytes	Penetrated %	Mean no. of sperm per penetrated egg
'a'	66	47.0[a]	1.03
'b'	63	92.0[b]	1.53

[a,b]Values in the third column with different superscripts differ significantly ($p < 0.05$).

verify whether the fertilisation medium with PVA and D-penicillamine, which has nearly no capacity to stimulate penetration, has the potency to maintain the spermatozoa for 5 h in a healthy state and is potentially able to induce sperm penetration later, in a medium with other capacitation activating substances. For this purpose, the spermatozoa were treated for 5 h with FM, as in experiment 2 (either with glucose and PVA only or supplemented with heparin or D-penicillamine or caffeine or caffeine + D-penicillamine), as shown in Table 5. The fully reversible penetration capacity of the spermatozoa which were incubated with D-penicillamine (86.7%) showed the capability of D-penicillamine to maintain the fertile life span of spermatozoa. This corresponded with the high (55%) motility of these spermatozoa after 5 h of incubation (Table 7). The spermatozoa in a control medium which were incubated only with PVA and glucose penetrated 13.8% oocytes. However, slightly increased penetration activity was shown also in spermatozoa incubated with heparin and caffeine (42.3% and 37.3%, respectively). The combination of caffeine with D-penicillamine was surprisingly more harmful for the fertile life span of incubated spermatozoa.

In experiment 4, the fertilisation protocol of Keskintepe & Brackett (1996), differing mainly in the swim-up technique and composition of capacitation active substances ('a'), was compared with our fertilisation procedure ('b'). The data in Table 6 show positive fertilisation results in both the experimental group 'a' (47%) and control group 'b' (92%). However, in group 'a' capacitation and fertilisation was stimulated by D-penicillamine and caffeine in protein-free medium, in conditions which in our fertilisation protocol (experiment 2) did not result in sperm penetration.

Sperm quality evaluation

The percentage of motile spermatozoa and spermatozoa positively labelled with ACR.4 was evaluated at the beginning and during sperm manipulation, after 5 h and 10 h of incubation, and in media with different capacitation activating substances (the same as for the fertilisation experiments). The more precise evaluation of progressive motility was possible only in media with less agglutinated sperm, namely before incubation and after incubation in those containing D-penicillamine. In sperm incubated for 5 h and 10 h, the best motility was observed in FM + PVA + D-penicillamine. BOS induced higher agglutination than PVA even with D-penicillamine. The data are shown in Table 7.

The high labelling of acrosomes with ACR.4 as well as high proportion of motile spermatozoa was observed mainly in freshly thawed sperm, before and after swim-up. During 5 h and 10 h incubation, the percentage of labelled acrosomes decreased irregularly in

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Table 7 Qualitative evaluation of frozen-thawed spermatozoa treated and incubated as for fertility tests (see Tables 1–5)

Incubation (h)	Treatment of sperm	% motile not agglutinated		% positive to ACR4[a]		% unstained (vitality)[a]	
	glucose	+	–	+	–	+	–
0	Thawed washed	65	60	71	80	80	74
0.5	Swim-up, sedimented	40*	35**	28**	56	80	50**
0.5	Swim-up, from supernatant	75	65	78	72	84	74
5	PVA + heparin[1]	15***	5***	58	65	–	–
5	PVA + D-p[2]	55	50	44*	66	–	–
5	PVA + caffeine[3]	5***	1***	65	57	–	–
5	PVA + caf/D-p[4]	30**	25**	42*	45**	–	–
5	PVA – control[5]	20**	1***	51*	50*	75	67
5	BOS + heparin[1]	5***	5***	54	55	–	–
5	BOS + D-p	25**	10***	51*	49*	–	–
5	BOS + caffeine[3]	5***	5***	45*	46*	–	–
5	BOS + caf/D-p[4]	10***	10***	43*	43*	–	–
5	BOS – control[5]	10***	5***	37*	49*	–	–
5	PVA + heparin[1]						
+5	BSA + heparin[6]	5***	–	57	–		
5	PVA + D-p[2]						
+5	BSA + heparin[6]	50	–	37*	–		
5	PVA + caffeine[3]						
+5	BSA + heparin[6]	1***	–	47*	–		
5	PVA + caf/D-p[4]						
+5	BSA + heparin[6]	5***	–	53	–		
5	PVA – control[5]						
+5	BSA + heparin[6]	5***	–	51	–		

[a]See Materials and Methods.
[1]4 IU/ml heparin; [2]0.5 mg/ml D-penicillamine; [3]1 mg/ml caffeine; [4]1 mg/ml caffeine + 0.5 mg/ml D-penicillamine; [5]without capacitation supplements; [6]4 IU/ml heparin + 3 mg/ml BSA.
Statistical evaluation of the variability between treatments comparing with residual variability by ANOVA:
Motility rate: FM + glucose: $n = 3$, mean SD = 8.39, $p < 0.0001$
FM – glucose: $n = 3$, mean SD = 6.92, $p < 0.0001$
ACR.4 treated: FM + glucose: $n = 5$, mean SD = 16.97, $p = 0.0048$
FM – glucose: $n = 7$, mean SD = 14.64, $p < 0.0001$
Vitality (% unstained): FM + glucose: $n = 3$, mean SD = 6.51, $p = 0.426$
FM – glucose: $n = 3$, mean SD = 5.79, $p = 0.0022$
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with 'swim-up' samples as control (post-test between selected pairs of groups).

all tested samples. The high variability between single experiments of groups incubated for 5 or 10 h with glucose or 5 h without glucose (Table 7) created difficulties in detecting more significant differences between differently treated and incubated groups.

The percentage of live spermatozoa stained by Hoechst 33258 was evaluated after thawing and swim-up and (for a rough estimate only) after 5 h incubation in FM + PVA (control) medium. The vitality staining of sperm showed suspiciously high proportions of unstained spermatozoa, in FM with glucose (Table 7).

Time-dependent changes in protein tyrosine phosphorylation in media with different capacitation activating substances

The biochemical analyses of tyrosine phosphorylation activity of spermatozoa in relation to the modification of capacitation activating molecules and time of capacitation shown in Fig. 1*A*, *B* and in the first 12 lanes of Fig. 1*C* were performed according to the previous fertilisation protocol of experiment 2. For analyses of tyrosine phosphorylation of the last five lanes of Fig.

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1*C*, the sperm samples were treated as for experiment 3. A number of protein bands labelled with the anti-phosphotyrosine antibody are visible already in frozen-thawed bull sperm before incubation, and the same pattern is seen after a 15 min swim-up. After 5 h incubation a new, approximately 90 kDa protein appeared in sperm samples treated with caffeine in FM with BOS as well as in FM with PVA (Fig. 1*A*, *B*). In FM with PVA and absence of glucose (Fig. 1*B*), only a very weak band appeared in the region of 90 kDa. In contrast to caffeine, heparin stimulated the phosphorylation of this protein only in the presence of BOS. As seen from all three blots (*A*, *B*, *C*) of Fig. 1, BOS stimulatedd high tyrosine phosphorylation of the 90 kDa protein of 5 h incubated sperm in all capacitation media with the exception of control FM and FM with D-penicillamine, where the 90 kDa protein was observed only in FM with BOS but without glucose (Fig. 1*B*). The control samples of non-incubated either cryopreserved or freshly ejaculated or epididymal semen of other bulls (Fig. 2), and all combinations of non-incubated control samples (including FM + BOS), showed no labelled band in the mentioned area. The last five lanes of Fig. 1*C* show the sperm samples which in the first 5 h interval were incubated in FM + PVA + glucose and either heparin or D-penicillamine or caffeine or caffeine with D-penicillamine (as in Fig. 1*A*). For the second 5 h incubation interval, heparin + BSA was added to all samples (as in experiment 3). Overall, the 10 h incubation resulted only in the phosphorylation of the 90 kDa protein in a sample incubated in the first 5 h interval with PVA + caffeine (Fig. 1*C*). However, there were several another bands of tyrosine phosphorylated protens visible in practicaLly all analysed sperm samples, independently of the time of their incubation and their treatment or preservation. (Figs. 1*A*, *B*, *C* and 2).

Discussion

Heparin and caffeine have been proved to be the basic independently acting capacitators of bovine spermatozoa (see reviews in the Introduction). In contrast to capacitation stimulating drugs, glucose was proved to be a capacitation retarding molecule (Parrish *et al*., 1989, 1994). On the other hand, the recent data of Tajik & Niwa (1998) and also the present experiments demonstrated that glucose could have a positive effect on the fertilisation rate and not an adverse effect on the kinetics of sperm penetration (as evaluated by pronuclei formation) in FM + BOS + heparin or caffeine, or in FM + PVA + heparin or caffeine (Tables 1, 2). Similar results were also shown by Tajik *et al*. (1994). However, Parrish *et al*. (1988, 1989, 1994) capacitated the spermatozoa at 25 time higher concentration (compared with our procedures). The high sperm concentration in medium during capacitation in the presence of glucose could create anaerobic surroundings (more suitable for the Embden-Meyerhof pathway) leading to increased lactate production and decreased pH, creating conditions less optimal for capacitation. Taken together, the slightly positive effect of glucose on sperm penetration in our experiments was probably caused by its very low concentration during incubation and high pH (7.7) of FM. In addition, the absence of mineral oil on the surface of the sperm suspension in our experiments probably created aerobic conditions with higher O_2 entry. The most positive effect of glucose was on the fertilisation rate and kinetics of fertilisation only in the media with heparin and BOS. BOS, like BSA, probably plays a pivotal role in destabilising the plasma membrane by removing the cholesterol (Go & Wolf, 1985; Langlais & Roberts, 1985). The role of BSA in chemically defined media could be substituted by PVA only partially. As documented by Tajik *et al*. (1993), successful fertilisation in protein-free medium needs supplementation with granulosa cells, secretions of which probably substitute the proteins in the process of capacitation. Soluble acrosome reaction (AR)-inducing factor was detected in COC (Boatman & Robbins, 1991). Higher sperm concentration in protein-free media can substitute for the absence of granulosa cells also only partly (Tajik *et al*., 1994).

In contrast to the results of Andrews & Bavister (1989) and Keskintepe & Brackett (1996), D-penicillamine in our experiments was not shown to be a capacitation-stimulating drug. However, the combination of FM with PVA and D-penicillamine and swim-up of unwashed frozen-thawed spermatozoa according to the protocol of Keskintepe & Brackett (1996) resultled in fertilisation also in our hands (Table 6). Using this method, the capacitating activity of glycerol or other sperm extender molecules (at the time of swim-up) could be calculated.

On the other hand, our experiments showed D-penicillamine had a significantly protective effect on the fertile life span of spermatozoa. This has been recently documented on the cryopreserved semen (including capacitation chlortetracycline test) of two other bulls (Pavlok, 2000).

However, the mechanism of action of D-penicillamine is not known. According to Wolf & Ruocco (1987), the involvement of d-penicillamine is most likely in the formation of drug–cysteine instead of cysteine–cysteine bonds. The formation of D-penicillamine–cysteine complexes is probably not a terminal reaction but rather the primary initiating step of a chain reaction involving continued reduction by various thiol reductants. The reducing action of D-penicillamine could eliminate the action of the reactive oxygen species which probably play a capacitation-stimulating role, as documented by Bize *et al*. (1991)

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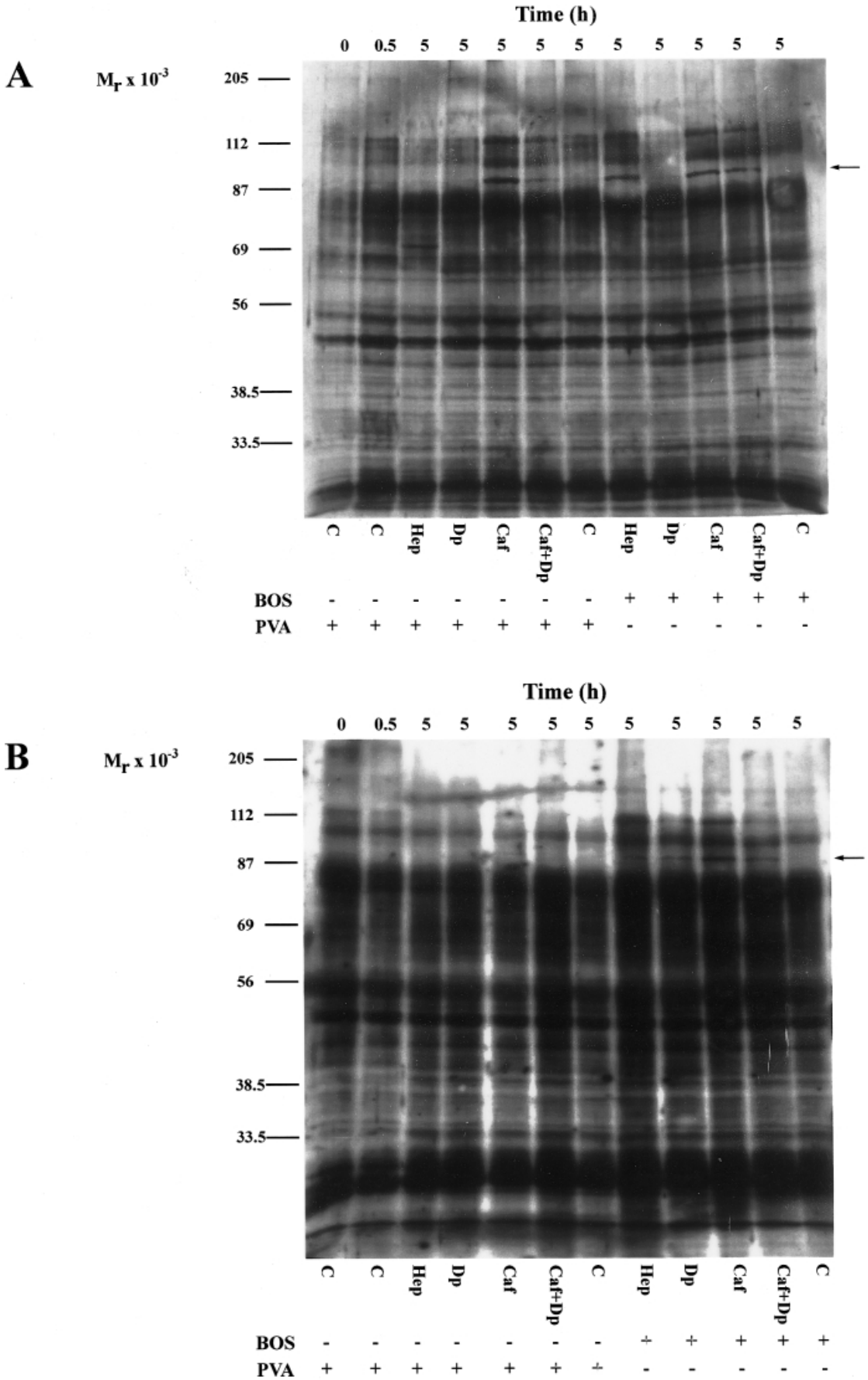

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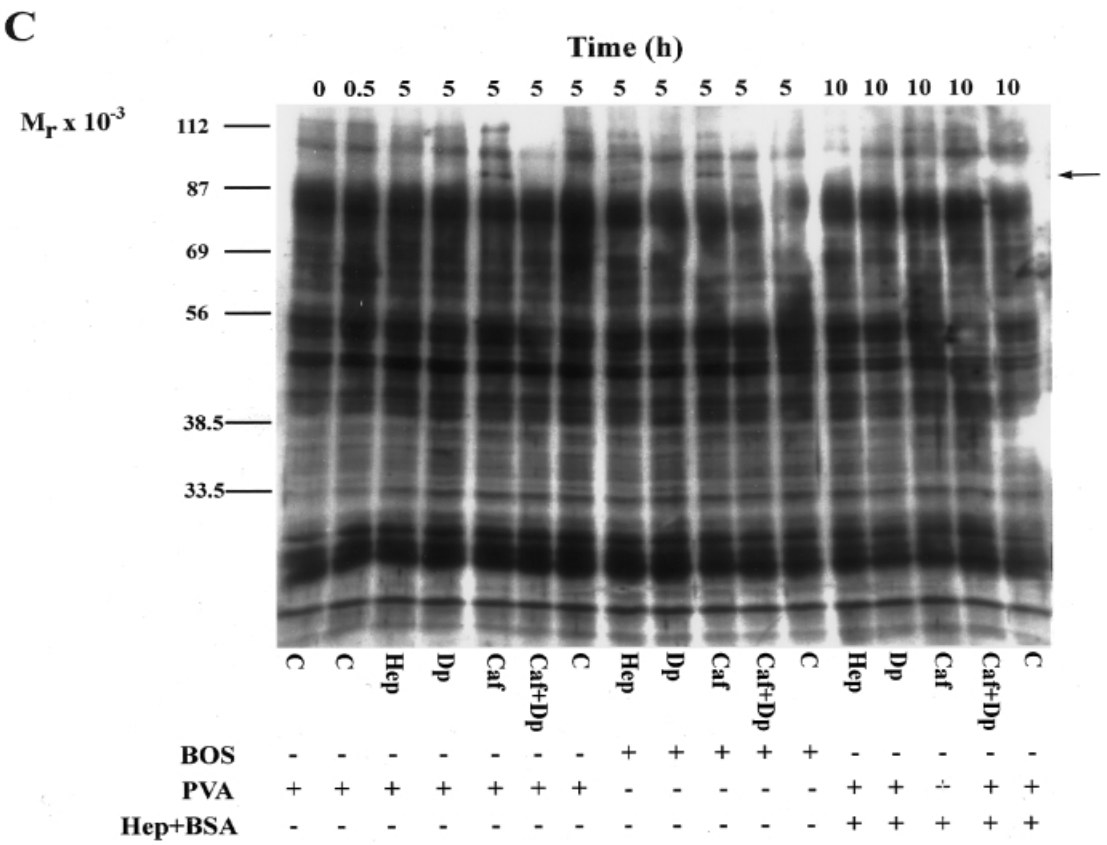

(Left and above)

Figure 1 The effects of heparin, D-penicillamine and caffeine on the one hand and BOS, PVA and glucose on the other hand on protein tyrosine phosphorylation in frozen-thawed bovine sperm. Sperm were incubated in either FM without glucose (*B*) or modified FM with 13.9 mM glucose (*A*, *C*) supplemented either with 10% (v/v) BOS, or 1 mg/ml PVA and different capacitation activating substances as shown in the Materials and Methods section. The arrows to the right of all gels indicate the position of 90 kDa protein, which become tyrosine phosphorylated in the indicated sperm samples. (*A*) Sperm samples were prepared (thawed and washed) in FM with glucose and PVA. The non-incubated samples (0 h) or those after swim-up (0.5 h) served as the control (C). The other samples were incubated for 5 h either with BOS or PVA, supplemented with heparin (Hep), D-penicillamine (Dp), caffeine (Caf) or the combination of caffeine and D-penicillamine (Caf+Dp) and then dissolved and washed in the SDS sample buffer. Equal numbers of sperm per lane were separated by SDS-PAGE and analysed by immunoblotting with anti-pY antibody. (*B*) Sperm were prepared under the same conditions as (*A*), except that FM (without glucose) was used for all treatment and incubations. (*C*) For the first 5 h of incubation the sperm samples were prepared exactly the same way as in (*A*); the last five lanes show the sperm samples which were incubated for an additional 5 h in the modified FM supplemented with heparin and BSA.

and de Lamirande & Gagnon (1995), in hamster and human sperm, respectively. Reactive oxygen species also upregulate protein tyrosine phosphorylation (Leclerc *et al*., 1997).

When compared with freshly ejaculated sperm, it is supposed that frozen-thawed spermatozoa have lower viability and a shorter fertile life span, either *in vitro* or in the female genital tract. The effects of cryopreservation on the capacitation status and some other functional and biochemical parameters have been studied in more detail in ram spermatozoa (Slavik, 1987; Gillan *et al*., 1997) and bull spermatozoa (Boerlegui *et al*., 1997). An effect of freezing on the capacitation of bovine spermatozoa was also observed in our preliminary experiments, such as a higher sensitivity to lysolecithin (unpublished data). This test was successfully applied to freshly collected and capacitated bull sperm in several papers (Parrish *et al*., 1988, 1994). However, from the point of view of the more convenient practical utilisation of frozen sperm (especially for fertilisation *in vitro*), it seems to be very important to have some more data concerning their fertile life span and the possibilities of its regulation. In this respect, D-penicillamine seems to be a very promising drug.

Monoclonal antibody ACR.4 recognises specifically the 28 kDa intra-acrosmal protein of bull spermatozoa before and during capacitation. These changes can be

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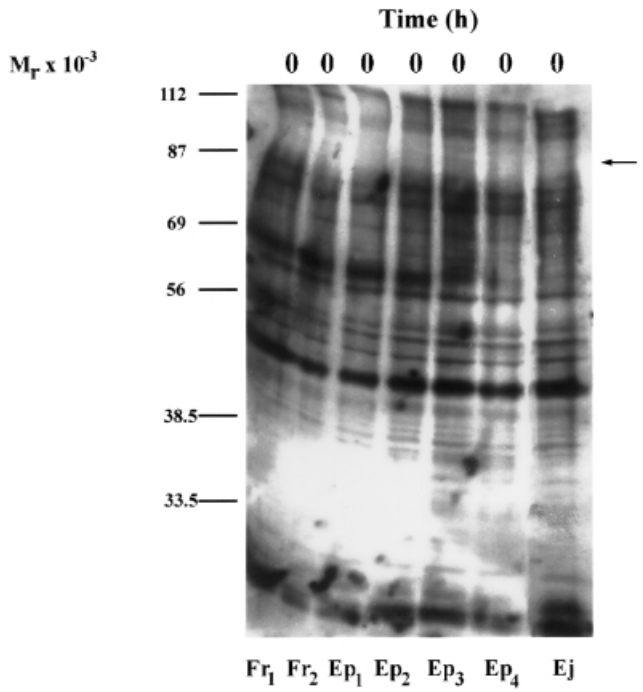

Figure 2 Protein tyrosine phosphorylation of control non-incubated sperm samples of cryopreserved sperm of two bulls (lanes 1 and 2), epididymal sperm of four bulls (lanes 3–6) and freshly collected ejaculated sperm (lane 7). Equal numbers of sperm per lane were separated by SDS-PAGE and analysed by immunoblotting with anti-pY antibody as the samples in Fig. 1. The 90 kDa protein was not detected (the arrow to the right).

connected either with the procedure of physiological preparation of spermatozoa for sperm–egg interaction (Pěknicová *et al*., 1994), or with the fact that different drugs could affect the resistance of plasma membrane and partly induce spontaneous AR. The high variability between identical sperm samples of 5 and 10 h incubated spermatozoa labelled by ACR.4 was most probably caused by lower mechanical resistance of the plasma membrane of frozen-thawed and incubated spermatozoa after centrifugation and smear-preparation procedure. Comparing the ACR.4 positive sperm with the results of vitality staining by Hoechst 33258, we can conclude that the reaction of ACR.4 with acrosomes is probably more sensitive to the changes in some of the vital functions of spermatozoa than is Hoechst 33258.

As already mentioned, both capacitation and the increase in protein tyrosine phosphorylation are downstream of the cAMP pathway (Visconti *et al*., 1985*a*, b), probably through activation of protein kinase A (Visconti *et al*., 1997). Capacitation-associated tyrosine phosphorylation is also probably an important regulatory pathway connected with induction of hyperactive motility, in mouse semen through protein kinase A anchoring protein AKAP82, which is the major protein of the fibrous sheath of sperm flagellum (Visconti *et al*., 1997).

Inhibitors of cyclic nucleotide phosphodiesterase activity, such as caffeine, and cAMP stimulators, such as heparin (Parrish *et al*., 1994), stimulated in our experiments not only sperm penetration but also tyrosine phosphorylation of the 90 kDa protein of bovine frozen-thawed 5 h capacitated sperm. The additive effect of BOS on tyrosine phosphorylation may be caused by cholesterol binding (cited above). Galatino-Homer *et al*. (1997) have observed tyrosine phosphorylation of several proteins also mainly in the area of 90 kDa in freshly ejaculated bull sperm in relation to the time of capacitation, and the presence of cAMP analogues, phosphodiesterase inhibitors and heparin. However, in contrast to our results they observed an inhibitory effect of glucose on tyrosine phosphorylation. The presence of glucose in a chemically defined medium (with PVA) with caffeine has, in our experiments, a stimulating effect on tyrosine phosphorylation, as well as on the fertilizing capacity. As already mentioned, the reason for the discrepancies between our experiments and literature data might be the substantially different sperm concentrations used for incubation. A tyrosine phosphorylated 55 kDa protein described by Vijayaraghaven *et al*. (1997) as a motility-associated protein is probably also present in all our sperm samples – however, already before incubation.

The spectrum of tyrosine phosphorylated proteins as documented in the first two lines of Fig. 1 (*A*, *B*, *C*) correlated very well with the spectrum of washed frozen, epididymal or freshly ejaculated spermatozoa from other (control) bulls (Fig. 2). The relatively broad spectrum of proteins phosphorylated on tyrosine in all tested sperm samples could also be caused by extensive washing of a small amount of spermatozoa in a medium with a high bicarbonate composition stimulating adenylyl cyclase activity (Garty & Salomon, 1987), and perhaps by stimulation of a higher Ca^{2+} influx after plasma membrane destabilisation (Harrison *et al*., 1993). However, the recent results of Kaláb *et al*. (1998) documented the paradoxical inhibition of protein tyrosine phosphorylation in boar semen by extracellular calcium.

The main reason for discrepancies between the fertilisation results and tyrosine phosphorylation of the 90 kDa protein may be mainly the heterogeneity of the sperm population. While for the detection of phosphorylation activity a significant proportion of sperm population should take part in this reaction, only a very small proportion should take part at fertilisation. The second reason may be that while capacitation is a process progressing mostly independently to the presence of the female gamete, a very limited proportion of spermatozoa take part at fertilisation, activate their fer-

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tilising capacity (including AR) in interaction with the secretion of granulosa cells and/or directly on the surface of zona pellucida and penetrate the egg membranes. From this point of view *in vitro* fertilisation seems to be one of the most objective tests of sperm fertilising capacity, but with the ability to recognize even a very small proportion of fertile spermatozoa present in the tested sperm sample.

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