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·*Original Article*·

Effect of genistein on acrosome reaction and zona pellucida binding independent of protein tyrosine kinase inhibition in bull

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Abstract

Aim: To investigate if the phytoestrogen, genistein, affects essential functions of cryopreserved bovine spermatozoa. **Methods:** The effect of genistein upon motility was assessed by computer-assisted motion analysis. Hemizona assay was performed to detect the ability of spermatozoa binding to the zona pellucida. The inducibility of the acrosome reaction using progesterone and ZP3-6 peptide was analysed by fluorescein-conjugated Pisum sativum agglutinin (FITC-PSA)/Hoechst 33258 double staining. Capacitation after incubation with genistein was assessed by the chlortetracycline (CTC) assay. Immunoblots showed the pattern of protein tyrosine phosphorylation of cryopreserved bovine spermatozoa. **Results:** Immunodetection of tyrosine-phosphorylated proteins showed that genistein did not affect tyrosine phosphorylation in cryopreserved bovine spermatozoa. However, genistein significantly reduced the progesterone- and ZP3-6 peptide-mediated induction of the acrosome reaction and led to a dose-dependent inhibition of sperm-zona pellucida binding; while sperm motility and capacitation were not affected by this phytoestrogen, as indicated by computer-assisted sperm motion analysis and the CTC assay, respectively. **Conclusion:** Our results suggest that in cryopreserved bovine spermatozoa, genistein affects a protein tyrosine phosphorylation-independent signal transduction pathway that is involved in sperm capacitation, the acrosome reaction and sperm-zona pellucida binding. *(Asian J Androl 2007 Sep; 9: 650–658)*

Keywords: genistein; sperm motility; acrosome reaction; capacitation; tyrosine phosphorylation; cryopreservation; zona pellucida

1 Introduction

Sperm motility, sperm capacitation, the acrosome reaction and tight binding of spermatozoa to the zona pellucida are crucial events in the process of fertilization [1]. The disturbance of one of these functions can cause male infertility. High concentrations of phytoestrogens are known to affect fertility [2]. Interest in the effect of phytoestrogens on male fertility has increased in recent years as it has been demonstrated that estrogens play an important role in the male reproductive system [3]. Phytoestrogens bind to estrogen receptors and are present in plants used as feed or plant-derived food (e.g. soybean, fava beans, lupines and clover). Genistein, an isoflavonoid, shows estrogen activity [2] and inhibits protein tyrosine kinases (PTK), which phosphorylate tyrosyl residues of membrane-bound receptors involved in signal transduction [4].

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Tyrosine phosphorylation has been reported to play a key role in various aspects of sperm function. The increase in protein tyrosine phosphorylation appears to be closely related to the process of sperm capacitation [5]. However, decapacitation initiated by epididymal proteins inhibits protein tyrosine phosphorylation [6], and seminal plasma diminishes the subpopulation of cells exhibiting hyperactivated motility in ejaculated human sperm via the decrease in phosphorylation of tyrosine residues [7]. Treatment with PTK inhibitors can also block zona pellucida- and progesterone-induced exocytosis and sperm-zona pellucida penetration [8–10].

Semen cryopreservation is an important tool for assisted reproduction. However, the fertility of frozen-thawed spermatozoa is reduced, possibly because of precocious capacitation-like changes that are known to occur [11]. In a series of previous studies, we evaluated whether aliquots of pooled post-thaw bovine semen are suitable for examining essential sperm functions [12, 13]. Using cryopreserved bull spermatozoa as test cells, we demonstrated that a number of essential sperm functions can be measured. We assessed cell viability, motility, the acrosomal status, the inducibility of the acrosomal exocytosis and sperm binding to the zona pellucida in post-thaw bull semen. Our results demonstrated that cryopreserved spermatozoa can be integrated as test cells for the *in vitro* screening of substances that might interfere with male reproductive function.

A recent study suggests different mechanisms for induced capacitation of fresh bull spermatozoa (in the presence of heparin) and cryocapacitation [11]. It has been demonstrated that the regulation of capacitation by protein tyrosine phosphorylation differs in frozen-thawed spermatozoa compared with fresh-extended cells, and that a subpopulation of cryocapacitated spermatozoa appears to be evident immediately after thaw.

In a preliminary study using the phytoestrogen genistein as a test substance for essential sperm functions we found the first evidence demonstrating that genistein concentrations below 1 μg/mL decrease sperm-zona pellucida binding [14]. In this investigation, we explored signal transduction pathways affected by genistein and possible altered sperm functions that are involved in bull sperm capacitation induced by cryopreservation (cryocapacitation).

2 Materials and methods

2.1 Bovine spermatozoa

A total of 12 ejaculates were collected from 10 young Holstein Friesian/black and white bulls (aged 14–16 months) with the aid of an artificial vagina (inside temperature 43ºC). Fresh ejaculates were transported to the laboratory and used within 2-h of collection. Semen processing for cryopreservation was performed by holding the ejaculate in a water bath at 28ºC. Ejaculates were diluted at room temperature using appropriate extenders free of animal proteins and based on soybean lecithins [12, 15], split up into aliquots (0.25 mL Cassou ministraws, IMV, L'Aigle, France; final concentration of 20×10^6 spermatozoa per dose) and cooled to 4ºC. Finally, semen was cryopreserved with liquid nitrogen vapor using a freezing processor (Type K; Heede-Nielsen, Copenhagen, Denmark). Thawing was performed by carefully moving the straws in a water bath at 38ºC for 25 s.

2.2 Incubation of spermatozoa with genistein

Frozen/thawed spermatozoa were diluted in pre-warmed (38.5ºC) Ham F-10 medium (Sigma, Munich, Germany) containing 0.3% bovine serum albumin (BSA) (fraction V; Sigma, Munich, Germany) and washed twice by centrifugation at $300 \times g$ for 5 min at room temperature. After the second wash, the pellet was layered with Ham F-10 medium containing 0.3% BSA and sperm were allowed to swim up for 45 min at 38.5ºC in an incubator with 5% CO_2 in air. After the swim-up, the supernatant was collected, and the concentration of spermatozoa was adjusted to 20×10^6–30×10^6 spermatozoa/mL. The sperm suspension was split, and each aliquot was incubated with three different concentrations of genistein (0.074 μmol/L, 0.74 μmol/L and 7.4 μmol/L) (Sigma, Munich, Germany). One additional aliquot incubated with the 0.02% genistein solvent dimethyl sulfoxide (DMSO) served as negative control. After 2.5-h incubation, the sperm suspension was centrifuged for 5 min at $400 \times g$ to remove genistein. The pellet was resuspended in the same volume of medium and incubated at 38.5ºC and 5% CO_2 for 10 min for recovering sperm motility.

2.3 Motility assessment

Sperm motility was assessed directly after the addition of genistein or DMSO (0 h) and after 1, 2, 5, and 6 h of incubation at 38.5ºC and 5% CO_2 in air. At each of these time points, an aliquot from each sample was transferred to a 10-μm thick Makler chamber pre-warmed to 38.5ºC. Analysis of sperm motility (200 spermatozoa in at least four different fields) was carried out using a Cell

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Motion Analyzer (CMA; Medical Technologies Montreux SA, Clarens/Montreux, Switzerland). The parameter settings were adjusted for bovine sperm as recommended by the supplier.

2.4 Hemizona assay

Bovine ovaries were obtained from the local slaughter house. Bovine oocytes were recovered from follicles with a diameter of 4–7 mm. Each oocyte was separated from cumulus cells by thoroughly washing with phosphate-buffered saline (PBS).

The hemizona assay was performed as described in a previous study for bovine gametes [12]. Briefly, denuded oocytes were placed in a droplet of Ham F-10 medium and equally microbisected using a micromanipulator (Zeiss, Göttingen, Germany). For the bovine hemizona assay, bovine spermatozoa were prepared after 2.5-h of incubation in the presence and absence of genistein as described above and adjusted to a final count of 5×10^5 motile spermatozoa/mL. Each hemizona was placed in a 100 µL sperm suspension droplet on a Petri dish under heavy white mineral oil. After 4-h spermatozoa-hemizona co-incubation (at 38.5ºC in 5% CO_2 in air), each hemizona was removed and rinsed five times in Ham F-10 medium to remove loosely attached spermatozoa. The number of spermatozoa tightly bound to the outer surface of each hemizona was counted using an inverted microscope (Axiovert100; Zeiss, Göttingen, Germany). Finally, the hemizona index (HZI) was calculated as the number of test spermatozoa bound per hemizona × 100 divided by the number of control spermatozoa bound per hemizona.

2.5 Induction of the acrosome reaction

The acrosome reaction was assessed using capacitating conditions for cryopreserved bovine semen reported to be effective in bovine *in vitro* test systems [12]. Progesterone was dissolved in DMSO as a 5 mmol/L stock solution and stored at –20ºC until use. Aliquots were thawed and diluted with Ham F-10 medium supplemented with 0.3% BSA. ZP3-6 peptide was dissolved in distilled water with a concentration of 1 mg/mL and then diluted with Ham F-10 medium to 10 µmol/L. The solution was stored at –20ºC until use.

After incubation with genistein (7.4 µmol/L), as described above, 100 µL aliquots of spermatozoa were stimulated with either progesterone (final concentration 1 µmol/L in 0.02% DMSO), ZP3-6 peptide (final concentration 1 µmol/L in Ham F-10 medium), or solvent control (0.02% DMSO). The samples were incubated for 25 min at 38.5ºC and then processed for the analysis of viability and acrosomal status. The numbers of live and dead spermatozoa and their acrosomal status were evaluated essentially according to previously described methods [12]. The acrosomal status of 200 spermatozoa was assessed. Slides were read blindly by a single observer and were routinely checked by another observer.

2.6 Assessment of capacitation

In vitro capacitation of bovine spermatozoa was evaluated using the chlortetracycline (CTC) fluorescence assay as described by Adeoya-Osiguwa *et al.* [16]. Slides were prepared by placing 10 µL of the fixed sperm suspension on a slide (two slides per sample) and mixing carefully with one drop of Citifluor (Plano, Wetzlar, Germany) to retard fading of fluorescence. Cells were assessed at × 400 magnification on a Zeiss microscope equipped with phase contrast and epifluorescent optics (Axioskop; Zeiss, Göttingen, Germany). Each cell was first observed under ultraviolet illumination (emission 365 nm) for determination of live/dead status. Cells showing bright blue staining of the nucleus were considered to be dead and not counted. Live cells were then observed under blue-violet illumination (emission 450–490 nm) for CTC patterns.

Two hundred live cells in each sample (100 sperm in each slide) were classified according to CTC staining patterns as described in a previous study [16]. The three patterns are: F, uncapacitated cells; B, capacitated sperm; and AR, acrosome-reacted cells.

2.7 Protein tyrosine phosphorylation patterns during capacitation

The biophysical profiles of phosphotyrosine-containing proteins from cryopreserved spermatozoa were identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting [17]. Before (0 h) and after 2 h and 4 h of incubation in Ham F-10 medium with or without the addition of heparin (10 µg/mL), dbcAMP (1 mmol/L) and IBMX (100 mmol/L), and with or without genistein (7.4 µmol/L), aliquots of 5×10^6 cells from sperm suspensions were centrifuged (22 000 × *g*) at room temperature in PBS (pH 7.4) supplemented with 0.2 mmol/L Na_3VO_4 and protease inhibitors (1 mmol/L EDTA, 10 mmol/L benzamidine, 2 mmol/L DTT and 0.2 mmol/L PMSF). Sperm proteins were extracted in

solubilization buffer (2% SDS; 62.5 mmol/L Tris-HCl, pH 6.8; 5% glycerol; 2% bromophenol blue) by heating for 5 min at 100ºC followed by centrifuging (22 000 × *g*) for 3 min. Thereafter, β-mercaptoethanol was added to the supernatants (final concentration, 10%); samples were then heated for 5 min at 100ºC and finally centrifuged (22 000 × *g*) for 1 min. Solubilized proteins were stored at –20ºC until separation on 10% SDS-PAGE minigels and subsequently electrotransferred onto nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany) using an electrophoretic blotting system (BioRad, Munich, Germany). Electrophoretic protein transfers were performed in blotting buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, 0.01% SDS, pH 8.3–8.5) at 70 V constant for 1.5 h at room temperature.

Nonspecific binding sites were saturated by soaking membranes with 5% (v/v) teleostean gelatine in T-TBS, pH 7.4 (10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature. Blots were incubated with the primary monoclonal anti-phosphotyrosine antibody (clone 4G10; Upstate Technologies, Biomol, Hamburg, Germany) diluted 1:1 000 in T-TBS + 1% BSA for 1 h at room temperature, then washed three times (5 min per wash) in T-TBS to remove excess antibody. Peroxidase-conjugated sheep anti-mouse IgG (Sigma, Munich, Germany) was used as the secondary antibody and incubated with the blots at a dilution of 1:3 000 in T-TBS + 1% BSA for 30 min. Excess secondary antibody was removed by washing the blots five times (10 min per wash) in T-TBS. Phosphotyrosine-containing bands were detected with an enhanced chemiluminescence kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions.

2.8 Statistical analysis

Data were presented as mean ± SEM. Percentage data were analyzed after angular transformation by the formula y = arcsine [square root (×/100)]. When two means were compared, statistical analysis was carried out using the unpaired *t*-test. Two-way ANOVA and Tukey's test were applied in order to compare the means from the motility or acrosome reaction. $P < 0.05$ were considered statistically significant.

CTC results were analyzed using a modification of Cochran's χ^2 test that compares responses within replicates. A significant difference requires responses within replicates to be consistent and of a reasonable magnitude [16].

3 Results

3.1 Assessment of sperm motility after incubation with different concentrations of genistein

We sought to investigate differences in motion parameters of cryopreserved semen after treatment with different concentrations of genistein over time. As shown in Table 1, at all concentrations tested, genistein had no effect on total sperm motility. The differences in motility between samples were not statistically significant ($P > 0.05$). Similar results were obtained when hyperactive and linear motilities were assessed (data not shown). However, it was observed that, over time, the total motility decreased significantly in test and control spermatozoa. After 5 h and 6 h of incubation, the motility was significantly lower than that at time points 0, 1 and 2 h in all samples ($P < 0.01$).

3.2 Hemizona assay after incubation with different concentrations of genistein

Possible differences in the capacity of cryopreserved bull sperm to bind to homologous zona pellucida after treatment with different concentrations of genistein were evaluated by using the bovine hemizona assay. We determined that pre-treatment of spermatozoa with genistein affects zona pellucida binding in a dose-dependent manner. As depicted in Table 2, hemizonae incubated with spermatozoa pre-treated with 7.4 μmol/L or 0.74 μmol/L genistein

Table 1. Motility (%) of cryopreserved bovine spermatozoa after the incubation with different concentrations of genistein. Data are expresed as mean ± SEM ($n = 4$). $P < 0.01$ when a *vs.* b in each row. DMSO: dimethyl sulfoxide.

Treatment	Time points				
	0 h	1 h	2 h	5 h	6 h
Control (0.02% DMSO)	87.05 ± 2.23	85.53 ± 1.72[a]	87.53 ± 1.64[a]	49.18 ± 8.06[b]	35.93 ± 6.66[b]
Genistein (0.074 μmol/L)	89.45 ± 0.98[a]	82.78 ± 1.92[a]	82.25 ± 1.94[a]	33.15 ± 5.16[b]	36.78 ± 4.09[b]
Genistein (0.74 μmol/L)	87.85 ± 3.56[a]	88.63 ± 1.52[a]	86.75 ± 0.60[a]	42.23 ± 4.04[b]	38.55 ± 5.47[b]
Genistein (7.4 μmol/L)	85.50 ± 4.47[a]	87.33 ± 0.86[a]	80.45 ± 4.84[a]	32.90 ± 3.04[b]	28.05 ± 7.86[b]

Table 2. Binding capacity of cryopreserved bovine spermatozoa to zona pellucida after the incubation with different concentrations of genistein. HZI, hemizona index; *n*, number of oocytes used for hemizona assay.

Genistein concentration (μmol/L)	No. of test sperm bound to hemizona	No. of control sperm bound to hemizona	HZI (%)	*n*	*P* value
7.4	47.18 ± 3.88	82.45 ± 6.40	59.13 ± 4.37	11	< 0.0001
0.74	56.20 ± 10.68	74.50 ± 10.75	71.89 ± 5.41	10	0.0006
0.074	69.44 ± 10.62	72.11 ± 11.44	101.08 ± 6.93	9	0.88

revealed an average of 47.18 ± 3.88 and 56.20 ± 10.68 bound spermatozoa, respectively; whereas the corresponding hemizonae incubated with control spermatozoa yielded a mean value of 82.45 ± 6.40 and 74.50 ± 10.75 tightly bound cells, respectively. The calculated HZI (mean values of 59.13 ± 4.37 for 7.4 μmol/L and 72.11 ± 11.44 for 0.74 μmol/L) and the statistical analysis revealed a significant inhibition of sperm binding to the zona pellucida caused by genistein as compared to control cells ($P < 0.0001$ and $P < 0.001$, respectively). The results with test spermatozoa pre-incubated with 0.074 μmol/L genistein revealed no significant decrease of sperm-zona pellucida binding ($P > 0.05$).

3.3 Inducibility of acrosome reaction after the incubation with genistein

To evaluate the influence of genistein upon the inducibility of the acrosome reaction, capacitated genistein-treated spermatozoa and control spermatozoa were incubated with progesterone (1 μmol/L) or ZP3-6 peptide (1 μmol/L). A concentration of genistein that yielded clear effects upon sperm-zona pellucida interactions (7.4 μmol/L) was chosen for this set of experiments. The results of these experiments are depicted in Figure 1. The percentage of live, acrosome-reacted spermatozoa after incubation with genistein or DMSO (vehicle control) was 14.5 ± 0.5% and 12.6 ± 2.0%, respectively. After incubation of control spermatozoa with progesterone, the percentage of live, acrosome-reacted spermatozoa was increased to 26.2 ± 2.9% and almost identical results were obtained with ZP3-6 peptide (increased to 24.4 ± 2.4%). The differences observed were statistically significant for progesterone and for ZP3-6 peptide compared with the results before their treatment ($P < 0.01$). When spermatozoa were incubated in the presence of 7.4 μmol/L genistein, the induction of the acrosome reaction by progesterone or ZP3-6 peptide was inhibited to 15.3 ± 1.9% and 12.1 ± 1.5%, respectively.

3.4 Effect of genistein upon capacitation assessed by CTC

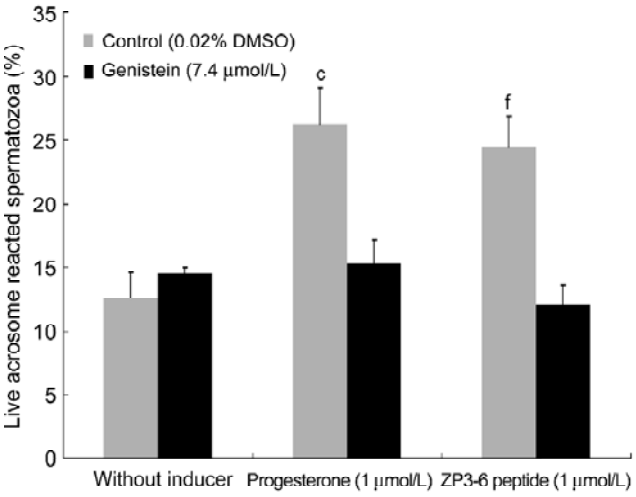

Figure 1. Inducibility of acrosome reaction in cryopreserved bovine spermatozoa after the incubation with 7.4 μmol/L genistein. Data are expressed as mean ± SEM ($n = 7$). $^{c}P < 0.01$, $^{f}P < 0.01$, compared with sample without inducer.

The distribution of the various CTC patterns (F, uncapacitated sperm; B, capacitated sperm; AR, acrosome reacted sperm) in cryopreserved genistein-treated and control spermatozoa are shown in Table 3. A change in the distribution of CTC patterns was observed at the end of the incubation time (T_1). During the incubation under capacitation conditions, both control and genistein-treated spermatozoa underwent capacitation as indicated by a time-dependent increase in the percentage of CTC pattern B ($P < 0.01$). The rise of the percentage of spermatozoa with pattern B was accompanied by a significant decline of the percentage of cells with pattern F over time ($P < 0.01$). The proportion of cells with the AR pattern increased slightly in the control sample ($P < 0.05$) and in the genistein sample (not significant). No differences in the distribution of CTC patterns between control and genistein samples could be determined.

Table 3. Capacitation status of cryopreserved bovine spermatozoa before (T_0) or after (T_1) the incubation with 7.4 μmol/L genistein. Data are expressed as mean ± SEM (%, n = 4). In each column a *vs.* c and b *vs.* d ($P < 0.01$); e *vs.* f ($P < 0.05$). CTC, chlortetracycline; F, uncapacitated cells; B, capacitated sperm; AR, acrosome-reacted cells.

Incubation time	Treatment	CTC pattern		
		F	B	AR
T_0	Control	84.75 ± 3.57[a]	11.25 ± 2.39[a]	4.00 ± 1.23[e]
	Genistein (7.4 μmol/L)	85.25 ± 3.15[b]	9.38 ± 3.01[b]	5.38 ± 1.21
T_1	Control	62.38 ± 6.81[c]	28.13 ± 6.26[c]	9.50 ± 1.06[f]
	Genistein (7.4 μmol/L)	63.63 ± 3.17[d]	27.63 ± 3.35[d]	8.75 ± 1.32

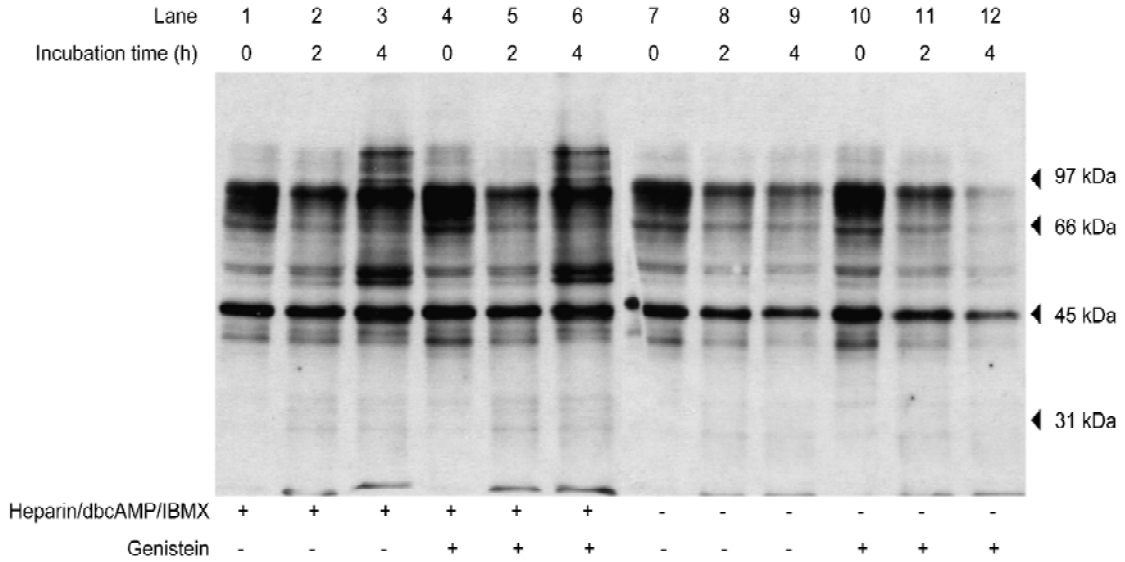

Figure 2. Profile of phosphotyrosine-containing proteins associated with capacitation of cryopreserved bovine spermatozoa after incubation with genistein and capacitation-enhancing substances (heparin, dbcAMP and IBMX). Molecular weight markers (kDa) are indicated to the right of the blots. A representative experiment is shown (n = 3).

3.5 Protein tyrosine phosphorylation patterns during capacitation

Tyrosine-phosphorylated proteins with apparent molecular masses of 20–210 kDa were immunodetected following incubation of cryopreserved bovine spermatozoa for 0, 2 and 4 h in capacitation medium (Ham F-10 supplemented with 0.3% BSA, with or without heparin, dbcAMP and IBMX). The profiles of phosphotyrosine-containing proteins associated with capacitation in frozen-thawed spermatozoa are depicted in Figure 2. Four observations were made: (1) The addition of genistein to the capacitation medium had no obvious effect upon the extent of tyrosine phosphorylation of proteins from cryopreserved bovine spermatozoa, independent of whether the sperm cells were incubated in the presence of the capacitation- and tyrosine phosphorylation-enhancing substances heparin, dbcAMP and IBMX; (2) For a number of sperm polypeptides (114, 105, 94, 56, 54, 45 and 43 kDa), the addition of heparin, dbcAMP, and IBMX to the capacitation medium led to an increase in tyrosine phosphorylation; (3) Incubation of cryopreserved bovine sperm without heparin, dbcAMP and IBMX yielded inverse effects for three polypeptides (56, 45 and 43 kDa; i. e. a decrease in the intensity of tyrosine phosphorylation), while the intensity of the immunostaining of another protein band remained stable (54 kDa). The decrease of intensity of protein phosphorylation could be related to an unequal blotting of proteins. However, almost identi-

cal protein loads in each lane could be visualized after Ponceau staining (not shown); (4) The extent of phosphorylation of only a few tyrosine-phosphorylated protein bands was not affected by heparin, dbcAMP and IBMX. The intensities of phosphorylation remained stable over time and were also not affected when these agents were omitted from the capacitation medium.

4 Discussion

It has been demonstrated that PTK activity plays a major role in human, mouse and bovine sperm functions [8, 17, 18]. Our results obtained with cryopreserved bovine spermatozoa show that the phytoestrogen genistein, which is known to inhibit PTKs, affects tyrosine phosphorylation-independent signal transduction pathways that play an important role in sperm function.

We sought to investigate whether genistein affects essential functions of cryopreserved spermatozoa. All functional experiments were performed with cryopreserved spermatozoa that were solely capacitated using Ham F-10 medium supplemented with 0.3% BSA for 4 h. We used this capacitation protocol because we found that by using cryopreserved semen effects on functions such as motility, capacitation as evaluated by the CTC method, induction of the acrosome reaction, or sperm-zona pellucida binding were evident without the addition of further capacitation-enhancing additives [12–15].

Our results, as evaluated by computer-assisted sperm motion analysis, revealed that the incubation of cryopreserved bovine spermatozoa with genistein over the range of concentrations from 0.074 μmol/L to 7.4 μmol/L showed no significant change in sperm-motion parameters. In cat spermatozoa, similar results were obtained. Genistein did not affect sperm percentage motility, forward progressive motility, or sperm motility index (SMI) when spermatozoa were treated with concentrations similar to that used in this study [10]. However, when higher concentrations of genistein are used, its effect on sperm motility is controversial, as discussed in the published literature. Bajpai *et al.* [19] showed an inhibitory effect of genistein (400 μmol/L) upon human sperm motility, while Uma *et al.* [20] showed no effect of 500 μmol/L genistein upon epididymal hamster spermatozoa. Mahony *et al.* [21] showed that treatment with genistein (10 μmol/L) had no effect on hyperactivated motility in the absence of caffeine and dbcAMP in cynomolgus monkey spermatozoa, but the tyrosine kinase inhibitor significantly decreased caffeine- and dbcAMP-stimulated hyperactivation in a dose-dependent manner. The differing results might be explained by different species or grade of sperm maturation, the different concentrations of genistein tested, or different test protocols including supplements to the media that were applied.

We also found that the CTC assay did not indicate that genistein affects capacitation of cryopreserved bovine spermatozoa because, in the presence of genistein, CTC data revealed an increase in spermatozoa with pattern B after an incubation time of 4 h. In epididymal mouse spermatozoa, genistein increases the capacitation process as revealed by the CTC assay, most probably via stimulation of adenylate cyclase/cAMP and not through inhibition of protein tyrosine phosphorylation [16].

In examining the induction of the acrosome reaction in cryopreserved-thawed bovine spermatozoa, we found that genistein clearly inhibits progesterone- and ZP3-6 peptide-induced acrosomal exocytosis. We also found that in capacitation medium devoid of heparin, dbcAMP and IBMX, progesterone and ZP3-6 peptide induce the acrosome reaction in cryopreserved bovine spermatozoa. In a recent study we demonstrated that the ZP3-6 peptide-induced acrosome reaction can be blocked by pretreatment of spermatozoa with pertussis toxin, while progesterone-induced acrosomal exocytosis is not affected [13]. Despite the fact that a G-protein-regulated, seven transmembrane-spanning sperm receptor for ZP3 is yet unknown, we concluded that pertussis toxin-dependent G proteins are involved in the ZP3-6 peptide-induced acrosomal exocytotic event. The progesterone-induced acrosome reaction, however, was not affected by the toxin [13]. It is important to note that both the progesterone and the ZP3-6 peptide-induced acrosome reactions are decreased by the addition of genistein. Therefore, our results suggest that genistein affects a pathway leading to the acrosome reaction upstream of pertussis toxin-sensitive G-proteins.

Furthermore, we demonstrated that a second crucial sperm function, sperm-zona pellucida binding as assayed by the HZI, was inhibited when spermatozoa were pretreated with genistein. It has to be mentioned that data obtained by using the HZI mainly reflect primary binding of spermatozoa to the zona pellucida. This event of primary loose binding occurs in the very beginning of sperm-oocyte interaction and exclusively involves spermatozoa that are not acrosome reacted. The observation that genistein initially decreases sperm-zona pellucida bind-

ing and, subsequently, inhibits the induction of the acrosomal exocytosis suggests that genistein possibly affects two distinct signal transduction pathways involved in sperm-egg interaction. Similar results were obtained with cat spermatozoa [10]. Exposure of spermatozoa to genistein, which did not influence sperm motility, markedly inhibited the ability of spermatozoa from normospermic cats to undergo the zona pellucida-induced acrosome reaction and to penetrate zona pellucida-intact oocytes. However, this effect of genistein was reported to be accompanied by an increase in tyrosine phosphorylation of two cat sperm proteins (95 kDa and 160 kDa) during capacitation.

In this study we show that in cryopreserved bovine spermatozoa, the addition of heparin, dbcAMP and IBMX induced an increase of polypeptides that are tyrosine phosphorylated, but the incubation with genistein does not prevent this phosphorylation. The protein tyrosine phosphorylation pattern was very similar to that previously described for fresh spermatozoa capacitated in the presence of heparin, dbcAMP and IBMX [17]. When cryopreserved spermatozoa were used and heparin, dbcAMP and IBMX were omitted from the capacitation medium, some of these proteins did not become tyrosine phosphorylated, or pre-existing phosphorylation decreased during capacitation. Under these conditions no effect of genistein upon phosphorylation could be observed. Recent results published by Cormier *et al*. [11] showed that capacitation induced either by heparin in fresh bovine spermatozoa or by cryopreservation is associated with a different profile of phosphotyrosine-containing proteins. Using fresh spermatozoa and heparin, two tyrosine-phosphorylated polypeptides (56 kDa and 114 kDa) that appeared after a 5-h capacitation period were identified, while there was no increase of intensity with or without heparin for nearly all of the phosphotyrosine-containing proteins in cryopreserved, thawed spermatozoa. We studied polypeptides of the same apparent molecular masses in cryopreserved semen and found that different phosphorylation patterns occurred when not only heparin but also dbcAMP and IBMX were added to the capacitation medium: tyrosine phosphorylation of several proteins including the 56 kDa and the 114 kDa polypeptides clearly increased independently of genistein. The omission of heparin, dbcAMP, and IBMX, however, resulted in the 114 kDa polypeptide, among other proteins, not becoming tyrosine phosphorylated or the intensity of tyrosine phosphorylation decreasing with the time of capacitation (including the 56 kDa protein). The results suggest that heparin-independent but cAMP-dependent pathways play a role in sperm protein tyrosine phosphorylation. The initial state of tyrosine phosphorylation in frozen-thawed spermatozoa could reflect capacitation-like changes that are similar to early membrane modifications that occur during physiological capacitation. Increase in protein phosphorylation in the presence of agents that elevate intracellular cAMP concentrations would support the hypothesis of Visconti *et al*. [5] that cryopreservation-dependent changes in membrane fluidity lead to an increase in calcium influx and thus stimulate adenylate cyclase, which initiates protein tyrosine phosphorylation. Interestingly, Cormier *et al*. [11] found a 35-kDa tyrosine-phosphorylated polypeptide in egg yolk-extended cryopreserved bovine spermatozoa that has not been observed in fresh spermatozoa. The authors stated that this 35-kDa protein most probably derives from the egg yolk extender because this protein could be immunodetected also in egg yolk alone. Our results support this assumption because we could not find this protein in immunoblots with spermatozoa that were cryopreserved in egg yolk-free extender. Our results strengthen the idea suggested in recent publications that the use of egg yolk-free extenders for biochemical experiments with cryopreserved semen might prevent artifacts because of egg yolk proteins that tightly adhere to cryopreserved spermatozoa [12, 15].

Our results support the hypothesis stated by Cormier *et al*. [11] that cryopreservation affects the regulatory mechanisms of capacitation. Cryopreserved bovine spermatozoa display an increase in capacitation (pattern B) as shown by the CTC assay despite the fact that no major increase in the intensity of protein tyrosine phosphorylation could be observed. Capacitation implies more than an increase in protein tyrosine phosphorylation. The increase in the number of spermatozoa showing pattern B (capacitated sperm) could be related to changes in the plasma membrane (e.g. removal of cholesterol through BSA), the influx of calcium, or other changes associated with capacitation that are not affected by genistein.

The direct effects of genistein upon spermatozoa can be manifold. As discussed above, inhibition of tyrosine kinases by genistein is inconsistent with our data. Effects on membrane fluidity cannot be excluded; however, submicromolar concentrations of this compound are not expected to have such effects. Another possibility could be a direct block of ion channels by genistein, as has been reported for

voltage-sensitive sodium channels in rat brain neurons [22] or for cardiac L-type calcium channels [23].

We know that genistein disturbs capacitation processes in fresh spermatozoa through PTK inhibition. However, the present study suggests that this effect is overcome by cryocapacitation. Sperm motility and capacitation as evaluated by the CTC assay are not affected by genistein. However, genistein still prevents induction of the acrosome reaction and sperm-zona pellucida binding, probably by triggering a process that is independent of its well-known function of PTK inhibition.

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