Involvement of intracellular calcium and *src* tyrosine-kinase in capacitation of cryopreserved bovine spermatozoa

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Summary

Increase of intracellular calcium concentration and tyrosine kinase involvement are pivotal in sperm capacitation. The aim was to determine the involvement of intracellular calcium and tyrosine kinases activity in frozen-thawed spermatozoa capacitated with heparin or quercetin. Genistein or PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo3,4, pyrimidine) were used to inhibit tyrosine kinases, and methoxyverapamil to inhibit voltage dependent calcium channels. Capacitation was determined by chlortetracycline and calcium by fluorescence spectrophotometry. Protein tyrosine-phosphorylation was determined by western blot. Pooled frozen semen samples from four bulls were used. In presence of heparin or quercetin capacitated spermatozoa percentage and intracellular calcium were greater ($P<0.05$) than in controls. Genistein, or PP2 addition in heparin treated samples decreased ($P<0.05$) capacitation and calcium, but not in quercetin treated samples. A differential inhibition of protein-phosphorylation pattern was observed in heparin or quercetin treated suspension in presence of genistein or PP2. Methoxyverapamil blocked calcium increase in heparin, but not in quercetin treated samples. Capacitated spermatozoa percentage and calcium increase were similar with both inductors suggesting that they are capable to provoke capacitation by different mechanisms that dependent or not dependent on voltage dependent calcium channels, thus different intracellular compartments may be involved. In cryopreserved bovine spermatozoa tyrosine kinases including SRC-isoform modulate capacitation.

Keywords: (calcium), (bovine capacitation), (SRC tyrosine kinases).

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Participación del calcio intracelular y src tirosina quinasas en la capacitación del espermatozoide bovino criopreservado.

Resumen

El incremento de calcio intracelular [Ca]i y la participación de tirosina quinasa son pasos cruciales en la inducción de la capacitación. El objetivo fue determinar en espermatozoides criopreservados, la variación [Ca]i y la actividad de tirosina quinasa en la capacitación con heparina o quercetina. Genisteina o PP2(4-amino-5-(4-clorofenil)-7-(t-butil)pirazolo3,4,d pirimidina) son inhibidores de tirosinas quinasas y de la isoforma SRC, respectivamente. Metoxiverapamil es un inhibidor de canales de calcio voltaje dependiente (CCVD). La capacitación, [Ca]i y fosforilación en tirosina se evaluaron con clorotetraciclina, espectrofluorometría y western blot, respectivamente. El porcentaje de espermatozoides capacitados y [Ca]i fueron mayores ($P<0.05$) en muestras con heparina o quercetina respecto a sus controles. Genisteina o PP2 disminuyeron ($P<0.05$) la capacitación y el incremento de [Ca]i en las muestras con heparina pero no en las tratadas con quercetina. Genisteina o PP2 inhibió diferencialmente la fosforilación de proteínas espermáticas con ambos inductores. Methoxiverapamil bloqueó el incremento de [Ca]i sólo en la muestras con heparina. Siendo los espermatozoides capacitados y [Ca]i similares con heparina o quercetina, sugiere que la capacitación con ambos inductores es inducida por mecanismos dependientes y no dependientes de CCVD, involucrando así diferentes compartimientos intracelulares. En bovino, las tirosinas quinasas, incluída la isoforma SRC, modulan la inducción de la capacitación.

Palabras clave: (calcio), (capacitación bovina), (SRC), (tirosina quinasa).

Introduction

During sperm capacitation, two relevant events occur the elevation of intracellular calcium and adenyl cyclase activation that provokes protein kinase A activation which phosphorylates a series of proteins[3]. During this sperm process, membrane proteins and lipid organization change dramatically[13].

During capacitation and fertilization, the main tyrosine- phosphorylated proteins are located into the flagellum, although tyrosine-phosphorylation of less abundant protein may also occur in sperm head[28]. Indeed, factors with a role in regulating capacitation also regulate tyrosine- phosphorylation, cAMP promotes sperm tyrosine-phosphorylation[15] and the cholesterol removal is regulated by cyclic AMP-dependent phosphorylation pathway[14]. Tyrosine phosphorylation of proteins in the flagellum has been linked to mammalian sperm hyperactivation[22], which is required to penetrate the cumulus and the zona pellucida of the oocyte[10]. Protein tyrosine-phosphorylation increases during capacitation of mice, humans, cattle spermatozoa[20, 15]. In human spermatozoa, a novel SRC isoform has been identified, which appears to be involved in regulating capacitation, calcium fluxes, tyrosine- phosphorylation and acrosome reaction[29]. In this regard, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo 3,4,d pyrimidine (PP2) is a potent and selective inhibitor of the SRC family tyrosine kinases and it is a useful tool for investigating the involvement of SRC family tyrosine kinases[18]. For fertilization, the sperm phosphorylation pattern and its regulation are interesting and relevant factors to be investigated.

Calcium signals regulate a number of functions in spermatozoa, such as capacitation[27] and hyperactivation[21]. In bovine, heparin capacitated spermatozoa; methoxyverapamil (specific inhibitor of voltage dependent calcium channel type L) addition provokes the inhibition of the intracellular calcium concentration increase[6]. Calcium - ATPase, present in the plasma membrane has been involved in the capacitation induction. The addition of quercetin as specific inhibitor of this enzyme provoked capacitation induction in mouse, human and bull spermatozoa[6, 11, 3]. However, the mechanisms and signal transduction pathways involved in capacitation have not been completely clarified yet. The aim of this work was to study, in cryopreserved bovine spermatozoa the participation of intracellular calcium variation, the role of SRC tyrosine kinase and voltage dependent calcium channels activation in heparin and quercetin capacitation induction.

Materials and methods

Unless otherwise indicated, chemicals used were purchased from Sigma Chemical Company (St Louis, MO, USA). Anti-phosphotyrosine antibody Clone 4G10 was from Upstate, Lake Placid, NY, USA and peroxidase conjugated goat anti-mouse antibody Inmun-Star Goat Anti-mouse HRP Conjugate was from Biorad, Hercules, USA. Western blot equipment was Mini Protean 3 cell from Bio-Rad Laboratories, Hertforshire, England.

Semen collection and freezing

Semen was collected by an artificial vagina from four Holstein bulls (4 to 5 yr old) of proven fertility. These bulls were routinely used to provide semen for artificial insemination; they were maintained under uniform nutritional and management conditions throughout the study[7].

Sperm suspension

Pooled frozen semen samples from the four bulls were thawed for 10 min at 37 ºC in F3MB medium[25]. Vigor and percentage of cells with progressive motility were evaluated at 38ºC using light microscopy. Samples with 60% average progressive motility and a vigor score of 3 to 4 (scale, 0 to 5) were considered suitable for the experiments. Sperm concentration was determined by hematocytometry in a Neubauer chamber. After the evaluation of motility, samples were centrifuged (600 x g for 5 min) and resuspended in F3MB at 38ºC for 15 min in the presence of no additional compounds (control), or with the addition of 60 µg mL -1 heparin[6, 12], or 50 µmol L-1 quercetin[11] (the latter was used as a specific calcium-dependent plasma membrane ATPase inhibitor). Control, quercetin or heparin treated spermatozoa were incubated at 38ºC during 15 min. The control was sperm suspension in F3MB medium with calcium and albumin without capacitation inductors.

Determination of sperm capacitation using chlortetracycline

Percentages of capacitated spermatozoa were determined by the epifluorescence chlortetracycline technique (CTC). To account for the percentage of spermatozoa with the capacitated pattern induced by freezing and thawing, the percentage of capacitated spermatozoa obtained at zero time was subtracted from results after incubation (for each treatment group)[2].

Sperm viability and acrosome integrity

An aliquot of the sperm suspension from each different treatment was incubated with an equal volume of 0.25 % (w/v) Trypan blue. Acrosomes from the different sperm samples stained with Trypan blue were evaluated by differential interference contrast (DIC) microscopy (200 spermatozoa per sample) to assess acrosome integrity in live and dead spermatozoa. To account for spontaneous damage, the value obtained at time zero was subtracted from the values obtained after treatment[7].

Isolation of sperm proteins, SDS-PAGE and Western inmunoblotting

Samples were prepared, separated and evaluated according to Satorre[24]. Frozen-thawed samples were centrifuged for 5 min at 600 x g to separate freezing extender or seminal plasma, and then spermatozoa were resuspended in Beltsville thawing solution (BTS) to a final concentration of 4 x 108 spermatozoa ml-1. Sodium orthovanadate (0.2 mmol L-1) was added to aliquots (115 µL) of sperm suspension and then samples were centrifuged to obtain sperm pellets (6 min, 11190 x g, 4 ºC), which were resuspended in sample buffer without β-mercaptoethanol and heated for 5 min at 100 ºC. The sperm suspension was centrifuged again (30 min, 11190 x g, 4 ºC) and β-mercaptoethanol (5%) was added to the supernatant. Samples were stored immediately at –18 ºC until electrophoresis was performed. Sperm protein samples were heated for 5 min at 95 ºC. Equivalent aliquots of control and treated spermatozoa were loaded on 12% SDS-polyacrylamide gels (6 x 106 spermatozoa, 16 µL per lane). Separated proteins were transferred electrophoretically to nitrocellulose membranes, checking the efficiency for transferring by staining with Red Ponceau (0.5% in 1% acetic acid). Non-specific protein binding sites on the membrane were blocked with 5% dry non-fat milk in Tris-buffered saline (TBS: 25 mmol L-1 Tris-HCl, 150 mmol L-1 NaCl) overnight at 4°C. Membranes were washed 1 x 15 min, 2 x 5 min with fresh TBS-Tween (0.1% Tween 20, 20 mmol L-1 Tris-HCl, 136 mmol L-1 NaCl). Blots were incubated during 60 min at room temperature with anti-phosphotyrosine antibody (Clone 4G10, Upstate, Lake Placid, NY, USA) diluted 1:1000 in 3% dry non-fat milk in TBS-Tween, washed 1 x 15 min, 2 x 5 min with TBS-Tween, and then incubated for 60 min at room temperature with peroxidase conjugated goat anti-mouse antibody (Inmun-Star Goat Anti-mouse HRP Conjugate, Biorad, Hercules, USA) diluted 1:1000 in 3% dry non-fat milk in TBS-Tween. Finally, blots were washed again 1 x 15 min, 4 x 5 min with TBS-Tween. Labeled tyrosine phosphoproteins were visualized using a chemiluminescence detection kit (ECL, Amersham Biosciences, Piscataway, NJ, USA). Then, the photographic film was scanned and quantified by measuring the intensities of each digitalized band using Image J version 1.240 software, (National Institutes of Health). The intensities of protein tyrosine phosphorylation were measured by converting them into peaks and were expressed as arbitrary units (AU). These units were calculated as the peak area of the band of the phosphoprotein visualized by chemiluminescence over the peak area of the band found in the same lane previously stained with Red Ponceau.

$$AU = \frac{\text{Peak area of the phosphoprotein determined by chemiluminiscence}}{\text{Peak area of the band stained with Red Ponceau}}$$

Voltage dependent calcium channel inhibition

Metoxyverapamil (60 µmol L^{-1})[6] was used as specific inhibitor of voltage dependent calcium channels type L during heparin or quercetin capacitation at 38ºC during 15 min.

Tyrosine kinase inhibition

PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo 3,4,d pyrimidine) (10 µmol L^{-1}) was used as a potent and selective inhibitor of the src family tyrosine kinases[18], Genistein (150 µmol L^{-1}) was used as specific tyrosine kinase inhibitor[20]. They were added with heparin or quercetin to the sperm suspensions and then were incubated at 38ºC during 15 min.

Intracellular calcium concentration

A fluorescence spectrophotometer (SHIMADZU, Tokyo Japan) was used for measurement of intracellular calcium with FURA 2AM (1µmol L^{-1}) during 45 min at 38°C at 340/380 nm excitation and 510 nm emission. Fluorescence was converted to intracellular calcium concentration according to the formula described by Grynkiewicz. Rmax and Rmin were

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determined in the presence of 0.01 % (wt/vol) digitonin and 15 mmol L^{-1} EGTA (ethylene-glycol-bis (beta aminoethyl-ether) N,N,N,N´-tetraacetic acid). Final sperm concentration was 1 x 10^{7} spermatozoa / ml in the measurement cuvette at 38 °C[17].

Statistical analysis

Differences among groups of percentages of capacitated spermatozoa, sperm viability, and intracellular calcium concentration were determined with ANOVA; Tukey's test was used as a post-ANOVA analysis to compare means (STATISTIX 7. 2000, Analytical Software for Windows, Version 7.0; Analytical Software, Tallahassee, Florida, United States). Intensity of protein tyrosine phosphorylation values were expressed as means ± SD. Bartlett's test was used to determine equal variances. For all analyses, $P<0.05$ was regarded as significant.

Results

A similar percentage of capacitated spermatozoa was observed with either heparin or quercetin; both were higher than the control value ($P < 0.05$). Capacitation was evaluated using the chlortetracycline fluorescence assay through the appearance of pattern B stained spermatozoa (Table 1). Furthermore, all the different treatments did not significantly modify the percentage of acrosome integrity and sperm viability respect to control (55.0 ± 10.0 %). Heparin and quercetin capacitation was completely inhibited by PP2 (10 µmol L^{-1}) (Table1). The inhibition of voltage dependent calcium channel by methoxyverapamil, (60 µmol L^{-1}), reduced the intracellular calcium increase in heparin capacitated spermatozoa but in heparin and quercetin treated spermatozoa, capacitation percentage decreased ($p<0.05$) (Table 2).

Intracellular calcium concentration was determined in heparin and quercetin capacitated spermatozoa. The heparin cellular action or the inhibition of calcium -ATPase induced a similar intracellular calcium increase which was significantly inhibited by PP2 (10µmol L^{-1}), a specific inhibitor of SRC tyrosine kinase (Table 1). In heparin capacitated spermatozoa, genistein (150µmol L^{-1}) and PP2 provoked a similar inhibition on intracellular calcium increase ($p>0.05$) (Table 1).

Capacitation induction by heparin or quercetin modified the pattern of protein tyrosine phosphorylation. Several bands of tyrosine phosphorylated proteins of 60, 55, 35 and 30 kDa were detected in all treatments (Figure 1). Tyrosine-phosphorylation of 55 and 35 kDa proteins was increased in heparin treated spermatozoa respect to quercetin treatment

Table 1: intracellular calcium concentration and capacitation percentage. PP2 and genistein inhibitory effects on capacitated spermatozoa percentage and intracellular calcium concentration was showed in this table. The percentage was determined by CTC technique. Bovine spermatozoa were capacitated with heparin or quercetin, with and without tyrosine kinase inhibitors. Superscript symbols and letters indicate significant differences between treatments ($P<0.05$). Data are expressed as mean (±SD; 7 replicates for each treatment).

Treatments	Intracellular calcium concentration (nmol L^{-1})	Capacitation percentage (%)
Calcium basal	130.8 ± 45.20 [a]	10.8 ± 3.6 *
Heparin	414.0 ± 108.0 [b]	38.3 ± 9.0 **
Heparin/ PP2	256.25 ± 16.61[c]	8.0 ± 3.1 *
Heparin /genistein	226.00 ± 74.00 [c]	11.0 ± 3.3 *
Quercetin	447.0 ± 118.0 [b]	35.6 ± 9.7 **
Quercetin/PP2	240.57 ± 52.25[c]	10.0 ± 2.0 *

Table 2: methoxyverapamil effect on intracellular calcium concentration and capacitation induction. Bovine spermatozoa were capacitated with heparin (60µg/mL) or quercetin (50 mmol L-1). Samples were treated with and without methoxyverapamil (M) (60µmol L-1). Data are expressed as mean (±SD; 7 replicates for each treatment). Within a column, entries without a common superscript letters (a-c) differ (P<0.05).

Treatments	Intracellular calcium concentration (nmol L^{-1})	Capacitation percentage (%)
Basal calcium	130.8 ± 45.20[a]	10.8 ± 3.6*
Heparin	414.0 ± 108.0[b]	38.3 ± 9.0**
Heparin/ methoxyverapamil	194.4 ± 33.3[a]	14.6 ± 2.6[a]
Quercetin	447.0± 118.0[b]	35.6 ± 9.7[b]
Quercetin/methoxyverapamil	443.5 ± 101.0[b]	23.0 ± 4.6[c]

and control, 35 kDA protein band decreased because of the inhibition effect of genistein or PP2. In the presence of quercetin, tyrosine phosphorylation of 55 and 35 kDa proteins has a different response to presence of genistein or PP2 (Figure 1). Capacitation induction by heparin or quercetin induced phosphorylation of 35 kDa protein which was inhibited by PP2 and genistein. In heparin capacitated spermatozoa, PP2 provoked a higher inhibition than genistein in the tyrosine phosphorylation in contrast to the effect of these inhibitors on quercetin treated spermatozoa. In quercetin treated samples, with both inhibitor of tyrosine kinases (PP2 and genistein), it was observed a significantly higher band intensity in 60 kDa in contrast to the intensity observed in the rest of sperm treatments (p>0.05) (Figure 1).

Discussion

In several mammalian species, there is an increase in the intracellular calcium concentration during sperm capacitation but all the mechanisms of regulation have not been fully elucidated yet. Cryopreservation provoked a precapacitated state in bovine spermatozoa and it has been demonstrated that quercetin and heparin induce a different metabolic pattern, with a low sperm oxygen consumption in contrast to the sperm respiratory burst produced by heparin capacitation, in bovine[7]. Futhermore, glucose is needed to support gamete fusion and to induce protein tyrosine phosphorylation during capacitation in mouse and bovine spermatozoa[26, 5]. So it was important to investigate how tyrosine kinases including SRC isoform, calcium and voltage dependent calcium channels (VDCC) activation would be involved in heparin and quercetin capacitation induction. Both inductors can induce capacitation and generate differential metabolic pathways as was demonstrated by Córdoba et.al[7]. Our data suggest that in bovine capacitated spermatozoa, heparin and quercetin increase intracellular calcium to similar levels but with different intracellular signals depend on tyrosine kinases including SRC isoform.

The present data demonstrated that intracellular calcium increase, induced by quercetin, is not dependent on VDCC activation but quercetin capacitation induction depends on this activation. In bovine capacitation, the initial flux of calcium is used to fill an intracellular calcium store located in the acrosome[23] this differential distribution of intracellular calcium may be responsible for the different pattern of tyrosine phosphorylation and different response to methoxyverapamil in bovine capacitation induction.

In bovine cryopreserved spermatozoa, heparin induces a different metabolic pattern

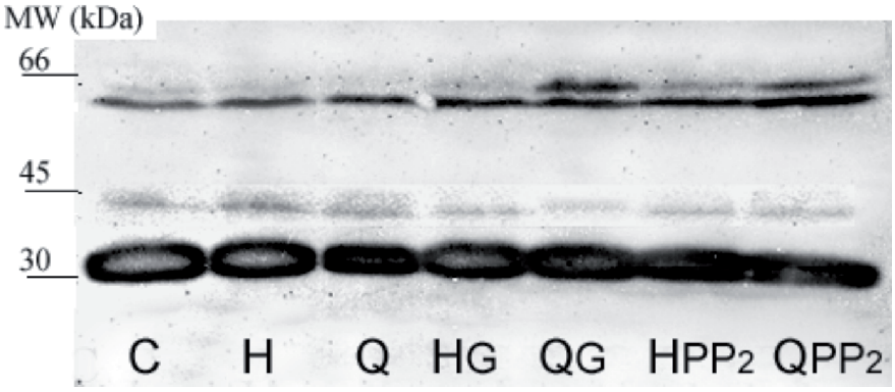

Figure 1: effect of different inductors with and without inhibitors of tyrosine phosphorylation in capacitation of cryopreserved bovine spermatozoa. Protein were extracted and used to determine sperm tyrosine- phosphorylation (western blot). In all cases, extracts corresponding to 6x106 spermatozoa were loaded per lane. Heparin (H) and quercetin (Q) were used as capacitation inductors. C (control). PP2 and genistein (G) were used as specific tyrosine phosphorylation inhibitors. Results are representative of three independent replicates.

and redox state levels respect to quercetin, furthermore differential activities of isocitrate dehydrogenase and malate dehydrogenase (NAD /NADPH) and lactate dehydrogenase are involved in heparin and quercetin capacitation[7, 8]. According to Travis et al, ATP was strictly required for protein tyrosine phosphorylation[26] and NADPH can modulate protein tyrosine phosphorylation in human[1] and mouse spermatozoa[28]. It is of interest that SRC tyrosine kinase activity can also be positively modulated by hydrogen peroxide consistent with a role for this kinase in the redox regulation of the signaling cascades of human sperm capacitation[1]. Therefore, several enzymes and metabolic processes seem to be involved in sperm protein tyrosine phosphorylation pattern, in the present study we have shown that heparin and quercitin, in accordance with their differential metabolic effect, induce a differential tyrosine phosphorylation patterns in capacitated bovine spermatozoa. It has been shown that, in bovine spermatozoa, hyperactivation is dependent primarily upon intracellular calcium and can occur separately to tyrosine phosphorylation[21]. Tyrosine phosphorylated proteins acquires the capacity to bind calcium during capacitation and may therefore play a role in calcium sequestration and release in the principal piece, establishing a link between tyrosine phosphorylation and calcium, regulating sperm hyperactivated motility[19]. We found several bands of tyrosine-phosphorylated proteins, of 60, 55, 35 and 30 kDa. Heparin induces an increase in tyrosine-phosphorylation of 55 and 35 kDa proteins and the 35kDa protein band decreased by genistein or PP2 inhibition. However, quercetin capacitated spermatozoa has a differential tyrosine phosphorylation of 55 kDa proteins respect to heparin capacitation. It is also different the response to genistein or PP2 inhibitors. In accordance with this data, tyrosine phosphorylated proteins are associated with the fibrous sheath; soluble proteins may also be associated with motility, as exemplified by the 55 kD, a tyrosine phosphorylated soluble protein linked to motility in bovine spermatozoa[30]. Quercetin capacitated spermatozoa, showed high band intensity in 60 kDa in the presence of tyrosine kinase inhibitors, it may be indicate that other tyrosine kinases not sensitive to PP2 or genistein are modulated the capacitation process. In bovine spermatozoa, heparin increased flagellar movements[4]. From the present data, we inferred that heparin and quercetin induce a distinct tyrosine phosphorylation pattern in spermatozoa with the same calcium concentration related to different calcium distribution and sperm metabolism conditions. In addition, the intracellular calcium increase

induced by heparin and quercitin was inhibited by approximately 50% in the presence of the tyrosine kinase inhibitor genistein or the more specific SRC inhibitor PP2. In these experiments the capacitation induction was totally blocked in the presence of these inhibitors. These results suggest that tyrosine kinases, including SRC enzymes are involved in the calcium signals regulation to allow bovine capacitation induction. It is possible that intracellular oxidative conditions induced by both inductors, may be involved in the different tyrosine phosphorylation protein bands observed, according with the influence that superoxide and hydrogen peroxide have on human sperm function and tyrosine phosphorylation[1, 20]. In this regard, quercetin and heparin capacitated spermatozoa had the same level of oxidative stress but the inhibition of NADPH oxidase blocked the capacitation induction in quercetin and heparin treated spermatozoa[8, 9].

Furthermore it has been demonstrated an inverse relationship between capacitation and apoptosis signal transduction related to caspase activation and disruption of the transmembrane mitochondrial potencial correlated negatively with the amount of tryrosine phosphorylation and the percentage of hyperactivated spermatozoa and positively with the number of non capacitated spermatozoa[16]. According with our data, intracellular calcium variation may be a crucial point related to tyrosine phosphorylation, metabolism and apoptosis signals in order to allow capacitation induction.

In conclusion, in cryopreserved bovine spermatozoa, the intracellular condition generated by heparin or quercetin play an important role to provoke different protein tyrosine phosphorylation and voltage calcium channels activations to allow capacitation induction with same level of intracellular calcium and SRC tyrosine kinase participation.

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