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Effect of xanthine-xanthine oxidase-catalase system on bovine sperm oxidative metabolism during capacitation induction

Efecto del sistema xanthine-xanthine oxidase-catalasa en el metabolismo oxidante de esperma bovina durante la inducción de la capacitación

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RESUMEN

El objetivo de este trabajo fue estudiar el efecto de heparina o el sistema xantina-xantino oxidase catalase (sistema generador de anión superóxido) en la inducción de la capacitación, consumo de oxígeno, la variación de la actividad de creatina quinasa B, y su regulación por tirosina quinasa y proteína quinasa C en presencia de heparina. Genisteina y GF 109203X fueron usados como inhibidores específicos de la tirosina quinasa y proteína quinasa C respectivamente. La actividad de creatina quinasa-B y la lipoperoxidación fueron registradas espectrofotométricamente. El consumo de oxígeno fue polarográficamente. Se evaluó viabilidad e integridad acrosomal por la coloración de azul tripán y microscopia óptica de Contraste Diferencial Interferencial y la capacitación por la coloración epifluorescente de Clorotetraciclina (CTC). Los datos fueron analizados por ANOVA y test de Tukey ($P<0,05$). La lipoperoxidación se modificó debido a los tratamientos ($P<0.05$). En la capacitación espermática con heparina la adición de genisteina o GF 109203X provocó una disminución de la actividad creatina quinasa B, toma de oxígeno y el porcentaje de capacitación ($P<0.05$). En la presencia del sistema xantina-xantino oxidase catalase, la capacitación y el consumo de oxígeno presentaron una disminución significativa a los 15 y 45 minutos, comparados con el valor del tratamiento con heparina, pero la actividad de la creatina quinasa B sólo disminuyó a los 45 minutos ($P<0.05$).

En conclusión, la capacitación con heparina está vinculada con burst respiratorio y con la disminución de la creatina quinasa B, ambos procesos dependientes de la proteína quinasa C. El anión superóxido induce la capacitación espermática y la lipoperoxidación lo cual provoca una alteración dependiente del tiempo de incubación. Este efecto deletéreo puede modificar el funcionamiento de la cadena respiratoria con la concomitante reducción de la actividad de la creatina quinasa B, enzima de la lanzadera citosólica-mitocondrial, impidiendo que el espermatozoide tenga la carga energética necesaria para adquirir su capacidad fertilizante.

Palabras clave: capacitación, metabolismo, espermatozoide bovino, anión superóxido

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ABSTRACT

The aim of this work was to study the effect of heparin or xanthine-xanthine oxidase-catalase system (superoxide anion generating system) on the induction of capacitation, sperm oxygen uptake, the variation in the activity of creatine kinase-B in bovine spermatozoa and tyrosine kinase and protein kinase C regulation with heparin. Genistein and GF 109203X were used as specific inhibitors of tyrosine kinase and protein kinase C, respectively. Creatine kinase-B activity and lipid peroxidation were registered spectrophotometrically. Oxygen uptake was measured polarographically. Capacitation was evaluated by chlortetracycline technique and viability by trypan blue stain. Data were analyzed by ANOVA and Tukey test. Lipid peroxidation was modified by treatments ($P<0.05$). In sperm capacitated with heparin the addition of genistein or GF 109203X, provoked a decrease in creatine kinase-B activity, oxygen uptake and capacitation rates ($P<0.05$). In the presence of xanthine-xanthine oxidase/catalase system, capacitation and oxygen uptake presented a significant decrease at 15 and 45 min compared to heparin treatment but creatine kinase-B activity only presented a decrease at 45 min ($P<0.05$).

To conclude, in cryopreserved bovine spermatozoa heparin capacitation is related to a respiratory burst and a decrease in creatine kinase-B activity, both processes dependent of tyrosine kinase and protein kinase C regulation. Superoxide anion induces sperm capacitation and lipid peroxidation, which can provoke mitochondrial membrane alteration, depending on the incubation time. This deleterious effect may modify the respiratory chain function with a concomitant reduction in creatine kinase-B activity, an enzyme of the cytosol- mitochondria shuttle, not allowing the sperm to reach the energetic state necessary to achieve fertilizing capability.

Key words: capacitation, metabolism, bovine spermatozoa, superoxide anion

INTRODUCTION

Capacitation is a process that prepares spermatozoa for acquiring fertilizing capability. Extensive research has been developed in order to elucidate the protein phosphorylation event during sperm capacitation and acrosome reaction wherein three pathways are mainly involved: cAMP/Protein kinase A (PKA), receptor tyrosine kinases, and non-receptor protein tyrosine kinases [1,2,3,4]. It is known that the phosphorylation of sperm proteins is an important aspect of capacitation and has been associated with sperm hypermotility, ZP binding and acrosome reaction [5,6]. In mammalian spermatozoa, capacitation is dependent on tyrosine kinase and protein kinase C activities and the variation of intracellular calcium concentration [7,8,9]. During the course of capacitation and fertilization, the main tyrosine- phosphorylated proteins are located in the flagellum, while they are less abundant in the sperm head[10].

Protein kinase C (PKC) roles in spermatozoa have been widely investigated in many species. This enzyme participates in many sperm functions, i.e. capacitation, acrosome reaction, and sperm motility[11]. It has been demonstrated that PKA activation leads to PKC inhibition[11]and phosphatidylinositol 3-kinase (PI3K) activation, allowing actin polymerization [12]. Moreover, a role for PI3K in sperm capacitation and acrosome reaction has been described [13].

Many of the intracelullar signals that occur during capacitation and acrosome reaction are regulated by the redox state of the sperm [14]. Futhermore, it was demonstrated that the induction of capacitation, acrosome reaction and hyperactivation are associated with superoxide anion and hydrogen peroxide [15,16,17]. Xanthine-xanthine oxidase / catalase system may be used to generate both types of ROS in bovine cryopreserved spermatozoa *in vitro* [18].

The excessive production of superoxide and hydrogen peroxide in the spermatozoa can initiate peroxidation. As a consequence, spermatozoa that have suffered oxidative stress

would be characterized by the accumulation of lipid hydroperoxides in their plasma membranes, which would be relatively stable until induced to decompose and liberate malondialdehyde upon addition of the ferrous ion promoter used in the TBA assay. The build-up of lipid hydroperoxides in the sperm plasma membrane clearly had a profound negative impact on sperm function [19].

There is evidence that capacitation is part of an oxidative process. In bovine spermatozoa, heparin induces capacitation and this process involves NADPH oxidase activity, intracellular calcium increase and a respiratory burst [17, 20]. It was also suggested that a certain ROS level and high respiratory activity in heparin-treated spermatozoa may be the factors associated with the alteration of the phosphocreatine/creatine shuttle function [8].

It has been demonstrated that in cryopreserved bovine spermatozoa, mitochondria preserve respiratory coupling for ATP synthesis for sperm function. Creatine kinase isoenzymes have been involved in the metabolism of bovine spermatozoa [21] and have been found to be specifically located at sites which have a high demand and production of energy [22].

In order to contribute to increase the knowledge of the different mechanisms involved in capacitation, the aim of the present work was to determine the influence of xanthine-xanthine oxidase -catalase system in this process, studying tyrosine kinase and PKC regulation, sperm oxygen uptake and creatine kinase-B activity variation, as an enzyme of the phosphocreatine/creatine shuttle, in cryopreserved bovine spermatozoa.

MATERIALS AND METHODS

Semen collection and freezing

Semen was collected by an artificial vagina from four Holstein bulls (4 to 5 years old) of proven fertility. These bulls were routinely used to provide semen for artificial insemination and they were maintained under uniform nutritional and management conditions throughout the study. The ejaculates were pooled and diluted in a buffer containing 0.20 M Tris, 0.06 M citrate, 0.13 M glycine, 0.06 M fructose, 20% egg yolk and 7% glycerol at a 2:1 ratio. A slow cooling curve at 5ºC (1ºC/min) was performed, and semen was then equilibrated at 5ºC for a further 90 min and then preserved at -196ºC in liquid nitrogen [21].

Sperm suspension

Pooled frozen semen samples collected from the four bulls were thawed for 10 minutes at 37ºC in TALP medium (99 mM NaCl, 3.1 mM KCl, 0.35 mM $NaH_2PO_4.H_2O$, 1.1 mM $MgCl_2.H_2O$, 25 mM $NaHCO_3$, 1 mM sodium pyruvate, 21.6 mM sodium lactate, and 10 mM HEPES) without bovine serum albumin (BSA) or calcium. The vigor score and the percentage of cells with progressive motility were evaluated at 38ºC using light microscopy. Samples with 60% average progressive motility and a 3 to 4 (scale 0 to 5) vigor score of were considered suitable for the experiments. Sperm concentration was determined by hematocytometry in a Neubauer chamber. Subsequent to the evaluation of motility, the samples were centrifuged (600 x *g* for 5 minutes) and resuspended in TALP with 2.1 mM calcium chloride and 6 mg/mL BSA. In order to induce capacitation, this sample suspension was incubated at 38ºC in the presence of no additional compounds (control), 60 µg/mL heparin 15 min [20,23] or 0.5 mM xanthine ; 0,05 mUI/mL, xanthine oxidase 100 mg/mL, catalase [24] at 15 and 45 min incubation.

Sperm viability and acrosome integrity

An aliquot of the sperm suspension taken from each different treatment was incubated with an equal volume of 0.25 % (w/v) Trypan blue in TALP for 15 minutes at 38ºC, centrifuged at 600 *g* for 10 minutes to remove excess stain and then fixed with 5% formaldehyde in PBS. Acrosomes obtained from the different sperm samples stained with Trypan blue were evaluated by differential interference contrast (DIC) microscopy (200 spermatozoa per sample) in order 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 each treatment [17].

Determination of sperm capacitation using chlortetracycline technique

The percentages of capacitation for the different treatments were determined by the epifluorescence chlortetracycline technique. 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) [25].

Preparation of extracts for the measurement of enzymatic activity

Suspensions of capacitated and control spermatozoa were centrifuged at 2000 rpm for 5 min. The pellets were resuspended in distilled water and an aliquot (20 µL) was used to determine sperm concentration. The final sperm concentration was adjusted to 1.0 x 10^8 spermatozoa/mL. The samples were frozen for 2 h at -20ºC, thawed at room temperature, refrozen (45 min) and thawed again at room temperature. Samples were then centrifuged (17000 rpm for 20 min at 4ºC). Supernatants were used to determine the enzymatic activity of creatine kinase-B. All replicates were processed by the same standard operating procedure to obtain enzymatic extracts; therefore, protein recovery was equivalent in all cases [8].

Determination of creatine kinase-B activity

Suspensions of capacitated spermatozoa and controls were centrifuged at 2000 rpm for 5 min. The pellets were resuspended in distilled water, and then treated as described in the section above. Supernatants were used to determine the enzymatic activities of creatine kinase-B at a final sperm concentration of 1.0 x 10^8 spermatozoa/mL in a measuring cuvette. Enzymatic activity was measured using 30 mM creatine phosphate as substrate, 2 mM ADP, 20 mM glucose, 2 mM NADP, 2500 U/L hexoquinase, 2000 U/L Glucose-6-phosphate-dehydrogenase, 10 mM Magnesium acetate, 5 mM AMP, 10 mM di-(adenosine 5')-phentaphophate and 20 mmol/L N-acetilcysteine, 200 U/L 6-phosphoglucolactonase, 400 U/L 6-phosphogluconate-dehydrogenase and 100 mM imidazol buffer and concentrations of monoclonal antibody capable of inhibiting 1000 U/L CKM or 2000U/L CKM. The activity was determined spectrophotometrically at 340 nm for 5 min by measuring the reduction of NADP**.** Enzyme activity was expressed in U/10^8 spermatozoa. One enzymatic unit of creatine kinase-B was defined as the enzyme quantity which catalyses the transfer of 1.0 mmol of phosphate from phosphocreatine to ADP per minute at pH 7.4 and 37ºC [26,27].

Oxygen consumption

Respiration of the sperm suspension was measured polarographically at 38°C with an oxygenelectrode modified Clark type and an Instech Laboratories (Philadelphia, PA, USA) oxygraph. The reaction cell had a 0.6 mL capacity and measured rapid changes in the oxygen consumption rate by the cell. The measurement cuvette was kept at 38°C so that the diffusion of atmospheric oxygen toward the solution could be negligible compared with the rate of oxygen uptake recorded with constant stirring. For evaluation of sperm respiration during capacitation, the final concentration in the cuvette was 1.0 x 10^8 spermatozoa/mL. Carbanyl-cyanide-m-chloro phenylhydrazone (CCCP; 0.42 mM) was used as a specific uncoupler of the respiratory chain and oxygen uptake was expressed as mLO_2/h/10^8 spermatozoa [17].

Protein kinase C inhibition

The GF 109203X, a specific inhibitor of protein kinase C was used at a 100 nM concentration[28]. The inhibitor and heparin or xanthine/xanthine-oxidase/catalase system were simultaneously added to the sperm suspension.

Tyrosine kinase inhibition

Genistein (150 mM) was used as specific tyrosine kinase inhibitor [29]. It was added along

with heparin or xanthine/xanthine-oxidase/ catalase system to the sperm suspensions and then they were incubated at 38ºC during 15 min.

Determination of Lipid peroxidation

Samples were incubated to enhanced lipid peroxidation at 37°C in the presence of 0.5 mM sodium ascorbate and 0.11 mM ferrorus sulfate for 2 h. Lipid peroxidation was evaluated as the mean of 2-thiobarbituric acid (TBARS) assay[16].

Statistical analysis

Differences of percentages of capacitated spermatozoa, sperm viability, CPK-B activities, oxygen consumption and lipid peroxidation levels between treatments were determined by ANOVA; a Tukey 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). For all analyses P<0.05 was regarded as significant.

RESULTS

In the present study, the influence of tyrosine kinase (genistein) or protein kinase C (GF 109203X) inhibitors on heparin capacitation induction as well as their respective oxygen uptake rate were evaluated on bovine spermatozoa. The results indicate that in spermatozoa treated with heparin, the presence of genistein or GF 109203X provoked a significant decrease in the capacitation percentage and sperm oxygen uptake compared to the values obtained with heparin treated samples (P < 0.05) Table 1. No differences were observed in the sperm viability with all treatments (P>0.05)

In sperm suspensions treated with xanthine -xanthine oxidase- catalase system, in both incubation times, spermatozoa were capacitated showing a lower respiration rate respect to heparin treated samples. Furthermore, in these conditions the capacitation decreased (P < 0.05) Table 1.

Table 1. Capacitation and oxygen uptake in bovine spermatozoa

Sperm samples	Capacitated spermatozoa	Sperm Viability (%)	Oxygen uptake (μlO_2 h/10^8spermatozoa)
Control	9.17 ± 2.00[a]	55,00 ±1,15°	9.00 ± 1.20 *
Heparin	37.00 ± 1.80[b]	50,75 ±2,98°	16.00 ± 1.80**
Heparin / Genistein	12.30 ± 2.40 [a]	58,8 ±2,38°	7.39 ± 1.73*
Heparin / GF109203X	16.70 ± 2.21 [a]	46,50 ±2,51°	5.26 ± 0.96*
X - XO / Catalase 15 min	30.08 ± 1.40 [c]	56,50 ±1,00°	7.38 ± 1.34*
X - XO / Catalase 45 min	30.00 ± 7.40 [c]	55,30 ±1,20°	4.31 ± 1.24*

Bovine spermatozoa were capacitated with heparin 60 µg/mL heparin or 0,5 mM xanthine -0,05 mUI/mL- xanthine oxidase (X-XO/Catalase)– 100 mg/mL catalase. GF 109203X (100 nM) (GF) or Genistein (150 mM) inhibitors of protein kinase C and tyrosine kinase respectively, were added simultaneosly with heparin. Data are expressed as means ± SD, n=7. Different superscripts and symbols indicate significant differences between treatments (P < 0.05).

A decrease in creatine kinase-B activity level was observed in the presence of heparin or xanthine -xanthine oxidase - catalase system in both incubation times respect to sperm control (P < 0.05) Figure 1.

The addition of genistein or GF 109203X, in order to demonstrate tyrosine kinase and protein kinase C modulation respectively on creatine kinase-B activity during heparin treatment, caused a significant inhibition and an undetectable level of creatine kinase-B activity respectively (P < 0.05) Figure 1.

A significant increase in lipid peroxidation was detected by the addition of xanthine-

xanthine oxidase/catalase at 45 min respect to controls, heparin treatments and xanthine-xanthine oxidase/catalase addition at 15 min ($P < 0.05$) Figure 2.

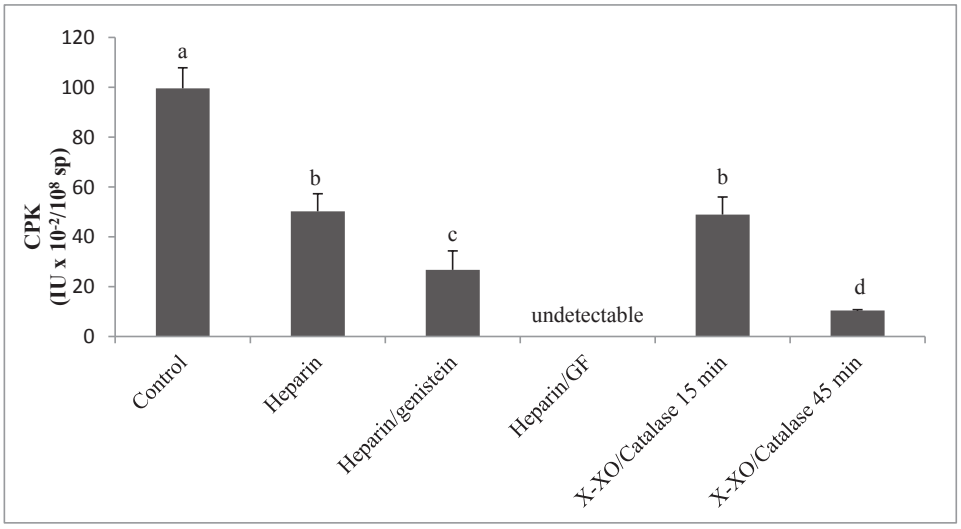

Figure 1. Creatine kinase-B activity variation

Data are expressed as means ± SD, n=7. Different superscripts and symbols indicate significant differences between treatments (P<0.05). GF 109203X (100 nM) (GF) or Genistein (150 mM) inhibitors of protein kinase C and tyrosine kinase respectively, were added simultaneosly with heparin. Xanthine-xanthine oxidase-catalase system (X-XO/Catalase) or heparin was used as capacitation inductors. The activity was determined spectrophotometrically at 340 nm over 5 min by measuring the reduction of NADP. Enzyme activity was expressed in International units per 10^8 spermatozoa (IU x $10^{-2}/10^8$ sp).

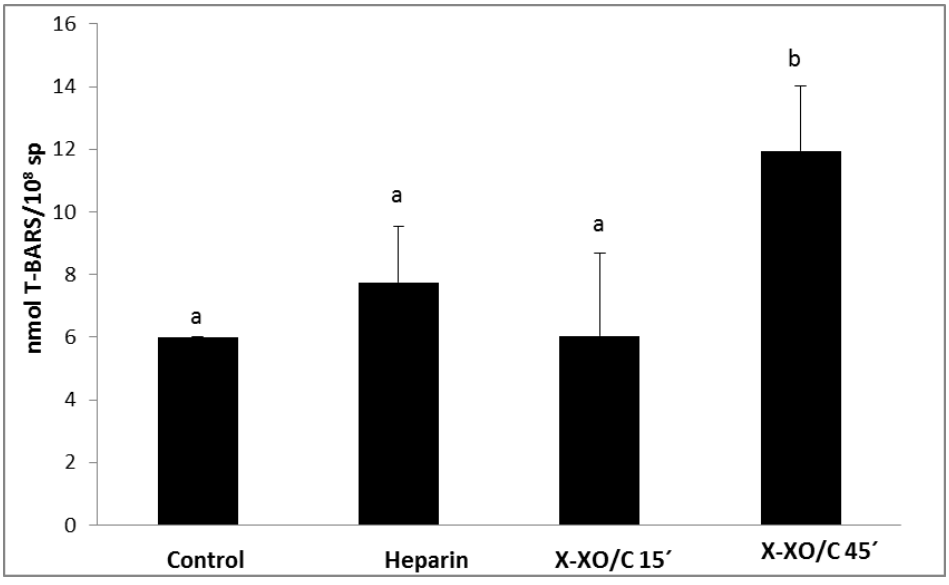

Figure 2. Lipid peroxidation variations in the presence of heparin or xanthine-xanthine oxidase-catalase system

Sperm samples were incubated to enhance lipid peroxidation at 37°C. Lipid peroxidation level was determined as means of 2-thiobarbituric acid (TBARS) assay. Data are expressed as means ± SD, n=7. Different superscripts and symbols indicate significant differences between treatments (P<0.05). Xanthine-xanthine oxidase-catalase system (X-XO/C).

DISCUSSION

Extensive research has started to elucidate the protein phosphorylation event during sperm capacitation and acrosome reaction, wherein three pathways are mainly involved: cAMP/PKA, receptor tyrosine kinases and non-receptor protein tyrosine kinases [1,2] which finally produce an increase in the phosphorylation of Tyr residues [3,4].

In cryopreserved bovine spermatozoa, pyruvate and lactate are sources of oxidative energy. Oxidative respiration provides the most efficient ATP generation pathway, while the major site of ATP consumption in the spermatozoa is the dynein ATPase, which is associated with the energy consumption in the flagellum [30].

It is known that the phosphorylation of sperm proteins is an important aspect of capacitation and has been associated with sperm hypermotility, ZP binding and acrosome reaction [5,6]. In several mammalian species, all the regulatory mechanisms of this event have not been fully elucidated yet.

Therefore, it is important to investigate how tyrosine kinase and protein kinase C (PKC) would be involved in heparin capacitation induction through sperm oxygen uptake and enzymatic activity to know the energy sources related to intracellular signals activation.

In this study, GF 109203X or genistein (PKC and tyrosine kinase inhibitors respectively) provoked a decrease in capacitation induction and in sperm oxygen uptake in heparin treated spermatozoa, so we infer that both kinases may modulate gamete oxidative metabolism related to mitochondrial function, which may be involved in heparin sperm capacitation signals. Identified Tyr-phosphorylated proteins in human sperm include ion channels, metabolic enzymes and structural proteins [31,32]. In cryopreserved bovine spermatozoa was studied that tyrosine kinases including SRC-isoform modulate capacitation, where the intracellular calcium variation may be a crucial point related to tyrosine phosphorylation [33].

Genistein inhibition of intracellular heparin mechanisms provoke metabolic changes that result in an energetic charge which is not enough to supply the conditions for glycosaminoglycan induction, inferred by a low oxygen consumption which indicates a decrease in oxidative phosphorylation as a potential source of ATP. This finding agrees with our previous research, which proposes that the heparin induced respiratory burst was produced mainly by mitochondrial activity, the main energy source for the sperm [17]. Another energy source for the sperm, the phosphocreatine/creatine shuttle, has been vinculated with oxidative phosphorylation and its regulation through cellular redox state variation has been proposed [34,8]. Creatine kinase (CPK) isoenzymes, specifically located at sites of energy demand and production, are linked by a phosphocreatine/creatine circuit [22]. Our data suggest that the heparin metabolic pathways including CPK shuttle are modulated by PKC and tyrosine kinase because of the significant CPK-B activity decrease, an enzyme involved in supporting cytosolic ATP, when both regulatory kinases were blocked. So CPK-B activity is required to supply energy for heparin induced capacitation in bovine spermatozoa.

Furthermore, CPK-B activity in heparin induced capacitation depends mainly on PKC modulation confirmed by the complete inhibition by GF 109203X, but CPK-B activity is also modulated (50%) by the activation of tyrosine kinase.

As explained above, the major energy requirement in the sperm is related to hypermotility. Preliminary studies on sperm PKC subspecies distribution revealed that they are involved in sperm flagellar motility in human [35] and in bovine spermatozoa [36]. It is also noteworthy that during the course of capacitation and fertilization, the main tyrosine-phosphorylated proteins are located in the flagellum [10] and it has been related to mammalian hyperactivated sperm motility [37].

CPK-B activity and oxygen uptake variations in heparin capacitated spermatozoa, due to the localization and compartmentalization of tyrosine kinase and PKC isoforms, may be

related to the phosphorylation of substrates in different sperm regions to regulate physiological functions connected with energy sources. So these results suggest that PKC and tyrosine kinase would be related with signaling pathways that maintain both CPK activity level and an active mitochondrial oxidative phosphorylation as energy sources for heparin induced capacitation in the bovine sperm.

The xanthine-xanthine oxidase-catalase system generates superoxide anion that can capacitate bovine sperm [18] and it has been demonstrated that it may improve sperm metabolism and oocyte fertilization in the mouse [38]. Data suggest that a lower sperm oxygen consumption induced by xanthine-xanthine oxidase-catalase system compared to respiratory burst produced by heparin, allowing a lower capacitation rate due to the energetic state generated by superoxide anion. As regards creatine kinase-B in the presence of xanthine-xanthine oxidase-catalase system, the increase in the incubation time had a negative effect in this enzyme activity, although the capacitation was similar with both incubation times. These results may be due to the deleterious effect of reactive oxygen species (ROS). It is known that despite their potential deleterious effect, ROS at low and controlled levels participate in cell signaling events in sperm physiology, allowing them to acquire fertilizing capability. In fact, in human and bovine sperm, hyperactivation and capacitation are triggered by ROS [18,8,39].

According to Koufen the decrease in mitochondrial CPK activity is caused by an increase in ROS [34], which was detected in the midpiece of sea urchin[40] and human spermatozoa [41]. The increase in ROS levels produces capacitation decrease in bovine sperm [8]. In relation with these observations, our data suggest that more incubation time (45 min) with xanthine-xanthine oxidase-catalase system produces a toxic effect on sperm, confirmed by the increase in lipid peroxidation that caused an alteration in oxygen consumption and CPK-B activity respect to heparin induction. Lipid peroxidation can provoke alterations in the plasma and mitochondrial membranes that may modify the respiratory chain function, with the concomitant reduction in CPK activity, not allowing the bovine sperm to capacitate because of a low energy charge for the process. In accordance with these changes in sperm metabolism, it has been demonstrated that heparin treatment is better than xanthine-xanthine oxidase-catalase system to induce highest rates early cleavage in bovine oocytes [42,43].

In conclusion, in cryopreserved bovine spermatozoa heparin capacitation is related to a respiratory burst and a decrease in CPK-B activity, both processes dependent of tyrosine kinase and protein kinase C regulation. In the conditions of this study, sperm capacitation and lipid peroxidation depending on the incubation time can provoke mitochondrial membrane alteration. This deleterious effect may modify the respiratory chain function, with the concomitant reduction in creatine kinase shuttle, not allowing the sperm to reach the energetic state required to achieve fertilizing capability.

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