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A membrane-associated adenylate cyclase modulates lactate dehydrogenase and creatine kinase activities required for bull sperm capacitation induced by hyaluronic acid

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ABSTRACT

Hyaluronic acid, as well as heparin, is a glycosaminoglycan present in the female genital tract of cattle. The aim of this study was to evaluate oxidative metabolism and intracellular signals mediated by a membrane-associated adenylate cyclase (mAC), in sperm capacitation with hyaluronic acid and heparin, in cryopreserved bull sperm. The mAC inhibitor, 2′,5′-dideoxyadenosine, was used in the present study. Lactate dehydrogenase (LDH) and creatine kinase (CK) activities and lactate concentration were determined spectrophotometrically in the incubation medium. Capacitation and acrosome reaction were evaluated by chlortetracycline technique, while plasma membrane and acrosome integrity were determined by trypan blue stain/differential interference contrast microscopy. Heparin capacitated samples had a significant decrease in LDH and CK activities, while in hyaluronic acid capacitated samples LDH and CK activities both increased compared to control samples, in heparin and hyaluronic acid capacitation conditions, respectively. A significant increase in lactate concentration in the incubation medium occurred in hyaluronic acid-treated sperm samples compared to heparin treatment, indicating this energetic metabolite is produced during capacitation. The LDH and CK enzyme activities and lactate concentrations in the incubation medium were decreased with 2′,5′-dideoxyadenosine treatment in hyaluronic acid samples. The mAC inhibitor significantly inhibited heparin-induced capacitation of sperm cells, but did not completely inhibit hyaluronic acid capacitation. Therefore, hyaluronic acid and heparin are physiological glycosaminoglycans capable of inducing *in vitro* capacitation in cryopreserved bull sperm, stimulating different enzymatic pathways and intracellular signals modulated by a mAC. Hyaluronic acid induces sperm capacitation involving LDH and CK activities, thereby reducing oxidative metabolism, and this process is mediated by mAC.

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1. Introduction

In mammals, ejaculated sperm requires a finite period of residence in the female reproductive tract to become competent for fertilization (Visconti et al., 1995a,b). Once oocytes are matured, it is important for these cells to be exposed to sperm that have already been capacitated or are undergoing capacitation (Parrish, 2014). In sperm capacitation several intracellular changes are known to occur, including an increase in membrane fluidity, cholesterol efflux, intracellular Ca^{2+} and cAMP concentrations and protein tyrosine phosphorylation, together with changes in

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swimming patterns and chemotactic motility (Breitbart and Naor, 1999).

Glycosaminoglycans are mucopolysaccharides that are present in the female genital tract (Lee and Ax, 1984). The exposure of bull sperm to glycosaminoglycans such as heparin or hyaluronic acid is positively correlated with its *in vitro* fertilizing ability, *in vitro* embryo developmental potential and embryonic gene expression (Kim et al., 2013). Heparin, the glycosaminoglycan used routinely to induce sperm capacitation prior to *in vitro* fertilization (IVF) in bull, binds to bull sperm as a typical receptor-ligand interaction, promoting capacitation (Parrish et al., 1988; Satorre and Córdoba, 2010).

Hyaluronic acid is a linear non-sulphated high molecular weight glycosaminoglycan which has been detected in uterine and oviductal fluids in ruminants (Lee and Ax, 1984). The localization of hyaluronic acid cell surface receptor CD44 in the sperm reservoir of bulls could indicate an intracellular signaling pathway generated by the CD44-hyaluronic acid binding, which would have a role in oocyte maturation, sperm storage, sperm capacitation and interactions linked to fertilization (Bergqvist et al., 2005). Human, boar, bull, and ram sperm contain hyaluronic acid receptor CD44 in the plasma membrane (Bain et al., 2002; Tienthai et al., 2003 Bergqvist et al., 2006; Vicente-Carrillo et al., 2015).

In several species, such as swine (Suzuki et al., 2002), hyaluronic acid has been used as a capacitation inducer during *in vitro* conditions. In dogs, this glycosaminoglycan accelerates calcium influx into the sperm cytoplasm and increases lactate dehydrogenase (LDH) activity and cAMP production provoking capacitation (Kawakami et al., 2006). It has previously been demonstrated that hyaluronic acid induces bull sperm capacitation *in vitro* with an optimal concentration of 1000 μg/ml and 60 min of incubation (Fernández and Córdoba, 2014a). Hyaluronic acid also was used successfully in IVF, early cleavage and blastocyst production percentages increased with hyaluronic acid and no significant differences were registered between this inductor and heparin (Gutnisky et al., 2007).

Male gametes have an active metabolism because these cells possess enzymes involved in metabolic pathways such as glycolysis, Krebs cycle, fatty acid oxidation and respiratory chain (Hafez and Hafez, 2002). Two of the most important enzymes that participate in sperm metabolism are LDH and creatine kinase (CK) (Duan and Goldberg, 2003; Córdoba et al., 2007; Córdoba et al., 2008; Gladden, 2004; Wallimann et al., 2011).

The LDH catalyzes the reversible conversion of pyruvate into lactate with NADH re-oxidation. Sperm almost exclusively contain an LDH isoenzyme (LDH-X or LDH-C4), which is not present in other tissues (Blanco et al., 1976; Duan and Goldberg, 2003). This enzyme has been localized in the mitochondrial and soluble fractions, thus demonstrating the presence of cytosolic and mitochondrial isozymes and suggesting the existence of a reduced equivalents shuttle between cytosol and mitochondria in mouse sperm (Burgos et al., 1995). It has also been reported that LDH-X can act in bull sperm in aerobic and anaerobic conditions (Kolb et al., 1970), therefore, it has been considered as a potentially important parameter for sperm motility and semen quality evaluation (Cui et al., 2015).

The CK catalyzes the reversible conversion of creatine into creatine phosphate using ATP and thereby releasing ADP. The CK isoenzymes, specifically localized in places where there is a demand and production of energy, are linked to a creatine/creatine phosphate shuttle (Wallimann et al., 1998). This CK shuttle is a source of extra-mitochondrial ATP and is responsible for the energy transfer between mitochondria and cytosol. The CK activity is an indicator of normal spermatogenesis and maturation and a predictor of fertilizing potential in human sperm (Huszar et al., 1997).

Sperm capacitation is a complex process modulated by intracellular signaling pathways, such as adenylate cyclase, protein kinase A and protein tyrosine kinases (Signorelli et al., 2012). Adenylate cyclase increases intracellular cAMP, capacitation and tyrosine protein phosphorylation in mouse sperm (Adeoya-Osiguwa and Fraser, 2000). A membrane-associated isoform of adenylate cyclase (mAC) produces cAMP regulating sperm capacitation in mammals (Fraser et al., 2005). The cAMP production was inhibited in mouse sperm by 2′,5′-dideoxyadenosine, a specific mAC inhibitor (Baxendale and Fraser, 2003). It was reported that bull sperm incubated with heparin displays a reproducible pattern of protein tyrosine phosphorylation regulated by a cAMP-dependent pathway (Galantino-Homer et al., 1997). In contrast, in previous research, it was suggested that hyaluronic acid capacitation would not be modulated by a mAC (Fernández and Córdoba, 2014a).

Until now intracellular effects of hyaluronic acid in sperm capacitation of bull remain unclear. Therefore, to compare metabolism and intracellular signaling between hyaluronic acid and heparin at the capacitation stage specifically, the aim of this study was to evaluate oxidative metabolism and intracellular signals mediated by mAC in sperm capacitation induced by hyaluronic acid and heparin in cryopreserved bull sperm, through the determination of LDH and CK activities and changes in lactate concentration in the incubation medium.

2. Materials and methods

2.1. Materials

All the chemicals used were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, USA).

2.2. Semen collection and cryopreservation

Semen was collected using an artificial vagina from Holstein bulls of proven fertility that routinely provide semen for artificial insemination. Bulls were 4 or 5 years of age and were maintained with uniform nutritional and management conditions throughout the study. Progressive sperm motility exceeded 70% in all ejaculates. Once a week, two ejaculates were obtained from each bull, and 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 (2:1 ratio) to a $3.0–4.5 \times 10^7$ sperm/ml final concentration. Diluted semen was slow-cooled (1 °C/min) to

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