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Expression, cellular localization, and involvement of the pentose phosphate pathway enzymes in the regulation of ram sperm capacitation

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ABSTRACT

Spermatozoa require substantially more ATP than other cells, not only for sustaining sperm motility but also for regulating protein phosphorylation during capacitation. In this study, we have reported for the first time the presence of the two key enzymes of the pentose phosphate pathway (PPP), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in ovine spermatozoa by indirect immunofluorescence, Western blotting, in-gel activity, and reverse transcription polymerase chain reaction analysis. We found that the activity of both enzymes significantly increased after *in vitro* capacitation in the presence of high-cAMP levels, with a concomitant increase in protein tyrosine phosphorylation and in the proportion of sperm-capacitated pattern assessed by the chlortetracycline staining. These results suggest that PPP is related with the progress of capacitation and that a relationship between calcium compartmentalization, protein tyrosine phosphorylation and PPP seems to exist. This is the first report that shows a connection between the PPP, cAMP/PKA signaling pathways and sperm capacitation. These findings can be of high-biological importance to improve our knowledge of the biochemical mechanisms involved in the acquisition of mammalian sperm functional competence and, ultimately, fertility.

1. Introduction

Energy metabolism is a crucial factor for supporting sperm function. Mammalian spermatozoa require energy in the form of intracellular ATP to sustain sperm motility, protein modifications, and other processes leading to egg fertilization. This ATP can be produced directly from glycolysis [1,2] or by mitochondrial oxidative phosphorylation [3,4]. Both pathways can provide energy, independently of one another. Depending on the species, spermatozoa can have a preference for either. For instance, although glycolysis appears to be the dominant pathway in mouse and oxidative phosphorylation in bull spermatozoa, ram spermatozoa seem to maintain motility by both parallel modes of energy generation (reviewed by [5]). Sugars from the oviductal fluid and the seminal plasma may play a role not only in the energetic metabolism and ATP production but would also act as modulators of a variety of functions such as phosphorylation of specific proteins as shown in dog spermatozoa [6]. Capacitation and the acrosome reaction [7,8] require lactate or pyruvate for ATP production by oxidative phosphorylation, whereas gamete fusion needs glucose to produce reduced nicotinamide adenine dinucleotide phosphate (NADPH) by the pentose phosphate pathway (PPP) [8]. An increased production of NADPH during capacitation has been found in human [9] and mouse [10] spermatozoa. This could be related to the involvement of either the PPP or the NADPH oxidase system [10,11] in redox regulation in spermatozoa. Although PPP has been

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shown to play an essential role in supporting human sperm capacitation [9], no PPP activity could be reported in either ejaculated or epididymal bull and ram spermatozoa [12–16]. These results would suggest important sperm metabolic differences between species that deserve investigation.

The aims of the present study were (1) to investigate the presence of the two enzymes involved in the oxidative phase of PPP, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), in ram spermatozoa by immunofluorescence, Western-blot and in-gel activity analysis; (2) to obtain the nucleotide sequences of these enzymes after RNA isolation and reverse transcription to confirm their presence; (3) to determine their kinetic characteristics, and (4) to report whether the PPP is involved in *in vitro* ram sperm capacitation using a high-cAMP induction medium very effective in initiating capacitation-associated responses [17]. It was revealed that the PPP operates in ram spermatozoa and that it is related to the progress of capacitation, regulating calcium compartmentalization, and protein tyrosine phosphorylation.

2. Materials and methods

2.1. Semen collection and processing

Semen was collected from nine 3 to 5 year-old *Rasa Aragonesa* rams using an artificial vagina. The rams, which belonged to the National Association of Rasa Aragonesa Breeding, were kept at the Veterinary School under uniform nutritional conditions, with an abstinence period of 2 days. Second ejaculates were pooled and used for each assay, to avoid individual differences [18]. All experimental procedures were performed under the supervision of the Ethics Committee of the University of Zaragoza. A seminal plasma-free sperm population was obtained by a dextran/swim-up procedure as already reported [19] with a swim-up medium devoid of $CaCl_2$ and $NaHCO_3^-$. The sperm concentration was calculated in duplicate using a Neubauer chamber (Marienfeld, Germany).

2.2. Indirect immunofluorescence

The localization of G6PD and 6PGD was investigated by indirect immunofluorescence (IIF) analysis. The sperm samples were diluted (2×10^6 sperm/mL) in (PBS, pH 7.2) and fixed in suspension for 20 minutes in 0.025% paraformaldehyde. After fixation and washing, the samples, processed in triplicate, were allowed to settle on Superfrost slides (Superfrost Plus, Thermo Scientific) and permeabilized with 0.5% Triton X-100 in PBS for 15 minutes. A second fixation was carried out for 5 minutes in 1.25% paraformaldehyde. The slides were then washed three times with PBS, and nonspecific binding sites were blocked with 5% BSA in PBS overnight at 4 °C in a wet chamber. After blocking, the slides were again washed three times with PBS and the corresponding antibody was added. The primary antibodies used in this study were rabbit polyclonal anti-G6PD and anti-6PGD antibodies (HPA000247 and HPA031314, respectively; Sigma–Aldrich, St. Louis, MO, USA) directed against G6PD and 6PGD recombinant protein epitope signature tags, 1/20 in PBS with 1% BSA. To check the specificity of the antibodies, the amino acid sequences from ovine G6PD and 6PGD obtained from Genebank were aligned (ClustalW 2.1, Multiple Sequence Alignment program) with the sequences of the immunogenic sequence used to raise the anti-G6PD and anti-PGD used antibodies. The high homology found between the sequences (93.6 and 92.6 for G6PD and 6PGD, respectively) suggests that these antibodies can be suitable for their use in ovine. The preparations were incubated overnight at 4 °C in a wet chamber, washed three times and incubated again with a chicken anti-rabbit Alexa Fluor 488-conjugated antibody (Molecular Probes Inc., Eugene, OR, USA) diluted at 1/600 in PBS with 1% BSA for 1 hour at room temperature (RT) in darkness. The slides were rinsed three times with PBS and, finally, 6 μl of 0.22 M 1,4-Diazabicyclo [2.2.2]octane (DABCO, Sigma–Aldrich, St. Louis, MO, USA) in glycerol:PBS (9:1) were added to enhance and preserve the cell fluorescence. The slides were then covered with a coverslip and sealed with colorless enamel. The cells were visualized with a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) under epifluorescence illumination using a B-2A filter (×1000). At least 200 cells per sample were evaluated and the percentage of cells displaying positive fluorescence was scored. Sperm cells incubated with rabbit serum instead of the primary antibodies were used as negative controls. Human hepatocytes (human hepatocellular carcinoma cells, cell line HepG2) were used as positive controls for both primary antibodies. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Eagle's Dulbecco's Modified *Eagle's* medium supplemented with 100-U/mL penicillin G, 100-μg/mL streptomycin, 200-mM glutamine, 100-mM sodium pyruvate, and heat-inactivated 10% fetal bovine serum. After trypsinization, the cells were allowed to settle on Superfrost slides and processed in parallel as indicated previously. Images were obtained using a microscope digital camera system (Sony 2CCD Color Video Camera and Sony Digital Still Recorder), and saved and edited with NIS-Elements Software (Nikon, Tokyo, Japan).

2.3. Extraction of ram sperm proteins

Aliquots of 3.2×10^7 spermatozoa were resuspended in 100 μl of the same extraction medium as previously used [17], composed of 2% sodium dodecyl sulfate (SDS) (w/v), 0.0626-mM Tris-HCl (pH 6.8), 0.002% bromophenol blue in 10% glycerol (final glycerol concentration 1%), and protease and phosphatase inhibitors 10% (v/v; Sigma–Aldrich, St. Louis, MO, USA). After incubation for 5 minutes at 100 °C, the samples were centrifuged at 7500 × *g* for 5 minutes at RT, the supernatant was recovered and 2-mercaptoethanol and glycerol were added to a final concentration of 5% and 1%, respectively. The lysates were stored at −80 °C. Protein concentration was assessed using the bicinchoninic acid (BCA) cooper reagent [20] (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Sheep liver tissue lysates

Sheep liver (300 mg) was homogenized with a T25 Digital Ultra-Turrax Homogenizer (IKA, Germany/Deutschland) with 3 mL of extraction medium (125-mM Tris-HCl, pH 6.8),