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Original Research

Effect of BAPTA-AM on Thawed Stallion Spermatozoa Extended in INRA 96 or Tyrode's Medium

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A B S T R A C T

We studied the effect of 1,2-*bis*-(o-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) on the outcome of cryopreservation of stallion spermatozoa and whether reextension of thawed sperm in a more physiological and Ca^{2+}-containing medium might improve the characteristics of thawed stallion spermatozoa. Individual ejaculates from six stallions were collected and split into three subsamples. The first two samples were supplemented with the membrane-permeable Ca^{2+} chelator BAPTA-AM at final concentrations of 5 and 10 μM, respectively, while the third subsample served as control. After 4 weeks of storage, samples were thawed in a water bath at 37°C and evaluated using flow cytometry and computer-assisted sperm analysis (CASA). In a second experiment, in order to determine whether restoring Ca^{2+} could improve sperm quality after cryopreservation, thawed semen was washed by centrifugation and resuspended in Tyrode's complete medium. BAPTA-AM supplementation did not modify the outcome of cryopreservation; however, changing the spermatozoa from INRA 96 to Tyrode's complete medium resulted in significant improvements in the percentages of live sperm and total motility post thaw.

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1. Introduction

Cryopreservation and other biotechnologies, such as sex sorting, cause differential damage that is either lethal or sublethal by nature to spermatozoa of all mammalian species [1-4]. Obviously, sperm death is the major factor explaining the lower fertility of thawed spermatozoa, but nonlethal modifications of surviving cells also account for their reduced life span, thus narrowing the window spermatozoa have for successful fertilization after artificial insemination [5,6]. One of the factors explaining this sublethal damage may be related to disruption of Ca^{2+} homeostasis that prematurely activates processes of capacitation and may even lead to cell death [7,8]. In addition, peroxidation of plasma membrane lipids (lipid peroxidation) leads to "apoptosis like changes" [9] and thus sublethal cryodamage [5,10]. The interaction between Ca^{2+} and oxidative stress under conditions of many different cellular pathologies and aging is well known [11]. This effect has been evaluated in spermatozoa using the chlortetracycline assay, results of which suggest that this cryocapacitation may be related to a sudden depletion of the intracellular Ca^{2+} stores in the acrosome [12]. Despite the major role of Ca^{2+} in sperm physiology, the study of the effect of cryopreservation on Ca^{2+} homeostasis has received little attention, although a recent study has demonstrated that maintenance of high intracellular Ca^{2+} level favors sperm survival after cryopreservation [13] and that cryopreservation causes Ca^{2+} depletion in dog sperm. Moreover, porcine studies indicate that Ca^{2+} supplementation after thawing can increase the fertility of cryopreserved sperm [14]; in addition, a recent report using avian sperm as a model indicates that chelation of calcium can inactivate sperm and then restoration of calcium can activate it, thus

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representing a "switch off-switch on" mechanism to preserve sperm [15]. In view of all these facts, the aims of the present study were to test the effect of the intracellular chelator 1,2-*bis*-(o-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) on the outcome of cryopreservation and to determine whether reextension of thawed sperm in a Ca^{2+}-containing medium may improve the characteristics of thawed stallion spermatozoa.

2. Material and Methods

2.1. Study Design: Experiment 1

Semen (four ejaculates per stallion) was obtained from six pure Spanish horses housed individually at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. The stallions were maintained according to institutional and European regulations, with semen being collected on a regular basis (two collections per week) during the 2009 breeding season. Ejaculates were collected using a Missouri model artificial vagina, lubricated and prewarmed to 45°C-50°C, fitted with an in-line filter to separate the gel fraction. The collected ejaculate was immediately transported to the laboratory for evaluation and processing. The filtered ejaculate was extended 1:2 (v/v) with INRA 96 (IMV, L'Aigle, France), and centrifuged at 600 *g* for 10 minutes. The resulting sperm pellet was reextended in freezing medium (Ghent, Minitüb Ibérica, Spain) to a final concentration of 100×10^6 spermatozoa per mL. Then, individual ejaculate samples were split into three subsamples; the first two were supplemented with the membrane-permeable Ca^{2+} chelator BAPTA-AM (Sigma, St. Louis, MO) at final concentrations of 5 μM (subsample 1) and 10 μM (subsample 2), respectively. The third subsample served as control. Spermatozoa were slowly cooled to 4°C within 1 hour, loaded in 0.5-mL plastic straws, and frozen horizontally in racks placed 4 cm above the surface of liquid N_2 for 10 minutes, after which they were directly plunged into liquid N_2 for storage. After at least 4 weeks of storage, straws were thawed in a water bath at 37°C for 30 seconds for analyses and extended in INRA 96 to 40×10^6 spermatozoa/mL. Motility was assessed using computer-assisted sperm analysis (CASA; ISAS Proiser, Valencia, Spain), and membrane permeability and integrity, acrosomal status, and mitochondrial membrane potential were analyzed by flow cytometry.

2.2. Study Design: Experiment 2

In order to determine whether a more physiological medium and restoring Ca^{2+} could improve sperm quality after cryopreservation, thawed semen samples were split, and one subsample was kept as control. The other subsample was washed by centrifugation and resuspended in Tyrode's complete medium [16,17] (96 mM NaCl, 3.1mM KCl, 2.0 mM $ClCa_2$, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 20 mM HEPES, 5mM glucose, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 15 mM $NaHCO_3$, and 3 mg of bovine serum albumin [BSA] ml^{-1}). Medium containing bicarbonate was equilibrated with 5% CO_2 in air. Tyrode's medium was maintained at 285 to 315 mOsm/kg and pH 7.4. Then, both samples were evaluated for motility using CASA and membrane integrity using flow cytometry. Intracellular calcium was also determined by flow cytometry.

2.3. Sperm Motility Analysis

Motility was measured using a CASA system based on examination of 25 consecutive digitized images obtained from a single field, using an ×10 negative phase-contrast objective in a light microscope (model CX41; Olympus, Tokyo, Japan). A minimum of 200 cells per sample was analyzed. Images were obtained with a time lapse of 1 second; the image capture speed was therefore one every 40 ms. The number of objects incorrectly identified as spermatozoa was minimized on the monitor by using the playback function. With respect to the setting parameters for the CASA program, spermatozoa with an average path velocity (VAP) of <10 μm/s were considered immotile, while spermatozoa with a velocity >15 μm/s were considered motile. Spermatozoa deviating by <45° from a straight line were designated linearly motile.

Absolute and recalculated kinematic parameters for sperm motion as measured by CASA included the following parameters: curvilinear velocity (VCL), which measures the sequential progression along the true trajectory (in μm/s); linear velocity (VSL), which measures the straight trajectory of the spermatozoa per unit of time (in μm/s); VAP), which measures the mean trajectory of the spermatozoa per unit of time (in μm/s); linearity coefficient (LIN), which is the percentage of VSL/VCL × 100; straightness coefficient (STR), which is the percentage of VSL/VAP × 100; wobble coefficient (WOB), which is the percentage of VAP/VCL × 100; average lateral head displacement (ALH), which measures the mean head displacement along the curvilinear trajectory (in μm); and the beat cross frequency, which is the number of times the sperm head crosses the mean path/second (in Hz).

2.4. Flow Cytometry

Flow cytometric analyses were carried out with an Epics XL (Coulter Corp. Inc., Miami, FL) flow cytometer equipped with standard optics, an argon-ion laser (Cyonics; Coherent, Santa Clara, CA) operating at 15 mW at 488 nm, and Expo 2000 software. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Nonsperm events (debris) were identified and eliminated from analysis as described previously [18]. Forward and sideways light scatter were recorded for a total of 10,000 events per sample (for YO-PRO-1, Arachis hypogaea lectin [PNA]) or 30,000 events for the lipophilic cationic compound 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolocarbocyanine (JC-1; Molecular Probes Europe, Leiden, The Netherlands). Samples were measured at a flow rate of 200 to 300 cells/s. Green fluorescence was detected in FL1 (525-nm band pass filter), red fluorescence was detected in FL3 (620-nm band pass filter), and orange fluorescence was detected in FL2 (570-nm band pass filter).

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