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In vivo fertility of bull semen following cryopreservation with an LDL (low density lipoprotein) extender: Preliminary results of artificial inseminations

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ABSTRACT

A semen extender made with low density lipoproteins (LDL) has been used instead of a standard extender that is already available on the market for the cryopreservation of bovine semen. However, in order to extend its use to artificial insemination centres, *in vivo* fertility studies were required. Semen was taken from three bulls and frozen-thawed in two extenders: the LDL extender and a standard Tris-egg-yolk (20%) extender used by AI centres. The quality of the semen was assessed prior to artificial insemination: motility was assessed using an image analyser (Computer Assisted Semen Analysis (Hamilton Thorne)), and the integrity of the plasma membrane was assessed using the hypo-osmotic test (HOS test). For the first time, gestations were obtained following the artificial insemination of cows in the field ($n = 193$) with semen that had been frozen-thawed in the LDL extender. No significant difference ($p > 0.05$) was detected between the success rates of AI between the semen that had been frozen-thawed in the LDL extender (59.2%) and the control extender, Tris-20% egg yolk (65.3%). In conclusion, the *in vivo* fertility of semen that has been frozen-thawed in the LDL extender is maintained since gestations are obtained following AI.

1. Introduction

The freezing process exposes the spermatozoa to thermal shock, which results in damage to the plasma membrane and acrosome (Woelders et al., 1997; Celeghini et al., 2007). Various extenders have been tested in an attempt to limit cellular injury. Egg yolk is the most widely used of these extenders by artificial insemination centres. In recent years, centrifugation techniques have enabled the isolation of the LDL that are responsible for the cryopreservative effect of egg yolk (Pace and Graham, 1974; Demianowicz and Strezek, 1996; Moussa et al., 2002). The incorporation of LDL in bovine extenders has given improved motility results in comparison with extenders containing egg yolk (Moussa et al., 2002; Amirat et al., 2004; Vera Munoz et al., 2009). However, to extend the use of this new LDL-based extender to insemination centres, a fertility study was essential. Fertility can either be assessed in the laboratory using *in vitro* fertilisation tests or by artificial insemination in the field (Larsson and Rodriguez-Martinez, 2000). The latter provides the most

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reliable means of assessing semen fertility following freezing and thawing. *In vitro* fertility results have already been published (Amirat et al., 2004); blastocysts were obtained after 7 days of *in vitro* culture from oocytes collected from abattoirs, matured, and then fertilised *in vitro* with spermatozoa that had been frozen-thawed in the LDL extender. An *in vitro* study is insufficient to assess the fertility of semen that has been frozen in the LDL extender, an *in vivo* field study was therefore necessary.

The objective of our study was to assess the *in vivo* fertility of bull semen that had been frozen-thawed in the LDL extender. A widely available, standard extender (Tris-egg yolk) was used as a control. Cows were thus inseminated with semen that had been frozen-thawed in the LDL extender; pregnancy diagnoses were undertaken to assess the maintenance of fertility.

2. Materials and methods

2.1. Preparation of the extenders

Two extenders were prepared and stored at −20 °C until use. (1) Tris-egg yolk extender (T-EY): 20 ml of chicken egg yolk, 2.42 g TRIS (base), 1.48 g citric acid, 1.00 g fructose, 6.4 ml glycerol, 25 mg gentamicin, 50,000 IU penicillin, and qsp 100 ml bidistilled water. (2) LDL extender: 8% LDL (w/v) prepared in our laboratory in accordance with the method described by Moussa et al. (2002) (patent no. 0100292): 2.42 g Tris, 1.48 g citric acid, 1.00 g fructose, 6.4 ml glycerol, 25 mg gentamicin, 50,000 IU penicillin, and qsp 100 ml bi-distilled water. The osmolarities were measured using an osmometer (Hermann Roebling microosmometer, type 13/13 DR auto cal Germany): 1365 and 1370 mOsm/kg for LDL and Tris-egg yolk, respectively. The extenders were thawed on the day of sampling and maintained at 37 °C.

All of the products used to prepare the extenders were produced by Sigma–Aldrich Chemicals, St. Quentin Fallavier, France.

2.2. Semen collection and freezing

Three bulls belonging to the La Crespelle insemination centre and that had been approved for public use, were used. These bulls were housed in individual boxes and fed on hay and concentrates; their semen was collected once weekly. Bull 1 was a 4-year-old Holstein, Bull 2 was a 4-year-old Red Holstein, and Bull 3 was a 10-year-old Charolais. All three had a recorded progeny. Using the Laicophos® extender, the artificial insemination centre had a non-return rate at 60–90d of 64.7% for Bull 1, 57.2% for Bull 2, and 72.2% for Bull 3. The semen was collected using an artificial vagina. To excite the bulls, they were teased with a Normandy cow for 30 min prior to sampling. The semen was collected into a glass tube that had been previously warmed to 37 °C. Following collection, the ejaculates were immediately placed in a water bath at 37 °C. Each ejaculate was divided into two equal fractions. Each fraction was immediately diluted to 100×10^6 spz/ml with the two extenders that had been previously warmed to 37 °C, and then subjected to progressive cooling from 37 °C to +4 °C over 1 h 30 min in a refrigerated unit before being placed into straws (0.25 ml Polyvinyl Chloride Straws, IMV, L'Aigle, France). The semen was maintained in equilibrium for 4 h at +4 °C. The straws were held for 10 min at +4 cm from the surface of the liquid nitrogen (−120 °C) before being immersed then stored in liquid nitrogen (−196 °C).

2.3. Post-thaw semen analysis

2.3.1. Evaluation of motility: automated semen analysis

Three straws of each extender were immersed for 30 s in a water bath at 37 °C. Each straw was wiped, the extremities cut with a pair of scissors and the contents emptied into a cryotube placed in a thermostatically controlled water bath at 37 °C. The semen was analysed using the Hamilton-Thorne sperm analyser with the CEROS 12 software program, Hamilton-Thorne biosciences, Inc., Beverly, USA. The machine had been previously configured for the analysis of bovine semen. Five microliters were placed in a 20 μm-deep counting cell (Leja Products, B.V., Nieuw Vennep, Holland). Five fields were chosen at random and analysed five times over. The following parameters were studied: motility (% mobile spermatozoa), straight line velocity: VSL (μm/s), curvilinear velocity: VCL (μm/sec.), the linearity index: LIN (=VSL/VCL × 100), amplitude of lateral head displacement: ALH (μm), and average path velocity: VAP (μm/s). VAP, VSL, STR, and LIN provide information about the progressive movements of the spermatozoa, VCL and ALH characterise the lateral movements, and BCF (Beat Croix Frequence) provides information about the frequency of movements.

2.3.2. Assessment of plasma membrane integrity: HOS test (hypoosmotic swelling test)

A 100 mOsm/kg H_2O hypo-osmotic solution was prepared: 49.95 mM fructose, 17 mM Tri Sodium Citrate in 100 ml of distilled water. The solution was stored at +4 °C until use. Three straws were thawed in a water bath at 37 °C for 30 s. One hundred microliters of semen were added to 1 ml of hypo-osmotic solution that had been previously warmed to 37 °C; the mixture was then placed in a water bath at 37 °C for 1 h. Five microliters of the solution were placed on an oil-free slide. After spreading, the slide was read using a phase-contrast microscope. The spermatozoa were classified as positive or negative. Positive spermatozoa: tail swollen and/or curled: plasma membrane intact. Negative spermatozoa: tail not curled: plasma membrane damaged.

2.4. Artificial insemination in the field

Three inseminators with 25 years of experience collaborated in the study. One hundred and ninety-three females from 83 different herds were inseminated. The females included in the study were from dairy or suckler herds with a Calving to First Insemination Interval (CFI) of more than 60 days, heifers over 18 months old, and first inseminations only. For each insemination, the following data was recorded: date of insemination, herd number, the animal's identification number, breed, lactation or calving index, date of the previous calving if relevant, condition score, the bull used, and the extender used. The pregnancy diag-