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# Development of extender based on soybean lecithin for its application in liquid ram semen

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**Abstract**

The soybean lecithin is used as a phospholipids source for the commercial extenders available for freezing bull semen which allows replacing the traditional membrane protective of animal origin (egg yolk). These extenders have been tested for freezing semen in various livestock species but specific adjustments cannot be made due to trade protection. The aim of the present study was to develop a soybean-based extender analyzing the optimal conditions of preparation, handling, and storage in order to optimize its use in liquid ram semen. Its effect on the quality of liquid ram semen was also studied. Different TES-Tris-Fructose-based extenders were prepared using two soybean types (S20 and S95) differentiated by their lipid composition (complex or simple, respectively). These extenders were made up in two temperatures: 20 °C (PT20) or 37 °C (PT37); centrifuged and filtered at 20 °C and stored at 15 °C or 5 °C (ST15 and ST05) for several periods (from 6 hours to 7 days). Three different concentrations of soybean (0.5%, 2%, and 3.5%) were evaluated for each extender. The amount and nature of phospholipids present in the extender were evaluated by high performance liquid chromatography (HPLC) method according to the different parameters applied in their preparation. In general, the highest quantity of phospholipids is observed in S20 extender. Centrifugation-filtration process during the extender preparation reduces by 50% the quantity of phospholipids in medium for different experiments. The quantity of phospholipids was not affected significantly by preparation temperature in S20 extender. Storage temperature affects the phospholipids present in the extender (S20 and S95) with minimum values for the storage at 5 °C. As for the storage time, both extenders (S20 and S95) showed a stable quantity of phospholipids in the course of the time, for 2 days at 15 °C and for 7 days at 5 °C. The extender obtained with a higher concentration of soybean (3.5%) showed a higher content of phospholipids under different conditions tested. Finally, sperm motility and viability in new extenders were analyzed. We observed that the sperm quality is not affected by storage temperature for S20 extender. Sperm motility was higher in S20-2% extender and control (UL). Our results suggest that a soybean lecithin extender obtained from S20 soybean at 20 °C, centrifuged and filtered, preserve the sperm motility and viability at 15 °C and 5 °C as an egg-yolk extender.


*Keywords:* Ram sperm; Extender; Soybean lecithin; Phospholipids.

## 1. Introduction

The modern cattle industry worldwide is based on the use of artificial insemination (AI) and frozen semen that has permitted an accelerated rate of genetic selection and improvement in animal production. AI is less commonly used in ram than in other domestic species because of the difficulty in applying frozen-thawed sperm [1]. The AI commercial programs can be classified in two categories [2]: those using refrigerated semen (15 °C) by intra-cervical deposition (vaginal) and

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those using thawed sperm by intrauterine deposition (laparoscopy). Thus, ovine AI fertility depends on the interaction between the sperm preservation method and the application technique [3].

At present, the most useful method for ovine intracervical AI is the application of refrigerated semen at 15 °C during 6–8 h. However, liquid semen must have a minimum shelf-life of between 2 and 4 days in order to be used in distant locations and to cover a large number of ewes in a short period of time [4]. Egg yolk-based extenders are known to be practical and efficient in protecting ram spermatozoa against cold shock during storage before AI [1,5,6]. Nevertheless, the wide variability in their composition and the risk of microbiological contamination and disease transmission are important handicaps to be resolved [7]. Furthermore, the greater viscosity and the presence of particulate debris in extenders due to egg yolk globules interferes in the microscopic assessment of the sample [8] and could be the cause of reduced fertility [9]. Therefore, a well-defined and pathogen-free substitute of non-animal origin for egg yolk should be included in the extender composition.

An alternative to egg yolk in extenders for ram semen could be soybean lecithin [2], which is present in several commercial extenders developed for bull semen. Some authors have tested these commercial extenders of bull semen for the cryopreservation of ram semen [7,9,10,11]. The fertility using these extenders have been tested for liquid storage of equine semen [12] and for freezing semen in bull [13,14,15,16] and ram [17,18,19] but it is not possible to make specific settings in your composition due to trade protection.

One problem when soy lecithin is added to a base extender is obtaining a homogeneous solution, because soy lecithin is insoluble in water solutions and originates emulsions. This could prove to be a handicap when the extender is used after hours or days of storage at 5 °C and 15 °C, which is required in the field when AI is applied with refrigerated semen. Temperature conditions, preparation methods, and storage time could affect the emulsion formation and phospholipids availability in the extender.

The aim of the present study was to develop an extender based on soybean lecithin to replace traditional egg yolk extenders analyzing the optimal conditions of preparation, handling, and storage in order to optimize its use in liquid ram semen. We analyzed the extenders and storage conditions assessing the quantity and nature of phospholipids and the motility and viability in liquid ram semen. These data could provide the basis for new extenders on those species that lack specific formulas.

## 2. Materials and methods

To obtain a stable soy lecithin-based extender with high phospholipids concentration, we used two soybean types (S95 and S20) comparing different preparation temperatures (37 °C and 20 °C), various storage temperatures, and time (at 15 °C for up to 48 h and at 5 °C for up to 7 d) and testing three concentrations of soybean lecithin (0.5%, 2%, and 3.5%). All the chemicals, unless otherwise specified, were obtained from Sigma-Aldrich® (Madrid, Spain) in the reagent grade.

### 2.1. Preparation of the extenders

We tested two types of soy: Soy 95% (L-α-Phosphatidilcholine, soy 95%; S95) and Soy 20% (L-α-Phosphatidilcholine, soy 20%; S20) both from Avanti Polar Lipids® (Alabaster, Alabama, USA). S95 was composed of phosphatidylcholine (PC) and a minority phospholipid, lysophosphatidylcholine (LPC). Soy 20% was composed of PC, phosphatidylinositol (PI), phosphatidylethanolamine (PE), and LPC.

A TES-Tris-Fructose buffer solution [TES solution (325 mOsm/kg) titrated to pH 7.2 with Tris solution (325 mOsm/kg), and with 4% final volume of D-fructose solution (325 mOsm/kg)] was used as base extender according to Anel et al [20] and, except for the concentration experiment, 2% solutions of S95 and S20 were prepared.

### 2.2. Quantitative and qualitative analyses of phospholipids (chromatography)

The quantitative and qualitative analyses were carried out by high performance liquid chromatography (HPLC) method. We tested each samples three times in the HPLC equipment (Alliance Waters 2690 Separation Module) which consists of a LC-10A pump, an injector with a 20 μL sample loop, and an ultraviolet detector working at a wavelength of 203 nm (Waters 996 Photodiode Array Detector; Waters, Millford, MA). The chromatographic data were acquired by chromatography working station (Millenium$^{32}$ version 3.05.01, Waters, Millford, MA). The separation was performed on a 150 × 4.6 mm column packed with 5 μm home made silica. The column (Tracer Excell 120) and precolumn were obtained from TEKNOKROMA® (Barcelona, Spain).

The samples were diluted in chloroform (1:0.250 v/v), washed four times by centrifugation at 3500 × g for 10 min, and the pellet was then analyzed. A mixture