Animal Reproduction Science xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Comparison of plant- and egg yolk-based semen diluents on *in vitro* sperm kinematics and *in vivo* fertility of frozen-thawed bull semen

E.M. Murphy[a,b], C. O'Meara[b], B. Eivers[b], P. Lonergan[c], S. Fair[a,*]

[a] Laboratory of Animal Reproduction, Department of Biological Sciences, School of Natural Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, V94 T9PX, Ireland
[b] National Cattle Breeding Centre, Naas, Co Kildare, W91 WF59, Ireland
[c] School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, D04 N2E5, Ireland

ARTICLE INFO

Keywords:
Sperm
Frozen-thawed semen
Diluents
Artificial insemination
Non-return rates

ABSTRACT

Diluents using components of plant origin have been developed as an alternative to animal based extenders for the dilution of bull semen, however, it is unclear if use of these diluents results in *in vivo* fertility rates similar to those that occur with use of traditional egg yolk-based diluents. The aim of this study was to assess the effect of semen diluent on 60-day non-return rate (NRR) following artificial insemination (AI) with frozen-thawed bull semen. The effect of semen dilution in one of three different commercial diluents (BullXcell – egg yolk-based, OptiXcell – plant-based or AndroMed – plant-based) on post-thaw total and progressive motility as well as kinematic parameters (Experiment 1) and field fertility (Experiment 2, $n = 1{,}480$ inseminations) was assessed. Semen stored in OptiXcell had greater post-thaw total and progressive motility than AndroMed ($P < 0.05$) but did not differ from BullXcell. Semen stored in BullXcell had a greater beat cross frequency and straight line velocity compared to semen stored in AndroMed ($P < 0.05$) but did not differ when compared with use of OptiXcell; while values for these variables when using OptiXcell and AndroMed did not differ from each other ($P > 0.05$). There was no difference in any other sperm kinematic parameters ($P > 0.05$). There was no effect of diluent on 60-day NRR (71.5%, 67.8% and 70.6% for BullXcell, OptiXcell and AndroMed, respectively). In conclusion, while diluent significantly affected post-thaw sperm motility and kinematics, no effect on 60-day NRR was observed. Given that OptiXcell and AndroMed are animal protein-free media these diluents may be a suitable alternative to BullXcell for the storage of frozen-thawed bull semen.

1. Introduction

The extensive use of artificial insemination (AI) within the dairy industry can be attributed in part to the development of suitable diluents for both fresh and frozen-thawed semen (Foote et al., 2002). Cryoprotectants, predominately glycerol and egg-yolk, are added to extenders to protect sperm from damage during the cryopreservation process. Since the discovery of the protective properties of egg yolk in relation to the preservation of bull semen (Phillips and Lardy et al., 1940), the addition of egg yolk (a non-permeable cryoprotectant) is regarded as one of the most essential components of diluents (Vishwanath and Shannon et al., 2000, Crespilho et al. 2012). It is widely acknowledged that low-density lipoproteins (LDLs) are the main component in egg yolk extenders offering protection, primarily functioning through increasing the cholesterol/phospholipid ratio, thus preventing a loss of membrane

* Corresponding author.
E-mail address: sean.fair@ul.ie (S. Fair).

https://doi.org/10.1016/j.anireprosci.2018.02.010
Received 4 January 2018; Received in revised form 30 January 2018; Accepted 13 February 2018

phospholipids, increasing chilling tolerance and reducing cold shock injuries (Medeiros et al. 2002, Muiño et al. 2007).

The use of egg yolk, however, is not without its disadvantages as it renders microscopic semen assessment more difficult, particularly when using computer-assisted sperm analysis techniques (CASA; Singh et al. 2012). Furthermore, being a protein of animal origin, egg yolk may introduce the risk of exotic disease transmission, such as avian influenza (Yildiz et al. 2013), or microbial contamination leading to increased widespread health concerns over its use in semen diluents (Aires et al. 2003). Moreover, there is growing demand for full product tractability and increasing emphasis on biosecurity issues in government legislation regarding animal based products (Layek et al. 2016). In addition, egg yolk is difficult to standardise, with significant potential for variation from batch to batch (Bousseau et al. 1998), posing problems for quality assurance in laboratories. Alternatives to components of animal origin in semen extenders such as soya-lecithin in plant-based diluents are, therefore, of interest (Gil et al. 2003, Akhter et al. 2012, Ansari et al. 2016), primarily due to the traceability and the reduced health risk associated with animal protein-free media and would represent a valuable contribution to the AI industry, however, these diluents are still not universally accepted due to concerns over reduced fertility (Leite et al. 2010, Layek et al. 2016).

Plant-based extenders contain a natural mixture of phosphatidylcholine and a number of fatty acids such as stearic, oleic and palmitic acid which are known to confer structural stability to cells (Oke et al. 2010, Chaudhari et al. 2015). Due to this composition, plant-based extenders have been used to substitute for egg yolk extenders as alternative diluents for commercial semen with studies reporting comparable *in vitro* assessment results in a number of species including cattle (Aires et al. 2003, Stradaioli et al. 2007, Miguel et al. 2008), ovine (Gil et al. 2003, Forouzanfar et al. 2010) and horses (Papa et al. 2010). In addition, comparable fertility rates have been reported in buffalo (Akhter et al. 2012). A number of other studies have, however, reported a reduction in semen quality when comparing plant-based versus egg yolk-based extenders (Muiño et al. 2007) with some studies also reporting a reduction in fertility (Van Wagtendonk-de Leeuw et al. 2000, Crespilho et al. 2012). The exact mechanism through which plant-based extenders protect sperm from cryo-injury is not well understood. It is believed that exogenous phospholipids and liposomes composed of different lipids protect sperm by reversibly binding lipids and phospholipids as well as fusing liposomes with the sperm plasma membrane, thus stabilising the membrane during the freezing and subsequent thawing process (Ansari et al. 2016). Furthermore, Zeron et al. (2002) reported that the fusion of liposomes with sperm membranes decrease the lipid phase transition of bull sperm resulting in decreased sensitivity of sperm to cryopreservation.

The aim of this study was to compare three commercially available diluents for frozen-thawed bull semen, one egg yolk-based and two plant-based, in terms of sperm functional parameters *in vitro* and fertility following AI. Importantly, ejaculates were split such that each treatment was represented in each ejaculate, eliminating any potential confounding affects.

2. Material and methods

2.1. Semen collection and processing

Semen was collected from Holstein-Friesian bulls ($n = 5$) at a commercial AI centre on four different occasions (occasion = replicate; total of 20 ejaculates) from early April to the end of April 2017. Upon collection, the raw ejaculate was split into three equal parts and partially diluted in 2 mL (approximately 1:1) of each of the pre-warmed (37 °C) diluents, namely, BullXcell (egg yolk-based; IMV Technologies, L'Aigle, France), OptiXcell (plant-based; IMV Technologies) and AndroMed (plant-based; Minitube, Tiefenbach, Germany) for transport. All diluents were prepared as per the manufacturer's instructions. Semen from each bull was kept separate throughout processing and ejaculates were split such that each bull was represented in each treatment. The ejaculate was then placed into a temperature-regulated cooler box at 18 °C and transported to the laboratory (approximately 3 h transport). At the time of arrival, the ejaculate was assessed for weight, sperm concentration using a coulter counter (Z Series, Beckman Coulter, Clare, Ireland), total motility (%) and gross motility on a 5-point scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard (results not shown). Microscopic assessments were conducted by the same experienced technician and initial quality control cut-off values were a total and gross motility of $\geq$ 70% and a score of $\geq$ 3, respectively; any ejaculates failing to meet these criteria were rejected and thus not used in the study.

Following *in vitro* assessment, each acceptable ejaculate was fully extended in the respective diluents to achieve a concentration of 15×10^6 sperm per 0.25 mL insemination dose. The final dilution ratio was dependent upon the ejaculate volume and sperm concentration per mL within each ejaculate. Semen straws were filled, printed and sealed as per routine procedures, gradually cooled to 4 °C and frozen using the following protocol: −5 °C per min from +4 to −10 °C, −40 °C per min from −10 to −100 °C and thereafter −20 °C per min from −100 to −140 °C (Murphy et al. 2018) in a programmable freezer (IMV Technologies), followed by submersion and storage in liquid nitrogen at −196 °C until use.

2.2. Experiment 1: in vitro analysis of the effects of semen diluent on frozen-thawed semen

The aim of this experiment was to assess the effects of three commercially available diluents on the motility and kinematics parameters of frozen-thawed bull sperm using the IVOS-II CASA system driven by software version 14 (Hamilton Thorne Inc, Beverly, USA). Samples from the three treatments were assessed in a randomised sequence to remove bias as a result of sampling order. Straws ($n = 4$ per ejaculate) were thawed at 37 °C for 30 s and each sample was diluted at a 1:3 ratio in EasyBufferB (IMV Technologies). A drop (3 µL) of diluted semen was placed in a pre-warmed chamber (37 °C; Leja counting chambers, depth 20 µm; Microptics, Barcelona, Spain) and analysed for sperm motion and kinematic characteristics immediately post-thaw. A minimum of 1000 sperm