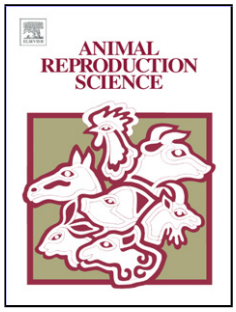

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"Revised"

Title

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

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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.

Keywords:

Sperm; Frozen-thawed semen; Diluents; Artificial insemination; Non-return rates

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 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 1940), the addition of egg yolk (a non-permeable cryoprotectant) is regarded as one of the most essential components of diluents (Vishwanath and Shannon 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 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 ºC) 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 ºC 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 x 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 ºC and frozen using the following protocol: -5 ºC per min from +4 to -10 ºC, -40 ºC per min from -10 to -100 ºC and thereafter -20 ºC per min from -100 to -140 ºC (Murphy et al. 2018) in a programmable freezer (IMV Technologies), followed by submersion and storage in liquid nitrogen at -196 ºC 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 ºC for 30 sec 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 ºC; 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 were analysed in at least eight microscopic fields with 30 frames acquired per field at a frame rate of 60 Hz. Objects incorrectly identified as sperm were edited out using the playback function. The CASA-derived motility and kinematic characteristics assessed were total motility (%), progressive motility (%), proximal and distal droplets (%), as well as average path velocity (VAP above 10 µm/s), straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF; Mortimer 2000). Regarding analysis settings, the CASA was set to standard factory

settings for bull semen and sperm with straightness of >80% and VAP >50 μm/s were considered progressively motile.

2.3. Experiment 2: Field fertility of frozen-thawed semen diluted in BullXcell, OptiXcell and AndroMed

The aim of this experiment was to assess the effect of frozen-thawed bull semen diluent on 60-day non return rate (NRR) following AI. Semen from the same Holstein-Friesian bulls (n = 5) and same batches of semen as in Experiment 1 (15 x 10^6 sperm per 0.25 mL dose) were used in the field trial. Each batch of semen was clearly labelled and distributed for insemination after 30 days of quarantine as per European regulations (Irish Status Book, 2004). Inseminations were conducted in May 2017 (coinciding with the peak dairy breeding season) in Irish dairy herds (n = 255). The majority (97%) of inseminations were in Holstein-Friesians (n = 1,433) but a small number of other breeds were represented including Jersey, MRY, Ayrshire, Montbeliarde, Norwegian Red and Shorthorn. Technicians (n = 22) were blind to treatments and received equal number of straws from each of the three treatments from each bull. For each insemination, the AI technician recorded the bull code, cow tag number and the straw code on an electronic handheld device. Inseminations and NRR data were captured using the Irish Cattle Breeding Federation (ICBF; Bandon, Co. Cork, Ireland) database by cross-referencing the technician name with the bull code and semen type used on each date within the trial period. Obvious errors (n = 115) were extracted from the dataset and data were then interrogated to remove animals based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or treatments, or cows which were not of a dairy breed. If a dairy cow or heifer received two inseminations from the same bull with the same diluent treatment within 5 days of each other, however, the record was kept and the second date was assumed to be correct.

Post editing, a total of 1,480 inseminations (n = 576, 547 and 357 for BullXcell, OptiXcell and AndroMed, respectively) consisting of 280 heifers and 1,200 multiparous dairy cows remained.

Cow characteristics such as parity, days in milk (DIM) and fertility sub-index were also included in the model. Fertility sub index is a key component of the Economic Breeding Index (EBI) comprising ~35% of the total EBI (ICBF, 2017). The EBI is an estimate of the economic value of an animal's genetic merit. It was established to combat a decrease in reproductive performance by providing farmers with a profit index enabling the selection of elite sires to breed replacement heifers with increased milk yield, reproductive performance and improved health traits (Berry et al. 2005).

2.4. Statistical Analysis

Data from Experiment 1 were examined for homogeneity of variance and analysed using the general linear model (GLM) repeated-measures procedure with a compound symmetry covariance structure in Statistical Package for Social Science (SPSS, Version 22.0; IBM, Chicago, USA). In Experiment 2, NRR data were compared using Pearson's Chi-square procedures in SPSS. The dependent variable in the analysis was NRR (1 = pregnant, 0 = not pregnant). In addition, a GLM for binomial data was used to assess the influence of a number of fixed effects on NRR including diluent treatment, bull, parity, cow breed, cow fertility sub-index, DIM, herd and technician. Each fixed effect was assessed for an interaction with treatment. All *post-hoc* tests were conducted using the Bonferroni test and results are reported as the mean ± the standard error of the mean (sem) in Experiment 1 and as the estimated marginal means in Experiment 2, to adjust for imbalance between the number of inseminations in each treatment. Data were considered to differ at $P < 0.05$.

3.1. Results

3.1. Experiment 1: In vitro effects of semen diluent on frozen-thawed semen

There was an effect of diluent on post-thaw total and progressive motility as assessed by CASA ($P < 0.01$; Table 1). Semen diluted in OptiXcell had greater post-thaw total and progressive motility (59.0 ± 4.52% and 45.7 ± 4.09%, respectively) scores than that diluted in AndroMed ($P < 0.05$) but did not differ from BullXcell which were intermediate (Table 1). There was an effect of treatment on BCF and VSL ($P < 0.05$) as semen diluted in BullXcell exhibited superior BCF and VSL compared to semen diluted in AndroMed ($P < 0.01$) but did not differ from OptiXcell ($P > 0.05$). Semen diluent did affect any other kinematic motility parameter (ALH, LIN, STR, VAP, VCL and WOB) or the percentage of sperm with proximal and distal droplets (Table 1).

3.2. Experiment 2: Field fertility of frozen-thawed semen diluted in BullXcell, OptiXcell and AndroMed

Diluent did not affect field fertility of frozen-thawed semen, with a 60-day NRR of 71.5%, 67.8% and 70.6% for BullXcell, OptiXcell and AndroMed, respectively; Figure 1). There was no bull, breed, parity, cow fertility sub-index, DIM, herd or technician by treatment interaction with NRR. Cows which were less than 60 DIM had a reduced NRR (65.4%) in comparison with those greater than 60 DIM prior to insemination (76.3%). There was no difference in NRR between maiden heifers (71.4%), primiparous (72.5%) and multiparous dairy cows (68.5%). As expected, NRR varied between individual herds and technicians ($P < 0.01$). There was no effect of bull, breed, cow fertility sub-index or parity on NRR ($P > 0.05$).

4. Discussion

The continuous health concerns within the AI industry relating to the use of animal proteins in semen extenders have led to the development of alternative diluents free of animal-derived products. Split ejaculates and a combination of *in vitro* and *in vivo* assessments were used in the present study in a comprehensive attempt to identify the optimal semen diluent for frozen-thawed bull semen. The main findings in the present study were that semen diluted in plant-based extenders OptiXcell and AndroMed resulted in similar total and progressive motility *in vitro* and NRR following AI compared to egg yolk-based extender BullXcell.

Motility is one of the most important parameters associated with semen fertilising capacity and has, therefore, been recognised as essential for sperm transport and fertilisation in the female reproductive tract (Verstegen et al. 2002). The presence of egg yolk globules has been shown, however, to interfere with microscopic evaluation (Vishwanath and Shannon 2000) with the use of plant-based extenders resulting in greater sample visualisation and a lesser bacterial load (Meena et al. 2010). There are, therefore, marked advantages in using plant-based over animal-based extenders. The efficacy of the use of plant-based extenders is, however, still a matter of debate with studies reporting contradictory *in vitro* and *in vivo* results. Some studies report no difference when comparing plant-based with egg-yolk extenders (Bousseau et al. 1998, Gil et al. 2003) whereas others found beneficial (Aires et al. 2003, Chaudhari et al. 2015) or damaging effects (Crespilho et al. 2012, Veerabramhaiah et al. 2015). In the current study, diluent did not affect the majority of sperm kinematic parameters (ALH, LIN, STR, VAP, VCL and WOB), nevertheless, it is noteworthy that extender had an effect on VSL and BCF with BullXcell having a greater value than semen diluted in AndroMed. A greater BCF value indicates that BullXcell may be more effective at preserving flagellar structures or stimulating ATP production and consequently sperm tail

beat frequency (Celeghini et al. 2008) compared to AndroMed; but there was no difference between BullXcell and OptiXcell. Ansari et al. (2016) and Aires et al. (2003) reported that sperm motility increased when semen was diluted in OptiXcell and AndroMed, respectively, compared to an egg yolk-based diluent. Furthermore, Kumar et al. (2015) reported that semen diluted in plant-based liposome extenders had improved kinematic parameters than semen diluted in egg yolk extenders while Crespilho et al. (2012) reported that use of egg yolk-based extenders resulted in greater total and progressive motility scores compared to use of a lecithin-based extender. The results of the current study are inconsistent with these previous findings as there was no overall significant difference observed when use of plant-based and egg yolk-based extenders on sperm motility was compared.

Continuous debate exists surrounding the cryo-protective capabilities of plant-based extenders compared to egg yolk-based extenders (Leite et al. 2010, Layek et al. 2016). Previous studies have reported a reduction in motility, viability and membrane integrity when semen was diluted in soy-lecithin-based extenders compared to egg yolk-based extenders (Celeghini et al. 2008, Papa et al. 2010, Beran et al. 2012, Crespilho et al. 2012) as well as greater protective capacity of egg yolk extenders resulting in greater *in vivo* fertility (Thun et al. 2002, Veerabramhaiah et al. 2015). In contrast other studies have reported greater sperm total motility, acrosomal integrity (Aires et al. 2003, Amirat et al. 2005, Chaudhari et al. 2015) and greater (Akhter et al. 2012) or similar (Bousseau et al. 1998, Gil et al. 2003) fertility rates when cryopreserved semen was diluted in lecithin-based extenders as compared with egg yolk extenders. It has also previously been reported that greater viscosity and the presence of particulate debris in egg yolk-based extenders may cause reduced fertility (Van Wagtendonk-de Leeuw et al. 2000); however, in the current study there was no difference in 60-day NRR of semen diluted in either egg yolk- or lecithin-based diluents. The results also are inconsistent with those of Ansari et al. (2016) and Aires et al. (2003), based on

approximately 100 and 9,000 inseminations per treatment, respectively, where it was reported that there were greater fertility rates when semen was diluted in OptiXcell and AndroMed compared to egg yolk extenders. The absence of a diluent effect on NRR observed in the present study suggests that plant-based diluents are as effective at protecting sperm cells during cryopreservation process as egg-yolk extenders. While it is widely accepted that cow characteristics such as parity, fertility sub-index and DIM have a role in fertility (Murphy et al. 2016, Murphy et al. 2017), semen diluent did not negate the effects of these on NRR.

5. Conclusion

In conclusion, the chemically defined plant-based commercial extender, OptiXcell, was more efficient than the egg yolk-based extender BullXcell in conserving post-thaw total and progressive motility; however, there was no effect of diluent on the 60-day NRR. Given that the use of plant-based extenders such as OptiXcell or AndroMed provides substantial advantages compared to egg-yolk extenders by reducing health risks, increasing standardisation and ease of preparation and assessment, the use of plant-based extenders should be considered as a viable alternative to egg yolk based diluents.

Declarations of interest

The authors declare that they have no financial or personal relationship with other people or organisations that could inappropriately influence or bias the paper entitled "Comparison of plant- and egg yolk-based semen diluents on *in vitro* sperm kinematics and *in vivo* fertility of frozen-thawed bovine semen".

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Table 1

Effect of semen diluent on post-thaw motility and kinematic parameters in bull semen as assessed by computer assisted sperm analysis (Experiment 1)

Parameters	Treatment mean ± sem			*P* value
	BullXcell	OptiXcell	AndroMed	Effect of Treatment
Total Motility (%)	51.9 ± 1.76^{ab}	59.0 ± 4.52^{b}	41.93 ± 3.61^{a}	< 0.05
Progressive Motility (%)	41.8 ± 1.82^{ab}	45.7 ± 4.09^{b}	31.7 ± 2.97^{a}	< 0.05
ALH (μm)	7.5 ± 0.85	6.6 ± 0.58	7.2 ± 0.67	ns
BCF (Hz)	29.2 ± 0.02^{a}	25.3 ± 1.22^{ab}	24.5 ± 1.24^{b}	< 0.05
LIN (%)	43.1 ± 1.51	44.0 ± 1.48	39.2 ± 1.70	ns
STR (%)	78.7 ± 0.99	79.8 ± 1.52	75.1 ± 2.21	ns
VAP (μm/s)	90.5 ± 5.68	75.5 ± 4.86	72.6 ± 4.19	ns
VCL (μm/s)	174.3 ± 13.76	141.8 ± 10.41	143.7 ± 8.32	ns
VSL (μm/s)	71.0 ± 4.26^{a}	61.1 ± 4.29^{ab}	55.4 ± 4.11^{b}	< 0.05
WOB (%)	53.7 ± 1.24	54.3 ± 0.97	51.4 ± 0.75	ns

Proximal Droplets (%)	2.2 ± 0.33	1.6 ± 0.36	2.5 ± 0.33	ns
Distal Droplets (%)	0.7 ± 0.14	0.5 ± 0.10	0.8 ± 0.08	ns

[abc]Values with different superscripts differ within row ($P < 0.01$; values are mean ± sem); ALH = amplitude of lateral head displacement, BCF = beat cross frequency, LIN = linearity, STR = straightness, VAP = average path velocity, VCL = curvilinear velocity, VSL = straight line velocity, WOB = wobble; ns = non-significant

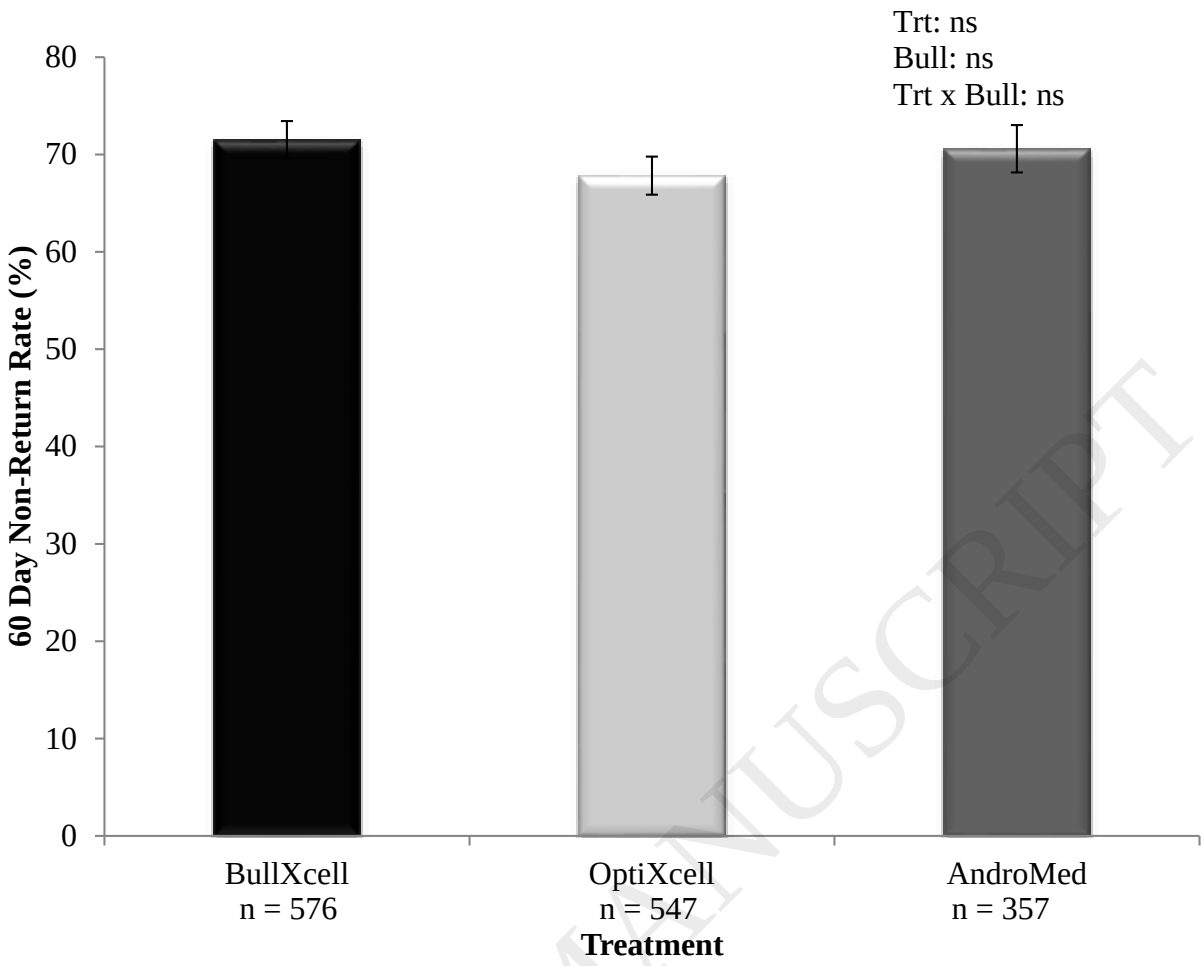

Fig. 1. Effect of semen diluent on 60-day non-return rate of frozen-thawed semen in dairy cows and heifers (Experiment 2); Vertical bars represent 95% confidence intervals; *n* = number of inseminations; Trt = treatment; ns = non-significant

References

Aires, V.A., Hinsch, K.D., Mueller-Schloesser, F., Bogner, K., Mueller-Schloesser, S., Hinsch, E., 2003. In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. Theriogenology 60, 269-279.

Akhter, S., Ansari, M., Andrabi, S., Rakha, B., Ullah, N., Khalid, M., 2012. Soya-lecithin in Extender Improves the Freezability and Fertility of Buffalo (Bubalus bubalis) Bull Spermatozoa. Reprod. Domest. Anim. 47, 815-819.

Amirat, L., Anton, M., Tainturier, D., Chatagnon, G., Battut, I., Courtens, J.L., 2005. 'Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reprod. 129, 535-543.

Ansari, M., Rakha, B., Akhter, S., Ashiq, M., 2016. OPTIXcell improves the postthaw quality and fertility of buffalo bull sperm. Theriogenology 85, 528-532.

Beran, J., Stádník, L., Bezdíček, J., Louda, F., Čítek, J., Ducháček, J., 2012. Effect of sire and extender on sperm motility and share of live or dead sperm in bulls' fresh ejaculate and in AI doses after thawing. Arch. Tierz, 55, 207-218.

Berry, D., Shalloo, L., Cromie, A., Olori, V., Amer, P., 2005. Economic breeding index for dairy cattle in Ireland. Irish Cattle Breeding Federation: Bandon, Cork.

Bousseau, S., Brillard, J., Marquant-Le Guienne, B., Guerin, B., Camus, A., Lechat, M., 1998. 'Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents', Theriogenology 50, 699-706.

Celeghini, E.C.C., de Arruda, R.P., de Andrade, A.F.C., Nascimento, J., Raphael, C.F., Rodrigues, P.H.M., 2008. Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. Anim. Reprod. Sci. 104, 119-131.

Chaudhari, D., Dhami, A., Hadiya, K.., Patel, J., 2015. Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (− 196 C) of buffalo semen. Vet World 8, 239.

Crespilho, A., Sá Filho, M., Dell'Aqua, J., Nichi, M., Monteiro, G., Avanzi, B., Martins, A., Papa, F.O., 2012. Comparison of in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. Livest. Sci. 149, 1-6.

Foote, R.H., 2002. The history of artificial insemination: Selected notes and notables. J. Anim. Sci. 80, 1-10.

Forouzanfar, M., Sharafi, M., Hosseini, S., Ostadhosseini, S., Hajian, M., Hosseini, L., Abedi, P., Nili, N., Rahmani, H., Nasr-Esfahani, M., 2010. In vitro comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. Theriogenology 73, 480-487.

Gil, J., Rodriguez-Irazoqui, M., Lundeheim, N., Söderquist, L., 2003. Fertility of ram semen frozen in Bioexcell® and used for cervical artificial insemination. Theriogenology 59, 1157-1170.

Irish Cattle Breeding Federation (ICBF). Economic Breeding Index (EBI), 2017. https://www.icbf.com/wp/?p=5852 (accessed 16 May 2017).

Irish Statue Book. European Communities (Trade in Bovine Breeding Animals, Their Semen, Ova and Embryos) (Amendment) Regulations 2004), 2004. http://www.irishstatutebook.ie/eli/2004/si/499/made/en/print (accessed 25 September 2017).

Kumar, P., Saini, M., Kumar, D., Balhara, A., Yadav, S., Singh, P., Yadav, P., 2015. Liposome-based semen extender is suitable alternative to egg yolk-based extender for cryopreservation of buffalo (Bubalus bubalis) semen. Anim. Rreprod. Sci. 159, 38-45.

Layek, S., Mohanty, T., Kumaresan, A., Parks, J., 2016. Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. Anim. Rreprod. Sci. 172, 1-9.

Leite, T.G., do Vale Filho, V.R., de Arruda, R.P., de Andrade, A.F.C., Emerick, L.L., Zaffalon, F.G., Martins, J.A.M., de Andrade, V.J., 2010. Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. Anim. Reprod. Sci. 120, 31-38.

Medeiros, C., Forell, F., Oliveira, A., Rodrigues, J., 2002. Current status of sperm cryopreservation: why isn't it better?. Theriogenology 57, 327-344.

Meena, G., Raina, V., Gupta, A., Mohanty, T., Bishist, R., 2010. Comparative performance of biociphos and egg yolk based extenders for buffalo semen cryopreservation. Indian J. Anim. Sci. 80, 414.

Miguel, C.T., Pérez-Garnelo, S., Breña, P.B., Palasz, A., De la Fuente, J., Rodriguez, A., Hidalgo, C., 2008. 162 Freezing of semen from endangered asturiana de la montana bulls in zwitterionic lipids-based extenders. Reprod. Fert. Develop. 20, 161-161.

Mortimer, S.T., 2000. CASA—practical aspects. J. Androl. 21, 515-524.

Muiño, R., Fernandez, M., Peña, A., 2007. Post-thaw Survival and Longevity of Bull Spermatozoa Frozen with an Egg Yolk-based or Two Egg Yolk-free Extenders after an Equilibration Period of 18 h. Reprod. Domest. Anim. 42, 305-311.

Murphy, C., Holden, S.A., Murphy, E.M., Cromie, A.R., Lonergan, P., Fair, S., 2016. The impact of storage temperature and sperm number on the fertility of liquid-stored bull semen. Reprod. Fert. Develop. 28, 1349-1359.

Murphy, E.M., Murphy, C., O'Meara, C., Dunne, G., Eivers, B., Lonergan, P., Fair, S., 2017. A comparison of semen diluents on the in vitro and in vivo fertility of liquid bull semen. J Dairy. Sci, 100, 1541-1554.

Murphy, E.M, Eivers, B., O'Meara, C., Lonergan, P., Fair, S., 2018. Effects of increasing equilibration time of diluted bull semen up to 72 hours prior to freezing on sperm quality parameters and calving rate following artificial insemination. Theriogenology 108, 217-222.

Oke, M., Jacob, J.K., Paliyath, G., 2010. Effect of soy lecithin in enhancing fruit juice/sauce quality. Food Res. Int. 43, 232-240.

Papa, F., Felıcio, G., Melo, C., Vita, B., Avanzi, B., Aqua, J., 2010. Effect of substituting soybean lecithin for egg yolk in an extender used for the cryopreservation of stallion semen. Anim. Reprod. Sci. 121, 71-72.

Phillips, P.H., Lardy, H.A., 1940. A Yolk-Buffer Pabulum for the Preservation of Bull Semen. J. Dairy. Sci. 23, 399-404.

Singh, A., Singh, V., Narwade, B., Mohanty, T., Atreja, S., 2012. Comparative Quality Assessment of Buffalo (Bubalus bubalis) Semen Chilled (5° C) in Egg Yolk-and Soya Milk–Based Extenders. Reprod. Domest. Anim. 47, 596-600.

Stradaioli, G., Noro, T., Sylla, L., Monaci, M., 2007. Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. Theriogenology 67, 1249-1255.

Thun, R., Hurtado, M., Janett, F., 2002. Comparison of Biociphos-Plus® and TRIS-egg yolk extender for cryopreservation of bull semen. Theriogenology 57, 1087-1094.

Van Wagtendonk-de Leeuw, A., Haring, R., Kaal-Lansbergen, L., Den Daas, J., 2000. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. Theriogenology 54, 57-67.

Veerabramhaiah, K., RAO, V., ST, V.R., 2015. Efficacy of the Tris and Biociphos Plus extenders on the freezability of Punganue bull semen. The Indian J. Anim. Reprod. 32, 1-4.

Verstegen, J., Iguer-Ouada, M., Onclin, K., 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57, 149-179.

Vishwanath, R., Shannon, P., 2000. Storage of bovine semen in liquid and frozen state. Anim. Reprod. Sci. 62, 23-53.

Yildiz, C., Bozkurt, Y., Yavas, I., 2013. An evaluation of soybean lecithin as an alternative to avian egg yolk in the cryopreservation of fish sperm. Cryobiology 67, 91-94.

Zeron, Y., Tomczak, M., Crowe, J., and Arav, A., 2002. The effect of liposomes on thermotropic membrane phase transitions of bovine spermatozoa and oocytes: implications for reducing chilling sensitivity. Cryobiology 45, 143-152.