Livestock Science 149 (2012) 1–6

Contents lists available at SciVerse ScienceDirect

Livestock Science

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

Comparison of *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders

A.M. Crespilho [a], M.F. Sá Filho [b], J.A. Dell'Aqua Jr.[c], M. Nichi [b], G.A. Monteiro [c], B.R. Avanzi [c], A. Martins [d], F.O. Papa [c,*]

[a] School of Veterinary Medicine, Santo Amaro University, UNISA, São Paulo, SP, Brazil
[b] Department of Animal Reproduction, São Paulo University, São Paulo, SP, Brazil
[c] Department of Animal Reproduction and Veterinary Radiology, São Paulo State University, Botucatu, SP, Brazil
[d] Department of Clinic, Surgery and Animal Reproduction, São Paulo State University, Araçatuba, SP, Brazil

ARTICLE INFO

Article history:
Received 7 February 2011
Received in revised form
7 May 2012
Accepted 11 May 2012

Keywords:
Bull semen
Semen extender
Egg yolk
Soy lecithin
Cryopreservation
Artificial insemination

ABSTRACT

One of the main factors known to influence quality and fertility of bovine cryopreserved semen is the extender used. In this matter, a great worldwide interest has been directed to the development of chemically defined media, free of animal origin products. The objective of the present study was to compare the efficacy of three bovine semen extenders: Tris–fructose (TRIS, control with 20% egg yolk), Botu-Bov® (BB; 20% egg yolk), and Botu-Bov®-soy lecithin (BB-L; 1% soy lecithin). Towards this aim, post-thaw computer assisted sperm analysis (CASA), sperm membrane and acrosome integrity were evaluated (Experiment 1). Additionally, cryopreserved samples were used in a fixed time artificial insemination program aiming to evaluate *in vivo* fertility (pregnancy per insemination—P/AI; Experiment 2). Despite the higher straightness and linearity found for samples cryopreserved in BB and BB-L when compared to those cryopreserved in TRIS, egg yolk based extenders provided higher total and progressive motilities, percentage of rapid sperms and intact membrane cells ($P < 0.05$). Furthermore, P/IA was higher in samples cryopreserved in egg yolk based extenders when compared to soy lecithin [TRIS=59.26^{a} (64/108), BB=62.37^{a} (58/93), and BB-L=36.45^{b} (35/96)]. Although soy lecithin represents an alternative for the development of chemically defined extenders with decreased risk of biological contamination, egg yolk based extenders are more efficient on the preservation of bovine post-thaw sperm viability and fertility.

1. Introduction

The two main concerns of cattle production systems regarding artificial insemination are the control of pathogens transmission by semen contamination, and the total quality control of the batches produced (Wangtendonk-de Leeuw et al., 2000). Biological safety of semen production is influenced by several factors such as the efficiency of the antibiotics used in the extenders, the quality control of reagents, the hygiene during semen processing, and the quality of the extenders (Ruigh et al., 2006). There is an increased global concern regarding microbiological safety. Therefore, recent studies are in progress aiming to develop chemically defined extenders, free of compounds of animal origin.

Commonly used semen extenders contain egg yolk, skim milk powder, or the combination of both ingredients, as primary source of lipoproteins, important to membrane stabilization during the freeze-thawing process (Bousseau et al., 1998). Despite the significant

* Corresponding author. Tel.: +55 14 3811 6249.
E-mail address: papa@fmvz.unesp.br (F.O. Papa).

1871-1413/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.livsci.2012.05.011

benefits of egg yolk and milk on semen cryopreservation, such components of animal origin may represent a potential microbiological risk, compromising the quality of cryopreserved semen and standardization.

As a consequence, the World Organization for Animal Health (OIE), recommended in the 2003 Terrestrial Animal Health Code, that animal origin products used in semen processing should be free of any biological risk or processed in order to insure the safety of such compounds (Marco-Jiménez et al., 2004).

An alternative to replace the components of animal origin in semen extenders is the soy lecithin, a natural mixture of phosphatidylcholine and several fatty acids such as stearic, oleic, and palmitic. Such fatty acids, the prevailing phospholipids in most of mammalian biological membranes, are known to confer structural stability to cells (Oke et al., 2010). Due to such composition, studies aiming to evaluate the efficiency of soy lecithin as a primary source of lipoproteins in semen extenders were performed in bovine (Aires et al., 2003; Bousseau et al., 1998; Vera-Munoz et al., 2009), buffalo (Akhter et al., 2010), ovine (Gil et al., 2003) and equine (Papa et al., 2010). However, results obtained using lecithin as a substitute to egg yolk are still a matter of debate (Leite et al., 2010). Furthermore, due to the reduced technological innovations on semen cryopreservation over the last years (Celeghini et al., 2008), the Tris–egg yolk–fructose extender is still the most commonly employed worldwide.

The present study aimed to compare the efficiency of soy lecithin based extender with that containing 20% egg yolk based on post-thaw sperm viability and field fertility under a fixed-time artificial insemination program (FTAI).

2. Materials and methods

2.1. Semen collection

Semen was collected from 20 Nellore bulls (*Bos taurus indicus*; from 24 to 30 months old, selected by semen quality from a group of 28 animals) by electroejaculation. In experiment 1, only samples showing motility higher than 70%, major and minor defects lower than 20%, and total sperm defects lower than 25% were used.

In *Experiment 2*, two bulls were selected based on phenotypic traits and used for semen collection, which were performed by artificial vagina. Two ejaculates from each bull were pooled ($n=4$) and cryopreserved.

2.2. Semen processing

Immediately after collection, ejaculates were subjectively evaluated for sperm motility and vigor by light microscopy. Sperm concentration was estimated using a Neubauer counting chamber.

Ejaculates were then submitted to cryopreservation using three different semen extenders: Tris–egg yolk–fructose (TRIS; 30 g of Tris-[hydroxymethyl-aminomethane], 17 g of citric acid, 12.5 g of fructose, 0.20 g amikacin sulphate, 2 mL of emulsifier Orvus WA Paste® (Procter and Gamble, Cincinnati, Ohio, USA) and 200 mL of egg yolk for 1000 mL of extender) and Botu-Bov® (BB; Botupharma, Botucatu, São Paulo, Brazil), both containing 20% of egg yolk (Control Groups), or Botu-Bov-Soy Lecithin (BB-L; Botupharma, Botucatu, São Paulo, Brazil), in which the only modification, when compared to the Botu-Bov®, was that egg yolk was completely replaced by 1% of soy lecithin. All extenders were produced in two fractions; fraction I with no cryoprotectant and fraction II containing 12.4% of glycerol (Merk Pharmaceutical, Darmstadt, Germany).

A concentration of 30×10^6 sperm per straw was used for all extenders. Semen samples extended in fraction I were maintained under a refrigerated environment (5 °C) for three hours. After the initial refrigeration period, the second fraction was added drop-wise to a final concentration of 6.2% of glycerol. Extended semen samples were loaded into 0.5 mL straws (IMV® Technologies, L'Aigle Cedex, France) after a two hour glycerolization period. Samples were then cryopreserved using a computerized freezing machine (Digit Cool®, IMV® Technologies, L'Aigle Cedex, France) using a previously tested freezing curve for bovine semen (from 4 °C to −10 °C at 5 °C/min; from −10 °C to −100 °C at 40 °C/min; and from −100 °C to −140 °C at 20 °C/min).

2.3. Experiment 1

In the first experiment, the effect of semen extender on cryopreserved semen was evaluated *in vitro* using the computerized sperm motility analysis system (CASA, Hamilton Thorn Research IVOS-12, Beverly, MA, USA) and the evaluation of sperm plasma membrane and acrosome integrities.

2.3.1. CASA

Immediately after thawing in water bath (37 °C/30 s), samples were loaded into pre-heated Makler chamber, which was inserted into the CASA system. Five aleatory fields with at least 150 sperm per field were considered.

Sperm variables assessed by the CASA and considered in the present study were: total sperm motility (MOT, %), progressive motility (MP, %), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), straightness (STR, %), linearity (LIN, %), and rapid velocity (RAP, %). Additionally, the proportion of semen batches showing acceptable sperm motility (AM) was also calculated (percentage of batches ≥ 50% MOT).

2.3.2. Plasma and acrosomal membrane integrity

Sperm viability was assessed using the association between the fluorescent probes Propidium iodide (PI) and fluorescein isothiocyanato-labeled Pisum sativum agglutinin (FITC-PSA), according to protocol adapted from Way et al. (1995) and Celeghini et al. (2008).

Briefly, 50 μL of each straw were transferred to 37 °C 1.5 mL microcentrifuge tubes containing 50 μL sodium citrate 2.94%. Propidium iodide (3 μL; 50 mg/mL in PBS) and FITC-PSA (30 μL; 100 μg/mL in PBS) were added to the samples which were then incubated at 38 °C for 10 min. Samples were loaded onto glass slides, covered with a 24 × 32-mm cover glass and observed under epifluorescence microscope (1000 ×; LEICA®, Solms,

Germany) through a single fluorescent filter (465–495 nm excitation and 515–555 nm emission). Two hundred sperm were evaluated and classified. Only the percentage of sperm with both an intact plasma membrane and an intact acrosome were included in the IPMA data and considered in statistical analyses.

2.4. Experiment 2

Semen pooled from two bulls and cryopreserved in TRIS, BB and BB-L were used for timed artificial insemination ($n=349$). Multiparous and lactating Nellore or crossbred Nellore cows were maintained under pasture of *Brachiaria brizantha*, with free access to mineral salt and water. Synchronization protocol followed by FTAI was performed during the south hemisphere summer period (December 2007/January 2008). All cows were maintained in the same commercial farm in Mato Grosso do Sul, Brazil (20°14′26″ latitude; 56°22′42″ longitude) and inseminations was performed in two random replicates.

2.4.1. Synchronization protocol for FTAI

In a random day of estrous cycle, all animals received 2.0 mg of Estradiol Benzoate (Estrogin®/Farmavet, São Paulo, Brazil) associated to the insertion of an intravaginal progesterone device of 2nd or 3rd use (CIDR-B®, Pfiser Saúde Animal, São Paulo, Brazil), which were removed after eight days. On the same day devices were removed, 300 IU of intramuscular eCG (Folligon®, Intervet-Schering Plough, Boxmeer, Netherlands), 25 mg of Dinoprost-Trometamine (Lutalyse®, Pfiser Saúde Animal, São Paulo, Brazil), and 0.6 mg of Estradiol Cypionate (ECP®, Pfiser Saúde Animal, São Paulo, Brazil) were administered. Artificial inseminations were performed 52–56 h after progesterone device removal (D10).

2.4.2. Ultrasound examination

All cows were submitted to three ovarian ultrasound examinations (Aloka-SSD 500, 5 MHz probe, Tokyo, Japan) and at the moment of progesterone device removal and on the day of FTAI, in order to determine synchronization rates and two days after artificial insemination, in order to confirm ovulation, i.e., disappearance of follicles ≥ 8.0 mm in two consecutive evaluations (Gimenes et al., 2008).

Pregnancy diagnoses were performed by ultrasound 40 day after FTAI. Cows were considered pregnant when embryonic vesicle was present and fetal heartbeats were visible.

2.5. Statistical analysis

In Experiment 1, the explanatory variable that was included in the statistical model was the treatment, bull and interaction between bull and treatment. Dependent variables (i.e., CASA parameters and IPMA) were analyzed by the GLIMMIX procedure of SAS. Response variables were tested according to their homogeneity and normality of variances using Guide Data Analysis from SAS. The Log transformation of VCL parameter was subjected to analyses. The results are presented as the means ± SE.

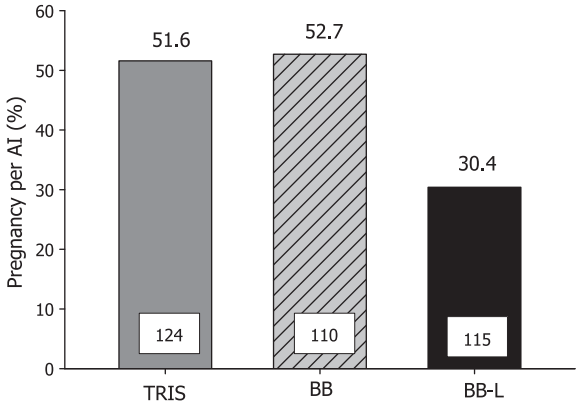

Fig. 1. Pregnancy per AI determined by ultrasound 40 days after the timed artificial insemination (TAI) in suckled *Bos indicus* cows according to the type of extenders (TRIS: Tris–egg yolk–fructose extender; BB: Botu-Bov®; BB-L: Botu-Bov® with 1% of soy lecithin). A significant effect of treatment ($P=0.0009$) on pregnancies per artificial insemination was found. Numbers in boxes indicate number of animals bred.

Results of the percentage of AM were analyzed by contingency tables using Fisher's exact test ($P<0.05$).

A binomial distribution was assumed for the categorical response variable. The P/AI was analyzed using the GLIMMIX procedure of SAS, with cows as a random effect. In Experiment 2, the variables that were initially included in the models were the treatments (TRIS, BB, or BE), BCS at the first day of the synchronization protocol (BSC; 1 to 5 scale, where 1=emaciated and 5=obese; Ayres et al., 2009), breeding group, type of P4 device (2nd or 3rd use) and interactions. For the final logistic regression model, variables were removed through backward elimination based on the Wald statistics criterion when $P>0.20$. Only the treatment was included in the final model for analysis.

3. Results

3.1. Experiment 1

A significant effect was found between the extenders used in the present study for total motility (58.52%, 61.24% and 31.43%, respectively for TRIS, BB and BB-L), progressive motility (TRIS=40.48%; BB=49.24%; BB-L=24.90%), percentage of rapid sperm (TRIS=52.57%; BB=57.29%; and BB-L=29.19%) and for the percentage of semen samples showing acceptable sperm motility post-thawing (TRIS=80%; BB=80%; and BB-L=20%), demonstrating improvement on motility variables when using extenders with 20% egg yolk ($P<0.0001$; Table 1). Regardless the fact that sperm cryopreserved in soy lecithin based extender showed higher straightness and linearity when compared to the TRIS egg yolk extender, a decrease on post-thaw sperm integrity was observed on lecithin cryopreserved samples when compared to both egg yolk based extenders ($P=0.0005$).

3.2. Experiment 2

Mean ovulation rates observed in the present study (85.10%, 297/349) were similar to previous study using

Table 1
Post-thaw sperm motility parameters and acrosome and membrane integrities in samples cryopreserved in Tris–egg yolk–fructose (TRIS), Botu-Bov® (BB), and Soy lecithin based extender (BB-L).

Sperm parameters	TRIS	BB	BB-L	Treatment	Bull	Treatmen × bull
MOT (%)	58.52 ± 2.98^{a}	61.24 ± 3.18^{a}	31.43 ± 3.87^{b}	< 0.0001	0.0407	0.7769
PROG (%)	40.48 ± 1.83^{a}	49.24 ± 2.49^{b}	24.90 ± 3.22^{c}	< 0.0001	0.0369	0.6717
VCL (μm/s)	136.80 ± 5.50^{a}	123.26 ± 5.00^{a}	139.73 ± 5.49^{a}	0.0563	0.0009	0.4440
ALH (μm)	5.83 ± 0.22^{a}	4.88 ± 0.18^{b}	5.45 ± 0.23^{a}	0.0044	0.0038	0.4690
STR (%)	80.76 ± 0.76^{a}	86.62 ± 0.69^{b}	86.70 ± 0.86^{b}	< 0.0001	0.1094	0.3393
LIN (%)	49.86 ± 0.85^{a}	58.19 ± 0.96^{b}	56.55 ± 1.09^{b}	< 0.0001	0.0499	0.8517
RAP (%)	52.57 ± 2.73^{a}	57.29 ± 3.25^{a}	29.19 ± 3.72^{b}	< 0.0001	0.0135	0.6700
IPMA (%)	8.21 ± 1.34^{a}	9.88 ± 1.41^{a}	2.53 ± 0.49^{b}	0.0005	0.1047	0.7826
AM (n/n) (%)	$(16/20)^{a}$ 80%	$(16/20)^{a}$ 80%	$(4/20)^{b}$ 20%	0.0004	–	–

Different letters on the same line indicate the statistical differences between treatments ($P < 0.05$).

Table 2
Number of inseminated cows (n), number of cows ovulating per treatment (nOV), ovulation rate (OvR, %) and pregnancy per ovulated cows (POV) according to bull semen extender used to sperm cryopreservation.

Extender	n	nOV	OvR	POV (%)
TRIS	124	108	0.87	59.26^{a}
BB	110	93	0.85	62.37^{a}
BB-L	115	96	0.83	36.46^{b}

Different letters on the same column indicate the statistical differences between treatments ($P < 0.05$). TRIS: Tris–egg yolk–fructose extender; BB: Botu-Bov®; BB-L: Botu-Bov® with 1% of soy lecithin.

the same synchronization protocol. Conception rates observed after AI using the extenders TRIS, BB or BB-L were 51.61% (64/124), 52.73% (58/110), and 30.43% (35/115), respectively ($P=0.0009$). BCS at the first day of synchronization protocol ($P=0.9265$), Fig. 1 breeding group ($P=0.7752$), type of P4 device ($P=0.7378$) and interactions were not associated with P/AI after FTAI.

When only cows that responded to the stimulation protocol were considered, conception rates were 59.26%, 62.57%, and 36.46%, respectively for the semen pooled and cryopreserved in TRIS, BB and BB-L ($P=0.0002$, Table 2).

4. Discussion

The advantages of lecithin based bovine semen extenders over milk and/or egg yolk regarding sanitary issues are unquestionable. According to Bousseau et al. (1998), the use of lecithin may prevent the contamination with bacteria and mycoplasm. However, the hypothesis that cryoprotectant capacity of soy lecithin is similar to that provided by egg yolk was not confirmed in the present study.

The efficacy of lecithin based extenders is still a matter of debate. Previous studies report higher sperm total motility and plasma and acrosomal integrity (Aires et al., 2003; Amirat et al., 2005) with similar or even higher fertility rates (Akhter et al., 2010; Bousseau et al., 1998; Gil et al., 2000) for semen cryopreserved using lecithin based commercial extenders, developed for use with plant-derived compounds.

Since the composition of semen extenders strongly influences sperm survival (Chaveiro et al., 2006), it is improbable that the higher results found for soy lecithin commercial extenders are related solely to the cryoprotectant effect of the lecithin. Commercial formulations are not liable to specific modifications (Paz et al., in press) aiming to evaluate the effect lecithin alone. In the present study, soy lipoproteins were used to replace the conventionally used egg yolk using the same formulation of Botu-Bov®, an egg yolk based bovine semen extender of proven efficacy (Celeghini et al., 2008; Crespilho et al., 2006). Therefore, the effect of soy lecithin on sperm viability and fertility could be isolated.

International standards postulate that a minimum of 50% of sperm should be motile after thaw (Zhang et al., 1999). In the present study, only 20% of semen samples cryopreserved in egg yolk based extenders did not reach the required minimum limit, while 80% of the samples cryopreserved using the BB-L ($P=0.0004$) showed sperm quality below the standards required for AI programs. Post-thaw values for MOT, PROG and RAP were, on average, 50% lower for samples cryopreserved using BB-L when compared to samples processed with TRIS or BB (Table 1). Similar results were found by Celeghini et al. (2008), in which sperm had higher percentages of total and progressive motilities when cryopreserved using Botu-Bov® as compared to those cryopreserved in commercial lecithin based extender.

The higher values of STR and LIN found for the extender BB-L were similar to those found by Thun et al. (2002). According to these authors, such results could be due to the lower viscosity found in lecithin based extenders when compared to the Tris–egg yolk–sodium citrate. The lipid particles found on egg yolk based extenders could also play a deleterious role on progressive motility, acting as a physical barrier for spermatozoa, influencing natural sperm trajectory. However, no differences were found on STR and LIN between BB and BB-L, which could be due to the common origin of the different extenders (the only difference was the lipoproteins source). Furthermore, in the final process of Botu-Bov® production, a centrifugation step is included, which may insure the solution clarification (personal communication).

The percentage of viable sperm in a semen sample may be defined as the amount of cells showing membrane

(Graham and Mocé, 2005; Graham, 2001) and acrosome stability and integrity (Dayem et al., 2009). This variable may therefore, reflect the efficacy and adequacy of cryopreservation protocol. In the present study, inferior results were found for IPMA when using BB-L, demonstrating the superiority of egg yolk on maintaining plasma and acrosomal membrane integrity, similarly to previous studies (Muiño et al., 2007; Thun et al., 2002).

Such differences on the efficacy of each extender may reflect the protection conferred for the different components during the cryopreservation process (Celeghini et al., 2008). The higher viability rates after thawing are probably due to the action of lipoproteins and phospholipids found in the egg yolk, which may have protected sperm membrane through the increase on the proportion cholesterol/phospholipids, reducing the cold shock (Medeiros et al., 2002) and improving cell viability (Kulaksiz et al., 2010).

Several studies indicate a significant individual variability on fertility indexes (i.e., bull effect), which justify the use of semen pooled from several bulls, especially in *in vivo* studies aiming to compare the efficacy of different techniques of sperm preservation. In this regard, results found in the preset study indicate the superiority of egg yolk based extenders, which increased significantly the probability of P/AI (Table 2). Furthermore, in addition to the low efficacy of soy lecithin as a primary source of lipoproteins, essential for sperm protection during cryopreservation, previous studies indicate that soy lecithin lipids may bound irreversibly to the equine sperm membrane leading to impairment of the sperm capacitation process (Papa et al., 2010), which, in turn, would result in lower conception rates. Thus, phospholipids incorporation might explain the differences found on egg yolk and soy lecithin based extenders on bovine sperm cryopreservation.

In conclusion, soy lecithin protective capacity is limited during sperm cryopreservation resulting in impaired post-thaw sperm viability and decrease on P/AI when compared to egg yolk based extenders.

Conflict of interest statement

The authors declare they have no actual or potential conflict of interest that could inappropriately influence in this work.

Acknowledgments

The present study received financial support in the form of Grant from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo, Brazil; Grant number 06/61153-5).

References

Aires, V.A., Hinsch, K.D., Schloesser, F.M., Bognera, K., Schloesser, S.M., 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.

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. Reproduction 129, 535–543.

Akhter, S., Ansari, M.S., Rakha, B.A., Andrabi, S.M.H., Iqbal, S., Ullah, N., 2010. Cryopreservation of buffalo (*Bubalus bubalis*) semen in Bioxcell® extender. Theriogenology 74, 951–955.

Ayres, H., Ferreira, R.M., Torres-Júnior, J.R.S., Demétrio, C.G.B., De Lima, C.G., Baruselli, P.S., 2009. Validation of body condition score as a predictor of subcutaneous fat in Nellore (*Bos indicus*) cows. Livest. Sci. 123, 175–179.

Bousseau, S., Brillard, J.P., Marquant-Le-Guienne, B., Guérin, 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., Arruda, R.P., 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.

Chaveiro, A., Machado, L., Frijters, A., Engel, B., Woelders, H., 2006. Improvement of parameters of freezing protocol for bull sperm using two osmotic supports. Theriogenology 65, 1875–1890.

Crespilho, A.M., Papa, F.O., Alberti, K., Siqueira Filho, E.R., Martins Jr., A., Novaes, J.L.C., Dell'Aqua Jr., J.A., 2006. Comparative efficiency of two freezing extenders for bovine semen based on sperm motility and membrane integrity. Ars Vet. 22, 229–235.

Dayem, A.A.M.H., Mahmoud, K.G.H.M., Nawito, M.F., Ayoub, M.M., Scholkamy, T.H., 2009. Fertility evaluation in Egyptian buffalo bulls using *zona pellucida* binding and in vitro fertilization assays. Livest. Sci. 122, 93–198.

Gil, J., Lundhein, N., Söderquist, L., Martínes-Rodríguez, H., 2003. Influence of extender, temperature, and addition glycerol on post-thaw sperm parameters in ram semen. Theriogenology 59, 1241–1255.

Gil, J., Söderquist, H., Martinez-Rodriguez, H., 2000. Influence of centrifugation and different extenders on post-thaw sperm quality of ram semen. Theriogenology 54, 93–108.

Graham, J.K., Mocé, E., 2005. Fertility evaluation of frozen/thawed semen. Theriogenology 64, 492–504.

Graham, J.K., 2001. Assessment of sperm quality: a flow cytometric approach. Anim. Reprod. Sci. 68, 239–247.

Gimenes, L.U., Sá Filho, M.F., Carvalho, N.A., Torres-Junior, J.R., Souza, A.H., Madureira, E.H., Trinca, L.A., Sartorelli, E.S., Barros, C.M., Carvalho, J.B., Mapletoft, R.J., Baruselli, P.S., 2008. Follicle deviation and ovulatory capacity in *Bos indicus* heifers. Theriogenology 69, 852–858.

Kulaksiz, R., Cebi, C., Akçay, E., Daskin, A., 2010. The protective effect of egg yolk from different avian species during the cryopreservation of Karayaka ram semen. Small Rumin. Res. 88, 12–15.

Leite, T.G., Vale Filho, V.R., Arruda, R.P., Andrade, A.F.C., Emerick, L.L., Zaffalon, F.G., Martins, J.A.M., 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.

Marco-Jiménez, F., Puchades, S., Mocé, E., Viudes-de-Cartro, M.P., Vicente, J.S., Rodriguez, M., 2004. Use of powdered egg yolk vs fresh egg yolk for the cryopreservation of ovine semen. Reprod. Dom. Anim. 39, 438–441.

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

Muiño, R., Fernández, M., Peña, A.I., 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. Dom. Anim. 42, 305–311.

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.O., Felício, G.B., Melo, C.M., Alvarenga, M.A., De Vita, B., Avanzi, B.R., Dell'Aqua, J.A., 2010. Effect of substituting soybean lecithin for egg yolk in an extender used for the cryopreservation of stallion semen. Anim. Reprod. Sci. 121S, 71–72.

Paz, C.P., Esteso, M.C., Alvarez, M., Mata, M., Chamorro, C.A., Anel, L., 2010. Development of extender based on soybean lecithin for its application in liquid ram semen. Theriogenology, in press.

Ruigh, L., Bosch, J.C., Brus, M.C., Landman, B., Merton, J.S., 2006. Ways to improve the biosecurity of bovine semen. Reprod. Dom. Anim. 41, 268–274.

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.

Vera-Munoz, O., Amirat-Briand, L., Diaz, T., Vásquez, L., Schmidt, E., Desherces, S., Anton, M., Bencharif, D., Tainturier, D., 2009. Effect of semen dilution to low-sperm number per dose on motility and

functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: comparison to Triladyl1 and Bioxcell1. Theriogenology 71, 895–900.
Way, L.A., Henault, M.A., Killian, G.J., 1995. Comparison of four staining methods for evaluating acrosome status and viability of ejaculated and cauda epididymal bull spermatozoa. Theriogenology 43, 1301–1316.
Wangtendonk-de Leeuw, A.M., Haring, R.M., Kaal-Lansbergen, L.M.T.E., Deen-Dass, J.H.G., 2000. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. Theriogenology 54, 57–67.
Zhang, B.R., Larsson, B., Lundeheim, N., Håård, M.G.H., Rodriguez-Martinez, H., 1999. Prediction of bull fertility by combined in vitro assessments of frozen-thawed semen from young dairy bulls entering an AI-programe. Intern. J. Androl. 22, 253–260.