Egg yolk saline soluble fraction was as efficient as ammonium sulfate insoluble yolk fraction in cryopreservation of bull semen in comparison with whole egg yolk

I. ASHRAFI[1], H. DAGHIGH KIA[1]*, J. PARRISH[2]

[1]*Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran*
2Department of Animal Sciences, University of Wisconsin-Madison, Madison, USA

**Corresponding author: daghighkia@tabrizu.ac.ir*

SUMMARY

Egg yolk is one of the first cryoprotective agents in semen freezing extenders, but it has some disadvantages to use in industrial scale. The objective of this study was to compare the whole egg yolk, Egg yolk saline soluble fraction (SS) and ammonium sulfate insoluble yolk fraction (ASI) in freezing extender. In this study bull semen was extended in extender containing one of the different dosages of egg yolk (15, 20 and 25%), SS (20 and 25%) and ASI (6, 8 and 10%). After freeze-thawing, no significant differences were observed between ASI treatments (6, 8 and 10%). Inclusion of 20% SS in semen freezing extender improved motility, viability and plasma membrane integrity parameters of cryopreserved bull sperm compared to 25% SS. Although there was no significant difference between 20% and 25% of egg yolk but, 20% egg yolk increased some motility parameters in comparison with 15% egg yolk. Inclusion of different dosage of ASI or 20% SS improved motility, viability and plasma membrane integrity of cryopreserved bull sperm compared with egg yolk. Different dosage of ASI improved viability and plasma membrane integrity of cryopreserved bull sperm over 20% SS but, the extra time required to prepare ASI does not warrant the small increase in sperm cryosurvival. No significant differences were detected in acrosome abnormality of sperm with different treatment groups. 20% SS was as efficient as ASI in terms of motion parameters for freezing bull semen, but compared with egg yolk, both ASI and SS had higher value.

Keywords: Bull semen; Cryopreservation; Whole egg yolk; Saline soluble fraction; Ammonium sulfate insoluble yolk fraction

RÉSUMÉ

La fraction saline soluble du jaune d'oeuf est aussi efficace que la fraction insoluble dans le sulfate d'ammonium pour la cryopréservation du sperme de taureau en comparaison avec le jaune d'oeuf entier.

Le jaune d'œuf est l'un des premiers cryoprotecteurs utilisé dans les extenseurs de congélation du sperme. L'objectif de cette était de comparer le jaune d'œuf entier, la fraction soluble saline du jaune d'œuf (SS) et de la fraction de jaune d'œuf insoluble dans le sulfate d'ammonium (ASI). Dans cette étude, du sperme de taureau a été congelé dans un extenseur contenant différentes doses de jaune d'œuf entier (15, 20 et 25 %), de SS (20 et 25 %) et d'ASI (6, 8 et 10%). Après la congélation-décongélation, aucune différence significative n'a été constatée entre les traitements ASI (6, 8 et 10%). L'inclusion de 20 % de SS dans l'extenseur améliore la motilité, la viabilité et les paramètres d'intégrité de la membrane plasmatique des spermatozoïdes par rapport à 25 % de SS. L'inclusion de différentes doses d'ASI ou de 20 % de SS augmente la motilité, la viabilité et intégrité de la membrane plasmique par rapport au jaune d'œuf. Différents dosages d'ASI améliorent la viabilité et l'intégrité de la membrane plasmique des spermatozoïdes, mais le temps supplémentaire nécessaire à la préparation de l'ASI ne semble pas justifié. Aucune différence significative n'a été décelée dans les anormalités acrosomique des spermatozoïdes dans les différents groupes. En conclusion, 20% de SS sont apparus aussi efficace que l'ASI pour la congélation du sperme de taureau ; SS et ASI sont apparus supérieurs au jaune d'œuf entier.

Mots-clés : Sperme de taureau ; Cryopréservation ; jaune d'œuf ; fractionnement

Introduction

Philip reported the first advantageous effects of egg yolk from chickens on bull semen preservation in 1939 [31]. Egg yolk was accepted swiftly as a protective factor to increase the ability of sperm to resist cold shock [13]. Egg yolk also has additional effects on sperm cryopreservation [13]. Cryopreservation media containing egg yolk has been used in the rabbit [36], ram [5], bull [4], gazelles [19] and horse [24]. Several potential concerns with the use of egg yolk include: 1) egg yolk is a biological substance obtained from chickens and as such could be contaminated with bacteria and/or viruses [14, 2] the beneficial part of the egg is the yolk [13] and as such the whites are discarded making preparation of egg yolk time consuming, 3) the diet of the hen may have an impact on yolk quality [32, 4] some ingredients of egg yolk such as granules, minerals and high density lipoproteins could limit the sperm respiration and may lead to reduce the motility of the sperm [2, 43]. Because of the above reason, it is important to consider the exchange of whole egg yolk with its cryoprotective compounds to potentially increase the efficacy.

Plasma and egg granules are two fraction of the egg yolk which their composition has been reported and these two fraction could be isolated via density gradient centrifugation [16, 17, 34]. Plasma contain mainly 85% low-density lipoprotein (LDL) and 15% protein components. Granules

are composed of 70% HDL (high-density lipoprotein), 16% phosvitin and 12% low-density lipoproteins [25].

The cryoprotective action of the egg yolk is related to the LDL found in yolk [29]. This has been reported also in ram [42], boar [22], dog [10], stallion [32] and bulls [21]. The low density lipoprotein particles appear to stick to the sperm plasma membrane and influence phospholipids and cholesterol content of the plasma membrane [11, 38].

Egg yolk has been fractionated into different components using density gradient centrifugation for obtaining saline soluble fraction (SS) and then precipitation of protein compounds with 40% ammonium sulfate to preparing ammonium sulfate insoluble yolk fraction (ASI) [27]. The methods of Mousa and et al., 2002, provide for 2 fractions from egg yolk that are rich in LDL and beneficial for cryopreservation of bovine sperm. Based on our knowledge, no previous study has compared the ASI and SS fractions of egg yolk to that of egg yolk in semen extender against cryodamage to semen. The present study was therefore conducted to determine the effect of different level of ASI (6, 8 and 10%) and SS (20% and 25%) in comparison with whole egg yolk (15%, 20% and 25%) on motion parameters, viability, plasma membrane integrity and morphology parameters of cryopreserved bull spermatozoa.

Materials and Methods

EGG YOLK: WHOLE, SALINE SOLUBLE AND AMMONIUM INSOLUBLE

Density gradient centrifugation has been generally used for extraction of saline soluble fraction [29]. Fresh eggs were manually broken. The yolks were separated from the albumen. The yolks were diluted with an isotonic saline solution (NaCl 0.9%) (w/w) and stirred for 1 h before being centrifuged at 10,000 x g for 45 min at 4 °C and supernatant, (saline soluble component) was separated from the sediment, [27]. The plasma was centrifuged again to remove all granules.

The saline soluble component was next mixed with 40% ammonium sulfate to precipitate out the protein components. After 1 h of stirring at 4–8 °C, the mixture was centrifuged at 10,000 x g for 45 min. The sediment was discarded and the supernatant was dialyzed against distilled water to eliminate the ammonium sulfate. Then the solution was centrifuged (10,000 x g, 45 min, 4–8°C) and supernatant was collected. This extraction technique has been described under the patent no. 0100292 held by the ENVN and INRA of Nantes [9, 27]. The isolated fraction will be called the ammonia sulfate insoluble component.

SEMEN SOURCE AND PREPARATION

This trial was performed at Northwest and West genetic material production Center, Tabriz, Iran. Ejaculates were collected from three fertile Holstein bulls, 5-6 years old. Bulls were regularly used for breeding purpose and had acceptable in vivo fertility. Semen samples were collected twice a week by an artificial vagina (45°C). Two ejaculated were collected from each bull in each replication of experiment. The ejaculates were transferred immediately to the laboratory and submerged in a water bath (34°C), until semen evaluation. The volume of ejaculates was estimated in a conical tube graduated at 0.1 ml intervals. The sperm concentration was determined by means of an Accucell photometer (IMV, L'Aigle, France). The ejaculates were evaluated and accepted into the study if the following criteria were met: volume between 5-12 ml; sperm concentration of > 1×109 sperm/ml; percentage of motile sperm > 70% and ≤ 10% abnormal sperm. The ejaculates were then mixed in a pool for balancing the sperm contribution of each male [6].

The base extender was 2.42 g Tris, 1.00 g fructose, 1.48 g citric acid, 6.4 ml glycerol, 250 µg gentamicine. All further additions to extenders were on a v/v basis. To the base extender, egg yolk was added at 15%, 20% or 25%; the saline soluble fraction of egg yolk was added as at 20% or 25%; the ammonium precipitate fraction was added at 6%, 8% or 10%. Each pooled ejaculate was split into eight equal aliquots and diluted in the semen extender supplemented with treatment, for a final concentration of 50×106 spermatozoa per ml. Then, the semen was cooled slowly to 5°C over 2 hours. Semen was packaged in 0.5 ml straws, sealed and were frozen by a semi-automatic freezer machine (mini tube, Germany) with liquid nitrogen vapor. The straws were cooled at approximately -15°C / min from +5 to -150°C. Then straws were submerged in liquid nitrogen, transferred to a semen storage tank and maintained under liquid nitrogen until evaluated. Frozen straws were thawed individually at 37°C for 30 s in a water bath for microscopic evaluation [28].

SEMEN EVALUATION POST THAW

Viability, motility and morphology parameters

The viability of spermatozoa in samples was assessed by means of the nigrosin–eosin stain method [7] . The composition of the stain was: Eosin-Y 1.67 g, nigrosine 10 g, and sodium citrate 2.9 g, dissolved in 100 ml distilled water. Sperm suspension smears were prepared by mixing a drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide. Viability was assessed by counting 400 sperm cells under bright field illumination, 1000× magnification and with immersion oil. Sperm displaying partial or complete purple staining were considered non-viable; only sperm showing strict exclusion of stain were counted as viable [7].

The sperm motility was assessed after thawing the samples, for about 5 min at 37°C. A computer-assisted sperm motility analysis system (CASA, video test, sperm 3.1, Russia) was used to analyze sperm motion characteristics. Semen was diluted (5 µl semen +45 µl extender) in a citrate-based extender (without egg yolk) and evaluated immediately

after dilution. A 4 µl sample of diluted semen was put onto a pre-warmed microscope plain slide and covered by a cover slip. Sperm motility characteristics were determined using phase-contrast microscopy (Labomed LX400) with a 10× objective at 37°C. The following motility values were recorded: Total motility (TM, %), progressive motility (PM, %), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), beat cross-frequency (BCF, Hz), lateral head displacement (ALH, µ), sperm track straightness (STR, %) and linearity (LIN, %). For each evaluation, five microscopic fields were analyzed to include at least 400 sperm cells per field.

For the evaluation of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution [35]. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosome and total abnormalities were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (1000× magnification, oil immersion).

Assessment of membrane integrity

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This was performed by incubating 30 µl of semen with 300 µl of a 100 mOsM hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37°C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. Four hundred sperm were examined at 400x magnification using bright-field microscopy. Sperm with swollen or coiled tails were recorded as viable [15].

STATISTICAL ANALYSIS

Aanalysis was conducted as a completely randomized design with 5 replicates. For each replicate, three straws were thawed and pooled for evaluation of sperm parameters. When the analysis showed statistical differences, the mean of the treatments was compared using Duncan's multiple range tests. All data were expressed as mean ± SD. Differences were considered significant at p< 0.05.

Results

Effects of different freezing extender on post-thaw motion parameters and other microscopic parameters (viability, plasma membrane integrity (HOST), total abnormality and acrosome abnormality) of bull sperm are presented in Table I and II, respectively. Inclusion of 20% egg yolk increased (P<0.05) ALH, VSL and VAP parameters of freeze-thawed sperm in comparison with 15% egg yolk. There was no difference between 20% and 25% of egg yolk in all motion parameters of cryopreserved bull sperm. No differences were observed between 15% and 25% egg yolk in motion parameters of cryopreserved bull sperm. As shown in table II, there was no difference among different dosage of egg yolk (15%, 20% and 25%) on post thawed bull sperm viability, plasma membrane integrity, total abnormality or acrosome abnormality.

Inclusion of 20% SS in the semen freezing extender improved TM, PM, VCL, ALH, viability and plasma membrane integrity parameters of cryopreserved bull sperm compared to 25% SS (P<0.05). There was no difference between 20% and 25% SS in total abnormalities or acrosome abnormalities of cryopreserved bull sperm.

There was no effect among ASI treatments (6, 8 and 10%) on post-thaw sperm motion parameters, viability, plasma

Motility parameters	Freezing extender							
	EY15%	EY20%	EY25%	SS20%	SS25%	ASI6%	ASI8%	ASI10%
TM (%)	45.3±6.5^{d}	59.9±14.9^{c}	55.5±17.20^{cd}	73.4±2.9^{b}	56.5±14.0^{cd}	87.8±3.2^{a}	83.1±6.4^{ab}	87.8±4.6^{a}
PM (%)	18.70±4.2^{c}	28.6±9.2^{b}	22.7±7.7^{bc}	42.6±4.8^{a}	29.0±10.1^{b}	48.6±4.3^{a}	47.7±3.5^{a}	49.0±7.2^{a}
STR (%)	0.76±0.02^{b}	0.77±0.02^{b}	0.77±0.02^{b}	0.79±0.00^{ab}	0.78±0.02^{b}	0.82±0.03^{a}	0.79±0.00^{ab}	0.79±0.01^{ab}
VCL (µm/s)	43.5±6.9^{b}	57.0±14.0^{b}	47.2±12.7^{b}	83.5±13.9^{a}	56.8±15.9^{b}	90.4±6.0^{a}	90.9±7.74^{a}	94.2±12.5^{a}
ALH (µ)	0.72±0.07^{c}	1.08±0.29^{b}	0.89±0.26^{bc}	1.56±0.26^{a}	0.95±0.29^{bc}	1.72±0.08^{a}	1.73±0.14^{a}	1.86±0.21^{a}
VSL (µm/s)	16.6±3.4^{c}	26.1±7.2^{b}	21.2±6.1^{bc}	38.4±6.0^{a}	28.7±9.0^{b}	39.8±2.9^{a}	40.9±3.8^{a}	41.0±5.7^{a}
VAP (µm/s)	19.7±3.1^{c}	29.5±8.2^{b}	24.1±7.0^{bc}	43.3±6.6^{a}	31.8±9.9^{b}	46.1±3.4^{a}	47.0±3.7^{a}	47.4±6.5^{a}
LIN (%)	0.315±0.031^{b}	0.367±0.047^{a}	0.360±0.046^{ab}	0.387±0.015^{a}	0.395±0.055^{a}	0.413±0.031^{a}	0.397±0.013^{a}	0.396±0.032^{a}
BCF (Hz)	14.0±1.2^{c}	14.2±0.6^{bc}	14.8±0.5^{abc}	14.6±0.5^{abc}	14.9±0.5^{abc}	15.5±0.5^{a}	15.1±0.2^{ab}	15.1±0.4^{ab}

Abbreviations were: TM, total motility (%); PM, progressive motility (%); LIN, linearity (%); STR, sperm track straightness (%); ALH; lateral head displacement (µ); VSL; straight linear velocity (µm/s); BCF; beat cross frequency (Hz); VCL; curvilinear velocity (µm/s); VAP; average path velocity (µm/s); ASI, ammonium sulfate insoluble yolk fraction; SS, egg yolk saline soluble fraction; EY, egg yolk.
^{a,b}, Different superscripts within rows differ, p < 0.05.

Table I: Mean (±SD) comparison of motion parameters of freeze–thawed bull spermatozoa with freezing extender containing different dosage of ASI, SS and egg yolk.

Parameters	Freezing extender							
	EY15%	EY20%	EY25%	SS20%	SS25%	ASI6%	ASI8%	ASI10%
Viability	49.7±7.2[e]	64.3±13.6[dc]	59.0±16.9[de]	76.3±2.8[bc]	60.1±13.0[de]	90.7±3.3[a]	88.0±6.1[ab]	91.2±3.1[a]
HOST	46.8±7.4[e]	61.5±13.7[cd]	56.0±16.5[de]	74.2±2.3[bc]	57.6±14.0[de]	88.5±2.8[a]	85.5±6.5[ab]	88.4±2.8[a]
Total Abnormality	7.1±0.6	7.0±0.7	6.1±0.6	6.1±1.2	7.5±0.7	6.8±1.0	6.7±0.5	6.3±0.8
Acrosome Abnormality	2.5±0.6	2.7±0.7	2.2±0.3	2.1±0.6	2.1±0.5	2.9±0.4	2.7±0.3	2.4±0.5

Abbreviations were: HOST, Hypo Osmotic Swelling Test; ASI, ammonium sulfate insoluble yolk fraction; SS, egg yolk saline soluble fraction; EY, egg yolk.
[a,b], Different superscripts within rows differ, $p < 0.05$.

Table II: Mean (±SD) comparison of viability, plasma membrane integrity, total abnormality and acrosome abnormality parameters of freeze–thawed bull spermatozoa with freezing extender containing different dosage of ASI, SS and egg yolk.

membrane integrity, total sperm abnormalities or acrosome abnormality parameters of bull sperm.

Inclusion of various concentrations of ASI in semen extender increased ($p < 0.05$) all motion parameters of freeze-thawed sperm except LIN and BCF rather than different dosage of egg yolk. All motion parameters except TM did not differ ($p > 0.05$) between LDL groups and the once frozen in extender supplemented with 20% SS while TM decreased ($p < 0.05$) in the dose of 20% SS rather than 6% and 8% ASI. Except STR, LIN and BCF, all motion parameters in 25% SS have significantly low value rather than ASI groups and all motion parameters in 20% SS have significantly high value rather than egg yolk groups. Sperm track straightness in 6% ASI has significantly high value rather than three dosage of egg yolk and 25% SS. Linearity and BCF reached the lowest value by inclusion of 15% egg yolk in the extender.

Inclusion of 20% SS in semen freezing extender improved viability and membrane integrity of freeze-thawed bull sperm rather than 15% and 25% egg yolk. The highest percentage of viability and membrane integrity were reached by inclusion of 6 and 10 % of ASI and the lowest percentage were achieved by usage of 15% egg yolk in freezing extender. No significant differences ($p > 0.05$) were detected in total abnormality and acrosome abnormality of sperm with different treatment groups.

Discussion

The replacement of egg yolk in the cryopreservation extender with ASI or 20% SS improved cryosurvival in terms of post thaw motility, motion parameters, viability and membrane integrity. While ASI improved viability and membrane integrity parameters over 20% SS, the extra time required to prepare this component does not warrant the small increase in sperm cryosurvival.

Egg yolk is an important cryoprotectant in addition to glycerol in the protection of bull sperm during semen freezing [41]. However, egg yolk has some adverse effects such as decreasing sperm cell respiration, motility, acrosomal intrigity and fertilizing ability of the spermatozoa [26], so it is reasonable to consider the use of just the cryoprotective fraction of egg yolk in the extender. Our result showed that ASI and SS has more protective effect in motion, viability and plasma membrane integrity of freeze-thawed sperm rather than whole egg yolk. Based on our knowledge, there is not any study comparing the ASI and SS fraction on freeze-thawing procedure. Previous studies reported that most effective compounds of egg yolk which have protective ability is low density lipoprotein [12]. But the exact mechanism of protective effect of LDL is still unclear. Parks and Grams, 1992 suggested that egg yolk LDL could enhance spermatozoa resistance against cold shock via preventing membrane phospholipids [30]. Another researcher proposed that LDL safeguard the plasma by making a protective layer on plasma membrane [1]. Bellin et al., indicated that LDL has protective action via linking with the sperm plasma membrane [8]. Another hypothesis is that the phospholipids of LDL could replace some phospholipids in the sperm plasma membrane and decrease the ice crystal formation by reducing phase transition temperature [27]. Recent studies have focused on protective effect of LDL binding phospholipid to the sperm membrane and challenge with seminal plasma protein. Although seminal plasma proteins are required for sperm capacitation but cause cholesterol and phospholipids efflux from membrane by binding to the sperm membrane [12, 23, 40]. So, seminal plasma proteins have adverse effect on sperm preservation. Vera Munoz et al, suggested that LDL could bind to the seminal plasma proteins rapidly and protect the sperm against detrimental effects of seminal plasma proteins. Binding LDL to seminal plasma proteins is very stable and could protect the sperm cell even after freeze thawing process [39].

Our results confirm that there is not much difference between 15, 20 or 25% egg yolk in the extender but that 20% egg yolk is slightly better for some post thaw sperm motion parameters (Table I) and is thus the standard percentage of egg yolk for bull semen freezing extender [33]. Moussa et al. [27] reported that using the same ASI fraction we did as an LDL replacement in the extender improved motion parameters in comparison with 20% egg yolk in the bull freezing process. However, the optimum ASI fraction was 8% while others suggest 9% may be optimal [21]. Our results suggest there was no difference between 6, 8 and 10% ASI in frozen bull semen extender.

In this study, the percentage of viability and plasma membrane integrity in ASI groups were significantly higher than 20 % whole egg yolk group. Our results are in the agreement with some previous studies [20, 21] while other researchers reported that a similar ASI fraction of egg yolk did not introduce more plasma membrane integrity during the cryopresevation procedure than those produced in the presence whole egg yolk [3, 27].

Our result found no differences for any of the extender modifications to replace egg yolk on post-thaw total abnormality morphology and acrosome abnormal morphology parameters of bull sperm. Corcini et al, [18] reported that normal sperm morphology did not differ among extenders containing 20% egg yolk and a similar SS fraction of egg yolk in frozen-thawed dog sperm. Souza et al [37], indicated that different dosages of ASI fractions did not affect sperm morphology when compared to a 20% egg yolk extender in the collared peccary sperm cryopreservation.

In conclusion, usage in the extender of ASI or 20% SS improved, motion parameters, viability and plasma membrane integrity of cryopreserved bull sperm compared with egg yolk. The extender with ASI had improved viability and plasma membrane integrity of cryopreserved bull sperm over 20% SS. But, 20% SS was as efficient as ASI in terms of motion parameters. Egg yolk saline soluble in extender is a clear ready-to-use medium and a fraction without detrimental factors. The extra time required to prepare ASI does not warrant the small increase in sperm cryosurvival. It is necessary that future studies compare the fertility ability of freeze-thawed semen which are extended with ASI and SS.

Acknowledgements

The authors thank Nahadehaye Dami Jahed Company, Tabriz and Tehran, Iran for supplying the semen samples and the equipment for cryopreservation.

References

1. - AKHTER S., ANSARI M.S., RAKHA B.A., ANDRABI S.M., KHALID M., ULLAH N.: Effect of low density lipoproteins in extender on freezability and fertility of buffalo (Bubalus bubalis) bull semen. *Theriogenology*, 2011, **76**, 759-764.
2. - AMIRAT L., TAINTURIER D., JEANNEAU L., THORIN C., GERARD O., COURTENS J.L., ANTON M.: Bull semen in vitro fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl, a commercial egg yolk extender. *Theriogenology*, 2004, **61**, 895-907.
3. - AMIRAT L., ANTON M., TAINTURIER D., CHATAGNON G., BATTUTAND I., COURTENS J L.: Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction*, 2004, **61**, 895-907.
4. - ASHRAFI I., KOHRAM H., ARDABILI F.F.: Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. *Anim. Reprod. Sci.*, 2013, **139**, 25-30.
5. - ASHRAFI I., KOHRAM H., NAIJIAN H., BAHREINI M., MIRZAKHANI H.: Effect of controlled and uncontrolled cooling rate on motility parameters of cryopreserved ram spermatozoa. *BMC. Res. Notes*, 2011, **4**, 547.
6. - ASHRAFI I., KOHRAM H., TAYEFI-NASRABADI H.: Antioxidant effects of bovine serum albumin on kinetics, microscopic and oxidative characters of cryopreserved bull spermatozoa. *SPAN. J. AGRIC. RES.,* 2013, **11**, 695-701.
7. - BALESTRI F., GIANNECCHINI M., SGARRELLA F., CARTA M.C., TOZZI M.G., CAMICI M.: Purine and pyrimidine nucleosides preserve human astrocytoma cell adenylate energy charge under ischemic conditions. *Neurochem. Int.*, 2007, **50**, 517-523.
8. - BELLIN M.E., OYARZO J.N., HAWKINS H.E., ZHANG H., SMITH R.G., FORREST D.W., SPROTT L.R., AX R.L.: Fertility-associated antigen on bull sperm indicates fertility potential. *J. Anim. Sci.*, 1998, **76**, 2032-2039.
9. - BENCHARIF D., AMIRAT-BRIAND L., LE GUILLOU J., GARAND A., ANTON M., SCHMITT E., DESHERCES S., DELHOMME G., LANGLOIS M.L., DESTRUMELLE S., VERA-MUNOZ O., BARRIERE P., TAINTURIER D.: Canine-chilled sperm: study of a semen extender made with low-density lipoproteins from hen egg yolk supplemented with glutamine. *Reprod. Domest. Anim.*, 2013, **48**, 258-266.
10. - BENCHARIF D., AMIRAT L., ANTON M., SCHMITT E., DESHERCES S., DELHOMME G., LANGLOIS M.L., BARRIERE P., LARRAT M., TAINTURIER D.: The advantages of LDL (low density lipoproteins) in the cryopreservation of canine semen. *Theriogenology*, 2008, **70**, 1478-1488.
11. - BERGERON A., CRÊTE M. H., BRINDLE Y., MANJUNATH P.: Low-Density Lipoprotein Fraction from Hen's Egg Yolk Decreases the Binding of the Major Proteins of Bovine Seminal Plasma to Sperm and Prevents Lipid Efflux from the Sperm Membrane. *Biol. Reprod.*, 2004, **70**, 708-717.
12. - BERGERON A., CRETE M.H., BRINDLE Y., MANJUNATH P.: Low-density lipoprotein fraction from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. *Biol. Reprod.*, 2004, **70**, 708-717.
13. - BOGART R., MAYER D.T.: The effects of egg yolk on the various physical and chemical factors detrimental to spermatozoan viability. *J. Anim. Sci*. 1950, **9**, 143-152.
14. - BOUSSEAU S., BRILLARD J.P., MARGUANT-LE GUIENNE B., GUERIN B., CAMUS A., LECHAT M.: 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*, 1998, **50**, 699-706.

15. - BUCKETT W.M., LUCKAS M.J., AIRD I.A., FARQUHARSON R.G., KINGSLAND C.R., LEWIS-JONES D.I.: The hypo-osmotic swelling test in recurrent miscarriage. *Fertil. Steril.*, 1997, **68**, 506-509.
16. - BURLEY R.W., COOK W.H.: Isolation and composition of avian egg yolk granules and their constituent alpha- and beta-lipovitellins. *Can. J. Biochem Physiol.*, 1961, **39**, 1295-1307.
17. - CAUSERET D., MATRINGE E., LORIENT D.: Ionic Strength and pH Effects on Composition and Microstructure of Yolk Granules. *J. Food. Sci.*, 1991, **56**, 1532-1536.
18. - CORCINI C.D., GOULARTE K.L., BONGALHARDO D.C., LUCIA T., JARDIM R.D., VARELA JUNIOR A.S.: Effect of egg yolk plasma on dog sperm cryopreservation. *Andrologia*, 2016, **48**, 114-115.
19. - GARDE J.J., DEL OLMO A., SOLER A.J., ESPESO G., GOMENDIO M., ROLDAN E.R.: Effect of egg yolk, cryoprotectant, and various sugars on semen cryopreservation in endangered Cuvier's gazelle (Gazella cuvieri). *Anim. Reprod. Sci.*, 2008, **108**, 384-401.
20. - HU J.H., JIANG Z.L., LV R.K., LI Q.W., ZHANG S.S., ZAN L.S., LI Y.K., LI X.: The advantages of low-density lipoproteins in the cryopreservation of bull semen. *Cryobiology*, 2011, **62**, 83-87.
21. - HU J.H., LI Q.W., ZAN L.S., JIANG Z.L., AN J.H., WANG L.Q., JIA Y.H.: The cryoprotective effect of low-density lipoproteins in extenders on bull spermatozoa following freezing-thawing. *Anim. Reprod. Sci.*, 2010, **117**, 11-17.
22. - JIANG Z.L., LI Q.W., HU J.H., LI W.Y., ZHAO H.W., ZHANG S.S.: Improvement of the quality of boar cryopreservation semen by supplementing with low density lipoprotein in diluents. *Cryobiology*, 2007, **54**, 301-304.
23. - MANJUNATH P., NAUC V., BERGERON A., MENARD M.: Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. *Biol. Reprod.*, 2002, **67**, 1250-1258.
24. - MARTIN J.C., KLUG E., GUNZEL A.R.: Centrifugation of stallion semen and its storage in large volume straws. *J. Reprod. Fertil. Suppl.*, 1979, 47-51.
25. - MCCULLY K.A., MOK C.-C., COMMON R.H.: PAPER ELEGTROPHORETIC CHARACTERIZATION OF PROTEINS AND LIPOPROTEINS OF HEN'S EGG YOLK. *Can. J. Biochem. Phys.*, 1962, **40**, 937-952.
26. - MORENO D., BENCHARIF D., AMIRAT-BRIAND L., NEIRA A., DESTRUMELLE S., TAINTURIER D.: Preliminary Results: The Advantages of Low-Density Lipoproteins for the Cryopreservation of Equine Semen. *J. Equine. Vet. Sci.,* 2013, **33**, 1068-1075.
27. - MOUSSA M., MARINET V., TRIMECHE A., TAINTURIER D., ANTON M.: Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology*, 2002, **57**, 1695-1706.
28. - OLFATI KARAJI R., DAGHIGH KIA H., ASHRAFI I.: Effects of in combination antioxidant supplementation on microscopic and oxidative parameters of freeze-thaw bull sperm. *Cell. Tissue. Bank*, 2014, **15**, 461-470.
29. - PACE M.M., GRAHAM E.F.: Components in egg yolk which protect bovine spermatozoa during freezing. *J. Anim. Sci.*, 1974, **39**, 1144-1149.
30. - PARKS J.E., GRAHAM J.K.: Effects of cryopreservation procedures on sperm membranes. *Theriogenology*, 1992, **38**, 209-222.
31. - PHILLIPS P.H.: PRESERVATION OF BULL SEMEN. *J. Biol. Chem.*, 1939, **130**, 415.
32. - PILLET E., DUCHAMP G., BATELLIER F., BEAUMAL V., ANTON M., DESHERCES S., SCHMITT E., MAGISTRINI M.: Egg yolk plasma can replace egg yolk in stallion freezing extenders. *Theriogenology*, 2011, **75**, 105-114.
33. - REHMANA F.U., ZHAOA C., SHAHA M.A., QURESHIB M.S., WANGA X.: Semen extenders and artificial insemination in ruminants. *Veterinaria*, 2013, **1**, 1-8.
34. - SAARI A., POWRIE W.D., FENNEMA O.: Isolation and Characterization of Low-Density Lipoproteins in Native Egg Yolk Plasma. *J. Food. Sci.*, 1964, **29**, 307-315.
35. - SCHAFER S., HOLZMANN A.: The use of transmigration and spermac stain to evaluate epididymal cat spermatozoa. *Anim. Reprod. Sci.*, 2000, **59**, 201-211.
36. - SI W., HILDEBRANDT T.B., REID C., KRIEG R., JI W., FASSBENDER M., HERMES R.: The successful double cryopreservation of rabbit (Oryctolagus cuniculus) semen in large volume using the directional freezing technique with reduced concentration of cryoprotectant. *Theriogenology*, 2006, **65**, 788-798.
37. - SOUZA A.L., LIMA G.L., PEIXOTO G.C., DE SOUZA CASTELO T., OLIVEIRA M.G., DE PAULA V.V., SILVA A.R.: Sperm characteristics following freezing in extenders supplemented with whole egg yolk and different concentrations of low-density lipoproteins in the collared peccary (Pecari tajacu). *Reprod. Biol.*, 2015, **15**, 223-228.
38. - THERIEN I., MOREAU R., MANJUNATH P.: Bovine seminal plasma phospholipid-binding proteins stimulate phospholipid efflux from epididymal sperm. *Biol. Reprod.*, 1999, **61**, 590-598.
39. - VERA-MUNOZ O., AMIRAT-BRIAND L., DIAZ T., VASQUEZ L., SCHMIDT E., DESHERCES S., ANTON M., BENCHARIF D., TAINTURIER D.: 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 Triladyl and Bioxcell. *Theriogenology*, 2009, **71**, 895-900.
40. - VISHWANATH R., SHANNON P., CURSON B.: Cationic extracts of egg yolk and their effects on motility, survival and fertilising ability of bull sperm. *Anim. Reprod. Sci.*, **29**, 185-194.
41. - WATSON P.F.: The roles of lipid and protein in the protection of ram spermatozoa at 5 degrees C by egg-yolk lipoprotein. *J. Reprod. Fertil.*, 1981, **62**, 483-492.
42. - WATSON P.F., MARTIN I.C.: The influence of fractions of egg yolk on the survival of ram spermatozoa at 5 degrees C. *J. Reprod. Fertil.*, 1973, **32**, 310.
43. - WATSON P.F., MARTIN I.C.A.: Influence of Some Fractions of Egg-Yolk on Survival of Ram Spermatozoa at 5 Degreesc. *Austr. J. Biol. Sci.*, 1975, **28**, 145-152.