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Effect of low-density lipoprotein addition to soybean lecithin-based extenders on bull spermatozoa following freezing-thawing – preliminary results

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ABSTRACT: Soybean lecithin-based extenders represent an alternative to extenders containing egg yolk, but there has been discussion about their cryoprotective efficacy. Low-density lipoprotein (LDL) was proved as a cryoprotective agent, which can replace egg yolk in extenders. The aim of this study was to investigate the effects of LDL addition to the soybean extenders on their cryoprotective properties. The effect of the LDL of our production was verified using commercial egg yolk extender BULLXcell®, 6%, 8%, and 10% LDL (v/v) as an egg yolk replacement. The effects of LDL addition to the soybean lecithin-based extenders in concentrations of 4%, 6%, and 8% (v/v) were tested using extenders AndroMed® and Bioxcell®. In total, 64 samples from eight bulls were evaluated. Kinematic parameters of spermatozoa, resulting from Computer Assisted Sperm Analysis, and their viability, evaluated by fluorescent technique, were assessed immediately after thawing and after 2 hours. The quality of LDL compared to other studies was confirmed, and the beneficial effects of egg yolk replacement by LDL were proved in extender BULLXcell®. 8% LDL provided the best values for the majority of kinematic parameters ($P < 0.05$), without effect on total motility ($P > 0.05$). Furthermore, addition of 4%, 6%, and 8% LDL to the soybean lecithin-based extender Bioxcell® showed a positive effect on the majority of kinematic parameters of spermatozoa ($P < 0.05$) at both times of incubation. However there was no significant influence on total motility ($P > 0.05$). Viability was higher after thawing in the case of 8% LDL ($P < 0.05$). However, there was no consistent effect of LDL addition to the AndroMed® extender. In conclusion, cryoprotective properties of the semen extenders based on a soybean lecithin can be improved by the addition of LDL.

Keywords: bull sperm; cryopreservation; sperm motility; viability; cryoprotectant

INTRODUCTION

Artificial insemination (AI) is a reproductive biotechnology method that has been intensively used worldwide, especially in dairy cattle breeding. For AI in cattle breeding, cryopreserved insemination doses (ID) are used (Zhang et al. 2015) and, consequently, their quality plays an important role

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in the success of fertilization (Beran et al. 2013). However, cryopreservation represents a limiting factor in several of the advantages of AI due to its impacts on structural integrity and the physiological processes of spermatozoa (Amirat-Briand et al. 2009; Dzyuba et al. 2015; Sieme et al. 2015; Meamar et al. 2016).

To ensure spermatozoa protection against harmful effects of cryopreservation, such as the "cold shock" (Drobnis et al. 1993; Stadnik et al. 2015), crystal formation (Koshimoto et al. 2000) or reactive oxygen species generation (Alomar et al. 2016), the collected semen has to be diluted with a suitable freezing extender. As many authors have demonstrated, optimization of extender composition can have a positive effect on the undesirable changes in spermatozoa caused by cryopreservation (Spalekova et al. 2014; Muchlisin et al. 2015). Egg yolk and glycerol are the cryoprotectants that are most often utilized in the field of bovine semen cryopreservation (Aires et al. 2003). However, there have been efforts to replace the egg yolk in extenders by another substance due to possible risks connected with bacteria or mycoplasma contamination and for standardization of the extender composition (Crespilho et al. 2012). Additionally, egg yolk contains detrimental constituents, such as high-density lipoprotein or granules, which can inhibit physiological processes in the spermatozoa (Wall and Foote 1999).

Soybean lecithin, which generally consists of phosphatidylcholine and a mixture of fatty acids, is an alternative to egg yolk (Vishwanath and Shannon 2000). However, studies evaluating the efficiency of bovine semen extenders based on soybean lecithin or egg yolk have shown varied results, in favour of the latter type of extender (e.g. Gil et al. 2000; van Wagtendonk-de Leeuw et al. 2000; Thun et al. 2002; Aires et al. 2003; Beran et al. 2012; Crespilho et al. 2012). Another alternative to egg yolk in extenders is its substitution only by the compound responsible for its cryoprotective properties. In 1974 it was discovered that this compound is the major fraction of egg yolk plasma, i.e. low-density lipoprotein or LDL (Pace and Graham 1974). Different cryoprotective properties of LDL, that positively and directly affected spermatozoa structure (Bergeron et al. 2004) and/or extracellular conditions, were found (Hu et al. 2011). The positive effects of egg yolk substitution by LDL in semen extenders on qualitative parameters of spermatozoa have been repeatedly demonstrated (Moussa et al. 2002; Vera-Munoz et al. 2009; Amirat-Briand et al. 2010; Hu et al. 2010, 2011). The question then arises on whether the cryoprotective properties of soybean lecithin-based extenders could be improved by LDL addition. Furthermore, none of the above-mentioned studies describes any form of LDL preservation, which limits the practical use of LDL. However, the shelf-life of produced LDL may be stabilized and prolonged using sodium azide.

Therefore, the aim of this study was to investigate the effects of LDL addition to the soybean extenders on their cryoprotective properties.

MATERIAL AND METHODS

LDL extraction. Low-density lipoprotein with 97% purity was prepared according to the methodology of Moussa et al. (2002). Hen eggs were obtained from the controlled breeding program of BIOPHARM Czech Republic. In comparison to the methodology established by Moussa et al. (2002), there were slight modifications in our study. Sodium azide (0.1%) was used for preservation of the produced LDL and to minimize sanitary risks. Before using the LDL, sodium azide was removed by extensive dialysis against phosphate buffer saline (PBS).

Preparation of the extenders. Extenders were prepared at the beginning of the experiments. To verify the quality of LDL, the egg yolk extender BULLXcell® was used (IMV Technologies, L'Aigle, France). The standard composition according to the producer's manual with egg yolk served as a control, and in experimental variants, the egg yolk was substituted by 6%, 8%, and 10% LDL (v/v).

Soybean lecithin-based extenders, AndroMed® (Minitübe, Tiefenbach, Germany) and Bioxcell® (IMV Technologies) were used for testing the effect of LDL addition. These media were prepared in accordance with the manufacturer's instructions, and then LDL was added in concentrations of 4%, 6% or 8% (v/v) to each of the aforementioned extenders. Extenders without LDL were used as a control.

Collection and processing of semen. The semen was collected from eight bulls in a standard way at the insemination centre (Natural Ltd., Hradišťko pod Medníkem, Czech Republic). Each ejaculate was submitted to the basic assessment done by

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trained laboratory technician from the insemination centre. The following parameters were evaluated: ejaculate volume, sperm concentration, and percentage of motile sperm. Only ejaculates that conformed to the limits of sperm concentration ($\geq 0.7 \times 10^9$/ml) and percentage of motile spermatozoa (≥ 70%) were used in this study. Semen was divided into equal fractions in relation to the number of tested variants and diluted to a final concentration of 120×10^6 spermatozoa/ml. Diluted semen was placed into polyvinyl straws (0.25 ml) and equilibrated at 5°C for 2 h. After this period, straws were cryopreserved using a computerized freezing machine (DigitCool®; IMV Technologies) with the standard freezing curve for bovine semen and then immersed directly into liquid nitrogen (–196°C) for storage. Straws were analyzed at least one week after the cryopreservation. Before each evaluation, the straws were thawed in the standard way in water bath (37°C/30 s). All analyses were performed 10 min after thawing ("incubation 0 h") and after a 2-hour thermoresistance test ("incubation 2 h") at the same temperature 37°C.

Evaluation of sperm motility. Sperm motility was assessed with the Computer Assisted Sperm Analysis (CASA) module NIS Elements Ar 4.20 (Laboratory Imaging Ltd., Prague, Czech Republic), using a DMK 23UM021 camera (The Imaging Source Europe GmbH, Bremen, Germany) with a frame rate of 60 images/s and a stereo microscope Nikon Eclipse E600 (Nikon Corp., Tokyo, Japan) with a heated plate. Thawed samples were transferred into the 1.5 ml plastic microtubes and diluted with physiological saline (pH 6.8) to a final concentration of $20–40 \times 10^6$ spermatozoa/ml, according to Verstegen et al. (2002). Next, 3 µl of the sample were evaluated in a calibrated Leja® counting chamber (Leja, Nieuw-Vennep, The Netherlands) with a depth of 20 µm in six different fields per sample. At least 200 trajectories per field were analyzed. The parameter total motility (TM) was evaluated on the basis of threshold of motile spermatozoa VAP > 20 µm/s. Selected kinematic parameters were analyzed: curvilinear velocity (VCL, µm/s), velocity of average path (VAP, µm/s), straight line velocity (VSL, µm/s), linearity (LIN, %), and amplitude of lateral head displacement (ALH, µm). For each sample, a total of 41 frames were captured at aforementioned frequency of camera (60 frames/s).

Evaluation of sperm viability. For the evaluation of sperm viability, a fluorescent technique according to Harrison and Vickers (1990) was used. Briefly, sperm samples were diluted with physiological saline (ph 6.8) to 950 µl to a final concentration of $1–10 \times 10^6$ spermatozoa/ml. This suspension was further supplemented with 20 µl of 5(6) carboxyfluorescein diacetate (CFDA) (Sigma Aldrich, St. Louis, USA) working solution (0.46 mg CFDA/ml dimethyl sulfoxide), 20 µl propidium iodide (PI) (Sigma Aldrich) working solution (0.5 mg PI/ml physiological saline), and 10 µl of 0.3% formaldehyde. The samples were then incubated in the dark at 37°C for 10 min. After incubation, 7 µl of the sample was transferred to a microscopic slide and mounted under a coverslip using nail polish. Evaluation was performed using a fluorescent microscope Nikon Eclipse E6000 (Nikon Corp.) at 400× magnification. Live spermatozoa with functional esterases emitted green fluorescence, while dead spermatozoa were red due to the intercalation of propidium iodide. For each sample, three replicates were evaluated, which means that 600 spermatozoa were counted per sample.

Statistical analysis. The dataset was evaluated using the SAS software (Statistical Analysis System, Version 9.3, 2011). To evaluate the indicators, a relevant model was selected using the REGG procedure and STEPWISE method. The effect of LDL concentration in each extender was assessed separately for incubation. The effect of bull in regression was added into the equation. The differences between samples and groups were evaluated using the GLM procedure and the Tukey-Cramer test.

Model equation. The following model was applied:

$$y_{ij} = \mu + a_i + b^*(BULL) + e_{ij}$$

where:

- y_{ij} = dependent variable (amplitude of lateral head displacement, linearity, velocity of average path, curvilinear velocity, straight line velocity)
- μ = mean value of dependent variable
- a_i = fixed effect of LDL concentration (BULLXcell®: $i = 0$, $n = 5225$; $i = 6$, $n = 6321$; $i = 8$, $n = 6118$; $i = 10$, $n = 6420$; Bioxcell®: $i = 0$, $n = 5696$; $i = 4$, $n = 4637$; $i = 6$, $n = 5434$; $i = 8$, $n = 5213$; AndroMed®: $i = 0$, $n = 6202$; $i = 4$, $n = 7116$; $i = 6$, $n = 6834$; $i = 8$, $n = 6581$)
- $b^*(BULL)$ = linear regression for bulls
- e_{ij} = random error

The significance was evaluated at the level $P < 0.05$.

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RESULTS

Effect of egg yolk replacement with LDL on motility and viability of spermatozoa. After thawing (T0) and 2 h of incubation (T2) there was no significant influence of LDL on TM ($P > 0.05$) (Table 1). The influence of egg yolk substitution with various concentration of LDL in the BULLXcell® extender on sperm motility and viability at T0 and T2 is shown in Table 1. All the chosen concentrations of LDL (6%, 8%, and 10% v/v) significantly influenced the kinematic parameters of motility at T0 as well as at T2 ($P < 0.05$). Substitution of egg yolk with particular concentrations of LDL in the BULLXcell® extender significantly increased the values of VAP, VCL, and VSL at T0 and T2 ($P < 0.05$). Viability of sperm was negatively influenced at T0 ($P < 0.05$) when egg yolk was substituted with 6% LDL; however, this effect was not observed after 2 h of incubation (T2).

Effect of LDL addition to soybean extenders on motility and viability of spermatozoa. There were no significant differences ($P > 0.05$) in TM between concentrations of LDL added to extender Bioxcell®. There was a significant positive effect ($P < 0.05$) of all added LDL concentrations on VAP, VCL, VSL, and ALH of spermatozoa treated with Bioxcell® extender at T0 (Table 2). These kinematic parameters were significantly higher ($P < 0.05$) when 4%, 6%, and 8% LDL (v/v) was added to Bioxcell® compared to the control. The same effect was seen after 2 h of incubation (T2) in VAP, VCL, and VSL ($P < 0.05$). Significant differences ($P < 0.05$) between individual concentrations of LDL were seen at T0 and T2. Addition of 8% LDL significantly ($P < 0.05$) increased the percentage of viable spermatozoa compared to the Bioxcell® control at T0. However, the proportion of viable spermatozoa after 2 h of incubation was the same for Bioxcell® with and without the addition of LDL ($P > 0.05$).

The effect of various concentrations of LDL on TM was also insignificant ($P > 0.05$), however the influence on evaluated kinematic parameters of spermatozoa was not as distinct in AndroMed® as in the case of Bioxcell® (Table 3). The effect of LDL (4%, 6%, and 8% v/v) addition to the AndroMed® extender was not beneficial, although differences between individual concentrations in ALH, LIN, VCL, and VSL were significant ($P < 0.05$) at T0. After 2 h of incubation (T2), spermatozoa in AndroMed® containing 8% LDL demonstrated significantly higher ($P < 0.05$) values of ALH, LIN, VAP, VCL, and VSL parameters compared to the AndroMed® control without LDL. A significantly higher ($P < 0.05$) number of viable spermatozoa were present at T0 in samples containing 4% LDL compared to the AndroMed® control.

Table 1. Effect of egg yolk replacement by low-density lipoprotein (LDL) on motility and viability of frozen-thawed spermatozoa

		Viability	TM	ALH	LIN	VAP	VCL	VSL
Incubation 0 h								
BULLXcell®		51.23 ± 2.32^{A}	56.71 ± 5.12	4.96 ± 0.06^{a}	44.11 ± 0.34^{a}	57.81 ± 0.88^{a}	107.67 ± 1.72^{a}	50.76 ± 0.87^{a}
	6%	41.57 ± 2.08^{B}	50.46 ± 4.67	5.32 ± 0.06^{b}	37.91 ± 0.31^{b}	64.42 ± 0.88^{b}	135.68 ± 1.60^{b}	54.94 ± 0.81^{b}
LDL	8%	50.86 ± 2.131	54.20 ± 4.33	5.81 ± 0.05^{c}	39.01 ± 0.29^{b}	73.98 ± 0.76^{c}	157.89 ± 1.48^{c}	63.12 ± 0.75^{c}
	10%	48.95 ± 2.077	42.89 ± 4.67	5.29 ± 0.05^{b}	39.02 ± 0.29^{b}	67.15 ± 0.77^{b}	139.73 ± 1.49^{b}	57.86 ± 0.76^{b}
Incubation 2 h								
BULLXcell®		36.70 ± 1.91	38.98 ± 5.12	5.17 ± 0.05	50.91 ± 0.34^{a}	66.11 ± 0.87^{a}	116.14 ± 1.43^{a}	116.14 ± 1.43^{a}
	6%	32.02 ± 1.91	48.26 ± 4.67	5.07 ± 0.05^{a}	47.24 ± 0.30^{b}	74.62 ± 0.76^{b}	136.20 ± 1.25^{b}	136.20 ± 1.25^{b}
LDL	8%	32.42 ± 1.96	43.73 ± 4.33	5.38 ± 0.05^{b}	48.43 ± 0.34^{b}	83.83 ± 0.86^{c}	153.41 ± 1.42^{c}	153.41 ± 1.43^{c}
	10%	35.10 ± 1.87	39.35 ± 4.67	5.24 ± 0.05	46.80 ± 0.32^{b}	77.30 ± 0.81^{b}	144.66 ± 1.32^{d}	144.66 ± 1.32^{b}

TM = total motility (%), ALH = lateral head displacement, LIN = linearity, VAP = average velocity path, VCL = curvilinear velocity, VSL = straight-line velocity

values are presented as Least Squares Means + SE

$^{A,B,a–d}$values within the same column and time of incubation with different letters mean significant differences ($P < 0.05$)

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Table 2. Effect of low-density lipoprotein addition to soybean lecithin-based extender Bioxcell® on motility and viability of frozen-thawed spermatozoa

		Viability	TM	ALH	LIN	VAP	VCL	VSL
Incubation 0 h								
Bioxcell®		61.36 ± 2.91^{A}	51.67± 4.67	3.95 ± 0.04^{a}	50.26 ± 0.32^{a}	61.23 ± 0.80^{a}	109.07 ± 1.26^{a}	56.29 ± 0.79^{a}
LDL	4%	66.87 ± 3.09	54.01 ± 5.12	4.64 ± 0.05^{b}	51.56 ± 0.38	74.45 ± 0.95^{b}	127.02 ± 1.50^{b}	69.02 ± 0.94^{b}
	6%	67.59 ± 2.99	52.27 ± 4.67	4.68 ± 0.05^{b}	51.82 ± 0.36^{b}	75.41 ± 0.91^{b}	127.25 ± 1.43^{b}	69.14 ± 0.90^{b}
	8%	74.20 ± 2.91^{B}	50.34 ± 4.67	4.37 ± 0.05^{c}	50.53 ± 0.35	70.58 ± 0.88^{c}	120.02 ± 1.39^{c}	64.99 ± 0.87^{c}
Incubation 2 h								
Bioxcel®		46.05 ± 2.34	28.91 ± 4.67	3.37 ± 0.04^{a}	45.58 ± 0.41	45.52 ± 0.74^{a}	85.95 ± 1.10^{a}	40.76 ± 0.75^{a}
LDL	4%	48.83 ± 2.64	44.16 ± 5.12	3.84 ± 0.04^{b}	45.00 ± 0.41	54.47 ± 0.74^{b}	99.94 ± 1.11^{b}	49.29 ± 0.76^{b}
	6%	46.77 ± 2.34	42.04 ± 4.67	3.72 ± 0.04^{b}	44.87 ± 0.36^{a}	50.87 ± 0.65^{c}	97.00 ± 0.98^{c}	45.93 ± 0.70^{c}
	8%	54.60 ± 2.48	45.90 ± 4.67	3.67 ± 0.04^{b}	46.43 ± 0.39^{b}	53.66 ± 0.71^{b}	97.78 ± 1.05^{c}	49.43 ± 0.72^{b}

TM = total motility (%), ALH = lateral head displacement, LIN = linearity, VAP = average velocity path, VCL = curvilinear velocity, VSL = straight-line velocity

values are presented as Least Squares Means + SE

$^{A,B,a–c}$values within the same column and time of incubation with different letters mean significant differences ($P < 0.05$)

Table 3. Effect of low-density lipoprotein addition to soybean lecithin-based extender Andromed® on motility and viability of frozen-thawed spermatozoa

		Viability	TM	ALH	LIN	VAP	VCL	VSL
Incubation 0 h								
Andromed®		56.20 ± 2.85^{A}	52.60 ± 5.72	5.13 ± 0.05	42.10 ± 0.29	72.75 ± 0.77^{a}	146.21 ± 1.50^{a}	64.41 ± 0.81^{a}
LDL	4%	68.76 ± 2.94^{B}	61.14 ± 4.67	5.18 ± 0.04^{a}	41.43 ± 0.26^{a}	69.84 ± 0.69	140.24 ± 1.35^{b}	61.52 ± 0.72^{b}
	6%	60.15 ± 2.94	62.87 ± 4.67	4.96 ± 0.05^{b}	41.81 ± 0.29	68.73 ± 0.76^{b}	140.29 ± 1.48^{b}	60.72 ± 0.79^{b}
	8%	62.31 ± 2.72	54.45 ± 4.05	5.03 ± 0.05	42.68 ± 0.28^{b}	71.32 ± 0.76	132.45 ± 1.46^{c}	70.10 ± 0.79^{c}
Incubation 2 h								
Andromed®		45.67 ± 2.25	40.58 ± 5.72	4.50 ± 0.04^{a}	41.63 ± 0.33	59.76 ± 0.72^{a}	118.13 ± 1.25^{a}	52.32 ± 0.74^{a}
LDL	4%	44.49 ± 2.20	44.27 ± 4.67	4.38 ± 0.04^{a}	41.07 ± 0.32^{a}	56.42 ± 0.71^{b}	112.86 ± 1.23^{b}	49.54 ± 0.73^{b}
	6%	44.23 ± 2.65	53.32 ± 4.67	4.59 ± 0.04^{a}	42.61 ± 0.30	62.18 ± 0.66^{c}	121.55 ± 1.15^{c}	55.21 ± 0.67^{c}
	8%	45.80 ± 2.32	45.43 ± 4.05	4.74 ± 0.04^{b}	42.77 ± 0.31^{b}	63.34 ± 0.69^{c}	123.59 ± 1.18^{c}	56.45 ± 0.70^{c}

TM = total motility (%), ALH = lateral head displacement, LIN = linearity, VAP = average velocity path, VCL = curvilinear velocity, VSL = straight-line velocity

values are presented as Least Squares Means + SE

$^{A,B,a–c}$values within the same column and time of incubation with different letters mean significant differences ($P < 0.05$)

DISCUSSION

Specific difficulties in using complete egg yolk in extenders (Wall and Foote 1999) led researchers to perform experiments to examine the possibility of egg yolk replacement in extenders with LDL. In most studies, substitution of egg yolk with 8% LDL improved the qualitative parameters of frozen-thawed spermatozoa immediately after thawing in comparison to extenders with whole egg yolk (Moussa et al. 2002; Hu et al. 2010, 2011; Stadnik et al. 2015) and soy bean lecithin-based

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extenders as well (Amirat et al. 2005; Vera-Munoz et al. 2009).

In our study, we modified the protocol of LDL preparation by addition of sodium azide for the long-term preservation of LDL. To the best of our knowledge, other authors (e.g. Moussa at al. 2002; Hu et al. 2010) did not use any preservative in their studies. We achieved similar results as the above-mentioned authors, i.e. a comparable TM, proportion of viable spermatozoa, and better kinematic parameters after substitution of egg yolk with LDL preserved with sodium azide. Moreover, 8% LDL improved the kinematic parameters of spermatozoa, which is in agreement with the results of other authors (e.g. Moussa et al. 2002; Hu et al. 2011). Generally, in these studies, spermatozoa parameters were assessed only immediately after thawing. Our results also confirmed the positive effects of LDL after a 2-hour thermoresistance test. The consensus with other published experiments and, moreover, the positive effects after prolonged spermatozoa incubation under stressful conditions point out standard quality of our own produced LDL. In summary, our modification means an extended period of LDL storage is possible, which is advantageous for its practical use. Moreover, sodium azide should minimize any microbial risk (Hyldgaard et al. 2014), which has been a debatable issue (Aires et al. 2003).

The main objective of our study was to determine the influence of LDL addition on the cryoprotective properties of the two soybean lecithin-based extenders. In the case of the first one (Bioxcell®), our results showed a positive effect of 8% LDL on sperm viability immediately after thawing. Regarding spermatozoa motility, 4%, 6%, and 8% LDL had a beneficial effect on the majority of sperm kinematic parameters immediately after thawing and after the 2-hour thermoresistance test. Nevertheless, the second lecithin-based extender (Andromed®) did not show these trends. Although the addition of 4% LDL had a positive effect on spermatozoa viability after thawing, this effect was not seen in other groups or after the thermoresistance test. Furthermore, the sperm motility parameters did not show any obvious positive tendency as in the Bioxcell® extender. It was shown that there could be a different level of specific interactions between LDL and soy bean lecithin. These differences may be caused by different compositions of the extenders tested in our study, when they may probably differ in percentage of soybean lecithin content; unfortunately more detailed information about this is not available. Low-density lipoprotein, as well as soybean lecithin, acts mainly due to its phospholipid content (Thun et al. 2002). As Moussa et al. (2002) noted, there is a possible interference between the concentration and the positive influence of LDL. In their study, more than 10% LDL as the replacement for egg yolk in the extender led to a decrease in the sperm motility after thawing. The situation was similar in our study, in which 10% LDL in the egg yolk extender BULLXcell® decreased the majority of the sperm kinematic parameters in comparison to the optimal 8% concentration of LDL. Moussa et al. (2002) hypothesized that this was due to precipitation of fructose and salts in the extender. Thereby, the positive effects of LDL addition to already present lecithin in the extender might be also limited.

CONCLUSION

Based on the results of this study it could be concluded that produced LDL might be preserved using sodium azide. And especially that the cryoprotective properties of the semen extenders based on a soybean lecithin could be improved by egg yolk LDL addition. Nevertheless, the identification of the limits or patterns of this synergism are beyond the scope of this study. Additional experiments should be performed to determine optimal LDL concentrations for individual types of soybean lecithin-based extenders, as well as to evaluate the clinical significance of the positive effects observed.

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