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INFLUENCE OF LDL ADDITION ON CRYOPROTECTIVE PROPERTIES OF BOVINE SEMEN EXTENDERS

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Abstract

The cryopreservation process and subsequent thawing of insemination doses (ID) can impair spermatozoon structures, especially sperm acrosome, nucleus and plasma membrane. These alterations affect post-thaw sperm motility, which is considered as the most important indicator in terms of fertilization capability (quality) of ID. The above-mentioned changes can be minimized through the usage of an appropriate cryoprotective compound in semen extender. Generally effective egg yolk has a number of disadvantages. Nevertheless, low density lipoprotein (LDL) - responsible for its cryoprotective effects - could improve the properties of the currently used non-yolk extenders.
The objective of this work was to evaluate the effect of the combination of LDL, processed from egg yolk plasma, with the commercially produced extender Andromed® (Minitübe, Germany) containing plant phospholipids. LDL was added to the extender at concentrations of 4%, 6 %, 8 %. Sperm motility was assessed using the selected Computer Assisted Sperm Analysis parameters (VCL, VAP, VSL) after 2 hours of incubation at 37 °C. After addition of LDL, the evaluated parameters reached significantly higher values compared with the control samples before cryopreservation and post-thawing as well. However, no differences were found among added LDL concentrations.
It can be concluded that the addition of LDL to an extender with plant phospholipids can have positive effects on sperm motility.
Keywords: LDL, spermatozoa, motility

Introduction

During the process of freezing and thawing of insemination doses (ID) the viability and fertility of sperm cells are reduced mainly due to the negative effects on sperm structure and physiological processes within the cells (Amirat et al., 2004).

The causes of these changes are represented by a combination of influences, such as congenital low resistance to the effects of cryopreservation, the technique of dilution and the semen freezing protocols (Holt, 2000; Medeiros et al., 2002). The structure of the sperm which is primarily affected during cryopreservation is the plasma membrane (Hammerstedt et al., 1990). Structural changes in the plasma membrane during freeze-thawing play an important role in the reduction of fertilizing capacity during long-term storage of spermatozoa (Parks and Graham, 1992). The main goal of freezing protocols is to prevent these harmful effects, which can be achieved by, among other things, the usage of appropriate cryoprotective agents (Amirat et al., 2004).

In common practice different types of semen extenders are used, these can be divided according to the type of cryoprotective substance into skimmed-milk extenders, extenders on

a base of plant phospholipids, with the last group including extenders containing egg yolk (Vishwanath and Shannon, 2000). On the basis of the study by Pace and Graham (1974) it has been found that the component of egg yolk which is responsible for its cryoprotective properties is Low Density Lipoprotein (LDL). LDL consists of spherical particles mainly present in egg yolk plasma (85%), composed of a core made up of triglycerides and cholesterol esters surrounded by an outer layer consisting of phospholipids and apoproteins (Anton et al., 2003). The mechanism of the protective action of LDL has not yet been completely elucidated. However, the positive effects of LDL reported by Bergeron and Manjunath (2006) consist in the formation of strong and stable bonds with the major protein fraction of the bovine seminal plasma BSP protein family (BSP–A1/A2, BSP–A3 and BSP–30-kDa). These proteins trigger choline phospholipids and cholesterol efflux from the plasma membrane, thereby causing a decrease in the integrity and consequently an increase in sperm susceptibility to cold shock. Further beneficial effect of this substance is a positive correlation with the anti-oxidative system activity of sperm cells as demonstrated by authors Hu et al. (2011). Moreover the replacement of egg yolk only by its active fraction LDL can prevent microbiological risks, because, the results of the study Bousseau et al. (1998) showed that in spite of the antibiotic content in the composition of commercially produced egg yolk extenders, there was significant bacterial contamination. Simultaneously this would also eliminate the negative effects of other components in egg yolk (Pace and Graham, 1974).

The possibility of egg yolk replacement by the LDL fraction in commercially produced extenders and their effects on the motility of sperm cells during the freezing-thawing process has already been verified by several studies (Moussa et al., 2002; Amirat et al., 2004; Amirat et al., 2005; Amirat-Briand et al., 2010; Hu et al., 2010; Hu et al., 2011) with positive impact of 8% LDL concentration in all cases. Further, egg yolk extenders with the yolk replaced by the fraction LDL were compared with extenders on the basis of plant phospholipids (the both - IMV, L’Aigle, France): Bioxcell (Vera-Munoz et al., 2009) and Biociphos (Moussa et al., 2002; Amirat et al., 2005). However these studies did not show a significant difference.

The objective of this study was to assess the effect of the addition of LDL to the commercially manufactured plant phospholipid-based extender AndroMed® (Minitübe, Germany) on selected motility parameters of spermatozoa in native ejaculate and during the freezing – thawing process.

Materials and methods

LDL extraction

Low Density Lipoprotein (LDL) was prepared in accordance with the methodology of Moussa et al. (2002). Hen eggs were obtained from Biopharm Inc., and production of the fraction of LDL was ensured by the company Henna Inc.

Firstly, egg yolk had to be separate from albumen, manually, by rolling on filter paper in order to remove the endosperm and chalazae. Then the vitellin membrane was cut with a scalpel, and the egg yolks were collected in a beaker kept refrigerated by ice at 4 °C. In this way egg yolk plasma obtained was diluted by 0.17 M NaCl solution and then mixed at 4°C/1h. Afterwards centrifugation 10,000 x g for 45 min at 4 °C was performed and after supernatant removal, the procedure was repeated. The main purpose of this centrifugation was to achieve the separation of egg yolk granules from plasma. The obtained egg yolk plasma was stored at 4 °C. For removal of livetines 20.5 g of ammonium sulphate was added to100 ml of plasma, and this was stirred for 1 hour at pH 8.7 and temperature 4 °C. Precipitated livetines were subsequently separated by centrifugation at 10,000 x g/45 min. Supernatant rich in LDL was dialyzed further 10 h for the purpose of ammonium sulphate elimination and selective coagulation of LDL. At the end of this dialysis the mixture was centrifugated at

10,000 x g/45 min, and the resulting sediment (pellets) represented LDL with a purity of 97%, which had to be stored at 4 °C.

Preparation of the extenders

Two extenders were prepared just before the beginning of the experiments. The commercially produced non-egg yolk extender AndroMed® (Minitübe, Germany) was used as the control, composition: plant phospholipids, Tris, citric acid, sugars, antioxidants, buffers, Tylosin, Gentamicyn, Spectinomycin, Lincomycin (the quantity of antibiotics in accordance with regulation EC 88/407). It was prepared by the standard method according to the instructions of the producer, deionized water preheated to 30-32 °C was added to the concentrated solution of the extender at the ratio 4 : 1. The extender enriched by LDL was prepared in the same manner only with the difference of 4%, 6%, or 8% LDL addition.

Collection and processing of semen

The semen was collected randomly from 7 bulls at the insemination centre in the standard way. It was submitted to the assessment of ejaculate volume, sperm concentration (≥ 0.7 x 10^9/ml), and percentage of motile sperm (≥ 70 %). All samples conformed to the limits. Afterwards the ejaculate was transported in a cooling box at a temperature of 6-8 °C to the laboratory for further processing.

The ejaculate was diluted to a concentration of approximately 30 x 10^6/ml, which has been proven for optimal evaluation of motility using Computer Assisted Sperm Analysis (CASA) (Verstegen et al., 2002). Cryopreservation was carried out in the laboratory; straws were filled by a pipette in the styrofoam box at 6-8 °C and sealed by preheated pean. After filling, the straws were stored in a refrigerator for a time of equilibration - 4 hours. Then the straws were inserted into the styrofoam box, adjusted for cryopreservation, frozen in nitrogen vapor (4 cm above the surface) for 10 min at a temperature of approximately -120 °C and subsequently immersed in liquid nitrogen (-196 °C) for storage.

Evaluation of sperm motility

Sperm motility was assessed with the CASA module (NIS Elements Ar 3.2.), using the camera JENOPTIK ProGres CT1 (30 fps) and stereo microscope (Nikon Eclipse E600) with heated plate (Tokai Hit). A 3 µl volume of the sample was evaluated in a calibrated counting chamber Leja ® (depth 20 µm) in 6 fields per one sample. The native ejaculate samples as well as the freeze-thawed ones were assessed after 5-minute tempering (“0 hour”) and 2-hour incubation in a water bath at 37 °C. In both cases 3 selected motility parameters were assessed - curvilinear motility (VCL, µm/s) average path velocity (VAP, µm/s) and straight line velocity (VSL µm/s).

Statistical analysis

Statistical analysis was performed with STATISTICA CZ 10, multi-factorial ANOVA method and subsequent Scheffe’s post-hoc test was used.

Results and discussion

In our study we tested the effect of LDL addition to the composition of a commercially manufactured plant phospholipid-based extender in proportional ratios 4%, 6% and 8%. The values of monitored motility parameters VCL, VAP, and VSL were significantly higher (P < 0,05) at the LDL enriched samples in comparison with the control numbers of samples diluted in pure AndroMed® (Minitübe, Germany) under our conditions. This effect was determined in diluted native semen as well as in freeze-thawed samples (Table 1 and 2).

No statistically significant differences across the concentration range of added LDL were found. The values of monitored parameters of post-thaw motility obtained in our study were lower in contrast to the studies by Moussa et al. (2002), Amirat et al., (2004), or Amirat et al. (2005). This difference could be related to the different length of the incubation period or can be associated with prolonged exposure to BSP proteins before semen processing, as these can have a negative effect on sperm motility (Manjunath et al., 2002; Bergeron et al., 2004). Differences may also be related to the fact that cryopreservation process was rather simplified under laboratory conditions without the appropriate freezer. However, in spite of this fact, positive effects of LDL fraction addition were conclusive. Due to the lack of studies on LDL effects dealing with sperm motility before freezing, it is difficult to comment this part of our experiment. Nevertheless, we can say that the LDL positive effect was found even before the freezing-thawing process.

Table 1
Sperm movement characteristics of bull native ejaculate extended with the commercial plant phospholipid-based extender Andromed with different portion of egg-yolk LDL and incubated at 37 °C (mean ± SD)

Extender	**0% LDL**		**4% LDL**		**6% LDL**		**8% LDL**	
Incubation period	0 hour	2 hours	0 hour	2 hours	0 hour	2 hours	0 hour	2 hours
VCL (μm/s)	102,1 ± 0,917 ^{ac1}	53,7 ± 0,846 ^{ABC1}	114,1 ± 0,863 ^{ab2}	91,9 ± 0,823 ^{A2}	104,3 ± 0,882 ^{b}	97,2 ± 0,824 ^{B}	108,7 ± 0,869 ^{c4}	94,8 ± 0,833 ^{C4}
VAP (μm/s)	54,1 ± 0,492 ^{a1}	26,3 ± 0,453 ^{ABC1}	60,1 ± 0,463 ^{a2}	48,9 ± 0,441 ^{A2}	56,5 ± 0,473 ^{3}	50,1 ± 0,442 ^{B3}	59,0± 0,466 ^{4}	48,1 ± 0,467 ^{C4}
VSL (μm/s)	43,7 ± 0,461 ^{1}	20,8 ± 0,425 ^{ABC1}	47,8 ± 0,434 ^{2}	39,2 ± 0,414 ^{A2}	45,2 ± 0,444 ^{3}	39,5 ± 0,414 ^{B3}	47,2 ± 0,437 ^{4}	36,9 ± 0,419 ^{C4}

$^{a, A, 1}$ -values in the raw signed with the same superscript differ significantly at P < 0,05

Table 2
Sperm movement characteristics after freeze-thawing processing of bull ejaculate extended with the commercial plant phospholipid-based extender Andromed with different portion of egg-yolk LDL and incubated at 37 °C (mean ± SD)

Extender	**0% LDL**		**4% LDL**		**6% LDL**		**8% LDL**	
Incubation period	0 hour	2 hours	0 hour	2 hours	0 hour	2 hours	0 hour	2 hours
VCL (μm/s)	33,0 ± 0,649 ^{abc}	30,5 ± 0,639 ^{ABC}	47,9 ± 0,642 ^{a2}	37,1 ± 0,625 ^{A2}	46,1 ± 0,646 ^{b}	41,8 ± 0,605 ^{B}	47,6 ± 0,641 ^{c4}	37,6 ± 0,645 ^{C4}
VAP (μm/s)	17,2 ± 0,341 ^{abc}	14,4 ± 0,336 ^{ABC}	24,7 ± 0,337 ^{a2}	17,8 ± 0,328 ^{A2}	24,1 ± 0,191 ^{b3}	20,2 ± 0,318 ^{B3}	25,3 ± 0,340 ^{c4}	18,0 ± 0,339 ^{C4}
VSL (μm/s)	14,5 ± 0,320 ^{abc1}	11,5 ± 0,315 ^{ABC1}	20,1 ± 0,317 ^{a2}	14,5 ± 0,308 ^{A2}	19,8 ± 0,319 ^{b3}	16,3 ± 0,298 ^{B3}	21,0 ± 0,316 ^{c4}	14,4 ± 0,318 ^{C4}

$^{a, A, 1}$ -values in the raw signed with the same superscript differ significantly at P < 0,05

The cryoprotective properties of LDL were verified mainly in studies using LDL as a substitute for the yolk in egg-yolk-based extenders (Moussa et al., 2002; Amirat et al., 2004; Amirat et al., 2005; Amirat -Briand et al., 2010; Hu et al., 2010; Hu et al., 2011). Those papers usually concluded, through testing the post-thaw effect of different concentrations, that extender with 8% of LDL is optimal and even better than control egg-yolk-extender. These effects were gradually confirmed not only on the basis of sperm motility evaluation but also by analysis of other sperm cells quality indicators. These findings are in accordance with results of Pace and Graham (1974) who found a detrimental effect of other components present in egg yolk on sperm motility. Also the proven optimal concentration is noteworthy because egg yolk naturally contains 7% LDL, thus very close to the experimentally defined optimum.

Plant phospholipid-based extenders Biociphos or Bioxcell (both - IMV, L'Aigle, France) were included in the studies by Moussa et al. (2002), Amirat et al. (2005) and Vera-Munoz et al. (2009), but only as control samples. The results showed no significant difference compared with egg-yolk extenders in which the yolk was replaced by LDL fraction. But the effect of LDL addition to the plant phospholipid-based extenders has not been tested yet. With regard to phospholipid content in these extenders, without any chance to remove it from

composition, we chose besides the recommended 8% LDL concentration also lower ones (4%, 6%) within our study. It is noteworthy that under our experimental conditions even these lower concentrations of LDL significantly increased the level of monitored parameters of sperm motility in the case of native ejaculate and post-thawed as well. Although we did not prove any significant differences in effect among LDL concentrations tested, slightly better results could be observed at 6 % LDL.

The positive effect of LDL on sperm motility, found also in our study, can be explained by more properties of this fraction: Hu et al. (2011) demonstrated a positive effect of LDL on sperm motility by supporting the activities of glutathione peroxidase (GSH – Px), reduced glutathione (GSH) and catalase (CAT) which are part of the sperm antioxidative system (Aitken and Baker, 2004). Furthermore, as shown by Manjunath et al. (2002) and Bergeron et al. (2004) LDL has an ability to form very stable complexes with the major bovine seminal plasma proteins - BSP proteins (BSP - A1 / A BSP - A3 BSP -30 kDa). These mentioned proteins can stimulate the efflux of cholesterol and choline phospholipids from the plasmatic membrane, thereby subsequently influencing on membrane integrity. Simultaneously an increase in lipid content in the plasma membrane was also demonstrated and therefore the possibility of incorporation of part or of whole LDL molecules into this sperm cell structure. The dose-independent effect of even low LDL level observed in our study might be related to a simple increase of general phospholipid content to the maximum effective level as well as to a combination of egg-yolk LDL and plant phospholipid properties.

Conclusion

We found a positive influence of egg yolk LDL fraction addition to a plant phospholipid-based extender on sperm motility before cryopreservation as well as after cryopreservation and subsequent thawing. This beneficial effect was demonstrated in all selected LDL concentrations. There is a need, however, for further studies to assess the optimal concentration in plant phospholipid-based extenders and also to verify the LDL interaction with various kinds of these extenders. Further studies are also needed to elucidate the precise mechanisms of the LDL effect.

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