https://doi.org/10.17221/223/2022-CJAS

# Low-density lipoprotein supplementation improves the quality of Holstein bulls' insemination doses

*Jan Pytlík*, Radim Codl, Jaromír Ducháček, Filipp Georgijevič Savvulidi, Marek Vrhel, Luděk Stádník*

*Department of Animal Science, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic*

**Corresponding author: pytlik@af.czu.cz*

**Citation:** Pytlík J., Codl R., Ducháček J., Savvulidi F.G., Vrhel M., Stádník L. (2023): Low-density lipoprotein supplementation improves the quality of Holstein bulls' insemination doses. Czech J. Anim. Sci., 68: 64–71.

**Abstract:** Despite the routine use of cryopreservation in dairy cattle, the subsequent deterioration in the quality of bull insemination doses (IDs) is an incentive to refine cryopreservation protocols, including the composition of the ID. The aim of this study was to examine, using a flow cytometry assay, whether the selected concentrations of low density lipoproteins (LDLs) extracted from hen-egg yolk may improve the post-thaw quality of the ID diluted with a plant-based extender. In total, 30 ejaculates from five sires (six trials per animal) were collected and processed at the Artificial Insemination Centre. The effect of the low density lipoprotein supplementation to the AndroMed® diluent at 4 and 8% (v/v) on the cryopreservation efficiency was tested. The effects of the modified extenders were determined on different sperm subpopulations by simultaneous quadruple staining for flow cytometry as the percentage of sperm with intact plasma membrane and acrosome (PMAI), PMAI sperm showing high mitochondrial membrane potential (HMMP), sperm with plasma membrane damage (PMD), and sperm with acrosome damage (AD). It was observed that the 8% LDL treatment was more effective ($P < 0.05$) in preserving all the analysed parameters than AndroMed® without any supplementation. The supplementation of the AndroMed® extender with 8% LDL resulted in the most optimal values of the PMAI (30.61 ± 1.13), HMMP (68.81 ± 1.25), PMD (68.69 ± 1.14), and AD (38.36 ± 1.13) compared to the control treatment (24.86 ± 1.13 for PMAI, 47.79 ± 1.25 for HMMP, 74.77 ± 1.14 for PMD, and 42.83 ± 1.13 for AD. The results of the study also demonstrated a synergistic positive effect of the LDL and soybean lecithin-based diluent on the spermatozoa post-thaw quality and resilience, based on 2 h long incubation. In conclusion, the soybean based semen extender treatment with LDL represents a beneficial tool to mitigate the detrimental effect of cryopreservation.

**Keywords:** cryopreservation; spermatozoa; bovine; post-thaw quality; flow cytometry

Artificial insemination with frozen-thawed insemination doses has entirely revolutionised the animal breeding industry since its introduction during the 20th century (Lonergan 2018). Cryopreservation and artificial insemination enabled the spread of elite bull genetic material, and,

Supported by the Ministry of Education, Youth and Sports of the Czech Republic ("S" grant), the Ministry of Agriculture of the Czech Republic (NAZV Project No. QK22010270) and the Czech University of Life Sciences Prague (SGS Project No. SV21-6-21320).

https://doi.org/10.17221/223/2022-CJAS

through such improved cattle production and phenotype traits, at an, until then, absolutely unimaginable speed (Yangman et al. 2021). For that and other reasons, this biotechnology currently dominates the available assisted reproductive technologies in farm animals in terms of use, as the majority of dairy cattle are bred by this method in developed countries (Lonergan 2018). Despite the undisputable benefits, the cryopreservation process deeply harms the spermatozoa quality and fertility capacities, as a large portion of the spermatozoa is unable to survive conventional freezing and thawing. Cryopreservation may also implicate other undesirable effects, such as excessive reactive oxygen species (ROS) production, an increased amount of DNA fragmentation, and disruption in the plasma membrane phospholipid structures, which can induce acrosome exocytosis or premature capacitation (Layek et al. 2016; Makarevich et al. 2022). The most cryo-impaired cell structure is the plasma membrane, followed by the acrosome, mitochondria, and chromatin, the key elements of a sperm's fertility potential (Khalil et al. 2018).

Therefore, considerable scientific efforts are being made in order to reverse this detrimental state (Miguel-Jimenez et al. 2020). There are two main strategies, a controllable offensive and defensive one, with many possible approaches that have been used to reverse the adverse effects of cryopreservation. The semen extender supplementation with various substances seems to possess the greatest potential (Hezavehei et al. 2018). Only suitable semen diluent components are prerequisites of a sperm's survival either in a fresh or frozen state. These include glycerol and egg yolk, traditional bovine sperm cryoprotectants, which are added to the semen diluents to protect the sperm from various cryo-injuries (Akhter et al. 2011). Egg yolk is regarded as one of the most essential constituents as it contains low density lipoproteins (LDLs), a fraction primarily responsible for the cryoprotective effects (Simonik et al. 2016), which simultaneously positively and directly affect the spermatozoa structure and/or extracellular conditions (Hu et al. 2011). The use of whole egg yolk has its disadvantages though, like a worsened microscopic assessment, unknown inter-species protein interactions, problematic standardisation, or microbial contamination potential. Therefore, non-animal-based alternatives to semen diluents, including soybean lecithin or liposomes, have emerged as replacement with great potential (Miguel-Jimenez et al. 2020). In addition to not posing a hygiene risk, or standardisation failure, they also eliminate several disadvantages of animal-derived diluents. Nevertheless, the results obtained using such substitutes to egg yolk are still a matter of debate (Layek et al. 2016). Since one of the main techniques to improve semen preservation is to adjust the extender composition, the use of the egg yolk LDL fraction only as a supplementation to a soybean lecithin based extender may represent a viable option to improve the bull insemination dose quality. The synergistic cryoprotective activities of both LDL and soybean lecithin were proven previously (Beran et al. 2013; Stadnik et al. 2015). However, previous studies have relied, to varying degrees, on a subjective sperm analysis. Therefore, the main goal of this study was to verify, using flow cytometry, the possibility of improving the efficiency of bull semen cryopreservation using LDL as the supplementation in a soybean lecithin based extender.

## MATERIAL AND METHODS

### Semen collection and processing

Five Holstein bulls housed at the private Artificial Insemination Centre (Hradišťko, Central Bohemian Region, Czech Republic) were used in this study. All the animals were maintained under the same nutrition and management practices. The sires were of a similar age (4 ± 0.5 years old), with no signs of health problems, and were routinely used for semen collection. Semen samples were collected from each bull with an artificial vagina in weekly intervals for a time period of six weeks. The obtained ejaculates were evaluated immediately after the semen collection using an electronic precision scale and the AndroVision® computer assisted sperm analysis (CASA) system (Minitübe GmbH, Tiefenbach, Germany). Only ejaculates with a volume ≥ 1.5 ml, sperm concentration ≥ 700 × $10^6$ sperm/ml, and motility ≥ 70% were further processed and cryopreserved. After the initial evaluation, the samples were divided into three parts and diluted to 40 × $10^6$ spermatozoa/ml in a commercial extender AndroMed® (Minitübe GmbH, Tiefenbach, Germany), supplemented with 0%, 4%, or 8% (v/v) concentrated LDL prepared according to Simonik et al. (2019). The diluted se-

https://doi.org/10.17221/223/2022-CJAS

men was then automatically packaged into 0.25 ml French straws (IMV Technologies, L'Aigle, France), slowly cooled down to a temperature of +4 °C, and equilibrated in a cooling box for 4 hours. Afterwards, the straws were frozen afterwards applying a two-phase freezing rate as recommended by Dolezalova et al. (2016) using a programmable DigitCool® freezer (IMV Technologies, L'Aigle, France). The frozen doses were then plunged into liquid nitrogen and stored there until the thawing process. The thawing was performed in a water bath at 38 ± 1 °C for 30 s (Dolezalova et al. 2016). After thawing, each sample was transferred into an Eppendorf tube and kept inside of an INB 400 incubator (Memmert GmbH, Schwabach, Germany) in the dark at 38 °C for 2 hours.

## Flow cytometry

The post-thaw sperm analysis was performed as described by Pytlik et al. (2022) using a NovoCyte digital flow cytometer, model number 3000 (Acea Biosciences, part of Agilent, Santa Clara, CA, USA). The assessed sperm subpopulations were as follows: the spermatozoa with intact plasma and acrosomal membrane (PMAI), PMAI with high mitochondrial membrane potential (HMMP), spermatozoa with plasma membrane damage (PMD), and spermatozoa with acrosome damage (AD). The sperm variables were determined immediately (< 15 min designated as 0 h) and 2 h after thawing.

In particular, an analysed sample was diluted to 0.8 × $10^6$ spermatozoa/ml with Dulbecco's phosphate-buffered saline without divalent cations (Biosera Europe, Nuaille, France) and the following fluorescent probes were added to a 20 µl aliquot of semen: 2 µl Hoechst-33342 (H-342; Sigma Aldrich, St. Louis, MO, USA; 16.2 µM), 2 µl propidium iodide (PI; Sigma Aldrich, St. Louis, MO, USA; 12 µM); 2 µl fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Thermo Fisher Scientific, Waltham, MA, USA; 0.5 µg/ml), and 4 µl MitoTracker Deep Red (MTR DR; Thermo Fisher Scientific, Waltham, MA, USA; 80 nM). The samples were then incubated at 38 °C for 10 minutes. The samples were run at a low speed (14 µl/min), and the fluorescence from 30 000 events was recorded for each sample. Each thawed straw was analysed by the flow cytometer twice. A rinse procedure was performed after every sample acquisition. NovoExpress software, v1.3.0 (Acea Biosciences, part of Agilent, Santa Clara, CA, USA) was used for the automated cytometer set-up and performance tracking, as well as the data acquisition. The same software was also used to analyse the acquired flow cytometry data. No compensation was required with the optical filter set-up used. The flow cytometer was quality-controlled on a daily basis using polystyrene microspheres (ACEA NovoCyte QC Particles; Acea Biosciences, part of Agilent, Santa Clara, CA, USA) providing routine calibration control.

## Statistical analysis

The acquired data ($n$ = 360) were assessed using the SAS v9.3. statistical software (SAS Institute Inc., Cary, NC, USA). The data for the sperm variables were examined for normal distribution (Shapiro-Wilk test) and for homogeneity of variance (Bartlett's test), then were analysed using a generalised linear model (PROC GLM) afterwards. The best model was selected based on the Akaike information criterion. The Tukey-Kramer method was used to evaluate the differences of the least square means. The following model equation was used:

$$Y_{ijk} = \mu + A_i + B_j + C_k + (BC)_{jk} + e_{ijk} \quad (1)$$

where:

$Y_{ijk}$ – dependent variable (PMAI, HMMP, PMD, AD);
µ – average value of the dependent variable;
$A_i$ – fixed effect of the bull: $i$ (1, $n$ = 72; $i$ = 2, $n$ = 72; $i$ = 3, $n$ = 72; $i$ = 4, $n$ = 72; $i$ = 5, $n$ = 72);
$B_j$ – fixed effect of the LDL supplementation: $k$ (control, $n$ = 120; 4% LDL, $n$ = 120; 8% LDL, $n$ = 120);
$C_k$ – fixed effect of the incubation time: $j$ (0, $n$ = 180; 2, $n$ = 180);
$BC_{jk}$ – interaction between the fixed effect of the LDL supplementation and the incubation time;
$e_{ijkl}$ – residual error.

Significant differences between groups were considered at $P < 0.05$.

## RESULTS

The model equation was statistically significant ($P < 0.001$) and explained from 37.24% up to 47.66%

https://doi.org/10.17221/223/2022-CJAS

of the variability for the PMAI and HMPP variables. All the described effects and interactions in the model equation were statistically significant ($P < 0.05$), except the interaction between the incubation time and the LDL supplementation for the PMAI and PMD parameters. The evaluated sperm variables for each bull are listed in Table 1. The greatest percentage of PMAI sperm was measured in bull No. 4, while the bull No. 1, oppositely, scored the lowest values ($P < 0.05$). The largest proportion of HMMP was observed in bull No. 5, which significantly differed ($P < 0.05$) from the other sires with the exception of bull No. 4. The lowest PMD was obtained in bull No. 4 ($P < 0.05$). Bull No. 4 also showed the lowest AD, which significantly ($P < 0.05$) differed from the rest of the bulls.

The sperm variables significantly differed ($P < 0.05$) depending on the LDL supplementation, as evident from Table 2. Compared to the control, all the post-thaw sperm variables were improved ($P < 0.05$) in the presence of the LDL in the extender except for the AD parameter at the 4% concentration. In detail, the LDL increased the values of the samples by scoring a greater PMAI by +6.59% and +5.75% for 4% LDL and 8% LDL, respectively, when compared to the control. The improving effect of the LDL addition was also detected in the HMMP parameter, where the control showed lower values by –18.9% and –21.02%, respectively, compared to 4% LDL and 8% LDL. Samples supplemented with LDL also statistically differed in the PMD variability in comparison to the control, as 4% LDL showed lower values by –7.07% while 8% LDL displayed a –6.08% difference from the control. In the AD parameter, the only difference between the control and 8% LDL (–4.47%) was observed in favour of the LDL supplemented samples.

The incubation length affected ($P < 0.05$) the evaluated sperm characteristics differently (Table 3), while the PMAI and PMD variables were positively influenced (+2.75% and +3.26%, respectively), the HMMP and AD variables were adversely impacted (–13.06% and –12.54%, respectively). In more detail, no time dependent changes, within each treatment, were found in the PMAI and PMD variables throughout the 2 h incubation period, even though the absolute figures fluctuated (Table 4). On the other hand, incubation-mediated

Table 1. Comparison of the bulls' post-thaw sperm variables ($n$ per bull = 72)

| Bull | PMAI (%) | HMMP (%) | PMD (%) | AD (%) |
|---|---|---|---|---|
| 1 | 15.71 ± 1.462$^{a}$ | 58.27 ± 1.616$^{a}$ | 83.86 ± 1.474$^{a}$ | 48.72 ± 1.452$^{a}$ |
| 2 | 28.19 ± 1.462$^{b}$ | 59.72 ± 1.616$^{a}$ | 71.44 ± 1.474$^{b}$ | 42.08 ± 1.452$^{b}$ |
| 3 | 29.02 ± 1.462$^{b}$ | 58.93 ± 1.616$^{a}$ | 70.27 ± 1.474$^{b}$ | 40.87 ± 1.452$^{b}$ |
| 4 | 43.57 ± 1.462$^{c}$ | 61.98 ± 1.616 | 55.48 ± 1.474$^{c}$ | 27.79 ± 1.452$^{c}$ |
| 5 | 28.37 ± 1.462$^{b}$ | 66.58 ± 1.616$^{b}$ | 70.88 ± 1.474$^{b}$ | 44.70 ± 1.452$^{a}$ |

AD = percentage of sperm with acrosome damage; HMMP = percentage of sperm with intact plasma membrane and acrosome showing high mitochondrial membrane potential; PMAI = percentage of sperm with intact plasma membrane and acrosome; PMD = percentage of sperm with plasma membrane damage

$^{a–c}$Different letters indicate differences between the bulls within a column ($P < 0.05$)

Results are presented as the least square means ± standard error of the mean

Table 2. Effect of the different LDL supplementation ($n$ per treatment = 120) on the post-thaw sperm variables after a 2 h of incubation

| Bull | Treatment | PMAI (%) | HMMP (%) | PMD (%) | AD (%) |
|---|---|---|---|---|---|
| All bulls | control | 24.86 ± 1.133$^{a}$ | 47.79 ± 1.252$^{a}$ | 74.77 ± 1.142$^{a}$ | 42.83 ± 1.125$^{a}$ |
| | 4% LDL | 31.45 ± 1.133$^{b}$ | 66.69 ± 1.252$^{b}$ | 67.70 ± 1.142$^{b}$ | 41.31 ± 1.125 |
| | 8% LDL | 30.61 ± 1.133$^{b}$ | 68.81 ± 1.252$^{b}$ | 68.69 ± 1.142$^{b}$ | 38.36 ± 1.125$^{b}$ |

AD = percentage of sperm with acrosome damage; HMMP = percentage of sperm with intact plasma membrane and acrosome showing high mitochondrial membrane potential; LDL = low density lipoprotein; PMAI = percentage of sperm with intact plasma membrane and acrosome; PMD = percentage of sperm with plasma membrane damage

$^{a,b}$Different letters indicate differences within a column ($P < 0.05$)

Results are presented as the least square means ± standard error of the mean

https://doi.org/10.17221/223/2022-CJAS

Table 3. Effect of the incubation time ($n$ per group = 180) on the sperm characteristics of the frozen-thawed samples

| Incubation time (h) | PMAI (%) | HMMP (%) | PMD (%) | AD (%) |
|---|---|---|---|---|
| 0 | 27.60 ± 0.925[a] | 67.63 ± 1.022[a] | 72.02 ± 0.933[a] | 34.56 ± 0.919[a] |
| 2 | 30.35 ± 0.925[b] | 54.57 ± 1.022[b] | 68.76 ± 0.933[b] | 47.10 ± 0.919[b] |

AD = percentage of sperm with acrosome damage; HMMP = percentage of sperm with intact plasma membrane and acrosome showing high mitochondrial membrane potential; PMAI = percentage of sperm with intact plasma membrane and acrosome; PMD = percentage of sperm with plasma membrane damage

[a,b]Different letters indicate differences within a column ($P < 0.05$)

Results are presented as the least square means ± standard error of the mean

Table 4. Effect of the low density lipoprotein (LDL) supplementation and incubation time on the flow cytometrically assessed parameters of the frozen-thawed sperm samples from Holstein bulls

| Parameter | 0 h | | | 2 h | | |
|---|---|---|---|---|---|---|
| | control | 4% LDL | 8% LDL | control | 4% LDL | 8% LDL |
| PMAI (%) | 24.22 ± 1.602[a] | 30.05 ± 1.602 | 28.52 ± 1.602 | 25.49 ± 1.602[a] | 32.85 ± 1.602[b] | 32.69 ± 1.602[b] |
| HMMP (%) | 60.34 ± 1.770[a] | 73.52 ± 1.770[b] | 69.02 ± 1.770[b] | 35.24 ± 1.770[c] | 59.85 ± 1.770[a] | 68.60 ± 1.770[b] |
| PMD (%) | 75.45 ± 1.615[a] | 69.42 ± 1.615 | 71.18 ± 1.615 | 74.09 ± 1.615[a] | 65.98 ± 1.615[b] | 66.20 ± 1.615[b] |
| AD (%) | 41.21 ± 1.591[a,c] | 34.31 ± 1.591[b] | 28.17 ± 1.591[b] | 44.44 ± 1.591[a] | 48.31 ± 1.591[a,d] | 48.55 ± 1.591[a,d] |

AD = percentage of sperm with acrosome damage; HMMP = percentage of sperm with intact plasma membrane and acrosome showing high mitochondrial membrane potential; PMAI = percentage of sperm with intact plasma membrane and acrosome; PMD = percentage of sperm with plasma membrane damage

[a–d]Different letters indicate differences within a column ($P < 0.05$)

Results are presented as the least square means ± standard error of the mean

changes were proven ($P < 0.05$) in the HMPP and AD parameters. The greatest drop in the HMMP variable was detected in the control samples (–25.1%) compared to the 4% or 8% LDL samples (–13.67% and –0.42%, respectively). The lowest increase in the AD parameter was observed in the control samples (+3.23%), while the greatest increase was observed in the LDL supplemented ones (+14% and +20.38%, in the 4% LDL and 8% LDL, respectively). Significant ($P < 0.05$) differences in the obtained results were found depending on the treatment. In detail, the improving effect of the LDL supplementation on the sperm variables, in comparison to the control, was mostly observed after 2 h of incubation. Thus, a significantly lower PMAI ($P < 0.05$) was detected in the control samples compared to the 4% and 8 % LDL ones (+7.36% and +7.2%, respectively) after 2 h of incubation. Similarly, the HMMP was affected by the LDL supplementation, as the control samples showed lower values by –24.61% and –33.36%, when compared with the 4% and 8% LDL samples, respectively. In the case of the PMD parameter, the differences between the control and the LDL supplemented samples were also in favour ($P < 0.05$) of the LDL (ranging from +7.89% to +8.11%). However, no difference was proven between the control and the LDL supplemented samples in the AD variable after 2 h of incubation.

## DISCUSSION

In the current study, several variables of the bull sperm quality, previously determined (Sellem et al. 2015) as fertility predictors, were analysed. Differences in the post-thaw sperm variables between the bulls were also detected. This phenomenon of the differing response of bulls to the same cryopreservation protocol has been reported in a number of studies, as discussed by Bezdicek et al. (2021). This variability is of a complex nature and partially depends on the protective seminal plasma protein specificity of an individual, the differing composition of the membrane structures and the metabolic properties of the sperm cells (Indriastuti et al. 2020). The results also demonstrated that the post-thaw bull semen quality might be positively affected if a soybean lecithin-based diluent is supplemented with LDL. In particular, we

https://doi.org/10.17221/223/2022-CJAS

found the beneficial effect of the LDL on the plasma membrane and acrosome integrities at both studied concentrations (4% and 8%) compared to the control. The sperm membrane intactness is of an utmost importance for its role in the cell integrity and successful fertilisation (Ansari et al. 2014). Similarly to our results, Akhter et al. (2011) observed a higher PMAI in the extender containing LDL (8% and 10%) over the control. On the contrary, no differences between the LDL supplemented (6% and 8%) samples and AndroMed-diluted samples were observed previously (Simonik et al. 2016). As discussed by Kroemer et al. (2007), sperm with mitochondrial dysfunction lose the capacity to perform their functions as they face the first manifestation of cell death. Such spermatozoa are unable to perform their metabolic pathways or motion properly and concurrently negatively affect the intact cells by reactive oxygen species production (Roca et al. 2016). Therefore, the mitochondrial functionality, expressed in the HMMP parameter, is one of key elements to be measured. In the present study, the HMMP was higher in the extender containing LDL compared to the control. Simonik et al. (2019) also observed a beneficial effect of 6% LDL supplementation on the HMMP in samples diluted with AndroMed®, while no such effect was found in Bioxcell®, another soybean lecithin-based diluent. The unimpaired plasma membrane maintains cell homeostasis, and many other functions, in which some of them are related to the fertilisation potential (Singh et al. 2018). The detected occurrence of plasma membrane damage was lower with the LDL supplementation compared to the control. This finding is in agreement with Akhter et al. (2011) and Hu et al. (2011) who also found lower PMD in favour of the LDL supplemented samples rather than the control. However, the previous work of Simonik et al. (2019) is in disagreement as no effect of the LDL addition, in terms of plasma membrane integrity preservation, to AndroMed® or Bioxcell® was proven. The significance of the acrosome integrity lies in its role in the successful fertilisation, as any structural or functional abnormality may impair the gamete fusion (Khawar et al. 2019). Moreover, the acrosome integrity is usually hampered during conventional cryopreservation processes which induce capacitation-like changes (Varela et al. 2020). Our results showed that the acrosome membrane was preserved unequally after cryopreservation with different LDL concentrations. While the 8% LDL supplementation significantly stabilised the acrosomal membrane in comparison with the control, no such effect was found after the 4% LDL supplementation. Similarly, Simonik et al. (2019) observed no impact on the acrosome integrity when a 6% LDL addition was used. Concomitantly, Anand et al. (2017) did not record any change in the AD when the samples were treated by 7% or 10% LDL, while the 8% and 9% supplementation proved beneficial. Perumal et al. (2016) also proved the beneficial effect of LDL given at 8% on the sperm acrosomal integrity, while no such effect was proven at 10% LDL or with the control. However, these mentioned findings are in disagreement with El-Sharawy et al. (2012) who found a beneficial effect of 4% to 15% LDL supplementation on acrosome integrity in comparison to the control.

Our study showed that the incubation-mediated changes differed between the sperm variables. The PMAI variable was significantly increased during incubation. Yet, this is not in line with Anzar et al. (2011) who observed no decrease over a 2 h incubation time-course. Contradictory findings were published by Ansari et al. (2014) and Bucher et al. (2019), who detected an 18% and 15% incubation-mediated drop in the PMAI, respectively. Similarly, a significant decrease in the HMMP was observed during incubation. The adverse effect of incubation on the mitochondrial functions and integrities was also found by Bucher et al. (2019). Oppositely, an incubation-mediated increase in the HMMP variable was detected by Anzar et al. (2011). The PMD variable was increased, which is in disagreement with Anzar et al. (2011) who observed no change over the monitored time course. Our finding is also in disagreement with Sellem et al. (2015) and Simonik et al. (2016) who detected a drop in the plasma membrane integrity during incubation. The AD parameter showed a significant change in time, similar to the recent work of Sellem et al. (2015) or Pytlik et al. (2022), where the acrosome damage was exacerbated.

Our results also confirmed the positive effects of LDL during the post-thaw incubation. It was also observed that immediately after thawing, the PMAI and PMD remained the same in the samples containing LDL as well as in the control ones. Whereas after 2-h incubation, the majority of the parameters were significantly superior in the

https://doi.org/10.17221/223/2022-CJAS

samples supplemented by LDL compared to the control. This indicates the crucial role of LDL at a concentration of 4% or 8% for the spermatozoa quality and resilience. It is suggested that the phospholipids, cholesterol, and other substances present in the LDL interact with the plasma membrane, which is imparted to be more structure-resistant during the challenging freezing-thawing process (Amirat et al. 2005). A similar improving effect was described for acrosomes by Hu et al. (2011) as the exchange or repair of acrosomal membrane phospholipids was attributed for its greater integrity. It is known that whole egg yolk contains substances of both sperm-deleterious and sperm-protective nature. Sperm respiration, which is closely related to spermatozoa motility, belongs among the hampering functions. Various harming components are, however, absent in the LDL fraction, providing better conditions for mitochondria unspoiled metabolism. Additionally, it was determined that LDL increases the antioxidant activity, securing a less stressful environment for the mitochondria, which are susceptible to the higher levels of ROS (Hu et al. 2011; Simonik et al. 2019).

## CONCLUSION

The samples supplemented with selected LDL concentrations gave higher proportions of plasma membrane-intact sperm, acrosome-intact sperm, and spermatozoa showing high mitochondrial potential as detected by the objective evaluation using flow cytometry, thereby improving the quality of the frozen-thawed semen compared to the samples diluted with the soybean lecithin extender only. Particularly, the most beneficial supplement was with the 8% LDL supplementation. The LDL addition to the extender's composition was also beneficial for the spermatozoa incubation resilience. In conclusion, the LDL supplementation represents a potential way to improve the post-thaw spermatozoa quality, and, therefore, even ameliorate the probability of fertilisation. Nevertheless, this should be proven in large-scale field fertility experiments.

### Conflict of interest

The authors declare no conflict of interest.

## REFERENCES

Akhter S, Ansari MS, Rakha BA, Andrabi SMH, Khalid M, Ullah N. Effect of low density lipoproteins in extender on freezability and fertility of buffalo (Bubalus bubalis) bull semen. Theriogenology. 2011 Sep 1;76(4):759-64.

Amirat L, Anton M, Taiturier D, Chatagnon G, Battut I, Courtens JL. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reproduction. 2005 Apr 1;129(4):535-43.

Anand M, Yadav S, Sing V, Vaswani S, Shukla PK. Cryoprotective effect of low-density lipoproteins on post thaw semen quality in Hariana bull. Indian J Anim Sci. 2017 Nov 1;87(11):1340-4.

Ansari MS, Rakha BA, Malik MF, Andrabi SMH, Ullah N, Iqbal R, Holt WV, Akhter S. Effect of cysteine addition to the freezing extender on the progressive motility, viability, plasma membrane and DNA integrity of Nili-Ravi buffalo (Bubalus bubalis) bull spermatozoa. J Appl Anim Res. 2014 Dec 20;44(1):36-41.

Anzar M, Kroetsch T, Boswall L. Cryopreservation of bull semen shipped overnight and its effect on post-thaw sperm motility, plasma membrane integrity, mitochondrial membrane potential and normal acrosomes. Anim Reprod Sci. 2011 Jun;126(1-2):23-31.

Beran J, Simonik O, Stadnik L, Rajmon R, Duchacek J, Krejcarkova A, Dolezalova M, Sichtar J. Effect of bull, diluter and LDL-cholesterol concentration on spermatozoa resistance against cold shock. Acta Univ Agric Silvic Mendelianae Brun. 2013 Nov;61(6):1575-81.

Bezdicek J, Nesvadbova A, Makarevich A, Kubovicova E. Negative impact of heat stress on reproduction in cows: Animal husbandry and biotechnological viewpoints: A review. Czech J Anim Sci. 2021 Aug;66(8):293-301.

Bucher K, Malama E, Siuda M, Janett F, Bollwein H. Multicolor flow cytometric analysis of cryopreserved bovine sperm: A tool for the evaluation of bull fertility. J Dairy Sci. 2019 Dec;102(12):11652-69.

Dolezalova M, Stadnik L, Biniova Z, Duchacek J, Stupka R. Equilibration and freezing interactions affecting bull sperm characteristics after thawing. Czech J Anim Sci. 2016 Nov;61(11):515-25.

El-Sharawy ME, El-Shamaa IS, Ibrahim MAR, Abd El-Razek IM, El-Seify EM. Effect of low density lipoproteins in extender on freezability and fertility of egyptian buffalo bull semen. Anim Sci Series D. 2012 Oct;55:114-20.

Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, Shahverdi A. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. Reprod Biomed Online. 2018 Sep;37:327-39.

https://doi.org/10.17221/223/2022-CJAS

Hu JH, Jiang ZL, Lv RK, Li QW, Zhang SS, Zan LS, Li YK, Li X. The advantages of low-density lipoproteins in the cryopreservation of bull semen. Cryobiology. 2011 Feb; 62(1):83-7.

Indriastuti R, Ulum MF, Arifiantini RI, Purwantara B. Individual variation in fresh and frozen semen of Bali bulls (Bos sondaicus). Vet World. 2020 May;13(5):840-6.

Khalil WA, El-Harairy MA, Zeidan AEB, Hassan MAE, Mohey-Elsaeed O. Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultrastructural insights. Int J Vet Sci Med. 2018 Nov;6:S49-56.

Khawar MB, Gao H, Li W. Mechanism of acrosome biogenesis in mammals. Front Cell Dev Biol. 2019 Sep 18;7: 12 p.

Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev. 2007 Jan;87(1):99-163.

Layek SS, Mohanty TK, Kumaresan A, Parks JE. Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. Anim Reprod Sci. 2016 Sep 1;172:1-9.

Lonergan P. Historical and futuristic developments in bovine semen technology. Animal. 2018 Dec;12(suppl 1):4-18.

Makarevich A, Spalekova A, Kubovicova E, Bezdicek J, Chrenek P. Cooling storage of ram sperm in presence of antioxidant glutathione. Czech J Anim Sci. 2022 Sep; 67(9):356-64.

Miguel-Jimenez S, del Alamo MMR, Alvarez-Rodriguez M, Hidalgo CO, Pena AI, Muino R, Rodriguez-Gil JE, Mogas T. In vitro assessment of egg yolk-, soya bean lecithin- and liposome-based extenders for cryopreservation of dairy bull semen. Anim Reprod Sci. 2020 Apr 1; 215: 106315.

Perumal P, Srivastava SK, Ghosh SK, Baruah KK, Bag S, Rajoria JS, Kumar K, Rajkhowa C, Pande M, Srivastava N. Effects of low-density lipoproteins as additive on quality parameters and oxidative stress following cryopreservation of mithun (Bos frontalis) spermatozoa. Reprod Domest Anim. 2016 Jul;51(5):708-16.

Pytlik J, Savvulidi FG, Duchacek J, Codl R, Vrhel M, Nagy S, Stadnik L. Effect of extender on the quality and incubation resilience of cryopreserved Holstein bull semen. Czech J Anim Sci. 2022 Mar;67(3):75-86.

Roca J, Parrilla I, Gil MA, Cuello C, Martinez EA, Rodriguez-Martinez H. Non-viable sperm in the ejaculate: Lethal escorts for Contemporary viable sperm. Anim Reprod Sci. 2016 Jun;169:24-31.

Sellem E, Broekhuijse MLWJ, Chevrier L, Camugli S, Schmitt E, Schibler L, Koenen EPC. Use of combinations of in vitro quality assessments to predict fertility of bovine semen. Theriogenology. 2015 Jul;84(9):1447-54.

Simonik O, Rajmon R, Stadnik L, Sichtar J, Beran J, Duchacek J, Hodek P, Trefil P. Effect of low-density lipoprotein addition to soybean lecithin-based extenders on bull spermatozoa following freezeing-thawing – Preliminary results. Czech J Anim Sci. 2016 Dec;61(12):560-7.

Simonik O, Sichtar J, Beran J, Manaskova-Postlerova P, Tumova L, Dolezalova M, Folkova P, Stadnik L, Rajmon R. Low density lipoprotein – Important player in increasing cryoprotective efficiency of soybean lecithin-based bull semen extenders. Animal Reprod. 2019 Oct;16(2):267-76.

Singh AK, Kumar A, Honparkhe M, Kaur S, Kaur H, Ghuman SPS, Brar PS. Comparison of in vitro and in vivo fertilizing potential of buffalo bull semen frozen in egg yolk-, soya bean lecithin-, and liposome-based extenders. Reprod Domest Anim. 2018 Oct;53(1):195-202.

Stadnik L, Rajmon R, Beran J, Simonik O, Dolezalova M, Sichtar J, Stupka R, Folkova P. Influence of selected factors on bovine spermatozoa cold shock resistence. Acta Vet Brno. 2015 Feb;84(2):125-31.

Varela E, Rojas M, Restrepo G. Membrane stability and mitochondrial activity of bovine sperm frozen with low-density lipoproteins and trehalose. Reprod Domest Anim. 2020 Feb;55(2):146-53.

Yangman Y, Chapanya S, Vongpralub T, Boonkum W, Chankitisakul V. Effect of semen extender supplementation with sericin on post-thaw dairy bull quality and lipid peroxidation. Czech J Anim Sci. 2021 Jan;66(1):13-20.

Received: December 16, 2022
Accepted: January 19, 2023
Published online: February 20, 2023