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# EFFECT OF LDL ADDITION INTO SELECTED BULL SPERM DILUTERS ON RESISTANCE OF SPERMATOZOA AGAINST COLD SHOCK

Jan Beran[1], Ondřej Šimoník[2], Radko Rajmon[2], Luděk Stádník[1], Martina Doležalová[1], Adéla Krejcárková[2], Jaromír Ducháček[1], Jiří Šichtař[2]

[1] Department of Animal Husbandry, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6-Suchdol, Czech Republic
[2] Department of Veterinary Science, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6-Suchdol, Czech Republic

## Abstract

BERAN JAN, ŠIMONÍK ONDŘEJ, RAJMON RADKO, STÁDNÍK LUDĚK, DOLEŽALOVÁ MARTINA, KREJCÁRKOVÁ ADÉLA, DUCHÁČEK JAROMÍR, ŠICHTAŘ JIŘÍ. 2016. Effect of LDL Addition Into Selected Bull Sperm Diluters on Resistance of Spermatozoa Against Cold Shock. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis,* 64(2): 395–399.

The aim of work was to determine the effect of LDL cholesterol addition into selected diluters on the resistance of spermatozoa against cold shock and on their short-term survivability during cold test. The hypothesis was that the addition of LDL cholesterol will positively affects sperm resistance to cold shock and ensures a higher survivability of spermatozoa during short-term cold survival test. Four bulls of different breeds and ages, from the same sire insemination center were used. A total of eight semen collections were processed. Each ejaculate was divided into 6 portions (3 controls and 3 samples). Three commercially produced diluters, AndroMed®, Bioxcell®, and Triladyl® were used, each in standard and LDL enriched variants. In the case of AndroMed® or Bioxcell®, 6% of LDL was simply added. In Triladyl®, 10% of LDL replaced the standard egg yolk component. Spermatozoa resistance to cold shock was evaluated by the percentage of live sperm using Eosin-Nigrosine staining. The results showed the influence of bull individuality as an important factor. It is possible to recommend Bioxcell® with addition of LDL cholesterol in 6% concentration, which survivability was 69.17% at the beginning of the test, and 52.94% after 2 hours of incubation.

Keywords: AndroMed, Bioxcell, bull semen, LDL, Triladyl, sperm survivability

## INTRODUCTION

During the processing of semen, spermatozoa are exposed to many non-physiological changes, e.g. of temperature, pH, osmotic pressure induced by cryoprotectants, formation and dissolution of ice crystals and of course the absence of female genital secretions, with which the sperm are mixed after ejaculation (Beran *et al.*, 2014). Biochemical and anatomical characteristics of sperm can be altered during the freezing process, plasma membrane is primarily affected (Hammerstedt *et al.*, 1990). So, the success of cryopreservation depends on many factors, including the interaction between cryoprotectants, type of diluters (Stádník *et al.*, 2015), speed of freezing/thawing (Doležalová *et al.*, 2015), packaging and individuality of donor (Cooter *et al.*, 2005; Clulow *et al.*, 2008).

Composition of diluters affects the viability and fertilization ability of sperm in the insemination dose (Siddique *et al.*, 2006). Therefore, the most suitable protecting media are continuously looking for and developing.

Egg yolk improves sperm function and keeps their fertilization ability (Barak *et al.*, 1992). The phospholipids and low-density lipoprotein (LDL) permeating into membranes are basic fractions of egg yolk which provide protection for sperm during cooling and freezing (Medeiros *et al.*, 2002). Egg yolk can be replaced in diluters by plant phospholipids due to its inconsistent composition (Ansari

*et al.*, 2010) and risk of bacterial contaminations (Bousseau *et al.*, 1998). There is an assumption that LDL addition to commercially manufactured diluters (animal and/or plant phospholipid-based) can improve cryoprotective properties of them.

The potential fertility of sperm can be evaluated by several methods (Dhurvey *et al.*, 2012). Cold shock test was evaluated in relation to LDL addition to diluters (Beran *et al.*, 2013), but optimal LDL concentration increasing resistance of spermatozoa against cold shock was not suggested. Thus, the aim of this study was to determine the effect of the LDL addition to selected diluters on the rate of spermatozoa survival following cold shock and propose the optimal combination of the diluter and LDL concentration.

## MATERIALS AND METHODS

### Collection of Semen, Dilution and Processing

Three commercially available diluters – AndroMed®, Triladyl® (both from MiniTüb GmbH, Tiefenbach, Germany) and Bioxcell® (IMV, L' Aigle, France) were used. Control variants (CA, CB, and CT) of diluters were prepared according to the manufacturer's instructions. Experimental variants included 6% LDL cholesterol addition into the AndroMed® (A6) and Bioxcell® (B6) diluters, and 10% LDL addition into the Triladyl® (T10) diluter prepared without using egg yolk, which is normally its essential component. Increased concentration of LDL in Triladyl® replaced cryoprotective properties of egg yolk. The diluters used were prepared on the day of sampling and stored at the cooling box (4 °C).

Four bulls (bull A, B, C, and D) of different breeds (two bulls of Holstein breed, two bulls of Czech Fleckvieh breed), of ages from two to four years and the same frequency of collecting (once weekly) belonging to one sire insemination center were used. The bulls were proved and actively used for sperm production. Eight samples of ejaculate were obtained from selected bulls using an artificial vagina during the period of eight weeks. The samples of semen were then transferred to the university laboratory within one hour at 4 °C for next processing.

Samples of semen were pipetted using a sterile pipette to a sterile chilled (4 °C) tubes. Each sample of semen was immediately diluted to 50 000 spermatozoa/ml. The required amount of diluters was applied using sterile syringes directly to the tubes with samples. Thereafter tubes were sealed with sterile stoppers, mixed gently and placed into the cooling box (4 °C).

### Evaluation of Resistance Against Cold Shock

Three capillaries (0.1 ml) were gradually filled from each sample at 4 °C, closed at one end with plasticine and stored for ten minutes in a cooled bath (No Ice, Bibby Scientific, Ltd., Staffordshire, UK) at 0 °C. After the end of incubation, Eosin-Nigrosine staining was done: the capillaries content was gently mixed with 20 µl of Eosin by circular motion for 30 sec. Then 40 µl of Nigrosine was added, gently mixed and smear was done from this suspension. This procedure was repeated after 2 hours incubation of extended semen samples in a water bath at 37 °C. Smears (36 of each collecting day, 288 together) were examined after drying under a phase contrast microscope (Eclipse E200, Nikon®, Tokyo, Japan) at 1 000× magnification and with oil immersion by one evaluator. Minimum of 100 spermatozoa was classified as either dead (with red heads) or live (with white heads) and expressed as a percentage rate of live sperm.

### Statistical Analysis

The data set was analyzed using a generalized linear model in the statistical program SAS/STAT 9.1. (SAS Institute Inc., Cary, NC, USA). The following equation was used:

$$Y_{ijk} = \mu + BULL_i + SAMPLE_j + e_{ijk},$$

where

$Y_{ijk}$.....observed value of the dependent variable (percentage rate of live sperm at the beginning and the end of 2 hours incubation, difference between these measurements),

$\mu$.....average value of the dependent variable,

$BULL_i$.....fixed effect of the $i^{th}$ bull (i = bull A, n = 3; bull B, n = 3; bull C, n = 1; bull D, n = 1),

$SAMPLE_j$ ...fixed associated effect of the $j^{th}$ sample of diluter and LDL addition combination (j = A6, n = 48; B6, n = 48; T10, n = 48; CA, n = 48; CB, n = 48; CT, n = 48),

$e_{ijk}$.....residual effects.

The differences between the variables estimated were tested at the levels of significance $P < 0.05$ and $P < 0.01$.

## RESULTS

The basic characteristics of the model equation used are presented in Tab. I. The coefficient of determination ranged from $r^2 = 0.24$ to 0.84. The sire individuality had a significant effect ($P < 0.01$) on all investigated characteristics (ACT 0, ACT 2 and ACT 0–ACT 2). The effect of sample (diluter and LDL addition) was statistically higher significant ($P < 0.01$) only on sperm survivability after 2 hours of the test (ACT 2) and on the difference between sperm survivability in time 0 and 2 hours of the test (ACT 0–ACT 2).

The results of evaluation of the effect of the bull on the sperm survivability are presented in Tab. II. The highest survivability of sperm at the beginning of the test was found in bull A (81.62%), the lowest was detected in bull D (36.44%, $P < 0.01$). The highest sperm survivability after 2 hours of the test duration was found in bull C (61.25%, $P < 0.05$–0.01), whereas the lowest was detected in bull D

I: *Basic characteristics of the model equation used for data analysis*

| TRAIT | MODEL | | BULL | | SAMPLE | |
|---|---|---|---|---|---|---|
| | $r^2$ | P | F-test | P | F-test | P |
| **ACT 0** | 0.84 | < 0.01 | 598.33 | < 0.01 | 1.58 | 0.0813 |
| **ACT 2** | 0.56 | < 0.01 | 123.48 | < 0.01 | 4.18 | < 0.01 |
| **ACT 0–ACT 2** | 0.24 | < 0.01 | 21.97 | < 0.01 | 2.94 | 0.0003 |

ACT 0 = sperm survivability at the beginning of the test; ACT 2 = sperm survivability after 2 hours of the test duration; ACT 0–ACT 2 = difference between sperm survivability in time 0 and 2 hours of the test.

II: *The effect of bull on sperm survivability*

| | Bull A | Bull B | Bull C | Bull D |
|---|---|---|---|---|
| | LSM ± SE | LSM ± SE | LSM ± SE | LSM ± SE |
| **ACT 0** | $81.62 \pm 0.56^{CD}$ | $80.61 \pm 0.56^{CD}$ | $70.34 \pm 0.98^{ABD}$ | $36.44 \pm 0.98^{ABC}$ |
| **ACT 2** | $57.43 \pm 1.05^{D}$ | $56.47 \pm 1.05^{cD}$ | $61.25 \pm 1.82^{bD}$ | $20.31 \pm 1.82^{ABC}$ |
| **ACT 0–ACT 2** | $24.18 \pm 1.06^{CD}$ | $24.14 \pm 1.06^{CD}$ | $9.09 \pm 1.84^{ABD}$ | $26.12 \pm 1.84^{ABC}$ |

ACT 0 = sperm survivability at the beginning of the test in percent; ACT 2 = sperm survivability after 2 hours of the test duration in percent; ACT 0–ACT 2 = difference between sperm survivability in time 0 and 2 hours of the test in percent; upper script letters means significant difference among sires – a, b, c, d = P < 0.05; A, B, C, D = P < 0.01.

III: *The effect of the diluter and LDL addition on sperm survivability against cold shock*

| Sample | Label | ACT 0 | ACT 2 | ACT 0-ACT 2 |
|---|---|---|---|---|
| | | LSM ± SE | LSM ± SE | LSM ± SE |
| **A6** | A | $67.86 \pm 1.36$ | $47.12 \pm 2.52$ | $20.74 \pm 2.56^{D}$ |
| **B6** | B | $69.17 \pm 1.36^{e}$ | $52.94 \pm 2.52^{e}$ | $16.23 \pm 2.56^{e}$ |
| **T10** | C | $65.97 \pm 1.36$ | $44.53 \pm 2.52^{f}$ | $21.44 \pm 2.56^{f}$ |
| **CA** | D | $64.75 \pm 1.36$ | $47.85 \pm 2.52^{E}$ | $16.90 \pm 2.56^{AE}$ |
| **CB** | E | $65.61 \pm 1.36^{b}$ | $46.16 \pm 2.52^{bD}$ | $19.46 \pm 2.56^{bD}$ |
| **CT** | F | $67.41 \pm 1.36$ | $51.40 \pm 2.52^{e}$ | $16.01 \pm 2.56^{e}$ |

ACT 0 = sperm survivability at the beginning of the test in percent; ACT 2 = sperm survivability after 2 hours of the test duration in percent; ACT 0–ACT 2 = difference between sperm survivability in time 0 and 2 hours of the test in percent; A6 = AndroMed® with 6% LDL; B6 = Bioxcell® with 6% LDL; T6 = Triladyl® with 10% LDL; CA = control samples of AndroMed®; CB = control samples of Bioxcell®; CT = control samples of Triladyl®; upper script letters means significant difference among samples – a, b, c, d, e, f = P < 0.05; A, B, C, D, E, F = P < 0.01.

again (20.31%, P < 0.01). The smallest difference in sperm survivability (9.09%, P < 0.01) was reached by bull C, on the other hand the highest difference was determined in bull D (26.12%, P < 0.01). Results presented in Tab. II documented significant variability of sperm survival among selected sires at the beginning of observation, at the end of the test, as well as in sperm survival decline during the entire test performed.

The results of evaluation of the diluter and LDL addition effect on sperm survivability against cold shock are presented in Tab. III. Significantly the highest survivability of sperm at the beginning of the test was found in sample B6 (69.17%) compared to CB (65.61%; P < 0.05), and the lowest was detected in sample CA (64.75%; P > 0.05). Significantly the highest sperm survivability after 2 hours of the test duration was found again in sample B6 (52.94%) in comparison with CB (46.16%; P < 0.05) and significantly the lowest values were detected in sample T10 (44.53%) compared to CT (51.40%; P < 0.05). Some of other differences among evaluated diluters at the end of the test were significant as well (P < 0.05–0.01). The difference between the beginning and the end of the test duration was the largest in sample T10 (21.44%) and the lowest were detected in CT (16.01%; P < 0.05). The second lowest decline of sperm survival was detected in sample B6 (16.23%), significantly (P < 0.05) different from CB (19.46%). Some of measured differences of sperm survival decline were statistically significant to each other as well (P < 0.05–0.01).

## DISCUSSION

The experiment brought some interesting results. Using the extracted LDL – cholesterol from egg yolk has been investigated previously in various extenders (Vera-Munoz *et al.*, 2009), at different concentrations (Moussa *et al.*, 2002) and confirmed by thermal test or cryoconservation, but the cold shock test was used in this experiment for the first time. According to Anton *et al.* (2003) is known that egg yolk has a positive effect during sperm cryopreservation, because the components in egg yolk can create the absorption of oil and

water phase interface and make the oil droplets protective film. These abilities include membrane stabilization or cool shock protection. Egg yolk contains phospholipids; the largest amount is represented by LDL – cholesterol, which provides sperm cryoprotection during freezing or thawing by increasing the stability of the plasma membrane (Moussa *et al*., 2002). Watson (1995) stated that is important to test both, the effect of heat and cold on sperm survivability. Chantler *et al*. (2000) confirmed the loss of motility of sperm if the cryoprotective substances (e.g. egg yolk) were not added into ejaculate. Cold shock was also investigated in boar sperm survivability using egg yolk. Positive results attributed to the composition of egg yolk, natural source of LDL cholesterol (Hu *et al*., 2006).

Individuality of bulls has very important effect on sperm survivability. Effect of bull was significant ($P < 0.01$) on all monitored indicators. The significantly ($P < 0.01$) lowest sperm survivability was found in bull D in all observed characteristics. On the other hand the highest sperm survivability had bull A at the beginning of the cold test and bull C at the end of the test; bull C had simultaneously the lowest ($P < 0.01$) difference between the beginning and the end of the test. In general we can say that considerable individual differences in sperm survivability between bulls were determined. This is in accordance with the work of Beran *et al*. (2012 and 2013).

We can assume that the associated effect of diluter and LDL addition on sperm survivability against cold shock was highly significant ($P < 0.01$) only after 2 hours of the test. This is in accordance with Vera-Munoz *et al*. (2011) findings. They confirmed that samples with LDL showed better sperm motility and plasma membrane integrity even after 8 days of incubation. Similarly, Amirat *et al*. (2005) determined the lowest damage of sperm diluted with LDL after 4 hours incubation.

Based on our results, we can say that the samples of AndroMed® and Bioxcell® enriched by LDL achieved balanced or higher sperm survival (–0.73% to +6.78%) compared to control samples. Control Triladyl® contained 20% of egg yolk and provided higher level of sperm survival during the test (+1.44% and +6.87%) than 10% addition of LDL cholesterol as substitution of egg yolk. Concurrently, Bioxcell® with 6% LDL addition was the best variant for resistance of sperm following cold shock. This variant had the highest values of sperm survivability at the beginning (69.17%) and after 2 hours incubation (52.94%) compared to Triladyl® and AndroMed®. Bioxcell® with 6% LDL addition had the smallest decline during the entire test (16.23%) as well. However, significant differences were determined only to control variant of Bioxcell®. The combination of Bioxcell® and 6% of LDL was the most favorable for sperm due to the lower decline of motility compared to other experimental variants of diluters.

If we compare the control samples each other we can state that the best variant is the diluter Triladyl®. This variant has achieved the lowest decline (16.01%). This is in opposite with Vera-Munoz *et al*. (2009) who state that the LDL diluter had better results than Triladyl® and AndroMed®.

## CONCLUSION

Generally, we can recommend addition of 6% LDL into diluter Bioxcell® according to significantly higher sperm survival and its lowest decline in comparison with control variant of Bioxcell®.

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Contact information

Jan Beran: h.beran@seznam.cz
Ondřej Šimoník: simoniko@af.czu.cz
Radko Rajmon: rajmon@af.czu.cz
Luděk Stádník: stadnik@af.czu.cz
Martina Doležalová: dolezalovamartina@af.czu.cz
Adéla Krejcárková: krejcarkova@af.czu.cz
Jaromír Ducháček: duchacek@af.czu.cz
Jiří Šichtař: sichtar@af.czu.cz