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EFFECT OF BULL, DILUTER AND LDL-CHOLESTEROL CONCENTRATION ON SPERMATOZOA RESISTANCE AGAINST COLD SHOCK

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Abstract

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The objectives of this study were to determine and evaluate the effect of bull, diluter and addition of LDL in different concentration on the percentage rate of spermatozoa survival after cold shock. In total, four bulls were collected during a period of eight weeks. A total of 8 samples of fresh semen with required quality were processed. Three extenders were used for dilution of each sample; AndroMed®, Bioxcell® and Triladyl®, each in standard and LDL enriched variants. In the case of AndroMed® and Bioxcell®, 4, 6 and 8% of LDL were simply added. In Triladyl®, 6, 8 and 10% of LDL replaced the standard egg yolk component. Resistance of spermatozoa against cold shock (0 °C, 10 minutes) was evaluated by the percentage rate of live sperm using Eosin-Nigrosine staining immediately and 2 hours after heat incubation (37 °C). The results showed the influence of bull individuality as an important factor. Among diluters used it is possible to recommend AndroMed® and Bioxcell® due to significantly ($P < 0.01$) lower decline of live sperm proportion during the cold shock test than Triladyl® (–9.19, respectively –4.95%). The optimal LDL concentration increasing resistance of spermatozoa against cold shock was not determined, therefore subsequent research is necessary.

bull semen, sperm survival, cold shock, extender, LDL cholesterol

Fertility of dairy cows has declined over the past five decades as milk production per cow has increased (Zink *et al.*, 2012; Walsh *et al.*, 2011). The issue of cattle fertility was intensively studied in recent years from cow point of view and effects on their reproductive performance (Doležalová *et al.*, 2013; Roche *et al.*, 2011; Hanuš *et al.*, 2010; LeBlanc *et al.*, 2010). However, cow's reproduction results are also affecting by male component which is represented by the fertilization ability of bull ejaculate.

Sperm quality is influenced by many factors, e.g. by such internal factors as breed, variation between individuals, and age of sire (Beran *et al.*, 2011; Štolc *et al.*, 2009), and by such external factors as environmental conditions (Balic *et al.*, 2012), composition of the diet (Horký *et al.*, 2012) and frequency of collecting ejaculate (Kaya *et al.*, 2002). Collection of ejaculate and its subsequent processing present further potential risk factors for declined sperm quality. Phases of producing insemination doses (diluting of sperm, filling of straws, cooling and freezing) have a significant effect on sperm motility after thawing (Siddique *et al.*, 2006), and the properties of extender used are especially important (Beran *et al.*, 2012; Hegedüšová *et al.*, 2012), as well as method of thawing (Filipčík and Hanuláková, 2011).

Low Density Lipoprotein (LDL cholesterol – LDL) – a component of egg yolk – is believed to

be largely responsible for the egg yolk protective effects on spermatozoa during the freezing process (Pace and Graham, 1974) increasing their resistance against cold shock (Moussa *et al.*, 2002). There is an assumption that extenders containing LDL, which is extracted from egg yolk, can have better effects on spermatozoa membranes during the freezing than diluters commercially exploited (Moussa *et al.*, 2002).

Biological tests of ejaculate have been developed to assess the resistance and the fertilization ability of sperm using LDL addition into diluters, e.g. short-term heat test of sperm survival (Maurya and Tuli, 2003), hypo-osmotic swelling tests (Padrik *et al.*, 2012), however resistance of sperm against cold shock has not been evaluated as well in relation to addition of LDL.

Thus, the aim of this paper was to determine the effect of bull, diluter and addition of LDL in different concentration on the percentage rate of spermatozoa survival after cold shock.

MATERIAL AND METHODS

Semen collecting, dilution and processing

Ejaculate of four bulls (No. 1, 2, 3, and 4) belonging to one artificial insemination (AI) centre approved for public use, were sampled and evaluated. Samples of ejaculate were obtained using an artificial vagina during the period of eight weeks and tested immediately after collection. Volume of semen (VOL), density of sperm (DEN, and percentage of progressive motile spermatozoa above head (ACT) were evaluated by only one trained technician of AI centre. A total of 8 samples of fresh semen with required quality (minimum progressive motility 70% and sperm concentration 0.7 × 10^6 mm^{-3}) were then transferred at 4 °C to the university laboratory within one hour for next processing.

Three extenders were used for dilution of each sample; AndroMed®, Triladyl® (both from MiniTüb GmbH, Tiefenbach, Germany) and Bioxcell® (IMV, L' Aigle, France). Control variants of diluters (0% LDL addition) were prepared according to the manufacturer's instructions. Experimental variants included 4, 6 and 8% LDL addition into the AndroMed® and Bioxcell® diluters, and 6, 8 and 10% LDL addition into the Triladyl® diluter prepared without using egg yolk, which is normally its essential component. Higher concentrations of LDL in Triladyl® replaced cryoprotective properties of egg yolk. The extenders were prepared on the day of sampling and stored at the cooling box (4 °C) before ejaculate dilution.

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 successively filled from each sample at 4 °C, closed at one end with plasticine and stored at 0 °C into a cooled bath (No Ice, Bibby Scientific, Ltd., Staffordshire, UK) for ten minutes. After the end of cold incubation the capillaries content was gently mixed on preheated hour glass (37 °C) with 20 µl of Eosin by circular motion for 30 sec. and then Nigrosine was added at amount of 40 µl. A volume of 20 µl of the resulting suspension was added into a preheated glass slide and smear was done at the beginning of the test (time 0). This procedure was repeated after 2 hours heat incubation of extended semen samples in a water bath at 37 °C. After drying each smear (72 of each collecting day, 576 together) was examined under a phase contrast microscope (Eclipse E200, Nikon®, Tokyo, Japan) at 1 000x magnification and with oil immersion by only 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 at the beginning of the test (L0) and after 2 hours of heat incubation (L2).

Statistical analysis

The data were evaluated with statistical software SAS 9.3. (SAS/STAT® 9.3, 2011) using UNIVARIATE, CORR, and MIXED procedures. The following equation was used:

$$Y_{ijkl} = \mu + BULL_i + DIL_j + LDLC_k + b_1*(\text{VOL}) + b_2*(\text{DEN}) + b_3*(\text{ACT}) + e_{ijkl},$$

where:

Y_{ijkl} ..... observed value of the dependent variable (percentage rate of live sperm at the beginning of the test and after 2 hours heat incubation, difference between these two measurements),

$BULL_i$ ..... fixed effect of the i^{th} bull (i = 1, n = 216; 2, n = 216; 3, n = 72; 4, n = 72);

DIL_j ..... fixed effect of the j^{th} diluter (j = 1 – AndroMed®, n = 192; 2 – Bioxcell®, n = 192; 3 – Triladyl®, n = 192);

$LDLC_k$ ..... fixed effect of the k^{th} concentration of LDL (k = 0, n = 144; 4, n = 96; 6, n = 144; 8, n = 144; 10, n = 48);

$b_1*(\text{VOL})$ .... regression on volume of ejaculate;

$b_2*(\text{DEN})$ .... regression on density of sperm;

$b_3*(\text{ACT})$ .... regression on activity of sperm;

e_{ijkl} ..... residual effects.

The differences between the variables estimated were tested at the levels of significance $P < 0.05$ and $P < 0.01$. Pearson correlation coefficients were also determined.

RESULTS AND DISCUSSION

The basic statistical characteristics of observed data are shown in Tab. I. The volume of ejaculate

I: *Basic statistical characteristics of observed data*

Variable	Unit	Min	Max	Mean	SD
VOL	[g]	6.2	11.8	9.78	1.663
DEN	[10^6. mm^{-3}]	0.9	1.8	1.28	0.269
ACT	[%]	80	90	87.5	3.542
L0	[%]	23.27	93.2	74.12	16.245
L2	[%]	5.98	89.25	54.37	16.667
L0 – L2	[%]	0.61	54.62	19.68	11.528

Key: VOL = volume of ejaculate; DEN = density of sperm; ACT = activity of sperm; L0 = percentage rate of live sperm at the beginning of the test; L2 = percentage rate of live sperm after 2 hours of the test duration; L0 – L2 = difference between percentage rate of live sperm at time 0 and 2 hours of the test

II: *Pearson correlation coefficients r and related statistical significance P among evaluated traits*

		DEN [10^6. mm^{-3}]	ACT [%]	L0 [%]	L2 [g]	L0 – L2 [%]
VOL [g]	r	0.5886	0.7563	0.8524	0.6006	0.3445
	P	< .0001	< .0001	< .0001	< .0001	< .0001
DEN [10^6. mm^{-3}]	r		0.4616	0.5607	0.3876	0.2377
	P		< .0001	< .0001	<.0001	< .0001
ACT [%]	r			0.8063	0.6007	0.2914
	P			< .0001	< .0001	< .0001
L0 [g]	r				0.7565	0.3256
	P				< .0001	< .0001

Key: VOL = volume of ejaculate; DEN = density of sperm; ACT = activity of sperm; L0 = percentage rate of live sperm at the beginning of the test; L2 = percentage rate of live sperm after 2 hours of the test duration; L0 – L2 = difference between percentage rate of live sperm at time 0 and 2 hours of the test

ranged from 6.2 to 11.8 g in selected bulls. The volumes of ejaculates correspond to the findings of other authors, e.g. Louda *et al.* (2007) reported the range 3–12 g in sires kept in AI centre. Ball and Peters (2004) mentioned the closer range from 5 to 6 g.

The sperm density ranged from 0.9 to 1.8 × 10^6 mm^{-3}. Our results again agreed with those of Louda *et al.* (2007), who reported standard sperm density of bulls' ejaculates from 0.8 to 2.0 × 10^6 mm^{-3}. Ball and Peters (2004) observed a range from 0 in azoospermic bulls to 3 000 × 10^6 mm^{-3} in excellent sires, however especially mentioned substandard marginal bulls could not be used as widely applied sires.

The activity of sperm immediately after collection ranged from 80 to 90% due to determined threshold of 70% commonly used in Czech AI centers. These values correspond with those of Louda *et al.* (2007), who reported sperm activity 45–75% or more. Similarly, Ball and Peters (2004) reported that at least 60% of the spermatozoa should be shown straight progressive movement above head. This requirement was fulfilled within our observation.

Cold shock test belongs to the main evaluation method of sperm quality. We take a reflection about viability and fertility of ejaculate. The rate of live sperm ranged from 23.27 to 93.2% in the beginning and from 5.98 to 89.25% after 2 hours of the heat incubation.

Indicators mentioned above belong to the main characteristics of collected fresh semen (Hanuláková *et al.*, 2012) and determine the initial quality of ejaculate subsequently used for AI doses manufacturing (Vágenknechtová *et al.*, 2011). The last findings confirm that the initial quality of ejaculate determines final quality of AI dose (Beran *et al.*, 2012).

Tab. II contains Pearson correlation coefficients among the evaluated traits. Significant ($P < 0.01$) correlation coefficients ($r = 0.2377$ to 0.8524) were detected between all characteristics evaluated.

Further, the effects of bull, diluter and LDL addition have been evaluated in detail by the statistical model designed. Results of this evaluation are presented in Tab. III and IV. Coefficient of the whole model repeatability ranged from $r^2 = 0.408$ to $r^2 = 0.852$ during the evaluation of observed traits. Effect of bull was significant ($P < 0.01$) in relation to the whole evaluated traits. Effect of diluter was significant ($P < 0.01$) in relation to the percentage rate of live sperm after 2 hours of the heat incubation and difference between percentage rate of live sperm at the beginning and 2 hours of the heat incubation. Effect of LDL concentration in tested samples was insignificant to all evaluated traits ($P > 0.05$). The statistical model also included the effects of the initial quality parameters of ejaculate regression (volume, density and activity). Effect of volume of ejaculate was significant ($P < 0.01$) only in relation

III: *Effects of individual factors in statistical model*

TRAIT	MODEL		BULL		DIL		LDLC		VOL		DEN		ACT	
	r^2	P	F-test	P	F-test	P	F-test	P	F-test	P	F-test	P	F-test	P
L0	0.852	< 0.001	34.03	< 0.001	2.06	0.13	1.20	0.311	9.13	0.003	9.02	0.003	13.38	< 0.001
L2	0.697	< 0.001	62.70	< 0.001	26.61	< 0.001	0.13	0.969	1.20	0.274	9.44	0.002	3.11	0.08
L0 – L2	0.408	< 0.001	25.31	< 0.001	19.38	< 0.001	1.23	0.298	0.68	0.410	27.8	< 0.001	18.85	<0.001

Key: L0 = percentage rate of live sperm at the beginning of the test; L2 = percentage rate of live sperm after 2 hours of the test duration; L0 – L2 = difference between percentage rate of live sperm at time 0 and 2 hours of the test; BULL = effect of each bull; DIL = effect of each diluter; LDLC = effect of different concentration of LDL in tested sample; VOL = volume of ejaculate; DEN = density of sperm; ACT = activity of sperm

IV: *Effect of bull, diluter and concentration of LDL on sperm survivability after cold shock*

EFFECT	LEVEL	L0	L2	L0 – L2
		LSM ± SE	LSM ± SE	LSM ± SE
BULL	1	75.40 ± 1.193^{A}	64.05 ± 1.807^{A}	11.56 ± 1.747^{A}
	2	78.82 ± 1.083^{A}	56.00 ± 1.624^{B}	22.86 ± 1.571^{a,B}
	3	75.70 ± 1.625^{A}	61.84 ± 2.408$^{A, B}$	13.67 ± 2.328$^{b, A, B}$
	4	54.66 ± 3.447^{B}	14.60 ± 5.167^{C}	39.54 ± 4.996$^{b, B,C}$
DIL	AndroMed®	71.65 ± 1.053	54.25 ± 1.556^{A}	17.43 ± 1.504^{A}
	Bioxcell®	71.88 ± 1.053	50.02 ± 1.554^{B}	21.67 ± 1.502^{B}
	Triladyl®	69.91 ± 1.032	43.10 ± 1.531^{C}	26.62 ± 1.481^{C}
LDLC	0	69.87 ± 1.075	49.61 ± 1.597	20.12 ± 1.545
	4	71.27 ± 1.238	49.46 ± 1.840	21.51 ± 1.779
	6	71.26 ± 1.075	48.58 ± 1.587	22.76 ± 1.535
	8	72.18 ± 1.075	48.84 ± 1.597	23.20 ± 1.545
	10	71.16 ± 1.632	49.14 ± 2.404	21.95 ± 2.325

Key: BULL = effect of each bull; DIL = effect of each diluter; LDLC = effect of different concentration of LDL in tested sample; L0 = percentage rate of live sperm at the beginning of the test; L2 = percentage rate of live sperm after 2 hours of the test duration; L0 – L2 = difference between percentage rate of live sperm at time 0 and 2 hours of the test. Different superscript letters mean a significant difference within a column – a, b = P < 0.05; A, B, C = P < 0.01

to the percentage rate of live sperm at the beginning of the test. This is in accordance with physiological basis, due to amount of sperm and their supply with important substances from seminal plasma. Effect of sperm density was significant (P < 0.01) in relation to the whole evaluated traits. This result has also biological background, because density of sperm in ejaculate defines the average conditions for the cells functioning. The effect of sperm activity was significant (P < 0.01) to the percentage rate of live sperm at the beginning and difference between percentage rate of live sperm at time 0 and after 2 hours of the heat incubation. This is also logical as sperm activity represents an indicator of initial quality – viability of spermatozoa.

Significant differences (P < 0.05 – 0.01) were detected between bulls regardless of the used diluter or LDL concentration. At the beginning of the test was the best bull No. 2 (78.82 ± 1.083%), while after 2 hours had the best survivability bull No. 1 (64.05 ± 1.807%), including the smallest difference between the first and second measurement (11.56 ± 1.747%). Significantly (P < 0.01) the lowest values of sperm survivability during whole test had bull number 4 (54.66 ± 3.447, respectively 14.60 ± 5.167%). This bull also had significantly (P < 0.05–0.01) the highest sperm survivability decline between time 0 and 2 hours of the heat incubation (39.54 ± 4.996%). Our results confirmed that effect of bull is important, individual differences were found between them (Thara and Nair, 2007).

Focusing on the influence of diluter regardless of the concentration of LDL we can assume that AndroMed® (71.65 ± 1.053%) and Bioxcell® (71.88 ± 1.053%) had the best results at the beginning of the test and Triladyl® (69.91 ± 1.032%) reached the worst results. Triladyl® extender achieved significantly (P < 0.01) the worst results after 2 hours of the heat incubation (–6.92% less than Bioxcell® and –11.12% less than AndroMed®). This extender also had significantly (P < 0.01) the highest declined percentage rate of live sperm during the entire the test (26.62 ± 1.481%). Our results showed that better results of the cold shock test were achieved using the AndroMed® and Bioxcell®. This is in accordance with Jannet *et al.* (2005) or Stradaioli *et al.* (2007) who state that AndroMed®, respectively Bioxcell® are the most suitable extenders for cryopreservation of bull semen.

In Tab. IV are also shown results of LDL concentration influence on sperm survivability without specification of extender type. Differences between each concentration of LDL cholesterol were statistically insignificant ($P > 0.05$). The lowest value of percentage rate of live sperm (69.87 ± 1.075%) at the beginning of the test had control samples (0% LDL cholesterol). While these samples had the best results at the end of the heat incubation (49.61 ± 1.597%). These samples also had the smallest difference between time 0 and 2 hours of the test. There is need to say that all differences of sperm survivability detected in relation to concentration of LDL were insignificant ($P > 0.05$). The values of percentage rate of live sperm after 2 hours of the heat incubation were almost the same in all LDL concentrations. From these results we cannot clearly determine the most suitable concentration of LDL addition and subsequent research in this area is necessary.

CONCLUSION

Based on our monitoring we can assume that effect of bull is important and individual differences between selected sires in their sperm resistance against cold shock were detected. AndroMed® and Bioxcell® have been found as the more suitable extenders for cryopreservation of bull semen compared to Triladyl® due to lower decline of live sperm proportion during the cold shock test. Effect of LDL concentration added to extenders was insignificant ($P > 0.05$) and showed unclear results. We cannot recommend the optimal LDL concentration increasing resistance of spermatozoa against cold shock. Therefore, subsequent research of this topis is necessary.

SUMMARY

The aim of this study was to determine and evaluate the effect of bull, diluter and addition of LDL in different concentration on the percentage rate of spermatozoa survival after cold shock.

In total, four bulls were collected during a period of eight weeks. A total of 8 samples of fresh semen with required quality were processed. Three extenders were used for dilution of each sample: AndroMed®, Triladyl® (both from MiniTüb GmbH, Tiefenbach, Germany) and Bioxcell® (IMV, L' Aigle, France). In the case of AndroMed® and Bioxcell®, 4, 6 and 8% of LDL were simply added. In Triladyl®, 6, 8 and 10% of LDL replaced the standard egg yolk component. Resistance of spermatozoa against cold shock (0 °C, 10 minutes) was evaluated by the percentage rate of live sperm using Eosin-Nigrosine staining in time 0 and 2 hours of heat incubation (37 °C) after exposure to cold. In total, 576 smears were evaluated. Statistical software SAS 9.3., procedures UNIVARIATE, CORR, and MIXED were used for analyzing the data.

Effect of bull was significant ($P < 0.01$) in relation to the whole evaluated traits. Effect of diluter was significant ($P < 0.01$) in relation to the percentage rate of live sperm after 2 hours of the heat incubation and difference between percentage rate of live sperm at the beginning and 2 hours of the heat incubation. We can recommend AndroMed® and Bioxcell® due to significantly ($P < 0.01$) lower decline of live sperm proportion during the cold shock test than Triladyl® (–9.19, respectively –4,95%). Effect of LDL concentration in tested samples was insignificant to all evaluated traits ($P > 0.05$). We cannot recommend the optimal LDL concentration increasing resistance of spermatozoa against cold shock and subsequent research is necessary.

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