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Study on Cooling Zarabi Buck Semen with Different Concentrations of Low Density Lipoprotiens

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Abstract

Cooled semen is a cheap and easy alternative technique that can be used in breeding program instead of freezing technique which induces detrimental effects on sperms. The most important need is a suitable extender and preservation technique in which spermatozoa can be storage without affecting their fertilizing ability. Egg yolk (EY) is one of the most common non-penetrating cryoprotectant in extender, despite it is containing substances that may interfere with the cell metabolism, thus reducing sperm motility. Low densitylipoproteins (LDL) contained in egg yolk is largely responsible for sperm protection during cryopreservation. In this study we used different concentrations of LDL (4, 6, 8 and10%) and 2.5% egg yolk (control) on Tris citric acid based extender for preservation of four Zarabi buck semen for 120 h. at 5 °C. Semen samples of threee bucks with Progressive forward motility (>80%) and abnormalities (<10%) were collected and pooled twice weekly using electro-ejaculator. This experiment revealed that the best concentrations of LDL for preservation of Zarabi buck semen at 5 °C for 120 hr. are 6% and 8% LDL as they maintain sperm motility, viability, integrity of sperm plasma membrane and acrosomal integrity and decreased abnormalities of the sperm, but 8% LDL gave highest results of buck semen preservation compared to EY 2.5% (control), 4, 6 and 10% LDL.

Keywords: Buck semen; Cooling; Cryoprotectant; Low Density Lipoprotien

Introduction

The productive contribution of Egyptian goat is only 0.3 and 3.37% of local milk and meat (Khalifa *et al.*, 2009) [1]. Therefore, it is very important to improving goats genetically in Egypt (Khalifa *et al.*, 2009) [1]. Artificial insemination (AI) is one of the solutions in goat breeding, to control reproduction with accurate progeny testing, to increase the production of milk, hair and meat and to allow kidding at one season of the year. The success of an AI program needs proper handling of semen from collection and storage to use (Pauleuz *et al.,* 2005) [2]. The technique of frozen buck semen has advantages but it is slow in development due to the biological properties of buck's seminal plasma (Yunsheng, 2008) [3]. It induces adverse effects in terms of sperm ultra-structural, biochemical, and functional damage (El Bawab *et al.*, 2014) [4], so a reduction of motility, membrane integrity, acrosome integrity and fertilizing ability (Purdy, 2006) [5]. Cooled semen is a cheap and easy alternative technique that can be used in breeding program especially, when the animals live in a limited area and fertility is higher when using semen stored at room temperature or cooled at 5 °C than when frozen semen is used (Paulenz *et al.*, 2005) [2], but the cooling process also disturbs normal chemical and physical cellular processes (Martorana *et al.*, 2014) [6]. The most important need is a suitable extender and preservation technique in which spermatozoa can be storage without affecting their fertilizing ability.

Egg yolk (EY) is one of the most common non-penetrating cryoprotectant in extender. It is included in extenders to stabilize the sperm membrane (Purdy, 2006) [5] during cryopreservation and also during thawing (Aboagla *et al.*, 2004) [7], despite it is containing substances that may interfere with the cell metabolism, thus reducing sperm motility (Moussa *et al.,* 2002) [8]. A specific problem in the preservation of buck semen in extenders containing EY is the presence of enzyme called an egg yolk coagulase (EYCE), in the seminal plasma, it coagulates EY in the extender and hydrolyses lecithin (present in EY) to fatty acids and lysolecithins (spermicidal) resulting in loss of sperm membranes (Shipley *et al.,* 2007) [9]. Low density lipoproteins (LDL) contained in EY is largely responsible for sperm protection during cryopreservation (Al Ahmad *et al.,* 2008) [10]. Several studies have reported successful results with the addition and replacement of EY by LDL as a non-penetrating cryoprotectant in bulls (Hu *et al.*, 2010) [11], boars (Jiang *et al.*, 2007) [12] and dogs (Varela *et al.*, 2009) [13], ram (Silva *et al.*, 2014) [14] and stallion (Moreno *et al.*, 2013) [15].

The present study aimed to evaluate if substitution of chicken egg yolk (CEY) with LDL could improve and /or affect the quality of buck semen preserved at 5 °C for 120 hr. Also, to study if changing concentrations of LDL could affect the quality of buck semen preserved at 5 °C for 120 hr.

Materials and Methods

This study was carried out at the farm of Theriogenology department, Faculty of Veterinary Medicine, Alexandria University, December 2018. Two times weekly for a month, semen samples with volume (>0.5 ml), progressive motility (>80%), concentration (>2000x10^6 spermatozoa/ml) and total abnormalities (<10%) were collected using electro-ejaculator from four healthy adult Zarabi bucks. Semen samples were pooled before cooling to have suitable volume and 52- to diminish the buck effect and divided into five aliquots in pre-labeled plastic tube.

The buffer composed of 4.543 g Tris (hydroxymethyl aminomethane, Loba Chemie Pvt. Ltd., India), 2.382 g Citric acid (Loba Chemie Pvt. Ltd., India), 0.75 g Glucose (D-Glucose, Chemjet, Egypt) and 500 µl of Gentamycin 5% and LDL (4, 6, 8 and10%) to 100 ml bi-distilled water was used according to Salamon and Ritar (1982) [16]. The control group differed from other groups by replacing LDL with 2.5% EY.

The extraction of LDL from EY was done as described by Moussa *et al.* (2002) [8]. Extraction of LDL from hen egg yolk, fresh hen eggs were manually broken and yolk was separated from the albumin. EY was used to prepare the extender; one fraction of each EY was used for the control group extender and the other fraction for LDL extraction.

EY was diluted (1:2) with an isotonic saline solution (0.17 M NaCl) and stirred for one hour before centrifugation at 11,000 g for 45 min at 4 °C. The supernatant (plasma) was centrifuged again (at 11,000 g for 45 min at 4 °C) for complete removal of granules.

The plasma was mixed with 22.7% ammonium sulphate (Sigma-Aldrich, USA) to precipitate livetins. The mixture was centrifuged at 11,000 xg for 45 min at 4 °C, and under constant agitation the plasma's pH was corrected and fixed (8.7) with 0.1 M sodium hydroxide after one hour of stirring at 4 °C, the sediment was discarded and the supernatant was dialyzed using dialysis page (Sigma-Aldrich, USA) against distilled water for ≈20 h in order to eliminate ammonium sulphate. The solution was again centrifuged (11.000 g for 45 min at 4 °C) and the floating residue rich in LDL was collected. The final dialysis of plasma supernatant treated with ammonium sulphate caused the plasma to clump together, and when centrifuged, they floated due to their low density. The floating residue presented the principal bands of LDL.

Five diluents with lipoproteins concentrations were prepared and mixed with (Table 1) the base extender which was prepared first. A gentle swirling motion until the mixture appeared homogenous then mixture was poured through a funnel fitted with a folded milk filter (230 mm, Hygia Favorit, Universal Dairies GmbH, Germany) to remove any clumps. The extenders were stored at 5 °C until use.

Composition	EY (D1)	4%LDL(D2)	6%LDL (D3)	8%LDL (D4)	10%LDL (D5)
	2.5%	11gm	16.5gm	22gm	27.5gm

Table 1: The concentrations of EY and LDL in the five extenders

Each aliquot of semen (concentration >2000x10^6 spermatozoa/ml) was diluted with one of the five extenders at 37o at rate of 1:10. Extenders were added slowly to prevent osmotic shock. The diluted semen tubes were placed in a beaker containing 100 ml warmed water (37 oC) then transferred to the refrigerator (5 oC) to achieve steady cooling rate, and preserved for 120 h (Zhao *et al*., 2009) [17].

Every 24 hours the semen tubes were getting out from the refrigerator and shaken to re-suspension of semen then part of semen tube was taken for evaluation of: progressive motility %, viability %, abnormalities %, acrosomal integrity %, plasma membrane integrity %.

Sperm motility was determined by using phase contrast microscope (Olympus Optical Co., Tokyo, Japan) at 400x magnification. A drop of semen which was diluted by 2.9% Sodium Citrate dehydrate solution was placed on pre-warmed glass slide at 37 °C and covered by a cover slip. Randomly 200 spermatozoa were selected from four microscopic fields and examined. The mean of four successive estimations was considered the final motility (Memon *et al*., 2011) [18].

Sperm viability of the samples was achieved by means of Nigrosin-Eosin staining (Evans and Maxwell, 1987) [19]. A drop of the semen sample was placed on a warm clean slide then a 1 drop of Eosin 97-stain (5%) and 1 drop of Nigrosin stain (10%) was added, then they were mixed together and smears were made with different slides immediately. The viability was achieved by recording 200 cells under bright field microscope (Optech GmbH, Munchen, Germany) at 400x magnification in four microscopic fields. Partial or complete purple coloring sperm was considered dead and sperm showing exclusion of the stain was considered alive (Uysal and Bucak, 2007) [20].

The same slide prepared for viability was also used for counting abnormal spermatozoa. About 200 spermatozoa in four microscopic fields were counted in each slide (Mekasha, 2007) [21].

Acrosomal morphology it was assessed using Giemsa stain according to method of Watson (1975) [22]. A drop of diluted semen was smeared on a pre-warmed slide. The smears were fixed by immersion in 10% buffered formal saline for 15 minutes, and then washed. The smears were air dried and then immersed in Giemsa solution for 90 minutes then they were rinsed in distilled water and dried. The slides were examined under a light microscope using oil immersion lens and magnification of 1000x. The normal acrosome percentage was recorded for about 200 spermatozoa from four microscopic fields. The normal acrosome was considered when the stain was distributed over the equatorial segment of the spermatozoa.

Plasma membrane integrity was assessed by using Hypo-Osmotic Swelling test (HOST).The solution (125mOsm/ l) was prepared as Fonseca *et al*. (2005) [23]. 10µl semen was mixed gently with 1 ml of hypo-osmotic solution then incubated for 60 minutes in a water bath at 37 °C. After incubation, a drop of the mixture was placed on a glass slide, covered with a cover slip and examined using a phase contrast microscope at 400x magnification. A total of 200 spermatozoa were observed in four different fields. The percentage of spermatozoa positive to HOST (having swelled or curled tail) was determined as per Revell and Mrode (1994) [24].

Data was analyzed by ANOVA with repeated measures and Post Hoc test was assessed using Bonferroni adjusted in SPSS ver. 20.0

Results and Discussion

Parameter	Group	Hour						
		Hr.0	Hr.3	Hr.24	Hr.48	Hr.72	Hr.96	Hr.120
Motility %	D1		79.90^{Ba} ± 9.9	76.10^{Ca} ± 1.07	59.50^{Da} ±1.89	43.0^{Ea} ± 1.86	19.0^{Fa} ± 1.45	-
	D2	83.50^{Aa} ±1.50	53.0^{Bb} ± 0.82	36.0^{Cb} ± 1.25	16.50^{Db} ±1.30	-	-	-
	D3		81.30^{Ba} ±1.42	78.50^{Cac} ± 1.50	64.0^{Dc} ± 1.25	47.0^{Ea} ±2.0	28.0^{Fb} ± 1.53	-
	D4		82.50^{Aa} ±1.50	80.30^{Bc} ± 1.79	68.40^{Cd} ±1.23	52.50^{Db} ±1.12	32.50^{Ec} ±1.12	17.5^{F} ±1.12
	D5		62.50^{Bc} ±1.12	47.0^{Cd} ± 1.11	27.0^{De} ± 1.11	-	-	-
Viability %	D1		80.35^{Ba}±1.85	76.68^{Ca} ± 1.45	67.99^{Da} ±1.81	48.61^{Ea} ±2.01	24.13^{Fa}±1.29	-
	D2	84.20^{aA} ±1.62	57.25^{Bb}±1.01	40.53^{Cb} ± 1.37	20.41^{Db} ± 1.24	-	-	-
	D3		81.62^{Ba}±1.69	79.71^{Cac} ±1.58	69.71^{Dac} ±1.09	60.0^{Eb} ± 1.35	31.16^{Fb}±1.30	-
	D4		82.72^{Ba}±1.72	81.08^{Cc} ± 1.59	73.39^{Dc} ± 0.97	-	37.16^{Fc}±1.17	27.18^{G}±1.2
	D5		65.63^{Bc}±0.78	52.30^{Cd} ± 1.60	35.75^{Dd} ± 1.23	-	-	-
Acrosomal integrity %	D1		81.96^{Ba}±1.48	80.85^{Ba} ± 1.41	75.73^{Ca} ± 1.56	55.34^{Da} ±1.67	37.79^{Ea} ±1.04	-
	D2		57.20^{Bb}±0.97	35.45^{Cb} ± 1.82	16.97^{Db} ± 1.13	51.14^{Ea} ±1.58	29.64^{Fb} ±1.41	-
	D3		81.99^{Ba}±1.43	80.61^{Ca} ± 1.40	69.04^{Dc} ± 1.03	60.55^{Eb}±1.09	44.50^{Fc} ±1.27	-
	D4		83.40^{Ba}±1.49	81.53^{Ca} ± 1.46	73.91^{Da} ± 0.74	-	-	-
	D5		53.10^{Bc}±1.17	41.10^{Cc} ± 1.16	23.15^{Dd} ± 0.78	-	-	-
Plasma membrane integrity %	D1		79.80^{Ba}±1.44	74.25^{Ca} ± 1.36	63.78^{Da} ± 1.63	44.05^{Ea}±2.02	21.0^{Fa} ± 1.40	-
	D2	83.38^{Aa} ±1.42	59.76^{Bb}±0.72	37.07^{Cb} ± 1.23	17.57^{Db} ± 1.33	-	-	-
	D3		81.44^{Aa}±1.10	77.08^{Bc} ± 1.58	64.78^{Ca} ± 1.16	46.87^{Da}±1.60	26.43^{Eb} ±1.22	-
	D4		81.37^{Aa}±1.43	78.75^{Bc}± 1.28	70.64^{Cc} ± 1.36	54.25^{Db}±1.19	33.85^{Ec}±1.10	24.70^{F} ±1.27
	D5		63.37^{Bc} ± 1.0	49.93^{Cd} ± 1.54	31.93^{Dd} ± 1.15	-	-	-
Sperm abnormalities	D1		15.49^{Ba}±1.18	17.12^{Ba} ± 1.05	19.67^{Ca} ± 0.87	33.85^{Da} ±0.98	43.28^{Ea}±0.85	-
	D2	9.66^{Aa} ±1.0	31.97^{Bb}±1.17	36.41^{Cb} ± 1.20	40.78^{Db} ± 1.21	-	-	-
	D3		14.61^{Ba}±0.66	19.22^{Ca} ± 0.71	22.09^{Da} ± 0.58	31.41^{Ea} ±0.94	41.58^{Fa}±0.95	-
	D4		11.77^{Ac} ± 0.63	15.74^{Bc} ± 0.73	19.73^{Ca} ± 0.56	27.21^{Db}±0.84	36.35^{Eb}±0.81	48.32^{F}±0.7
	D5		17.72^{B}d±0.71	24.70^{Cd} ± 1.16	38.95^{Db} ± 0.91	-	-	-

Mean ± SEM in same row with different capital letters are significantly different ($p<0.05$)
Mean ± SEM in same column with different small letters are significantly different ($p<0.05$)
Dots (-) indicate sperm became non-motile
Table 2: Effect of different LDL concentrations on parameters of cooled buck semen

This research revealed that the best concentrations of LDL for preservation of Zarabi buck semen at 5 °C for 120 hr. are 6% and 8% LDL, they maintain the percentages of sperm motility, viability, plasma membrane integrity, acrosomal integrity and tertiary abnormalities of the sperm decreased, but 8% LDL gave highest results of buck semen preservation compared to EY 2.5% (control), 4, 6 and 10% LDL. LDL improve the spermatozoa motility as Moussa *et al*. (2002) [8] and Bencharif *et al.* (2008) [25] observed that HDL granules decreased significantly the spermatozoa motility compared to yolk. LDL gives the best protection of acrosomal

integrity, may be due to the substitution or repair of acrosomal phospholipids or due to the medium is less in progesterone than EY extender due to the effect of the dialysis membrane (Bencharif *et al*., 2010) [26]. The progesterone found in EY plays a role in the capacitation of spermatozoa in cattle (Yunsheng, 2008) [3] and other species. Moustacas *et al.* (2011) [27] found that LDL is adhered to the plasma membrane and repairing those lost by natural outflow so maintaining the membrane stability and protecting them against cold shock. LDLs build complex with specific binding proteins and causing them unavailable to act in the cell membrane (Acrosome reaction) (Bergeron *et al*., 2004) [28].

These results were in agreement with Al Ahmad *et al.* (2008) [10] who found that the best concentration of LDL for freezing buck semen is 8% which improved post thaw sperm progressive velocity and gave the lowest abnormal spermatozoa percentages. Tonieto *et al.* (2010) [29] said that extenders containing 100 mM trehalose and 8% LDL can maintain sperm motility and membrane integrity of frozen ram semen after thawing. Silva *et al.* (2014) [14] concluded that LDLs 8% LDL would be more beneficial for ram semen cryopreservation and improved the sperm viability percentages.

The optimum concentrations of LDL in freezing and cooling was different among different researches and different species as Yunsheng (2008) [3] found that 9% LDL was the concentration of choice in freezing and cooling of buck semen compared to the control 20% EY and different concentrations of LDL. Bencharif *et al.* (2008) [25] demonstrated that the percentage of motile spermatozoa in canine semen following freezing thawing in the 6% LDL dilution medium is significantly higher than other dilution media (5%, 7% and 20% EY medium). Valera *et al.* (2008) found no differences in sperm motility in canine semen among 6, 8 and 10% LDL in any period of storage at 5 °C but were significantly higher than control 20% EY.

Moreno *et al.* (2013) [15] reported that LDL at a concentration of 2% and 3% significantly increase sperm motility after freezing-thawing of equine semen in comparison with the 2% EY extender. Khlifaoui (2004) [30] has already shown the beneficial effect of LDL for the cryopreservation of equine sperm but at a concentration of 0.5%. El-Sharawy (2010) [31] showed clearly that the optimum concentration of LDL has been determined to be 12%, they found that the proportion of buffalo-bull spermatozoa with an intact plasma membrane and acrosomal integrity were superior than in the medium with EY. Jiang *et al.* (2007) [12] reported that that 8 or 9% gave the highest post thaw motility, plasma membrane integrity and acrosomal integrity in boar semen. Hu *et al.* (2008) [32] in boar semen found that 9% increased significantly the proportion of spermatozoa with an intact plasma membrane compared to the medium with EY.

In this study, 4% LDL extender was of low protection of buck sperms during cooling, may be because the phospholipids were insufficient to protect buck sperm during cooling processes causing cold shock and death of sperms (El-Sharawy, 2010) [31]. These results were in agreement with Al Ahmad *et al.* (2008) [10], who found that the motility percentages decreased at concentration of 4% compared to 8% of buck spermatozoa, El-Sharawy (2010) [31] found that the viability percentage of buffalo-bull spermatozoa decreased in 4%, 6% and 15% LDL extenders. Moreno *et al.* (2013) [15] reported that the LDL 4% and 5%, effect on plasma membrane of equine sperms and, and the percentage of anomalies increased. Silva *et al*. (2014) [14] reported during cooling of ram sperm diluted in 15% EY extender had higher percentage of progressive motility than 2% or 4% LDL extenders.

Also, in this study it was observed that the extender containing 10% LDL gave less protection of buck sperm during cooling may be because high level of phospholipids lead to production of high amount of lysolecithin which was liberated causing death of the sperm, due to an EYCE which coagulates EY and hydrolyses lecithin to fatty acids and spermicidal lysolecithin (Shipley et al., 2007) [9], also this enzyme can act on the ester bond of yolk phospholipids to release saturated fatty acid (Citric acid) and unsaturated fatty acids (oleic and linoleic), leading to a rapid decline in pH of about 6.0 , Under such circumstances the rate of sperm respiration decreases and inhibits motility (El Bawab et al., 2014) [4], another possibility they reported that the increase of LDL above 10% in an extender led to decrease in sperms performance due to increase granules of LDL lead to aggregation (Jiang et al., 2007) [12]. However, Yunsheng (2008) [3] found that EY, 8%, 9% and 10% LDL was more effective in protecting buck sperm and 15% LDL was less effective, also El-Sharawy (2010) [31] predicted that 12% LDL was the best concentration in preserving buffalo-bull sperms.

The difference in the optimum concentration of LDL between researches may be related to differences in the composition of the sperm plasma membranes among species and their susceptibility to cold shock (El Bawab et al., 2014) [4]. There are other reasons that may be due to different composition of EY and the extraction of LDL depends on human factor so different moisture in LDL.

Conclusion

Finally, based on the results of this research it was conclude that 8% LDL extender was optimum to maintain liquid Zarabi buck semen than 4, 6, 10% LDL and 2.5% EY during cooling at 5 °C for 120hr. this achievement in sperm survival would lead to decreasing dose of sperms which required per insemination, increasing the efficiency of AI.

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