EFFECT OF SUBSTITUTION EGG YOLK WITH DIFFERENT LEVELS OF SOYBEAN LECITHIN IN TRIS-BASED EXTENDERONFREEZING AND FERTILIZING CAPACITY OF HOLSTEIN BULL SEMEN

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SUMMARY

Soy bean lecithin has been attracted increasing attention and has been used to substitution egg yolk in the semen extender. Aim of the currentresearch articlewas to study the impact of substitutionof egg yolk with soybean-lecithin in tris- extender on freezability and fertilizing capacity of Holstein sperm in post-thawed semen. Semen from five Holstein bulls was frozen in tris-citric acid extender containing 15% egg yolk (EY, control) or 0.25, 0.5 and 0.75% soybean-lecithin (SBL). Percentages of motility, viability, plasma membrane integrity and intact acrosome spermatozoa were assessed in diluted, equilibrated and thawed semen. Results showed that0.5% SBL-extender had positive (P<0.05) effect on percentages of motility, livability, plasma membrane integrity and intact acrosome of sperm cellsafter dilution, equilibration and thawing.In post-thawed semen, the highest (P<0.05) spermrecovery rate of motility,livability, plasma membrane integrity and intact acrosome was obtained for 0.5% SBL-extender compared to other extenders.The highest conception rate (68.6%) of Holstein cows was obtained following insemination with semen extended with 0.5% SBL compared with other extenders (48&56%).

In conclusion, egg yolk could be substituted by soybean-lecithin in tris-based extender at a level of 0.5% to improve fertilizing capacity of cryopreserved Holstein semen.

Keywords: Holstein, frozen semen, sperm characteristics, fertility

INTRODUCTION

Choice of semen extender is an important aspect of semen processing for AI.The use of frozen semen may decrease the risk of breed and population extinction and reduce the cost of transportation (Stanishevskaya *et al*., 2021). Cryopreservation causes marked reduction in semen quality leading to low conception rate. Chilling temperatures are less detrimental to the integrity of the bull spermatozoa than freezing-thawing procedure, resulting in more damage to the sperm cells (Tarig *et al*., 2017).

The ease of which egg yolk (EY, commonly used in semen extenders) is affected by microbial contamination necessitates the development of alternatives to EY in semen extenders. Therefore, there are many attempts to develop chemically defined extenders, free of compounds that are of the animal sources (Crespilho *et al*., 2012). Apart from being linked to microbial contamination, EY-extenders are known to interfere with the microscopic examination. Thus, EY replacement with other compounds (plant source) without compromising the desired qualities of perfect extender is very much warranted (Ansari *et al*., 2012).

Furthermore, high density lipoprotein HDL in egg yolk is one factor that decreases the quality of semen by causing efflux of cholesterol from the sperm plasma membrane and resulted in a change in fluidity that increases the sensitivity to cold shock (Amirat *et al.*, 2005). However, improvement in the semen quality occurs after supplementing low density lipoproteins (LDLs) extracted from hen egg yolk (Akhter*et al.*, 2011a). The main effective component of egg yolk is LDLs fraction like lecithin, which protects the membrane phospholipids integrity during cryopreservation (Moussa *et al*., 2002; Amirat *et al*., 2004).

Scientists are working on uncovering new sperm protective agents that replaces EY. To avoid the addition of EY (Animal source), SBL as a plant source, is currently being evaluated (Chelucci *et al*., 2015).SBL at a range from 1.25 to 10% was used instead of EY to preserve the sheep sperm, and the effect of 1.25% concentration was considered much better (Zhao et *al*., 2018). It not only prevents the occurrence of health risks, but also improves the efficiency of semen preservation (Akhter *et al*., 2012). In this respect, SBL has the same EY components utilized for preventing animal semen from cold shock on frozen-thawed sperm (Aires *et al.*, 2003).

During semen cryopreservation, SBL has the capacity to protect the integrity of the phospholipid membrane (Nadri *et al*., 2019). It also acts as an antioxidant against free radical damage. It improved sperm viability and motility in post-thawed semen in bulls (Singh *et al.,* 2017). According to Fukui *et al.* (2008),SBL might have a greater safety function for the sheep sperm than that of the EYor other ingredients through cryopreservation procedure and thus, decrease the threat of introducing mycoplasma and bacteria in the frozen extenders.Zhang *et al.* (2009) reported that the addition of 6% SBL to the extender showed the best motility and plasma membrane integrity of sperm cells in post-thawed semen of boar. El-Sisy *et al*. (2016) reported SBL can effectively alternate EY as cryoprotective

additive for cryopreservation extender, without detrimental effects on post-chilling and post-thawing semen quality in cattle bull. Addition of 1.5% SBL improved acrosome integrity percentage of chilled bull semen compared with 20% EY(Tarig *et al*., 2017).

Therefore, the present study was designed to evaluate the effect of egg yolk substitution with alternative cryoprotectants such as plant-derived lecithin from soybean on sperm characteristics (motility, livability, plasma membrane integrity and intact acrosome spermatozoa), and fertility of cryopreservedHolstein semen.

MATERIALS AND METHODS

This study was conducted at the International Livestock Management Training Center (ILMTC), Sakha, belonging to the Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture.

Animals and feeding system:

Five sexually mature healthy Holstein bulls with 534-578 kg body weight and4-5years old were used for semen collection in this study. All bulls were healthy and clinically free of external and internal parasites. Bulls were housed individually under semi-open sheds and were fed on daily ration composed of 8 kg concentrate feed mixture (CFM), clover hay (4kg) and rice straw (4kg).The CFM was composed of 32% undecorticated cotton seed cake, 26% wheat bran, 22% yellow maize, 12% rice bran, 5% linseed meal, 2% vines, 0.5% limestone and 0.5% salt. Fresh water and minerals blocks were available for all bulls at all day times, while the ration was given individually to all bulls at 8.00 a.m. and 3.00 p.m.

Semen collection:

Semen was collected twice weekly from each bull and immediately held in a water bath at 37^{o}C before transferred to the lab. Ejaculates having good mass motility (≥70%) were pooled for each collection day for 10 weeks. On each collection day, semen was pooled and diluted with tris-extenders containing EY or different levels of SBL.

Preparation of experimental extender:

Tris-solution (TS)containing 3.025 g tris–(hydroxymethyl-aminomethane), 1.675 g citric acid, 0.75 g glucose, 7% glycerol, 0.25 g lincospectin, and 0.005 g streptomycin added to 100 ml distilled water was used. In the 1st extender (E1), 15% fresh EY (15 ml/100 ml TS)was added. Meanwhile, SBL (Sigma, St. Louis, MO, USA) was added at levels of 0.25, 0.5 and 0.75% to E2, E3, and E4, as a replacement of egg yolk, respectively. Semen was diluted with all extenders at a rate of 1:20.

Filling and freezing the straws:

Semen was extended with all types of extenders, gently mixed withsemen,wormed up to 37^{o}C in a water bath, and then cooled gradually in are frigerator at 5^{o}C for 4 hours (equilibration period). Post-equilibrated semenwas loaded in French straws (0.25 ml, 20 x10^{6} motile sperm) using a semenfilling machine.

During filling, extended semen in the straws was kept in ice water bath (5^{o}C) to keep its temperature. Straws were transported into processing canister and located horizontally in static nitrogen vapor (4 cm above the surface of liquid nitrogen for 10 minutes), and placed vertically in a metal canister and immersed completely in liquid nitrogen container, then stored at -196^{o}C for one month at least.

Semen evaluation:

After freezing, semen was thawed at a rate of 37^{o}C for 30 seconds by dipping the frozen straws into a water bath. Post- diluted, equilibrated, and thawed semen was evaluated using a hot microscope stage (37^{o}C) for determination of percentages of motility, livability, plasma membrane integrity, andintact acrosome of sperm cells.

Research microscope with warmed stage (37°C) was used to determine the percentageof sperm progressive motility under the high power magnification (400x) according to Amman and Hammerstedt (1980). The percentage of sperm livability was determined using eosin and nigros in mixture stain (Hackett and Macpherson, 1965).

The plasma membrane integrity of spermatozoa was assessed using the hyposmotic swelling test (HOS-t) according to Jeyendran *et al.* (1984). Percentage of acrosome integrity was conducted as indicated by Watson (1975). Spermrecovery rate (RR) of motility, livability, membrane integrity, and acrosome integrityin post-thawed semen was calculated as follows:

RR=Post-thawed sperm parameter(%)/ post-diluted sperm parameter(%)× 100

Fertility trail:

Total of 100Holstein cows in heat were divided into 4 groups (25 in each). Holstein cows in each group were inseminated with frozen/thawed semen extended with different types of extenders (0.25, 0.5and 0.75% soybean-lecithin compared with 15% egg yolk). Immediately post-thawing, gun of insemination was used to inseminate cows artificially. Pregnancy diagnosis was performed 50 days post insemination using rectal palpation.

Statistical analysis:

To study the effect of extender (E1-E4), data at each stage (dilution, equilibration, and thawing) were statistically analyzed by one-way ANOVA using SAS (2004). Duncan's New Multiple Range test was used to separate the differences among means. The percentage values were transformed to arcsine values before analysis of variance, then mean values were presented after being recalculated from the transformed ones.

RESULTS

Sperm parametersafterdilution:

Results presented in Table (1) showed that percentages of progressive motility, livability, and

acrosome integrity of spermatozoain post-diluted semen weresignificantly (P<0.05) improvedin E3, significantly (P<0.05) reduced in E2, and maintained in E4 as compared to control (E1).However, membrane integrity percentage was maintained in E3 closer to E1 and significantly (P<0.05) reduced in E2 and E4 in comparison with E1.

Table 1. Effect of soybean-lecithin extender on sperm parameters in Holstein bull semen after dilution

Sperm parameter (%)	E1 (15%EY)	Level of soybean-lecithin E2 (0.25%)	E3 (0.5%)	E4 (0.75%)
Progressive motility	73.25^{b}±0.55	66.50^{c}±0.90	76.50^{a}±0.64	72.75±0.77
Livability	74.80^{b}±0.79	69.95^{c}±0.91	77.25^{a}±0.58	73.60^{b}±0.75
Membrane integrity	74.55^{a}±0.55	68.75^{c}±0.86	76.40^{a}±0.48	72.15^{b}±0.97
Acrosomeintegrity	75.90^{b}±0.66	67.95^{c}±0.92	79.90^{a}±0.69	78.00^{b}±1.24

a,b and c: Significant mean differencesin the same row at P<0.05.E1-E4= Extenders 1 to 4.

Sperm parametersafterequilibration:

Data in Table 2 showed that progressive motility, livability, membrane integrity and acrosome integrity after equilibration were significantly (P<0.05) improved in post-equilibrated semen extended with E3, while decreased significantly (P<0.05) by E2 compared with E1 (control). At the same time,progressive motility and acrosome integrity significantly (P<0.05) decreased, while livability and membrane integrity were maintained in E4 as compared to E1.

Table 2. Effect of soybean-lecithin extender on sperm parameters in Holstein bull semen afterequilibration

Sperm parameter (%)	E1 (15% EY)	Level of soybean-lecithin E2 (0.25%)	E3 (0.50%)	E4 (0.75%)
Progressive motility	64.25^{b}±0.98	54.00^{d}±1.24	71.75^{a}±0.83	60.75^{c}±1.32
Livability	65.05^{b}±1.36	58.40^{c}±1.46	71.35^{a}±0.73	65.20^{b}±1.29
Membrane integrity	65.60^{b}±1.02	57.20^{c}±1.29	70.30^{a}±0.92	63.40^{b}±1.12
Acrosome integrity	67.65^{b}±1.28	56.35^{d}±1.16	75.45^{a}±1.18	61.70^{c}±1.21

a, b ….d: Significant mean differences in the same row at P<0.05.E1-E4= Extenders 1-4.

Sperm parametersof post-thawed semen:

Results presented in Table (3) revealed that percentages of progressive motility, livability, acrosome integrity, and membrane integrity of spermatozoa in post-thawed semen were significantly (P<0.05) increased in E3, significantly (P<0.05) reduced in E2, and did not differ in E4 as compared to E1.

Table 3. Effect of soybean-lecithin extender on sperm parameters in Holstein bull semen after thawing

Sperm parameter (%)	E1 (15% EY)	Level of soybean-lecithin E2 (0.25%)	E3 (0.50%)	E4 (0.75%)
Progressive motility	46.75^{b}±0.83	41.25^{c}±1.08	63.75^{a}±1.08	48.75^{b}±1.14
Livability	50.95^{b}±0.79	43.85^{c}±0.90	61.70^{a}±0.99	49.65^{b}±0.87
Membrane integrity	49.15±0.74^{b}	44.60^{c}±1.26	60.05^{a}±1.12	49.70^{b}±1.17
Acrosome integrity	53.40±1.03^{b}	45.55^{c}±1.31	65.50^{a}±1.76	50.90^{b}±1.45

a, b and c: Significant mean differences in the same row at P<0.05. E1-E4= Extenders 1-4.

Recovery rate*:*

Recovery rate of all sperm parameters (motility, livability, membrane integrity, and acrosome integrity) in post-thawed semen were significantly (P<0.05) improved by E3, maintained by E4, while decreased by E2 Table (3) revealed that percentages of progressive motility, livability, acrosome integrity, and membrane integrity of spermatozoa in post-thawed semen were significantly (P<0.05) increased in E3, significantly (P<0.05) reduced in E2, and did not differ in E4 as compared to E1 (Table 4).

Table 4. Effect of soybean-lecithin extender on recovery rate (%) of sperm parameters in post-thawed Holstein bull semen

Sperm recovery (%)	E1 (15% EY)	Level of soybean-lecithin E2 (0.25%)	E3 (0.50%)	E4 (0.75%)
Progressive motility	63.91^{b}±1.28	62.12^{c}±1.61	83.39^{a}±1.43	67.22^{b}±1.86
Livability	68.24^{b}±1.20	62.78^{c}±1.28	79.85^{a}±1.07	69.57^{b}±1.58
Membrane integrity	65.95^{b}±0.95	64.95^{b}±1.84	78.64^{a}±1.49	69.10^{b}±1.84
Acrosome integrity	70.47^{b}±1.49	67.05^{b}±1.76	82.13^{a}±2.39	65.72^{b}±2.37

a, b and c: Significant mean differences in the same row at P<0.05.

Fertility trail:

Data in Table 5 showed that conception rate was significantly (P<0.05) higher for Holstein cows inseminated with semen extended by E3than that with other SBL-extenders (48-52%) and control (56.0%).

Table 5: Effect of levels of soybean-lecithin extender on conception rate of Holstein cow

Item	E1	Level of soybean-lecithin		
	(15% EY)	E2 (0.25%)	E3 (0.50%)	E4 (0.75%)
Inseminated animals	25	25	25	25
Non-conceived animals	11	13	8	12
Conceived animals	14	12	17	13
Conception rate (%)	56.0[b]	48.0[b]	68.6[a]	52.0[b]

a and b: Significant mean differences in the same row at P<0.05.

DISCUSSION

For genetic resource preservation, genetic diversity maintenance, and increasing AI success, cryopreservation of semen is a useful tool (Thélie *et al*., 2019), but this tool is responsible for decreasing the sperm quality by physical or chemical damage during cryopreservation (Wang *et al*., 2020). Egg-Yolk is commonly used as a non-permeable cryoprotectantintris-based extenders. It protects spermatozoa against the temperature shock to maintain motility, acrosome integrity, and mitochondrial integrity (Moustacas*et al*., 2011). Egg-Yolk has cryoprotectant antagonists, inconsistent composition, high density lipoproteins (HDLs) and granules of EY interfere with sperm motility (Ansari *et al*., 2010). So, the present work was planned to evaluate tris-extenders containing different levels of SBL(0.25, 0.5 and 0.75%) in comparison with 20% EY on freezability and fertilizing capacity of frozen Holstein spermatozoa. According to the obtained results in our study, progressive motility, livability, acrosome integrity and membrane integrity of spermatozoa were improved after dilution, equilibration, and thawing the semen extended with tris-citric acid supplemented with SBL at a level of 0.5% as compared to other levels of SBL- and EY-extenders. Accordance with the present results, several authors reported an increase in sperm motility percentage when SBL (Biociphos Plus®) was used as compared to tris-EY based extender in frozen-thawed bovine semen (Amirat *et al*., 2005; Stradaioli *et al.,* 2007). Also, a marked increase in motility of cryopreserved spermatozoa in semen extended with SBL-extender (AndroMed®) as compared to EY-extender (Aires *et al*., 2003). Several reports confirmed that SBL can prevent sperm cells from cryodamage during cryopreservation by mitigating the efflux of cholesterol and phospholipids. Using 2% nano-SBL in the extender improved the sperm survival of cryopreserved goat semen, being better than any SBL suspension (Nadri *et al*., 2019). The SBL can prevent apoptotic cascades in goat and ram spermatozoa during cryopreservation (Masoudi *et al.*, 2016). In our study, sperm viability percentage was found to be higher with 0.5% SBL in diluted, equilibrated and thawed semen. Similarly, higher viable sperm cells in SBL- based extender (BiociphosPlus®, Amirat *et al*., 2005), and live sperm percentage was recorded as compared to egg yolk based extender in SBL-based extenders (AndroMed®, Biociphos Plus® and Bioxcell®; Nehring and Rothe, 2003) as compared to EY-based extender were reported on cryopreserved bovine spermatozoa.

Results in the present study on the percentage of acrosome and membrane integrity supported the obtained results of sperm progressive motility and livability. The percentage of acrosome integrity increased with increasing percentages of sperm motility and livability in semen extended with 0.5% soybean-lecithin.In accordance with our results, the addition of 1.5% SBLwith 2% virgin coconut oil (VCO) in Tris-based extender improved the quality of chilled semen such as motility, abnormality, viability, membrane integrity, and acrosome integrity as compared to 20% EY (Tarig *et al*., 2017). In buffaloes, motility and viability of sperm cells in semen stored at 5°C in SBL-based extender were improved as compared to semen extended with milk, tris-citric-EY, and EY-citrate extenders (Akhter *et al.,* 2011b). In this concern, improved membrane integrity of bovine spermatozoaof semendiluted with extenders containing SBL (Andro Med, Aires *et al*., 2003; Biociphos Plus, Amirat *et al*., 2005; Bioxcell, Stradaioli *et al*., 2007) was observed in comparison with EY-based extenders. Other authors found that lecithin-based extenders have higher motility rates compared with egg yolk-based extenders (LekshmiBhai *et al*., 2015).

The positive impact of SBL in our study may be due to its little viscosity and a lesser amount of debris (Zhang *et al.,* 2009). Also, the phospholipids plasma membrane of sperm cells may be replaced by the phospholipids of SBL, resulting in maintaining the structure and function of spermatozoa (Trimeche *et al.,* 1997). Furthermore, the phospholipids of SBL and sperm membrane may form a protective membrane against the harmful elements (Zhang *et al.,* 2009). On the other hand, Salmani *et al*. (2014) indicated that SBL was more efficient in protecting goat sperm against destructive lipid peroxidationduring cryopreservation compared to EY which can be attributed to composition of EY that is containing moreun saturated fatty acids susceptible to lipid peroxidation. Theyalso noticed a significant

reduction in malodialdhyde level in the goat semenextender containing different levels of SBL than extender containing EY.

The number of viable sperms per cryopreserved insemination dose affects fertility rate (Andrabi*et al*., 2006). Plasma membrane integrity of spermatozoa controls the metabolic exchanges with the surrounding medium (Silva and Gadella, 2006) and a biochemically active plasmalemma is required for the process of capacitation, acrosome reaction and the oocyte penetration (Jeyendran *et al*., 1984). Also, the acrosome integrity of sperm cells is important for the acrosomal reaction required to facilitate fertilization (Thomas *et al*., 1997) and its assessment can be an effective tool to predict the fertilizing ability. These findings were supported in the present study, since semen extended with 0.5% SBL-extender exhibiting the highest percentage of sperm parameters and the highest conception rate (68.6%) in comparison with other extenders. In buffaloes, SBL-extender was incapable of improving the fertility of frozen semen (Andrabi, 2009). The HDLs of EY interact with seminal plasma proteins and stimulate sperm capacitation, while the LDLs interact with seminal plasma proteins (Manjunath *et al*., 2002) to decrease the efflux of cholesterol and phospholipids from the plasma membrane of sperm cells and prevent premature capacitation and subsequent acrosome reaction (Bergeron *et al.,* 2004).

CONCLUSION

Based on the obtained results, sperm characteristics were improved in diluted, equilibrated and thawed semen extended with soy-lecithin at a level of 0.5%. In conclusion, soy-lecithin at a level of 0.5% as the lipid/lipoprotein plant source can be an alternative to egg yolk as an animal product in tris- extender preparation for cryopreserved semen to increase fertilizing capacity of cryopreserved Holstein bull semen.

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تأثير إستبدال صفار البيض بتركيزات مختلفة من ليسيثين فول الصويا فى مخفف الترس علي القدرة التجميدية والإخصابية للسائل المنوي لطلائق الهولستين

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تم إستخدام ليسيثين فول الصويا في مخفف الترس للسائل المنوي لطلائق الهولستين بديلا عن صفار البيض.الهدف من هذه الدراسة تقييم تأثير احلال تركيزات مختلفة من ليسيثين فول الصويا محل صفار البيض في المخفف علي القدرة التجمدية و الأخصابية للسائل المنوي.تم تجميد السائل المنوي من خمسة طلائق هولستين في مخفف الترس المحتوي علي ٠,٢٥ ، ٠,٥٠ ، ٠,٧٥ % ليسيثين الصويا (معاملة) و١٥% صفار بيض(المجموعة الضابطة). تم تقدير النسبة المئوية للحيوية والحيوا نات المنوية الحية وذات الأكروسوم السليم وسلامة الغشاء البلازمي بعد التخفيف وبعد فترة الموازنة وبعد الإسالة.وقد أشارت النتائج الي وجود زيادة معنوية في النسبة المئوية للحيوية والحيوانات المنوية الحية ذات الأكروسوم السليم وسلامة الغشاء البلازميبعد التخفيف وبعد فترة الموازنة وبعد الأسالة في المخفف المحتوي علي ٠.٥% ليسيثين الصويا وزيادة معدل الخصوبة (٦٨.٦%) في المخفف المحتوي علي(٠.٥%) ليسيثين الصويا مقارنة بالمخفف المحتوي علي صفار البيض (٥٦%). لذلك يمكن إستبدال صفار البيض بلسيثين فول الصويا في مخفف الترس عند مستوي (٠.٥%) لزيادة القدرة التجميدية والإخصابية للسائل المنوي لطلائق الهولستين.