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Use of whey Paneer Cheese as a New Dilute for Bovine Semen Cryopreservation

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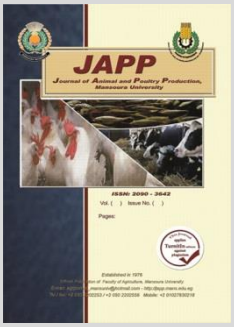

ABSTRACT

This study aimed to determine the utilizing whey resulting from making paneer cheese as a diluent for bovine semen. Semen was collected from 5 Friesian bulls, pooled, divided into three portions, and diluted (1:10) with three extenders; Tris-soybean lecithin (TSL), whey (W0), or whey+5% egg yolk (WEY), then equilibrated (5ºC for 4 h), and frozen (-196ºC). Semen was evaluated for progressive motility (PM), live sperm (LS), acrosome integrity (AI), abnormality (AB) and curled tail (CT) after dilution,equilibration and thawing using Computer Assisted Semen Analyzers (CASA) system. Results revealed that percentages of PM, LS, AI and CT of spermatozoa were higher (P<0.05), and AB percentage was lower (P<0.05) with WEY-extender than in other extenders either post- dilution or post-equilibration. In post-thawing semen evaluated by CASA, WEY-extender improved (P<0.05) percentages of progressive motility, total motility, rapid progress motility, functional sperm, and sperm immotility as compared to other extenders. WEY and W0 extenders increased (P<0.05) Linearity (LIN) compared with TSL extender. Straight line velocity (VSL) and straightness (STR) were higher (P<0.05) with WEY than TSL-extender, while did not differ between WEY and TSL-extender. Percentages of normal morphology form, abnormality in head, neck, and dual deformation, as well as teratozoospermic and sperm deformity indices were lower (P<0.05) in WEY-extender than in other extenders. Conclusion: Sperm freezability in Friesian bull semen can be improved during cryopreservation by using whey+5% egg yolk extender, as a promising extender, in comparing with whey without egg yolk and tris-citrate extenders.

Keywords: Beef, semen, whey, diluent, cryopreservation, sperm activity.

INTRODUCTION

In dairy breeding practices, artificial insemination (AI) is considered as an important tool for genetic improvement. Application of AI requires a good quality extender. Semen diluents have an important role during semen preservation for obtaining high quality of livability, motility, and integrity of acrosome and membrane of spermatozoa.

Extenders of semen composed of buffer system for maintaining pH value such as Tris, sodium phosphate, and citric acid; cryo-shock preservatives like glycerol, egg-yolk, soybean lecithin, milk); energy source such as fructose and antibiotics like streptomycin and penicillin (Rehman *et al*., 2013). Therefore, high quality extender should provide energy for sperm metabolism, and maintenance of the osmotic pressure and pH value of sperm medium (Salamon and Maxwell, 2000). Also, semen extenders supply sufficient nutrients to spermatozoa during preservation in the form of fructose. In addition, extender prevents spermatozoa from freezing shocks during cryopreservation in liquid nitrogen at -196 ºC (Foote, 2002).

Proteins in whey are naturally formed during the production of cheese and accountof 20% of all protein in milk (Pal *et al*., 2010), such as β-lactoglobulins, α-lactalbumin, immunoglobulin, lactoferrin, lactoperoxidase, glycomacro-peptide, bovine serum albumin and other proteins (Hulmi*et al*., 2010). Further proteins, such as a-lactalbumin, b-lactoglobulin, albumin, and lactoferrin, are soluble in milk and are collectively called whey proteins (Amio*et al*., 2002).Some researches available using whey as a diluent for semen storage (Ganguli *et al*., 1973). They observed that citric acid whey is a suitable diluent for preserving buffalo semen both at room and at refrigerated temperature, as revealed by motility studies and conception rate. A 35 to 40% recovery rate was obtained with buffalo spermatozoa frozen in citric acid whey with glycerol citric acid whey, egg yolk citrate and TRIS buffer have been used as the diluents by the Indian animal scientists (Elimwadi and Fluekiger, 1974, Ganguli, 1978; Vasanth, 1978) for the preservation of buffalo semen at -196°C.

Computer assisted semen analyzers (CASA) system give reliable results with high accuracy for most semen characteristics such as sperm motility parameters and several parameters of sperm velocity (Verstegen *et al*., 2002) by measuring the individual sperm cells. Also, CASA estimates individual motion parameters of sperm cells and it has more predictive indicator for fertilizing ability of semen (Mortimer, 2000).

The aimed of the current study was to determine the utilizing whey resulting from making paneer cheese as a diluent for cryopreserved bovine semen.

MATERIALS AND METHODS

This study was performed at Animal Production Research Station, Sakha, belonging to Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. The experimental period lasted during the interval August-December 2020.

Semen donors:

Five Frisian bulls, with 30-36 months old and live body weight averaging 435.9±2.29 kg, were used as semen donors in this study. Bulls were subjected to carful general clinical and

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andrological examination to determines that they are healthy and free from any disease in addition all bulls vaccinated against all infection disease. These animals were housed in a semi-open shaded yard and fed based on the recommendation of Animal Production Research Institute (APRI). Bulls were fed berseem hay (*Trifolium alexandrinum*), concentrate feed mixture and rice straw to cover their nutritional requirements based on their LBW.

Experimental semen extenders:

Soybean lecithin extender:

Tris-soybean lecithin extender (TSL) which was used as the control extender consisted of tris (0.3025 g), citric acid (0.1675 g), glucose (0.075 g), lecithin (0.5%), antibiotics (1000 IU penicillin and 1 mg streptomycin per ml).These ingredients were well mixed and dissolved in distilled water up to 100 ml. Thereafter, 7 ml glycerol (7 % v/v) was added to 93 ml of TSL-extender, and filtered through 0.45µm-Millipore filter (Milieux GV, millepore, Cooperation Bedford MOA).

Whey extender:

Citric whey was obtained during preparation of paneer cheese according to Chandan (2007). Cow skim milk was heated separately up to 90 °C for 10 min in a stainless-steel vat. The heated milk was allowed to cool to 70 °C and hot solutions of coagulants (1% citric acid) was added for milk with continuous stirring until pH value of 5.2 and a complete coagulation with clear whey separation. The coagulant curd was allowed to settle down for 5 min and the whey was allowed to drain using a muslin cloth used as dilution semen.

Citric acid whey as a by-product resulting from paneer cheese was used as dilution semen. The suspension was allowed to stand for 5-10 minutes, thenpH value of this diluent was adjusted to 6.8 with freshly prepared 10% sodium hydroxide solution. Physico-chemical characteristics of whey paneer cheese and whey-extender are shown in Table 1.

Table 1. Physico-chemical characteristics of whey paneer and whey paneer as an extender.

Constituent	Whey Paneer as by-product	Whey paneer as an extender
Total solids (%)	6.3	6.3
Fat (%)	0.29	0.29
Protein (%)	0.68	0.68
Ash (%)	0.7	0.7
Cholesterol (g)	0.001	0.001
Calcium (mg)	0.42	0.42
Sodium (mg)	0.29	0.29
pH value	4.5	6.8
Total bacterial count/g	1.1×10^2	ND
Yeast and mould count (cfu⁄g)	ND	ND
E. coli	ND	ND

ND: Not determined

Citric-whey as an extender was used without egg yolk or with 5% egg yolk.

Both citric-whey extenders were supplemented with7 % (v/v) glycerol, 1000 IU penicillin and one mg streptomycin per ml, and filtered through0.45µm-Millipore filter.

Semen collection:

Semen was collected once a week from each bull using an artificial vagina for 5 weeks.The ejaculateswere transferred to the laboratory and in a water bath at 37°C.Semen ejaculates were pooled to avoid individual variation.Ejaculates with individual motility more than 75% were used in this study.

Semen preservation:

On each day of semen collection, ejaculates were pooled and divided into three parts, diluted at a rate of 1:10withTSL, whey or whey+5% egg yolk extenders.After dilution, semen was preserved at 5ºCfor 4 h (as equilibration period) in a refrigerated unit before being placed into straws (0.25 ml French straws). The straws were held for 10 min at the surface of the liquid nitrogen vapor (-120°C) before being immersed then stored in liquid nitrogen (-196°C) for one month.

Semen evaluation:

Visual examination:

Semen extended with each type of extenders was visually evaluated after dilution and equilibrium for determining the percentage of sperm progressive motility, viability, abnormality, acrosomal integrity and membrane integrity (Hypo-osmotic swelling-test).

Sperm progressive motility:

Sperm progressive motility was evaluated after the methods of Fadl-Aya *et al*. (2019). A small semen drop was placed onto a pre-warmed glass slide and covered with a coverslip. The slides were investigated under high power microscope for calculation of motile sperm count in 200 spermatozoa, then sperm progressive motility percentage was calculated.

Sperm viability and abnormalities

By using eosin–nigrosin stains, sperm livability and abnormality were assessed (Evans and Maxwell, 1987). About 10 µL of semen was mixed with 10 µL of stains to prepare a smear on a glass slide, then air dried. In field of about 200 sperm cells, live and normal sperm cells were counted using microscope (×1000). Sperm cells without stain were considered live, while those stained with eosin–nigrosin were considered as dead. On the same slide, sperm cells with morphological defects in head, neck, mid-piece and tail were considered as abnormal spermatozoa. The percentages of sperm livability and abnormality were calculated.

Sperm acrosomal integrity

For sperm fixation, 50 µl of 1% formal citrate was added to 500 µl of semen according to Hancock (1959). The fixation solution was prepared by adding 1 ml commercial formaldehyde (38%) to 99 mL of 2.9% (w/v) sodium citrate. In microscopic field of 100 sperm cells, using oil immersion (1000x) of a phase-contrast microscope (Olympus, BX20), sperm cells with normal apical ridge were characterized as sperm with intact acrosome.

Hypo-osmotic swelling test (HOST)

Membrane integrity was determined in term of percentage of spermatozoa with curled tail in response to hypo-osmotic solution of 150 mOsm/l. According to Jeyendran *et al.* (1984), this solution composed of sodium citrate (7.35 g) and fructose (13.5 g) added to in 1000 ml of deionized water, then stored at 4^0 C untill using. About one ml of the hypoosmotic solution with a drop of semen was incubated (at 37^0 C for 1 hour). Post-incubation, a drop of semen was examined on a clean dry glass slide with cover slip and sperm with curled tails were counted in a field of 100 sperm cells by using microscope (200 x), then percentage of sperm cells positively responded by coiled or curled tails were calculated.

CASA analysis:

Computer assisted semen analysis (CASA, SPERMOLAB®, Cairo, Egypt) was used to evaluate semen with different extenders after thawing. The analyzed data included sperm motility parameters such as percentage of total, progressive, rapid and slaw progressive, and non-progressive motility, and immotility of spermatozoa, as well

as a percentage of sperm morphology parameters such as normal form,abnormalities (head, neck, and tail), deformation (mono, dual and tri) of spermatozoa in addition toteratozoospermic and sperm deformity indices.

Sperm kinetic parameters including curve linear velocity, straight linear velocity, average path velocity, linearity, straightness, and wobble were also determined.

Statistical analysis:

Data of all semen parameters were statistically analyzed by one-way ANOVA) using SAS (2004) after arcsine transformation.Duncan Multiple Range Test (Duncan, 1955) was used to separate the significant differencesbetween means.

RESULTS AND DISCUSSION

Results

Sperm characteristics after dilution and equilibration:

Sperm characteristics in post-diluted and equilibrated semen with different types of extender are presented in Table 2. Statically analysis revealed that the progressively motile sperm, live sperm, acrosome integrity and sperm cells with curled tails percentages were significantly (P<0.05), higher, while the percentage of sperm abnormality was significantly (P<0.05) lower with WEY-extender than in other extenders either after dilution or post-equilibrated.

Characteristics of spermatozoa in post-thawed semen:

Sperm motility:

Frozen Frisian bull semen diluted with WEY-extender significantly (P<0.001) improved most sperm characteristics, in terms of increasing percentages of total, progressive, and rapid progress motility, and functional sperm percentage, and decreasing immotile sperm percentagein comparing with other extenders. However, no significant (p<0.05) differences were detected among extender groups in non-progressive motility percentage (Table 3).

Table 2. Sperm characteristics in bull semen diluted with different types of extenders.

Item	Sperm characteristics (%)				
	Progressive motility	Livability	Abnormality	Acrosome integrity	Curled tail
			Post-Dilution:		
TSL	56.67±1.67^{c}	64.33±2.40^{c}	20.0±0.58^{a}	61.0±2.31^{b}	55.67±2.4^{c}
W0	69.67±0.33^{b}	74.0±1.56^{b}	15.67±0.88^{ab}	73.67±2.4^{a}	61.67±0.88^{b}
WEY	78.33±1.67^{a}	86.33±1.86^{a}	14.0±1.53^{b}	78.33±0.88^{a}	72.0±1.0^{a}
P-value	0.00	0.001	0.018	0.002	0.001
			Post- equilibrium:		
TSL	53.33±1.67^{c}	59.67±0.88^{c}	23.0±0.58^{a}	58.67±3.38^{b}	48.33±0.88^{b}
W0	63.33±1.67^{b}	69.67±0.67^{b}	19.33±0.88^{b}	64.67±3.76^{ab}	54.67±1.45^{ab}
WEY	73.33±1.67^{a}	78.67±0.88^{a}	18.33±0.88^{b}	75.33±2.19^{a}	59.33±1.45^{a}
P-value	0.000	0.000	0.014	0.027	0.003

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

Table 3. Effect of different extenders on sperm motility parameters in Frisian bull post-thawed semen. (mean ±SE)

Item	TSL	W0	WEY
Progress motility (%)	54.83±1.79^{c}	60.83±0.59^{b}	74.64±1.69^{a}
Non progress motility (%)	18.72±0.85	16.94±0.98	15.73±1.42
Total motility (%)	73.55±0.94^{c}	77.77±0.38^{b}	90.37±0.27^{a}
Immotile sperms (%)	26.46±0.94^{a}	22.23±0.38^{b}	9.64±0.27^{c}
Rapid progress motility (%)	33.05±0.03^{b}	33.46±2.19^{b}	59.42±2.81^{a}
Functional sperm(%)	33.05±0.03^{b}	33.50±2.19^{b}	59.40±2.83^{a}

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

Sperm morphology:

Frozen Frisian bull semen extended with WEY significantly (P<0.05) increased the normal form percentage and significantly (P<0.05) deceased percentages of abnormality in head and neck, dual deformation, teratozoospermic index (TZI), and sperm deformity index (SDI)as compared to other extenders. However, no significant (P<0.05) difference were detected among extenders in percentages of abnormal tail,), mono deformation, and tri deformation (Table 4).

Table 4. Effect of different extenders on sperm morphology parameters in post-thawed semen of Frisian bulls spermatozoa. (mean ±SE)

Item	TSL	W0	WEY
Normal form (%)	43.90±0.23^{b}	45.00±0.06^{b}	69.60±2.83^{A}
Abnormal head (%)	53.94±0.71^{a}	53.38±0.25^{a}	28.91±2.55^{a}
Abnormal neck (%)	29.41±1.34^{a}	23.94±5.99^{a}	3.54±1.45^{b}
Abnormal tail (%)	19.56±3.04	16.75±8.44	4.05±1.75
Mono deformation (%)	28.28±1.59	31.27±6.17	26.39±1.09
Dual deformation (%)	8.87±1.14^{a}	8.38±2.01^{a}	2.03±0.58^{b}
Tri deformation (%)	18.97±2.99	15.34±8.12	2.02±1.17
Teratozoospermic index	2.28±0.01^{a}	2.23±0.003^{a}	1.45±0.06^{b}
Sperm deformity index	1.28±0.01^{a}	1.23±0.003^{a}	0.45±0.06^{b}

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

Kinetic sperm parameters:

Frozen Frisian bull semen diluted with WEY and W0 increased (P<0.05) linearity compared with TSL-extender. Only semen with WEY-extender significantly (P<0.05) increased straight line velocity and straightness values as compared to TSL-extender**.** However, no significant effect of extenders on curvilinear velocity, average path velocity, and wobble value (Table 5).

Table 5. Effect of different extenders on sperm kinetic parameters in Frisian bull post-thawed semen. (mean ±SE)

Item	TSL	W0	WEY
Curvilinear velocity (VCL) (μm/sec)	20.25±4.30	24.43± 1.66	27.11±0.76
Straight line velocity (VSL) (μm/sec)	8.58±2.36^{b}	15.01±2.43^{ab}	16.42±0.25^{a}
Average path velocity (VAP) (μm/sec)	20.39±6.78	25.38±1.22	21.76±0.26
Linearity (LIN) (%)	40.40±3.06^{b}	60.22±5.86^{a}	60.79±2.64^{a}
Wobble (WOB) (%)	91.78±13.99	106.35±12.22	80.40±1.32
Straightness (STR) (%)	45.68±3.62^{b}	60.94±12.51^{ab}	75.51±2.04^{a}

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

Discussion

Respecting the problems of using several animal proteins in semen dilution and to optimal the contents in the semen extender, many chemicals defined semen extender were commercially used like dried coconut water ACP-111®, Tris based- Tris concentrate-Gibco BRL®, commercial egg-yolk based media-Botu-Bov®, BullXcell®, Bovidyl®, Triladyl® and skim milk based- Laciphos®. Soybean-lecithin-based extender is used as plant source. Lecithin in different cryoprotectants protect plasma membrane of spermatozoa by restoring phospholipids which get lost during dilution, cooling and freezing to protect cell viability (Layek *et al*., 2016).

Biocephos plus®, Bioxcell® and AndroMed® are animal protein free commercial diluents used for semen preservation (Bousseau and Brillard 1994; Muller-Schlosser 2005).

The 1st use of milk as an extender for preservation was reported by Koelliker in 1856. Before using milk as an extender, milk lactenin which has spermicidal properties (toxic to spermatozoa) should be isolate by heat treatment. Both milk based and egg-yolk citrate extenders were compared to be similar on sperm fertility (Foote, 1978). By changing osmotic pressure surrounding the sperm cells, milk lactose is non-permeating and prevents the formation intracellular ice crystals. whole milk (10%) or skimmed milk with glycerol (7%) and antibiotics were used in semen cryopreservation.

The obtained results in this study indicated the best results concerning sperm characteristics in post-diluted and post-equilibrated semen diluted by WEY, followed by W0-extender. During cryopreservation, non-penetrating cryoprotectant used which contains phosphatidylcholine (lecithin), phosholipids, lipid extracts, lipoprotein fractions and specific lipoproteins which provides protection from cold shock (Mayer and Lasley, 1945). A fraction, Phospho-lipid moiety of LDL, protects sperm cells from cold shock (Pace and Graham 1974). Egg yolk in extenders of cryopreserved bull semen is 15-30% (Van Demark *et al*., 1957). Egg yolk in tris-based extender at a level of 1-2.5% was used in cryopreserved buffalo semen (5 °C) yielded the highest survival rates of sperm cells after 72h (Sahni and Mohan 1990). Presence of whey protein in whey milk may indicated the superiority of W0 or WEY extenders in maintaining sperm characteristics after dilution and cooling during equilibration. The improvement by both whey-extenders may be due to rolesof both whey proteins and egg yolk phospholipid during frozen Frisian bull semen. In agreement with the present results, Ganguli *et al*. (1973) found that citric acid whey is a suitable diluent for preserving buffalo semen both at room and at refrigerated temperature, as revealed by sperm motility parameters and conception rate. Also, 35 to 40% recovery rate was obtained with buffalo spermatozoa frozen in citric acid whey with glycerol and the percentage of motile sperm and count of non-eosinophilic were higher ($P<0.01$) in citric acid whey (CAW) fortified with 0.1% and 0.2% cysteine after storage period of 96 h at 5ºC (Singh *et al*., 1989).

While, Rabie *et al*. (2018) reported significant increase in the mean percentage of progressively motile sperms (from 62.73 to 65.76%), which may be attributed to the amino acid content of whey proteins. Also, Rabie *et al*. (2018) observed that whey proteins did not have any negative impact on their fertility parameters. Moreover, this practice may even improve their fertility capacity through improving sperm motility. Whey protein represents 20% of milk proteins. It is derived from the watery portion of milk which was separated from the curds during the process of cheese production (Tsutsumi and Tsutsumi, 2014). Whey proteins contain high concentrations of branched-chain amino acids (BCAAs) Millward *et al*., 2008), which are easily digested and rapidly increase the postprandial plasma BCAA levels (Salehi *et al*., 2012). BCAAs stimulate muscle protein synthesis, prevent muscle protein breakdown, and may ameliorate exercise-induced muscle damage and pain (Volek *et al*., 2013). These properties of whey may proteins explain their common use in the exercise industry as a muscle-building supplement (Josse and Phillips, 2012). Whey protein supplementations have become a common practice among gym-goers due to their expected benefit regarding performance enhancement and muscle building (Samal and Samal, 2017). Although whey protein supplementations are generally considered safe for most adults when used appropriately (Tsutsumi and Tsutsumi, 2014).

CONCLUSION

Freezability of Friesian bull spermatozoa were remarkably improved by using whey+5% egg yolk during cryopreservation. Further studies are required on sperm fertilizing ability of cryopreserved bovine semen.

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إستحدام شرش جبنة البانير كمخفف جديد لحفظ السائل المنوى البقرى بالتجميد

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الهدف من هذه الدراسة هو استخدام شرش اللبن الناتج عن صناعة جبن البانير كمخفف للسائل المنوي البقري. تم جمع قذفات من 5 ثيران فريزيان وخلط السائل المنوي لهما وتقسيمه إلى ثلاث أجزاء وتخفيفه بمعدل 10:1 مع مخفف الترس ليسيسين فول الصويا ، الشرش ، أو الشرش + صفار البيض. ثم تقييم السائل المنوى بعد التخفيف والموازنه (5ºC لمدة 4 ساعات) والتجميد (-196ºC) من حيث الحركة التقدمية، الحياتيه، الشواذ، سلامة الأكروسوم، الذيل الملتوي للحيوانات المنويه، استخدم نظام تحليل السائل المنوي بالكمبيوتر (CASA). أظهرت النتائج الآتى: نسبة الحركة التقدمية و الحياتيه و سلامة الأكروسوم و الذيل الملتوى كانت أعلى ، ونسبة الشواذ كانت أقل ($P<0.05$) مع مخفف الشرش + صفار البيض مقارنة بالمخففات الأخرى إما بعد التخفيف أو بعد التوازن. بعد الذوبان تم تقييمة بواسطة CASA ، وتحسن مخفف الشرش + صفار البيض ($P<0.05$) النسب المئوية للحركة التقدمية ، والحركة الكلية ، وحركة التقدم السريع ، والحيوانات المنوية الوظيفية ، وعدم حركة الحيوانات المنوية بالمقارنة مع المخففات الأخرى. زادت مخففات الشرش + صفار البيض والشرش ($P<0.05$) الخطية مقارنة بمخففات الترس ليسيسين فول الصويا. كانت سرعة الخط المستقيم والاستقامة أعلى ($P<0.05$) مع مخفف الشرش + صفار البيض من مخفف الترس ليسيسين فول الصويا ، بينما لم تختلف بين مخفف الشرش + صفار البيض و مخفف الترس ليسيسين فول الصويا. وكذلك كان تشوه الحيوانات المنوية أقل ($P<0.05$) في مخفف الشرش + صفار البيض مقارنة بالمخففات الأخرى.

الخلاصة: تحسن خصائص السائل المنوي بشكل ملحوظ باستخدام شرش اللبن + صفار البيض بنسبة 5٪ مقارنة بالمخففات الأخرى أثناء عملية تجميد السائل المنوي للثيران الفريزيان.