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Using of Cottage Cheese Whey with or without Egg Yolk as a New Dilution for Cryopreservation of Ram Semen

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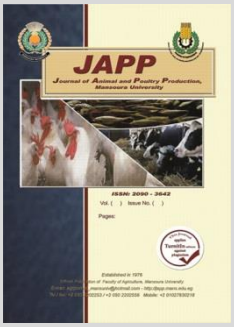

ABSTRACT

The aim of this study was to evaluate using whey of cottage cheese as a diluting agent for ovine semen. Semen from five rams were collected, pooled, and divided into 3 parts, and diluted (1:20) using three solvents: Tris-15% egg yolk extender (E1, control), whey-cetric-10% egg yolk extender (E2), and whey alone (E3). The diluted semen was then allowed to equilibrate (5 ºC for four hours), then frozen (-196 ºC). After thawing, semen was assessed by Computer Assisted Semen Analyzers (CASA) program for progressive motility (PM), live sperm (LS), acrosome integrity (AI), abnormality (AB), and DNA damage of spermatozoa. Results showed that the percentages of LS, and AI in post-thawed semen were higher (P<0.50) and the proportion of chromatin damage was lower (P<0.05) with E2 than in other extenders (E1 and E3). Comparing E2 to other extenders, post-thawing semen examined by CASA showed improved (P<0.05) percentages of progressive, rapid progressive, and total motility, and decreased immotility. E2 showed significantly (P<0.05) higher VSL than in E1 and E3 with the highest LIN and STR percentages. E3 showed significantly (P<0.05) higher VCL and VAP than in E1 and E2 with the lowest LIN and STR percentages. Conclusion: Cottage whey-10% egg yolk can be used as an extender for ram semen cryopreservation. It increased the freezing ability of ram spermatozoa by improving most sperm characteristics as compared to Tris-egg yolk extender.

Keywords: Ram, semen, whey, diluent, freezing, sperm activity.

INTRODUCTION

Sperm cryopreservation has many benefits beyond extended storage, such as cost savings (by reducing the herd's ram population, for example), ease of storage, prevention of genetic drift, and promotion of global genetic resource transfer, and interchange (Takizawa *et al*., 2018; Lv *et al*., 2019). While cryopreservation has many advantages, the quick structural changes that spermatozoa undergo after freezing can cause a variety of cell damage, such as modifications to the arrangement of lipids and proteins or a reduction in membrane fluidity (Crockett *et al*., 2001).

For the reasons mentioned above, freezing semen extenders (S-EXT) are commonly used to dilute semen before cryopreservation. They can be made with a number of ingredients, like Tris, energy source (glucose or fructose), glycerol, citric acid, antibiotics, and egg yolk (Barbas *et al*., 2009, Lv *et al*., 2019). A variety of compounds have been used to increase sheep S-EXT, including sugars, seminal plasma, fatty acids, enzymes, vitamins, amino acids, proteins, and different plant extracts (Allai *et al*., 2018).

In terms of the control, suppression, or prevention of reactive oxygen species (ROS) formation (Sikka *et al*., 2004; Maneesh *et al*., 2006), which has been clarified to directly impact the ability of spermatozoa to fertilize (Allai *et al*., 2018), the primary aim of these materials in almost cases, is reducing the oxidative process (Pietta *et al*., 2000). After thawing, maintaining the sperm membrane's integrity and functionality is essential for cryopreserved semen to be successful. The processes of freezing and thawing is characterized by abrupt changes in osmolality, intracellular ice crystal production, and significant temperature swings. These occurrences result in sperm function loss and cell damage (Bailey *et al*., 2000).

Recent research has shown that the fundamental cause of sperm cryodamage is most likely plasma membrane failure rather than intracellular ice crystal formation (Kim *et al*., 2011, Oldenhof *et al*., 2013). Additionally, preservation in liquid nitrogen at -196 ºC, extenders shield spermatozoa from freezing shocks (Foote, 2002). Hulmi*et al*. (2010) listed glycomacropeptide, immunoglobulin, β-lactoglobulins, α-lactalbumin, lactoferrin, lactoperoxidase, and bovine serum albumin as examples of these proteins. Other soluble proteins (whey proteins) found in milk include albumin, b-lactoglobulin, a-lactalbumin, and lactoferrin (Amiot*et al*., 2002). Whey is used as a diluent in certain experiments to preserve semen (Ganguli *et al*., 1973). Based on motility tests and conception rate, they found that citric acid whey is an appropriate solvent for storing cow buffalo semen at room temperature and in a refrigerator.

Computer-aided semen analyzers (CASA) are instruments that assess individual sperm cells and produce accurate and dependable findings for the majority of semen properties, such as various metrics for sperm velocity and sperm motility (Verstegen *et al*., 2002). Furthermore, according to Mortimer (2000), CASA provides a more accurate estimation of the fertilization capacity of semen and forecasts the particular mobility properties of sperm cells.

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Ram semen can be preserved using a variety of non-commercial and commercial extenders. There are two different types of semen diluents in use: those based on plant lipoprotein and animal lipoprotein sources, (Gil *et al* 2003; Kasimanickam *et al*., 2011). Soybean lecithin is the principal source derived from plants, while egg yolk is the predominant source from animals (Batellier *et al*. (2001). According to Batellier *et al*. (2001), Bergeron and Manjunath (2006) and Rahman (2014), semen can also be preserved with whole or skim milk. Rahman (2014) observed that when it comes to chilling and freezing ram semen, skim milk extenders provided superior protection during the preservation process than egg yolk-based diluents. However, after eight days of storage at 4 °C, tests on sperm motility, pH value, and livability revealed that a diluent of citrate-egg yolk had superior sperm protection properties than milk-based extenders (Soltanpour and Moghaddam, 2014).

In comparison to egg yolk-only and egg yolk-free alternatives, the study by Kulaksiz *et al*. (2012) demonstrated that the skimmed milk-egg yolk diluent had the best liquid storage and post-thaw motility. On the first day of storage, the overall percentage of aberrant spermatozoa in skim milk plus egg yolk extender was at its lowest. Although the cryopreservation of ram semen has improved due to the discovery and modification of storage diluents and procedures, fresh semen still has a much higher fertility than frozen-thawed sperm (Kulaksiz *et al*., 2012). Extensive research is needed to determine which extenders, and at what dosages, are suitable for a given species. If insemination is completed promptly, dilution and cooling of ram semen is preferable to frozen semen because the adverse consequences are not as severe as they would be with frozen and thawed semen.

The aim of this research was to evaluate the impact of Cottage cheese whey with or without egg yolk, as an extender, on maintaining sperm parameters in ram semen after cryopreservation.

MATERIALS AND METHODS

Sakha Animal Production Station, belonging to Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture, Egypt's, is where this study was carried out during the period from August 2023 until December 2023.

Animals:

Four Rahmani rams (2-4 years old; 50-60 kg) in good health and sexual maturity were selected as semen donors in this study. Before the trial began, rams were checked to make sure there were no reproductive problems, and being healthy during the experimental period. Rams were housed in a somewhat shaded yard. Based on their live body weight (LBW), APRI advised that rams be fed rice straw, concentrate feed combination and berseem hay to suit their nutritional demands.

Experimental semen extenders:

Tris-Egg Yolk Extender:

The control base extender used in our study was Tris-extender containing 15% fresh chicken egg yolk, 6% glycerol, 2,17 g Tris, 0.31 g glucose, 1,193 g citric acid, and 0.051 g Penicillin dissolved in 38 mL of sterile doubled-distilled.

Whey cottage extender:

LJJ-Tratnik *et al*. (2001) state that lactic whey was obtained throughout the Cottage cheese production procedure. In a stainless-steel vat, cow skim milk was separately heated to 90 °C for ten minutes. Following the addition of hot coagulant solutions containing 1% lactic acid, the heated milk was allowed to cool to 70 °C. Then, liquid was continuously stirred until the pH reached 5.4 and the whey was well separated. After five minutes of resting the coagulant curd, the whey was allowed to flow through cotton fabric, diluting the semen. Lactic acid whey, a cottage cheese byproduct, was used to dilute the semen. A freshly produced 10% sodium hydroxide solution was used to lower the pH of the diluent to 6.8 after allowing the suspension to sit for five to ten minutes. The physical and chemical properties of cottage cheese whey and whey-diluent are listed in Table 1.

Table 1. Physical and chemical properties of Cottage cheese whey and whey-extender.

Constituent	Cottage cheese whey	Whey-extender
Total solids (%)	6.5	6.5
Fat (%)	0.3	0.3
Protein (%)	0.6	0.6
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	4.6	6.8
Total bacterial count ($x10^2$/g)	1.2	ND
Yeast and mould count (cfu/g)	ND	ND
E. coli	ND	ND

ND: Not determined

Citric-whey was used as an extender with 10% egg yolk (E2) or without egg yolk (E3). Following filtration via a 0.45μm Millipore filter, the two citric-whey extenders were supplemented with 6% (v/v) glycerol, 1000 IU penicillin, and 1 mg streptomycin per milliliter.

Semen evaluation:

Manual sperm analysis:

After thawing at 37 ºC for 30 s, semen was manually evaluated to determine the percentage of sperm livability and abnormality, as well as the percentage of acrosomal and chromatin integrities in the post-thawed semen extended with different extenders.

Sperm livability and abnormality:

Sperm livability and abnormalities were evaluated using eosin–nigrosin staining (Evans and Maxwell, 1987). Create a smear on a glass slide by combining around 10 μL of semen with 10 μL of stains, and then allow it to air dry. To detect live and healthy sperm cells, a field of about 100 sperm cells was counted using a ×100 microscope (power). Sperm cells stained with eosin or nigrosin were regarded as dead, whilst cells that did not show stain were seen as living.

Sperm cells having abnormalities in the head, neck, mid-piece, or tail were identified as aberrant spermatozoa on the same slide. We computed the percentages of livability and abnormal sperm.

Acrosome status:

According to Almadaly *et al*. (2014), the staining procedure involved dripping semen onto a glass objet. Using a hot plate, the smear preparation was formed and heated to 37ºC. After 10 minutes of fixing with methanol, the preparation was washed under running water. Giemsa solution consisting of 3 milliliters of absolute Giemsa, 2 milliliters of PBS, and 35 milliliters of distilled water was used to stain it for three hours, while it was submerged in a

staining jar. It was again dried after being rinsed under running water. A 400x magnification light microscope was used to study 200 cells. Spermatozoa with undamaged acrosomes had a purple head, but those with damaged acrosomes had a pale lavender head. The number of spermatozoa with intact acrosomes divided by the total number of spermatozoa and multiplied by 100% was used to determine the acrosomal status percentage.

Chromatin integrity:

Results of the chromatin integrity was estimated by Acridine Orange Test (AOT). Briefly, five milliliters of saline buffered with phosphate was used to wash the frozen semen. After centrifugation, the sperm pellet was again suspended in 0.5 milliliters of pbs. Next, a little amount (50 µL) of the sperm solution was smeared onto a glass. According to Liu and Baker (1994), samples were allowed to air dry and three smears were formed on glass slides before being fixed overnight in Carnoy's solution (3:1 methanol/acetic acid solution). After rinsing and letting the slides air dry, freshly made acridine orange (AO) dye was applied to them for five minutes in the manner described below: Ten milliliters of 1% AO and 2.5 milliliters of 0.3 M $Na_2HPO_47H_2O$ were mixed to make 40 milliliters.

CASA Analysis:

After the semen had thawed, it was evaluated using computer assisted semen analysis (CASA, SPERMOLAB®, Cairo, Egypt). On a heated slide (37ºC), a 10 µL drop of diluted semen was inserted and covered with a coverslip. Five photo repetitions were made for every sample. The following standards were assessed: the percentage of progressive (slow and rapid), non-progressive, and total motilities as well as immotilities, straight-line velocity (VSL), curvilinear velocity (VCL), the linearity (LIN %), wobbles (WOB %), and straightness (STR %).

Statistical Analysis:

After arcsine transformation, data for all semen parameters were statistically evaluated using SAS (2004) and one-way ANOVA. The statistically significant mean differences were distinguished using the Duncan Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Results

Sperm characteristics in post-thawed ram semen:

Manual analysis

Sperm livability and abnormality:

Table 2 displays the features of the sperm in post-thawing semen with various types of extenders. Following freezing, statistically significant variations were seen in the percentage of sperm livability, being significantly (P<0.05) higher with E2 than with E1 and E2. However, sperm abnormality percentage was not affected significantly by extender type.

Table 2. The impact of extender type on the percentage of livability and abnormality in post-thawed semen (Mean±SE)

Extender	Sperm livability (%)	Sperm abnormality (%)
E1	52.40±0.50^{b}	28.60±1.12
E2	60.00±0.54^{a}	26.00±1.58
E3	51.60±0.59^{b}	25.40±2.01
P-value	0.0001	0.361

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

Acrosome integrity and chromatin damage:

Table 3 demonstrated that acrosome integrity percentage was significantly (P<0.05) the highest in E2, moderate in E1, and the lowest in E3. However, percentage of chromatin damage showed insignificant change in E2 and significantly (P<0.05) increased in E3 as compared to E1.

Table 3. The impact of extender type on the percentage of intact acrosome and chromatin damage in post-thawed semen

Extender	Intact acrosome (%)	Chromatin damage (%)
E1	39.00±0.70^{b}	41.00±2.64^{b}
E2	47.40±1.50^{a}	44.00±2.12^{ab}
E3	31.00±1.40^{c}	49.80±1.59^{a}
P-value	0.0001	0.040

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

CASA analysis

Sperm motility parameters

Table 4. displays the features of the sperm motilities in post-thawing semen with various types of extenders. Following freezing, statistically significant variations were found in the percentage of progressive, rapid progressive, total motility, and immotility parameters. However, slow progressive motility and non-progressive motility were not affected by extender type.

Results showed that dilution of ram semen with E2 had significantly (P<0.05) beneficial impacts on increasing the percentage of progressive, rapid progressive, and total motility, while decreasing immotility percentage, in comparing with other extenders (E1 and E3).

Table 4. The impact of extender type on the percentage of sperm motility parameters in post-thawed semen (Mean±SE)

Extender	Sperm motility (%)					Immotility (%)
	Progressive	Rapid	Slow	Non-progressive	Total	
E1	43.20±0.66^{b}	34.60±0.50^{b}	8.60±0.40	7.20±0.86	50.40±0.50^{b}	49.60±0.50^{a}
E2	51.40±0.97^{a}	41.80±0.37^{a}	9.60±0.92	6.60±1.07	58.00±0.54^{a}	42.00±0.54^{b}
E3	43.00±0.70^{b}	35.00±0.70^{b}	8.00±0.31	6.60±1.20	49.60±0.50^{b}	50.40±0.50^{a}
P-value	0.0001	0.0001	0.215	0.899	0.0001	0.0001

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

Sperm velocity and kinetic indexes:

Table 5 illustrates that the extender type had a significant effect on all sperm velocities (VCL, VSL, and VAP) and kinetic indexes (LIN, STR, and WOB).

E2 showed significantly (P<0.05) higher VSL than in E1 and E3 with the highest LIN and STR percentages, but did not differ significantly regarding VCL, VAP, and WOB as compared to E1.

On the other hand, E3 showed significantly (P<0.05) higher VCL and VAP than in E1 and E2 with the lowest LIN and STR percentages, but did not differ significantly regarding VSL.

Table 5. The impact of extender type on the percentage of sperm motility parameters in post-thawed semen (Mean±SE)

Extender	Sperm velocity (µm/s)			Sperm kinetic index (%)		
	VCL	VSL	VAP	LIN	STR	WOB
E1	98.30±0.25[b]	52.32±0.85[b]	81.04±0.39[b]	53.21±0.83[b]	64.57±1.28[b]	82.43±0.41[a]
E2	97.72±0.40[b]	56.76±0.60[a]	80.76±0.50[b]	58.08±0.77[a]	70.28±0.89[a]	82.64±0.44[a]
E3	128.52±0.64[a]	52.88±0.73[b]	95.74±0.77[a]	41.15±0.75[c]	55.24±0.99[c]	74.49±0.70[b]
P-value	0.0001	0.002	0.0001	0.0001	0.0001	0.0001

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

Linearity (LIN) is the linearity of the curvilinear trajectory calculated as VSL/VCL × 100. STR is the LIN of the sperm average path calculated as VSL/VAP × 100. Wobbling (WOB)= VAP/VCL

Discussion

In the commercial sector, a range of chemicals that fall under the category of semen extenders were used to maximize the contents in the product and solve the issues associated with using multiple animal proteins in semen dilution. Some examples of these materials are ACP-111® dry coconut water, Gibco BRL® tris concentrate, Botu-Bov®, BullXcell®, Bovidyl®, Triladyl® commercial egg-yolk based media, and Laciphos® skim milk based media. The plant source is an extender made from soybean lecithin. By replenishing phospholipids lost during dilution, chilling, and freezing to preserve cell viability, lecithin in various cryoprotectants shields the spermatozoa's plasma membrane (Layek *et al*., 2016). In the present study, we evaluated the impact of Cottage cheese whey with or without egg yolk, as an extender, on maintaining sperm parameters in ram semen after cryopreservation. We assessed the parameters of sperm motility and motion, living sperm with intact acrosome integrity, and spermatozoa DNA damage.

It is well known that extender components interact with several spermatozoa components, causing osmotic and toxic stress as well as biochemical alterations in cell metabolism right away following dilution (Domingo *et al*., 2019, Yaniz *et al*., 2019). Semen extenders supply the energy necessary to maintain sperm motility. Spermatozoa may suffer harm from the freeze-thaw cycle, leading to undesirable alterations in their morphology, motility, motion parameters, acrosome status and livability (Gangwar*et al*., 2018). We found that the whey-based extender supplemented with egg yolk (E2) increased motility parameters and the livability percentage of sperm cells with intact acrosomes and DNA integrity in comparison with the Tris-based extender (E1) and whey-based extender without egg yolk (E3). Numerous experiments have been carried out employing various semen extenders and additives to safe guard the power of ram sperm to fertilize during the freeze-thaw process (Nur *et al*., 2009).

Furthermore, extenders based on egg yolk and skim milk is frequently employed to preserve ram semen (Dayanikli *et al*., 2022). According to Rahman *et al*. (2018), the use of skim milk-based extender coupled with 10% egg yolk is preferable to Tris-based extender when it comes to the liquid preservation of ram semen. Whey protein in whey milk may be a sign that E2 and E3 are good extenders for preserving sperm traits after dilution, equilibration, and thawing. The enhancement resulting from the use of E2 could be attributed to the participation of both phospholipids found in egg yolks and whey proteins in frozen semen as found in buffalo semen by El-Hamady and Elgaml (2021) and Ganguli *et al*. (1973). They reported that sperm motility metrics and conception rate indicate that citric acid whey is an acceptable diluent for storing buffalo semen at room temperature and in a refrigerator. Additionally, buffalo spermatozoa frozen in citric acid whey with glycerol yielded a sperm recovery rate of 35–40% for motile sperm in the citric acid whey.

When assessing the potential fertilizing capability of sperm, it is important to keep in mind that each spermatozoon is a multi-compartmental cell that needs to have a range of qualities in order to fertilize an egg (Graham *et al*., 2005). The CASA analysis has been the accepted laboratory method for evaluating kinematic and motility parameters for a long period (Mortimer *et al*., 2015). Increasing sperm velocity-related metrics in semen extended with E2 and E3 can be used as a proxy for fertility and mitochondrial function (Nesci *et al*., 2020).

CONCLUSION

Ram spermatozoa's ability to freeze was significantly increased when Cottage whey-10% egg yolk was used during cryopreservation. Also, Cottage why without egg yolk had nearly similar impacts as Tris-egg yolk on freezing ability of ram semen. The potential of cryopreserved ovine semen to fertilize sperm needs more investigations.

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إستخدام شرش الجبنة القريش مع او بدون صفار البيض كمخفف جديد لحفظ السائل المنوى للكباش

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الملخص

هدفت هذه الدراسة إلى تقييم استخدام شرش اللبن الناتج من الجبن القريش كمخفف للسائل المنوي للأغنام. تم جمع عينات السائل المنوي من خمسة كباش وتقسيمها الى ٣ أجزاء لتخفيفها بمعدل تخفيف (١:٢٠) باستخدام ثلاثة مخففات: صفار البيض ١٥٪ (E1 االكنترول) - شرش اللبن + ١٠٪ صفار البيض (E2) - شرش اللبن (E3). تم عمل فترة موازنة للسائل المنوى المخفف على ٥ درجة مئوية لمدة أربع ساعات ثم تم تجميده على درجة (-١٩٦ درجة مئوية) فى النيتروجين السائل. تم تقييم السائل المنوي بعد التجميد و الاسالة بواسطة برنامج تقييم السائل المنوي بمساعدة الكمبيوتر(CASA) للحركة التقدمية وتقييم الحياتية وسلامة الأكروسوم ونسبة الشواذ وسلامة الحمض النووي. أظهرت النتائج أن النسب المئوية للحركة التقدمية وسلامة الأكر وسوم في الحيوانات المنوية أعلى فى مخففات شرش اللبن (E2-E2) بالمقارنة بالكنترولE1 وكانت نسبة الحمض النووي في الأشكال الشاذة أقل مع المخف (E2). عند مقارنته بالمخففات الأخرى أظهرت النتائج ان السائل المنوي بعد الاسالة باستخدام CASA تحسنا ملحوظا فى نسب الحركة التقدمية والحركة الكلية والحركة التقدمية السريعة. سرعة الحيوان النوى الخطية (LIN) عند مقارنة مخفف E2 بمخففات E1 وE3 و كانت سرعة الحيوان المنوي في خط مستقيم (VSL) و (STR) أعلى مع مخفف E2 من مخفف E1. الخلاصة: أدى استخدام شرش اللبن + صفار البيض بنسبة ١٠٪ (E2) إلى زيادة ملحوظة في قدرة الحيوانات المنوية على التجميد حيث تأثرت جودة الحيوانات المنوية بعد الاسالة بشكل إيجابي في جميع المجموعات باستخدام مخففات السائل المنوي ومع ذلك كان التأثير أكثر وضوحًا مع المخفف الذي يحتوي على جبن شرش اللبن + ١٠٪ من صفار البيض (E2) مقارنة بالمخفف (E1).مع العلم انه مازال هناك حاجة الى مزيد من الابحاث لدراسة استخدام شرش اللبن فى مخففات السائل المنوي فى الاغنام . تحسن خصائص السائل المنوي بشكل ملحوظ باستخدام شرش اللبن + صفار البيض بنسبة ١٠ ٪ مقارنة بالمخففات الأخرى أثناء عملية تجميد السائل المنوي للكباش.