OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Using Bee Bread Extract as Natural Source of Energy, Antibiotics and Antioxidants Instead of Synthetic Sources During Freezing Steps of Ram Semen Extender

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Abstract | This study was conducted to assess the probabilities of using the ideal level of bee bear extract (BBE) up to 0.5, 1.0, 1.5, and 2.0 % for ram semen extender during freezing steps. Semen was collected by an artificial vagina from five adult Rahmani rams twice-weekly for five weeks. Semen was pooled, split into five replicates, and prepared to be frozen. In 1st replicate, semen was extended in Tris egg yolk (TEY) is contained synthetic sources of energy (fructose), antibiotics (penicillin and streptomycin), and antioxidants (vitamin E), which served as control extender (E0). However, 2nd, 3rd, 4thand 5th replicates were serviced as trial extenders as E1, E2, E3, and E4, which supplied 0.50, 1.00, 1.50, and 2.00 % of BBE as natural sources of energy, antibiotic and antioxidant, respectively. Results indicated that adding BBE to E1, E2, E3, and E4 extenders has increased (P<0.05) in all sperm characteristics and biochemical activity of extender as compared to E0 extender through semen extender preservation. The best level of BBE is marked when added up to 1.5% in the E3 extender which improves all characteristics compared to E0 and other BBE extenders. Also, E3 indicated post-thawing sperm parameters as motility, live, normal, and integrity acrosome up to 47.50, 65.10, 65.66, and 69.67%, but it was 37.22, 49.78, 55.89, and 59.22% relative to E0 extender, respectively. Furthermore, ATP, BC, and LPO levels post-thawing were 0.35µM, 255.40 CFU/ml, and 2.61 µM with E3 extender however, 0.19 µM, 276.00 CFU/ml, and 2.89 µM with E0 extender, respectively. Then, all of their previously enhanced post- thawing semen characteristics reflected higher positive values on its fertility (46.15 and 53.85%) and productive performance (53.85 and 69.23%) rates in E0 and E3, respectively. In conclusion, these results suggest that a ram semen extender adding 1.5% of BBE was optimum to obtain improved semen preservation.

Keywords | Rams, Semen extender, Bee bread extract, Sperm function, Fertility.

Received | July 17, 2022; **Accepted** | August 08, 2022; **Published** | August 25, 2022
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Email: tarekmahdy22@yahoo.com
Citation | Khalifa EI, Desoky ALI, Elbadawy AA, El-Emam GI, Mahdy TMM (2022). Using bee bread extract as natural source of energy, antibiotics and antioxidants instead of synthetic sources during freezing steps of ram semen extender. Adv. Anim. Vet. Sci. 10(9): 2031-2042.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.9.2031.2042
ISSN (Online) | 2307-8316

INTRODUCTION

Semen extenders are a solution for protecting spermatozoa to enable fertilization and maintain preserve sperm metabolic processes such as providing energy and providing other characteristics to control bacterial transmission and contamination (Khalifa et al., 2016a). These characteristics maintain the storage, transport, and utilization of sperm *in vitro* artificial insemination (AI) (Alvarez et al., 2019). Spermatozoa that have just been ejaculated require an energy metabolism that produces energy quickly to sustain the significant quantity of energy needed by spermatozoa to activate for exiting the ejaculation point (Khalifa and Khalil 2016b). Obviously, the energy requirements of

sperm in the oviduct through the uterus are very different from those shortly following ejaculation (Mohamed et al., 2019). In addition, adenosine triphosphate (ATP), which provides energy for major functions of spermatozoa throughout two metabolic pathways, oxidative phosphorylation, and glycolysis (happens in sperm cells' membrane and supplies energy for sperm metabolism) (Hernández-Avilés et al., 2020). Generally, Although fructose or glucose are thought to be the main sources of energy for sperm cells, fructose is the best sugar for preserving functional membrane integrity and sustaining appropriate sperm motility and tonicity following thawing (Bustani and Baiee, 2021). Microbial contamination may have an impact on both the sperm quality and final production yield (Waberski et al., 2019). Antibiotics must be added to semen extenders in order to avoid bacteriospermia's negative effects on sperm quality and disease transmission in animals through AI. Semen extenders were given various antibiotics, including penicillin and streptomycin. (Banday et al., 2019) to prevent risk of bacterial infection. In this context, Anel-Lopez et al. (2021) reported that higher ($P < 0.05$) sperm motility, integrity membrane, metabolic activity, fertility rate and lower bacteriological assessment in ram extended semen has penicillin and streptomycin up to 500 UI and 625µg/ml, respectively than the control extender without antibiotic after 2 h of incubation at 37◦C. Antioxidants are very important in semen extenders, El-Nagar (2017) found that antioxidants such as vitamins C or E presented in semen extender of mammals had against protection of sperm cells from lipid peroxidation (LPO) and maintenance of cell membrane. The natural energy, antibiotic and antioxidant sources used in this work are bee bread extract. The term "bee bread (BB)" is referred to the collected bee pollen (BP) that is processed by bees and fermented and stored inside a bee hive where it undergoes lactic acid fermentation (Al-Amery and Banana, 2020). Also, BB as one of the bee products, is overlooked with more highly acclaimed honey. It is generally known that bees' gland-derived enzymes, such as amylases, which are in charge of starch hydrolysis, as well as bacteria, primarily lactic acid bacteria and some yeasts derived from bees' saliva and surfaces of pollen loads, are essential for BP fermentation and BB formation (Kieliszek et al., 2018). Combarros-Fuertes et al. (2020) demonstrated that extracts made from BB had significantly better antibacterial potential than extracts from BP, and both products effectively inhibited the development of *Staphylococcus aureus* in water suspensions. Brudzynski et al. (2021) confirmed that BB and BP might be inhibited antimicrobial activity were grouped into the genus *Bacillus* spp., and 5 distinct species were identified: *B. safensis, B. pumilus, B. licheniformis, B. altitudinis, and B. subtilis*. Regarding to energy in BB, Mohammad et al. (2020a) found that the sugar contents in BB were fructose, glucose, sucrose and maltose which had average concentration up to 0.99, 11.55, 1.49, 1.24 g/100g, respectively. On the other hand, Dranca et al. (2020) defined that physicochemical parameters of BB such as fat, protein, carbohydrate and energy were 5.15, 18.6, 72.38 % and 412.07 k cal/100g however, free sugars such as fructose, glucose, sucrose, melibiose and raffinose were 19.73, 8.82, not detected, 0.97 and 0.96 %, respectively. Because it contains a lot of beneficial substances, such as vitamins, fatty acids, macro and micro-elements, amino acids, and several groups of phytochemicals, mostly significant thought of BB as functional antioxidants (Asadpour et al., 2021).

The present study aimed to investigate the ability to use natural energy, antibiotic and antioxidant existent from bee bread extract instead of synthetic energy, antibiotic and antioxidant sources in ram semen extender during the process of freezing steps.

MATERIAL AND METHODS

All experimental procedures were carried out at El-Serw Experimental Research Station. The animal herd belongs to Animal Production Research Institute (*APRI*), Agriculture Research Center, Ministry of Agriculture, Egypt. This study work was managed during breeding season in 2021. The pregnancy and lambing rate were done in May 2021.

Collection of raw bee bread samples

Bee bread (BB) samples were collected from commercial local bee farms in several regions of the Egyptian Nile Delta Governorates (Dakahlia, Sharkeia, and Ismaila) from April to May 2021. The samples of BB were stored at -20 C° until further analysis post- preparing of bee bread extract (BBE) / each geographical location.

Preparing of bee bread extract (BBE) / geographical location

20 g of drying and crushing raw BB / geographical location were completed with 80 mL of 95% ethanol. The ethanolic BB / geographical location were put on a shaker at 200 rpm at 37 °C for 24 h individually. The top layers of ethanolic BB / geographical location were decanted, and bee bread extract (BBE) / geographical location was centrifuged at 3000 rpm for 30 min. Supernatant of BBE / geographical location was evaporated at 40°C in the rotary evaporator to expel the smelling ethanol. The preparing BBE / geographical location was stored in the dark bottle at 5°C until analysis of carbohydrates, protein, fat, fructose and glucose (at least four samples / geographical location).

Animals

A total of 5 sexually mature Rahmani rams (live body weight of 70 -75 kg with 3-3.5 years of age) were used to collect semen in this study. All experimental rams were

healthy and free of diseases, housed individually under semi-open sheds. Rams were fed individually on a daily diet containing 60% concentrate fed mixture (CFM) + 40% roughage (25% corn silage (CS) and 5% rice straw (RS). The freshwater was available all day time of the study. The chemical analysis of daily diet is shown in Table (2).

Preparing of bee bread extract (BBE) used in semen extender

20 g of drying and crushing raw BB from all geographical location were mixed as one sample and completed with 80 mL of 95% ethanol. The ethanolic solution was shaken for 24 hours at 37 ˚C and 200 rpm. After decanting, the top layer BBE was centrifuged at 3000 rpm for 30 minutes. Then, the rotary evaporator was used to evaporate the BBE supernatant at 40˚C in order to remove the odorous ethanol. The preparing BBE were stored in the dark at 5 ˚C until analysis (at least four samples) and used in semen extenders.

Semen collection

Semen was collected twice a week from all rams for 5 weeks (50 ejaculates from all rams) using a warm artificial vagina at 42°C with a teaser ewe for mating on days of semen collection. Semen samples were collected before feeding at 7-8 am; the ejaculates were taken immediately to the laboratory and kept in the water bath at 37°C for evaluation. The semen ejaculates with more than 80% of individual motility were pooled to study the steps of the freezing processes (after dilution, equilibration, and thawing).

Semen evaluation of experimental rams

Immediately after semen collection, parameters like semen volume, sperm motility, live sperm, normal sperm, damaged acrosome, and sperm cell concentration of experimental rams were recorded in Table (3).

Semen extenders

After semen samples were collected, they were divided into five extenders as E0 (as control), but E1, E2, E3, and E4 were supplied with different levels of BBE up to extension rate 1semen:6 dilutions. The E0, E1, E2, E3, and E4 extenders had the pH value up to 6.92, 6.85, 6.80, 6.76, and 6.71, also, osmolarity was 270, 281, 290, 300, and 310 mOsm/l, respectively. The chemical composition of semen extenders included E0, E1, E2, E3, and E4 were prepared as presented in Table (4).

Evaluation of semen characteristics

Sperm characteristics included progressive motility, livability, normality ,and intact acrosome were done immediately after dilution, equilibrated (up to 4 hours at 5°C), and thawing, according to Khalifa and Mahdy (2019).

Freezing regime

Tubes containing the expanded semen were immediately gently shaken and placed in a warm water bath (37 °C). Semen was diluted at a ratio of 1:6 (semen/extender) with E0, E1, E2, E3, and E4 extenders. Post-dilution, Semen was stored in the refrigerator (5 °C) to cool for 4 hrs as an equilibration period. Equilibrated semen was frozen by the method of packaging in 0.5ml French straws. The straws were placed in a vertical position into a basket of liquid nitrogen container. Then, the basket was exposed to nitrogen vapours at a level 4 cm distance above the surface of liquid nitrogen, where the cooling temperature was from -90 to -100 °C up to 10 minutes. The basket was dipped directly into liquid nitrogen at -196 °C.

Thawing regime

At evaluation times, the frozen semen straws for each extender were thawed by holding the straws at a closed edge (not the plugged end), which sealing with polyvinyl alcohol powder then the straws were dipped in a water bath at 37 °C up to 60 sec.

Assaying of ATP, BC, and LPO in semen extenders

Assaying ATP, BC, and LPO parameters were measured for at least five samples / each extender type after dilution, equilibration, and thawing as following:

Assaying of ATP in semen extenders: The ATP concentrations in seminal plasma were determined colorimetrically using commercial kits. The ATP kits as Colorimetric / Fluorometric ab83355 this kit can detect as low as 1 µM of ATP in samples.

Assaying of BC in semen extenders: The preparation of semen bacterial counting was described by the methods of Khalifa et al. (2016a). The final results were expressed as CFU/ml according to Qureshi et al. (1993).

Assaying of LPO in semen extender: The level of LPO was estimated by measuring the level of malondialdehyde acid (MDA) using commercial kit LPO-586 (Oxis Research, Burlingame, CA, US) with sensitivity at 0.5µM and 0.5 to 4.0 µM as a range curve.

Fertility and productive performance

After assessing the best BBE level in the above trial, semen was collected again, diluted, equilibrated, and frozen in two French straws 0.5ml contained the control extender group (E0) and the optimal BBE addition (E3) to prepare for artificial insemination (AI). The AI technique is used in the Rahmani ewes (n = 26), which housed under similarly condition and used for cervical insemination by post-thawing semen frozen. Ewes included 13 ewes/extender types as E0 or E3 synchronized with a Controlled Internal Drug

Table 1: Analysis of bee bread based on different geographical locations.

Geographical locations	Analysis of bee bread					
	*Carbohydrate g/100g	*Protein g/100g	*Fat g/100g	**Fructose g/100g	**Glucose g/100g	***Energy Kcal/100g
Dakahlia	71.20±0.39	17.26±0.15	1.85±0.02	12.43±0.26^{ab}	6.36±0.30^{a}	307.47±2.08
Sharkeia	70.81±0.40	17.22±0.23	1.84±0.01	11.55±0.27^{b}	5.37±0.03^{b}	368.79±2.57
Ismaila	71.26±0.52	17.25±0.14	1.82±0.02	13.34±0.37^{a}	5.35±0.07^{b}	370.06±2.32

Mean ±SE with different letters in the same column are statistically different (P>0.05).

*Carbohydrates, fat and protein composition of bee bread were determined by (AOAC, 2016).

**Free sugars using the internal standard (IS, melezitose) methodology described by Barros *et al.*, 2013).

***Energy (kcal) = 4× (g protein + g carbohydrates) + 9× (g fat).

Table 2: Chemical analysis of basal experimental daily diet as CFM, CS, and RS.

Items	Chemical composition (% on dry matter basis)						
	DM	OM	CP	EE	CF	NFE	Ash
CFM*	88.70	92.83	14.15	2.31	11.06	65.31	7.17
CS	36.24	86.89	9.16	2.55	24.66	50.52	13.11
RS	89.22	83.78	3.85	1.76	36.72	41.45	16.22

*The CFM was composed of un-corticated cotton seed cake (25%), coarse wheat bran (44%), corn (15%), extracted rice bran (8.5%), molasses (3%), limestone (3%) and sodium chloride (1.5%).

Table 3: Physical semen characteristics in fresh ejaculates of Rahmani rams.

Experimental rams	Semen characteristics					
	Volume (ml)	Motility (%)	Live sperm (%)	Normal sperm (%)	Damage acrosome (%)	Sperm-cell concentration (n×10^{9}/ml)
Ram1	1.05 ±0.09	88.55 ±1.24	89.95 ±0.85	92.45 ±0.59	9.75 ±0.65	3.97 ±0.39
Ram2	0.97 ±0.08	89.70 ±2.02	87.90 ±0.97	90.40 ±0.47	8.79 ±0.82	3.98 ±0.43
Ram3	1.03 ±1.01	87.65 ±1.34	91.50 ±0.99	91.59 ±0.69	8.80 ±0.83	3.99 ±0.42
Ram4	1.04 ±0.08	89.26 ±1.28	87.78 ±0.69	90.35 ±0.44	9.55 ±0.67	3.93 ±0.28
Ram5	1.20 ±1.09	88.75 ±1.54	90.80 ±0.95	91.63 ±0.66	8.56 ±0.68	3.89 ±0.15

Table 4: Chemical composition of experimental extenders.

Ingredients	Semen extender types				
	E0	E1	E2	E3	E4
Tris (g)	3.63	3.63	3.63	3.63	3.63
Citric acid(g)	1.99	1.99	1.99	1.99	1.99
Fructose (g)	0. 50	-	-	-	-
BBE (ml)	-	0.50	1.00	1.50	2.00
*Vitamin E (mg)	50.00	-	-	-	-
Egg yolk (ml)	15.00	15.00	15.00	15.00	15.00
Glycerol (ml)	6.00	6.00	6.00	6.00	6.00
Penicillin (IU)	500.00	-	-	-	-
Streptomycin (µg)	500.00	-	-	-	-
Distilled water added up to	100ml	100ml	100ml	100ml	100ml

* Vitamin E: solution 50 mg/ml in ampoule 1ml (Sigma. Co.). According to João et al. (2018).

Release for 14 days, 58 hrs prior AI, the sponges were removed, and ewes were injected 500-600 IU IM equine chorionic gonadotropin. Each ewe from either E0 or E3 groups received two thawing semen samples within interval time up to 12 hours at began observed estrus behaviors. Then, pregnancy was sureness if ewe did not come back to the oestrus cycle after passed two estrous cycles.

As an indicator of fertility and productivity, Pregnancy (PR) and lambing rate (LR) were estimated as the follows:
PR = No gravid ewes / No inseminated ewes × 100.
LR = No lambing ewes / No inseminated ewes × 100.

Statistical Analysis

Statistical evaluation of the significant difference between means (mean ± SEM) was performed by ANOVA followed by the Duncan to determine significant differences in all the parameters among all energy addition types using the SPSS/PC program (SPSS Statistics version 2020). PR and the LR were tested by Chi-square. Then, the significance level considered was $P<0.05$. The model in statistical analysis was: $Yij = \mu + Gi + eij$ Where: Yij = an observation; μ = overall means; Gi = effect of treatment (i = E0, E1, E2, E3, & E4); and eij = random error

RESULTS AND DISCUSSION

Analysis of bee bread ethanolic extracts (BBE):

The BBE composition results are shown in Table (5). The analysis of BBE collected from different geographical locations demonstrated that carbohydrates (71.09 g/100 g) followed by proteins (17.24 g/100 g) were the main macronutrients in raw BB. The minor contents were found that fat and revealed an energetic contribution were 1.85g/100 g and 369.77kcal/100g, respectively. These results were supported nearly by (Bakour et al., 2019) who reported that carbohydrates (g/100g), protein (g/100g), fat and energetic value (kcal/100 g) were 74.82, 19.96, 1.90 and 396.20, respectively. Confirming our results in the study with Dranca et al. (2020), who indicated that carbohydrates and protein were 72.82 and 18.60% but fat and energetic values increased slightly from our study it was 5.15% and 412.07 kcal/100, respectively. On the contrary, Kieliszek et al. (2018) found that BBE has a few concentrations of carbohydrates (24-34%), proteins (14-37%) and lipids (6-13%). Also, Othman et al. (2019) found that the lowest levels of carbohydrates, protein and fat in BBE samples ranged up to 32.74-59.55, 17.22-18.37 and 21.7-4.80g/100g, respectively. on the other hand, Mohammad et al. (2020a) recorded that averages of carbohydrates, protein and fat in BBE samples were 58.16, 22.26 and 5.34g/100g while, energetic value was around our study (369.67 kcal/100 g). The physicochemical analysis displays that carbohydrate was the most available macronutrient in BB then, carbohydrate was above the minimum level required by bee pollen (40 g/100 g) set by Campos et al. (2008). Furthermore, our results were found that sugar profile content of glucose and fructose up to 5.69 and 12.44 g/100g in BBE, respectively. Nearly to our study, Bakour et al. (2019) who found that free sugars included fructose and glucose were 11.80 and 5.70 g/100g, respectively. Contrary, Dranca et al. (2020) confirmed higher fructose (19.73%) and glucose (8.82 %) concentration in BBE than our results in this study. Also, Urcan et al. (2017) recorded that fructose is the largest amount (57.51% of total fresh weight) followed by glucose (42.59% of total fresh weight). Furthermore, Mohammad et al. (2020a) defined that average of glucose amount was higher than fructose amount; it reached 11.55 and 1.99 g/100g, respectively. Then, the previous authors defined that carbohydrate content in bee bread is increased due to the addition of nectar, honey and sucrose (1.49g/100g) and maltose (1.24g/100g) with the fresh pollen basal of bee bread. Regarding mineral composition of bee bread (Table 5), the most minerals in the sample were phosphorus (246.96 mg/100 g of BB), followed by calcium (189.55 mg/100 g of BB) and zinc (3.37mg/100 g of BB). Our results agree with those of Bakour et al. (2019) who reported that analysis minerals of BBE included Ca, Zn and P up to 198.00, 3.31, and 251.00 g/100g, respectively. Also the same authors defined that anther minerals included Na, K and Mg which reached to 14.20, 338.00 and 61.00 mg/100 g BBE, respectively. In a study of Andjelkovic et al. (2012) mineral content of BBE revealed that the average predominant mineral was potassium (0.74%) and phosphorus (0.65%) followed by calcium (0.65%). Also they reported that the average value minerals of Mg, Fe, Zn and Mn were 0.27, 121.99, 44.09, and 29.92 mg/kg, respectively. Furthermore, Adaškevi˘ciute et al. (2019) indicated that the highest average mineral levels in BBE were P (2265 mg/kg) followed by K (1142mg/kg) and Ca (558 mg/kg) while, average minerals were 345.00, 51.00, 28.00, 19.00, 22.00, 8.80, 0.50, 0.02, 1.00 and 0.23mg/kg for Mg, Fe, Na, Mn, Zn, Cu, Cr, Cd, Ba, and Pb, respectively. According to Mohammad et al. (2020a) found that the average mineral contents as Ca, Fe, K, Mg, Mn, Na, Zn, P and Se were 1547.31, 126.43, 652.49, 1635.40, 61.66, 139.70, 60.62, 6402.28 and 0.26 mg/kg, respectively. Table (5), shows that amounts of vitamins E and C were 89.24 and 1043.60 mg/100g in BBE, respectively. Similarly, Del-Risco et al. (2012) recoded that range average of vitamin C was (6 -2000 mg/100g) however, Hryniewicka et al. (2016) found that range average of vitamin E was 34.6 to 103.6 mg/100g in BBE then, those findings have supported the results in this study. Furthermore, the different findings of vitamins values between samples could be attributed to the different floral sources (Bleha et al., 2019). From Table (5), it can be seen that BBE is contained conditionally essential amino acids such as arginine (2.33g/100g). Similarly, Mo-

hammad et al. (2020a) indicated that average concentrations of arginine in BBE of different BBE samples ranged between 1.959 and 2.660 g/100g which agrees with our study. Also, the previous authors indicated that the average of essential amino acids including phenylalanine, valine, histidine, methionine, isoleucine, leucine, threonine and alanine was 2.288, 1.083, 0.995, 0.445, 0.851, 1.690, 1.751 and 1.028 g/100g, conditionally essential amino acids as tyrosine, glycine, proline was 1.397, 1.314 and 1.573 g/100g and non-essential amino acids such as hydroxyproline, serine, glutamic acid, aspartic acid and lysine up to 0.456, 2.043, 1.632, 1.430 and 0.702 g/100g, respectively. Othman et al. (2019) recorded that the lowest arginine values ranged from 0.74 to 0.90 g/100g also, some amino acid contents such as alanine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, throsine and valine were ranged as 0.86-0.91,1.45-1.50, 2.00-2.10, 0.66-0.77, 0.35-0.49, 0.13-0.20, 0.65-0.74, 1.06-1.20, 0.85-0.93, 0.34-0.39, 0.67-0.77, 0.83-1.56, 0.74-0.81, 0.63-0.71, 0.35-0.48 and 0.78-0.84g/100g, respectively. Regarding the antimicrobial impact of the BBE sample is presented in Table (5). The findings indicated that all bacterial Spp. in ethanolic BBE with bactericidal concentration (MIC) values ranging between 0.027 mg/mL and 0.172 mg/mL for bacterial strains. The findings obtained from this study agreed with data from (Bakour et al., 2019), who reported that all bacterial strains were sensitive to the hydromethanolic BBE with MIC values ranging between 0.04 mg/mL and 0.175 mg/mL. Also, the previous authors revealed that a methanolic extract of bee bread samples showed antimicrobial effect against 4 bacterial strains included 2 Gram-positive (*Staphylococcus aureus* and *Bacillus thuringiensis*) and the other 2 Gram-negative (*Salmonella enterica* and *E. coli*). Also, the authors suggested that the antimicrobial action of bee bread is probably correlated to antioxidants compounds like phenolic compounds, especially flavonoids. On the other hand, Sobral et al. (2017) and Urcan et al. (2017) suggested that BBE may inhibit the growth of gram positive and negative bacteria resistant to antibiotics. All BBE samples against all tested bacteria had antibacterial effects (Pełka et al., 2021) revealed that the existence of lactic acid bacteria such as *Lactobacillus kunkeei* and *L. plantarum* in BB can produce metabolites (such as bacteriocins) that have strong antibacterial ability.

Sperm characteristics in post-diluted, equilibration, and thawing

Data is presented in either Figure 1 or Tables (6) or (7) discussed sperm characteristics post-diluted, equilibration, and thawing, respectively. Thus, sperm characteristics, including motility percentages, live, normal, and integrity acrosome post-diluted, which extended with E1, E2, E3,

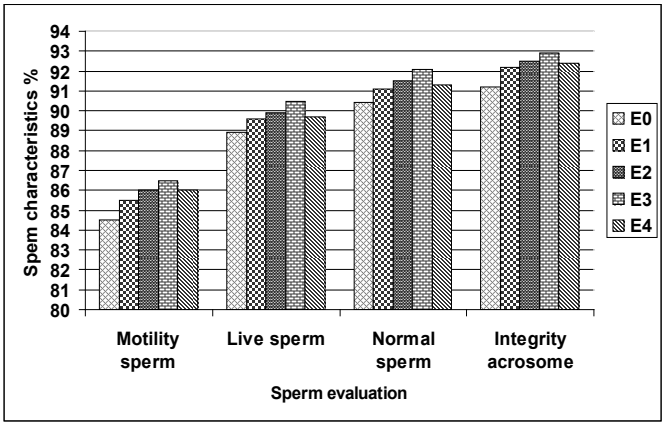

Figure 1: Sperm characteristics post- diluted with different levels of BBE

and E4, did not differ significantly from control semen extended (E0). However, semen extended in E1, E2, E3, and E4 extenders resulted marked increase (P>0.05) in all characteristics as compared to E0 extenders after dilution (Figure 1). Then, these results indicated to save the usage of BBE supplemented with 0.5, 1.0, or 1.5%; however, increasing the level of BBE to 2.0% in E4 extender harmed previous sperm characteristics. In post-equilibration and post-thawing proceeding, all sperm characteristics were significantly (P<0.05) higher than control extender (E0). Still, the best sperm functions were observed in E3 extender compared to E1, E2 and E4 extenders (Tables 6 and 7). Our results revealed that adding BBE at 1.5% can improve sperm quality post-diluted, equilibration and thawing. The success of semen cryopreservation depends on type semen dilution. Hence, Khalifa and Mahdy (2019) recorded that the beneficial effects of Tris egg yolk (TEY) extender in dilution of ram semen. However, the observed maintenance of sperm characteristics post-diluted, equilibration and thawing with BBE is not able to be used or obtained. In this respect, the best significantly increased mean of sperm function in ram semen extended with TEY extender containing 1.5 ml of BBE compared to 0.0, 0.5,1.0 and 2.0 % may be related to the exact focus of natural free sugars (Jesús et al., 2020) and essential amino acids (Omar et al., 2021). Also, Kaya et al. (2019) observed that arginine could increase sperm motility in the ram because of the mechanism action of arginine could be attributed to elevate ATP synthesis that supplied spermatozoa by energy. Furthermore, Mohammed et al. (2020a) suggested that arginine acts as antioxidant substance through its ability to remove different types of free radicals like reactive oxygen species (ROS) included superoxide anion (O_2^-), peroxide radical (H_2O_2), hydroxyl radical (OH^-) and activate generation of enzymatic antioxidant such as GPx, SOD and CAT that improved sperm function. On the other hands, macro- elements in BBE can be able to activate sperm function (Kerns et al., 2018) who revealed that elements are known to play an

Table 5: Analysis of bee bread ethanolic extracts which using in semen extenders

Ingredients				Values	
Carbohydrates, g/100g				71.09±0.24	
Fat,g/100g				1.85±0.009	
Protein, g/100g				17.24±0.09	
Energy, Kcal/100g				369.77±1.23	
Free sugars g/100g					
Fructose				12.44±0.11	
Glucose				5.69±0.17	
*Macro- elements mg/100g					
Ca^{++}				189.55±0.23	
Zn^{++}				3.37±0.05	
**Vitamins mg/100g					
Vitamin E (as Tocopherol)				89.24±0.47	
Vitamin C				1043.60±23.32	
***Conditionally essential amino acids, g/100g					
Arginine				2.33±0.04	
****Antibacterial activity by minimal bactericidal concentration (MBC), mg/mL					
B. cereus	*S. aureus*	*L. monicytogenes*	*E. coli*	*En. cloacae*	*S. typhimurium*
0.027±0.003	0.172±0.006	0.171±0.0007	0.23±0.008	0.170±0.0007	0.171±0.0001

*Mineral composition of BBEwas determined by (AOAC, 2016).
** The methods of vitamins analysis were described by Hryniewicka et al. (2016).
*** The conditionally essential amino acids suggested by the methods of Kieliszek et al. (2018).
****The antibacterial activities were evaluated according to the procedure of Akhir et al. (2017).

Table 6: Sperm characteristics post- equilibration time with different levels of BBE

Sperm characteristics	Types of extenders with different levels of BBE				
	E0	E1	E2	E3	E4
Motility	81.50±1.30^{bc}	83.50±0.76^{ab}	84.50±0.89^{a}	85.50±0.50^{a}	83.00±0.82^{b}
Live	83.90±0.50^{bc}	85.40±0.43^{b}	86.20±0.33^{b}	86.60±0.60^{a}	85.10±0.38^{b}
Normal	84.40±0.64^{bc}	85.90±0.28^{b}	86.60±0.34^{b}	88.20±0.44^{a}	85.40±0.31^{b}
Integrity acrosome	86.10±0.37^{bc}	87.90±0.60^{ab}	88.60±0.69^{ab}	89.70±0.73^{a}	87.20±0.57^{b}

Means denoted within the same row with different superscripts are significantly different at P<0.05.

Table 7: Sperm characteristics post-thawing with different levels of BBE

Sperm characteristics	Types of extenders with different levels of BBE				
	E0	E1	E2	E3	E4
Motility	40.22±1.88^{b}	42.50±0.83^{b}	43.50±1.51^{b}	47.50±1.12^{a}	39.55±1.38^{bc}
Live	49.78±1.88^{d}	56.90±0.98^{bc}	61.10±1.23^{b}	65.10±1.09^{a}	55.60±1.00^{bc}
Normal	55.89±1.29^{bc}	60.70±1.41^{c}	63.60±1.01^{ab}	65.66±1.08^{a}	58.80±1.06^{c}
Integrity acrosome	59.22±1.09^{d}	64.10±1.22^{bc}	67.15±1.17^{ab}	69.67±0.95^{a}	61.83±0.96^{cd}

Means denoted within the same row with different superscripts are significantly different at P<0.05.

Table 8: Assaying of ATP, BC, and LPO in semen extenders after dilution

Item levels	Types of extenders with different levels of BBE				
	E0	E1	E2	E3	E4
ATP, µM	0.25±0.00^{d}	0.51±0.00^{c}	0.56±0.00^{b}	0.66±0.01^{a}	0.67±0.00^{a}
BC, CFU/ml	210.80±1.14^{a}	204.60±0.55^{b}	203.60±0.51^{b}	202.20±0.58^{c}	201.40±0.81^{c}
LPO, µM	1.27±0.03^{a}	1.25±0.00^{a}	1.23±0.00^{b}	1.21±0.00^{c}	1.20±0.00^{c}

Means denoted within the same row with different superscripts are significantly different at P<0.05.

Table 9: Assaying of ATP, BC, and LPO in semen extenders after equilibration

Item levels	Types of extenders with different levels of BBE				
	E0	E1	E2	E3	E4
ATP, µM	0.27±0.01[c]	0.57±0.01[b]	0.63±0.02[b]	0.69±0.01[a]	071±0.00[a]
BC, CFU/ml	238.00±1.18[a]	228.60±2.46[b]	225.60±0.53[b]	220.80±0.97[c]	220.20±1.28[c]
LPO, µM	1.91±0.02[a]	1.79±0.02[b]	1.75±0.01[b]	1.71±0.01[c]	170.00±0.00[c]

Means denoted within the same row with different superscripts are significantly different at P<0.05.

Table 10: Assaying of ATP, BC, and LPO in semen extenders after thawing

Item levels	Types of extenders with different levels of BBE				
	E0	E1	E2	E3	E4
ATP, µM	0.19±0.01[c]	0.27±0.01[b]	0.31±0.02[b]	0.35±0.01[a]	0.36±0.01[a]
BC, CFU/ml	276.00±1.55[a]	269.80±1.93[a]	264.60±2.54[b]	255.40±2.44[c]	254.20±3.64[c]
LPO, µM	2.89±0.03[a]	2.71±0.02[b]	2.68±0.01[b]	2.61±0.04[c]	2.59±0.02[c]

Means denoted within the same row with different superscripts are significantly different at P<0.05.

Table 11: Effect of the E0 or E3 extenders on fertility and productive performance after AI of semen thawing

Assaying of pregnancy and lambing rates	Extender groups	
	E0	E3
Fertility rate		
Total ewes inseminated	13	13
Pregnancy rate, %	6/13= 46.15[b]	7/13=53.85[a]
Productive performance		
No. of ewes birth single	5	5
No. of ewes birth twins	1	2
No. of male lambs	4	5
No. of female lambs	3	4
Total No. of lambs	7	9
Lambing rate, %	7/13=53.85[b]	9/13=69.23[a]

E0 is a control extender with 0.00% of BBE. E3 is a trial extender with 1.5% of BBE.
Means denoted within the same column with different superscripts are significantly different at P<0.05.

important role in reproduction performance also, zinc is a crucial element for reproduction, and its lack can result in degenerative changes sperm activity. Also, Zakošek et al. (2021) had shown that enrichment of a sperm extender with calcium (Ca^{++}) to raise sperm quality post-hawing and consequently improves fertility by decreasing cryopreservation-induced sperm damage. In addition, Pinto et al. (2020) extender's supplementation with Vitamin C has shown positive impacts on sperm motility and preservation of plasma and acrosomal membranes throughout semen cryopreservation. Also, Espina-Ávila et al. (2021) reported that Adding vitamins immediately to semen extenders enhanced the properties of the semen after thawing.

Hence, this study proved negative values when the concentration of BBE increased up to 2.00 ml (in E4 dilute) which may be due to either increasing level of carbohydrates that affect the osmotic pressure of dilute. These findings are in close agreement with Herdis et al. (2019) who reported that lowering the solution osmotic pressure used in the preparation of the extender had a considerable positive impact on the plasma membrane integrity and the quality of the cryopreserved semen compared to the highest osmotic pressure. Thus, these authors recorded that average of ram plasma membrane intact post-thawing was 62.33, 58.50 and 56.40% in dilute which has sucrose concentration up to 2, 4 and 6 g /100ml, respectively. On the other hand, Mughal et al. (2018) recorded that plasma membrane integrity post-thawing with osmotic pressures at 275 and 295 mOsm/kg was 61.42 and 56.67 %, respectively. Also, minerals play significant roles in many physiological functions but required in small amounts (Shahin et al., 2020) proved that supplementation of minerals is

a useful strategy for enhancing spermatozoal integrity for cryopreservation steps. Additional of minerals with limited required during cryopreservation steps had improved physiological sperm functions including viability, motility and morphology of post-thaw semen (Bustani et al., 2021). Supplementing vitamins up certain level to the extender could ameliorate the quality sperm by protecting spermatozoa against damages during cryopreservation. However, sperm factors, including motility, morphology, viability and DNA integrity were usefully decreased following adding unsuitable levels of vitamins because of high levels of vitamins may be affected on sperm quality negatively. In agreement with our results of Asadpour et al. (2021), who demonstrated that vitamins levels at 0, 5 and 10 ng/ml could obtain the percentage sperm viability after freeze-thaw up to 57.00, 45.67 and 37.67%, respectively. Otherwise, the highest level of amino acids (like arginine) may be affected by the spermatozoa lifespan. Actually, arginine metabolism resulted from the effect arginase enzyme that leads to production of nitric oxide (No) and urea hence, it has been reported that low concentration of arginine increases sperm activity, whereas high arginine concentration decreases sperm activity. In confirmation, Omar et al. (2021) determined that percentage of individual motility was 56.66, 52.50 and 40.00% and percentage of dead sperm was 27.00, 31.83 and 37.50% after dilution ram semen with arginine levels at 0.001, 0.100 and 1.000µmol, respectively. Also, the same authors defined that ram sperm motility was 30.00, 19.11 and 13.33% and dead sperm was 42.00, 54.33 and 65.00% after cooling at 5°C up to 72 hours when arginine was added to extended semen at 0.001, 0.100 and 1.000µmol, respectively.

Assaying of ATP, BC, and LPO in semen extenders

Assaying of ATP, BC, and LPO parameters in different semen extenders after dilution, equilibration, and thawing are explained in Tables (8), (9), and (10), respectively. This work showed significant change (P<0.05) with E1, E2, E3, and E4 extenders in ATP, BC, and LPO concentrations after dilution, equilibration, and thawing when compared to E0 semen extender. However, after dilution, equilibration, and thawing, the ATP level was significantly higher (P<0.05), but BC and LPO were (P<0.05) lower in either E3 or E4 extenders than in either E1 or E2 or E0 semen extenders. Hence, it should be emphasized that ATP, BC, and LPO parameters in E3 or E4 had significant (P<0.05) change with increasing BBE in semen dilution at 1.5 and 2.0%, respectively. Hence, the present study indicated that supplementation of the extender with different levels of BBE amplified the level of ATP in E1, E2, E3, and E4 extenders compared to E0 extender during freezing processing. The ATP level depends on the dose of energy sources which addition to extender (Table 5). These results align with the findings of several authors (Bakour et al., 2019; Dranca et al., 2020; Mohammad et al., 2020b). The previous authors reported positive effects of BBE on ATP activity of solutions. Otherwise, the superior to all BBE extenders specially E3 and E4 compared to E0 extender may be due to the presence of Ca^{++} in BBE which has positive effect on the mitochondria metabolic rate thus enhancing the enhancement of generating a higher ATP. These results are equivalent to the current study that recorded increasing in Ca^{++} concentration in semen extender enhances cyclic guanosine monophosphate (cGMP) synthesis in the spermatozoa mitochondria thus leading to an enhancement of the metabolic rate which had a higher ATP level (Zakošek et al., 2021). Moreover, it has been reported that arginine in BBE has significant increase in ATP, which is thought to be associated with an increase in glycolysis rate resulting in higher ATP rates (Omar et al., 2021). Regarding to antimicrobial activity, it may be seen that all the extracts under study possessed antimicrobial activity. The results observed that a significant reduction in the number of viable bacteria after BBE addition to E1, E2 , E3 and E4 extenders as compared with the E0 extender. Differently from the results hereby reported and to the described studies mentioned above, Combarros-Fuertes et al., (2020), who found that BBE exhibits antimicrobial properties against diverse pathogens such as bacteria and fungi. Then, the antimicrobial resistance might be attributed to the synergy of more than one antimicrobial compounds within bee bread (Brudzynski et al., 2021). Also, De Arrudaa et al. (2021) found that these biological properties are attributed to physical and chemical compounds such as high phenolic and flavonoid contents. In addition, Pełka et al. (2021) observed that the ethanolic extract inhibited *S. aureus* at 90%, inhibited the microorganisms *Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella spp* and 5% of BB (in 70% ethanol) was efficient against on *Streptococcus mutans, Streptococcus salivarius, Streptococcus mitis, and Lactobacillus casei*. Our data discusses the effect of BBE addition to extender on malondialdehyde (MDA) as a LPO marker in cryopreserved ram spermatozoa. It revealed that BBE at levels 0.50, 1.00, 1.5 and 2.00% has a negative severe effect on LPO concentration during processing frozen steps of ram semen extender compared to E0 extender. Further, the positive effect of BBE as an antioxidant activity on semen extender may be attributed to the existence of amino acid (arginine) and vitamins (E and C) in BBE (Table 5). It has been proposed that the beneficial effects of arginine are linked to nitric oxide thereby decreasing lipid peroxidation (Badr et al., 2020). Also, Omar et al. (2021) showed that arginine prevents lipid peroxidation under different peroxidation conditions and it acts as an antioxidant by absorption of inactivating superoxide (O_2) anions, thereby scavenging free radicals. Also, vitamins of BBE play an important role as antioxidant activity in semen extender

(Asadpour et al., 2021). The previous authors defined that vitamins influence generation of oxygen free radicals and improve antioxidant defenses. In addition, Shahin et al. (2020) stated that mineral can also neutralize the effects of ROS and increases antioxidant enzymes as mediated through glutathione peroxidase enzyme activity, which is an important antioxidant.

Fertility and productive performance

Table (11) revealed the fertility rate and reproductive performance of the thawed ram semen without BBE (extender E0) or plus 1.5% of BBE (extender E3). Hence, the concentration of 1.5% BBE in semen extender was used in a fertility experiment because it was the highest treatment in sperm characteristics post-thawing (Table 7). Also, treatment of E3 extender containing 1.5% BBE is considered the second best treatment in ATP, BC, and LPO after thawing (Table 10). Therefore, the E3 extender showed a significantly ($P<0.05$) higher effect on the pregnancy rate of ewes. It was up to 53.85% than the E0 extender, which reached 46.15%. Also, there was a significantly ($P<0.05$) lower lambing rate of E0 (which has 7 lambs and a lambing rate of 53.85%) than E3 extender (which has 9 lambs and a lambing rate of 69.23%). Meanwhile, adding 1.5% of BBE in the E3 extender could improve semen motility, viability, and fertilizing ability of post-thawing ram semen but maintain the acrosomal integrity compared to the control semen in E0 extender. These results are in agreement with the findings of several authors (Khalifa et al., 2019; Shahin et al., 2020; Badr et al., 2020; Espina-Ávila et al., 2021). They emphasized that the principal factor for AI was the best semen extenders which refluxed the most sperm characteristics and led to the greatest fertility rate and productive performance.

CONCLUSION

The results of this study demonstrate that supplying semen extender with 1.5% of BBE can only make available energy, antibiotic and antioxidant sources successfully during the preservation of ram semen without any adverse effects on sperm characteristics. Also, 1.5% of BBE has benefical effects on the spermatozoa's quality and fertilizing potential. Hence, these constructive effects of BBE appear due to energy supply and the domination of antibacterial or antioxidant activities by reinforcing ATP and lessening rates of bacterial count or lipid peroxidation of preserved ram spermatozoa.

Ethics statement

All research procedures were carried out in compliance with the standards set forth guidelines for the care and use of experimental animals by the Animal Ethics Committee of APRI, ARC, Egypt.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

NOVELTY STATEMENT

An attempt to find natural and safe sources of energy, antibiotics, and antioxidants as bee bread extract instead of synthetic sources in the ram's semen extender during the freezing process.

AUTHOR'S CONTRIBUTION

E.I. Khalifa conceived the presented idea and carried out the experiments, collected and cured the data. A.I.L. Desoky developed the theory, has revised the experimental design, and wrote the manuscript with input and support from all authors. A.A. Elbadawy has planned the study, performed the experimental procedures, cured the data, performed the data analysis, prepared and revised the manuscript. G.I. El-Emam worked out almost all of the technical details, and performed the numerical calculations for the suggested experiment. T.M.M. Mahdy supervised the experimental procedures, revision the manuscript and paraphrase some paragraphs. All authors discussed the results, provided critical feedback, helped shape the research, and contributed to the final manuscript.

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