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Research article

Preservability of buffalo bull semen in tris-citrate extender enriched with bee's honey

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Abstract

Honey produced by *Apis mellifera* lamarckii is a natural supersaturated sugar solution with important nutritive ingredients. The present study aimed to improve semen preservability through the use of honey as an extender additive expressed in chilled and frozen semen of native buffalo bulls. Three mature buffalo bulls were used for semen collection. Semen samples were diluted with TRIS extender (control) and TRIS-Honey bee extender (THB-1%, THB-2%, THB-3%, THB-4%, THB-5%). Extended semen was slowly cooled, packed into 0.25 ml straws and frozen in liquid nitrogen. A fraction of extended semen from control TCFY and each concentration of THB were kept at 5°C for 7 days (chilling) and sperm motility was evaluated daily. The parameters studied were subjective semen characteristics (motility, alive, abnormality and sperm membrane integrity using hypoosmotic swelling test (HOST) %). Results revealed that sperm motility % of a THB-1% was the best after 7 days of chilling (60.00%), while THB-2% concentration was the best after thawing (55.00%). Finally, it is concluded that, addition of 1% (in chilling) and 2-5% (in freezing) of honey solution to semen extender enhanced the main sperm characteristical parameters of buffalo bull semen.

Key words: Honey, buffalo, Semen, Cryopreservation, Natural extender.

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1. Introduction

Semen as a male high valued product had likely inspired the investigators to found methods for its preservation for a long-term survival to be used on demand [1]. Nowadays, Sperm cryopreservation and storage are of a great challenge for conserving the super genetic origins of the males, the expansion of artificial reproductive technologies such as artificial insemination (AI) and in vitro fertilization (IVF) [2] and clinical medicine. AI with frozen semen is essential in breeding and selection schedules contributing to increase production of domestic species. In the Holy Qur'an, honey was mentioned as curative for people" *And your Lord inspired to the bee, "Take for yourself among the mountains, houses, and among the trees and [in] that*

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which they construct. Then eat from all the fruits and follow the ways of your Lord laid down [for you]."There emerges from their bellies a drink, varying in colors, in which there is healing for people. Indeed in that is a sign for a people who give thought. [Surah an-Nahl (the Bee), Versus 68-69]". According to his teachings (hadith), Muhammad [O Allah confers blessing and peace upon him] strongly recommended honey for healing purposes [3. Investigators had reported [4] that honey produced by the honey bee is a natural supersaturated sugar solution, which is derived from Nectar [specialized cell groups named *nectaries* or *floral,* reviewed in Ball [5], mainly composed of a complex mixture of carbohydrates, proteins, enzymes (invertase, glucose oxidase, catalase, phosphatases), amino acids, organic acids (gluconic acid, acetic acid), lipids, vitamins (ascorbic acid, niacin, pyridoxine), volatile chemicals, phenolic acids, flavonoids and minerals. This chemical composition depends on the plant species visited by the honeybees, the environmental, processing and storage conditions [6]. Some of the components were used solely as a source of energy in a semen extender (glucose, fructose and sucrose) [7-9], some as antioxidants (catalase, amino acids) [10-12]. Ascorbic acid was also tried [13]. Then, the present study aimed to improve semen preservability through the enrichment of a basal semen extender with variant concentrations of honey and their effects on buffalo bull spermatozoa main characteristics during chilling and after freeze-thawing.

2. Materials and Methods

TRIS Base Extender

Tris-citric acid-fructose egg yolk (TCFY) diluent was used as control extender [14-15]. TRIS-Honey Bee (*Apis mellifera* lamarckii) Extender (THB): Honey solution was prepared by adding 1 honey to 9 distilled water (v:v) to obtain a honey solution of 10% concentration. The later solution is added to TCFY (v/v) in concentrations 0.5/4.5 (THB-1%), 1/4 (THB-2%), 1.5/3.5 (THB-3%), 2/3 (THB-4%), 2.5/2.5 (THB-5%) ml (v/v) to obtain a final volume of 5 ml in each tube [16].

Semen Collection and Initial Evaluation

Three mature buffalo bulls maintained at Semen Freezing Center, General Organization for Veterinary Services, Ministry of Agriculture, Abbasia, Egypt, were used as semen donors. Ejaculates were collected using a bovine adapted artificial vagina, early in the morning, at weekly intervals for 3 weeks. Semen samples were transferred in seconds to the lab. to be evaluated for subjective sperm motility and sperm concentration. Ejaculates fulfilling minimum standard of 70% sperm motility and sperm morphology, are pooled in order to have sufficient semen and to eliminate the bull effect. The semen was given a holding time for 10 minutes at 37°C in the water bath before the extension.

Semen Processing

Semen samples were extended with TCFY extender (control) and other aliquots of pooled semen samples were extended with the different concentrations of THB solution in order to provide a final concentration of 60 million sperm/ml for the extended semen. The later was slowly cooled (approximately for 2 hrs) to 5°C and equilibrated for a further 2 hrs. Semen was packed into 0.25 ml polyvinyl French straws. After equilibrium periods, the straws were horizontally placed on a rack and frozen at 4 cm above liquid

nitrogen (in its vapour) for 10 minutes and then dipped patiently in liquid nitrogen. A fraction of extended semen from control TCFY and each concentration of THB were kept at 5°C for 7 days (chilling) and sperm motility was evaluated daily.

Assessment of Semen Quality Parameters:

The assessment was undertaken on after freeze thawing of bull spermatozoa. Also, sperm motility was evaluated for raw semen, 2 hours after cooling and chilled semen daily up to 7 days. Frozen straws were thawed at 37°C for 1 minute. The parameters studied were subjective semen characteristics (motility, alive, abnormality and sperm membrane integrity using hypo-osmotic swelling test (HOST) %) [17].

Statistical Analysis:

Statistical analysis data were analyzed using the SPSS [18] computerized program v. 14.0 to calculate the analysis of variance (ANOVA) for the different parameters between control and additives replications. Significant differences between means was calculated using Duncan's multiple range test at $p<0.05$ [19].

3. Results and Discussion

After 7 days of chilling, THB-1% proved its priority on the other THB concentrations as it maintained sperm motility % (60.00±2.89%) significantly ($P<0.0001$) higher than the control and other THB concentrations exclusively [Table 1]. On the other hand, the use of THB-2% had significantly enhanced the percentage of motility, intact sperm membranes and alive sperms (55.00 ± 2.89% ($P<0.0325$), 83.33±1.67% ($P<0.0001$) and 71.11 ± 1.11% ($P<0.0001$), respectively) after thawing, meanwhile, THB-3%, THB-4% and 5% had maintained sperm motility percentage significantly ($P<0.0325$) higher than the control and THB-1% treatments. Also, the THB-3% improved the sperm membrane integrity (83.89 ± 2.00%) compared to the control (74.38±2.37%) [Table 2]. Concerning the after thawing abnormal sperm percentage, the control had recorded significantly the best (9.46±0.54) result compared to the other enrichments [Table 2].

Table 1. Effect of Honey enriched Tris diluent on the motility of cooled and chilled buffalo bull extended semen

Time after 0 hours of chilling / Treatment	2 Hours	7 days
Control	78.75 ± 5.54^{A}	41.67 ± 1.67^{B}
THB-1%	88.75 ± 2.39^{A}	60.00 ± 2.89^{A}
THB-2%	82.50 ± 1.44^{A}	26.67 ± 4.41^{C}
THB-3%	91.25 ± 2.39^{A}	23.33 ± 3.33^{C}
THB-4%	86.25 ± 3.15^{A}	0.00 ± 0.00^{D}
THB-5%	78.75 ± 3.15^{A}	0.00 ± 0.00^{D}
F-value	2.56	79.43
P<	0.064	0.0001

Means with different superscripts are significantly different using Duncan' multiple range test at $P<0.05$.

Table 2. Effect of Honey enriched Tris diluent on the after thawing characters of buffalo bull extended semen

Parameter / Treatment	Motility %	HOST %	Alive %	Abnormality %
Control	37.50 ± 4.79^{B}	74.38 ± 2.37^{B}	71.94 ± 1.94^{A}	9.46 ± 0.54^{C}
THB-1%	35.00 ± 5.00^{B}	54.44 ± 0.56^{C}	49.58 ± 0.42^{C}	21.67 ± 1.67^{B}
THB-2%	55.00 ± 2.89^{A}	83.33 ± 1.67^{A}	71.11 ± 1.11^{A}	19.07 ± 1.60^{B}
THB-3%	47.50 ± 4.33^{AB}	83.89 ± 2.00^{A}	61.11 ± 1.11^{B}	25.00 ± 2.89^{B}
THB-4%	47.50 ± 4.79^{AB}	76.67 ± 1.67^{B}	62.78 ± 1.47^{B}	34.22 ± 3.73^{A}
THB-5%	45.00 ± 2.04^{AB}	77.06 ± 1.51^{B}	71.98 ± 1.14^{A}	21.41 ± 1.41^{B}
F-value	3.15	39.09	47.63	13.02
P<	0.0325	0.0001	0.0001	0.0002

Means with different superscripts are significantly different using Duncan' multiple range test at P<0.05.

The artificial insemination is a branch of biotechnology that proved its enduring foot till now in the animal reproduction and fertility domain. The incorporation of natural additives in semen extender, to get a powerful extender, blow life into this field of investigation as it takes special interest in the development of semen diluent [20-24]. The use of honey was one of those additives. The present study exhibited good sperm motility for chilled semen on using THB-1%, so it can be used in AI up to 7 days of chilling. While, Addition of honey solution in a concentration of 2% and 3% (THB-2% and THB-3%, respectively) followed by 4-5% (THB-4% and THB-5%, respectively) gave superior post thawing semen characteristics as represented by post thawing motility and a percent of intact sperm membranes. The previous results could be related to the strong antioxidant property of honey as it contains a mixture of carbohydrates, proteins, enzymes, amino acids and organic acids, vitamins, phenolic acids and flavonoids [4, 25] and also to the cryoprotectant properties of glucose, fructose and sucrose used at a percent of 1% up to 2% maximum [26]. In a human investigation, Fakhrildin and Alsaadi [27] reported that the use of 10% enriched honey cryoprotectant had maintained the semen quality after thawing as good. They ascribed their results to the presence of fructose, glucose and sucrose and other sugars in honey that may protect sperm during cryopreservation and enhanced sperm parameters. They also concluded that 5% enriched honey cryoprotectant didn't differ than the control cryoprotectant group. In another study, the addition of 1-2% honey to a boar semen extender (based on glucose, sodium bicarbonate, sodium citrate and EDTA) proved its effectiveness in preserving boar semen [28]. These results were in agreement with our results. This may be due to the high energy content in honey sugars (glucose, fructose, sucrose, maltose) which represent 80% of the honey's content, besides, the content of antioxidants and antimicrobial agents.

Israili [29] reviewed that the clinical studies revealed the broad-spectrum antimicrobial properties of the honey. This may be attributed to the acidity, osmotic effect, high sugar concentration, presence of bacteriostatic and bactericidal factors (hydrogen peroxide, antioxidants, lysozyme, polyphenols, phenolic acids, flavonoids, methylglyoxal, and bee peptides), this illustrated that honey enriched extender is in no need of external antimicrobial addition. A previous study was conducted on the cattle semen preservation with THB with the same concentrations [16]. There was a difference in the response of the semen to THB extender between the two species of animals that may be attributed to the difference between them. The best result in cattle was coincided with the addition of 1% honey to the extender and its best results obtained in chilled and frozen extended semen. In conclusion, the addition of honey solution [1% (in chilling) and 2-5% (in freezing)] to tris-citrate semen extender enhanced the sperm parameters of buffalo bull semen.

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