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Does natural honey act as an alternative to antibiotics in the semen extender for cryopreservation of crossbred ram semen?

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Summary

Antibiotics are added to semen extenders to take care of heavy microbial load, however, their continuous use poses a constant threat of developing antibiotic resistance by the common microbes present in the semen. Our hypothesis was that natural honey, having antibacterial activity and rich in fructose could replace the use of antibiotics and fructose in the semen extender. Twenty-four ejaculates from six crossbred rams were obtained and extended with tris-based extender without (control) and with honey at 2.5% (T1), 5% (T2) and 7% (T3). Sperm quality was measured in terms of percentage sperm motility, live sperm count, intact acrosome and hypo-osmotic swelling test (HOST) reacted spermatozoa. The semen samples at post-thaw were also evaluated for total viable count (colony forming units/ml). At post-thaw, control exhibited significantly ($P<0.05$) higher sperm motility in comparison to T2 and T3. The percent of live sperm count, intact acrosome and HOST reacted spermatozoa were significantly higher ($P<0.05$) for control than all other treatment groups at post-thaw. Among treatment groups, T1 maintained significantly higher ($P<0.05$) percentage of live sperm count, intact acrosome and HOST reacted spermatozoa than T2 and T3. The total viable count at post-thaw was significantly lower ($P<0.05$) for control than all the treatment groups. In conclusion, honey cannot be used as an alternative to antibiotics to take care of heavy microbial load in semen, however, levels up to 2.5% may be supplemented to semen as an energy source.

Key words: Antibiotics, Colony forming units, Cryopreservation, Honey, Ram

Introduction

Artificial insemination (AI) with frozen semen has been the most widely used reproductive technique to extend the use of superior sires to bring about rapid genetic improvement in livestock species (El-Sheshtawy *et al*., 2016). However, its success is related to the quality of semen used (Hossain *et al*., 2011). The microbials gain access to the raw semen from the reproductive tract during collection and processing (Althouse and Lu, 2005). If left unchecked, their presence can severely affect quality through competition for nutrients with the spermatozoa or through production of harmful products (Catry *et al*., 2010; Morrell and Wallgren, 2014). Higher bacterial load in the extended semen results in reduced sperm viability and motility, increases in the proportion of spermatozoa with defective acrosome (Althouse *et al*., 2000; Ortega-Ferrusola *et al*., 2009; Kustar and Althouse, 2016). In addition, there may be early embryonic death, endometritis or systemic ailment in the animal to be inseminated with such contaminated frozen semen (Maes *et al*., 2008). Therefore, antibiotics are added to the semen extender to limit the growth of bacterial contamination in the ejaculate prolonging the shelf life of semen and reducing the transmission of pathogenic microbes into the female tract (Morrel and Wallgren, 2011; Schulze *et al*., 2016). However, their continuous use could be detrimental as the bacterial population may gradually become resistant, which may lead to deterioration of sperm quality and disease in the female (Schulze *et al*., 2016). It has been recently reported that more than 90% of the bacteria isolated from the extended semen sample are resistant to the routinely used antibiotics in the semen extender (Bolarin, 2011; Bussalleu and Torner, 2013). Therefore, agents or strategies other than antibiotics may prove useful in limiting microbial population in semen (Morrel and Wallgren, 2011). Physical removal of the bacteria by Single Layer Centrifugation (SLC) (Morrell and Wallgren, 2014) and inclusion of antimicrobial peptides (AMP) in semen diluents (Bussaleu *et al*., 2017; Schulze *et al*., 2016) have been tried in order to limit the microbial population in the semen with limited success. Physical removal of bacteria either by SLC or density gradient centrifugation (DGC) is a tedious and stressful process affecting the keeping quality of spermatozoa over a long period of time. Further, inclusion of AMPs in diluents have been found cytotoxic at higher

concentration and lower concentrations were not effective in controlling microbial growth (Schulze *et al*., 2016). Moreover, some AMPs have shown good promise in maintaining viability but could not maintain progressive motility (Bussalleu *et al*., 2017) which is the most fundamental for sucessful fertilization. Therefore, supplementation of an agent in the extender that can overcome the threat of antibiotic resistance and at the same time would maintain the sperm motility by taking care of the bacterial load in the semen may prove a very useful strategy for successful preservation of semen for years to come. To this, honey, as a natural product has been studied as a supplement with different aims like antioxidant, cryoprotectant, and energy source for improvement of post-thaw semen quality in bull (El-Sheshtawy *et al*., 2014; Yimer *et al*., 2015), ram (Jerez-Ebenesperger *et al*., 2015), buck (Olayemi *et al*., 2011), buffalo bull (El-Nattat *et al*., 2016) and human (Tartibian and Maleki, 2012; Fakhrildin *et al*., 2014). Honey also possesses antibacterial activity (Molan and Russell, 1988; Aljady *et al*., 2000; Taormina *et al*., 2001; Al-Waili, 2004; Zoheir *et al*., 2015) besides containing high concentration of D-glucose and D-fructose (Nayik *et al*., 2015) as an energy source. The antibacterial activity of honey is attributed to the presence of bee defensin-1 and methylglyoxal (Molan, 1992; Kwakman and Zaat, 2012), presence of high content of sugars and more importantly to the generation of hydrogen peroxide when diluted due to conversion of glucose into hydrogen peroxide by glucose oxidase enzyme present in all types of honey (Aurongzeb and Azim, 2011). Thus its inclusion in the semen extenders may replace the use of fructose and antibiotics in the extender. This is a new kind of work in which suitability of honey as a replacement to antibiotics and fructose has been studied.

Materials and Methods

Chemicals and honey

Chemicals used were purchased from Merck (India Pvt Ltd.) and Himedia (India Pvt Ltd.). Natural pure honey harvested aseptically in a sterilized container was procured from State Agricultural Department (Srinagar, J&K, India).

Selection of animals, collection and cryo-preservation of semen

Crossbred rams (n=6) with good health condition were randomly selected during the breeding season and maintained under isomanagerial conditions throughout this study. Twenty-four ejaculates were collected in the morning by artificial vagina method. Bacterial contamination was minimized by thorough washing of the prepuce and trimming of preputial hair if any before semen collection on each day. Each collected semen sample was observed for consistency, volume, colour and presence of any foreign body. Normal ejaculates were initially diluted with the tris buffer (1:1) for recording of initial motility. Ejaculates that showed ≥70% initial sperm motility were pooled (total 6 pools) to eliminate the individual variation. For pre-freeze quality check, a portion of the pooled sample was taken in a separate tube and mixed with tris extender. The rest of the sample was quickly divided into four equal aliquots labelled as control, 2.5% (T1), 5% (T2) and 7% (T3). These aliquots were subsequently extended with tris extender without or with different levels of natural honey (Table 1). Natural pure sterile honey was first mixed with a portion of tris buffer and this mixture was then added to the final extender. The samples were then immediately placed in an ice chest. It took about 50 min for the semen samples to attain 4-5 °C and was considered as cooling. The distance between the divisional laboratory and frozen semen station is nearly 40 km and the time taken from the initial processing at the divisional laboratory to reach at the frozen semen station was about 3 h. The time excluding the period of cooling was included in the total equilibration period of 4 h and the amount of time left for equilibration was completed in cold handling cabinet at the station itself (Banday *et al*., 2017). After equilibration, the semen samples were loaded into French mini straws (0.25 ml) with an automatic filling and sealing machine (IMV Technologies, France). The concentration of sperm in each straw was set at 150 million. Vapour freezing of loaded straws was achieved in a Biological Programmable Freezer (IMV Technologies, France) with inside pressure maintained at 3 Bar. The freezing rate was set as: -5°C/min (from +4°C to -10°C), -40°C/min (from -10°C to -100°C) and -20°C/min (from -100°C to -140°C). The straws were then plunged deep in liquid nitrogen.

Table 1: Composition of tris extender for control and treatments

Components	Tris extender			
	Control	T1	T2	T3
Tris buffer	73 ml	70.5 ml	68 ml	66 ml
Fructose	1.25 g	-	-	-
Egg yolk	20 ml	20 ml	20 ml	20 ml
Glycerol	7 ml	7 ml	7 ml	7 ml
Penicillin G sodium	80000 IU	-	-	-
Streptomycin	100 mg	-	-	-
Honey	-	2.5 ml	5 ml	7 ml

Pre-freeze sperm quality evaluation

Sperm motility and live sperm count

A small drop (100 µL of diluted semen sample) was placed on a clean grease free slide kept on a biotherm stage and a cover slip was then placed on the drop. The slide was examined under ×400 magnification (phase contrast microscope, Nikon Eclipse E200, Japan). Three readings of three trained individuals were taken and an average value was considered as a final value of the sperm motility. The procedure for live sperm count by eosin nigrosin method is discussed elsewhere (Banday *et al*., 2017). After staining, a thin smear was prepared, air dried immediately as soon as possible and then observed at ×1000 magnification (phase contrast microscope, Nikon Eclipse E200, Japan). Colourless sperm were considered as live and completely or partially stained

were considered as dead. A total of 200 spermatozoa were counted and live sperm count percentage was determined.

Acrosomal integrity and hypo-osmotic swelling test (HOST)

The acrosomal integrity is generally evaluated by Giemsa staining method. The method is described in detail in our previous publication (Lone *et al*., 2012). The stained smear is observed at ×1000 magnification (Phase contrast microscope, Nikon Eclipse E200, Japan) to determine spermatozoa with percentage intact acrosome (PIA). Two hundred spermatozoa were counted randomly in different fields and PIA was determined. HOST was performed to assess the functional integrity of sperm plasma membrane. For complete procedure refer to Banday *et al*. (2017). After incubation, a small drop (15 µL) of sample was placed on a clean grease free slide, then a cover slip was put on top and it was immediately examined at ×400 magnification (Phase contrast microscope, Nikon Eclipse E200, Japan) for determination of HOST reacted spermatozoa. HOST reacted spermatozoa were identified by having highly coiled tails. Two hundred sperm were counted and percentage of HOST reacted spermatozoa was determined.

Post-thaw sperm quality evaluation

After a week, the straws were thawed individually in warm water bath maintained at 37°C for 30 s. The sperm quality was measured as in the case of pre-freeze. In addition to this, total viable count (CFU/ml) was also measured in post-thaw semen samples.

Total viable count (CFU/ml)

The microbial load of thawed semen samples from the control and treatment groups was determined as per the standard method described by Shukla (2005). Briefly, the semen straws were thawed in sterile water maintained at 37°C for 30 s. From each treatment, two straws were thawed to obtain semen volume of 0.5 ml. Each semen sample (0.5 ml) was diluted in 4.5 ml sterile normal saline. Ten (10) fold serial dilutions were made up to 10^{-4} dilutions and then 0.1 ml of sample from each dilution was inoculated in duplicate on sterile petri plates containing plate count agar. The petri plates were incubated for 24 h at 37°C followed by the subsequent examination of colony forming units. The plates were examined and the colonies were counted. Plates with 30-300 colonies were only considered and colony forming unit (CFU/ml) was determined as:

$$\text{Total viable count (CFU/ml)} = \text{average number of colonies formed} / (\text{dilution factor} \times \text{volume inoculated})$$

Statistical analysis

The percentage data of each group for sperm motility, live sperm count, intact acrosome, HOST reacted spermatozoa were first transformed to arc-sin data and then analyzed with one way ANOVA. However, non-transformed data of total viable count of treatment and control groups was also analyzed by one way ANOVA. Post hoc analysis was done by Tukey's HSD test. However, the difference in sperm quality between pre-freeze and post-thaw in each group for each parameter was analysed by paired t-test. All the statistical analysis was done by using SPSS, statistics-20. P-value ≤ 0.05 was considered significant. The data are presented in the tables as mean±SEM.

Results

Sperm motility and live sperm count

The sperm motility percentage at post-thaw for T1 was non-significantly (P>0.05) lower than control, but significantly (P<0.05) higher than other treatment groups. The sperm motility percentage declined significantly (P<0.05) from pre-freeze to post-thaw in all the treatment and control groups. The percentage of live sperm count at post-thaw for T1 was significantly (P<0.05) lower than control but significantly higher (P<0.05) than T2 and T3. The percentage of live sperm count declined significantly (P<0.05) from pre-freeze to post-thaw in all the treatment and control groups (Table 2).

Acrosomal integrity and hypo-osmotic swelling test

The PIA at post-thaw were significantly higher (P<0.05) for control than all the other treatment groups. Among the treatment groups, T1 had significantly higher (P<0.05) PIA than T2 and T3 at post-thaw. The PIA declined significantly (P<0.05) from pre-freeze to post-thaw in all the treatment and control groups. The percentage of HOST reacted spermatozoa at post-thaw was significantly higher (P<0.05) for control than all the other treatments. Among the treatment groups, T1 had significantly higher (P<0.05) number of HOST reacted spermatozoa than T2. The percentage of HOST reacted spermatozoa declined significantly (P<0.05) from pre-freeze to post-thaw in control and all the treatment

Table 2: Effect of different levels of honey on percentage of sperm motility and live sperm count (mean±SEM) of ram during cryopreservation

Treatments	Sperm motility (%)		Live sperm count (%)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Control	80.83 ± 1.53^{A}	55.83 ± 1.53^{aB}	85.08 ± 1.42^{A}	65.08 ± 2.00^{aB}
T1	80.83 ± 1.53^{A}	52.50 ± 1.11^{aB}	85.08 ± 1.42^{A}	57.25 ± 1.25^{bB}
T2	80.83 ± 1.53^{A}	37.50 ± 1.11^{bB}	85.08 ± 1.42^{A}	47.41 ± 1.55^{cB}
T3	80.83 ± 1.53^{A}	15.00 ± 1.29^{cB}	85.08 ± 1.42^{A}	30.08 ± 1.26^{dB}

Means with different superscripts in a column ($^{a, b, c, d}$) and row ($^{A, B}$) differ significantly (P<0.05)

Table 3: Effect of different levels of honey on PIA and HOST reacted ram spermatozoa (mean±SEM) of ram during cryopreservation

Treatments	Intact acrosome (%)		HOST reacted spermatozoa (%)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Control	93.16 ± 0.40^{A}	80.75 ± 0.62^{aB}	79.91 ± 1.34^{A}	60.33 ± 1.42^{aB}
T1	93.16 ± 0.40^{A}	76.50 ± 0.88^{bB}	79.91 ± 1.34^{A}	51.83 ± 2.27^{bB}
T2	93.16 ± 0.40^{A}	70.83 ± 1.06^{cB}	79.91 ± 1.34^{A}	42.91 ± 0.85^{cB}
T3	93.16 ± 0.40^{A}	67.75 ± 1.18^{dB}	79.91 ± 1.34^{A}	19.41 ± 0.85^{dB}

Means with different superscripts in a column ($^{a, b, c, d}$) and row ($^{A, B}$) differ significantly (P<0.05)

groups (Table 3).

Total viable count (CFU/ml)

At post-thaw, the total viable count was significantly lower (P<0.05) in control group (3500 ± 259) than all treatment groups (Fig. 1). Among treatment groups, T3 (7916 ± 219) maintained non-significantly (P>0.05) lower number of colonies than T2 (8316 ± 151) and T1 (8150 ± 158).

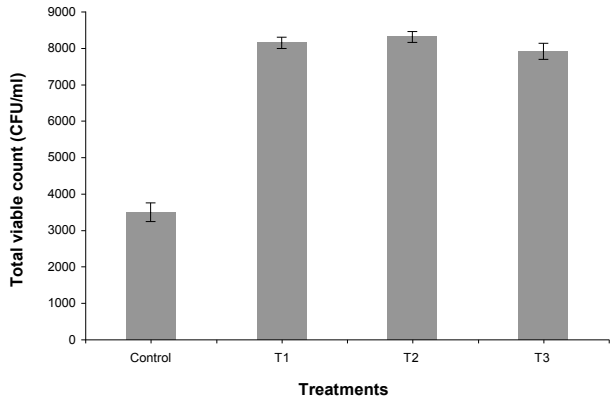

Fig. 1: Effect of different levels of honey on total viable count (CFU/ml) of ram semen at post thaw

Discussion

Scientists nowadays are making efforts to search for an alternative to antibiotics so as to stem the use of antibiotics in semen extenders. Honey, a naturally occurring product has potent antibacterial activity (Zoheir *et al*., 2015) and is a good source of energy to spermatozoa owing to the presence of glucose and fructose (Al-Waili, 2004). The percentage of sperm motility at post-thaw was significantly higher (P<0.05) for control than T2 (honey 5%) and T3 (honey 7%), but non-significantly higher than T1 (honey 2.5%). However, among treatment groups, T1 maintained significantly higher sperm motility and live sperm count at post-thaw than T2 and T3. The higher sperm motility in control may be ascribed to the presence of antibiotics which have inhibited the microbial growth. Microorganisms present in the semen reduce sperm motility, longevity and acrosomal integrity by directly competing with spermatozoa for nutrients or by the production of toxic products (Catry *et al*., 2010; Morrell and Wallgren, 2014). The results could not be compared due to lack of literature on this aspect. The authors have not come across any published report where honey has been used as an alternative to antibiotics. However, honey has been used as a cryoprotectant and a source of fructose in the semen extender for cryopreservation (Olayemi *et al*., 2011; Yimer *et al*., 2015). In the present study, T1 resulted in significantly higher sperm motility and live sperm count as compared to T2 and T3. The decrease in sperm motility and live sperm count at post-thaw in T2 and T3 group may be attributed to the higher concentration of honey which might have created an excessive hyperosmotic extracellular environment and subsequently led to an excessive dehydration of the sperm, resulting in their death (Yimer *et al*., 2015). Honey at 2.5% level might have maintained nearly isotonic environment around the sperm and provided energy as it is a rich source of fructose and glucose. The results of the present study are in close agreement with the study of El-Nattat *et al*. (2016) who also obtained better post-thaw sperm motility and viability with honey at the rate of 2% as compared to 3%, 4% and 5%. El-Sheshtawy *et al*. (2014) also reported better post-thaw sperm motility and live sperm count at 1% level for cryopreserved bull sperm and at 3% level for chilled bull semen and others (Yimer *et al*., 2015) also obtained better post-thaw sperm quality at 2.5% level as compared to 5% and 10%. However, our results are in disagreement with those of Fakhrildin *et al*. (2014) who reported better post-thaw sperm quality at 10% level. The reason could be the resilience of human sperm to high osmotic environments.

The percentage of intact acrosome and HOST reacted spermatozoa at post-thaw was significantly higher for control than T1, T2 and T3 group at post-thaw. Higher number of HOST reacted spermatozoa and spermatozoa with intact acrosome in control may be due to the presence of fructose and antibiotics in the extender. The higher number of PIA recorded in control is in concurrence with the previous studies in ram (Paulenz *et al*., 2002; Gundogan, 2009; Bohlooli *et al*., 2012; Rakha, 2013) and buck semen (Islam *et al*., 2006). However, among the treatment groups, T1 maintained better acrosomal integrity as compared to T2 and T3. Higher number of sperm with intact acrosome in T1 may be due to sugar content in the honey, as additions of sugars have been found to have a beneficial effect on acrosomal integrity (El-Sheshtawy *et al*., 2016). The lower values of PIA in T2 and T3 may be due to high concentration of honey, possibly resulting in osmotic shock and

disruption in plasma membrane integrity.

The total viable count at post-thaw were significantly lower for control than T1, T2 and T3, reflecting the role of antibiotics in the semen extender. Honey in any of the treatment groups studied could not take care of heavy microbial load in the semen and this may have led to decease in the sperm quality in the treatment groups as compared to control. The results could not be compared due to lack of available literature on this aspect. This appears to be the first report, where honey has been studied as an alternative to antibiotics and fructose in the semen extender. The permissible limit of colony forming units in frozen bull semen is 5000 CFU/ml as per OIE guidelines and minimum standard protocol for production of bovine semen (NDDB, 2012). However, such standard is lacking in ram semen as AI is not routinely practiced in sheep. Therefore, a greater number of studies need to be carried out on the maximum permissible limit of CFU/ml in frozen ram semen. In conclusion, natural honey could not take care of heavy microbial load in the semen, therefore, there are no benefits to its use as an alternative to antibiotics. However, levels up to 2.5% were not detrimental to crossbred ram spermatozoa, and hence may be used as an energy source in the semen extender.

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Conflict of interest

The authors declare that there is no conflict of interest that might inappropriately influence or bias their work.

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