The Effects of Stingless Bee Honey in Tris Extender on Semen Quality of Boer Goat at 37˚C

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Abstract

Tris extender supplemented with honey sourced from honey bees is widely researched. However, alternative usage of stingless bee honey (SBH) as a semen extender is still scarce. Therefore, the objective of this research is to determine the effect of different concentrations of SBH supplemented in Tris extender on the semen survivability of goats. Semen samples from three male Boer goats were collected using an artificial vagina and pooled before being diluted into Tris Buffer Solution (TBS) as control and three different concentration of SBH extenders (0.5%; SBH1, 1.0%; SBH2, 1.5%; SBH3 respectively). Samples were prepared by volume per volume (v/v) of pure SBH, respectively. Semen evaluations were conducted every 2 hours for the first 10 hours, once during the 24 hours, and every 12 hours thereafter. Semen samples from each treatment were then assessed for sperm individual progressive motility, sperm viability and sperm abnormality through microscopic observation and eosin-nigrosin staining procedure. Throughout this period, the extended semen was kept in a water bath set at 37°C. Sperm individual progressive motility and viability when supplemented with 0.5% stingless bee honey are comparable to the control treatment which contain TBS only, and both can preserve sperm motility and viability for up to 24 hours. However, the addition of different concentrations of SBH does not have any effect on sperm abnormality parameters. This concludes sperm treated at 0.5% concentration prolonged the survivability until 24 hours compared to the higher concentration of 1.0% and 1.5% of SHB supplementation when treated at 37˚C.

Keywords: buck, stingless bee honey, Tris extender, semen quality, *Trigona sp*

Introduction

Stingless bee honey (SBH) or known locally as *Madu Lebah Kelulut* which is from Melipona or Trigona genera tribe has recently received commercial attention in Malaysia (Abd Jalil *et al.,* 2017). The properties of the SBH such as the antimicrobial activity (Zainol *et al.,* 2013) against various types of bacteria, fungi, and viruses (Aggad and Guemour, 2014) are reported in manuka honey (Al Somal *et al.,* 1994) and *Tualang* honey which of the most potent and well-investigated honey for its antimicrobial and wound healing activities (Abdul Jalil *et al.,* 2017). For example, SBH produced by *Trigona laeviceps* also exhibits antimicrobial activity against several types of bacteria (*E. coli and S. aureus*), fungal strains and yeast (Chanchao, 2009). Same with *Trigona angustula* honey which has significant antimicrobial activity against several different bacterial strains, including *Bacillus cereus* (Gram-positive bacteria) and *Pseudomonas aeruginosa*

(Gram-negative bacteria). In addition, they are also shown to be against yeasts such as *Candida albicans* and *Saccharomyces cerevisiae* (DeMera and Angert, 2004). The high sugar content mainly glucose and fructose and other different kinds of sugars such as trehalose, ribose, raffinose, saccharose and galactose are used as an energy source for sperm cells are still actively researched (Zakiya *et al.,* 2020). In ruminants, fructose sugar-based extenders are proven most effective, with less negative effects as compared to others (Kasimanickam *et al.*, 2011). Research on the SBH in Malaysia is still scarce, and alternative usage of this type of honey may be of significant value. Therefore, the objective of this research was to determine the effect of different concentrations of SBH in Tris extender on sperm survivability of Boer goat.

Materials and methods

Animal and semen collection

The research was conducted at the Goat Rearing Facility, Faculty of Sustainable Agriculture (FSA), Universiti Malaysia Sabah (UMS) Sandakan Campus. Three (3) Boer bucks were used in this study, ranging from 2-5 years old, with a bodyweight of 50-75kg. The bucks were fed 3% of their body weight with a commercial total mixed ration pellet for goats. Water and mineral block were given *ad libitum*. Semen was collected using an artificial vagina. Five ejaculates obtained were subjected to sperm washing by diluting with Tris Buffer Solution (TBS) in a 1:15 ratio (v/v) and centrifuged for 15 minutes at 500 × g inside a 15 ml plastic tube to remove the supernatant. The remaining pellet obtained from sperm washing was subjected to control and three concentrations of stingless bee honey semen extender. The extended semen was placed in a water bath with a temperature of 37°C.

Preparation of semen extender

Honey used as a supplementation in TBS was produced from *Trigona sp.* bee. TBS was set as Control (C) and three different concentration of stingless bee honey (SBH) extenders (0.5%; SBH1, 1.0%; SBH2, 1.5%; SBH3) were prepared by volume per volume (v/v) of pure stingless bee honey, respectively. All the treatment extenders were prepared in 50 ml volumes (Table 1). The C extender consists of 2.4 g Tris, 1 g fructose, 1.4 g citric acid, 100 mg/ml Streptomycin and 100µg/ml Penicillin per 100 ml. The pH for the TBS was set at 6.8 and the pH value of the final SBH extenders was set at 7 to 7.2. After the sperm washing, the pellet obtained was suspended with TBS in a 1:8 ratio (v/v) (Salvador *et al.*, 2006). Approximately 0.3 ml of semen sample was dispensed into a sterilized plastic tube containing 3.0 ml of treatment extender.

Table 1. Composition of Tris Buffer Solution (TBS) and stingless bee honey (SHB) semen extender

Treatments	Ingredients	
	TBS (mL)	SHB(µL)
C (0%)	50	-
SBH1 (0.5%)	49.75	250
SBH2 (1.0%)	49.50	500
SBH3 (1.5%)	49.25	750

Semen quality assessment

The samples were incubated in the water bath at 37°C and analyzed for individual progressive motility, sperm viability, sperm mortality, and sperm abnormality at 0, 2, 4, 6, 8, 10, 24, 36 and 48 hours intervals. A 10 µL of extended semen was placed on the pre-warmed slide (37˚C) and covered by a coverslip. The individual progressive motility was determined by viewing five fields for each sample using an arbitrary scale of 0 to 10 (Blesbois *et al.*, 2008). A 10 µL of the extended semen from each treatment group

was mixed with 10 µl of eosin-nigrosin reagent and smeared on top of a glass slide and left to dry. The dried slides were observed under a bright-field microscope at 400× magnification. The viability and abnormalities percentage of the spermatozoa were counted at the same time after the staining. A minimum of 200 spermatozoa were examined from five different fields of view in each smear. Spermatozoa that appear transparent (non-stained) were considered alive while spermatozoa with a shade of purple (stained) were considered dead. At the same time, the same slides were used to count the percentage of abnormalities. Morphology was viewed under 400x magnification.

Statistical analysis

Two-way analyses of variance (SAS software version 9.4) and Duncan's Multiple Range Test (DMRT) were used to compare the differences between mean values. Statistical significance for the means was considered at $P<0.05$. The results were then presented as mean ± standard error mean (SEM).

Results and discussions

Sperm individual progressive motility

There was no interaction between treatment and hour intervals ($P>0.05$) for the percentage of sperm individual progressive motility. They were presented as per main effect ($P<0.05$). Figure 1 showed the main effect of time (hour) when the individual progressive motility is better on average when the SBH was not added into the extender. However, SBH1 showed a better response as compared to SBH2 and SBH3. The longer period applied during extension reduced the performance of progressive motility for each treatment.

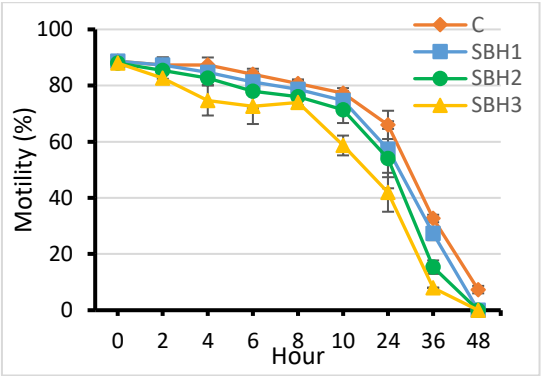

Figure 1. Individual progressive motility with different concentrations of stingless bee honey (SBH) across 48 hours intervals. *Note: C (Control = TBS); SBH1 (TBS+0.5% SBH); SBH2 (TBS+1.0% SBH); SBH3 (TBS+1.5% SBH).

The progressive motility of the buck semen supplemented with SBH is presented in Table 2. At the 4 hours interval, individual progressive motility in C was greater ($P<0.05$) than in SBH3. At 10 hours intervals, C, SBH1and SBH2 were significantly higher than SBH3. The mean values of C and SBH1 were also significantly ($P<0.05$) different from SBH2 and SBH3 at 36 hours intervals. At the 48 hours interval, only C was significantly ($P<0.05$) motile as compared to SBH1, SBH2 and SBH3. There was no difference among C, SBH1, SBH2 and SBH3 for evaluation time intervals at 0, 2, 6, 8 and 24 hours. As per the main effect of different concentrations of SBH mentioned, progressive motility percentages at 0, 2 and 4 hours were significantly higher ($P<0.05$) than 8, 24, 36 and 48 hours respectively when extended in C. Meanwhile, in SBH1 and SBH2, the sperm were more progressively motile ($P<0.05$) at the beginning of the experiment than at 10, 24, 36, and 48 hours. Additionally, in SBH3 percentage of progressive motility was significant ($P<0.05$) from each hour except at 2 hours.

Table 2. Mean (%) of sperm individual progressive motility with different concentrations of stingless bee honey (SBH) in Tris extender across 48 hours intervals.

	Evaluation time interval (hour)/ Mean ± SEM (%)								
Treatments	0	2	4	6	8	10	24	36	48
C	88.67 ±1.33^{aA}	87.33 ±2.67^{aA}	87.33 ±2.67^{aA}	84.00 ±2.00^{aAB}	80.67 ±1.33^{aAB}	77.33 ±1.76^{aB}	66.00 ±5.03^{aC}	32.67 ±1.33^{aD}	7.33 ±1.33^{aE}
SBH1	88.67 ±1.33^{aA}	87.33 ±2.67^{aAB}	84.67 ±2.40^{abAB}	81.33 ±3.71^{aAB}	78.67 ±3.53^{aAB}	74.67 ±3.33^{aB}	57.33 ±9.96^{aC}	27.33 ±2.40^{aD}	0.00 ±0.00^{bE}
SBH2	88.00 ±2.00^{aA}	85.33 ±2.91^{aAB}	83.33 ±2.40^{abAB}	78.00 ±6.11^{aAB}	76.00 ±3.06^{aAB}	71.33 ±4.67^{aB}	54.00 ±10.58^{aC}	15.33 ±2.40^{bD}	0.00 ±0.00^{bE}
SBH3	88.00 ±2.00^{aA}	82.67 ±1.76^{aAB}	74.67 ±5.33^{bB}	72.67 ±6.36^{aB}	74.00 ±2.00^{aB}	58.67 ±3.53^{bC}	42.00 ±6.93^{aD}	8.00 ±0.00^{cE}	0.00 ±0.00^{bE}

^{a,b,c} Mean with different superscripts within the same column are significant differences at P<0.05. ^{A,B,C,D,E} Mean with different superscript within the same row are significant difference at P<0.05.C (Control = TBS); SBH1 (TBS+0.5% Stingless bee honey (SBH); SBH2 (TBS+1.0% SBH); SBH3 (TBS+1.5% SBH).

Table 3. Mean (%) of live sperm with different concentrations of stingless bee honey (SBH) in Tris extender across 48 hours intervals.

	Evaluation time interval (hour) / Mean ± SEM (%)								
Treatment	0	2	4	6	8	10	24	36	48
C	88.00 ±2.08^{aA}	86.67 ±2.13^{aA}	82.50 ±2.25^{aAB}	76.00 ±1.04^{aBC}	72.17 ±1.36^{aCD}	66.50 ±2.60^{aD}	59.00 ±3.33^{aE}	24.67 ±4.48^{aF}	6.83 ±1.09^{aG}
SBH1	85.67 ±2.13^{aA}	84.17 ±1.01^{aA}	80.00 ±1.26^{aA}	71.50 ±2.36^{abB}	67.67 ±2.62^{abBC}	63.33 ±2.62^{abC}	49.17 ±5.13^{abD}	15.83 ±0.93^{bE}	0.33 ±0.33^{bF}
SBH2	88.83 ±2.73^{aA}	84.17 ±2.05^{aAB}	78.17 ±1.88^{abB}	68.67 ±2.17^{bC}	61.00 ±2.00^{bcCD}	55.17 ±3.90^{bcD}	41.67 ±4.42^{bcE}	17.67 ±2.35^{bF}	0.00 ±0.00^{bG}
SBH3	86.50 ±3.77^{aA}	81.33 ±2.08^{aA}	71.33 ±2.95^{bB}	66.33 ±1.45^{bB}	59.03 ±3.28^{cC}	52.50 ±1.26^{cC}	33.67 ±2.42^{cD}	8.83 ±1.20^{bE}	0.00 ±0.00^{bF}

^{a,b,c} Mean with different superscripts within the same column are significant differences at P<0.05. ^{A,B,C,D,E,F,G} Mean with different superscript within the same row are significant difference at P<0.05.C (Control = TBS); SBH1 (TBS+0.5% SBH); SBH2 (TBS+1.0% SBH); SBH3 (TBS+1.5% SBH).

Table 4. Mean (%) of abnormal sperm with different concentrations of stingless bee honey (SBH) in Tris extender across 48 hours intervals

	Evaluation time interval (hour) / Mean ± SEM (%)								
Treatments	0	2	4	6	8	10	24	36	48
C	10.50 ±0.58[a]	9.00 ±0.29[a]	11.67 ±0.73[a]	8.83 ±1.69[a]	8.50 ±0.58[a]	8.67 ±0.33[a]	10.00 ±0.58[a]	8.67 ±0.33[a]	8.83 ±2.20[a]
SBH1	9.83 ±2.03[a]	10.17 ±1.45[a]	8.33 ±0.33[b]	9.83 ±0.88[a]	10.33 ±1.86[a]	8.17 ±0.73[a]	8.17 ±0.33[b]	7.50 ±2.25[a]	10.17 ±1.17[a]
SBH2	9.83 ±1.36[a]	9.83 ±1.20[a]	9.83 ±0.88[ab]	9.00 ±1.04[a]	8.00 ±0.50[a]	9.50 ±1.61[a]	7.33 ±0.44[b]	9.67 ±0.88[a]	10.00 ±0.76[a]
SBH3	9.00 ±0.76[a]	8.17 ±0.88[a]	10.67 ±1.33[ab]	9.83 ±2.20[a]	8.17 ±0.44[a]	10.00 ±1.26[a]	8.17 ±0.33[b]	11.67 ±1.69[a]	11.33 ±1.59[a]

[a,b,c] Mean with different superscripts within the same column are significant differences at P<0.05. C (Control = TBS); SBH1 (TBS+0.5% SBH); SBH2 (TBS+1.0% SBH); SBH3 (TBS+1.5% SBH).

Sperm viability percentage

During the first two hours, no significant difference can be observed in sperm viable percentage between C, SBH1, SBH2, and SBH3 (Table 3). However, at 4 hours viable sperm were significantly ($p<0.05$) greater in C and SBH1 than in SBH3. The C remained viable at the highest percentage as compared to SBH2 and SBH3 at 6 hours intervals. During 8, 10 and 24 hours, C showed the highest viability percentage as compared to the treated extender with 0.5% and 1% of SBH ($p<0.05$). Viable sperm is reduced significantly in SBH3 at 36 hours as compared with C ($p<0.0.5$) when SBH1 and SBH2 were not significantly different from each other ($p>0.05$). A similar result was obtained in 48 hours when C had the highest viable sperm percentage as compared with all SBH extenders ($p<0.05$).

Sperm abnormality

The addition of stingless bee honey and control treatment were not significantly affected sperm abnormalities ($p>0.05$). Nevertheless, a significant difference was observed during the 4 and 24 hours During the 4 hours, sperm abnormality was significantly higher ($p<0.05$) in C (11.67±0.73%) than in SBH1 (8.33±0.33%). For the 24 hours, the mean percentage of abnormal sperm from C (10.00±0.58%) was significantly higher compared to all the SBH treatments while no significant difference was found among SBH1 (8.17±0.33%), SBH2 (7.33±0.44%), and SBH3 (8.17±0.33%).

Discussion

Sperm individual progressive motility

In this study, SBH seemed to affect the performance of sperm individual progressive motility when only sperm in C is still motile at 48 hours intervals. Starting from the 36 hours the motility was significantly reduced when the highest concentration of SBH was given. The same results were found in the fresh semen and post-chilled semen when local honey was given to different cattle breeds and the progressive motility percentage was reduced. However, they reported that the progressive motility was significantly improved when 1% of honey is added to the extender in comparison with the control treatment (Chung *et al.,* 2019). The latter result is contradicting our result when the C and 0.5% concentration of SBH extenders were significantly higher than the 1 % and 1.5% concentration of SBH extenders. The different results reported by them might be because of the usage of different types of honey and tris solution, breed and the time of sample taken. In their study, local honey produced from *Apis mellifera* bees with different concentrations was added to bull commercial extenders (Bioexcell™) and was analyzed on fresh, post-chilled and post-thawed semen (Chung *et al.,* 2019). Initially, our finding was in line with the research conducted by Yimer *et al.* (2015) whereby the effect of honey supplementation into Tris extender on cryopreservation of bull spermatozoa was studied. After cryopreservation in liquid nitrogen for 24 hours and thawed, bull semen that were extended in Tris and Tris + 2.5% honey showed no significant difference between each other, with progressive motility of 25.0±1.1% and 24.5±1.7% respectively. Both honey and stingless bee honey which contains reducing sugar of glucose and fructose (*Rao et al.*, 2016) may contribute to subsequent lower motile sperm due to increment of the osmotic pressure of the extender. Of the total sugar in honey, 32-38% is comprised of fructose making it the most prevalent monosaccharide in honey (Rao *et al.*, 2016). Both glucose and fructose are essential in providing energy to the sperm cell, thus maintaining their physiological function (Aires, 2003). However, other disaccharides and

oligosaccharides can also be found in honey, for example, sucrose, maltose, maltotriose, and panose (Rao *et al.,* 2016). Sugar such as lactose, mannose, raffinose, and trehalose is a non-penetrating cryoprotectant (Lemma, 2011), thereby if added to the semen extender will draw the fluid out from the sperm cell. Even though this characteristic is useful in protecting sperm cells from intracellular ice crystal formation following cryopreservation, excessive osmotic pressure could also contribute to severe dehydration of the sperm cell (Lemma, 2011), leading to sperm injury and loss of motility. Ram spermatozoa may exhibit significant cell damage and irreversible loss of motility when subjected to osmotic pressure higher than 600 mOsm (Curry and Watson, 1994). Less damage occurred when sperm is frozen in diluents with osmolarities ranging between 425-525 mOsm (Bowen *et al.,* 1988). In buck, the optimal tonicity of a Tris–glucose–citric acid for goat sperm medium would be 540 mOsm (Salamon and Maxwell, 2000). Similar observations were also found by Peterson *et al.* (2007), in which goat semen stored in liquid form progressively declined over time in motile sperm percentage, regardless of storage temperature of 4 or 18°C. In ram, extended spermatozoa deteriorated when preserved for more than 24 hours (Salamon and Maxwell, 2000). Depletion of vital nutrients or energy sources from the media, accumulation of metabolic by-products that affect the extracellular pH, or even due to microbial growth inside the extended semen are contributing factors to these changes (Althouse *et al.,* 2000).

Based on the result the Control group (without the SBH) extenders are the best choice to preserve semen motility compared to other treatments. Up until the 24th hour, semen extended with Tris and different concentrations of stingless bee honey (SBH1, SBH2, and SBH3) have no significant difference with C. This can provide an alternative to the TBS solution to preserve sperm motility for a short period. Beyond the 24 hours, semen extended with SBH starts to deteriorate rapidly compared to C and thus is not recommended for longer use.

Sperm viability percentage

In this study, viable sperm was affected by the addition of SBH. This result was in contrast with the research of Yimer et al. (2015) when the bull spermatozoa were cryopreserved for 24 hours in Tris plus the addition of 2.5% honey showed a better viability percentage compared to Tris extender alone. The contrasting result between the current research and the previous one may be attributed to the different types of honey used. The glucose, fructose, and sucrose content of SBH are generally lower compared to regular bee honey (Rao *et al.,* 2016). The lower concentration of sugar in SBH may explain the reduced mean percentage of viable sperm in this research compared to Yimer *et al.* (2015) when the concentration of honey used was at 2.5 %, 5% and 10%. As discussed previously, sugar such as glucose and fructose are essential in providing energy to the sperm cell which depends heavily on available energy source in an extender or seminal plasma in the form of adenosine triphosphate (ATP), which is produced from metabolism, and therefore required in constant supply for cell function and survival (Wattimena *et al.,* 2009). The highly concentrated nature of the SBH may increase the osmotic pressure of the semen extender, thereby causing cell damage and loss of motility (Curry and Watson, 1994). Another factor is the pH level of the SBH, which reported that the pH of semen extender is important in maximizing goat sperm respiration and motility. A pH range of 7.2 to 7.5 can produce maximal oxygen uptake of goat sperm which useful optimum range for sperm cell motility and helps the survival of

goat sperm *in vitro* when set at 7.2 (Fukuhara and Nishikawa,1973). The mean pH value of SBH is in the range of 3.05 to 4.55 (Souza *et al.,* 2006). This is well below the pH value of 7.2 stated by Fukuhara and Nishikawa (1973) which was considered optimal for goat sperm viability. By increasing the SBH content inside the semen extender, the resultant mixture will also be lower in pH, thereby affecting sperm viability as shown in this study. Purdy (2006) states that large changes in semen pH can lead to sperm damage, infertility, or sperm mortality. Liu *et al.* (2016) also showed that during long-term liquid storage of goat semen, both sperm motility and semen pH decreased gradually, and a strong correlation was observed between the two. The same study also found significant impairment in sperm motility when subjected to an artificial lowering of the pH. To overcome this matter, the SBH extender in this study was set at pH 7 to pH 7.2 before being used. Hence, the addition of 0.5% SBH produced the best result compared to other concentrations of SBH by preserving sperm viability for up to 24 hours. However, it is still inferior compared to C when SBH1 will lose its effectiveness after 24 hours. Therefore, it is crucial to keep the pH of the semen extender to an optimal value, and the addition of SBH should only be in small concentrations or the usage of SBH as an extender is not suitable.

Sperm abnormality

Morphological abnormalities from a normal fertile buck are usually less than 5%, however, it can reach more than 10% if the buck is below average or poor in fertility. Poor sperm morphology could be used as an indicator of decreased fertility when the percentage is exceeded 20 % (Purdy, 2006). The higher percentage of abnormal sperm in this research may be influenced by several factors such as genetic-make up, physiological stage of the animal, nutrition, climate, or presence of underlying disease (Dana *et al.,* 2000). In addition, the result from this research is in contrast with Yimer *et al.* (2015) where supplementation of 2.5% honey in Tris extender produced a better percentage of morphologically normal bull spermatozoa compared to Tris alone. Fakhrildin *et al.* (2014), also record a similar observation in human semen, where supplementation of 10% of the honey to cryoprotectant solution results in enhancement of post-thawing sperm quality. The use of different types of honey, higher concentration, and different components of Tris extender in both of these experiments may explain the contrasting result from our result. According to Erejuwa *et al.* (2012), honey is a novel antioxidant, containing pinocembrin, pinostrobin, vitamins, glucose oxidase, and diastase. It reduces lipid peroxidation and oxidative stress on the sperm cells from reactive oxygen species like superoxide and hydrogen peroxide. Research by Reda *et al.* (2014) also showed that the addition of 10% honey solution to semen extender improved sperm motility in chilled and frozen semen. This result is attributed 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 (Chua *et al.,* 2013). Many studies on semen extenders have been done using regular honey instead of SBH, therefore its effect on sperm quality parameters is really limited. SBH gave a detrimental effect on sperm abnormality, however, there were no significant differences between treatment and only significant at 4 and 24 hours.

Conclusion

In conclusion, 0.5% concentration is the optimum amount for SBH in Tris extender in preserving sperm viability for up to 24 hours.

Further studies should be carried out to determine the effect of *in vitro* SBH supplementation on the semen qualities of other ruminants and its effectiveness other than reproductive performance.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

Abdul Jalil, M.A., Kasmuri, K.R., and Hadi, H. 2017. Stingless bee honey, the natural wound healer: A Review. *Skin Pharmacol. Physiol.* 2017; 30:66–75. doi:10.1159/000458416.

Aggad, H. and Guemour, D. 2014. Honey antibacterial activity. *Med Aromat. Plants* 3: 152.

Aires, V. 2003. *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology* 60: 269–279. doi. org/10.1016/S0093-691X(02)01369-9.

Al Somal, N., Coley, K., Molan, P., and Hancock, B. 1994. Susceptibility of *Helicobacter pylori* to the antibacterial activity of manuka honey. *J. R. Soc. Med.* 87: 9-12.

Althouse, G.C., Kuster, C. E., Clark, S.G., and Weisiger, R. M. 2000. Field investigation of bacterial contaminants and their effects on the porcine semen. *Theriogenology* 53: 1167-1176.

Blesbois, E., Grasseau, I., Seigneurin, F., Mignon-Grasteau, S., Saint Jalme, M., and Mialon-Richard, M. M. 2008. Predictors of success of semen cryopreservation in chickens. *Theriogenology* 69(2), 252-261.

Bowen, J.A., Fonda, E.S., Kooyman, D.L., 1988. Ultrastructural study of goat spermatozoa frozen at different diluent osmolalities. In: Proceedings of the Western Section, American Society of Animal Science. *J. Anim. Sci.* (39) 312–315.

Chanchao, C. 2009. Antimicrobial activity by *Trigona laeviceps* (stingless bee) honey from Thailand. *Pak. J. Med. Sci.* 25: 364-369.

Chua, L. S., Rahaman, N. L. A., Adnan, N. A., and Eddie Tan, T.T. 2013. Antioxidant activity of three honey samples in relation with their biochemical components. *J. Anal. Methods. Chem.* 313798. doi.org/10.1155/2013/313798

Chung, E.L.T., Nayan, N., Nasir, N.S.M., Hing, P.S.A., Ramli, S., Rahman, M.H.A., Kamalludin, M.H. 2019. Effect of honey as an additive for cryopreservation on bull semen quality from different cattle breeds under tropical condition. *J. Anim. Health. Prod.*7(4):171-178. doi.org/10.17582/ journal.jahp/2019/7.4.171.178.

Curry, M. R., and Watson, P. F. 1994. Osmotic effects on ram and human sperm membranes in relation to thawing injury. *Cryobiology* 31(1): 39–46.

Dana, N., Tegegne, A. and Shenkoru, T. 2000. Feed intake, sperm output and seminal characteristics of Ethiopian highland sheep supplemented with different levels of leucaena (*Leucaena leucocephala*) leaf hay. *Anim. Feed Sci. Technol.* 86: 239-249.

DeMera, J. H., and Angert, E. R. 2004. Comparison of the antimicrobial activity of honey produced by *Tetragonisca*

angustula (Meliponinae) and *Apis mellifera* from different phytogeographic regions of Costa Rica. *Apidologie* 35: 411-417.

Erejuwa, O.O., Sulaima, S. A., and Ab Wahab, M.S. 2012. *Honey: A novel antioxidant. Molecules*. 17: 4400-4423. doi. org/10.3390/molecules17044400.

Fakhrildin, M.-B. M.-R., and Alsaadi, R. A.-R. 2014. Honey supplementation to semen freezing medium improves human sperm parameters post-thawing. *J. Family Reprod. Health* 8(1): 27–31.

Fukuhara, R., and Nishikawa, Y. 1973. Effects of pH, sperm concentration, washing and substrate concentration on respiration and motility of goat spermatozoa. *Japanese J. Zootech. Sci.* 44: 266–270.

Kasimanickam, R., Kasimanickam, V., Tibary, A., and Pelzer, K. 2011. Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4°C. *Small Rumin. Res.* 99: 208–213.

Lemma, A. 2011. Effect of cryopreservation on sperm quality and fertility. In: M Manafi (ed), *Artificial Insemination in Farm Animals*. Published online by In Tech, pp. 191-216.

Liu, C.-H., Dong, H.-B., Ma, D.-L., Li, Y.-W., Han, D., Luo, M.-J., Chang, Z.-L. and Tan, J.-H. 2016. Effects of pH during liquid storage of goat semen on sperm viability and fertilizing potential. *Anim. Reprod. Sci*. Vol 164: 47-56.

Peterson, K., Kappen, M.A.P.N., Ursem, P.J.F., Nothling, J.O., Colenbrander, B. and Gadella, B.M. 2007. Microscopic and flow cytometric semen assessment of Dutch AI-bucks: Effect of semen processing procedures and their correlation to fertility. *Theriogenology* 67: 863-871.

Purdy, P.H. 2006. A review on goat sperm cryopreservation. *Small Rumin. Res.* 63: 215-225.

Rao, P. V., Krishnan, K. T., Salleh, N., and Gan, S. H. 2016. Biological and therapeutic effects of honey produced by honeybees and stingless bees: a comparative review. *Rev. Bras. Farmacogn.* 26: 657-664.

Reda, I., El-Sheshtawy, Walid, S., El-Nattat, Hussein, A., Sabra and Amal Ali, H. 2014. Effect of honey solution on semen preservability of local breeds of cattle bulls. *World Appl. Sci. J.* 32 (10): 2076-2078.

Salamon, S. and Maxwell, W.M.C. 2000. Storage of ram semen. Anim. Reprod. Sci. 62, 77–111.

Salvador, I., Yaniz, J., Viudes-de-Castro, M. P., Gomez, E. A. and Silvestre, M. A. 2006. Effect of solid storage on caprine semen conservation at 5 degrees C. *Theriogenology* 66: 974–981.

Souza, B., Roubik, D., Barth, O., Heard, T., Enriquez, E., Carvalho, C., Villas-Boas, J., Marchini, L., Locatelli, J., and Persano-Oddo, L., 2006. Composition of stingless bee honey: setting quality standards. *Interciencia (*Caracas) 31: 867.

Wattimena, J., Parera, F., and Veerman, M. 2009. Effect of maltose concentration in Tris dilution on epididymal spermatozoa quality of Bali bull preserved at 5°C. *J. Indones. Trop. Anim. Agri.* 34.

Yimer, N., Muhammad, N., Sarsaifi, K., Rosnina, Y., Wahid, H., Khumran, A. M., & Kaka, A. (2015). Effect of honey supplementation into tris extender on cryopreservation of bull spermatozoa. *Mal. J. Anim. Sci.* 18(2), 47-54.

Zainol, M. I., Yusoff, K. M., and Yusof, M. Y. M., 2013. Antibacterial activity of selected Malaysian honey. *BMC Complement Altern. Med.* 13: 129.

Zakiya, N.A.H., Yanti, A.H., Setyawati, T.R. 2020. Viability of Peranakan Etawah liquid semen preserved in tris substituted with various energy sources. *JITV* 25(2): 68-73.