Effect of honey supplementation into Tris Extender on Cryopreservation of Bull Spermatozoa

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Abstract

The aim of this study was to evaluate the effects of using honey as a supplement into Tris extender in comparison with a commercial Bioxcell® extender on bull semen cryopreservation quality. Semen samples from four fertile bulls, which comprised of two Friesian Sahiwal, one Braford and one Kedah-Kelantan breeds, were collected using an electro-ejaculation method. Collected samples were freshly evaluated physically and microscopically before divided and diluted using four Tris-based extenders containing 0% (control), 2.5%, 5% and 10% honey, and a commercial Bioxcell® extender. The processed semen was equilibrated at 5°C for 3 h (chilling), packed into straws (0.25 ml), frozen and stored in a liquid nitrogen tank for 24 h before thawing and assessment of quality. Semen quality parameters used for evaluation and comparison after chilling and thawing included general and progressive motility (analyzed using a computer assisted semen analyzer), livability and sperm morphology (done using eosin-nigrosin staining technique). The experiment revealed that among all Tris-based extenders, improved post-cryopreservation semen quality was obtained from Tris+2.5% honey; comparable to Bioxcell® which was superior to all other extenders. Use of honey at concentrations of 5 and 10% however, were not suitable and showed significantly poor semen quality following cryopreservation. Therefore, Tris extender with 2.5% honey could be considered as a cheaper alternative to Bioxcell® as it could be prepared in the laboratory whenever needed.

Key words: Bulls, semen cryopreservation, honey, Tris extender, Bioxcell®

Introduction

Artificial insemination (AI), a useful assisted reproductive biotechnology, has been applied to achieve rapid livestock genetic improvement and production. Its success however, depends on several factors which include quality of frozen-thawed semen used for AI. The ability to produce good post-thawed semen quality in turn depends on many other factors which include the composition of the cryopreservation media used as semen extenders. The most common integral components of semen extenders used for cryopreservation include egg-yolk, buffer, nutrients, cryoprotectant (e.g., glycerol) and antibiotics, each providing a complex and important role in protecting the spermatozoa (Purdy, 2006).

Cryopreservation technique allows spermatozoa to be stored indefinitely under -196°C in liquid nitrogen, while retaining acceptable post-thawed fertilization rate (Lemma, 2011). However, cryopreservation is known to cause detrimental effects to spermatozoa partly during the freezing-thawing process (Watson, 2000). Semen extender is added in order to maintain spermatozoa metabolic demands, control pH changes in the extracellular environment of the spermatozoa, minimize cryogenic damage, and also control bacterial

contamination. Commonly added nutrients in semen extender are simple sugars such as glucose and fructose (Bearden *et al.,* 2004). Inadequate nutrients in the semen extender will reduce the metabolic activity of the spermatozoa and lead to higher number of dead spermatozoa (Gadea, 2003). Cryoprotectants are of low molecular weight and act intracellularly or extracellularly to protect spermatozoa from freeze damage by ice crystallization. Cryoprotectants act by decreasing the freezing point of a substance, reducing the amount of salts and solutes present in the liquid phase of the sample and by decreasing ice formation within the spermatozoa (Fakhrildin *et al.,* 2014). The addition of a cryoprotectant into the semen sample is needed in order to protect spermatozoa from cold shock.

Cryoprotectants can generally be classified into two types: penetrating and non-penetrating agents of sperm plasma membrane (Lemma, 2011). Among the non-penetrating cryoprotectants are simple sugars which act extra celullarly by altering the osmotic gradient of the extended semen and allowing water content from the spermatozoa to diffuse out (Fuller, 2004).

In vitro additions of various agents such as antioxidants and fatty acids as supplements have been reported to enhance post-thawed semen quality (Bathgate, 2011; Asadpour and Tayefi-Nasrabadi 2012; Yimer *et al.,* 2014). Honey is known to contain high amount of a variety of simple sugars (Fuller, 2004) which might serve both as a source of nutrition and non-penetrating cryoprotectant to sperm cells during cryopreservation. Honey also contains tiny amounts of several compounds thought to function as antioxidants, including chrysin, pinobanksin, vitamin C, catalase, and pinocembrin (Bogdanov *et al.,* 2008; Fakhrildin *et al.,* 2014). A review by Erejuwa *et al.* (2012) described honey as a novel antioxidant considering its effect on the health of the various organs of the body. Owing to the wide variety of properties of honey, its addition into a freezing medium is also expected to help spermatozoa protection during cryopreservation. However, no prior study has been conducted to test the effects of supplementing honey in vitro into Tris extender as bull semen freezing medium. Thus, this study was designed to determine the effects of adding different concentrations of honey into Tris extender in comparison with a commercial semen extender-Bioxcell® on bull semen quality after cryopreservation.

Materials and Methods

Animals

Four sexually mature bulls consisted of two Friesian Sahiwal, one Bradford, and one Kedah Kelantan bulls belonging to the Universiti Putra Malaysia's dairy and beef cattle farm were used for semen collection. The age of the bulls was within the range of 3 to 4 years old and with body weight between 300 to 400 kg. All of the bulls were kept semi-intensively, provided with a commercial feed known as palm kernel cake (PKC) which contains 10.5% ME and 16% CP. They were also allowed to graze on Napier grass during the day for about 12 hours. The bulls were herded back into pens for confinement when they are not needed for breeding. Commercial mineral block and water were also given *ad libitum*.

Semen collection and evaluation

A total of twelve semen samples (three samples per bull) were collected using an automatic electro-ejaculation method (Yimer *et al.,* 2011). Following ejaculation the semen was collected directly into an insulated graduated collection glass tube. The average time for each collection cycle was 4.5 min. The bulls were allowed to rest for

one week before the next collection. The semen samples were evaluated based on macroscopic and microscopic characteristics. Macroscopic evaluations included volume and color. Microscopic evaluation included spermatozoa concentration, general motility, progressive motility, morphology and livability. For the first three microscopic parameters, evaluation was made by using a Computer Assisted Semen Analyzer (CASA, IVOS System, Hamilton Thorne Inc., USA). Spermatozoa morphology and livability were assessed using eosin negrosin stain technique (Yimer *et al.*, 2014). A total of 200 spermatozoa were examined under a light microscope (400 x magnifications) for evaluation of live or dead spermatozoa based on their staining characteristics. Spermatozoa that stained pink-purple due to absorption of eosin-negrosin were considered dead while those that didn't take up the stain (remained white) were considered live. The same eosin-nigrosin stained slides were also used to determine sperm abnormality for which 200 sperm cells were examined for defects associated with sperm head (detached, tapered, giant and micro head), mid-piece (cytoplasmic droplets, bent, irregular shape) and tail region (broken, bent, coiled and looped tail).

Cryopreservation procedure

Extenders preparation

To prepare the Tris based diluents, 3.5 g of Tris (hydroxymethyl), 1.97 g of citric acid and 1.25 g of fructose (Sigma-Aldrich®) respective prepared extenders were determined and fresh semen was distributed into each of the 5 tubes with target final concentration of $20x10^6$ spermatozoa/0.25 ml of straw (Bearden *et al.*, 2004).

were weighed and dissolved in 100 mL of distilled water (mixture A) and mixed well with the aid of a magnetic stirring device. Then, 27 ml of mixture-A was substituted by 20 mL of egg yolk (20%) freshly separated from the egg albumin according to Yimer *et al.* (2014) and 7 mL glycerol (7%) forming mixture-B. Then, from mixture-B, 40 mL was taken and distributed into four different glass tubes (10 ml/tube). From the 3 tubes, another 0.25, 0.5 and 1 mL of the diluents were removed and replaced by respective amount of honey to have a final 2.5%, 5% and 10% honey concentrations while the 4^{th} tube was left as a control. The fifth tube that contained commercial extender (Bioxcell® , IMV Technologies, France) used in this study as a standard was prepared by mixing one part of a stock Bioxcell® solution to four parts of distilled water. The overall experimental groups used for comparison include Bioxcell®, Tris with 2.5% honey (Tris+2.5% H), Tris with 5% honey (Tris+5% H), Tris with 10% honey (Tris+10% H) and untreated control (Tris).

Semen dilution

Soon after collection and evaluation, dilution of semen was done at 37°C. Before extending the semen, antibiotic was added to the neat semen at the rate of 0.1 ml of Pen-Strep (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin) to 1 ml of neat semen and the mixture were left for 5 min. Based on initial sperm concentration, volume of the neat semen and motile sperm percentage, the required amounts of the Chilling, freezing and thawing procedures

The extended semen was chilled at 5 °C for three h. Then, the post-chilling semen quality was assessed. The extended semen was then, packed into 0.25 ml straws and sealed. The straws were subject to a slow gradual freezing process manually before

they were stored in a liquid nitrogen tank. After sealing, straws were placed horizontally on a cold rack (5°C) and lowered into nitrogen vapors (-50°C), 3-4 cm above the surface of liquid nitrogen contained in a polystyrene box. After 3 min, when the temperature reached -100°C, the frozen straws were transferred into goblets of appropriate size and transferred into a liquid nitrogen tank (-196°C). After 24 h of storage, the straws were thawed in water bath (37°C) for 45 sec (Lemma, 2011). Then, the straws were cut and the semen quality was again evaluated based on sperm general motility, progressive motility, normal spermatozoa morphology and livability.

Statistical analysis

The percentage values for fresh, post-chilled and post-thawed semen quality parameters using the five extenders are expressed as Mean±SEM. A one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test was applied to determine differences among the extenders using SPSS Statistical Software version 20.0. Differences with P value <0.05 were considered to be statistically significant.

Results and Discussion

There was a general decrease in post-chilled semen quality parameters compared to fresh semen for all extenders (Table 1). The decrease was much lesser for Bioxcell® and Tris+2.5% H. Among Tris based extenders, the highest post-chilled quality was obtained using Tris+2.5% H extender demonstrated by higher (P>0.05) percentage of most of the parameters than the Tris+5%H and Tris+10%H and a difference in magnitude compared to the control. Bioxcell® resulted in the best post-chilled semen quality compared to all Tris-based extenders tested with higher (P<0.05) percentage of all the parameters compared to Tris+5% H and Tris+10% H while the difference with Tris+2.5% H was comparable and insignificant.

Table 1: Mean and standard error of mean (SEM) of fresh and post-chilled semen quality parameters using five different extenders

Parameters (%)	Fresh sample	Extender type				
		Bioxcell®	Tris	Tris +2.5%H	Tris +5% H	Tris +10%H
General motility	86.0±1.8^{a}	82.0±3.5^{a}	77.3±4.1^{ab}	81.6±3.1^{a}	66.7±2.8^{b}	46.0±1.3^{c}
Progressive motility	48.9±2.3^{a}	36.8±1.7^{b}	30.5±0.9^{cd}	32.3±1.0^{bc}	26.3±1.1^{cd}	23.3±0.5^{d}
Normal morphology	85.6±1.8^{a}	83.9±1.7^{a,b}	78.0±0.9^{bc}	81.9±1.4^{ab}	75.8±1.4^{cd}	71.1±1.1^{d}
Livability	84.1±1.3^{a}	79.5±1.4^{ab}	78.1±1.8^{abc}	78.4±1.3^{abc}	71.2±1.8^{bc}	55.7 ±1.5^{d}

^{abc}Values with different superscripts across rows indicate significant differences (P<0.05). H - Honey

Similar to chilling, there was a decrease in fresh semen quality parameters for all extenders after cryo-preservation in liquid nitrogen and thawing which were significant as compared to the fresh semen (P<0.05, Table 2) . Among all Tris based extenders,

the highest post-thaw quality was achieved using again Tris+2.5% H. The difference compared to Tris-control was in magnitude only while it was lower (P<0.05) compared to the 5% and 10% H supplement (Table 2). After thawing, Bioxcell® continued to show its dominance significantly in all parameters compared to Tris+5% H and Tris+10% H, and in magnitude compared to Tris-control and Tris+2.5% H. Nevertheless the difference with Tris-control and Tris+2.5% H were insignificant (P>0.05). Although the difference between Tris-control and Tris+2.5% H, both after chilling and thawing were in magnitude only, compared to Bioxcell®, Tris-control showed lower (P<0.05) post–chilled progressive motility percentage while Tris+2.5% H resulted in comparable and insignificant reading (Tables 1 and 2).

Table 2: Mean and standard error of mean (SEM) of fresh and post-thawed semen quality parameter extended using five different extenders

Parameters (%)	Fresh sample	Extender type				
		Bioxcell®	Tris	Tris +2.5%H	Tris +5% H	Tris +10%H
General motility	86.0±1.8^{a}	45.7±2.4^{b}	44.3±1.6^{b}	44.4±1.0^{b}	31.6±0.9^{c}	28.2±1.0^{c}
Progressive motility	48.9±2.3^{a}	25.0±0.6^{b}	20.5±1.1^{bc}	24.5±1.7^{b}	15.8±0.3^{c}	15.5±0.7^{c}
Normal morphology	85.6±1.8^{a}	65.3±1.2^{b}	65.3±0.9^{b}	65.1±1.2^{b}	63.1±1.2^{c}	58.4±1.3^{c}
Livability	84.1±1.3^{a}	44.9±2.9^{b}	39.8±2.5^{bc}	43.5±1.5^{bc}	37.0±2.6^{bc}	35.3±1.1^{c}

^{abc}Values with different superscript across the rows indicate significant differences (P<0.05). H- Honey

During cryopreservation, sperm cells are subject to a number of physical and chemical stresses. Because of the deleterious effect of these factors, it is inevitable to have a reduction in the quality of sperm after cryopreservation (Watson 2000; Andrabi 2007*;* Lemma 2011), which is the same as what was observed in the present study for all types of extenders tested. How much the quality of semen deteriorates however, depends on several factors including the type of cryopreservation medium and its composition, and processing procedure.

The present study enabled to assess the effect of honey supplement into Tris-based extender compared with Tris alone and the commercial Bioxcell® extender. The study revealed that among all Tris-based extenders tested, the addition of honey at a concentration of 2.5% was the best to obtain higher semen quality both after chilling and thawing. However, when the amount of honey added increased to 5 and 10%, the semen quality parameters tended to deteriorate significantly from the fresh quality both after chilling and thawing implying that 2.5% H was the optimum level of honey to be incorporated to see a positive effect. A recent study by Fakhrildin *et al.*, (2014) reported enhancement of post-thaw quality of human sperm with addition of 10% honey into a commercial cryoprotectant solution. The improvement in semen quality gained with addition of 2.5% H might be attributed to the wide variety of properties that honey possessed. Sugar is one of the essential components of most semen extenders (Gadea, 2003; Bearden *et al.*, 2004; Purdy, 2006) and honey is known to consist primarily sugars such as monosaccharides, disaccharides, oligosaccharides and polysaccharides

(Bogdanov *et al.,* 2008) that can act as a source of energy to support spermatozoa survival and motility during cryopreservation.

Naturally, honey is also a highly concentrated product and has the potential hyperosmotic extracellular environment around sperm cells that enhances efflux of intracellular fluid thereby minimizing formation of ice crystals inside the sperm cytoplasm which has been linked to sperm damage during cryopreservation (Royere *et al.,* 1996; Fuller 2004; Fakhrildin *et al.,* 2014). This mechanism of protection gives honey the property of a non-permeable cryoprotectant. Moreover, honey has also been praised as a potent noble antioxidant in protecting cells of the various organs in the body from damage due to oxidative stress/reactive oxygen species (Erejuwa *et al.,* 2012). More specifically, in vivo supplementation of honey has been also reported to increase significant concentrations of different antioxidants and a decrease in oxidative stress biomarker present in seminal plasma of humans exposed to a stress factor compared to those who did not take honey (Tartibian *et al.,* 2011). Therefore, this could be additional mechanism by which the *in vitro* addition of honey at 2.5% into Tris extender improved quality by reducing percentage of sperm cells with abnormal morphology compared to other Tris based extenders tested. The decrease in post-cryopreservation semen quality with an increase in honey supplement of more than 2.5% however might be associated with excess hyperosmotic extracellular environment created due to high concentration of honey that can lead to excessive intracellular dehydration similar to effect of high concentration of non-penetrating cryoprotectants (Lemma, 2011). Bioxcell® is a commercial extender currently used by semen processing and AI centers in Malaysia. This extender produced the best semen quality compared to all other Tris based extenders tested in the present study. Nevertheless, its result was not significantly different from Tris+2.5% H in all of the parameters, reflecting their comparable protective effect to the spermatozoa during cryopreservation. Bioxcell® has been reported to be successfully utilized for semen cryopreservation in many species of domestic animals (Akhter *et al.,* 2010; Asr *et al.,* 2011; Daşkin *et al.,* 2011; Kulaksiz *et al.,* 2012). However, it is a costly product and usually has a short shelf-life that makes it difficult to use it effectively especially for AI centers processing semen at small scale.

In conclusion, the present study revealed that addition of honey to Tris extender at 2.5% was optimum to obtain improved semen cryopreservation result which was comparable to Bioxcell® extender that was superior to all other extenders. Use of honey at concentrations of 5 and 10% however, was not suitable as they resulted in significantly poor quality semen following cryopreservation. Therefore, Tris extender supplemented with 2.5% honey can be considered as a cheaper alternative to Bioxcell® and it can be prepared in the laboratory whenever needed.

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