

PHYSICAL CHARACTERISTICS AND MICROBIAL LOAD OF CHILLED HOLSTEIN BULL SEMEN DILUTED IN PROPOLIS CONTAINED EXTENDER

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SUMMARY

The current pilot study was carried out to evaluate the efficiency of supplementing bovine semen extender with propolis extract on cooled semen physical characteristics and microbial contamination. Six Holstein Friesian bulls aged 4-6 years, and average body weights of 1041.7 ± 58.3 Kg were used. Eighteen ejaculates were collected from the bulls (3 ejaculates each). Thereafter, the ejaculates were pooled and diluted. The pooled specimens were further split into the following five groups: control, 1ml/L streptomycin sulfate plus 1000000 IU penicillin, 5 μ L/ml propolis extract, 10 μ L/ml propolis extract, 20 μ L/ml propolis extract. All semen groups were subjected to cooling preservation for 48h at 5°C, during which sperm physical properties were evaluated. Formation of total colony forming unite (CFU) was also determined. The results showed that propolis extract supplementation improved ($P < 0.05$) all sperm physical characteristics, in a dose – depending manner, over preservation time. Further, the control group exhibited total CFU higher ($P < 0.05$) than all treated specimens. These results imply the possibility of using propolis extract as a sufficient alternative to conventional synthetic antibiotics in bovine semen extenders.

Keywords: Propolis, semen, extender, physical characteristics

INTRODUCTION

Artificial insemination (AI) has been widely used as reliable tool for reproduction in cattle under intensive animal production systems. In the meantime, successful AI depends not only upon maximizing the initial fertility of fresh ejaculates, but also its maintenance during chilled or frozen storage (Jha *et al.*, 2013). However, the fertilizing capacity of cooled spermatozoa has been reported to decrease over chilling-preservation time along with both sperm motility and morphology (Alam *et al.*, 2005 and Munsi *et al.*, 2007). Long preservation of chilled semen has been reported to expose sperms to the drastic effects of spontaneous lipid peroxidation, which is associated with loss of motility and increased sperm damage (e.g. loss of membrane fluidity and increase in DNA breakage) in different mammalian species (Foote *et al.*, 2002 and Agarwal *et al.*, 2003).

Microbial contamination of fresh and preserved semen represents another constrain in applying AI, as it may lead to rapid decline in sperm motility and subsequent fertility (Shukla, 2005 and Morrell, 2006). Semen contamination has been reported to occur at any time right from collection through the various steps involved in processing and preservation. Although zero risk does not exist in the biological world, but it should be as close to it as possible (Sannat *et al.*, 2015). The artificial vagina, the glass wares, semen extender and laboratory environment are some of the common sources that contribute to the bacterial load of semen during processing (Morrell, 2006).

Propolis is a natural resinous product that honeybees collect from several plants and mix it with beeswax and salivary enzymes (β -glucosidase) (Marcucci *et al.*, 1995 and Cardoso *et al.*, 2011).

Bees use propolis on their hives as protection against predators and microorganisms, to repair damage, as a thermal isolator, and to build aseptic locals to prevent microbial infection of larva (Marcucci *et al.*, 1995; Bankova, 2005 and Fokt *et al.*, 2010). Due to its chemical composition, propolis has been used by humans to meet the needs of health and food preservation (Umthong *et al.*, 2011). In the last years the interest in this complex natural product has increased due to its broad spectrum of biological properties (Fokt *et al.*, 2010). Propolis comprises complexity of compounds which play a role in antiviral protection as well as antimicrobial activity due to its major effect against several bacterial strains (Marcucci *et al.*, 1995; Kujumgiev *et al.*, 1999; Mirzoeva *et al.*, 1997 and Scazzocchio *et al.*, 2006). Recently, several studies have shown the effect of propolis from different geographic origin against different fungi, particularly of clinical interest. Additionally, the polyphenols in propolis extract has been reported to possess a potent antioxidant activity (Scalbert *et al.*, 2005; Almaraz-Abarca *et al.*, 2007 and Nader *et al.*, 2010). The current pilot investigation was undertaken to evaluate the efficiency of supplementing bovine semen extender with propolis ethanolic extract on chilling preservation of spermatozoa. The potential antimicrobial effect of propolis supplementation was also investigated.

MATERIALS AND METHODS

Animals and management:

The current study was carried out at the Artificial Insemination Center, belonging to the West Noubaria Rural Development Project, Alexandria, Egypt. Six Holstein bulls aged 4-6 years, and average body weights of 1041.7 ± 58.3 were used. The bulls were kept under intensive breeding management, where Egyptian clover, *Trifolium alexandrinum*, was provided *ad lib.*, and a concentrate mixture was presented to meet the bulls' protein and energy requirements (NRC, 2001). Before executing the experiment, the bulls were clinically examined and were found free of disease or reproductive disorders.

Semen extender and propolis extract:

Semen extender was freshly prepared prior to semen collection. Each 100 ml of the extender was composed of 3.025g tris (hydroxymethyl amino methane), 1.675g citric acid, 0.75g glucose and was further supplemented with 20% egg yolk to reach a final pH of 7.4.

Extraction of red propolis was carried out according to the method of Morsy *et al.* (2013). Filtration, purification and preservation of the propolis extract were conducted according to the method of Morsy *et al.* (2015).

Semen collection:

A total of 18 ejaculates were collected from the bulls (three ejaculates from each bull) during the experimental period. Collection of semen was conducted using an artificial vagina according to the method of Barszcz *et al.* (2012). Upon collection, the ejaculates that showed contamination with urine, strange color or odder were discarded.

Immediately after collection, the ejaculates were transported to a fully equipped laboratory in a warm water bath adjusted at 37°C.

The ejaculates of each collection session were pooled immediately after collection, and the pooled samples were further diluted 1:8 with the previously mentioned extender. Thereafter, the diluted semen specimens were split into the following five groups: control, 1ml/L streptomycin sulphate plus 100000 IU penicillin, 5µl /mL propolis extract, 10µl/ml propolis extract, 20µl/mL propolis extract. All control and treated semen groups were further stored at 5°C for 48h, during which semen physical properties were evaluated.

Semen evaluation:

Physical characteristics of all semen groups were evaluated throughout the 48 h of preservation at times 0, 24 and 48h, respectively. Percentage of sperm motility, dead spermatozoa and sperm abnormalities were recorded according to Salisbury *et al.* (1978). For acrosomal damage estimation, spermatozoa were fixed in a solution of 0.2% glutaraldehyde and integral acrosome percentage was estimated according to Johanson *et al.* (1976). Simultaneously with physical properties evaluation,

chilled semen of control and treated groups were analyzed for presence of microbial activity at 0, 24 and 48h of cooled storage. Determination of microbial activity was performed by counting the colony forming unite (CFU) as described by Sannat *et al.* (2015). The microbial activity was monitored on nutrient agar medium, and total CFU ($\times 10^3$) were counted.

Statistical analysis:

All parametric data were subjected to Analysis of Variance (ANOVA) using Minitab (Version 10, Minitab Inc., USA) statistical package. The effect model used was $Y_{ijk} = \mu + G_i + T_j + GT_{ij} + e_{ijk}$

Where;

Y_{ijk} = The observation taken on sperm criteria (ijk)

μ = Overall mean,

G_i = A fixed effect of the (i) treatment

T_j = A fixed effect of the (j) observation time

GT_{ij} = The interaction effect between treatment and observation time

e_{ijk} = Random error assumed to be normally distributed with mean = 0 and variance = σ^2 .

The comparisons between means were determined by Fisher's least significant difference (LSD) according to Steel and Torrie (1980). The data are presented as mean \pm SEM.

RESULTS AND DISCUSSION

Data for the effect of adding different levels of propolis extract to semen extender of Holstein bulls on cooled semen physical properties are expressed in Table (1). The results showed that mean values of sperm motility were significantly ($P < 0.05$) affected by the semen additive. The highest mean values of sperm motility were observed in both synthetic antibiotic and the high dose (20µl/ml) propolis extract semen groups, while the lowest mean values were observed in the low dose (5 µl/ml) semen group with values 93 ± 1.2 , 88 ± 1.8 and $76 \pm 3.7\%$, respectively. Moreover, time of preservation had a significant ($P < 0.05$) effect on sperm motility all semen treatment groups with highest ($P < 0.05$) values recorded at T_0 and lowest ($P < 0.05$) values at T_{48} (Table1). On the other hand neither mean values of live sperm (%), intact acrosome (%) normal sperm (%) were significantly ($P < 0.05$) affected by the treatment prior to cooling (T_0). However, all previously criteria were drastically declined ($P < 0.05$) with progression of preservation time (Table1). The results also showed that the control semen group exhibited colony forming unit (CFU) higher ($P < 0.05$) than all treated semen groups. However, no significant effect was observed among the different levels of propolis extract groups in the CFU (Figure 1).

The present pilot investigation is the first trial to address the effect of adding propolis extract to semen extender on physical properties and microbial load of chilled bull sperm. The results showed that propolis extract significantly affected all preserved sperm characteristics in a dose-depending manner. The highest mean values were recorded in the high dose

of propolis extract group, whereas the lowest values were observed in the low dose group with progression of storage time. The results also demonstrated the efficiency of adding propolis extract to semen extender in reducing the microbial load of cooled semen. Long preservation of chilled semen has been reported to expose sperms to the drastic effects of spontaneous lipid peroxidation, which is associated with loss of motility and increased sperm damage (Foote *et al.*, 2002 and Agarwal *et al.*, 2003).

Propolis has versatile biological and pharmacological activities, such as antibacterial, antioxidant, antiviral, antifungal, anti-inflammatory, antitumoral, and immunomodulatory (Alencar *et al.*, 2007 and Sforzin *et al.*, 2011). The biological activity of propolis may vary, together with its chemical composition, as it comprises numerous constituents. These comprise polyphenols (flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols, and ketones), sesquiterpene quinones, coumarins, steroids, amino acids, and inorganic compounds (Alencar *et al.*, 2007). The enhancement in semen properties observed in the high dose group in the current investigation could be attributed to

presence of isoflavonoids, which have been reported to manifest antioxidant, antimicrobial, antiprotozoal, and antifungal activities (Cattani *et al.*, 2012). Further, Marquede *et al.* (2005) reported that Brazilian propolis was determined to exhibit antioxidant effect. Meanwhile, propolis is known to be active against (gram-positive) bacteria, viruses, fungi, and parasites (Alencar *et al.*, 2007 and Morsy *et al.*, 2013). It has been proven to be 100 % effective against some parasites such as lethal protozoa and would also decrease inflammation associated with parasite infection (Higashi and de Castro, 1994). Several authors have confirmed the activity of propolis volatiles against different microorganisms such as Gram-positive bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus glutamicus*, *Bacillus subtilis*, *Bacillus cereus*, *Sarcinalutea*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Streptococcus faecalis* (Silva *et al.*, 2008; Falcão *et al.*, 2010; Popova *et al.*, 2010; Duran *et al.*, 2011; Ordóñez *et al.*, 2011 and Tran *et al.*, 2012), and also Gram-negative bacteria such as *Escherichia coli*, *Enterobacter cloacae*, *Klebsiellapneumoni*, *Pseudomonas aeruginosa* (Silva *et al.*, 2008 and Duran *et al.*, 2011).

Table 1. Effect of adding different levels of propolis extract to Holstein bulls' semen extender on cooled semen physical characteristics (mean \pm SEM)

Parameters	Time	Treatment groups				
		Control	Synthetic antibiotics	Propolis extract (20 μ l/ml)	Propolis extract (10 μ l/ml)	Propolis extract (5 μ l/ml)
Motility (%)	T ₀	94.0 \pm 1.0 ^{a,A}	93.0 \pm 1.2 ^{ab,A}	88.0 \pm 1.2 ^{ab,A}	86.0 \pm 1.9 ^{b,A}	76.0 \pm 3.7 ^{c,A}
	T ₂₄	60.5 \pm 0.5 ^{c,B}	87.0 \pm 1.4 ^{a,B}	78.0 \pm 0.5 ^{a,B}	73.5 \pm 1.3 ^{b,B}	59.0 \pm 2.8 ^{c,B}
	T ₄₈	27.0 \pm 1.2 ^{c,C}	81.0 \pm 1.8 ^{a,C}	68.0 \pm 1.2 ^{b,C}	61.0 \pm 1.0 ^{c,C}	42.0 \pm 2.0 ^{d,C}
Live Sperm (%)	T ₀	86.0 \pm 1.3 ^A	86.4 \pm 1.6 ^A	82.6 \pm 3.4 ^A	82.0 \pm 2.7 ^A	77.6 \pm 2.6 ^A
	T ₂₄	58.6 \pm 0.7 ^{c,B}	78.3 \pm 0.9 ^{a,B}	74.8 \pm 1.3 ^{a,AB}	66.9 \pm 1.5 ^{b,B}	57.5 \pm 3.0 ^{c,B}
	T ₄₈	31.2 \pm 1.2 ^{c,C}	70.2 \pm 0.7 ^{a,C}	67.0 \pm 2.3 ^{a,B}	51.8 \pm 0.9 ^{b,C}	37.4 \pm 3.4 ^{c,C}
Intact acrosome (%)	T ₀	85.6 \pm 1.5 ^A	83.2 \pm 1.9 ^A	86.8 \pm 1.2 ^A	86.4 \pm 1.6 ^A	84.8 \pm 1.9
	T ₂₄	63.8 \pm 0.8 ^{b,B}	80.0 \pm 1.0 ^{a,AB}	80.9 \pm 1.0 ^{a,AB}	78.7 \pm 1.0 ^{a,B}	66.4 \pm 3.1 ^b
	T ₄₈	42.0 \pm 1.2 ^{b,C}	76.8 \pm 1.8 ^{a,B}	75.0 \pm 1.5 ^{a,B}	71.0 \pm 1.0 ^{a,C}	46.0 \pm 4.0 ^b
Normal Sperm (%)	T ₀	88.6 \pm 1.2 ^A	89.2 \pm 1.1 ^A	84.6 \pm 0.4 ^A	85.4 \pm 1.2 ^A	84.2 \pm 1.7 ^A
	T ₂₄	70.1 \pm 1.0 ^{c,B}	82.9 \pm 0.5 ^{a,B}	81.7 \pm 0.5 ^{a,B}	77.9 \pm 0.6 ^{b,B}	77.6 \pm 2.1 ^{b,B}
	T ₄₈	51.6 \pm 1.0 ^{c,C}	76.6 \pm 0.4 ^{a,C}	78.8 \pm 0.7 ^{a,C}	70.4 \pm 0.2 ^{b,C}	71.0 \pm 4.0 ^{b,C}

^{a-d} Values in the same row with different superscript letters differ significantly (P < 0.05)

^{A-C} Values in the same column with different superscript letters differ significantly (P < 0.05)

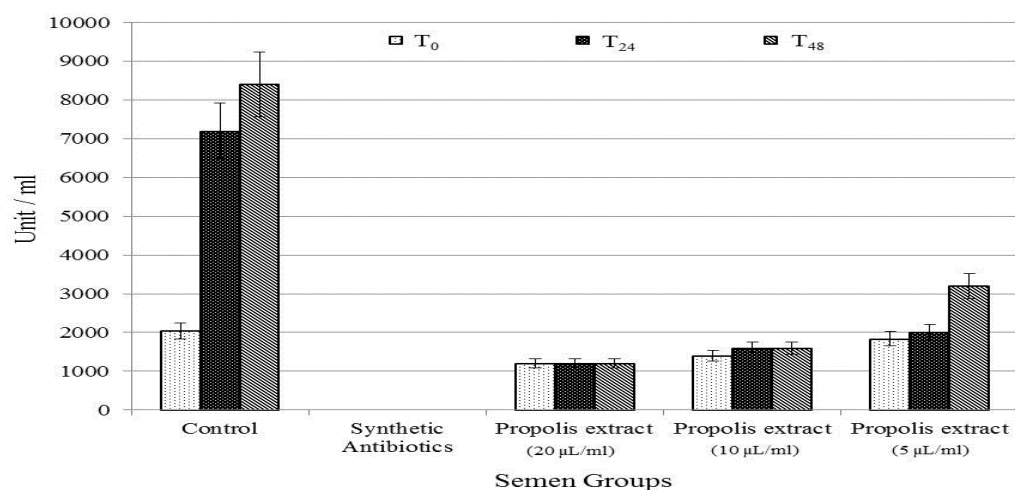


Figure 1. Effect of adding different levels of propolis extract to Holstein bulls' semen extender on colony-forming unit (CFU) formation during cooled preservation (mean \pm SEM)

CONCLUSION

The results of the current pilot study revealed the efficiency of adding propolis ethanolic extract to semen extender on enhancing cooled storage of bull sperm in regard to physical characteristics and microbial contamination. Further studies are needed to evaluate the influence of propolis extract supplementation on potential fertility of spermatozoa.

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تأثير تدعيم مخفف السائل المنوي للطلاق بمستخلص البروبليس علي الحمل الميكروبي والخصائص الطبيعية للسائل المنوي المحفوظ بالتبريد

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تم استخدام ستة طلائق هوليشتين فريزيان متوسط عمر ٤-٦ سنوات ومتوسط وزن 1041.7 ± 58.3 كجم تم جمع ١٨ قذفة منوية بمعدل ٣ قذفة / طلوقة وتم تخفيفهم وتقسيمهم الي خمس مجموعات تجريبية. استخدمت المجموعة الأولى كمجموعة مقارنة، بينما دعمت المجموعة الثانية بمضاد حيوي اصطناعي في صورة تجارية (١ مل استربتوميسين + ١٠٠٠٠٠٠ وحدة دولية بنسلين). المجموعات الثلاث الاخرى تم تدعيمها بمستخلص البروبليس بمعدلات ٥ و ١٠ و ٢٠ ميكروليتر / مل على التوالي. حفظت جميع المعاملات بالتبريد علي درجة حرارة ٥ درجة مئوية لمدة ٤٨ ساعة لدراسة تقييم اضافة مستويات مختلفة من مستخلص البروبليس علي الخصائص الطبيعية للحيوانات المنوية تحت التبريد، و كذلك تقييم كفاءة البروبليس كمضاد حيوي طبيعي في تثبيط النشاط الميكروبي في المخفف مقارنة بالمضادات الحيوية الاصطناعية التجارية. وتشير نتائج هذه الدراسة الي ان مستخلص البروبليس بمستوياته المختلفة ادي الي تحسن معنوي ($p \leq 0.05$) في الخصائص الطبيعية للحيوانات المنوية خلال ٤٨ ساعة تحت التبريد كذلك كان له تأثير معنوي ($p \leq 0.05$) علي تثبيط المستعمرات البكتيرية مما يعني امكانية استخدامه كمضاد حيوي طبيعي مقارنة بالمضادات الحيوية المخلقة الموجودة في صورة تجارية.