Egyptian Journal of Sheep & Goat Sciences, Vol. 12, No. 3, P: 13-23, December 2017

EFFECT OF PROPOLIS AS AN EXTENDR SUPPLEMENTATION ON RAM SEMEN QUALITY AND SPERM PENTRATION ABILITY

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ABSTARCT

Propolis as natural antibiotic has lot of flavonoids, organic compounds, fatty acids steroids, amino acids and others. These ingredients may affect fertility and productivity. This study was designed to determine the effects of various concentrations of propolis powder extract or glue compared with synthetic antibiotic on motility (%), live spermatozoa (%), normality (%) and acrosomal intact (%) traits of ram spermatozoa (as indicator for semen quality), enzymatic activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactic dehydrogenase (LDH); penetration ability and resistance to bacterial contamination in ram semen extender. Ethanolic extract of propolis was prepared by dissolving either powder or glue (10 grams/100 ml ethanol, w/v) incubated at 37 °C for 6 hrs. Seven semen extenders included M1 (control), M2: 400µl pen-sterp and M3: 600µl pen-sterp as synthetic antibiotic; M4: 400µl and M5: 600µl propolis powder; M6: 400µl and M7: 600µl propolis glue as natural antibiotic, respectively. The results showed that semen quality traits decreased significantly ($P<0.01$) with incubation time in all extenders. On the other hand, all sperm parameters and penetration ability were improved ($P<0.01$) while enzymatic activities (ALT, AST, ALP and LDH) were significantly ($P<0.01$) decreased in extenders treated groups [(synthetic and natural antibiotic, either propolis powder or glue)] compared with control group. Furthermore, a higher antimicrobial activity ($P<0.01$) for M5 and M7 extenders were observed compared with other semen extenders. From the present results, it could be concluded that supplementation of propolis extracts either powder or glue in extended ram semen as natural antibiotic led to positive effect on different sperm characteristics, enzymatic activities, penetration ability and protection extender from bacterial contamination.

Keywords: S*emen, propolis, incubation, penetration ability.*

INTRODUCTION

Propolis is produced naturally by honeybees. More than 300 compounds have detected in various propolis extracts that has beneficial pharmacological effects.

Propolis protects the colony from diseases because of its antiseptic efficacy and useful as antibacterial and anti-fungal. In addition, many studies reported that using propolis increased fertility and improved the blood criteria **(Bankova, 2005; Salatino *et al.*, 2005).**

The Egyptian brown propolis analysis showed high percentage of long chain fatty acids, such as palitic acid (24.42%) and flavonoids as 3-hydroxymethyl-1-Phenyl-1-Heptadecyn-3-Ol (13.7%) and flavones (10.15%). These major compounds may be associated with the biological and main activity of different varieties of propolis since isoflavones, flavonoids and fatty acids have been reported as an antioxidant, antimicrobial, antiprotozoal and antifungal activity **(Selem, 2012)**.

Pasing *et al.* (2013) showed that existence of bacterial contaminant in extended semen decreased acrosome reaction, increased sperm agglutination, this may cause infection, inflammation, endotoxins and reproductive disorders. In spite that when female reproductive tract expose to bacterial pollution from the male during natural mating, female tract has natural defense mechanisms prevent bacteria development in the reproductive system **(Gloria *et al.*, 2014)**. Simultaneously, addition of antibiotics to semen extenders is

required to prevent development and growth of bacterial contaminants **(Morrell, 2016).** Indeed, synthetic antibiotics perhaps toxic to spermatozoa and were shown to have an adverse effect on sperm motility, structure of the sperm, shortening viability and caused production of antibodies directed against the sperm glycocalyx complex **(Guimaraes *et al.*, 2015)**. Results substitute antioxidants and antibacterial to resist microorganisms in semen doses for AI would be profitable to improve sperm quality and sperm survival which cause the best rate of fertility. Later, the antioxidant activity of propolis recognized to be mainly attributed to its flavonoid contents, for instance, quercetin, flavones, isoflavones, flavonones, anthocyanins, catechins and isocatechins **(Alves and Kubota, 2013)** that are able to scavenge free radicals and thereby protect against lipid peroxidation **(Moraes *et al.*, 2014).** Conversely, **El-Battawy and Brannas (2015)** specified that semen extenders including propolis might achieve the best motility, act as cryoprotective agent and maintained sperm cells integrity. Additionally, **El-Sheshtawy *et al.* (2016)** announced that addition of bee product as honey and propolis solution to stallion semen extender could improve storing, frozen sperm activity and also attended superior conception rate. On the other hands, **Khalifa *et al.*, (2016)** worked on extended rams semen found greater elimination of bacterial contamination with propolis extract types such as powder or glue than synthetic antibiotics such as Pen-Strep, Alamycin and Vetrocin.

Thus, the targets of this study designed to estimate the efficiency of antibiotics (natural or synthetic) on extended semen quality, penetration ability and resistance to bacterial contamination through incubation at 37°C.

MATERIALS AND METHODS

Location of experiment

All semen samples were collected from Sids Research Stations belonging to Animal Production Research Institute (APRI). Animal Production Department, Faculty of Agriculture, Minia University, Egypt cooperated all extended work for experiment.

Extraction procedure of propolis

At room temperature, ten grams of either propolis powder or glue were mixed individually with 100 ml methanol (70%), kept for 24 hours then filtrated. Then, each filtrate was completed to 100 ml 70% ethanol. Finally, the filtrates of propolis types were transferred individually into two dark bottles and stored at 4°C until supplied to diluted semen.

Experimental animals and feeding

Three rams included in this study. They housed individually in barns and tested for quality of semen that prove acceptable fertility rates. They were mature and healthily and aged 2.2±0.12 years with average live body weight 62.0±1.16 kg. All rams were fed concentrate feed mixture with corn silage twice daily. Minerals and fresh water were available *ad libitum* through all daytime during experimental period.

Semen collection

Early between 8:00 and 9:00 am, in the presence of an estrus ewe used as a mounted animal, semen ejaculates collected using artificial vagina technique. Two ejaculates /ram/ day were obtained up to three weeks from experimental rams. Ejaculates had sperm motility higher than 75%, least 85% of spermatozoa showing normal morphology, live spermatozoa, acrosomal intact and concentration more than 2×10^9 /ml were considered.

Semen evaluation

Evaluation of semen characteristics (volume, motility, livability, normal morphology, acrosomal intact and concentration) were performed immediately after collection. Semen volume estimated directly in the calibrated tube. To estimate advanced motility, one drop of raw semen was mixed with 2 drops of NaCl 0.9 % solution, placed on a warm slide (37 °C); motility was shown at ×400 magnification. Sperm viability was obtained by Eosin-Nigrosin staining of live sperm cells, where intact cell membrane of live sperm does not take up the Eosin stain. While, dead sperm cells takes up the red stain. For more accuracy, 200 spermatozoa were assessed using ×400 magnification. The accepted stained slides were those detected normal morphology spermatozoa by counting a total of 200 sperm

for each stained smear under ×400 magnification. To examine the acrosomal intact (%), 100 µl of semen sample mixed with 500 µl 1% formal citrate lightly (consisted of 2.9g Tri-sodium citrate dehydrate and 1 ml 37% formaldehyde solution, then dissolved in 100 ml distilled water). Acrosomal intact (%) assessed by two drops of the last mixture which smeared and dried on clean slide then inspected under oil immersion ×100 magnifications. The hemocytometer slide was used to count sperm cells concentration at n ×10 /ml using Bulb Pipette for WBCs.

Supplementation of antibiotic to semen extender

After evaluation, pooled samples of raw semen were diluted in Tris extender at 1 ml of raw semen: 10 ml of Tris dilution. Each sample of pooled semen was split in the following seven extenders media in sterilized test tubes. The extender medium included M1 (free from antibiotic types). While, the other six semen extenders M2 and M3 contained 400 and 600µl /100ml extender of synthetic antibiotic as pen-sterp, M4 and M5 included 400 and 600 µl of propolis powder /100 ml extender and M6 and M7 supplied with propolis glue at 400 and 600 µl /100 ml extender, respectively.

Enzymatic Activities

Diluted semen samples centrifuged at 3500 rpm for 15 minutes and the supernatant was removed and used for enzymatic assay. Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactic dehydrogenase (LDH) were determined according to **Graham and Pace (1967).**

Microbiological evaluation

Semen bacterial count expressed as CFU/ml according to **Qureshi *et al.* (1993)**.

Penetration ability

Sperm penetration into cervical mucus tested as follow: cervical mucus obtained from normally cyclic ewes in heat. A portion of mucus was sucked into polyethylene-sealed tubes with 2 mm internal diameter to provide columns of 6 cm length. The extended semen with M7 (the natural antibiotic that had the best characteristics compared to M3, control synthetic antibiotic) incubated at 37°C for 0, 1, 2, 4, and 6 hours and then placed into 2ml cuvettes (1ml each). The tubes containing the mucus were inserted (open end) into cuvettes containing the semen and incubated at 37°C for one hour. Sperm penetration estimated according to score reported by **Hanson *et al.* (1982).**

Statistical analysis

The experiment designed according to the complete randomized design and statistical analyses performed with **SPSS (2013).** Duncan's Multiple Range Test **(Duncan, 1955)** done to detect the differences among means.

RESULTS AND DISCUSSION

Semen quality:

The effects of propolis as natural antibiotics on extended ram semen parameters incubated at 37 °C for up to 6 hours are shown in Tables (1&2). Sperm motility (%), live spermatozoa (Table, 1), sperm normality and acrosomal intact (Table, 2) were decreased with the advancement of incubation time in extended samples. The percentage of spermatozoa with damaged acrosomes showed significant increase following incubation of ram semen at 37 °C for 6 hours **(El-Gaafary, 1987)**. At the same time, propolis supplement in extended samples had significantly ($P<0.01$) higher sperm parameters than M1 extended samples. It was clear from Tables (1&2) that sperm parameters in samples treated by propolis powder or glue had the highest values followed by samples treated by pen-strep and the lowest one was control samples. Regarding propolis as natural antibiotic, the values of the sperm parameters for propolis glue were better than powder. These results are in harmony with **Khalifa *et al*. (2016)** and **Mahmoud (2017)** who reported that propolis, either powder or glue in diluted ram semen improved ram semen quality.

Propolis supplement in diluted semen may protect semen parameters as motility, viability and DNA integrity. Meanwhile, propolis reduces malondialdehyde concentration and provides minerals and vitamins to semen extenders, which keep high quality of viable sperm **(Akandi *et al.*, 2015)**. **Khalifa *et al.* (2017)** reported that non-traditional extender as

lecithin plus propolis extract were insignificantly affected semen characteristics during incubation at 37°C for 4 hours. They found that semen characteristics had the best values in treated extenders compared with control extender.

Table 1: Effect of propolis as an extender supplement on sperm motility (%) and live spermatozoa (%) of the ram semen incubated at 37 °C for 6 hours.

Extenders	Sperm motility (%)					Overall means
	Incubation time (hour)					
	0	1st	2nd	4th	6th	
M1	79.2±2.0^{b}	71.7±2.8^{c}	60.8±2.7^{c}	49.2±3.5^{d}	29.2±2.4^{d}	58.0±3.5^{c}
M2	81.7±1.7^{ab}	74.2±3.3^{bc}	65.8±3.0^{bc}	54.2±3.0^{cd}	44.2±3.0^{c}	64.0±2.8^{bc}
M3	82.5±1.7^{ab}	76.7±2.5^{abc}	69.2±2.4^{ab}	59.2±2.4^{bc}	49.2±2.4^{bc}	67.3±2.4^{ab}
M4	83.3±1.1^{a}	77.5±1.7^{abc}	70.0±2.6^{ab}	60.8±3.3^{abc}	52.5±4.2^{abc}	68.8±2.4^{ab}
M5	84.2±0.8^{a}	78.3±1.7^{abc}	70.8±2.4^{ab}	62.5±3.1^{abc}	55.8±3.9^{ab}	70.3±2.2^{ab}
M6	85.0±0.8^{a}	80.8±0.8^{ab}	75.0±1.3^{a}	67.5±1.1^{ab}	59.2±2.0^{a}	73.5±1.8^{a}
M7	85.8±0.8^{a}	81.7±1.1^{a}	75.8±1.5^{a}	69.2±2.4^{a}	61.7±3.3^{a}	74.8±1.8^{a}
Means	83.1±0.6^{A}	77.3±0.9^{B}	69.6±1.1^{C}	60.4±1.4^{D}	50.2±1.9^{E}	68.1
	Live spermatozoa (%)					
M1	80.50±1.34^{e}	74.17±1.14^{e}	53.17±1.45^{e}	40.50±1.54^{g}	27.83±1.51^{g}	55.2±3.7^{c}
M2	83.67±0.95^{de}	79.17±0.95^{d}	65.83±0.70^{d}	55.17±0.70^{f}	45.17±0.70^{f}	65.8±2.7^{b}
M3	85.83±1.72^{cd}	82.50±1.75^{c}	72.33±1.26^{c}	65.17±1.08^{e}	53.83±1.70^{e}	71.9±2.3^{ab}
M4	86.50±0.76^{bcd}	83.33±0.67^{c}	74.83±0.70^{c}	70.17±0.75^{d}	59.50±0.99^{d}	74.9±1.8^{ab}
M5	88.50±1.18^{bc}	85.33±1.26^{bc}	79.17±0.95^{b}	74.17±0.87^{c}	63.17±1.11^{c}	78.1±1.7^{ab}
M6	89.83±0.87^{ab}	87.17±0.87^{ab}	82.50±0.92^{a}	77.50±0.67^{b}	68.17±1.14^{b}	81.0±1.5^{a}
M7	92.17±0.95^{a}	89.33±0.84^{a}	85.17±0.95^{a}	80.83±0.83^{a}	72.50±0.85^{a}	84.0±1.3^{a}
Means	86.7±0.7^{A}	83.0±0.8^{A}	73.3±1.6^{B}	66.2±2.1^{C}	55.7±2.2^{D}	73.0

$^{a:e}$, Means with different superscripts, within each column, are significantly different (P<0.01).
$^{A:E}$, Means with different superscripts, within each row, are significantly different (P<0.01).
M1: Control, **M2**: 400µl pen-sterp, **M3**: 600µl pen-sterp, **M4**: 400µl propolis powder, **M5**: 600µl propolis powder, **M6**: 400µl propolis glue and **M7**: 600µl propolis glue.

Moraes *et al.* (2014) revealed that semen characteristics had better values with 1.25 g powder propolis/kg diet of rabbit. Results showed that propolis extracts either powder or glue supplemented in ram semen extender improved sperm parameters compared with control. M7 had the highest values and M1 was the lowest, other extenders came in between. From these results, propolis extract as natural antibiotics in the extenders had the highest ability to sustain sperm viability and motility. This may be related to ability of antibiotics to supply protection to nutrients afford to sperm cells, besides they inhibit microbial growth and improve physiologic condition. Hence, the reduction in sperm parameters with longer storage time could attribute to the gradual consumption of nutrients required for sperm metabolism during incubation. **Kasimanickam *et al*. (2006)** reported that decline in motility might be due to physiological reasons include extracellular oxidative stress, seminal plasma volume-constituents and endogenous free radical production.

Table 2: Effect of propolis as an extender supplement on sperm normality (%) and acrosomal intact (%) of the ram semen incubated at 37 °C for 6 hours.

Extenders	Sperm normality (%)					Overall means
	Incubation time (hour)					
	Zero	1st	2nd	4th	6th	
M1	86.5±1.5^{e}	74.5±2.6^{e}	63.8±2.1^{e}	56.3±2.1^{d}	46.3±2.3^{e}	65.5±2.7^{d}
M2	89.2±1.0^{d}	80.7±0.9^{d}	70.8±1.4^{d}	64.8±1.2^{c}	56.7±2.7^{d}	72.4±2.2^{c}
M3	90.8±0.9^{cd}	82.5±1.2^{cd}	71.5±1.9^{d}	66.2±2.5^{c}	60.3±2.1^{cd}	74.3±2.2^{bc}
M4	91.8±0.8^{bcd}	83.7±0.7^{cd}	74.2±0.6^{cd}	69.5±0.9^{bc}	65.2±1.4^{bc}	76.9±1.8^{ab}
M5	93.2±0.9^{abc}	85.3±1.0^{bc}	75.8±0.5^{bc}	71.8±1.2^{ab}	67.2±1.1^{ab}	78.7±1.8^{ab}
M6	94.5±0.4^{ab}	87.8±0.5^{ab}	78.7±0.7^{ab}	73.3±0.7^{ab}	69.3±0.7^{ab}	80.7±1.7^{ab}
M7	95.2±0.3^{a}	89.3±0.4^{a}	80.8±0.6^{a}	76.5±0.6^{a}	71.8±0.7^{a}	82.7±1.6^{a}
Means	91.6±0.5^{A}	83.4±0.8^{B}	73.7±0.9^{C}	68.4±1.1^{D}	62.4±1.4^{E}	75.9
	Acrosomal intact (%)					
M1	86.17±1.08^{c}	77.67±1.71^{c}	68.17±1.60^{d}	60.50±1.38^{d}	54.67±1.67^{e}	70.1±2.0^{c}
M2	89.83±0.75^{b}	83.67±0.49^{b}	77.50±0.43^{c}	68.50±0.62^{c}	60.17±1.08^{d}	75.9±2.0^{b}
M3	94.17±0.48^{a}	87.83±0.75^{a}	80.83±0.79^{b}	74.83±1.35^{b}	66.67±0.84^{c}	79.6±2.2^{ab}
M4	94.83±0.48^{a}	88.83±0.79^{a}	82.50±1.38^{ab}	76.67±1.26^{ab}	69.50±1.18^{bc}	80.8±2.2^{ab}
M5	95.17±0.70^{a}	89.67±0.61^{a}	83.67±0.67^{ab}	77.17±1.47^{ab}	71.67±1.56^{ab}	81.2±2.1^{ab}
M6	95.50±0.22^{a}	90.17±0.65^{a}	84.33±1.02^{a}	78.83±0.54^{a}	73.17±1.14^{ab}	82.6±2.0^{a}
M7	95.67±0.33^{a}	90.67±0.67^{a}	85.33±0.88^{a}	80.17±1.08^{a}	75.17±1.28^{a}	83.3±1.8^{a}
Means	93.0±0.6^{A}	86.9±0.7^{B}	79.9±0.9^{C}	73.4±1.0^{D}	62.1±0.5^{E}	79.1

$^{a:e}$, Means with different superscripts, within each column, are significantly different (P<0.01).
$^{A:E}$, Means with different superscripts, within each row, are significantly different (P<0.01).
M1: Control, **M2**: 400µl pen-sterp, **M3**: 600µl pen-sterp, **M4**: 400µl propolis powder, **M5**: 600µl propolis powder, **M6**: 400µl propolis glue and **M7**: 600µl propolis glue.

Moreover, propolis in semen extender as antioxidants plays a major role in preventing the formation of free radicals, which are responsible of many oxidative processes leading to cell damage. Many studies showed that propolis possesses antioxidant activity. This may be due to the free radical scavenging activity of propolis that protects sperm membrane from the harmful action of oxidative raids and decrease the formation of barbituric acid reactive substances **(Russo *et al*., 2006)**.

Enzymatic activities (U/10^{9} spermatozoa):

The obtained data in Tables (3&4) indicate the effect of propolis as natural antibiotic on enzymatic activities (ALT, AST, ALP and LDH) during incubation at 37 °C for 6 hours.

Results showed that propolis extenders especially M7 significantly (P<0.01) lowered the release of ALT, AST, ALP and LDH enzymes into the extracellular medium than the control during incubation period. Overall means of ALT, AST, ALP and LDH enzymes differed significantly (P<0.01) among extenders, either due to antibiotic types or incubation times (0, 1, 2, 4 and 6 hours).

The highest values recorded with M7 (14.4, 30.7, 80.9 and 211.6 U/10^{9} spermatozoa) followed by M6 (15.1, 31.5, 83.0 and 213.7 U/10^{9} spermatozoa); M5 (16.2, 32.5, 83.7 and 214.4 U/10^{9} spermatozoa) and M4 extender (16.8, 32.7, 85.2 and 215.9) for ALT, AST, ALP and LDH enzymes, respectively.

Table 3: Effect of propolis as an extender supplement on alanine aminotransferase and aspartate aminotransferase enzymes activity of the ram semen incubated at 37 °C for 6 hours.

Extenders	Incubation time (hour)					Overall means
	Zero	1st	2nd	4th	6th	
Alanine aminotransferase (U/10^9 spermatozoa)						
M1	17.0±0.1^{a}	16.9±0.1^{a}	19.3±0.2^{a}	21.9±0.3^{a}	27.6±0.4^{a}	20.6±0.9^{a}
M2	16.6±0.2^{b}	16.8±0.1^{a}	19.5±0.2^{a}	20.5±0.3^{b}	24.5±0.3^{b}	19.6±0.7^{a}
M3	15.6±0.2^{c}	15.9±0.1^{b}	18.5±0.2^{b}	20.0±0.2^{b}	23.9±0.2^{b}	18.8±0.7^{a}
M4	14.2±0.2^{d}	14.2±0.1^{c}	16.2±0.1^{c}	18.5±0.2^{c}	20.9±0.3^{c}	16.8±0.6^{b}
M5	14.1±0.1^{d}	13.9±0.1^{d}	15.9±0.1^{c}	17.4±0.1^{d}	19.7±0.1^{cd}	16.2±0.5^{bc}
M6	12.0±0.1^{e}	12.6±0.1^{e}	15.1±0.1^{d}	17.0±0.1^{de}	18.9±0.2^{de}	15.1±0.6^{bc}
M7	11.4±0.1^{f}	11.9±0.1^{f}	14.3±0.1^{e}	16.4±0.1^{e}	18.0±0.1^{e}	14.4±0.6^{c}
Means	14.4±0.4^{D}	14.6±0.4^{D}	17.0±0.4^{C}	18.8±0.4^{B}	21.9±0.6^{A}	17.4
Aspartate aminotransferase (U/10^9 spermatozoa)						
M1	35.0±0.4^{a}	35.2±0.5^{a}	40.7±0.6^{a}	42.7±0.6^{a}	48.2±0.5^{a}	40.3±1.3^{a}
M2	33.8±0.4^{b}	32.8±0.4^{b}	36.7±0.5^{b}	39.3±0.5^{b}	46.0±0.4^{b}	37.7±1.1^{a}
M3	31.3±0.5^{c}	30.5±0.3^{c}	34.2±0.3^{bc}	37.5±0.5^{c}	41.0±0.8^{c}	34.9±0.9^{b}
M4	29.2±0.3^{d}	28.6±0.4^{d}	32.3±0.6^{cd}	35.3±0.5^{d}	38.0±0.4^{d}	32.7±0.8^{bc}
M5	29.0±0.4^{d}	28.4±0.4^{de}	32.0±0.4^{cd}	35.0±0.4^{de}	38.3±0.6^{d}	32.5±0.9^{bcd}
M6	28.3±0.2^{de}	27.4±0.2^{de}	30.7±0.2^{d}	34.0±0.4^{de}	37.0±0.4^{de}	31.5±0.8^{cd}
M7	27.7±0.2^{e}	26.7±0.2^{e}	29.8±0.1^{d}	33.2±0.3^{e}	36.2±0.3^{e}	30.7±0.8^{d}
Means	30.6±0.5^{D}	30.0±0.6^{D}	33.8±0.8^{C}	36.7±0.6^{B}	40.7±0.9^{A}	34.3

$^{a:f}$, Means with different superscripts, within each column, are significantly different (P<0.01).
$^{A:D}$, Means with different superscripts, within each row, are significantly different (P<0.01).
M1: Control, **M2**: 400µl pen-sterp, **M3**: 600µl pen-sterp, **M4**: 400µl propolis powder, **M5**: 600µl propolis powder, **M6**: 400µl propolis glue and **M7**: 600µl propolis glue.

On the other hand, the pen-strep antibiotic extenders (M3 & M2) and M1 had the lowest (P<0.01) values (18.8, 34.9, 88.9 and 219.7 U/10^9 spermatozoa); (19.6, 37.7, 91.9 and 222.6 U/10^9 spermatozoa) and (20.6, 40.3, 96.3 and 227.0 U/10^9 spermatozoa) for ALT, AST, ALP and LDH enzymes, respectively.

The incubation at 37 °C for 6 hours increased the quantity of ALT, AST, ALP and LDH enzymes released into the extracellular medium. The same trend was reported by **Zeidan *et al.* (2004).** They also reported that the continuous increase in leakage of ALT, AST, ALP and LDH enzymes into the extracellular medium through incubation or storage might reflect the breakdown of the cellular sperm membrane during incubation and storage. **S**uch increase in the enzyme activity after incubation and preservation, also, might be a sign for increasing the cell break which occurred during storage process **(Zeidan, 1994).**

In mammals, the acrosome includes digestive enzymes (containing hyaluronidase and acrosin). Those enzymes destroy the outer membrane of the ovum (zona pellucida), allowing the haploid nucleus in the sperm cell to joint with the haploid nucleus in the ovum.

Sperm penetration into cervical mucus:

Figure 1 shows that, the penetration ability into ewe cervical mucus was better, but insignificantly, in the extended ram semen with natural antibiotics (M7) than with synthetic antibiotics (M3) during incubation time.

Table 4: Effect of propolis as an extender supplement on alkaline phosphatase and lactic dehydrogenase enzymes activity of the ram semen incubated at 37 °C for 6 hours.

Extenders	Incubation time (hour)					Overall means
	0	1st	2nd	4th	6th	
Alkaline phosphatase (U/10^9 spermatozoa)						
M1	81.6±0.5^{a}	82.4±0.6^{a}	91.7±0.7^{a}	100.4±0.7^{a}	125.5±0.9^{a}	96.3±3.8^{a}
M2	77.1±0.2^{b}	82.2±0.5^{a}	87.6±0.9^{b}	100.7±0.6^{a}	111.8±0.7^{b}	91.9±2.9^{ab}
M3	76.6±0.3^{b}	81.7±0.3^{ab}	85.7±0.9^{b}	98.7±0.4^{a}	102.0±0.9^{c}	88.9±2.3^{bc}
M4	74.7±0.2^{c}	79.6±0.3^{bc}	83.0±0.2^{c}	89.5±0.2^{b}	99.3±0.2^{cd}	85.2±2.0^{bcd}
M5	73.8±0.1^{c}	77.4±0.2^{cd}	80.2±0.1^{d}	88.3±0.3^{b}	98.8±0.4^{d}	83.7±2.0^{cd}
M6	73.5±0.1^{c}	77.1±0.1^{d}	79.8±0.1^{d}	87.7±0.2^{bc}	96.8±0.1^{d}	83.0±1.9^{cd}
M7	70.9±0.1^{d}	75.8±0.4^{d}	78.6±0.4^{d}	86.0±0.2^{c}	93.0±0.3^{e}	80.9±1.8^{d}
Means	75.5±0.6^{E}	79.5±0.5^{D}	83.8±0.9^{C}	93.0±1.2^{B}	103.9±2.0^{A}	87.1
Lactic dehydrogenase (U/10^9 spermatozoa)						
M1	193.2±1.1^{a}	194.4±1.6^{a}	216.7±1.7^{a}	245.4±1.7^{a}	285.5±2.0^{a}	227.0±8.0
M2	188.6±1.2^{b}	194.2±1.1^{a}	212.6±1.9^{b}	245.7±1.6^{a}	271.8±1.7^{b}	222.6±7.3
M3	188.1±1.3^{b}	193.7±1.3^{ab}	210.7±1.9^{b}	243.7±1.4^{a}	262.0±1.9^{c}	219.7±6.6
M4	186.3±1.2^{c}	191.6±1.2^{bc}	208.0±1.2^{c}	234.5±1.2^{b}	259.3±1.2^{cd}	215.9±6.3
M5	185.4±1.1^{c}	189.4±1.2^{cd}	205.2±1.1^{d}	233.3±1.3^{b}	258.8±1.4^{d}	214.4±6.4
M6	185.1±1.1^{c}	189.1±1.1^{d}	204.8±1.1^{d}	232.7±1.2^{bc}	256.8±1.1^{d}	213.7±6.3
M7	182.5±1.1^{d}	187.8±1.4^{d}	203.6±1.2^{d}	231.0±1.2^{c}	253.0±1.2^{e}	211.6±6.1
Means	187.0±0.6^{E}	191.5±0.5^{D}	208.8±0.9^{C}	238.0±1.2^{B}	263.9±2.0^{A}	217.8

$^{a:e}$, Means with different superscripts, within each column, are significantly different (P<0.01).

$^{A:E}$, Means with different superscripts, within each row, are significantly different (P<0.01).

M1: Control, **M2**: 400µl pen-sterp, **M3**: 600µl pen-sterp, **M4**: 400µl propolis powder, **M5**: 600µl propolis powder, **M6**: 400µl propolis glue and **M7**: 600µl propolis glue.

However, incubation time decreased significantly (P<0.01) the penetration score. Sperm that are unable to go properly through the acrosome reaction will not be able to fertilize an egg **(Miyazaki *et al.*, 1990).** Results in this study concerning sperm penetration (Fig. 1) may be due to that presence of propolis in seminal plasma of extended semen causes increase the release of amino acid oxidase, an enzyme responsible for reduction of sperm motility and survivability **(Martinus *et al.*, 1991). Aitken and Kelly (1985)** found a close correlation between spermatozoa movement in human semen and their penetration ability into cervical mucus.

Total bacterial count

The effect of synthetic and natural antibiotics compared with control extenders on total bacterial count for incubation at 37 °C for 6 hrs are shown in Figure (2).

Total bacterial count increased with incubation time used for all extenders. However, the type of extender significantly (P<0.01) affected the bacterial count. It is evident from the bacterial count that extenders with propolis, as natural antibiotic, had the lowest values and the best protective film against bacterial contamination followed by pen-strep as synthetic antibiotic and the least one was the control extender. Concerning propolis extenders, values of total bacterial count for the propolis glue were better than the powder.

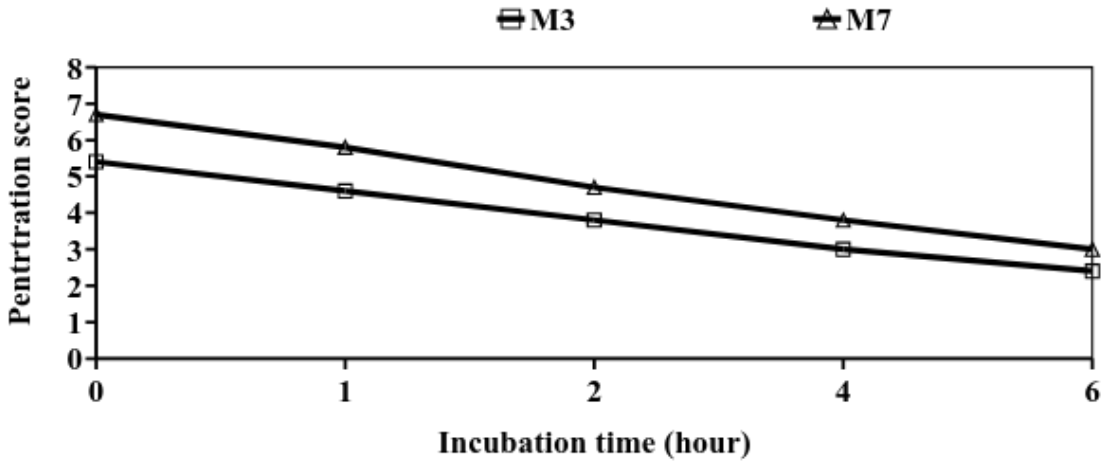

Figure 1: Penetration ability of extended ram's semen into ewe cervical mucus.

There were significantly (P<0.01) higher inhibition for Gram-positive and negative bacteria in extenders treated by propolis than either synthetic antibiotic or control in extended ram semen. These results are in agreement with those reported by **Takaisi and Schilder (1994)** who showed that propolis involving several mechanisms such as the formation of pseudo-multicellular streptococci; disorganization of the cytoplasm, the cytoplasmatic of membrane and the cell wall; partial bacteriolysis and inhibitation of protein synthesis. Furthermore, **Khalifa *et al*. (2016)** reported that semen extender with propolis displayed more effectiveness on inhibitory bacterial zone and reduced bacterial count compared with other extenders and that propolis may use to restrain the bacterial infection and improve semen quality. **Khalifa *et al*. (2017)** reported also that propolis addition to the extender instead of synthetic antibiotics resisted bacterial contamination, which improved sperm viability during incubation or storage.

Probst *et al.* (2011) noted that essential oils of propolis glue contribute to fights the bacteria and it has different mechanism of action that are important for the antimicrobial activity.

The antibacterial action of propolis on different bacterial strains has been observed by several authors **(Bankova, 2005; Khalifa *et al*., 2016).** Additionally, **Itavo *et al*. (2011)** confirmed that propolis has bacteriostatic activity against gram-positive and some gram-negative bacteria, *via* changes in the bioenergetic status, which inhibits bacterial motility.

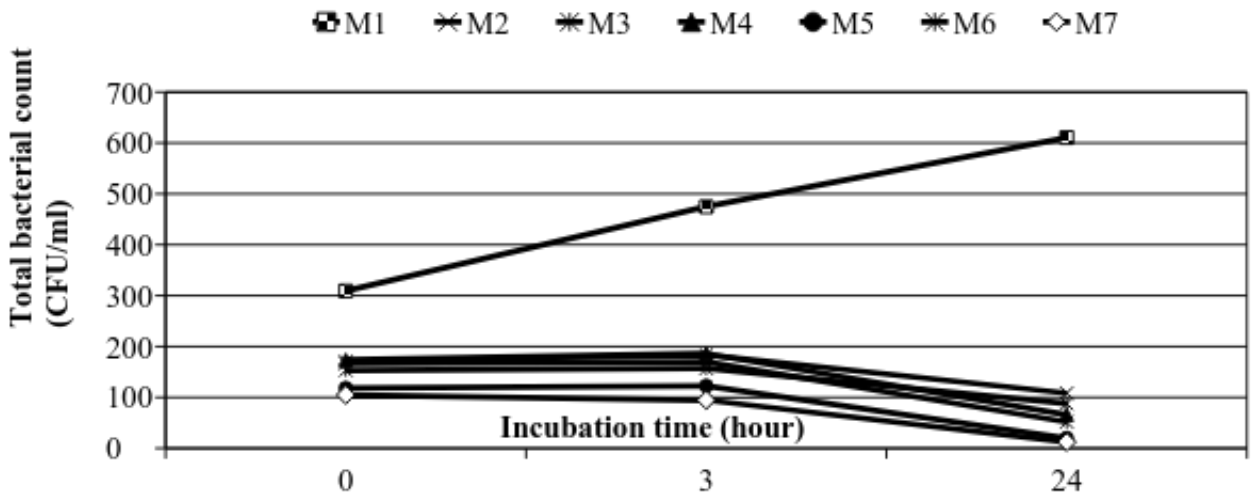

Figure 2: Effect of propolis as an extender supplement on total bacterial count (CFU/ml) of ram semen during incubation at 37°C

M1: Control, **M2**: 400µl pen-sterp, **M3**: 600µl pen-sterp, **M4**: 400µl propolis powder, **M5**: 600µl propolis powder, **M6**: 400µl propolis glue and **M7**: 600µl propolis glue.

CONCLUSION

From current results, it was concluded that supplementation of propolis extracts either powder or glue as natural antibiotic to ram semen extender improved sperm characteristics and enzymatic activities compared to either pen-strep as synthetic antibiotic or control extenders. Sperm characteristics and penetration ability with propolis glue extenders were better than propolis powder. In addition to, added 600µl of propolis glue in ram semen extender was the best compared with other extenders and it can be used to protective the extender from bacterial contamination.

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تأثير إضافة البروبوليس الى مخفف السائل المنوي للكباش على بعض صفاته الطبيعية والكيميائية

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الملخص العربي

البروبوليس كمضاد حيوي طبيعي يمتلك كثير من الفلافونيدات ، المركبات العضوية ، الاحماض الدهنية الاستروبدية، الأحماض الأمينية وأخرى . هذه المكونات تؤثر على خصوبة الحيوان وأداءه الإنتاجي وهذا البحث يهدف إلى تقييم التركيزات المختلفة لمستخلص البروبوليس في صورة بودر أو صمغ بالمقارنة بالمضادات الحيوية المخلقة وتأثير ذلك على حركة الحيوانات المنوية (%)، الحيوانات المنوية الحية (%)، الحيوانات المنوية الطبيعية (%)، الأكروسوم السليم (%) خلال التحضين كدليل على جودة السائل المنوي ، النشاط الأنزيمي (ALT, AST, ALP and LDH) ، درجة الاختراق والنفاذ داخل مخاط عنق الرحم للنعاج ومقاومة التلوث البكتيري. تم اعداد المستخلص الاثيري للبروبوليس عن طريق إذابة المسحوق أو الصمغ في الإيثانول (10 جم لكل 100 مل إيثانول ، وزن/حجم) والتحضين على درجة 37 درجة مئوية لمده 6 ساعات. كان هناك سبعة معاملات للسائل المنوي 1M =الكنترول ، 2M = 400 مل بنسلين ستربتومايسين ، 3M =600 مل بنسلين ستربتومايسين ، 4M = 400 مل مسحوق البروبوليس ، 5M =600 مل مسحوق البروبوليس ، 6M =400 مل صمغ بروبوليس و 7M =600 مل صمغ بروبوليس كمضادات حيوية طبيعية.

أوضحت النتائج أن صفات جودة السائل المنوي أنخفضت عند مستوى معنوية (P<0.01) مع زيادة فترة التحضين لكل المعاملات. من ناحية أخري تحسنت قياسات السائل المنوي وقيم اختراق الحيوانات المنوية لمخاط عنق الرحم عند مستوى معنوية (P<0.01). كما ان النشاط الأنزيمي (ALT, AST, ALP and LDH) أنخفض عند مستوى معنوية (P<0.01) بمقارنة المعاملات الأخري بالمعاملة الكنترول (مضادات حيوية مخلقة ، طبيعية، مسحوق أو صمغ البروبوليس) . وكانت المعاملات 5M، 7M الأفضل في النشاط الميكروبي عن المعاملات الأخري. من النتائج الحالية يمكن استنتاج ان اضافة مستخلص البروبوليس سواء البودر أو الصمغ لمخفف السائل المنوى كمضاد حيوي طبيعي له اثار ايجابية على خصائص السائل المنوى المختلفة وكذلك النشاط الانزيمي وقدرة اختراق الحيوان المنوي لمخاط عنق الرحم كما انه يقوم بحماية المخفف من التلوث البكتيري.