

EFFECT OF BEE HONEY-INCLUDED EGG YOLK BASED EXTENDERS ON MOTILITY, VIABILITY AND FERTILIZING ABILITY OF FROZEN RABBIT SEMEN

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The proportion of bee honey, added to Tris-based extender with or without dimethylsulfoxide (DMSO) for extension of New Zealand White (NZW) rabbit buck semen conserved as frozen form was studied. Semen were collected from ten sexually mature NZW rabbit bucks, pooled and diluted with 16 different extenders based on Tris-citric-glucose with or without DMSO. Honey was added in increasing concentrations (v/v) 0, 1, 2, 3, 4, 5, 7.5, 10% and decreasing concentrations of egg yolk 20, 19, 18, 17, 16, 15, 12.5 and 10% (v/v). Evaluations for percentages of progressive motility, dead and abnormal spermatozoa were done immediately after dilution and one month of freezing in liquid nitrogen. Fertilizing ability was evaluated as pregnancy rate and litter size of 20 females inseminated artificially for each extender, which showed $\geq 50\%$ sperm progressive motility after thawing. Extenders contained 2 and 3% honey showed the best values for sperm motility, livability and abnormalities, also for pregnancy rate and litter size.

***Conclusively,** the results of the present study suggested that the addition of bee honey to egg yolk extenders improves the motility and viability of rabbit spermatozoa and may improve cryopreservation of rabbit spermatozoa. However, this effect is concentration dependent and higher concentrations of honey may have negative effect on sperm quality or fertilizing ability.*

Key words: Honey, egg yolk, extenders, motility, fertilizing ability, rabbit, semen.

Artificial insemination (AI) has been postulated to have the potential of increasing productivity, enhancing biosecurity and aiding the genetic management of animals. A.I. has been introduced in industrial rabbitries mainly to improve breeding management (Castellini, 1996). A.I. is usually performed with fresh diluted semen resulted in obtaining a satisfactory

fertility rate or similar to that obtained from natural insemination (El-Gaafary and Marai, 1994) and (Harkness *et al.*, 2010), especially during unfavorable times of the year (Carluccio *et al.*, 2004).

The major target for the rabbit breeder is how to preserve the fertilizing capacity of stored rabbit semen extenders are certain ingredients added to the ejaculated semen to sustain and protect the spermatozoa thereby preserving its fertility until they are used for insemination (Geoffrey *et al.*, 1992). Egg yolk based extender has been the common used extender, unfavorably, egg yolk represent a good medium for the growth of microorganisms. The use of honey to partly replace egg yolk in egg yolk based extenders is the focus of this study since honey has antibacterial activity against some microorganisms, which could be resistant to the common antibiotics used in the extender. Additionally, honey contains high level of metabolizable energy in form of glucose and fructose (Molan and Russell, 1988; Al-Waili, 2004). Gelam honey has the potential to increase the fertility of male rats by increasing sperm count and number of spermatozoa with normal morphology (Syazana *et al.*, 2011). The addition of honey to egg yolk extenders improves motility and live dead ratio and thus viability of liquid goat semen.

Therefore, the aim of the present work is to study the effect of pure bee honey addition to egg yolk based extender on motility, viability, abnormalities and fertilizing ability of NZW rabbit spermatozoa stored in liquid nitrogen.

MATERIALS AND METHODS

The present study was carried out during winter season 2012 at the Intensive Rabbit Production Unit, Faculty of Agriculture, Ain Shams University, Cairo, Egypt and a Private Rabbit Farm, Kalioubia, Egypt.

Experimental animals:

Ten sexually mature New Zealand White (NZW) rabbit bucks, aged 15 months with average body weight 3.8 kg, were selected for high quality semen (reaction time < 2 minutes, color white only, volume \geq 0.2 ml, density creamy and milky only, concentration > 60 million/ml semen, dead-sperm percentage < 20%, and sperm abnormalities < 20%). For testing the fertilizing ability of frozen diluted semen, 100 hybrid nonparous females at a private rabbit farm, Kalioubia, Egypt, were used in the experiment (20 females per each experimental group). All experimental animals were housed individually in flat deck cages and fed a commercial concentrate pelleted diet according to their reproductive condition and fresh water was provided *ad libitum*.

Semen collection and evaluation:

Semen was collected from each buck twice weekly using an artificial vagina. Two female rabbits were used as a teaser. Immediately after semen collection, gel plug was removed. Only ejaculates with white color and good mass motility (≥ 3 on a 0–5 scale) were used for semen processing. After collection, semen from ten bucks was pooled together as described by Safaa *et al.* (2012). The parameters were examined to evaluate semen quality of rabbit bucks as described by El-Sherbiny (1987) and Madhuri *et al.* (2012). Pooled semen was diluted by adding one volume of semen to five volume extender (1 semen : 5 extenders v/v) at 37°C.

Percentages of progressive motility, dead and total abnormalities of spermatozoa were evaluated immediately after semen extension, and one month later after conservation in liquid nitrogen.

Semen dilution:

Tris-citric-glucose with or without 1.75 M DMSO, as described by Vincente and Viudes-de-Castro (1996) was used in the present study. Tris-citric-glucose was the basic diluent and had the following composition: 0.25M of Tris buffer (hydroxymethyl) aminomethan, 0.87M of citric acid monohydrate, 0.47M D (+) glucose, 100000 IU Penicillin and 100mg streptomycin sulfate (Viudes-de-Castro and Vincente, 1996 and Si *et al.*, 2006), All previous components were dissolved in glass bid stiller water and completed total volume to 100 ml. Honey was added to the diluent in increasing concentrations (v/v) 0, 1, 2,3, 4, 5, 7.5 and 10% and decreasing concentrations of egg yolk 20, 19, 18, 17, 16, 15, 12.5 and 10% (v/v). The honey-included egg yolk percentage was 20% (v/v) of diluent.

pH and osmotic potential of extenders used in the present study:

After preparation of different extenders, pH and osmotic pressure were calculated (Table 1). Values of extenders pH were calculated using comparative pH papers ranging from 6.3 to 8.1. Extender osmotic potential was calculated using osmometer (Osmomat 030, Gonotec, Germany) as megapascal (Mpa); using the next formula: OP (MPa) = - c (mosmol/kg) x 2.58×10^{-3} , where c is the osmolarity of the sap (Bagatta *et al.*, 2008).

Semen freezing:

Semen freezing was performed with honey-included egg yolk extenders as described by Mocé and Vincente (2009). The period between semen extension and completion of semen freezing didn't exceed 90 minute.

Table 1. pH and osmotic potential of semen extenders

Extender	pH	Osmotic potential (MPa)
<i>Tris-citric-glucose +:</i>		
20%egg yolk	6.8	- 0.408
1%honey+19%egg yolk	6.5	- 0.535
2%honey+18%egg yolk	6.6	- 0.539
3%honey+17%egg yolk	6.6	- 0.630
4%honey+16%egg yolk	6.7	- 0.717
5%honey+15%egg yolk	6.2	- 0.788
7.5%honey+12.5% egg yolk	6.5	- 0.859
10%honey+10% egg yolk	6.6	- 1.180
<i>DMSO +Tris-citric-glucose+:</i>		
20%egg yolk	6.9	- 5.237
1%honey+19%egg yolk	6.8	- 5.272
2%honey+18%egg yolk	6.8	- 5.418
3%honey+17%egg yolk	6.8	- 5.480
4%honey+16%egg yolk	6.6	- 5.614
5%honey+15%egg yolk	6.7	- 6.099
7.5%honey+12.5% egg yolk	6.7	- 6.388
10%honey+10% egg yolk	6.8	- 6.632

Semen freezing was performed with honey-included egg yolk extenders as described by Mocé and Vincente (2009). Semen freezing was performed with extenders as follows: Directly after semen dilution, diluted semen vials was kept in a glass of water at 37°C, so that diluted semen level in vials kept below the surface of water; then, quickly the vials of diluted semen were transferred in a refrigerator to be cooled down to +5°C. Extended semen was left at 5°C for 45-60 minutes. Vials were stirred well by applying a rotative motion before backing. The extended semen was sucked immediately into 0.5 ml cooled French straws. The straws were filled incompletely so that a column of air 0.5-1 cm in length was left in each straw. The straws were then plugged by pressing their free end into a ½ cm thick layer of polyvinyl

alcohol powder. The plugged straws were put into a glass of water maintained at 4-5°C to remove excess of powder and to ensure good plugs.

After the completion of packing, the straws were removed from the water glass and placed in a horizontal position into a special cooled rack; then, the rack was transferred quickly into a rectangle foam box which contain liquid nitrogen at a height of about 1cm, where the straws were exposed to nitrogen vapor for 8 minutes as follows: one minute at about 10 cm distance above the surface of the liquid nitrogen, 4 minutes at about 8 cm distance above the surface of the liquid nitrogen, 3 minutes at about 3 cm distance above the surface of the liquid nitrogen, then straws removed from the rack and dipped directly into the liquid nitrogen canister.

The period between semen extension and completion of semen freezing don't exceed 90 minutes as semen pH dose not became stable until 60-90 minute from the start of dilution.

Examination of frozen-thawed semen:

Frozen straws were thawed in a water bath at 37°C for 30 seconds (Si *et al.* 2006). After thawing, semen samples were examined for percentage of progressive motility, dead and total abnormal spermatozoa.

Artificial insemination:

Females chosen for insemination were thought to be sexually receptive (had red color of vulva lips). In order to induce ovulation, females were injected intramuscularly with 0.3 ml receptal (GnRH analogue, 1.26µg of busereline acetate; intervet, Cairo, Egypt). Thawed diluted semen showed progressive motility less than 50% didn't use for AI. Then, each doe was inseminated artificially with 0.5 ml diluted semen (containing approximately 30×10^6 sperms) just after GnRH injection. Pregnancy was detected by trans-abdominal palpation 14 days post-insemination to determine pregnancy rate. Litter size was determined for each doe directly after kindling.

Statistical analysis:

Analysis of variance was performed using SAS program (SAS, 2011) to test the significance of treatments using one way ANOVA. Differences among experimental groups were tested by Duncan's Multiple Range test (Duncan, 1955). The statistical model was:

$$Y_{ij} = \mu + t_i + e_{ij}$$

Where: μ is the overall mean, Y_{ij} is the observation of the studied trait of j^{th} animal of i^{th} treatment, t_i is the fixed effect of treatment ($i = 1, 2, \dots, 16$), e_{ij} is the individual error.

For pregnancy rate trait, **Catmod** and Chi-Square test for homogeneity of variance was performed using SAS program (SAS, 2011).

RESULTS AND DISCUSSION

Effect of bee honey on some physical characteristics of diluted NZW rabbit semen before and after freezing:

The impact of adding bee honey to replace part of egg yolk to rabbit semen extenders was studied on progressive motility, viability and sperm abnormalities before freezing and after thawing. The results represented in Table (2) showed that honey improved percentage of progressive motility significantly ($P \leq 0.05$) as compared with basal Tris-citric-glucose + 20% egg yolk. On the other hand, after freezing and thawing, percentage of progressive motility of NZW rabbit spermatozoa diluted with extenders supplemented honey was significantly ($P \leq 0.05$) higher than that obtained for Tris-citric-glucose with or without DMSO. This finding proved that bee honey may had such effect to improve the **cryoprotectant** capacity of DMSO.

The inclusion of honey as a source of glucose (Molan and Russell, 1988; Al-Waili, 2004) in an egg yolk buffer diluting medium used in the present study supported by the report of Smith *et al.* (1954) that the addition of small amounts of glucose to an egg yolk buffer increases and prolongs active motility of spermatozoa.

The higher concentrations of honey (7.5 and 10%) did not sustain the motility of diluted rabbit semen either before or after freezing suggesting that the effect of honey on sperm motility might be dependent on concentration and that higher concentrations of honey may have negative effect on sperm motility and viability.

Values obtained for both percentages of dead and abnormal spermatozoa immediately after dilution were low and the differences between experimental groups were slightly lower for Tris-citric-glucose with or without DMSO + 20% egg yolk extenders compared with those of honey, except for extenders containing 1 and 2% honey. The differences between extenders on percentages of dead and abnormal spermatozoa were significant ($P \leq 0.05$). However, adding honey to rabbit semen extenders decreased percentages of dead and abnormal spermatozoa after freezing and thawing.

The results showed that the effect of honey is concentration dependent for all physical semen characteristics studied and that concentrations of 2% honey + 18% egg yolk and 3% honey + 17% egg yolk showed higher

progressive motility, lower dead and abnormal spermatozoa compared to the other experimental group.

In this study, the inclusion of honey in egg yolk based extenders was found to sustain both sperm motility and livability. However, this effect was found to be dependent on the ratio of honey to egg yolk in the extender. This is in agreement with the findings of Olayemi *et al.* (2011), on goat semen.

Effect of bee honey on fertilizing ability of frozen NZW rabbit semen:

Thawed semen in straws showed more than 50% progressive motility after thawing, were used to inseminate 100 females artificially (20 rabbit does for each group).

The results obtained for pregnant females inseminated artificially with diluted semen of the five previous groups after one month of conservation in liquid nitrogen was 12, 14, 16, 16, 10/20, respectively (Table, 3). Whereas, conception rate (CR%) values were 60, 70, 80, 80 and 50%, respectively. The effect of treatments (extenders) on CR% was highly significant ($p \leq 0.000$). Values of litter size were 6.0, 6.7, 7.6, 7.4 and 5.6 litters/doe, respectively. The better values for fertilizing ability parameters were for the concentrations of 2 and 3% honey. The differences between the five extenders used in conception rate and litter size were highly significant at $P \leq 0.001$ (Table 3). Syazana *et al.* (2011) studied the effect of Gelam honey on sperm quality and testis of rats, and suggested that honey has the potential to increase the fertility of male rats by increasing sperm count and number of sperm with normal morphology.

The use of honey to replace egg yolk in part for egg yolk based extenders is the focus of this study which showed that the semen quality after thawing was better than Tris-citric-glucose +DMSO without honey. This effect could be due to the antibacterial activity of honey against some microorganisms resistant to the common antibiotics used in the extender and also honey is a good source of glucose and fructose (Molan and Russell, 1988). On the other hand, DMSO+Tris-citric-glucose extender in the presence of 1, 2 or 3% honey showed better action for cryopreservation of rabbit semen. The results are in agreement with the findings of Sawada and Chang (1964) and Si *et al.* (2006).

Tris-citric-glucose extender with or without honey showed low efficacy to sustain freezing effect on diluted rabbit semen. Roca *et al.*, (2000) mentioned that Tris-citric-glucose extender is effective for dilution and storage of rabbit semen at 15°C. The results of the present study showed that

Table 3. Effect of Honey on fertilizing ability of frozen NZW rabbit semen

	Fertilizing ability		
	Pregnant Females	CR%	Litter Size
<i>DMSO+Tris-citric-glucose +</i>			
20% egg yolk	12/20	60	6.0±0.13 ^d
1% honey+19% egg yolk	14/20	70	6.7±0.15 ^b
2% honey+18% egg yolk	16/20	80	7.6±0.13 ^c
3% honey+17% egg yolk	16/20	80	7.4±0.13 ^{bc}
4% honey+16% egg yolk	10/20	50	5.6±0.16 ^a
Significance	**	**	**

Means within a column with different superscripts differ significantly ($P < 0.05$).

** $P < 0.01$.

addition of honey to some rabbit semen extenders may improve sperm motility, viability (Table, 2) and fertilizing ability (Table, 3) of diluted rabbit semen stored frozen in liquid nitrogen and may improve cryopreservation of rabbit spermatozoa. These results suggested that honey-included egg yolk extenders could be used for the freezing conservation of diluted rabbit semen.

In conclusion, the results of this study suggested that the addition of bee honey to egg yolk-based extender improved motility, viability and fertilizing ability of NZW rabbit semen conserved frozen in liquid nitrogen. However, this effect is concentration dependent and higher concentrations of honey may have negative effect on sperm motility and viability.

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تأثير اشتغال مخففات صفار البيض لعسل النحل على الحركة والحيوية والقدرة الإخصابية للحيوانات المنوية المجمدة فى الأرانب

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تم دراسة نسبة إضافة عسل النحل و صفار البيض و مخفف تريس-ستريك-جلوكوز فى وجود أو عدم وجود الداى ميثيل سلفوكسيد (DMSO) لتخفيف الحيوانات المنوية للأرانب النيوزيلندى الأبيض و تجميدها. تم استخدام مائة أنثى بكر لغرض التلقيح الإصطناعى. تم جمع السائل المنوى من عشرة ذكور أرانب نيوزيلندى أبيض، و بعد تجميع السائل المنوى تم تخفيفه بستة عشر إضافة مرتكزة على مخفف التريس-ستريك-جلوكوز فى وجود أو عدم وجود DMSO. تم إضافة العسل بتركيزات تصاعديّة (حجم / حجم) ٠ و ١ و ٢ و ٣ و ٤ و ٥ و ٧,٥ و ١٠% مع تركيزات تنازلية من صفار البيض ٢٠ و ١٩ و ١٨ و ١٧ و ١٦ و ١٥ و ١٢,٥ و ١٠% (حجم / حجم). تم تقييم النسب المئوية للحركة التقدمية للحيوانات المنوية والحيوانات المنوية الميتة والحيوانات المنوية الشاذة بعد التخفيف مباشرة، وكذلك بعد شهر من الحفظ مجمدا فى الأزوت السائل. تم تقدير القدرة الإخصابية المتحصل عليها من تلقيح عدد ٢٠ أنثى / إضافة، كمعدل الحمل و عدد الخلفة لكل أم، تم فقط استخدام الإضافات التي أظهرت $\leq 50\%$ حركة تقدمية للحيوانات المنوية بعد الإسالة فى التلقيح الإصطناعى.

أظهرت النتائج أن إضافة ٢ و ٣% عسل أظهرت أفضل قيم للحركة التقدمية للحيوانات المنوية وكذلك قلت نسبتي الحيوانات المنوية الميتة و الشاذة و كذلك معدل الحمل و عدد الخلفة / أم.

التوصية: يتضح من نتائج هذه الدراسة أن إضافة عسل النحل إلى مخففات صفار البيض قد حسن من حركة الحيوانات المنوية و كذلك حيويتها و من الممكن أن تحسن خصائص الحفظ الحيوى للحيوانات المنوية. مع ذلك فإن هذا التأثير مرتبط بتركيز عسل النحل، و أن التركيزات العالية من العسل من الممكن أن يكون لها تأثير سلبى على جودة السائل المنوى و قدرته الإخصابية.