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EFFECT OF BEE HONEY AND/OR ROYAL JELLY ON THE FERTILIZING CAPACITY OF DILUTED RABBIT SEMEN STORED AT 5ºC

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The effect of bee honey and/or royal jelly (RJ) addition to tris-based extender on the motility, viability, abnormalities and fertilizing capacity of New Zealand White (NZW) rabbit spermatozoa was studied. Pooled semen was processed in tris-based extenders containing ascending concentrations (v/v) of bee honey (0, ½, 2, 3, 4, 5, 7.5 and 10% of diluent) and descending concentrations (v/v) of egg yolk (20. 19, 18, 17, 16, 15, 12.5 and 10%). RJ was added to tris-buffer extender with 2% bee honey and 18% egg yolk (EY), the concentrations (w/v) of RJ were 10, 20, 30, 40, 50 and 100 mg/6 mL diluent. Dilution rate was 1: 6 (1 semen + 5 extender) with final concentration of 30 × 10^6 spermatozoa / 0.5 mL diluted semen. Diluted semen samples were stored in aliquots at 5ºC up to 216 h (9 days). Diluted semen was evaluated at 0, 2, 4, 6, 24, 48, 72, 96, 120, 144, 168, 192 and 216 h during cooled storage. Fertilizing capacity represented in conception rate% and litter size at birth/doe of stored diluted semen was examined at 0, 24, 72, 120 and 168 h after storage at 5ºC.

Obtained results showed that NZW rabbit semen diluted with extenders having either 10 or 20 mg RJ / 6 mL tris based extender containing 2% bee honey and 18% EY could be used beneficially within a week when stored at 5ºC for artificial insemination (AI), which would facilitate commercial distribution. In conclusion, the addition of bee honey and RJ in rabbit semen diluents improved both semen quality and fertilizing capacity when stored at 5ºC.

***Conclusively**, the results of the present study suggested that the addition of bee honey and RJ to rabbit semen diluents improved both semen quality and fertilizing capacity during storage at 5ºC. These results revealed that NZW rabbit semen diluted with extenders that contained 10 or 20 mg RJ / 6mL tris-basal extender*

having 2% bee honey and 18% EY beneficially used within a week when stored at 5ºC for AI. The previous results showed a significant positive effect on the quality of stored semen, which would facilitate commercial distribution.

Key words: Cooling, egg yolk, extender, fertilizing capacity, honey, rabbit, royal jelly, semen.

Artificial insemination (A.I.) in rabbits is usually performed using fresh diluted semen (on the day of semen collection) yielded pregnancy rates similar or less than those would be achieved by natural mating (El-Gaafary and Marai, 1994, Morrel, 1995 and Harkness *et al.,* 2010). A.I. has been introduced in industrial rabbitries mainly to improve breeding management (Castellini, 1996).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).The use of honey to partly replace honey in egg yolk based extender (El-Sherbiny, 2013a). Additionally, honey contains high level of metabolizable energy in the form of glucose and fructose (Molan and Russell, 1988 and Al-Waili, 2004).

Royal jelly (RJ) has been used for centuries for its extraordinary properties and health effects (Pavel *et al*., 2011). Royal jelly is one of the most studied bee products; however, many researches are needed to reveal its biochemical and biological activities. On the other hand, the chemical and bioactive compounds of RJ are not sufficiently known. RJ has a multitude of pharmacological activities (Mărghitas, 2008). The overall composition of RJ is 67% water, 12.5% crude protein (including small amounts of many different amino acids) and 11% simple sugars (monosaccharide), also including a relatively high amount (5%) of fatty acids. It also contains many trace minerals, some enzymes, antibacterial agents, antibiotic components and trace amounts of vitamin C (Graham, 1992). RJ has immunomodulatory activities (Gasic *et al*., 2007).

El-Sherbiny (2013b) concluded that RJ addition to tris basal extender which contained *dimethysulfoxide (DMSO),* 2% honey bee and 18% EY improved semen quality and fertility of diluted NZW rabbit semen before and after storage at -195ºC. On buffalo, *in vitro* study by Abd-Allah (2012) suggested that treating buffalo sperm with 0.4% RJ in combination with heparin is effective not only to induce sperm acrosome

reaction but also is effective for *in vitro* fertilizing capacity of the cryopreserved buffalo spermatozoa. For rabbit semen**,** fast cooling rates from room temperature to 5°C are likely possible due to the peculiarities of the sperm membrane in this species (high cholesterol: phospholipid ratio) as showed by Mocé and Vicente (2009). Tris-buffer extenders were effective at preserving fertility for two days when stored at 15ºC (Roca *et al*., 2000). However, attempts are underway to preserve fertility in semen stored for several days. Commercial semen distribution is still of low significance in rabbits due to semen precipitation and energy expenditure movement during storing. Further experimentation (*in vitro*, in animal's research) and validation would be needed to prove any useful benefit and action mechanism of native bee honey and RJ and their isolated compounds, as well.

Therefore, the aim of the present study was to investigate the effect of bee honey and/ or RJ addition to tris-buffer extender on the quality and fertilizing capacity of diluted rabbit semen after preservation at 5ºC up to 216 hours.

MATERIALS AND METHODS

The present study was carried out during winter season 2013/2014 (from December 2013 to February 2014) at the Intensive Rabbit Production Unit, Faculty of Agriculture, Ain Shams University, Cairo, Egypt and a Private Rabbit Farm, Kalioubia, Egypt.

Experimental animals:

A total of twenty sexually mature New Zealand White (NZW) rabbit bucks, aged 18 months with average body weight 4.1 kg, were selected for high quality semen (reaction time < 2 minutes, color white only, volume ≥ 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 capacity of cooled diluted semen, 460 hybrid nonparous females (5-6 months old) at a private rabbitry, Kalioubia, Egypt, were used in the experiment (10 females each time per each extender). All animals were housed individually in flat deck cages and fed a commercial concentrated pelleted diet according to their physiological and reproductive condition according NRC allowances (NRC, 1977). Fresh water was provided *ad libitum.*

Semen collection and evaluation:

Semen was collected from each buck twice weekly using an artificial vagina (960 ejaculates were used). Two rabbit does 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 semen quality parameters of rabbit bucks were evaluated (percentages of sperm progressive motility, dead and abnormal spermatozoa) as described by El-Sherbiny (1987) and Madhuri *et al*. (2012).

Semen dilution:

Tris-citric-glucose extender was the basic diluent and had the following composition: 0.25M of Tris buffer (hydroxylmethyl) aminomethan, 0.87M of citric acid monohydrate, 0.47M D (+) glucose, 100000 IU Penicillin and 100 mg streptomycin sulfate (Viudes-de-Castro and Vicente, 1996 and Si *et al.*, 2006), All previous components were dissolved in double distilled water up to 100 mL. Honey was added to the diluent in ascending concentrations (v/v) 0, 1, 2,3, 4, 5, 7.5 and 10% and descending concentrations of EY 20, 19, 18, 17, 16, 15, 12.5 and 10% (v/v), for extenders 1, 2, 3, 4, 5, 6, 7 and 8, respectively. Fresh EY was added just before semen collection (Chen *et al.,* 1989 and El-Kelawy *et al.,* 2012). The bee honey-included EY percentage was 20% (v/v) of diluent according to El-Sherbiny (2013a). RJ was added to tris-buffer extender with 2% bee honey and 18% EY; the concentrations (w/v) of RJ were 10, 20, 30, 40, 50 and 100 mg/6 mL of diluent according to El-Sherbiny (2013b), for extenders 9, 10, 11, 12, 13 and 14, respectively. The dilution rate of pooled semen was one volume of semen to five volume of extender (v/v) at 37^{o}C. Semen samples were stored in aliquots in a refrigerator at 5^{o}C up to 216 h (9 days).

Semen cooling:

Total sperm-cell concentration (Conc.) was 363.08 million spermatozoa / mL. Hence, Conc. after dilution was 60.51 million spermatozoa / mL. The diluted semen was packed into aliquots (1 mL), then dipped in a glass beaker of water at room temperature (18-22^{o}C) and stored in a refrigerator at 5^{o}C. The elapsed time between semen dilution and the beginning of storage didn't exceed 30 minute.

Post cooling examination of diluted semen:

Cooled aliquots were warmed in a water bath at 37°C for 10 seconds (Kashiwazaki *et al.,* 2006). After warming, diluted semen samples were examined for percentage of sperm progressive motility, dead and total abnormal spermatozoa. Diluted semen was examined at 0, 2, 4, 6, 24, 48, 72, 96, 120, 144, 168, 192 and 216 h after cold storage at 5^{o}C**.**

Artificial insemination:

Rabbit does 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). 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 x 10^6 sperms) just after GnRH injection (Chen *et al.*, 1989). Pregnancy was detected by trans-abdominal palpation 14 days post-insemination to determine conception rate% (El-Kelawy *et al.*, 2012; EL-Sherbieny *et al.*, 2012 and Safaa *et al.*, 2012). Litter size was determined for each doe directly after kindling. Fertilizing capacity of stored diluted rabbit semen was examined at 0, 24, 72, 120 and 168 h after storage at 5ºC.

Statistical analysis:

Data of percentages of sperm progressive motility, dead and abnormal spermatozoa of diluted NZW rabbit semen were analyzed using Two Way Analysis of Variance (ANOVA). Litter size data were analyzed using One Way ANOVA. Whereas, for conception rate trait, Catmod procedure and Chai-square test for homogeneity of variance were performed. All statistical analysis for the different traits was realized using SAS program (SAS, 2011). Differences among experimental groups were tested by Duncan's Multiple Range test (Duncan, 1955).Two Way ANOVA was according to the following model:

$$y_{ijk} = \mu + trt_i + time_j + (trt * time)_{ij} + e_{ijk}$$

Where: µ is the overall mean, y_{ijk} is the observation of the studied trait of k^{th} animal of i^{th} trt and j^{th} time, trt_i is the effect of i^{th} trt (i = 1, 2, …….14), $Time_{ej}$ is the effect of j^{th} time (j = 1, 2, 3 and 4), $(trt*time)_{ij}$ is the effect of the interaction between trt and time, e_{ijk} is the individual error. One way ANOVA was according to the following model:

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

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 …., 14), e_{ij} is the individual error.

RESULTS AND DISCUSSION

Physical characteristics of diluted NZW rabbit semen:

Estimated physical characteristics of NZW rabbit crude semen before dilution are summarized in Table 1.

Table 1: Some physical characteristics (Means ± SE) of NZW rabbit semen.

Parameter	Volume (ml)	pH	PM%	DS%	AS%	Conc (million/ml)
N	40	40	40	40	40	40
Mean	**0.6**	**7.1**	**89.5**	**7.4**	**10.6**	**363.1**
±SE	±0.05	±0.05	±0.06	±0.01	±0.03	±46.31

PM: Progressive motility; DS: Dead spermatozoa; AS: Abnormal spermatozoa; Conc: Sperm-cell concentration; N: number of evaluated ejaculates.

The effect of bee honey and/ or royal jelly on sperm progressive motility of diluted NZW rabbit semen stored at 5ºC is presented in Table 2. Results showed that the effects of either extender or storage time on percentage of sperm progressive motility (PM) were highly significant ($P<0.01$). On the other hand, PM was decreased with the increasing of storage time for all extenders used in the present study.

Addition of bee honey to the extender improved PM, but this improvement was concentration dependent. This finding is in agreement with those obtained by El-Sherbiny (2013a). Honey had a significant ($P<0.01$) positive effect when added in concentrations of 2, 3 and 4% (extenders 3, 4 and 5) compared to Tris-basal extender (extender 1). The inclusion of bee honey as a source of glucose (Molan and Russell, 1988 and Al-Waili, 2004) in EY 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 EY buffer increases and prolongs active motility of spermatozoa. This effect could be due to the antibacterial activity of bee honey against some microorganisms resistant to the common antibiotics used in the extender, additionally, honey bee is a good source of glucose and fructose (Molan and Russell, 1988).

In the present study, the inclusion of honey in EY 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 EY in the extender. This is in agreement with the findings of Olayemi *et al*. (2011) on goat semen.

Tris-citric-glucose extender was used as a basal extender in the present study. Roca *et al.*, (2000) mentioned that Tris-citric-glucose extender is effective for dilution and storage of rabbit semen at 15^{o}C.

El-Gaafary (1994) showed that storage and incubation time decreased ($P<0.01$) sperm motility and increased ($P<0.01$) the percentages of spermatozoa with damaged acrosomes and amounts of lactic dehydrogenase

released into the extracellular medium. While, the addition of RJ enhanced PM all over the periods of diluted semen examination from 0h to 216 h at 5ºC. The present results proved that the addition of 10 and 20 mg RJ / 6 mL of diluent, showed better PM than the other extenders used. These two RJ concentrations showed not only more than 50% of PM till 168 h of storage at 5ºC, but also maintained PM to 216 h of storage (33 and 22%, respectively).

Results represented in Table 3 showed the effect of bee honey or/ and RJ on percentage of dead spermatozoa (DS). These results indicated that, percentage of DS increased significantly ($P<0.01$) with advanced time for all extenders examined. The impact of either extender or time on DS was significant at $P<0.01$; while, bee honey addition in concentrations of 2, 3 and 4% showed DS significantly ($P<0.01$) lower than the other honey-extenders and Tris-basal extender. Whereas, RJ addition to extender revealed the lowest values ($P<0.01$) of DS all the storage time at 5ºC, especially in concentrations of 10 and 20 mg RJ / 6 mL of diluent.

Table 4 represents the effect of bee honey or/ and RJ on percentage of abnormal spermatozoa (AS) stored at 5ºC. The results of the present study indicated that both extender and storage time had significant ($P<0.01$) effect on AS. The lower values obtained for AS were for concentrations of 2, 3 and 4% of honey, but the lowest values were for RJ concentrations of 10 and 20 mg/6 mL of diluent. The results of the present study showed that rabbit semen preservation for 48 hours at 5°C did not affect spermatozoa characteristics, after that, all parameters studied and fertility depreciated. This is in agreement with the results obtained by Echegaray-Torres *et al*. (2004).

Dead and abnormal percentages of spermatozoa were significantly ($P<0.01$) increased, while sperm progressive motility decreased significantly ($P<0.01$) with the increasing of storage time. That may be due to the concentration of toxic substances in the extender during storage, stress from continuous motility of sperm and low energy sources available. Therefore, extenders are suitable for rabbit semen within the first two or three days of storage (Roca *et al.,* 2000 and Echegaray-Torres *et al.,* 2004).

Fertilizing capacity of diluted NZW rabbit semen:

Conception rate (CR) and litter size at birth (LS) of the 14 extenders among the different storage time are represented in Table 5. At 0 h, differences among different extenders in both conception rate and litter size at birth were significant ($P<0.05$). The results showed that insemination of nonparous rabbit does artificially with diluted semen of 2,

3 and 4% bee honey extenders realized more CR and LS than those inseminated by semen diluted with tris-citric-glucose extender alone at 0 h. On the other hand, RJ extenders showed the highest values of CR and LS compared to other extenders at 0 h. The differences among extenders in CR and LS were highly significant ($P<0.01$) when examined at 24, 72, 120 and 168 h of storage at 5ºC. Extenders contained less than 5% honey improved fertilizing capacity of diluted NZW rabbit semen until 5^{th} day of storage at 5ºC (Table 4).

Addition of RJ to extenders significantly ($P<0.05$) improved fertilizing capacity of diluted rabbit semen stored at 5ºC till 7^{th} day of storage. Concentrations of 10 and 20 mg RJ/6 mL diluent (extenders 9 and 10) showed the best values of CR and LS compared to the other extenders used in the present study.

Roca *et al.* (2000) obtained 75.1 % fertility and 7.68 kids/ kindled doe when does inseminated at zero time with rabbit semen diluted in TCG extender. Also, Viudes-de-Castro and Vicente (1997) reported 91% fertility which is close to that obtained in the present study, and 9.4 kids/ kindled doe for the same time and extender type. Roca *et al.* (2000) suggested that sperm concentration in rabbit insemination dose could be slightly less than 15 million; in the present study, insemination dose had 30 million spermatozoa. Fertility test also, showed that rabbit semen fertilizing capacity deteriorated over storage time more quickly (Echegaray-Torres *et al*., 2004).

Conclusively, the results of the present study suggested that the addition of bee honey and RJ to rabbit semen diluents improved both semen quality and fertilizing capacity during storage at 5ºC. These results revealed that NZW rabbit semen diluted with extenders that contained 10 or 20 mg RJ / 6mL tris-basal extender having 2% bee honey and 18% EY beneficially used within a week when stored at 5ºC for AI. The previous results showed a significant positive effect on the quality of stored semen, which would facilitate commercial distribution.

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تأثير عسل النحل و الغذاء الملكى على القدرة الإخصائية للسائل المنوى المخفف للأرانب المخزن على درجة حرارة 5ºC مئوية

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قسم الإنتاج الحيوانى – كلية الزراعة – جامعة عين شمس – القاهرة – مصر

تم دراسة تأثير إضافة عسل و/ أو غذاء ملكات النحل إلى مخفف تريس-ستريك-جلوكوز على الحركة التقدمية والحيوية ونسبة الحيوانات المنوية الميتة والشاذة والمقدرة الإخصابية للسائل المنوى المخفف للأرانب النيوزيلندى البيضاء والمحفوظ على درجة حرارة 5ºC مئوية. تم تجميع السائل المنوى و إضافته للمخففات التى أساسها مخفف تريس-ستريك-جلوكوز و التى تحتوى على تركيزات تصاعدية من عسل النحل (٠ و١ و٢ و٣ و٤ و٥ و٧,٥ و١٠ %) و تركيزات تنازلية (حجم/ حجم) من صفار البيض (٢٠ و١٩ و ١٨ و١٧ و١٦ و١٥ و١٢,٥ و١٠%). تم إضافة غذاء ملكات النحل لمخفف التريس المحتوى على ٢% عسل نحل و١٨% صفار بيض، و قد كانت تركيزات غذاء ملكات النحل (وزن/ حجم) ١٠ و٢٠ و٣٠ و٤٠ و٥٠ و١٠٠ مجم / ٦ ملليلتر من المخفف. نسبة التخفيف كانت ١: ٦ (١ سائل منوي + ٥ مخفف) بحيث يحتوى كل ٠،٥ ملليلتر من السائل المنوي المخفف على ٣٠ مليون حيوان منوى. تم حفظ عينات السائل المنوي المخفف في عبوات محكمة على درجة حرارة 5ºC مئوية لفترات تصل إلى ٢١٦ ساعة (٩ أيام)، تم تقييم السائل المنوي المخفف قبل الحفظ (الوقت ٠) ثم بعد ٢ و٤ و٦ و٢٤ و٤٨ و٧٢ و٩٦ و١٢٠ و١٤٤ و١٦٨ و١٩٢ و٢١٦ ساعة من الحفظ المبرد. تم تقدير المقدرة الإخصابية (معدل الحمل % وعدد الخلفة عند الميلاد لكل أم) بعد ٠ و٢٤ و٧٢ و١٢٠ و١٦٨ ساعة من التخزين على درجة حرارة 5ºC مئوية.

أظهرت النتائج أن السائل المنوى المخفف للأرانب النيوزيلندي البيضاء والمضاف إليه ١٠ أو ٢٠ مجم غذاء ملكات النحل/ ٦ ملليلتر من مخفف التريس المحتوى على ٢% عسل نحل و١٨% صفار بيض، يمكن استخدامه بأمان خلال فترة أسبوع من الحفظ المبرد على درجة حرارة 5ºC مئوية لغرض التلقيح الإصطناعى، والذي قد يسهل عملية النقل والتداول التجاري للسائل المنوى المخفف.

التوصية: يتضح من نتائج هذه الدراسة أن إضافة عسل النحل وغذاء ملكاته إلى مخففات سائل منوي الأرانب يمكن أن يحسن كلا من الخصائص النوعية للسائل المنوي والمقدرة الإخصابية له عند حفظه وتخزينه على درجة حرارة الثلاجة (5ºC مئوية).