Effect of dietary pumpkin (Cucurbita moschata) seed oil supplementation on reproductive performance and serum antioxidant capacity in male and nulliparous female V-Line rabbits

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ABSTRACT

Pumpkin seed (PS) oil is antioxidant that can provide a scavenging effect on reactive oxygen species produced during gametogenesis. The current study aimed to investigate the effect of 0.5% dietary PS oil supplementation on rabbit reproductive performance. A total of 20 mature bucks and 48 nulliparous does were used in a 72 days experiment. Rabbits were divided into two groups: Control group (CON-M = 10 bucks; CON-F = 24 does) and PSO group (PSO-M = 10 bucks; PSO-F = 24 does), blood samples were collected and analysed for serum total antioxidant capacity (TAC), superoxide dismutase (SOD), and malondialdehyde (MDA) levels. Bucks and does were cross-mated on d30 to have 4 female groups: (1) CON-C (CON-F/C2 CON-M), (2) CON-P (CON-F/C2 PSO-M), (3) PSO-C (PSO-F/C2 CON-M) and (4) PSO-P (PSO-F/C2 PSO-M). Doe serum oestra-diol and progesterone were assessed on days 0, 14, 26 and 32 after mating. Bucks serum testosterone and oestradiol were also assessed. Reproductive performance and litter size and weight were recorded, semen samples were collected from bucks for 6 successive weeks and assessed for semen quality. PS oil increased the level of TAC (p = .05) and decreased total MDA (p = .04) in both sexes. The percentage of sperm abnormality was decreased in PS oil-fed bucks (p = .04), PS oil did not alter the reproductive performance of the does including litter size and litter birth weight (p > .05). In conclusion, PS oil improved buck fertility. The antioxidant effect of PS oil was remarkable in both sexes.

HIGHLIGHTS

- Pumpkin seed (PS) oil improved the semen quality of rabbit bucks.
- PS oil increased antioxidant capacity in both rabbit buck and doe.
- PS oil did not impact the reproductive performance of rabbits.

Introduction

Increasing the need of the rapidly growing populations for a nutritious and cheap animal protein source necessitates the need to increase white meat production. Rabbit is identical animal species to be used to fulfil those needs, where one female rabbit (doe) can give up to 4.7 litters (Castellini et al. 2010) during her reproductive lifespan. Optimising rabbit reproductive potential requires the improvement of the fertility rate of both rabbit doe and buck (Castellini et al. 2010).

Improving the antioxidant status of the animal is associated with improving its reproductive performance (Markó et al. 2011). One of the well known natural antioxidants is pumpkin seed (PS) oil (Shaban and Sahu 2017). As formerly reported, pumpkin seed oil and vitamin E supplementation improved the testosterone level and semen quality in a rat model (Hashemi 2013), in addition, PS oil in combination with black seed oil improved semen quality, antioxidant activity, and testosterone level in New Zealand white rabbit (Ragab et al. 2016).

Even more, pumpkin seed ethanolic extract increased the production of oestradiol (the main form of oestrogen; regulates the reproductive activity of rabbit does) in MCF7, BeWo, and Jeg3 cell lines.
(Richter et al. 2013). To our knowledge, there are no data available about the effect of PS oil on fertility and reproductive performance of healthy rabbit doe and buck. We hypothesise that dietary PS oil will enhance rabbit fertility through its antioxidant properties. Therefore, the objective of this study is to investigate the influences of dietary pumpkin seed oil supplementation on oxidative status, fertility, and reproductive performance of rabbit buck and doe.

Materials and methods

Ethical approval

The current experiment was conducted in accordance with the American Veterinary Medical Association (AVMA) guidelines. The Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, Cairo University (VET-IACUC) have approved the protocol of the experiment, approval# (Vet-CU230120201111). Blood sample collection protocol from ear-vein was in line with Parasuraman et al. (2010).

Experimental design

This study was conducted in the rabbit husbandry unit of the Physiology Department of Cairo University. A total of 20 mature (6-month-old) V-Line bucks of average body weight ± SE (3210 ± 23.42), and 48 nulliparous does (never pregnant before; 5 to 6-month-old) of average body weight ± SE (2651.875 ± 34.81) were used in the current experiment. The total experimental period was 7 days adaptation period, and 65 days experimental period. In the experimental period, rabbits were divided into two equal groups as follows: Control group (CON-M = 10 bucks; CON-F = 24 does) fed on a basal diet, and PSO group (PSO-M = 10 bucks; PSO-F = 24 does) fed on a diet supplemented with 0.5% pumpkin seed oil, the pellet was around 3–4 mm.

On experimental day 30, the does were cross-mated naturally by fertile bucks as follows: (1) rabbit does fed on the basal control diet and mated with bucks fed on basal control diet (CON-C, n = 12 females), (2) rabbit does fed on the basal control diet and mated with buck fed on PS oil containing diet (CON-P, n = 12 females), (3) rabbit does fed on pumpkin seed oil and mated with bucks fed on basal control diet (PSO-C, n = 12 females), and (4) rabbit does fed on a diet supplemented with pumpkin seed oil and mated with rabbit bucks fed on pumpkin seed oil supplemented diet (PSO-P, n = 12 females).

Housing and diet

Soybean oil was added to the CON diet to so the diets are iso-nitrogenous and iso-caloric, diet was formulated to meet or exceed rabbit nutrient requirement of the National Research Council (NRC 1977), experimental diet and its chemical composition is provided in Table 1.

Rabbits were housed individually in cages, equipped with automatic drinkers and j-feeders. Feed and freshwater were available all time. The light program was 16 h light and 8 h dark, the temperature was 15–20 °C, cage size was 0.4 x 0.6 x 0.35 m³ and was equipped with kindling nest-box (0.43 x 0.26 x 0.26 m³) (Minuti et al. 2020).

Mating and pregnancy diagnosis

Buck to does ratio was 1:5, each doe admitted to the bucks’ cage twice with 30-min intervals, receptive females showed lordosis and allowed mating immediately. Does were palpated for pregnancy after 12 days of mating (Attia et al. 2009).

Semen samples collection and evaluation

Semen samples were collected once a week for 6 successive weeks (days 28, 35, 42, 49, 56 and 63 using artificial vagina and female teasers similar to Abdelatty et al. (2020a,b). Semen samples were immediately assessed for pH (pH probe pH600, Milwaukee), semen

| Item | % (As-fed) |
|------|------------|
| Barley | 29.70 |
| Soybean meal (44%) | 10.40 |
| Hay | 32.80 |
| Wheat bran | 22.10 |
| Sunflower meal | 3.10 |
| Di-calcium phosphate | 0.70 |
| Limestone | 0.50 |
| Sodium chloride | 0.40 |
| Premix* | 0.30 |

Chemical composition

Crude protein 16.23
Ether extract 2.50
Crude fibre 14.02
Calcium 0.80
Phosphorus 0.54
Methionine 0.25
Lysine 0.60

Vitamin and mineral premix supplied per kilogram of diet: vitamin A 11,000 U; vitamin D3 2,000 U; vitamin E 200 mg; vitamin K 2.5 mg; vitamin B1 0.01 mg; vitamin B2 4 mg; vitamin B6 1.25 mg; vitamin B12 0.01 mg; biotin 0.06 mg; niacin 15 mg; folic acid 0.30 mg; pantothenic acid 10 mg; choline 600 mg; Mn 60 mg; Cu 3 mg; Fe 50 mg; Zn 15 mg; I 0.5 mg; Co 0.5 mg.

Note. Digestible energy = 2571 Kcal/Kg.
volume, mass motility using a light microscope with thermostatically controlled hot stage adjusted at 38–40°C (Olympus, USA; and score 0–5 was used), individual motility (%), sperm viability (%), sperm abnormalities (%), and sperm concentration (The number of spermatozoa present on both sides of Neubauer haemocytometer slide (Marienfeld, USA). The semen evaluation process is detailed in our former report (Bakeer et al. 2021).

**Blood samples collection and assessment**

Blood samples were collected from rabbit bucks on days 15 and 30 of the experiment. Blood samples were collected from rabbit does on days 15, 30 (immediately after mating), 45 (mid-pregnancy) and 60 (late pregnancy).

A well-trained person collected the blood samples. Blood was collected from the marginal ear-vein using a 26 G needle, after topical application of Xylocaine 4% anaesthetic cream (AstraZeneca, India) as recommended by (Parasuraman et al. 2010). Blood samples were processed for serum separation similar to (Abdelatty et al. 2019), briefly, Blood samples were left for 30 min at room temperature then centrifuged at 1000 x g for 15 min, serum was then separated and stored in aliquots at −20°C until assessment.

**Antioxidant biomarkers assessment**

Serum total antioxidant capacity (TAC) was assessed by TAC assay kit (Cell biolabs INC., San Diego, CA, USA). Serum total superoxide dismutase (SOD) and Malondialdehyde (MDA) were assessed using HPLC (Agilent HP1200 Series, CA, USA), analysis protocol and HPLC condition were detailed in our former study (Abdelatty et al. 2020).

**Reproductive hormones assessment**

Buck serum testosterone assessed using enzyme immunoassay kits (Cat# MBS704954, MyBiosource, Inc. CA, USA), and serum oestradiol level in both bucks and does was assessed using enzyme immunoassay kits (Cat# MBS2601037, MyBiosource, Inc. CA, USA), and finally, doe serum progesterone level assessed using enzyme immunoassay kits (Cat# MBS2602502, MyBiosource, Inc. CA, USA) following the manufacturer instructions.

**Reproductive performance**

At the time of mating, the doe was scored receptive when its vulva was red-violet and turgid (Eiben et al. 2007). Sexual receptivity rate was considered as the percentage of does that accepted mating/total number of observed does at the first mating trial. The fertility rate was considered as the percentage of pregnant does (palpated at day 12 of insemination)/total number of does that accepted mating. Kindling rate was considered as the percentage of kindled does from the total number of does that accepted mating (Hoy 2018). After parturition, the total number of kits, number of born alive kits, and weight of litter were recorded per each doe.

**Statistical analysis**

The UNIVARIATE procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, United States, 2014) was used for semen quality and serum oestradiol and testosterone concentrations between CON-M and PSO-M groups in V-Line rabbit bucks, and litter weight and size of total and born-live offspring. Serum antioxidant biomarkers between CON-M, CON-F, PSO-M, and PSO-F groups in V-Line male and female rabbits were analysed using the MIXED procedure of SAS where the individual rabbit was the experimental unit. The model included the fixed effects of treatment, sex, and treatment x sex interaction.

The MIXED procedure of SAS was used for repeated measures analysis of the serum level of oestradiol and progesterone in female V-Line rabbits during the course of pregnancy where the individual rabbit was the experimental unit. The model included the fixed effects of treatment, time, and treatment x time interaction. Significance was declared at p ≤ .05. Pearson’s Chi-square procedure was used for the analysis of categorical data including sexual receptivity rate, fertility rate, and kindling rate. Significance was declared at p ≤ .05.

**Results**

The effect of PS oil on semen quality is shown in Table 2. Where dietary supplementation of PS oil improved the semen quality of rabbit bucks through decrease the percentage of abnormal spermatozoa (p = .04). Neither serum oestradiol levels nor testosterone levels were affected (p > .10) by dietary PS oil supplementation to rabbit bucks (Figure 1). The effect of dietary PS oil supplementation on serum antioxidant metabolites is shown in Table 3. There was a marked overall treatment effect of PS oil on serum antioxidant metabolites of the rabbits (regardless of the sex), where TAC increased in PS oil-fed rabbits (p = .05),
which was associated with a decrease in the level of MDA ($p = .04$) in the same groups.

The effect of PS oil on rabbit doe reproductive hormones (oestradiol and progesterone) is presented in Figure 2. There was no negative effect of PS oil on serum oestradiol and progesterone level ($p > .05$) in any stage of pregnancy.

The effect of PS oil on litter size and weight is presented in Table 4. There was no deleterious effect of PS oil supplementation in the diet of male and/or female rabbits on the reproductive performance of the doe, including the sexual receptivity, fertility, and kindling rate ($p > .05$). Additionally, none of the total or born alive litter sizes were affected by the treatment ($p > .05$), litter weight was similar between all groups ($p > .05$).

**Discussion**

Due to its well-documented antioxidant properties (Xanthopoulou et al. 2009), PS oil was used as a dietary supplement for several animal species including rats, mice, and rabbits (Eraslan et al. 2013; Hashemi 2013; Tabari et al. 2016). The process of gametogenesis elaborates a lot of reactive oxygen species (ROS) resulting in different degrees of oxidative stress which affects male and female fertility (Agarwal et al. 2006). Therefore, dietary supplementation with nutraceutical possessing antioxidant properties is crucial to improve the oxidative status and enhance fertility. PS oil was formerly used as a dietary antioxidant to improve male fertility in rats with testicular dysfunction due to oxidative damage induced by aluminium oxide (Hamdi 2020). Additionally, PS oil in combination with vitamin E alleviated testicular injury in the rat (Hashemi 2013). However, there are no former reports on the effect of PS oil on rabbit fertility (including male and female).

In the current study, PS oil dietary supplementation improved semen quality through decrease the percentage of abnormal sperm similar to Aghaei et al. (2014) and Hashemi (2013) where pumpkin seed extract improved sperm abnormality in the rat model. The antioxidant activity of PS oil reported in our study explains the decrease in sperm abnormality level due to the protective effect of PS oil against ROS that are normally produced during spermatogenesis (Sikka 1996). However, in the current experiment, there was

| Table 2. Effect of dietary pumpkin seed oil on semen quality of rabbit buck.1 |
|-------------------------|--------|--------|--------|--------|--------|
| Item                    | CON-M  | PSO-M  | SEM    | p-Value |
| Volume (mL)             | 0.611  | 0.746  | 0.060  | .190    |
| pH                      | 7.441  | 7.462  | 0.140  | .930    |
| Mass motility (score)2  | 3.291  | 3.745  | 0.260  | .130    |
| Individual motility (%) | 79.734 | 84.541 | 2.640  | .180    |
| Sperm viability (%)     | 82.265 | 85.694 | 2.720  | .210    |
| Sperm abnormalities (%) | 11.110 | 9.304b | 0.660  | .040    |
| Sperm concentration (10^6/mL) | 301.603 | 304.100 | 2.200  | .460    |

1Values are least square mean; 2Mass motility score (0–5).
CON-M: Control male rabbit group ($n = 10$); PSO-M: Pumpkin seed oil male rabbit group ($n = 10$); SEM: Standard error of the mean.
Note. Different superscript letters in the same raw denote $p < .05$ between treatments.

| Table 3. Effect of dietary pumpkin seed oil on antioxidant biomarkers in rabbit buck and doe.1 |
|-------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Item                    | CON-M  | CON-F  | PSO-M  | PSO-F  | SEM    | p-Value |
| TAC (μm/L)              | 2.490a | 2.942b | 2.992a | 3.253a | 0.180  | .050    |
| MDA (nmol/mL)           | 15.100a| 15.243a| 13.331b| 12.841b| 3.150  | .040    |
| SOD (U/mL)              | 112.000| 117.401| 114.750| 117.250| 1.870  | .470    |

1Values are least square mean.
CON-M: Control male rabbit group ($n = 10$); CON-F: Control female rabbit group ($n = 24$); PSO-M: Pumpkin seed oil male rabbit group ($n = 10$); PSO-F: Pumpkin seed oil female rabbit group ($n = 24$); SEM: Standard error of the mean; TAC: Total antioxidant capacity; MDA: Malondialdehyde; SOD: Superoxide dismutase.
Note. Different superscript letters in the same raw denote $p < .05$ between treatments.

![Figure 1](image1.png)

Figure 1. Effect of dietary pumpkin seed oil on serum oestradiol and testosterone levels in V-Line rabbit bucks. There was no difference in serum oestradiol and testosterone levels between control bucks (CON-M) and 0.5% pumpkin seed oil treated bucks (PSO-M).
no effect on serum testosterone level, unlike this observed by Aghaei et al. (2014) in the rat model, the reason for this contradiction might be due to species difference. Similar to our findings, PS oil improved antioxidant activity through increase serum TAC in the rat model as reported by Rouag et al. (2020). However, contrary to our findings, PS extract did not have any effect on MDA in the rat (Aghaei et al. 2014) and PS oil did not affect MDA level in mice (Eraslan et al. 2013) models. This difference in MDA level between different studies indicates that PS oil has a species-specific antioxidant effect.

Oestradiol is the major form of oestrogen in both males and females (DeMayo et al. 2002), both oestradiol and progesterone play a key role in the regulation of reproduction and sexual maturity (DeMayo et al. 2002). In the current study, no negative effect of PS oil was observed on oestradiol and progesterone levels in both male and female rabbits, these findings are contrary to the former report that investigated the effect of pumpkin seed ethanolic extract on oestradiol production from different cell lines (Richter et al. 2013), where pumpkin seed ethanolic extract increased the production of oestradiol in MCF7, BeWo, and Jeg3 cells. The difference between the oil and the ethanolic extract components might explain their different effects on the oestradiol level, where the ethanolic extract is very rich in phytoestrogens (Richter et al. 2013) that might be the reason behind the increase in the level of oestradiol. Furthermore, PS oil did not affect any of the reproductive performance indices, which is contrary to a former study investigating the effect of 1.5% and 3% dietary inclusion of PS oil on the reproductive performance of Japanese quail (Al-Salhie et al. 2017), this contradiction could be explained by the difference in animal species and PS oil dose used.

**Conclusions**

The dietary supplementation of PS oil at a dose of 0.5% decreases the percentage of abnormal sperm and improves antioxidant activity. While in female rabbits, only the antioxidant effect of PS oil was remarkable, with no negative effect on female fertility or litter size and weight. Therefore, we recommend the use of PS oil at this rate to improve buck semen quality and antioxidant activity of buck and doe, and it is

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**Figure 2.** The effect of dietary pumpkin seed oil on serum oestradiol and progesterone levels in female rabbits during the course of pregnancy (immediately after mating, mid-pregnancy, late pregnancy, and after parturition). CON-C is group of rabbit does fed on basal control diet and mated with bucks fed on basal control diet; CON-P, rabbit does fed on basal control diet and mated with bucks fed on PS oil containing diet; PSO-C is group of rabbit does fed on pumpkin seed oil and mated with bucks fed on basal control diet; and PSO-P is group of rabbit does fed on diet supplemented with pumpkin seed oil and mated with rabbit bucks fed on pumpkin seed oil supplemented diet; TRT is the treatment effect.

**Table 4.** Effect of dietary pumpkin seed oil on reproductive performance of rabbit doe.1

| Item                        | CON-C | CON-P | PSO-C | PSO-P | SEM   | p-Value |
|-----------------------------|-------|-------|-------|-------|-------|---------|
| Sexual receptivity (%)      | 79    | 83    | 85    | 87    | n/a   | .460    |
| Fertility rate (%)          | 77    | 80    | 84    | 86    | n/a   | .350    |
| Kindling rate (%)           | 76    | 79    | 84    | 86    | n/a   | .250    |
| Total litter size (no.)     | 7.000 | 7.410 | 7.331 | 8.500 | .960  | .680    |
| Alive at birth (no.)        | 6.170 | 7.000 | 6.663 | 7.750 | .930  | .610    |
| Litter birth weight (g)     | 382.500 | 397.500 | 362.500 | 435.000 | 41.160 | .650    |

1Values are least square mean.

CON-C: Doe fed a control diet and mated with buck fed a control diet (n = 24); CON-P: Doe fed a control diet and mated with buck fed pumpkin seed oil diet (n = 24); PSO-C: Doe fed a pumpkin seed oil diet and mated with buck fed a control diet (n = 24); PSO-P: Doe fed a pumpkin seed oil diet and mated with buck fed pumpkin seed oil diet (n = 24); SEM: Standard error of the mean for non-categorical data, and it is not available (n/a) for categorical data (sexual receptivity, fertility rate, and kindling rate).

Note. Chi-square df for sexual receptivity, fertility rate, and kindling rate = 2.54, 3.26 and 4.11, respectively.
not profitable to be used to improve the fertility of rabbit doe.

**Study limitation**

Due to fund limitation, the number of does used in this study might not be enough to properly reflect the reproductive traits, further study with a higher number of females still needed to affirm our findings. The fatty acid profile of pumpkin seed oil could have explained some effects of fatty acids on reproductive traits, but it was not assessed in the current study.

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**Author contributions**

Manal R. Bakeer and Sohair Y. Saleh: conceptualisation, methodology, validation, investigation, supervision, resources, and writing – review & editing. Gazia: Validation, investigation, and writing – review & editing. Ahmed A. Elolimy and Hisham A. Abdelrahman: formal analysis, resources, and writing – review & editing. Alzahraa A. Abdelatty: conceptualisation, validation, resources, visualisation, writing – original draft, and writing – review & editing.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Data and model availability statement**

None of the data were deposited in an official repository. All relevant data are within the manuscript.

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