INCLUSION OF PROPOLIS IN RABBIT DIETS AND SEMEN CHARACTERISTICS

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MORAES, G. V. de; MATAVELI, M.; MOURA, L. P. P. de; SCAPINELLO, C.; MORA, F.; OSMARI, M. P. Inclusion of própolis in rabbits diets and sêmen characteristics. **Arq. Ciênc. Vet. Zool. UNIPAR**, Umuarama, v. 17, n. 4, p. 227-231, out./dez. 2014.

ABSTRACT: This experiment was carried out with the objective of evaluating different levels of powder propolis in rabbit diets and their effect on semen characteristics. A total of 36 New Zealand White male rabbits were used, randomly distributed into six groups, corresponding to six propolis levels (0, 0.25, 0.50, 0.75, 1.0 and 1.25 g propolis/kg of ration). Semen was collected twice a week, using an artificial vagina. Semen volume, progressive spermatic motility, spermatic vigor, spermatic concentration and spermatic morphology were analyzed. General linear models were used for statistical analysis. The inclusion of powder propolis in the diet increased normal spermatozoa percentage and reduced spermatozoa abnormalities. The powder propolis did not affect the progressive spermatic motility, spermatic vigor or spermatic concentration. The values were considered normal for rabbits. However, a small reduction in semen volume was observed, without any negative effect on the other semen characteristics evaluated. Thus, it is possible to observe better semen quality with the inclusion of 1.25 g powder propolis/kg in the diet for reproducer rabbits.
KEYWORDS: Reproduction. Reproducer. Semen evaluation.

INCLUSÃO DE PRÓPOLIS NA DIETA DE COELHOS E CARACTERÍSTICAS DO SÊMEN

RESUMO: Este experimento foi realizado com o objetivo de avaliar a influência de diferentes níveis de própolis em pó na ração de coelhos sobre as características do sêmen. Utilizaram-se 36 coelhos machos, adultos, Nova Zelândia Brancos, divididos aleatoriamente em seis grupos, consumindo cinco níveis de própolis (0; 0,25; 0,50; 0,75; 1,0 e 1,25 g de própolis/kg de ração). Coletou-se sêmen duas vezes por semana, utilizando vagina artificial. Verificou-se o volume, a motilidade espermática progressiva, o vigor espermático, a concentração espermática e a morfologia espermática. As análises estatísticas foram realizadas utilizando os modelos lineares generalizados. A adição da própolis na ração elevou a porcentagem de espermatozóides normais e reduziu os anormais. Todavia, foi observada uma pequena redução no volume do sêmen com o aumento do nível de própolis na dieta, sem afetar as demais características do sêmen. A motilidade progressiva, vigor espermático e concentração espermática não foram influenciados pelos diferentes níveis de própolis, valores considerados normais para coelhos. Conclui-se que a melhor qualidade do sêmen de coelhos reprodutores ocorreu com a adição de 1,25 g de própolis/kg de ração.
PALAVRAS-CHAVE: Avaliação de sêmen. Reprodução. Reprodutores.

INCLUSIÓN DE PROPÓLEOS EN LA DIETA DE CONEJOS Y CARACTERÍSTICAS DEL SEMEN

RESUMEN: Este experimento se llevó a cabo para evaluar la influencia de diferentes niveles de polvo de propóleos en la dieta de conejos, bajo las características del semen. Se utilizó 36 conejos machos, adultos, Nueva Zelanda Blancos, divididos al azar en seis grupos, consumiendo cinco niveles de propóleos (0; 0,25; 0,50; 0,75; 1,0 y 1,25g de propóleos/kg en el alimento). Se recogió semen dos veces a la semana, utilizando vagina artificial. Se encontró el volumen, motilidad espermática progresiva, el vigor de espermático, la concentración espermática y la morfología espermática. Los análisis estadísticos se realizaron utilizando modelos lineales generales. La adición de propóleos en la dieta aumentó el porcentaje de espermatozoides normales y redujo los anormales. Sin embargo, se ha observado una pequeña reducción en el volumen del semen con el aumento de propóleos en la dieta, sin afectar las demás características del semen. La motilidad progresiva, vigor espermático y concentración de espermatozoides no se vieron afectados por los diferentes niveles de propóleos, valores considerados normales para conejos. Se concluye que la mejor calidad del semen de conejos reproductores ocurrió con la adición de 1,25g de propóleos / kg en el alimento.
PALABRAS CLAVE: Evaluación del semen. Reproducción. Reproductores.

DOI: https://doi.org/10.25110/arqvet.v17i4.2014.5021
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Introduction

Propolis is a natural resin of complex chemical composition and its principal role is to maintain an antiseptic environment in the bee hive and to enable the bee colony health. It is a substance obtained from several parts of the plant such as buds, floral buds, leaves, branches and barks, in which the composition varies according to the flora of each region visited by the bees. Propolis generally contains 40 to 70% of resins and balsams, 20 to 35% of wax, 3 to 5% of essential oils and 5% of pollen grains (BOGDANOV; BANKOVA, 2014). It has been demonstrated that propolis provides protection against infertility by improving sperm production, motility, count and quality, and increased the process of steroidogenesis and hence testosterone production (YOUSEF; SALAMA, 2009), and could help in productions variables like body weight and feed intake due to high content of flavonoids (YOUSEF et al., 2010).

The results showed by Mahmoud and Elsoadaa (2013) indicated that the use of biopropolis combined with $AlCl_3$ in diets rats were ascertained to alleviate the harmful effects of $AlCl_3$ in biochemical parameters induced by Al intoxication.

Carvalho et al. (2002) noted that under normal conditions, the sperm cells contain antioxidant substances in their cytoplasm, however, the sperm, during the maturation period, loses most of their cytoplasm, and thus, loses some of the endogenous antioxidants, becoming vulnerable to the action of free radicals, that is one of the major causes of reduction in sperms viability and fertility (MARTINEZ et al., 2002; RUSSO et al., 2006).

The antioxidant activity of propolis is mainly attributed to its flavonoid contents, such as quercetin, flavones, isoflavones, flavonones, anthocyanins, catechins and isocatechins (ALVES; KUBOTA, 2013), that are capable of scavenging free radicals and thereby protection against lipid peroxidation (YOUSEF; SALAMA, 2009).

In this case, the experiment was carried out with the objective of analyzing the influence of different levels of propolis powder added to rabbits ration on rabbits` semen characteristics.

Material and Methods

The experiment was conducted in the rabbit sector of the Experimental Farm of Maringá State University – PR, from September 12th to December 14th, 2005, in a total of 94 days of propolis supplementation. An amount of 36 New Zealand White male rabbits were used with an initial mean age of seven months, adults, and initial average weight of 3.44 kg, divided into 6 groups with 6 animals each.

The animals were housed, individually, in cages of galvanized wire of 40cm x 60cm x 45cm (length, width and height, respectively), and the cages were equipped with automatic watering and semi-automatic feeder.

Before initiating the experiment, the animals received a diet formulated with the same addition of propolis for eight weeks (Table 1). After this period six treatments were tested with different concentrations of powder propolis standardized on the product SL 49 (IP Patent 0506396.0). The treatments were: 1) control; 2) animals that received 0.25 g of propolis/kg of ration; 3) animals that received 0.50 g of propolis/kg of ration; 4) animals that received 0.75 g of propolis/kg of ration; 5) animals that received 1.00 g of propolis/kg of ration; and 6) animals that received 1.25 g of propolis/kg of ration.

Table 1: Composition in the natural matter of the New Zealand White rabbits ration, used in the experiment

Ingredients	Quantity (%)
Ground Corn	25,27
Soybean Meal	14,00
Cotton Meal	24,00
Alfalfa Hay	23,30
Coast cross Hay	10,00
Salt	0,40
Dicalcium Phosphate	0,80
Limestone	1,00
Methionine – DL	0,60
Bacitracin1	0,05
Robenidine Cicostat 1	0,08
Nuvital2	0,50
TOTAL	100,00

[1]= Alfarma Laboratory; [2]= Composition per kg of product: vitamin A 600.000 IU, vitamin D 100.000 IU, vitamin E 8.000 mg, vitamin K3 200 mg, vitamin B1 400 mg, vitamin B2 600 mg, vitamin B6 200 mg, vitamin B12 1.200 mg, cobalt 200 mg, manganese 8.600 mg, zinc 1.200 mg, iodine 64 mg, selenium 16 mg, methionine 120.000 mg, antioxidant 20.000 mg.

The basic ration and the ingredients that were added to it were mixed for 13 minutes in a "Y" shaped mixer, and later processed in pellets with 0.5cm in diameter and 1.0cm in length and then air-dried and stored in bags identified for each treatment. The chemical composition of the ration was considered to be the one determined by Andreazzi et al. (2004), since the ingredients' composition percentage was the same (Table 2). Water and food were provided *ad libitum*.

Table 2: Chemical Composition in the natural matter of the New Zealand White rabbits ration, used in the experiment

Ingredients	Quantity
Dry Matter (%)	90,07
Starch (%)	17,04
Crude Protein (%)	16,95
Neutral Detergent Fiber (%)	35,97
Acid Detergent Fiber (%)	17,79
Ether Extract (%)	3,65
Digestible Energy (Kcal/kg)	2.770,20
Calcium (%)	1,00
Phosphorus (%)	0,56
Methionine + Cysteine (%)	0,60
Lysine (%)	0,84

The collection of semen was performed twice a week for five weeks, totalizing 10 samples per animal. The

semen collection was done with an artificial vagina, made of plastic tube 8 cm long and 4 cm in diameter, coated internally with a membrane of a non-lubricated condom and a graduated collection cup, at a temperature of 44º C, developed by the Animal Reproduction Laboratory, from Maringa State University (SCAPINELLO et al*.,* 1997), using a female rabbit (doe) in estrus to collect the semen. After the collection of the samples, the volume (V) with and without gel, the progressive spermatic motility, spermatic vigor, spermatic concentration in mm^3 and spermatic morphology were verified. The volume was observed in the graduated collection cup; the progressive spermatic motility and spermatic vigor were observed in an optical microscope slide, with coverslip from a drop obtained from the dilution of one drop of semen (0.03mL) with five drops of dehydrated sodium citrate 2,94% (0.03mL), and taken to the phase contrast microscope 40 X and subjectively evaluated.

For the progressive spermatic motility a score from 0 to 100% was used and for spermatic vigor a score from 0 to 5 points, which in this case the highest value represents higher displacement speed. To determine spermatic concentration, the semen was diluted in buffered saline formaldehyde (HANCOCK, 1957), utilizing Shalli pipette, in a 1:100 dilution. Once the dilution was done, the improved Neubauer chamber was filled by capillarity, and the sperms were counted in five large squares of the chamber and then added together and divided into 80 small squares, multiplied by 400 small squares, the dilution and the height of the chamber, obtaining the amount of sperm per mm^3 of semen. The spermatic morphology was assessed based on two smears prepared from the dilution made to evaluate the progressive spermatic motility and the spermatic vigor and stained by the Williams Method (1920), modified by Lagerlöff (1934). It was used a completely randomized design and the response variables were submitted to the procedure Generalized Linear Models (Dobson, 1990) using the SAS' procedure Genmod (2001), whereas the errors had different probability distributions, with canonical link function.

Results and Discussion

When analyzing sperm morphology, it was observed differences ($P<0.05$) on normal and abnormal sperms in relation to the different levels of propolis used in the animal ration (Figure 1). As the content of propolis in the diets increased, the percentage of normal sperms increased as well (Figure 1).

The same response was observed by Hatamoto et al. (2006), when using vitamin E injection in dogs, added to the improved progressive spermatic motility, attributing this fact to the antioxidant action of this supplement. This was also observed by Yousef et al. (2010), in rabbits, by administering daily, 50 mg/kg of body weight of propolis orally, for 12 weeks. Perhaps, the antioxidant properties of flavonoids (VASCONCELLOS et al., 2007; SYAZANA et al., 2011) found in propolis, among other factors, have contributed to improve sperm morphology, acting as antioxidant elements, protecting the sperm membrane. Russo et al. (2006) found that propolis protects the sperm DNA from oxidative damage caused by the thiobarbituricacid-reactive substances (TBARS).

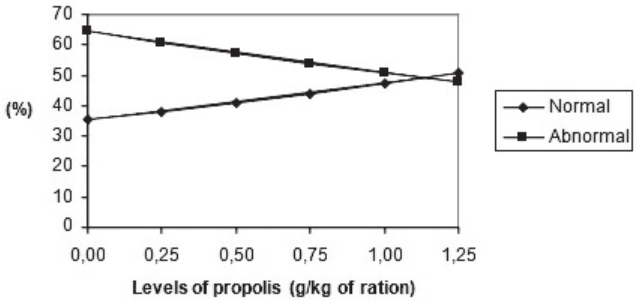

Figure 1: Percentages os normal and abnormal sperm in relation to diferrent levels of propolis supplied by SL49, used in the ration of the New Zeland White rabbits fed for 94 days. Normal: $y = e^{3,5655+0,2908(x)}$, Abnormal: $y = e^{4,1689-0,2444(x)}$

The semen volume decreased ($P<0.05$) as the levels of propolis in the diets of rabbits increased (Figure 2).

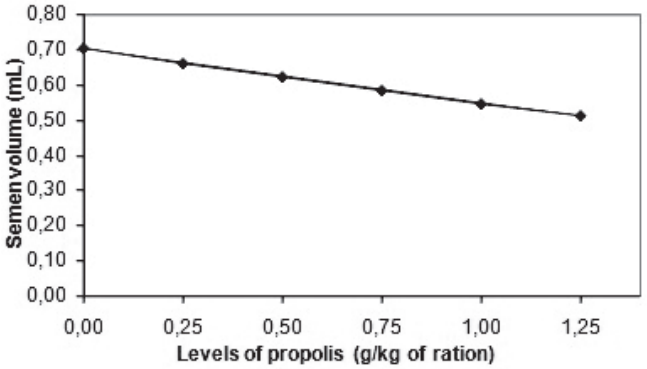

Figure 2: Semen volume compared to the levels of propolis (SL49) used in the ration of the New Zealand White rabbits, which were fed with treatment ration for 94 days. Volume: $v = e^{4,2542-0,2529(x)}$

However the gradual reduction of the volume of the semen did not cause reduction in the sperm concentration nor in the total number of sperm, which is a favorable factor. It means that, also physiologically, there was an improvement in sperm concentration.

In Table 3, in addition to concentration, it shows the mean values and the confidence intervals of the progressive spermatic motility, the sperm vigor, the number of sperm per ejaculation and rates of primary and secondary abnormalities, which were not influenced by the levels of propolis provided. Though, the mean values estimated for these parameters, at the time of the year in which the experiment was developed, are considered normal for rabbits (CASTELLINI et al., 2002; ALVAREZ et al., 2006).

These results are at variance with those found by Yousef and Salama (2009), when administering orally, in male rats, 50 mg/kg of body weight of propolis for 70 days and Yousef et al. (2010), when also administered orally, in rabbits, 50 mg/kg of body weight of propolis for 12 weeks. Both authors found significant improvement in semen quality of propolis treated-animals compared to controls. Moreover Yousef and Salama (2009) have observed increased weight of the testicles, seminal vesicles and epididymis in animals which received propolis compared to the control, but there was no increase in the prostate.

Table 3: Estimated and predicted average and the interval of confidence from the semen characteristics of the New Zealand White rabbits during 94 days of experiment

Parameters	Estimated and predicted average	Probability Distribution	Confidence Interval
Progressive motility (%)	42,88	Negative binormal	33,95 - 54,16
Spermatic vigor (points)	2,90	Poisson	2,51 - 3,41
Sperm concentration (sperm⁄ml x108)	2,92	Negative binormal	2,30 - 3,71
Number of sperm per ejaculate x 108	1,46	Negative binormal	1,20 - 1,78
Primary Abnormalities (%)	32,38	Negative binormal	28,35 - 36,97
Secundary Abnormalities (%)	15,60	Negative binormal	13,23 - 18,41

Syazana et al. (2011) by providing honey in rats diets, which is a product rich in flavonoids, found that honey is potentially useful in increasing sperm count, percentage of normal sperm and reducing the percentage of sperm head and tail abnormalities. Therefore, improvement in motility and spermatozoa vigor of the animals fed with propolis were expected. However, it is believed that the addition of propolis in the diet has benefited breeders with some of these properties or perhaps all, especially factors that led to reduction of total sperm abnormalities. However, when Castilho et al. (2009) evaluated the freezing goat semen and adding lyophilized propolis to extenders, found toxic effect of propolis to sperm concentrations of 0.25 and 0.5%.

The favorable results to the production of semen in rabbits, tested in this study, may be related to Guerra et al. (2004) in relation to the antioxidant properties of vitamins C and E, which provided a higher percentage of gametes with normal acrosomes, intact mitochondrial structures, increased motility and increased concentration of sperm in the ejaculate. Perhaps, such properties may be present in the elements that constitute the propolis, the flavonoids in particular, as pointed out by Russo et al. (2006), Vasconcellos et al. (2007), Yousef and Salama (2009) and Yousef et al. (2010).

Conclusions

It can be concluded that the addition of propolis to the rabbit diets improved sperm morphology, and reduced semen volume. It did not alter the motility, sperm vigor or sperm concentration. Therefore, based on these results, it is possible to indicate the use of 1.25 g of propolis / kg of ration to breeding rabbits and to develop further research to find the optimal level of propolis to be added to rations. However, more studies are necessary to understand the results showed in this research.

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Recebido em: 01.11.2014
Aceito em: 29.12.2014