World Rabbit Science

World Rabbit Sci. 2018, 26: 209-215
doi:10.4995/wrs.2018.10154
© WRSA, UPV, 2003

THE EFFECT OF CAROB (*CERATONIA SILIQUA*) BEAN EXTRACT ON MALE NEW ZEALAND WHITE RABBIT SEMEN

ATA A.*, YILDIZ-GULAY O.†, GÜNGÖR S.*, BALIC A.‡, GULAY M.S.‡

*Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Theriogenology and Artificial Insemination, Burdur, Turkey.
†Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Physiology, Burdur, Turkey.
‡Sakarya Toyota Hospital, Sakarya, Turkey.

Abstract: The carob tree (*Ceratonia siliqua*) grows naturally in the Mediterranean region. The empiric use of carob cures for their aphrodisiac properties is very common in Turkey. Thus, the experiment was conducted to determine the effects of carob bean extracts on some reproductive parameters in male New Zealand White rabbits. During the adaptation period (stage 1), 6-8 mo old rabbits were trained in semen collection for 30 d. At the beginning of the treatment period (stage 2), rabbits were assigned randomly to 2 groups of 8 animals each. For a period of 49 d (1 spermatogenesis duration), one group was treated with a daily oral dose (10 mL) of carob extract and the other group received the corresponding volume of tap water. Semen was collected weekly. Semen samples taken at week 1 and 7 were analysed separately. At the beginning of stage 2, no differences were observed in the volume and pH of the ejaculate, sperm concentration, percentage of motility, percentage of live spermatozoa, percentage of sperm plasma membrane integrity, plasma concentration of testosterone, and seminal plasma protein levels between the control and carob extract treated animals. Similarly, at the end of stage 2, there were no differences in the volume and pH of the ejaculate, motility percentage, the percentage of live spermatozoa, percentage of sperm plasma membrane integrity, and the seminal plasma protein levels between the control and the carob extract treated animals. However, sperm concentration ($P<0.05$), plasma concentration of testosterone ($P<0.05$), and percentage of change in spermatozoa concentration ($P<0.02$) between groups were affected at the end of stage 2. The data suggested that the use of carob cures prepared by boiling carob fruit could have beneficial influences on sperm concentration in rabbits.

Key Words: carob, rabbit, sperm parameters, reproduction, testosterone.

INTRODUCTION

The carob tree (*Ceratonia siliqua*) is an evergreen tree native to the Mediterranean region found in Turkey, Libya, Spain, Italy, Portugal, Morocco, and Greece, and cultivated for its edible seed pods. Several products are produced from the seedpods and these products have a promising importance in healthy and balanced feeding because of the high nutrient contents (Karkacier and Artik, 1995; Marakis, 1996).

Previous studies have shown that carob juice is rich in potassium, sodium, calcium, magnesium, iron, copper and manganese in addition to zinc, which is a nutrient vital to the healthy functioning of the male reproductive system. Furthermore, a potent antioxidant element, gallic acid, is the most abundant phenolic compound (3.27 mg/g) present in carob fruit (Ayaz *et al.,* 2007). High tannic acid (10.2 mg/dL) content of the carob could reduce blood cholesterol levels (Würsch, 1979).

Correspondence: Gulay M.S., msgulay@mehmetakif.edu.tr. Received *May 2018* - Accepted *June 2018.*
https://doi.org/10.4995/wrs.2018.10154

Carob cures are traditionally used for their aphrodisiac properties and are believed to increase sperm count in men (Saracoglu, 2011). As a result, the empiric use of carob cures prepared by boiling the carob fruit to treat low sperm count is very common. Although carob cures are commonly consumed for their aphrodisiac properties, there is no pre-planned study available in the literature on the effects of carob on the reproductive system. Thus, the purpose of the current study was to determine whether the traditional use of carob bean extract has any effects on some reproductive parameters in male New Zealand White rabbits.

MATERIALS AND METHODS

Animals and diets

This study was approved by the ethics committee of Suleyman Demirel University (08/12/2009.27.04-171531123157011-139) and care was taken to minimise the number of animals used. A total of 16 male New Zealand White rabbits (6-8 mo old) weighing 2.5 and 3.0 kg were used in the study. Rabbits were housed individually in galvanised cages (50×50×50 cm) during the experiment. The animals were kept under standard laboratory conditions (12 h dark: 12 h light and 24±4°C) during the entire experimental period.

Feed and water were provided *ad libitum*. The rabbits were fed standard commercial rabbit pellets (Ekinciler Food Company, Burdur, Turkey; 2600 kcal/kg of metabolisable energy) with 88% dry matter, 9% ash, 16% crude protein, 15% crude fibre, 1.8% mineral mixture.

Preparation of carob bean extract

Carob bean samples were collected from the same plant in Antalya, Turkey. Extracts of carob bean were prepared daily in the way used traditionally for the treatment of low sperm count. Carob beans (150 g) were cut into 2 cm pieces and boiled in 500 mL of tap water for 3 min (Saracoglu, 2011). After cooling the mixture for 20 min, carob fruits were removed from the water by centrifugation at 3000 rpm for 10 min and filtered through cheesecloth.

Experiment and sampling

Rabbits were divided randomly into 2 groups of 8 rabbits each and trained for semen collection prior to the experiment for 30 d (adaptation period). The experimental period was 49 d (spermatogenesis duration). The traditional dose of carob extract for increasing sperm count in man is 250 mL/d. Thus, rabbits in the treatment group were exposed to 10 mL of carob extract prepared daily for 7 wk. Rabbits in the control group received the same amount of tap water daily. Throughout the experimental period, semen samples were collected twice a week with 2- to 3-d intervals between each collection. The samples were obtained by means of an artificial vagina and immediately transferred to the laboratory for further processing. Semen samples at day 1 (initial) and 49 of the experiment were analysed separately. At the end of the experiment, blood samples from each rabbit were collected from the ear artery. Blood samples were centrifuged at 1457×*g* for 30 min and the serum from each sample was stored at –20°C until assayed. One day after blood sample collection, the rabbits were euthanised and wet weights of testes, epididymides and accessory sex glands (seminal vesicles, prostate and bulbourethral glands) as a whole were recorded.

Ejaculates collection and semen evaluation

All bucks were previously adapted to the semen collection procedure. Before semen collection, bucks were allowed one false mount and at the subsequent mounting, the artificial vagina was suitably positioned for penis intromission. Libido was also evaluated during semen collection based on the time of introducing the female to the male rabbits until the male rabbit starts to mount and ejaculate into the artificial vagina. Semen evaluations were performed by a single researcher in single-blinded fashion. Semen samples were immediately assessed for physical parameters of aspect, colour, volume and pH. Immediately after collection of ejaculates, the spermatozoa concentration, motility, morphology, sperm plasma membrane integrity, number of live cells and acrosomal status were determined on the raw semen.

Spermatozoa concentration

The amount of ejaculate without gel (mL) and the semen concentration (number of sperm per mL) were recorded using a graduated tube and a haemocytometer, respectively. Each ejaculate was processed to estimate the sperm concentration in duplicate by direct microscopic examination using two haemocytometer chambers. Sperm counts were made in the sperm suspension in formalin saline solution (4% formalin in 0.9% saline; a ratio of 1:100 semen:formalin), with the aid of a Thoma haemocytometer (Marienfeld GmbH & Co. KG, Lauda Königshofen, Germany) at ×400 magnification (Ata *et al.,* 2007).

Semen pH

Initial hydrogen ion concentration (pH) of semen samples was determined just after collection using a pH cooperative paper ranging from 5.5 to 9.0 with 0.5 grades (pH-Indikatorpapier Neutralit pH 5.5-9.0; Merck, Darmstadt, Germany).

Sperm motility

The percentage of motile sperm was estimated by visual examination under ×400 magnification using a phase-contrast microscope with heated stage (Ata *et al.,* 2007). Semen samples were diluted at the rate of 1:10 with isotonic phosphate-buffered saline buffer at 37.8°C. Motility estimations were performed from 3 different fields in each sample. The mean of three successive estimations was used as the final motility score.

Sperm morphology

Structural detail visualisation of sperm (major morphological abnormalities) was recorded by fixing the cells in buffered formalin saline, then viewing the unstained cells as a wet mount with phase-contrast microscopy at ×1000 magnification. At least 200 spermatozoa were evaluated for the presence, type, and incidence of each morphologic defect. Specific morphological defects, such as knobbed acrosomes, proximal protoplasmic droplets, complete separation of the acrosome and galea capitis, complete separation of the head, swollen midpieces and coiled tails were recorded (Ata *et al.,* 2007).

Sperm viability

Sperm viability was determined using an eosin-nigrosin staining mixture (EET). Spermatozoa that were not stained were classified as 'vital' and those that showed any pink or red colouration were classified as 'dead' (Ata *et al.,* 2007).

Sperm membrane integrity test (hypo-osmotic swelling test)

The percentage of sperm membrane response was evaluated using the hypo-osmotic swelling test (HOS), as previously explained by Ata *et al.* (2007). A minimum of 200 cells was observed, and spermatozoa with typically coiled tails were recorded as HOS (+).

Seminal plasma protein

Total seminal plasma protein values were measured by a refractometer (Atago, SPR-N, Japan). Semen samples (200 µL) from each collection were centrifuged at 800×*g* for 20 min. Seminal plasma was separated from the sperm cells. Ten µL of seminal plasma were placed into the refractometer for total seminal plasma protein readings.

Histopathology

After euthanasia, left testicles were placed in buffered formalin for histological examinations. All tissue samples were routinely processed into paraffin; 5-µm thick sections were stained with haematoxylin and eosin (H&E). The slides were coded and examined in a single-blind fashion by a pathologist. The minor diameter of 15 seminiferous tubules per testis measured for quantitative purposes.

Testosterone

Serum testosterone levels were measured using a rabbit testosterone ELISA kit (CSB-E06927Rb, Cusabio Biotech CO., LTD Wuhan, Hubei, China). Intra- and inter assay precision of the assay were <15%. The detection range of the kit was 0.625-10 ng/mL. Measurements were performed according to the manufacturer's instructions.

Statistical analyses

Results are presented as mean±standard error. The data on seminiferous tubules diameters were analysed by one-way ANOVA. The rest of the data were analysed by Proc T-Test procedure. As the age and age*treatment interactions were not significant, they were excluded from the statistical model. All statistical analyses were carried out using SAS statistical package. The minimum level of significance was set at $P<0.05$.

RESULTS

Data on the reproductive organ weights of control and the carob extract treated animals at the end of the experiment are shown in Table 1. Towards the end of the experiment, the right, the left and paired testis weights in the carob extract treated animals significantly increased compared to controls. Similarly, paired epididymides weights were altered in the carob extract treated animals ($P<0.05$). However, no changes in the accessory sex gland weights were observed between the 2 groups (Table 1). No histopathological lesions were detected in either group. However, the diameters of the seminiferous tubules were significantly increased in the carob extract treated animals compared to controls (Table 1). Furthermore, serum testosterone concentrations were significantly increased in the carob extract treated animals (Table 1). Libido was normal in all groups.

Data on the changes observed in the semen characteristics of the carob extract treated animals at day 1 (at the beginning of the experiment) and day 49 (at the end of the experiment) are shown in Table 2 and 3, respectively. Initial values for ejaculate volume, pH, progressive motility, head defect, tail defect, sperm concentration, percentage of live spermatozoa, percentage of sperm plasma membrane integrity and total seminal plasma protein levels between the groups did not differ significantly on day 1 of the experiment (Table 2). At the end of the experiment, oral daily administration of rabbits with carob bean extracts caused a significant increase in sperm concentration ($P<0.05$). Treatment effect on sperm concentration was more pronounced when percentage changes in sperm concentrations [(sperm concentrations at the end of experiment / sperm concentrations at the beginning of the experiment)×100] were compared between the 2 groups (Table 3; $P<0.02$). Similarly, percentage changes in progressive motility [(progressive motility at the end of experiment / progressive motility at the beginning of the experiment)×100] was also affected positively due to the treatment (Table 3; $P<0.02$). However, no notable changes in ejaculate volume, pH, progressive motility, head defect, tail defect, percentage of live spermatozoa, percentage of sperm membrane response and total seminal plasma protein levels were observed in the control or carob extract treated animals at the end of the experiment (Table 3).

DISCUSSION

Throughout the ages, nutrients have been linked to reproductive performance in man (Rickard *et al.,* 2010). There have been studies suggesting that the prophylactic use of some nutrients may be useful in enhancing male

Table 1: Effects of carob bean treatments on average feed intake, body and organ weights and serum testosterone concentration in New Zealand White male rabbits after oral gavages for 49 d (mean±standard error of mean).

	Treatments		
Parameters	Control	Carob	*P*
Body Weight (g)	2738±97	2729±79	NS
Testis weight (g)			
Right	3.63±0.07	3.93±0.05	0.02
Left	3.66±0.07	3.97±0.06	0.02
Pair	7.29±0.19	7.92±0.14	0.03
Epididymides (pair,g)	7.02±0.06	7.20±0.06	0.03
Accessory sex glands (g)	3.27±0.05	3.34±0.08	NS
Seminiferous tubules diameter (µm)	201.5±3.57	211.6±4.16	0.05
Testosterone (ng/dL)	338.8±3.3	391.6±5.6	0.05

NS=not significant.

Table 2: Ages and sperm parameters (mean± standard error of mean) of New Zealand White male rabbits prior to the experiment.

Parameters	Treatments		
	Control	Carob	*P*
Age (months)	7.21±0.12	7.12±0.16	NS
Ejaculate volume (mL)	0.71±0.06	0.64±0.03	NS
Ejaculate pH	7.01±0.02	7.00±0.01	NS
Head defect (%)	2.17±0.83	1.65±0.80	NS
Tail defect (%)	7.50±0.88	8.87±1.08	NS
Live spermatozoa (%)	82.5±1.25	78.3± 3.06	NS
Sperm plasma membrane integrity (%)	79.0±1.21	74.9±2.54	NS
Seminal plasma protein levels (g/dL)	2.6±0.3	2.9±0.3	NS
Progressive motility (%)	78.9±1.44	79.1±0.91	NS
Sperm concentration ($x10^6$mL)	347.5±45.1	380.9±29.4	NS

NS=not significant.

fertility (Turk *et al.,* 2008; Robbins *et al.,* 2012). In the current study, some histological characteristics and semen parameters of carob bean extracts were evaluated in male New Zealand rabbits. To our knowledge, this is the first study demonstrating that daily consumption of traditional use of carob cures for 7 wk caused increased sperm concentration and serum testosterone levels in male rabbits.

On gross pathological examination, no changes were observed in any of the reproductive organs examined. The histopathological examination of the reproductive organs in the control and treated groups also showed no differences, suggesting that carob bean extracts at the dose tested did not result in any adverse toxicological effects on the reproductive organs.

In the current study, the testis and epididymis weights were increased and testosterone levels were elevated significantly when compared to the control group. Increased testis end epididymis weights corresponded to greater testosterone levels in treated rabbits. Moreover, parallel to the changes in testis weights, sperm concentration between the groups was also altered by the treatment. Spermatogenesis is known to be controlled by testosterone produced from Leydig cells. Any decrease in Leydig cell numbers or testosterone levels could adversely affect germinative epithelial cell numbers (Agnes and Akbarsha, 2003). Furthermore, changes in the testosterone

Table 3: Effects of carob bean extract treatments on some sperm parameters (mean±standard error of mean) of New Zealand White male rabbits at the end of the experiment.

Parameters	Treatments		
	Control	Carob	*P*
Ejaculate volume (mL)	0.68±0.06	0.63±0.03	NS
Ejaculate pH	7.06±0.03	7.02±0.02	NS
Head defect (%)	2.41±0.19	2.53±0.13	NS
Tail defect (%)	13.6±1.22	11.4±1.17	NS
Live spermatozoa (%)	80.1±3.51	80.4±2.41	NS
Sperm plasma membrane integrity (%)	77.5±2.37	80.9±2.54	NS
Seminal plasma protein levels (g/dL)	2.6±0.19	2.4 ±0.25	NS
Progressive motility (%)	78.0±2.7	84.0± 1.41	NS
Per cent changes in progressive motility (%)[1]	98.7±1.85	106.4±2.88	0.05
Sperm concentration ($\times10^6$ mL)	337.6±43.4	460.7±44.1	0.05
Per cent changes in spermatozoa concentrations (%)[2]	98.9±6.38	122.9±6.56	0.02

[1][(Progressive motility at the end of the experiment / progressive motility at the beginning of the experiment)×100].

[2] [(Sperm concentrations at the end of the experiment / sperm concentrations at the beginning of the experiment)×100].

NS=not significant.

concentration are associated with the secretory activity of the testes (Katsiya *et al.,* 1989; Abd El-Ghany, 2007). Thus, the elevated levels of serum testosterone may be one of the mechanisms underlying the effect of carob bean extract on the improvement of testicular weight and sperm concentration.

Micronutrient deficiencies have been associated significantly with high reproductive risks. Furthermore, insufficient mineral intake can cause deleterious effects on sperm production (Robbins *et al.,* 2012). Minerals and natural antioxidants such as zinc, gallic acid and tannic acid can protect sperm DNA from oxidative stress, and hence may improve sperm motility and male fertility (Hala, 2011).

Carob is rich in minerals and phenolic compounds important for a healthy lifestyle, spermatogenesis and sperm motility. Thus, carob cures have been used empirically for its clinical properties (Würsch, 1979; Inouce *et al.,* 1994; Amico and Source, 1997; Merzouki *et al.,* 1997). The antioxidant and free radical scavenging activity of phenolic compounds and zinc have already been reported (Tokeshi *et al.,* 2007; Osaretin and Gabriel, 2008). Zinc can work against oxidation by binding sulphydryl groups in proteins. It can engage the binding sites for copper and iron in DNA, proteins and lipids (Zago and Oteiza, 2001). Zinc is capable of normalising deficient sperm counts and increasing sperm motility (Feng *et al.,* 2002). Zinc also acts as an antioxidant through the protection of proteins and enzymes against free radical attack or oxidation and the prevention of free radical formation. Loss of zinc from biological membranes increases their susceptibility to oxidative damage and impairs their function (Takeda *et al.,* 2005; Osaretin and Gabriel, 2008). It has been shown that tannic and gallic acids possessed strong O2- scavenging activity (Tokeshi *et al.,* 2007). They can act as an antioxidant and help protect spermatozoa against oxidative damage. Therefore, it is possible that antioxidant properties of carob bean extracts could have had a positive effect on decreased ROS activity in the treatment group and caused improvement in spermatological parameters.

In the current study, daily oral administration of carob bean extracts increased the sperm concentration and motility without increasing the rates of abnormal sperm cells. Therefore, it may be concluded that carob bean extracts could improve serum testosterone concentration and semen quality of male New Zealand White rabbits without any adverse effect on the reproductive performance of the male animal.

REFERENCES

Abd El-Ghany M.A. 2007. The relation of antioxidants and sodium nitrite on the oxidation reduction system and reproductive ability of male rats. *Egypt J. Nutr., 2: 33-64.*

Agnes V.F., Akbarsha M.A. 2003. Spermatotoxic effect of aflatoxin B1 in the albino Mouse. *Food. Chem. Tox., 41: 119-130. https://doi.org/10.1016/S0278-6915(02)00171-0*

Amico F.P., Source E.G. 1997. Medical plants and phytotheraphy in Mussomeli area (Caltenisseta, Scily, Italy). *Fitoterapia, 68: 143-159.*

Ata A., Hatipoglu F.S., Yildiz-Gulay O., Gulay M.S. 2007. Protective role of ascorbic acid on subacute sperm toxicity in male New Zealand White Rabbits treated with endosulfan. *Drug Chem. Toxicol., 30: 181-195. https://doi.org/10.1080/01480540701374896*

Ayaz F.A., Torun H., Ayaz S., Correia P.J., Alaiz M., Sanz C., Gruz J., Strand M. 2007. Determination of chemical composition of Anatolian carob pod (*Ceratonia siliqua* L.), Sugars, amino and organic acids, minerals and phenolic compounds. *J. Food Quality, 30: 1040-1055. https://doi.org/10.1111/j.1745-4557.2007.00176.x*

Feng P., Li TL., Guan Z.X., Franklin R.B., Costello L.C. 2002. Direct effect of zinc on mitochondrial apoptogenesis in prostate cells. *Prostate, 52: 311-318. https://doi.org/10.1002/pros.10128*

Hala M.A.W. 2011. Protective effect of *Nigella sativa*, linseed and celery oil against testicular toxicity induced by sodium valproate in male rats. *J. Am. Sci., 7: 687-693.*

Inouce M., Suzuki R., Koide T., Sakaguchi N., Ogihera Y., Yabu Y. 1994. Antioxidant, gallic acid, induces apaptosis in HL- 60RG cells. *Biochem. Res. Commun., 204: 898-904. https://doi.org/10.1006/bbrc.1994.2544*

Karkacier M., Artik N. 1995. Determination of physical properties, chemical composition and extraction conditions of carob bean (*Ceratonia siliqua* L.). *Gida, 20: 131-136.*

Katsiya G.V., Todua T.N., Gorlushkin V.M., Chirkov A.M., Goncharov N.P. 1989. Effect of immobilization stress on the gonadotropic function of the hypophysis in male hamadryas baboons (*Papio hamadryas*). *Biol. Medit., 107: 231-234.*

Marakis S. 1996. Carob bean in food and feed: current status and future potentials: a critical appraisal. *J. Food Sci. Technol., 33: 365-383.*

Merzouki A., Ed-Derfoufi F., El Allau A., Molero-Mesas J. 1997. Wild medicinal plants used by local Bouhmed population (Morocco). *Fitoterapia, 68: 444-460.*

Rickard I.J., Holopainen J., Helama S., Helle S., Russell A.F., Lummaa V. 2010. Food availability at birth limited reproductive success in historical humans. *Ecology, 91: 3515-3525. https://doi.org/10.1890/10-0019.1*

Robbins W.A., Xun L., FitzGerald L.Z., Esguerra S., Henning S.M., Carpenter C.L. 2012. Walnuts Improve Semen Quality in Men Consuming a Western-Style Diet: Randomized Control Dietary Intervention Trial. *Biol. Reprod., 87: 101-108. https://doi.org/10.1095/biolreprod.112.101634*

Osaretin A.T., Gabriel A.A. 2008. Effect of zinc deficiency on memory, oxidative stress and blood chemistry in rats. *Adv. Med. Dent. Sci., 2: 74-82.*

Saracoglu A.I. 2011. Şifa Kitabı, 1st ed. *Haygrup Publishers, Istanbul, Turkey*.

Takeda A., Tamano H., Tochigi M. 2005. Zinc homeostasis in the hypocampus of zinc deficient young adult rats. *J. Neurochem. Int., 46: 221-225. https://doi.org/10.1016/j.neuint.2004.10.003*

Tokeshi I., Yoshimoto T., Muto N., Nakamura S., Ashizawa K., Nakada T., Tatemoto H. 2007. Antihyaluronidase action of ellagic acid effectively prevents polyspermy as a result of suppression of the acrosome reaction induced by sperm-zona interaction during *in vitro* fertilization of porcine oocytes. *J. Reprod. Develop., 53: 755-764. https://doi.org/10.1262/jrd.18173*

Turk G., Sonmez M., Aydin M., Yuce A., Gur S., Yuksel M., Aksu E.H., Aksoy H. 2008. Effects of pomegranate juice consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats. *Clin. Nutr., 27: 289-296. https://doi.org/10.1016/j.clnu.2007.12.006*

Würsch P. 1979. Influence of tannin-rich carob pod fiber on the cholesterol metabolism in the rat. *J. Nutr., 109: 685-692. https://doi.org/10.1093/jn/109.4.685*

Zago M.P., Oteiza P.I. 2001. The antioxidant properties of zinc: interactions with iron and antioxidants. *Free Rad. Biol. Med., 31: 266-274. https://doi.org/10.1016/S0891-5849(01)00583-4*