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Effects of Using Hydro-Alcoholic Extract of Carob (*Ceratonia siliqua*) Seed on Semen Quality and Blood Parameters of Rams

Research Article

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ABSTRACT

Carob belongs to the Fabaceae family, which has been studied for its medicinal properties, showcasing antioxidative properties and the ability to enhance sexual and immune system functions. This study aimed at examining the effect of adding hydro-alcoholic extracts of carob seeds to the diet on steroid hormones, sperm parameters and blood parameters of Lori-Bakhtiari rams. In this study, carob seeds were extracted using the percolation method. Thirty Lori-Bakhtiari rams were selected with a mean weight of 50 ± 5 kg and mean age of 2.5 years old. They were equally divided into three groups: (1) control (without carob extract), treatment (1) 150 mg/kg body weight (BW), and treatment (2) 300 mg/kg of carob seed extract in the diet for 4 weeks (2021-2022) for adaption process. Then semen and blood were analyzed for four months. Sperm motility, viability and the hypo-osmotic swelling (HOS) tests were evaluated at pre-freezing and post-thawing by the Computer Assisted Semen Analysis (CASA) software, Eosin-Nigrosin staining and a phase-contrast microscope, respectively. Furthermore, blood samples were collected for evaluation of fasting blood sugar (FBS), cholesterol (CH) and triglyceride (TG), serum creatinine (sCr), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and testosterone hormone (T). The results of this study showed that the sperm parameters like motility and viability in the treatment groups increased significantly ($P<0.05$) in pre-freezing and post-thawing evaluations. Additionally, the results of blood parameters showed that FBS, CH, TG, LDL and sCr were significantly decreased by using the carob extract in a diet ($P<0.05$), while the testosterone level significantly increased during the experiment ($P<0.05$). In conclusion, the use of 300 mg/kg carob extract in the diet showed a more positive effect on sperm and blood parameters at Lori-Bakhtiari rams.

KEY WORDS blood parameter, carob (*Ceratonia siliqua*), Lori-Bakhtiari ram, sperm parameter.

INTRODUCTION

The most important domestic ruminants in the world include cattle, sheep and goats, which supply milk and meat, and success in the livestock industry depends in some way on the success of the fertility of domestic animals to increase their pregnancy rates (Davis and White 2020; Simoes *et al*. 2021). Artificial insemination is used to fertilize domestic animals, which requires high quality sperms. Today, for this purpose, sperm collection centers collect, freeze and package the sperm and provide it to livestock centers. One of the important goals of the livestock production physiology department is to be able to increase the quality of fertility. Sperm quality as related to fertility potential has been studied and reviewed over many decades (Rodriguez-Martines, 2007; Emanizadeh *et al*. 2023).

Infertility is one of the important problems in several species, including human beings, and farm animals. Numerous studies have reported that antioxidants and vitamins A, B, C, and E in the diet can protect sperm. Evidence suggests that medicinal plants, such as carob can have positive effects on increasing fertility (Priolo *et al*. 2000; Mokhtari *et al*. 2012; Aghajani *et al.* 2019).

Ceratonia siliqua, a member of the Fabacea family, is an evergreen tree which is grown in Mediterranean countries, such as Italy, Spain, Turkey as well as Iran. It has been evaluated as a medicinal plant to treat infertility. Various parts of plants, such as fruit, peel and seed, demonstrated various chemical compounds (Motalebipour and Pirestani, 2022).

The extracts of carob, which are rich in phenolic compounds, showed a significant antioxidant activity. Its carbohydrates' content is 40%, its fat is about 1% and its protein is 3 to 4%. Furthermore, it contains a lot of tannins, fiber, minerals such as calcium, potassium, sodium, iron and phosphorus, as well as vitamins E, D, C, B6, niacin, folic acid and selenium (Youssef *et al*. 2013; Najafi *et al*. 2017; Rtibi *et al.* 2021).

Carob extract has been used as feed for laboratory animals in order to identify the potential changes in sexual hormones, semen quality, sperm production, and also the spermatogenesis process as well as total antioxidant capacity (TAC) (Vafaei *et al*. 2018; Soleimanzadeh *et al.* 2020; Aghajani *et al.* 2021; Iranmanesh *et al*. 2023). One of the properties of carob is the treatment and improvement of diabetes symptoms due to its fiber, tocopherol, polyphenols, antioxidants and plant sterols (Mokhtari *et al*. 2012). Carob seed powder has the effect of reducing blood cholesterol (Duha *et al*. 2008) one of the obstacles to feeding carob pods for domestic animals is the presence of tannin (Obeidat *et al.* 2011).

High levels of tannins in feed can potentially reduce the nutritional value of the feed, especially if tannins bind to proteins and make them unavailable to the animal (Gobindram *et al*. 2015).

Therefore, based on recent researches, the appropriate amount of carob can be used up to 300 mg without affecting its nutritional valu. The biological and pharmacological efficacy of *Ceratonia siliqua* has not been made known in ram. Therefore, the aim of this study was to determine the effects of hydro-alcoholic extract of carob (*Ceratonia siliqua*) seed in diet on Semen and blood parameters of Lori-Bakhtiari rams.

MATERIALS AND METHODS

All chemical reagents were obtained from Merck (Darmstadt, Germany), unless otherwise noted.

Blood and semen collection, evaluation, and sample preparation

Sperm samples were obtained from thirty (n=30) mature Lori-Bakhtiari rams (aged 2.5 years, weighing 50±5.0 kg) maintained at the Animal Breeding Center Farm, Isfahan branch, Islamic Azad University, Iran. The rams were daily fed based on NRC (2007). Water and high quality hay were supplied *ad libitum.* Adaptation period was set for four weeks, after which sampling was done. They were divided equally into three groups (n=10 rams/group), which included: control (without extract), treatment 1: containing 150 mg/kg of the carob seed extract in the diet, and treatment 2: containing 300 mg/kg of the carob seed extract in the diet for four months.

Semen samples were collected from the rams using the artificial vagina (*imv*, Frances). It was performed in December, January, February, and March (2021-2022). Sperm was collected from all treated and control groups during the experiment. Semen collection was done twice a week during the study for each ram. After semen collection, the fresh semen samples were transferred to the laboratory immediately, and kept in a water bath at 37 ˚C for examination. Sperm concentration was measured with a photometer (*imv*, Frances). Semen samples were pooled to eliminate individual differences. Spermatozoa that showed >60% progressive motility and concentration of 2.5×10^9 spermatozoa/ml were selected for experiments (Gil *et al*. 2003). However, at the same time of semen sampling, blood samples were collected from a jugular vein and their serums were separated in a laboratory for evaluation of fasting blood sugar (FBS), cholesterol (CH) and triglyceride (TG), serum creatinine (sCr), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and testosterone hormone (T).

Extract preparation

Carob seeds were ground to a fine powder and extracted using cold percolation at room temperature for 72 hours with 60 percent hydro-alcohol combination (50:50 ratio). The extract was filtered and reduced concentration at 50 ˚C using a rotary evaporator. The concentrates were dried in an oven, and the dried extract was stored at −20 ˚C (Mokhtari *et al.* 2012).

Extender preparation

An extender (10 mL) was prepared in autoclaved double distilled water (prepared in a Transgenesis Center of Excellence, Islamic Azad University of Isfahan) containing 0.244 g Tris, 0.136 g citric acid, 0.082 g D-fructose and 20 % v/v egg yolk (Yaniz *et al.* 2011).

Experimental design

In this study, semen samples were collected from each group in December, January, February and March (2021-2022). Three replicates from each experiment were also gathered. Then were added to an extender and freeze using the protocol of Jha *et al.* (2019). The freeze semen straws were analyzed after thawing at 37 ˚C for 30 s.

Sperm parameters were evaluated by the Computer Assisted Semen Analysis (CASA) software, Papanicolaou and Eosin-Nigrosin (E&N) staining was also carried out as pre-freezing. Afterwards, semen samples were freeze and one week later was thawed and sperm parameters were analyzed again.

Blood parameters

A 10-cc blood sample was collected. Immediately after blood collection, test tubes containing blood were placed in a centrifuge at a speed of 3000 rpm for 15 minutes to separate their plasma. Then the extracted plasma was transferred to the laboratory and FBS, Ceratinine, TG, HDL, LDL and testosterone hormone were evaluated with a biochemical kit. In current study, Delta Darman Part kit was used for FBS, Ceratinine, TG analyzed and Demeditec Diagnostics GmbH was used for HDL, LDL and testosterone analyzed.

Sperm motility

Sperm motility was evaluated using the computer automated semen analysis (CASA analyzer, video sperm test 2.1), and an Olympus BX40 microscope under 10× magnifications on a warm stage with 37 ˚C (Kumar *et al.* 2007). The sperm motility parameters were assessed in 10 microscopic fields from each slide which include at least 300 sperm cells as: fast forward moving sperm (A motility), slow forward moving sperm (B motility), not forward moving sperm (C motility), non-moving sperm (D motility), percentage of progressive motility (PM %) and percentage of total motility (TM %).

Hypo-osmotic swelling tests

The hypo-osmotic swelling (HOS) test was performed by a traditional HOS (Jeyendran *et al.* 1984) with minor modifications (Shirazi *et al.* 2006). Physiological serum containing 0.9% sodium chloride was mixed with an equal volume of distilled water until its osmolality reaches 150 mOsm/kg. Then, the prepared sperm was mixed in a 1:2 ratio with the prepared hypo-osmolar liquid at 37 ˚C for 30 min. After incubating at 37 ˚C for 30-60 min, the wet preparation was examined under a phase contrast microscopy (1000 Olympus BX 40, Olympus Optical Co. Ltd, Japan). The ratio of spermatozoa with swollen tails was expressed as a percentage of the total count (mean of 3 replicates). A total of 100 spermatozoa were assessed in each replicate.

Sperm viability

Eosin and Nigrosin (E&N) staining was carried out according to the Kruger *et al.* (1993). Briefly, a mixture of 1% eosin and 10% nigrosin were prepared in distilled water and a 1:2 admixture of semen to the prepared eosin (v/v) was made successively. After 30 seconds, an equal volume of nigrosin was added to this mixture, then thin smears were prepared and observed by the light microscopy at 20X magnification. Viable sperm remained colorless while nonviable sperm stained red.

Statistical analysis

Data analysis was performed using the SPSS ver. 21 software package (SPSS, 2010). Statistical analysis was carried out using the ANOVA procedure and the mean comparison test was performed by the LSD test. A P-value < 0.05 was considered as statistically significance.

RESULTS AND DISCUSSION

The effect of using carob seed extract on blood parameters of rams was also investigated in this study (Table 2). The concentrations of FBS, CH, TG, sCr and LDL were significantly decreased by using carob extract in the diet of ram, while HDL and testosterone significantly increased during the experiments.

Additionally, a significant difference was observed regarding FBS between the treatment and control groups ($P<0.05$). There was a statistically significant difference in the CH level between the treatment and control groups ($P<0.05$). Also, there was a statistically significant difference in the TG level between the treatment and control groups ($P<0.05$). No significant difference was also found regarding sCr (mg/dL) between the treatment and control groups. The HDL level was significantly higher in the treatment group (carob 300 mg/kg) compared to the other treatment and control groups; whereas the LDL level was significantly lower in the treatment group (carob 300 mg/kg) compared to the other treatment and control groups. Using carob seed extract in the diet of rams showed a significant increase in the testosterone (ng/mL) level in both of the treatment groups (carob 150 and 300 mg/kg).

In this study, the effect of adding hydro-alcoholic extract of Carob (*Ceratonia siliqua*) seed to the diet was evaluated on sperm motility, and viability and the HOS test of semen samples in both pre-freezing and post-thawing stages (Table 1, Figure 1).

Total motility in the treatment groups (diet with 150 and 300 mg/kg carob) was significantly higher than that of the control group ($P<0.05$) in both pre-freezing and post-thawing evaluations. Furthermore, no statistically significant difference was found regarding the total motility in the beginning of the experiment (December) in both control

and treatment groups in pre-freezing and post-thawing evaluations. In the current study, the total motility percentages in all treatment groups (diet with 150 mg carob and 300 mg carob) were significantly increased compared to the control group ($P<0.05$, Table 1). Total motility was significantly increased ($P<0.05$) in the treatment group with 300 mg/kg carob compared to the treatment group with 150 mg/kg carob. Overall, as shown in Table 1, the total motility improved by increasing the dose of the carob extract in diet.

As shown in Table 1, all of the treatment groups either received 150 mg/kg carob or 300 mg/kg carob in their diet had a significantly higher viability rate compared to the control group. Treatment groups had no significant difference compared to the control group at the beginning of the diet. However, after one month of the carob diet, the highest sperm viability was observed in pre-freezing stage at the beginning of the experiment (December) followed the second month of usage (January). Moreover, the sperm viability rate showed no significant difference between the treatment and control groups in post-thawing evaluation, while it had significantly increased in the last two months of the experiment (February and March).

HOS test values of spermatozoa in the different treatment groups (150 mg/kg and 300 mg/kg carob in diet) are given in Table 1. The HOS test values from pre-freezing and post-thawing evaluation were significantly higher in the treatment groups compared to the control group. The highest values of the HOS test response were found in the treatment group (300 mg/kg carob) which showed significant difference ($P<0.05$). The HOS test values significantly increased at the end of the experiment in the treatment groups (150 mg/kg and 300 mg/kg carob).

Blood parameters were significantly improved in the treatment groups compared to the control group. Recent studies found that carob has a significant and positive impact on blood parameters of animal (El-Manfalotyand Ali, 2014). The similar results were observed by Macho-Gonzalez *et al.* (2019) and Rtibi *et al.* (2021). Carob extract in the diet significantly reduced the levels of FBS, TG and CH, which could be caused by pathways of hepatic metabolism directly.

Furthermore, the results of the present study were similar to the results of a study conducted by Jaffari *et al.* (2020) which showed that carob significantly lowers TG, CH, LDL, and increases HDL levels.

Carob, bind bile salts and acids inside the intestinal lumen consequently lead to decreased absorption of cholesterol and fatty acids (El-Manfalotyand Ali, 2014). Furthermore, some flavonoids, especially proanthocyanidins can increase the insulin levels which due to acting on hepatocytes can lead to a higher LDL-receptor density on the cell surface (Macho-González *et al.* 2019). The low levels of cholesterol, TG and LDL in this study may be explained by above-mentioned reasons and can affect the hepatic metabolism directly. Further research needs to be conducted to evaluate these impacts.

The results of the present study showed that the extract of carob seeds increased the level of testosterone hormone; this could be due to the positive effect of the carob seed hydro-alcoholic extract on the hormones of the pituitary-testicular axis and improvement of sperm production. The results of present study are in agreement with the results of Tabatabaee (2011) study.

According to the Patel *et al.* (2018), sperm total motility percentages are the main criteria for selecting sperm for fertility analysis in both pre-freezing and post-thawing stages. Total motility of sperm was significantly increased during the pre-freezing than post-thawing evaluations after using carob extract in diet for a period of four months. Usage of carob extract showed that this plant has a positive effect on sperm motility of Lori-Bakhtiari rams. Recent studies have been shown that the carob seed extract contains high antioxidants and phenolic compounds and thus it has free radical scavenger activity. This extract is capable of protecting proteins and enzymes against free radical attack, or oxidation. Furthermore, antioxidant property of that leads to an increase in the lipids of the sperm membrane, the sperm resists the freezing-thawing process and preserves parameters related to sperm fertility (Mokhtari *et al.* 2012). Therefore, these results can be a good reason for increased total sperm motility in the treatment groups. The results of similar studies revealed that carob extract plays an important role in semen quality recovery with regard to sperm concentration (Ebuehi and Akande, 2009; Pirestani and Ziya Motalebipour, 2022). These findings are in agreement with the results of the present study in which total motility of sperm increased in the treatment groups.

In this study, viability was significantly higher in the treatment groups compared to the control group. The anti-inflammatory and antioxidative effects of carob may have an important role in semen quality, which could improve the shape and concentration of spermatozoa.

In addition, the level of viability of sperms in pre-freezing and post-thawing can be due to cold shock in the plasma membrane of sperm which were improved by using carob in diet and plasma membranes were protected in the freezing-thawing process (Barbas and Mascarenhas, 2009).

In the pre-freezing and post-thawing samples, the spermatozoa of the treatment groups showed a higher increase in their response to hypo-osmotic solution compared to the control group.

Table 1 Comparison of total motility, viability and the Hypo-osmotic swelling test (HOST) of semen samples in both pre-freezing and post-thawing stages

Item		Total motility			Viability			Hos test		
	Collection time	Control	Carob (150 mg/kg)	Carob (300 mg/kg)	Control	Carob (150 mg/kg)	Carob (300 mg/kg)	Control	Carob (150 mg/kg)	Carob (300 mg/kg)
Pre- freezing	December	52.0±2.5	54.4±2.8	67.4±1.7	37.8±1.8	42.3±2.7	48.6±1.4	38.6±1.6	48.3±1.4	53.4±1.3*
	January	48.0±2.3	76.4±1.6*	79.6±1.4*	34.4±1.5	50.2±1.8*	49.3±2.2*	36.2±1.2	48.4±1.7	65.6±1.8*
	February	44.0±1.8	88.9±3.1*	88.2±1.9*	33.6±1.8	45.3±2.1	56.8±1.6*	32.1±1.7	44.2±1.6	58.9±2.1*
	March	48.0± 1.8	98.7±3.5*	98.8±2.8*	38.2±1.5	52.6±1.6*	62.7±1.8*	38.4±1.5	52.0±1.6*	67.3±1.5*
Post-thawing	December	50.6± 1.5	48.78±1.3	68.9±1.7*	41.6±1.3	43.7±1.8	46.3±1.9	45.3±1.3	56.7±1.8*	64.2±2.4*
	January	41.6±1.7	47.9±1.2	56.3±1.8*	44.17±1.6	45.7±1.7	47.7±2.1	43.2±1.9	45.7±2.1	48.8±2.2
	February	39.6±1.8	56.4±1.6*	65.4±2.1*	36.2±1.8	38.6±2.1	48.6±1.9	38.2±2.2	48.8±1.9	56.6±1.3*
	March	48.7±1.7	69.8±1.6*	78.1±2.3*	35.8±2.4	48.7±1.9*	46.9±2.2	36.4±1.7	57±2.3*	56.8±1.7*

* (P<0.05).

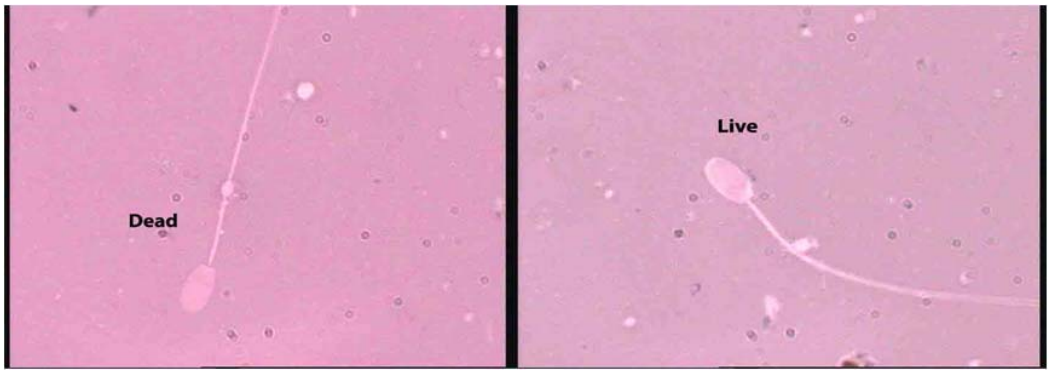

Figure 1 Ram sperm viability by eosin and nigrosin (E&N) staining

Table 1 Blood parameter in different experimental groups

No	Blood parameters	Collection time	Control	Carob (150 mg/kg)	Carob (300 mg/kg)
1	FBS (g/dL)[1]	December	76±1.9	68±1.8*	66±1.8
		January	70±1.7	70±2.2	63±1.9*
		February	76±2.1	65±1.9*	72±1.6
		March	71±1.9	58±1.9*	59±1.8*
2	CH (mg/dL)[2]	December	48±1.6	45±1.6	44±1.3
		January	48±1.7	44±1.8	44±1.9
		February	50±2.1	45±1.7	43±1.7
		March	49±1.3	43±1.6*	42±1.5*
3	TG (mg/L)[3]	December	20±1.6	14±1.7*	15±1.3*
		January	23±1.8	13±1.8*	13±2.3*
		February	30±2.1	26±1.9	18±2.1*
		March	25±1.3	19±1.9*	20±1.9*
4	sCr (mg/dL)[4]	December	1.46±1.6	1.30±1.8	1.36±1.8
		January	1.58±1.6	1.58±1.4	1.34±1.7
		February	1.34±1.9	1.37±2.0	1.33±1.6
		March	1.30±1.7	1.30±1.9	1.33±1.9
5	HDL (mg/L)[5]	December	31±1.7	28±2.0	38±1.8
		January	28±1.8	29±2.4	28±1.7
		February	21±1.9	25±2.1	25±2.1
		March	20±1.9	29±1.8*	32±2.2*
6	LDL (mg/L)[6]	December	42±1.6	30±1.7*	26±2.2*
		January	29±1.5	22±1.6*	18±2.3*
		February	22±1.8	16±1.9*	16±1.8*
		March	27±1.9	25±2.1	21±1.9*
7	T (ng/mL)[7]	December	16±1.6	21±1.6*	25±1.9*
		January	19.0. ±1.5	18.0±1.8	19±1.8
		February	15±1.4	18±1.7	20±2.1*
		March	14±1.3	26±1.6*	33±1.9*

FBS: fasting blood sugar; TAG: triglyceride; CH: cholesterol; sCr: serum creatinine; HDL: high-density lipoprotein; LDL: low-density lipoprotein and T: testosterone.

The results evinced that the carob extract in the ram diet brought about a significant increase in the viability and motility of spermatozoa. HOS test is a good observation of the integrity of the spermatozoa membrane and observing individual normal and abnormal sperms (Prochowska *et al.* 2022). Thus, increase of the HOS test response indicates increase of membrane integrity. This is in line with the results of the present study in which the carob extract in the ram diet makes the spermatozoa membrane more resistant to cryodamage.

A substantial reduction in the HOS test response was seen in the cryopreserved semen in comparison to the pre-freeze semen. This can be attributed to the mechanical damages, which increase the membrane permeability and subsequent loss of plasma integrity associated with osmotic and chemical changes (Harshan *et al.* 2006).

CONCLUSION

According to our results, the increased mobility and viability of sperm were observed by adding carob seed extract in diet of the treated groups. The treatment group of 300 mg/kg carob extract showed a high level of motility parameters in pre-freezing and post-thawing processes. The findings this study indicated that blood parameters were significantly improved in the treatment groups during the experiment. Carob is a type of bile salt and binds fatty acids and cholesterol in the intestines to reduce their absorption. Carob binds bile salts and acids inside the intestinal lumen. This leads to decreased absorption of cholesterol and fatty acids. In addition, due to the antioxidant property of carob, it leads to an increase in the lipids of the sperm membrane, and as a result, the resistance of the sperm to the freezing-thawing process and maintaining the parameters related to sperm fertility.

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