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Effects of dietary basil (*Ocimum basilicum*) supplementation on reproductive hormones, semen parameters, and testicular development in Zandi male lambs

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ABSTRACT

This study investigated the potential impact of feeding whole plant basil on sperm quality and the concentration of certain reproductive hormones in male lambs. A total of 18 Zandi male lambs with an initial weight of 28.8 ± 2.03 kg were included in a completely randomized design with three treatments and six repetitions. The experimental treatments included: 1) control (basal diet without basil), 2) diet containing 12.5 % basil, and 3) diet containing 25 % basil. The results showed that feeding basil to male lambs significantly increased testosterone concentration and decreased blood cortisol levels ($P < 0.05$). Additionally, feeding high levels of basil significantly improved sperm concentration, motility, and viability in the experimental samples, while reducing the level of complete abnormalities and malondialdehyde concentration ($P < 0.05$). The findings suggest that dietary supplementation of 25 % whole plant basil could be a useful strategy to improve sperm quality and increase testosterone secretion while reducing cortisol levels in male lambs.

1. Introduction

The use of medicinal plants in livestock feed has attracted the attention of researchers due to their strong antimicrobial and antioxidant properties and significant nutritional content of minerals, phenols, and carotenoids (Azzaz et al., 2016; Özcan et al., 2005; Suhaj, 2006). These by-products can be used as feed for farm animals, especially since the prices of common feeds such as alfalfa and wheat straw are constantly increasing. The improvement of digestibility and nutritional value of aromatic plants (such as basil) may be due to increased digestion and metabolism activity of rumen microbiome as a direct effect of active compounds (phenols and carotenoids) of basil by-products (Calsamiglia et al., 2007). Additionally, the strong antimicrobial and antioxidant properties of basil may play an important role in inhibiting the growth of rumen methane-producing bacteria (Archaea) and consequently increase digestion efficiency and reduce energy loss in feed for greater production (Patra & Saxena, 2010). Therefore, dietary manipulation by including stress-relieving compounds (such as enzymes, minerals, vitamins, and active medicinal compounds with antioxidant properties) is recommended under high metabolic pressure conditions such as environmental stresses like heat, or high production and growth conditions (Chauhan et al., 2014; Ramnath et al., 2008). For example, antioxidants like vitamin E and selenium decrease oxidative stress and improve animal health (Chauhan et al., 2014). There are also criticisms about the use of chemicals and artificial feed additives due to high cost, consumption restrictions, and especially the presence of residual effects (Jahejo et al., 2019).

Basil (*Ocimum basilicum*) is rich in essential oils such as eugenol, carvacrol, linalool, and caryophyllene, as well as terpenes such as ursolic acid and flavonoids, and contains antioxidant enzymes (catalase, guaiacol peroxidase, ascorbate peroxidase, superoxide dismutase, polyphenol oxidase) (Ramnath et al., 2008). Oxidative stress plays an important role in impaired testicular function and testosterone reduction (Chauhan et al., 2014) and causes testicular destruction and weak sperm production (Hamden et al., 2008). Specifically, there is limited research on the effects of whole plant basil or basil extract on reproductive parameters in rams. Previous studies have explored basil leaf extract in mice (Zaini et al., 2020), finding improvements in sperm count, motility, and morphology. However, the potential for dietary basil foliage to benefit ram fertility has not been investigated.

We hypothesized that the array of bioactive compounds in basil would enhance testicular development, reproductive hormone levels, semen quality, and antioxidant status in male lambs. The innovation of using the entire basil plant, allows for synergies between the wide range

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of phytochemicals and nutrients to optimize sperm parameters and male fertility.

2. Material and methods

2.1. Chemicals and animal ethics

Unless otherwise indicated, all of the chemicals utilized in the study were obtained from the Merck company located in Darmstadt, Germany. Additionally, the Animal Research Committee at the University of Tehran granted permission for the animal study to be conducted under approval number UT-1399.

2.2. Animal management

In this experiment, 18 male lambs of Zandi breed, 6 months of age with an initial weight of 28.8 ± 2.03 kg were used in a completely randomized design with 6 repetitions. The lambs were kept in individual cages during the experiment period. The experiment started with an adaption period (14 day) which was followed by a 70-day period. Before starting the experiment, they were treated with antiparasitic drug, niclosamide. The experimental diets consisted of 1) control (basal diet without basil plant), 2) diet containing 12.5 % basil plant, and 3) diet containing 25 % basil plant (Table 1). Diets were fed as total mixed rations (TMR) and formulated according to NRC (2007) recommendations for growing lambs. The experimental diets were offered twice (08:00 and 16:00 h) daily for ad libitum intake. The lambs had continuous access to clean and fresh water. All lambs were weighed once a week throughout the experimental period.

Quantities of feed offered and refused were recorded daily for each lamb throughout the experiment to calculate feed intake for each animal. Fresh samples of TMR were collected weekly and immediately stored at −20 °C for future analyses. Feed samples were weighted, oven dried at 65 °C for 48 h to measure DM content, and then were ground in a Wiley mill (standard model 4; Arthur H.Thomas Co., Philadelphia, PA, USA) to pass 1-mm screen. The dietary contents of crude protein (CP), Calcium and phosphorus were determined using AOAC methods. The neutral detergent fiber (NDF) contents were analyzed by the Fibertec System (1010 Heat Extractor, Tecator, Sweden) according to Van Soest et al. (1991).

Blood samples were collected on days 0, 30, and 70 of the feeding trial via jugular venipuncture into vacutainer tubes containing EDTA. Samples were kept on ice and centrifuged at 2000 x g for 10 min within 30 min of collection to separate the plasma. The plasma was transferred to microcentrifuge tubes and stored at −20 °C until radioimmunoassay analysis. Plasma testosterone and cortisol concentrations were measured by commercially available radioimmunoassay (RIA) kits. To account for physiological fluctuations, blood sampling was conducted in the morning before feeding at the same time of day. The radioimmunoassay procedures were performed according to the manufacturer's protocol. Testicular length and breadth were assessed on day 70 of the feeding trial using a Vernier's caliper. After 70 days of feeding, 5 lambs from each group were randomly selected for slaughter and testicular analysis. The selection of 5 of the 6 animals allowed for testicular measurements and sperm parameters to be assessed while retaining one animal per treatment for additional trials. The remaining lambs were maintained on their assigned diets for possible long-term reproductive evaluations. After weighing, testes were transferred to the laboratory under refrigerated conditions. The method used for isolating sperm from the cauda segments of the epididymis was the one reported by Merati et al. (2018). Sperm were separated by suspending pieces of the cauda epididymis in a tris solution. The samples were then subjected to centrifugation at 700 g for 10 min to separate the sperm from tissue residues. Next, the sperm suspension was placed in fresh tris solution (The basic extender was composed of comprised 2.71 g Tris, 1. 4 g citric acid, and 1 g fructose in 100 ml distilled water (Mehdipour et al., 2016)) for 15 min for washing. In the end, the samples were prepared for subsequent tests. Sperm concentration was assessed using a Neubauer improved brightline hemocytometer (Smith & Mayer, 1955) (Paul Marienfeld GmbH & Co. KG, Germany). Briefly, semen samples were diluted 1:200 in saline solution. Ten microliters of the diluted semen were loaded into each chamber of the hemocytometer. Technical duplicates were performed for each experimental sample and averaged to obtain the final sperm concentration.

2.3. Radioimmunoassay analysis

All collected blood samples were analyzed for testosterone and cortisol concentrations using commercially available radioimmunoassay (RIA) kits with a gamma counter (2470 WIZARD2 Automatic Gamma Counter, PerkinElmer). Testosterone levels were measured using a validated RIA kit (Institute of Isotopes, Budapest, Hungary) (REF: RK-61M, LOT: 10416AB) and cortisol was assayed using a separate validated kit (REF: RK-240CT, LOT: 10425C). The RIA procedures were performed according to the manufacturer's instructions. Briefly, standards, controls, and treatments samples were incubated in antibody-coated tubes with labeled antigens for 2 h at 37 °C. After decanting the supernatant, tubes were counted for 1 min in the gamma counter. The average counts per minute (CPM) of the duplicates was used to calculate the hormone concentration based on the standard curve generated for each assay. The intra-assay coefficients of variation for the testosterone and cortisol RIAs were 9.28 % and 8.38 %, respectively. The inter-assay coefficients of variation were 10.68 % for the testosterone assay and 13.65 % for the cortisol assay.

2.4. Microscopic evaluation of sperm

To assess sperm motility, a 5 μL drop of diluted semen was placed on a slide at 37 °C and observed under a light microscope at 100× magnification. Individual fields sperm cells were categorized as progressive motile, non-progressive motile, or immotile. At least 200 sperm were categorized per sample. For the assessment of irregularities in sperm morphology, the sperm samples were initially fixed in Hancock solution, following the method described by Mehdipour et al. (2022).

Table 1
Ingredients and chemical composition of experimental diets.

Ingredients (percentage of dry matter)	Levels of basil			Chemical composition	Levels of basil		
	0	12.5	25		0	12.5	25
Barley	43	43	43	Crude protein	12.34	12.20	12.05
Basil	0	12.5	25	Metabolizable energy	2.69	2.68	2.67
Alfalfa	25	20	15	Neutral detergent fiber	38.4	37.5	36.6
Wheat straw	15	7.5	0	Calcium	0.73	0.62	0.52
Wheat bran	16	16	16	Phosphorus	0.45	0.47	0.49
Mineral vitamin premix	0.3	0.3	0.3				
Salt	0.2	0.2	0.2				
Dicalcium phosphate	0.5	0.5	0.5				

Levels of basil, ingredients and chemical compositions of experimental diets are expressed as percentage.

The Hancock solution consisted of 426 mM sodium, 21.4 mM formalin (37 %), 304.29 mM Na_2HPO_4, and 99.42 mM K_2HPO_4. Fifteen microliters of the sperm sample were mixed with 300 mL of the Hancock solution. A drop of this mixture was examined under a phase-contrast microscope (Labomed LX400; Labomed Inc., Culver City, CA) using a slide and cover slip, with a magnification of ×400. At least 200 sperm were evaluated, and the number of irregular sperm was recorded. To assess the membrane function, the hypo-osmotic swelling (HOS) test was selected, according to Abdalkarim Salih et al. (2021). Ten microliters of the sperm sample were combined with 100 microliters of hypo-osmotic solution (100 mOsm/kg; 1.9 mM sodium citrate, 5.0 mM fructose) and incubated at 37 °C for 30 min. A drop of this mixture was observed under a phase-contrast microscope (Labomed LX400; Labomed Inc., Culver City, CA) using a slide and cover slip, with a magnification of 400. Two hundred sperm were counted in 5 microscopic fields. To record the membrane function of the cells, the percentage of sperm with intact tails and coiled tails was defined as the sperm with active plasma membrane.

2.5. Malondialdehyde (MDA) assessment

The concentration of MDA in sperm samples was determined as an indicator of lipid peroxidation using the thiobarbituric acid (TBA) reaction, following the method described Mehdipour et al. (2020). To carry out the assay, 1 mL of sperm and its diluent were mixed with 1 mL of 20 % (w/v) trichloroacetic acid to precipitate the protein content. The mixture was then subjected to centrifugation at 960 g for 15 min, and 1 mL of the supernatant was mixed with 1 mL of 0.67 % (w/v) TBA in a boiling water bath at 100 °C for 10 min. After cooling, the absorbance of the samples was measured at 532 nm using a spectrophotometer (T80 UV/VIS PJ Instruments Ltd., UK). The levels of MDA were expressed in nmol/mL.

2.6. Statistical analysis

The data were analyzed Mixed procedure of SAS version 9.4. The model included treatment as the fixed effect and animal as the random term:

$$Y_{ij} = \mu + T_i + A_j + \varepsilon_{ij}$$

where Y_{ij} is the observed response, μ is the overall mean, T_i is the fixed effect of dietary treatment ($i = 1$–3), A_j is the random effect of animal, and ε_{ij} is the residual error term. When significant treatment effects were detected at $P < 0.05$, means separations were conducted using Tukey's HSD multiple comparison test. Data in figures with labeling of statistical significance reflect the results of the Tukey tests indicating differences.

3. Results

Dry matter intake (DMI) was 1477, 1505 and 1611 g/d for control, diet containing 12.5 % and 25 % basil plant, respectively. The lambs fed the diet containing 25 % basil plant had the highest DMI compared to other lambs. This may be related to the effect of basil's effective compounds which have a stimulating effect on appetite, fermentation and digestion in the rumen. The average daily gain for groups receiving 12.5 % (170.2 g/d) and 25 % (211.9 g/d) basil plant was significantly higher than control group (164.3; $P < 0.01$), which might be related to the improvement of rumen fermentation parameters and nutrient digestibility in the digestive system.

As evidenced in Fig. 1, increasing basil supplementation led to markedly elevated blood testosterone concentrations, with the 25 % basil diet eliciting a 90.5 % increase compared to the control ($P < 0.05$). Concurrently, a dramatic reduction in circulating cortisol was observed as basil inclusion rate augmented, with the 25 % group exhibiting a 94.9 % lower cortisol compared to 0 % basil ($P < 0.05$; Fig. 2). It was shown that MDA was significantly lowest in 12.5 % basil level group compared with the other groups ($P < 0.05$; Fig. 3).

Examination of semen attributes (Table 2) revealed beneficial effects of dietary basil on sperm concentration, motility, viability, membrane integrity, and morphology. The 25 % basil group showed the optimal values for total motility, progressive motility, viability, and membrane integrity. Sperm concentration and normal morphology were also superior in the 25 %. The intermediate 12.5 % basil level generated semen metrics between the highest and lowest groups. These results signify that escalating dietary basil intake enhances semen quantity.

4. Discussion

This study provides novel evidence that feeding whole basil plant, can beneficially modulate reproductive hormone levels and improve sperm quality in male lambs. The use of the complete basil plant as a natural feed additive represents an innovative approach to enhancing testosterone, sperm parameters, and testicular development. While previous research has explored individual compounds from basil, this study uniquely demonstrates that incorporating the entire basil plant into the diet can promote reproductive health in livestock. The using of the whole basil plant provides a rich array of bioactive compounds and

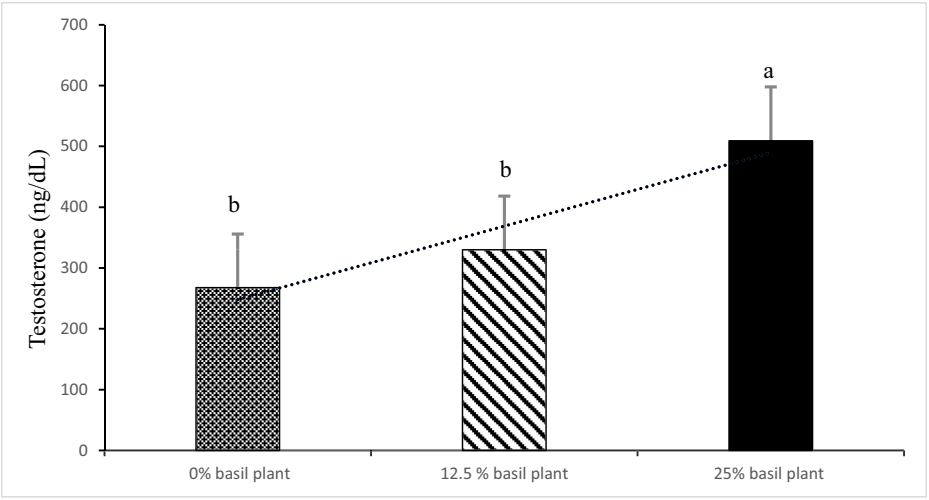

Fig. 1. Effect of feeding different levels of whole basil plant on testosterone concentrations in experimental lambs. Different letters indicate that treatments differ $P < 0.05$.

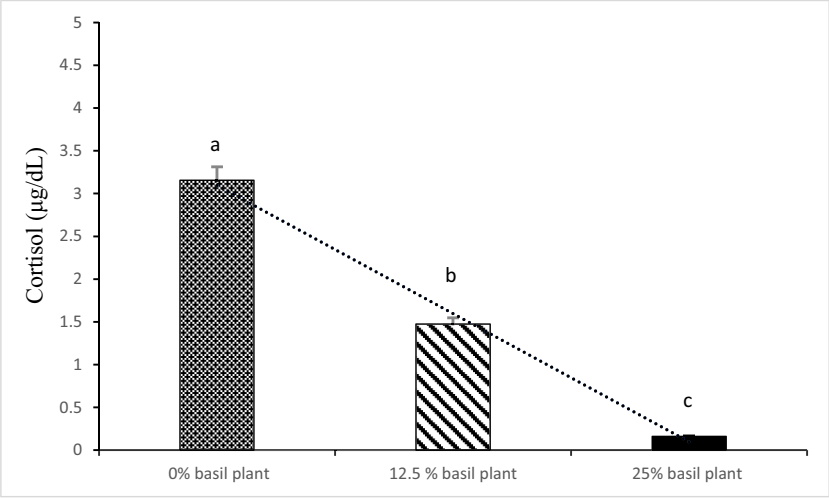

Fig. 2. Effect of feeding different levels of whole basil plant on cortisol concentrations in experimental lambs. Different letters indicate that treatments differ $P < 0.05$.

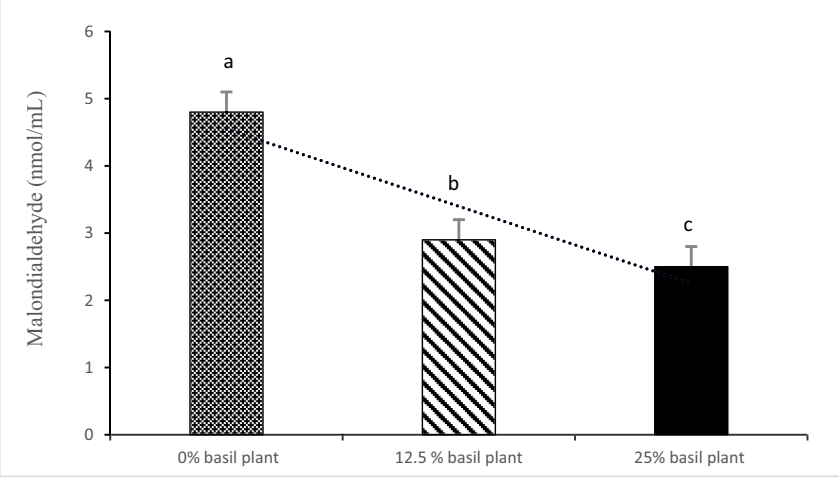

Fig. 3. Effect of feeding different levels of whole basil plant on Malondialdehyde in experimental lambs. Different letters indicate that treatments differ $P < 0.05$.

Table 2
Effect of feeding different levels of whole basil plant on sperm characteristics in experimental lambs.

Parameter	Levels of basil		
	0	12.5	25
Sperm concentration ($\times 10^9$ cells/mL)	2.1 ± 0.05^b	2.6 ± 0.06^a	2.7 ± 0.08^a
Total motility (%)	70.2 ± 1.98^b	79.2 ± 2.22^a	81.4 ± 2.01^a
Progressive motility (%)	31.8 ± 2.11^b	36.8 ± 2.01^{ab}	40.6 ± 2.06^a
Viability (%)	71.6 ± 2.31^b	81.4 ± 2.72^a	83.4 ± 2.24^a
Membrane integrity (%)	68.6 ± 2.16^b	76.5 ± 2.20^{ab}	79.6 ± 1.99^a
Total abnormality (%)	12.1 ± 0.25^a	10.7 ± 0.59^a	8.5 ± 0.72^b
Testicular length (cm)	10.3 ± 0.29	11.1 ± 0.48	11.3 ± 0.37
Testicular width (cm)	5.1 ± 0.19	5.5 ± 0.26	5.8 ± 0.15

Different letters indicate that treatments differ $P < 0.05$.

nutrients that may work synergistically to optimize male fertility. There is limited research on the effects of whole plant basil or basil extract on male reproductive health specifically. However, there have been some studies on the effects of other plant extracts on ram sperm quality and reproductive function, which may provide some insight into the potential effects of basil extract. In a study conducted by Golandam et al. (2020), the impact of a garlic extract supplement on the quality of ram sperm was examined. The findings of the study revealed a significant improvement in sperm count, motility, and morphology due to the garlic extract supplementation. In agreement with our study, Zaini et al. (2020), performed a study to investigate the effect of basil leaf extract on sperm quality in mice exposed to cigarette smoke. They indicated that the consumption of basil leaf extract improved sperm quality and reduced the number of abnormal and individual sperm in mice exposed to cigarette smoke. Thus, the study suggests that basil leaf extract may serve as a valuable herbal extract to enhance sperm quality.

Similar results were obtained in a study where a 14-day administration of 500 mg/kg of rosemary extract significantly increased body weight, testicular weight, serum testosterone levels, and antioxidant enzyme levels while decreasing MDA levels in testicular tissue in desert rats (Honari & Pouraboli, 2019). Researchers have shown that phenolic acids such as rosmarinic acid improve serum testosterone levels and sexual behaviors in male rats (Farzadi et al., 2011). Furthermore, caffeic acid has antioxidant properties (Fukumoto & Mazza, 2000) and can protect against testicular toxicity and oxidative stress-related male reproductive impairments (Akyol et al., 2015). Therefore, the observed

improvement in testicular weight in this study could be attributed to improved antioxidant status in the spermatogenic tubules and Leydig cells in the testicular tissue due to the extract's antioxidant content in basil. Basil, due to its phenolic and flavonoid compounds that are powerful natural antioxidants, likely prevents testicular tissue atrophy and improves testosterone secretion from Leydig cells by inhibiting oxidative stress and preserving the function of antioxidant enzymes. On the other hand, it has been shown that an increase in cortisol levels can directly reduce testosterone secretion by affecting the hypothalamic-pituitary-gonadal axis. Therefore, the positive effect of basil consumption on reducing cortisol levels may have led to improved testosterone secretion in this experiment (Mukherjee et al., 2018). The dose-dependent hormonal responses indicate that whole basil intake boosts androgenic status while mitigating stress in lambs.

Basil, as an adaptogenic or anti-stress plant, balances various processes in the body and improves response time and reduces anxiety, thus improving performance (Jothie Richard et al., 2016). These results signify that escalating dietary basil intake enhances semen quantity. Plant essential oils, in various concentrations, can also act as potent ROS inhibitors. This is important because sperm have limited inherent antioxidant defense mechanisms and are highly susceptible to oxidative stress (Aitken et al., 2016). It has been reported that adding plant polyphenols to sperm media has beneficial antioxidant effects on sperm quality (Gibb et al., 2013). Najafi et al. (2020), demonstrated that the polyphenol quercetin at 50 mM can improve sperm survival and motility and decrease MDA levels. In the present experiment, dietary supplementation of 25 % basil resulted in a decrease in MDA levels, which can reduce lipid peroxidation of sperm membrane and improve its integrity by reducing intracellular H_2O_2 concentration. Malondialdehyde, by inducing lipid peroxidation in the plasma membrane of sperm, leads to a decrease in sperm membrane integrity (Ball, 2008). It has been shown that sperm membrane integrity is directly related to sperm motility (Mehdipour et al., 2017). Therefore, improving antioxidant indexes in this experiment may improve other sperm quality parameters such as motility, viability, and morphology. Our findings suggest that using basil can lower cortisol levels, which may be connected to sperm quality. Additionally, the basil flower was found to reduce oxidative stress, indicating that the basil plant can also decrease oxidative stress and enhance sperm quality by reducing stress levels. Consistent with our results, Bhongade et al. (2015), demonstrated that psychological stress can increase cortisol levels, a hormone released by the adrenal glands in response to stress. High cortisol levels can lead to a decrease in testosterone synthesis, which can adversely affect sperm quantity and quality. Stress can also disrupt the hypothalamic-pituitary-gonadal (HPG) axis, which regulates reproductive hormones like follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This delicate hormonal balance can be disturbed by stress, leading to impaired male fertility. Nateghian, Aliabadi, and Aliabadi (2022) has also shown that psychological stress can exacerbate oxidative stress, which is associated with DNA damage in sperm. This can cause a decrease in semen volume, sperm concentration, and sperm motility, making it more difficult for sperm to reach and fertilize the egg.

5. Conclusion

The findings of this study demonstrate that incorporating whole basil plant into the diet can beneficially modulate reproductive hormone levels and improve semen quality in male lambs. Feeding lambs diets containing 12.5 % and 25 % basil significantly increased blood testosterone concentrations and decreased cortisol levels compared to the control. Additionally, supplementation with basil enhanced sperm concentration, motility, viability, and membrane integrity, while reducing sperm abnormalities and oxidative stress. The diet with 25 % basil elicited optimal improvements in sperm parameters.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

CRediT authorship contribution statement

Golnor Kosari: Methodology, Investigation, Data curation, Conceptualization. **Mohammad Ali Norouzian:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Behzad Khorrami:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Abouzar Najafi:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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