Effect of dietary *Satureja khuzistanica* powder on semen characteristics and thiobarbituric acid reactive substances concentration in testicular tissue of Iranian native breeder rooster

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Summary

Because of a paucity of information on the effect of *Satureja khuzistanica* in male chickens, this study was undertaken to determine the influence of dietary *S. khuzistanica* powder (SKP) on seminal characteristics and testes thiobarbituric acid reactive substances (TBARS) content in Iranian native breeder rooster. Thirty-six 40-week-old roosters were randomly allotted to 3 equal groups and received either a basal diet without SKP (T1 or control), or a diet containing 20 g/kg (T2) and 40 g/kg (T3) of SKP for 8-week-long experimental period. Semen samples were obtained weekly by abdominal massage to evaluate the seminal characteristics. At the end of the eighth week 18 birds (6 birds per each group) were randomly slaughtered, and sample was taken from right testes for TBARS evaluation. Administration of SKP improved all semen traits, except for sperm concentration. Likewise, TBARS content in SKP treatments did not significantly differ from the control ($P>0.05$). Seminal volume, live sperm percentage and plasma membrane integrity percentage in SKP-treated groups were higher than the control. Conversely, abnormal sperm percentages reduced in SKP-treated groups ($P<0.05$). Plasma membrane integrity in experimental treatments was significantly higher than the control in 2nd, 3rd and 7th weeks. However, at 6th and 8th weeks only T3 treatment was significantly different from the control. Notably, there was an increase in total sperm concentration in SKP-treated groups in compared to the control birds. In conclusion, this study indicated that addition of SKP in rooster diet improves sperm quality and also reduces their sperm membrane lipid peroxidation, which may lead to higher fertilization rate.

Key words: Breeder rooster, *Satureja khuzistanica* powder, Sperm quality, TBARS concentration

Introduction

Fertility is the first and most important requisite of poultry breeding. The number of fertile eggs produced for hatching dictates the ultimate profitability of hens. Infertility is a major economic loss in poultry industry. Although males and females both contribute to decreasing fertility, however, low fertility is thought to be largely a problem in males because the ratio of males to females in a flock is very low (Lin *et al*., 2005; Ommati *et al*., 2013). The deterioration in fertilizing ability has been attributed to many factors like age, weight and decline in semen quality (Khan, 2011). Plasma membrane lipids play a significant role in sperm fertilizing capacity (Scott, 1973). Avian sperm cell membranes have a much greater concentration of polyunsaturated fatty acids (PUFAs) than mammalian sperm cells and are therefore more susceptible to lipid peroxidation (LPO) in the presence of reactive oxygen species (ROS) (Fujihara and Howarth, 1978). Reactive oxygen species are reactive molecules produced during oxygen reduction that can deteriorate function and viability of the spermatozoa, if produced at higher than optimal concentrations (Aitken *et al*., 1989). Peroxidation of PUFAs in the sperm cell membranes is an autocatalytic, self-propagating reaction, which can cause cell dysfunction associated with the loss of the membrane function and integrity which finally leads to decreased fertilizing ability of spermatozoa (Alvarez and Storey, 1982). The high content of PUFAs makes sperm more susceptible to lipid peroxidation by ROS, associated with male infertility (Cerolini *et al*., 1997). Sperm is low in antioxidant capacity, but enzymatic and nonenzymatic antioxidants in seminal plasma protect sperm by scavenging ROS (Zini *et al*., 2009). It has been suggested that total antioxidant capacity of seminal plasma in infertile men may be lower than in fertile men (Lewis *et al*., 1995). In light of the results of such studies, several approaches have been introduced to enhance semen quality and the anti-oxidative capacity of seminal plasma, including the use of dietary sage extract (Ommati *et al*., 2013), dried tomato pomace (Saemi *et al*., 2012), dried ginger rhizome (Akhlaghi *et al*., 2014a) and dried apple pomace (Akhlaghi *et al*., 2014b). The mint family (Labiates) constitutes a large number of herbs, including rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) as well as *S. khuzistanica* that are known to have potent antioxidant activities (Haeri *et*

al., 2006; Ommati *et al*., 2013). *Satureja khuzistanica* Jamzad is an endemic plant that is widely distributed in the southern part of Iran. The genus *Satureja* belongs to the family Lamiaceae, subfamily Nepetoideae and the tribe Mentheae (Haeri *et al*., 2006). One of the diagnostic characteristics of the subfamily Nepetoideae is that its representatives contain more than 0.5% of essential oil (El-Gazzar and Watson, 1970). This plant has therapeutic value, such as analgestic and antiseptic, antibacterial, antiviral and antifungal properties (Sadeghi-Nejad *et al*., 2010). The main component of essential oil of *S. khuzistanica* is carvacrol. Other constituents identified in this plant are flavones, triterpenoids, steroids and tannins (Farsam *et al*., 2004). Both carvacrol and flavonoids have been found to have antioxidant properties (Vardar-Ünlü *et al*., 2003). Study on the effect of oral administration of essential oil from *S. khuzistanica* on male rat fertility has revealed significant improvement in all parameters of libido such as potency, fecundity, fertility index and litter size. Moreover, concentrations of FSH and testosterone as well as weight of testes, seminal vesicles and ventral prostate were significantly increased. Histopathological analysis showed that increased number of spermatogonia, spermatids, Leydig cells, spermatozoa and hypertrophic Sertoli cells (Haeri *et al*., 2006). As far as we know, the effect of *S. khuzistanica* powder (SKP) on the seminal characteristics in avian species has not been reported previously. Therefore, this study was conducted to evaluate the efficacy of dietary inclusion of SKP on conventional seminal attributes and testes lipid peroxidation in breeder roosters.

Materials and Methods

Birds and diets

Thirty-six 40-week-old Iranian native breeder roosters purchased from Research Center of Isfahan Native Chickens, Isfahan, Iran, were individually caged and randomly allocated to three groups. Each treatment included 3 replicates of 4 birds. Birds received a basal corn-soybean based diet (Table 1) with free access to diet. *Satureja khuzistanica* powder was obtained from Khoraman Daroo Co. (Khorram Abad, Iran). A bulk sample of the obtained essential oils was analyzed using the methods described by Hadian *et al*. (2011) and its composition is presented in Table 2. Proximate analysis of SKP is also shown in Table 3 and was added to the bird diet for 8 weeks. Birds' diet included either no SKP

Table 1: Ingredients and chemical composition of the basal diet fed to breeder roosters

Ingredient	%
Corn	51
Soybean meal	22
Barley	7
Wheat bran	8.79
Mono calcium phosphate	1.1
Calcium carbonate	9
Sodium chloride	0.3
Vitamin premix[A]	0.26
Trace-mineral premix[B]	0.26
DL-Met	0.11
Vitamin D3	0.1
Composition	
ME (kcal/kg)	2500
CP (%)	15
Ca (%)	3.2
P (%)	0.4
Lysine (%)	0.7
Met + cysteine (%)	0.6

[A] Supplied per kg diet: vitamin A, 15000 IU; vitamin E, 30 mg; vitamin K3, 4 mg; vitamin D3, 3000 IU; riboflavin, 7.5 mg; pyridoxine, 5.5 mg; vitamin B12, 25 mg; biotin, 50 mg; niacin, 50 mg; calcium pantothenate, 18 mg, and folic acid, 1.5 mg. [B] Supplied per kg diet: Fe ($FeSO_4.H_2O$), 90 mg; Mn ($MnSO_4.H_2O$), 90 mg; Zn (ZnO), 67.3 mg; Cu ($CuSO_4.5H_2O$), 10.9 mg, and Se (Na_2SeO_3), 0.18 mg

Table 2: The compositions of *Satureja khuzistanica* essential oil

Compound	RI [1]	Composition %	Identification [2]
α-thujene	925	0.24 ± 0.14	RI, MS
α-pinene	933	0.15 ± 0.05	RI, MS, CoI
Myreene	981	0.26 ± 0.19	RI, MS
α-terpinene	1013	0.24 ± 0.12	RI, MS, CoI
p-cymene	1017	1.26 ± 0.86	RI, MS, CoI
Limonene	1026	0.13 ± 0.04	RI, MS, CoI
(Z)-β-oeimene	1036	0.54 ± 0.08	RI, MS
γ-terpenene	1053	0.74 ± 0.23	RI, MS, CoI
trans-sabinene hydrate	1081	0.17 ± 0.02	RI, MS
Terpin-4-ol	1163	tr [3]	RI, MS
α-terpinole	1175	0.42 ± 0.45	RI, MS
Thymol	1266	tr	RI, MS, CoI
Carvacrol	1282	92.16 ± 0.46	RI, MS, CoI
Thymyl acetate	1329	tr	RI, MS
β-caryophyllence	1425	0.16 ± 0.01	RI, MS, CoI
α-humulene	1427	tr	RI, MS
β-bisabolene	1501	tr	RI, MS
Trans-β-bisabolene	1522	0.10 ± 0.01	RI, MS

[1] RI: Retention indices determined relative ton-alkanes (C6-C24) on a DB-5GC column, [2] RI: Retention indices, MS: Mass spectra, CoI: Co-injection, and [3] tr: Trace (<0.05%)

Table 3: Proximate analysis of *Satureja khuzistanica* powder

GE cal/g	ADF %	NDF %	Ca %	CF %	CP %
3866	32-34.5	1.37-2.38	3.8-3.9	10.5-14.5	6.8

(control or T1) or 20 g (T2) and 40 g/kg (T3) of SKP of diet.

Semen evaluation

After a 2-week adaptation period to the basal diet and to abdominal massage for semen collection (Burrows and Quinn, 1937), the birds were subjected to experimental treatments and seminal characteristics were determined weekly for another 8 weeks. Ejaculates obtained from the birds in each replicate (n=4) were pooled and evaluated as a single sample. Seminal volume was measured in graded collecting tubes. Sperm forward motility was assessed by placing a portion of ejaculate diluted with 2.9% sodium citrate solution (1:40) on a slide covered with a cover slip, using an Olympus compound light microscope (Shinjuku, Tokyo), (×400 magnification) after maintaining at 37°C. Sperm live/dead ratio and abnormality were evaluated, using a portion of ejaculate stained with warmed eosin-nigrosin solution. The stained diluted seminal smear was prepared in duplicate, and 200 spermatozoa in each slide were evaluated and unstained spermatozoa were considered as live. Spermatozoa with detached heads, abaxial heads, malformed heads, bent tails, coiled tails, double tails, and protoplasmic droplets were considered as abnormal (Pursel *et al*., 1972). Hypo osmotic swelling test (HOS-test) was used to evaluate functional sperm plasma membrane integrity. Briefly, a microtube containing 30 µL of diluted semen and 50-mOsm NaCl solution (150 µL) was placed in a water bath (39°C) for 10 min and then evaluated using light microscopy (×1,000 magnification). The percentage of spermatozoa with a swollen "bubble" around the curled flagellum (HOS-positive) was determined by counting of 200 cells per slide (Fonseca *et al*., 2005). Sperm concentration was determined in duplicate, using a Neubauer hemocytometer. Total sperm concentration (TSC) was expressed as:

TSC= semen volume × sperm concentration

Total concentrations of live, normal and motile sperm with healthy plasma membrane integrity (TLNMIS) were expressed as:

TLNMIS= TSC × (viability × normality × motility × plasma membrane integrity (HOS+))

TBARS concentration in testes

At the end of eighth week, 18 birds (6 birds per each treatment) were randomly slaughtered, and sample were taken from right testes and stored at -80° (within 30 days) for thiobarbituric acid reactive substances (TBARS) measurement. The amount of lipid peroxidation was indicated by the content of TBARS in the testes. Tissue TBARS was determined by following the production of TBARS as described by Subbarao *et al*. (1990). In short, testes were homogenized manually in cold phosphate buffer (pH = 7.4) and debris removed by centrifugation at 3500 g for 10 min; 40 µL of this homogenate was added to 40 µL of 0.9% NaCl and 40 µL of deionized H_2O, resulting in a total reaction volume of 120 µL. The reaction was incubated at 37°C for 20 min and stopped by the addition of 600 µL of cold 0.8 M hydrochloride acid, containing 12.5% trichloroacetic acid. Following the addition of 780 µL of 1% TBA, the reaction was boiled for 20 min and then cooled at 4°C for 1 h. In order to measure the amount of TBARS produced by the homogenate, the cooled samples were spun at 1,500 × g in a microcentrifuge for 20 min and the absorbance of the supernatant was spectro-photometrically read at 532 nm, using an extinction coefficient of 1.56 × 105/M cm. The blanks for all of the TBARS assays contained an additional 40 µL of 0.9% NaCl instead of homogenate as just described. TBARS results were expressed as nanomoles per milligram of tissue protein (nmol/mg protein). Protein content of tissue homogenates were determined by a colorimetric method of Lowry using bovine serum albumin as standard (Lowry *et al*., 1951).

Statistical analysis

The present study was designed in a completely randomized design. The data were then subjected to ANOVA, using Proc Mixed (SAS, 2002), where the time of semen sampling was included as the random effect. Body weight was included as a covariate for some traits for ANOVA. Data of TBARS content in testes were analyzed using mixed procedures. Mean separation was performed by the Tukey's test, and the level of significance was set at $P<0.05$.

Results

Effects of dietary administration of SKP on seminal characteristics in Iranian native breeder rooster are shown in Table 4. Administration of SKP improved all reproductive traits, except for sperm concentration (Table 4). The results showed that plasma membrane integrity (HOS reactive) is affected by interaction between experimental diets and time (Fig. 1). The effect of SKP on TBARS content in testes of birds has been summarized in Table 4. TBARS content in SKP treatments was not significantly different compared to the control ($P>0.05$). The percentage of sperm forward motility in SKP-treated groups was higher than the control in a dose-dependent manner, in which it was greater in T3 group than the T2 treatment. Seminal volume, live sperm percentage and plasma membrane integrity percentage increased significantly in the SKP-treated groups compared to the control birds. Although the abnormal sperm percentage for SKP-treated groups was lower than the control, however no difference was

Table 4: The effect of dietary administration of *Satureja khuzistanica* powder on seminal characteristics and testes TBARS concentration in Iranian native rooster, (least squares means ± SE)

Trait	Diet [1]			P-value		
	T1	T2	T3	Diet	Time	Diet × time
Seminal volume (ml)	1.6441±0.09935[b]	2.1190±0.08995[a]	2.0369±0.09798[a]	0.0034	NS	NS
Sperm forward motility (%)	75.2083±1.1347[c]	81.6667±1.1347[b]	86.6667±1.1347[a]	<.0001	NS	NS
Live sperm (%)	82.9778±0.8996[b]	87.1484±0.8145[a]	87.9987±0.8873[a]	0.001	0.0003	NS
Abnormal sperm (%)	5.1434±0.2672[a]	3.5974±0.2419[b]	2.8841±0.2635[b]	<.0001	0.0097	NS
Plasma membrane integrity (HOST) [2]	84.3750±0.6627[b]	88.7500±0.6627[a]	90.0417±0.6627[a]	<.0001	<.0001	0.0332
Sperm concentration ($\times 10^9$ cells/ml)	3.2691±0.1277	3.5254±0.1156	3.6971±0.1259	NS	0.0002	NS
TSC ($\times 10^9$ cells) [3]	5.3958±0.4818[b]	7.5658±0.4363[a]	7.6256±0.4752[a]	0.0034	0.0281	NS
TLNMIS ($\times 10^9$ cells) [4]	2.7689±0.2944[b]	4.6876±0.2944[a]	5.1456±0.2944[a]	<.0001	0.0006	NS
Testes TBARS (nmol/mg protein) [5]	7.7257±0.5566	7.2111±0.5566	8.3281±0.5566	NS	-	-

[a, b] and [c] values with different superscripts in each row differ significantly (P<0.05). [1] *Satureja khuzistanica* powder was included at 0 (control or T1), 20 g (T2), or 40 g (T3) per kg diet for 8 weeks, [2] The values represent the percentage of sperm with a swollen bubble around the curled flagellum in a hypoosmotic (HOS) solution (50 mOsm NaCl), [3] Total sperm concentration, [4] Total concentrations of live, normal and motile sperm with healthy plasma membrane integrity, and [5] Thiobarbituric acid reactive species concentration in testes. NS: Not significant

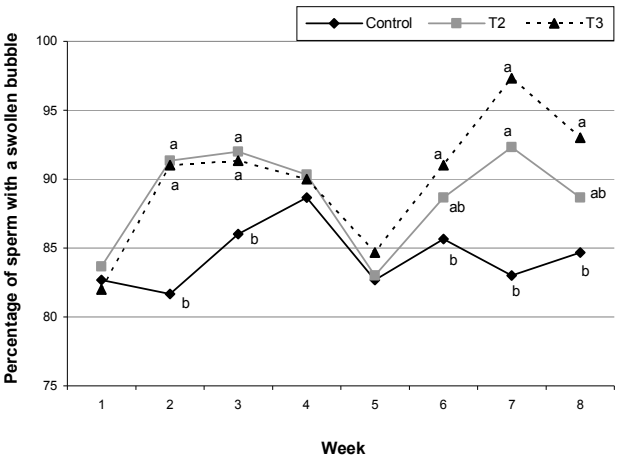

Fig. 1: Effect of diet × time (week) interaction on the percentage of sperm with a swollen bubble around the curled flagellum in a hypotonic solution (HOS) in roosters fed *Satureja khuzistanica* powder (pooled SE = 1.8745). *Satureja khuzistanica* powder was included at 0 (T1 or control), 20 g (T2), or 40 g (T3) per kg diet for 8 weeks. a, b: The means with different superscripts in each row differ significantly (P<0.05). No differences were found at week 1, 4 and 5

observed among groups (P<0.05). Although sperm concentration in T2 and T3 treatments did not show significant difference compared to control (P>0.05), total sperm concentration in SKP-treated groups was greater than the control group. Overall, there was a significant increment in the total concentration of live, normal and motile spermatozoa with healthy plasma membrane integrity in SKP-treated groups compared to the control (P<0.05, Table 4). Plasma membrane integrity percentage in SKP-treated group increased during experimental period time dependently, where plasma membrane integrity in T2 treatment was significantly higher than the control in 2nd, 3rd and 7th weeks. However, at the 6th and 8th weeks only T3 treatment was significantly different from the control (Fig. 1).

Discussion

Many studies of positive effects of herbal antioxidants on the quality of poultry semen are available. Recent studies have demonstrated that herbal antioxidants such as dried tomato pomace (Saemi *et al*., 2012), sage extract (Ommati *et al*., 2013), dried ginger rhizome (Akhlaghi *et al*., 2014a) and dried apple pomace (Akhlaghi *et al*., 2014b) improve semen quality in poultry. Several studies suggest that a complex of natural component such as phenolic compounds may act as antioxidant. *Satureja khuzistanica* contains compounds such as carvacrol and flavonoids, as the major phenolic compounds (Farsam *et al*., 2004). Along with the effectiveness of antioxidants in management of infertility, antioxidant properties of *S. Khuzestanica* have been established which could be proposed as reproduction stimulatory agent (Safarnavadeh and Rastegarpanah, 2010). Thus, we hypothesized that SKP may affect the roosters semen quality due to its possible antioxidant properties. In this regard, Haeri *et al*. (2006) demonstrated that administration of SK essential oil to the diet leads to improvement of reproductive traits, such as an increase in testicular weight, testosterone and FSH concentration in male rats. In our study, sperm motility in SKP-treated birds was higher than the control group. It has been indicated that sperm membranes are rich in PUFAS, which is necessary for sperm motility and fertilization (Zaniboni *et al*., 2006). On the other hand, membrane fluidity is a structural advantage, but causes the sperm to be sensitive to the effects of free radical and lipid peroxidation (Aitken *et al*., 1989; Kelso *et al*., 1996). The high concentration of free radicals induces lipid peroxidation and decreases sperm motility (Baumber *et al*., 2000). It is well-known that in the absence of antioxidant compounds (such as carvacrol), oxidative damage caused by oxygen free radicals and hydrogen peroxide produced in the reaction of xanthine-xanthine oxidase is increased, and therefore results in decreased sperm motility (De Lamirande and Gagnon, 1992). The antioxidant defense system contains enzymatic and non-enzymatic defenses (Zini *et al*., 2009). Basically, phenolic compounds destroy free radicals and probably, carvacrol in SKP acts as a non-enzymatic antioxidant and might protect sperm against oxidative damage. On the other hand, ROS attack PUFAS in sperm membrane and cause oxidative damage, which breaks fatty acid chains and produces

oxidative by-product, this reaction can damage cell membrane and even cause cell death (Reiter *et al*., 1995). Antioxidant compounds have the ability to scavenge free radical and led to increased viability and decreased abnormality in sperm (Saemi *et al*., 2012; Ommati *et al*., 2013). Consistent with this, SKP significantly increased percentage of plasma membrane integrity in T2 and T3 groups compared to control. Peroxidation of poly-unsaturated fatty acids in sperm cell membranes is an autocatalytic, self-propagating reaction, which can give rise to cell dysfunction associated with loss of membrane function and integrity (Sanocka and Kurpisz, 2004). Phenolic compound (such as carvacrol) scavenge free radicals and improve the percentage of functional plasma membrane integrity (as indicated by HOS-test). In our study, the plasma membrane integrity was affected by interaction between experimental diets and time. Considering the duration of the spermatogenic cycle in poultry (Etches, 1996), the differences found in the percentage of plasma membrane integrity (HOS) before 3 weeks, could not be attributed to the treatment effect. Sperm concentration was another measured parameter which was not affected by the experimental diets, but semen volume and total sperm concentration in SKP-treated groups was higher than the control. Possibly, the increase in semen volume in the treatment groups and subsequent increment of seminal plasma may be the reason why sperm concentration was not significantly different from control groups.

TBARS content in testes of birds did not show any difference in comparison with the control. Probably, the lack of any oxidative stress in the testicular tissue in the present work may be the reason why TBARS levels did not exhibit significant differences among groups during the experimental period.

In conclusion, our data demonstrated that SKP feeding improves total concentration of live, normal and motile sperm with healthy plasma membrane integrity (TLNMIS) in Iranian native breeder rooster. In addition, the current study showed a profound enhancement in seminal characteristics in SKP-fed roosters, which might be beneficial to improve fertility rate in parent stock.

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