Immune Response and Pasteurella Resistance in Rabbits Fed Diets Containing Various Amounts of Black Cumin Seeds

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Abstract: Problem statement: The consumption of black cumin (*Nigella sativa*) seed has immunomodulatory and anti-bacterial activity, but in rabbits this had not yet been tested. **Approach:** In the present studies, rabbits were fed diets without or with black cumin seed and antibody production, phagocytotic activity, hypersensitivity and resistance against Pasteurellosis were assessed. **Results:** Feeding black cumin seed significantly increased serum concentrations of antibodies in response to intramusculary injected serum bovine albumin. Blood derived from rabbits fed the diets containing either 15 or 20% black cumin seed significantly reduced the growth of *Staphylococcus aureus* on sheep-blood agar plates. Skin thickness as index of hypersensitivity towards tuberculin was significantly reduced at 48 h after intradermal injection of the agent. Ingestion of black cumin seed significantly extended survival time after intraperitoneal administration of *Pasteurella multocida*. **Conclusion:** The feeding of black cumin seed to rabbits stimulated their immune system, but did not enhance inflammation.

Key words: Rabbits, antibody production, phagocytosis, hypersensitivity, Pasteurellosis, black cumin

INTRODUCTION

The oil fraction of black cumin (*Nigella sativa*) seeds contains thymoquinone, which exerts anti-inflammatory, anti-bacterial and immunomodulatory effects (Swamy and Tan, 2000; Salem, 2005; Ragheb *et al*., 2009). The biological activity of the active principle explains the beneficial effects of black cumin seeds or extracts in the treatment of human patients with allergic diseases (Kalus *et al*., 2003; Işik *et al*., 2010), asthma (Boskabady *et al*., 2007) or tonsillopharyngitis (Dirjomuljono *et al*., 2008). In induced animal models, the administration of thymoquinone was effective against rheumatoid arthritis (Tekeoglu *et al*., 2007) and autoimmune encephalomyelitis (Ozugurlu *et al*., 2005; Mohamed *et al*., 2009). Studies with rats and mice have demonstrated that extracts of black cumin seed have anti-bacterial (Hanafy and Hatem, 1991), anti-viral (Salem and Hossain, 2000) and anti-parasite activity (Mahmoud *et al*., 2002; Abu El Ezz, 2005; Ayaz *et al*., 2007).

The influence of dietary black cumin seed on clinical laboratory serum values in rabbits has been described (El Bagir *et al*., 2010). However, the effects of black cumin seed on immune response and anti-bacterial activity have not yet been investigated in rabbits. Such studies may be considered relevant because rabbits can be laboratory, companion or production animals. In this study, we have determined the effect of feeding black cumin seed on antibody production, phagocytotic activity and hypersensitivity in rabbits. The influence on resistance against Pasteurellosis has also been assessed. This is especially relevant from a practical point of view as respiratory disease caused by *Pasteurella multocida* is responsible for decreased productivity and mortality of does.

MATERIALS AND METHODS

Animals and diets: Male and female rabbits, aged 3-4 months and weighing 1.1-1.2 kg were used throughout. All rabbits were apparently healthy. On arrival, they were housed in ground pens located in a shed with adequate ventilation and lighting. During the adaptation period of 2 weeks, the rabbits were fed lucerne and crushed sorghum.

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Table 1: Ingredient and calculated macronutrient composition of the experimental diets

	Dietary black cumin						
	Experiments 1, 2, 3				Experiment 4		
	0%	10%	15%	20%	0%	2%	9%
Ingredient, g/1000 g							
Lucerne	600.0	540.0	510.0	480.0	667.0	653.6	606.1
Sorghum	400.0	360.0	340.0	320.0	333.0	326.8	303.0
Black cumin seed	0.0	100.0	150.0	200.0	0.0	19.6	90.9
Macronutrient, g/100 g							
Crude protein	7.0	8.4	9.1	9.8	6.7	7.0	8.0
Crude fat	1.5	4.9	6.6	8.3	1.4	2.0	4.5
Crude fiber	6.0	6.0	5.9	5.9	6.4	6.4	6.3
Ash	2.8	2.9	2.9	3.0	2.9	2.9	3.0
Moisture	47.1	43.0	40.9	38.8	51.0	50.0	46.8
Carbohydrates (NFE)	35.6	34.8	34.6	34.2	31.6	31.7	31.4

Note: The composition of lucerne, sorghum and black cumin seed, respectively, was taken to be as follows (g/100 g product): crude protein, 5.4, 9.3, 21.3; crude fat, 0.6, 2.9, 35.5; crude fiber, 8.4, 2.4, 5.5; ash, 3.6, 1.5, 3.8; moisture, 70.0, 12.8, 5.5

The lucerne was harvested in the afternoon, stored in the animal house and fed the next morning. Feed left-overs were measured and feed intakes were calculated. Based on the feed intakes, it was decided that each animal would eat at least 150 g lucerne and 100 g sorghum day^{-1}.

After the adaptation period, the rabbits used in Experiments 1-3 were divided into four groups so that weight distributions between the groups were similar. In Experiment 4, the rabbits were allocated to one of three treatment groups. The groups were fed the diets shown in Table 1. The diets consisted of lucerne and crushed sorghum without or with black cumin seeds. The seeds were mixed with the sorghum. Each rabbit received a fixed amount of 250 g of feed day^{-1} and had free access to water.

Experimental designs and methods:
Experiment 1: Male rabbits were used and each group consisted of four animals. After feeding the experimental diets for 6 weeks, group-mean body weights were 1.31, 1.45, 1.56 and 1.34 kg for the control group and the groups fed increasing amounts of black cumin, respectively. All rabbits were immunized with 100 mg Bovine Serum Albumin (BSA). The BSA was dissolved in a mixture of 1.0 mL of complete Freund's adjuvant and 0.1 mL saline and then injected into the thigh muscle. The procedure was repeated 2 weeks later, but the opposite thigh muscle was now injected.

Blood samples were collected before immunization, at 10 days after the first injection and at 7 days after the second injection. Samples were taken by incision of the marginal ear vein into glass tubes without anti-coagulant. The blood was allowed to clot at room temperature for one hour and serum was collected by low speed centrifugation. Serum was kept frozen at-20°C until antibody analyses. BSA-specific antibodies in serum were measured by ELISA.

Experiment 2: In this experiment, the influence of dietary black cumin seed on phagocytosis of *Staphylococcus aureus* by rabbit blood was measured. There were four female rabbits per dietary group. After 6 weeks on the experimental diets, blood was collected from the marginal ear vein into tubes containing sodium fluoride as anticoagulant. The blood was mixed with saline containing a standardized concentration of cultured *Staphylococcus aureus* and incubated at 37°C for 1 h with frequent mixing. The mixture was then plated onto sheep-blood agar plates. The plates were kept at 37°C for 24 h under increased carbon dioxide tension. Then, the colonies were counted. For each blood sample the phagocytosis test was done in triplicate.

Experiment 3: Twelve female rabbits were used to assess the effect of ingestion of black cumin seed on hypersensitivity towards intradermal administration of tuberculin. There were three rabbits per dietary group. After feeding the diets for 6 weeks, a portion of the flank was shaved and cleaned with alcohol. A skin fold was taken and injected intradermally with 0.2 mL of saline containing tuberculin. Skin thickness was determined using a scaliper before the injection and 12 and 48 h afterwards.

Experiment 4: To determine the resistance against Pasteurella as modulated by black cumin seed, 9 male and 9 female rabbits were used. The rabbits were divided over three dietary groups, each group consisting of three male and three female rabbits. After the diets had been fed for 8 weeks, all animals were injected intraperitoneally with 0.1 mL of saline containing 0.13×10^6 freeze-dried *Pasteurella multocida*. For each rabbit the time after injection until death was recorded.

Data analysis: The data are presented as group means and SEM (Experiments 1-3) or group means and SD (Experiment 4). Statistically significant differences between group means were identified with the use of Duncan's multiple range test. The level of statistical significance was pre-set at $p < 0.05$.

RESULTS

Table 2 documents the anti-BSA antibody production after feeding the experimental diets. The feeding of black cumin seed caused significantly increased antibody concentrations in serum, both after the first and second inoculation. There was no dose effect of black cumin seed.

Table 2: Serum anti-BSA antibody production in rabbits fed the experimental diets for 6 weeks

	Dietary black cumin				
	0%	10%	15%	20%	SEM
Anti-BSA antibodies, optical density at 650 nm					
Pre-immunization	0.63[a]	0.75[b]	0.64[a]	0.76[b]	0.01
First inoculation	0.73[a]	1.06[b]	1.01[b]	1.00[b]	
Second inoculation	0.83[a]	1.04[b]	1.13[c]	1.09[b,c]	

Note: Group means within the same row not sharing the same superscript letter are significantly different

Table 3: Phagocytotic activity against *Staphylococcus aureus* in blood from rabbits fed the experimental diets for 6 weeks

	Dietary black cumin				
	0%	10%	15%	20%	SEM
Colony forming units	165[a]	166[a]	148[b]	147[b]	4.3

Note: Group means within the same row not sharing the same superscript letter are significantly different

Table 4: Hypersensitivity towards tuberculin in rabbits fed the experimental diets for 6 weeks

	Dietary black cumin				
	0%	10%	15%	20%	SEM
Skin thickness (cm)					
Pre-injection	0.21[a]	0.21[a]	0.20[a]	0.21[a]	0.002
12 h post injection	0.23[a]	0.25[b]	0.22[a]	0.23[a]	
48 h post injection	0.27[a]	0.24[b]	0.23[b]	0.24[b]	

Note: Group means within the same row not sharing the same superscript letter are significantly different

Table 5: Time until death due to Pasteurella injection in rabbits fed the experimental diets for 8 weeks

	Dietary black cumin		
	0%	2%	9%
Time until death (h)			
Males	5.6±0.27[a]	10.5±0.32[b]	14.1±0.39[c]
Females	6.5±0.30[a]	12.0±0.38[b]	15.9±0.63[c]

Note: Group means within the same row not sharing the same superscript letter are significantly different

Blood derived from rabbits fed the diets containing 15 or 20% black cumin seed significantly reduced the growth of *Staphylococcus aureus* on sheep-blood agar plates (Table 3). For the rabbits fed the diet with 10% black cumin, phagocytotic activity of blood was similar to that for the control rabbits.

Skin thickness as index of hypersensitivity towards tuberculin was not systematically influenced by dietary treatment at 12 h after injection (Table 4). However, at 48 h after injection, the diets containing black cumin seed had significantly reduced skin thickness when compared with the control diet. There was no dependency of skin thickness on the dietary inclusion level of black cumin seed.

Table 5 shows that the feeding of black cumin significantly prolonged the time until death after the intraperitoneal administration of *Pasteurella multocida*. Both in the female and male rabbits the time until death was longer for the diet containing 9% black cumin seed than for the diet with 2% black cumin seed.

DISCUSSION

The experimental diets were formulated by the addition of substantial amounts of black cumin seeds to the lucerne-sorghum base diet. This approach caused differences in the macronutrient compositions of the experimental diets. When compared with the control diet, the experimental diets with black cumin seed contained more protein and more fat. The control diet contained 13.2% protein in the dietary dry matter, which causes growth limitation in young rabbits (DeBlas *et al.*, 1981). Suboptimal protein supply may diminish immune function and resistance to infection (McMurray, 1984; Harbige, 1996). When interpreting the outcome of the present studies it should be realized that the differences in protein intake associated with different dietary concentrations of black cumin seed might have had an influence independent of the active principle in the seed.

The feeding of black cumin stimulated the production of antibodies in response to the intramuscular injection of BSA. This effect could by caused by the oil component of black cumin seed. In gamma-irradiated rats, the oral administration of *Nigella sativa* oil significantly raised hemolysis-antibody titers and produced regeneration lymphoid follicles in spleen and thymus gland (Assayed, 2010). The oral administration of *Nigella sativa* oil to rats infected with *Trichinella spiralis* was found to raise the level of antibody against the parasite (Abu El Ezz, 2005). In contrast, the administration of aqueous extracts of *Nigella sativa* did not influence the antibody

response to human serum in mice (Massadeh *et al.*, 2007).

Blood from donor rabbits fed diets containing either 15 or 20% black cumin seed had increased phagocytotic activity against *Staphylococcus aureus.* Similar observations have been made in human patients with allergic rhinitis. The treatment with allergen-specific immunotherapy plus intake of black cumin seed produced a significantly greater increase in phagocytic activity of polymorphonuclear leukocytes than immunotherapy alone (Işik *et al.*, 2010). It is likely that black cumin seed contains a factor that stimulates the polymorphonuclear leukocyte phagocytotic activity and the lymphocyte response to various mitogens. Evidence has been shown in studies with either human blood mononuclear cells (Haq *et al.*, 1995) or mouse splenocytes (Swamy and Tan, 2000).

It is clear that the feeding of black cumin seed elongated survival time of rabbits after the intraperitoneal administration of *Pasteurella multocida*. This observation corroborates an earlier study in mice infected with *Staphylococcus aureus*, showing eradication of the infection by the simultaneous injection of a diethyl-ether extract of *Nigella sativa* seeds (Hanafy and Hatem, 1991). Probably, the anti-bacterial activity of black cumin seed is explained by an enhanced phagocytotic activity.

The well-known anti-inflammatory effect of black cumin seed (Ragheb *et al.*, 2009) can also be demonstrated in rabbits. The feeding of black cumin seed was found to diminish the skin thickness seen after the intradermal administration of tuberculin. Various observations may explain the anti-inflammatory activity of black cumin seed. In a mouse model of allergic airway inflammation, the administration of *Nigella sativa* oil reduced the number of inflammatory cells in lung tissue (Abbas *et al.*, 2005). In a comparable mouse model, the intraperitoneal administration of thymoquinone inhibited the synthesis of both prostaglandin D2 and T-helper 2 cytokines (El Mezayen *et al.*, 2006). However, in splenic mononuclear cells isolated from allergen-sensitized mice given *Nigella sativa* oil, cytokine production was unchanged (Buyukozturk *et al.*, 2005). The exposure of mouse-bone marrow derived dendritic cells to thymoquinone compromised their maturation, cytokine release and survival (Xuan *et al.*, 2010). Possibly, dendritic cells regulate the anti-inflammatory action of black cumin seed.

Taken together the results of the four experiments, it may be concluded that black cumin seed at a dietary inclusion level of 10-15% produces a maximum effect on immune response and resistance against infection in rabbits. The dietary levels of 10, 15 and 20% black

cumin seed elicited similar effects on anti-BSA antibody production and tuberculin-mediated hypersensitivity. Phagocytotic activity against *Staphylococcus aureus* was not affected by 10% black cumin in the diet, but it was increased by the 15 and 20% inclusion level. Survival time after *Pasteurella* injection was increased after feeding the diet with 9% instead of 2% black cumin.

CONCLUSION

The studies described clearly show that the feeding of diets containing black cumin seed modulated the immune system in rabbits. The synthesis of antibodies and phagocytotic activity was increased after black cumin feeding. It would appear that black cumin seed did not overstimulate or dysregulate the immune system. In fact, after the intradermal administration of tuberculin the consumption of black cumin seed attenuated the inflammatory response.

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