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The effect of dietary grape pomace supplementation on epididymal sperm quality and testicular antioxidant ability in ram lambs

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ABSTRACT

Wine grape pomace (WGP) contains a rich source of polyphenols that can act as powerful antioxidants. The objective of this study was to investigate the effect of dietary WGP supplementation on antioxidative activity and epididymal sperm quality in rams. The rams were raised either under free-range or pen conditions, and the pen-raised rams were fed a WGP-containing diet (0, 5% and 10% of dry matter basis) for 74 days. An increase in the concentrations of reactive oxygen species (ROS, $P < 0.05$) and malondialdehyde (MDA, $P < 0.05$) were observed in the testes of rams subjected to restraint stress, and dietary WGP supplementation effectively decreased their contents ($P < 0.05$). Restraint stress reduced both weight and volume of testes, and impaired sperm quality. Dietary WGP supplementation increased testes weight, sperm concentration, motility and acrosomal integrity, and decreased sperm deformity in pen-raised animals ($P < 0.05$). The total antioxidative capacity (T-AOC) and catalase activity were decreased in the testes of pen-raised lambs ($P < 0.05$), and T-AOC, catalase, glutathione peroxidase 4 (GPx4) and superoxide dismutase (SOD) activity were increased when rams were fed the WGP-containing diet ($P < 0.05$). With the exception of *SOD* and *GPx4*, the mRNA contents of *catalase* and *nuclear factor-like-2 factor* (*Nrf2*) did not vary among the groups, and greater protein levels of SOD, catalase and GPx4 were observed in WGP-treated lambs ($P < 0.05$). Taken together, these results suggest that WGP can be used as a feed ingredient in rams to alleviate restraint induced oxidative stress and improve epididymal sperm quality.

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1. Introduction

Wine grape pomace (WGP), which consists mainly of stems, skins and seeds, is the major by-product of the red wine industry. WGP is rich in extractable polyphenols, including phenolic acid, flavonoids, procyanidins, resveratrol and anthocyanins [1], and possesses numerous health benefits, such as antimutagenic and anticarcinogenic activity, anti-inflammatory activities, and antimicrobial activities [2,3]. Importantly, WGP and its extract have even more effective antioxidative effects than vitamin C and vitamin E [4], and thus can be used as a promising natural feed additive in the animal industry [5,6].

China's lamb production areas are mainly located in the West and Northwest, and are characterized by fragile ecology [7]. An ecosystem conservation and restoration project was initiated in 2000, which includes the requirement of pen-raising for sheep. In rats, immobilization can induce oxidative stress, protein oxidation and lipid peroxidation [8]. Moreover, restraint stress results in a significant decrease in testes weight and spermatogenesis [9], and a decline in semen quality and sperm concentration [10]. Interestingly, grape seed procyanidin extract attenuates oxidative stress induced by nickel sulfate, and enhances sperm motility in rats [11]. Furthermore, resveratrol, a natural product derived from grape, protects against chronic immobilization stress in rat testis [12]. It is not clear whether pen-raising conditions induce oxidative stress and affect a ram's reproductive ability.

Based on data from the International Organization of Vine and Wine (OIV), China is ninth in a list of ten major red wine producing countries, suggesting the possible use of WGP in the lamb industry. The aim of this study was to investigate the effects of dietary WGP

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supplementation on sperm parameters and antioxidant capacity in the testes of ram lambs raised in pens.

2. Materials and methods

2.1. Feed preparation and chemical analysis

Wine grape pomace (obtained from a winery) was dried and milled, and nutrient composition is shown in Table 1. For nutrient composition analysis, the WGP were dried at 105 °C at least 8 h for dry mass (DM) analysis using the procedure described by AOAC. Ash was obtained via complete combustion in a muffle furnace at 600 °C for 6 h. The total nitrogen (N) was measured using the Kjeldahl method, the crude protein (CP) content was calculated by multiplying its nitrogen content by 6.25. The ether extract (EE) was determined via extraction in petroleum ether in a Soxhlet apparatus for 6 h. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined with the ANKOM A200i Filter Bag Digestion System (ANKOM Technology Corporation, Macedon, NY, US). The calcium (Ca) and phosphorus (P) were analyzed using the procedure described by AOAC. The feed was formulated based on the National Research Council's (NRC, 2007) recommendation as a pelleted mixed diet, and nutrient composition was determined as described above. The ingredients and nutrient contents are shown in Table 2.

2.2. Care and use of animals

All animal procedures were approved by the Shanxi Agricultural University Animal Care and Ethics Committee. A total of twenty-four 1/2 Dorper (♂) × 1/2 Small thin-tailed (♀) crossed ram lambs (4 months old, 25 ± 1 kg) were randomly selected; six lambs were raised under free-range conditions and the remaining lambs were housed in individual stalls (3 m × 0.8 m) equipped with feeders and a water source. The confined lambs were equally assigned into three groups in a randomized design, and received three levels of WGP supplementation (0%, 5% and 10%, based on air-dried matter). All animals were injected with ivermectin at a dosage of 0.2 mg/kg of body weight (BW) to eliminate parasites. Prior to the experiment, the animals were fed an experimental diet for 10 days *ad libitum* for adaptation, and the feeding experiment lasted for 74 days before sampling. The animals were individually fed twice daily at 8:00 a.m. and 18:00 p.m., and had free access to clean water and a salt block throughout the experiment, and increases in BW of approximately 200 g/day were expected.

At the end of the experiment, the lambs were anesthetized via CO_2 inhalation, exsanguinated, and the weights of the testes were recorded. The length, width, and thickness of the testes were measured, and both the volume (length × width × thickness) and the organ coefficient of the testes (testes weight, g/body weight, kg) were calculated. One small sample was obtained from the left testes and snap-frozen in liquid nitrogen for further evaluation, and another small sample from the right testis at the same anatomic position was obtained and fixed in 4% paraformaldehyde (PFA) for paraffin embedding.

Table 1
Nutrient composition of wine grape pomace (air-dry matter basis, %).

Items	%
Dry matter, DM	92.37
Crude protein, CP	11.50
Ether extract, EE	6.53
Crude ash, Ash	8.12
Neutral detergent fiber, NDF	43.83
Acid detergent fiber, ADF	35.12
Calcium	1.20
Phosphorus	0.05
Total phenols, TP	7.2

Table 2
Composition and nutrient level of diets (air-dry matter basis, %).

Dietary ingredient	Group		
	Free and 0% WGP[a]	5% WGP	10% WGP
Corn, %	29.00	27.00	24.95
Soybean meal, %	9.00	8.60	8.20
Wheat bran, %	4.00	4.00	4.00
Oil cake of flax seed, %	5.00	5.00	5.00
Mineral/vitamin premix, %	5.00	5.00	5.00
WGP, %	0	5.00	10.00
Naked oats straw, %	35.00	34.65	34.25
Potato rattan, %	13.00	10.75	8.60
Total	100.00	100.00	100.00
Nutritional level			
Dry matter (%)	88.43	88.57	88.51
Digestible energy (MJ/kg)	10.60	10.53	10.47
Crude protein (%)	11.73	11.90	12.21
Neutral detergent fiber (%)	42.85	43.20	43.38
Calcium (%)	0.40	0.40	0.39
Phosphorus (%)	0.25	0.26	0.26

[a] Wine Grape pomace.

2.3. Epididymal sperm analysis

Epididymal sperm counts and motility were determined as described previously with minor modification [13]. Briefly, distal cauda epididymis was removed and minced with scissors in 40 mL Tyrode's solution (pH 7.4) to release the epididymal fluid. Samples were balanced at 37 °C for 10 min to release the spermatozoa, and 10 μL of diluted epididymal fluid was placed in a Neubauer hemocytometer, and total, motile, and nonmotile sperm were microscopically counted (DMi8, Leica, Germany). For deformity and acrosomal integrity analysis, 10 μL of diluted epididymal fluid was spread onto a glass slide, fixed with 37% formaldehyde, and allowed to air-dry at room temperature. The smears were then stained with 0.75% Giemsa solution, left overnight and sperm morphology was assessed. Two different examiners counted 200 cells per smear using a microscope at a final magnification of 1000×, and the percentage of abnormal acrosomes and sperm malformations were calculated.

2.4. Enzyme activity, reactive oxygen species (ROS) and malondialdehyde (MDA) contents analysis

The snap-frozen testes samples were powdered in liquid nitrogen using a pestle and mortar. Testes powder (500 mg) was weighed and homogenized in 4.5 mL of 0.9% saline on ice, and then centrifuged at 2500×*g* for 10 min at 4 °C. The supernatant was used to analyze enzyme activity, reactive oxygen species (ROS) and malondialdehyde (MDA) contents using ELISA kits. Kit information was as follows: total antioxidant capacity (T-AOC, no. HY-60021), catalase (no. HY-60015), glutathione peroxidase 4 (GPx4, no. HY-60005), superoxide dismutase (SOD, no. HY-60001) and MDA (HY-60003) were from Beijing SINO-UK Institute of Biological Technology (Beijing, China); ROS (E004) was from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

2.5. Real-time quantitative PCR (q-PCR)

Total RNA was extracted using Trizol reagent (Sigma, Saint Louis,

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