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DIETARY GRAPE POMACE AFFECTS LIPID PEROXIDATION AND ANTIOXIDATIVE STATUS IN RABBIT SEMEN

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Abstract: The objective of this study is to evaluate the effect of dietary grape pomace (GP) on certain characteristics, mainly lipid peroxidation and the antioxidative status of rabbit buck semen. Twenty seven adult New Zealand White rabbit bucks (6 months of age) were divided into three homogeneous groups (n=9) and randomly submitted to one of the three investigated dietary treatments. Animals in the first treatment (control) group were given the basal diet. The diets of the second (GP-10) and third (GP-20) treatment groups contained 10 and 20% of GP, respectively. Bucks received the experimental diets for 10 continuous weeks. GP did not appear to have any significant effect on body weight gain. Bucks receiving 10 or 20% dietary GP had a higher semen volume, 32% above that of rabbits in the control group ($P<0.05$). The same trend was observed for sperm count. Dietary GP reduced the percentage of dead sperm and enhanced sperm motility. Interestingly, GP reduced lipid peroxidation in seminal plasma as indicated by TBARS, and significantly increased both the total antioxidant capacity and glutathione peroxidase activity ($P<0.05$). In conclusion, the use of antioxidant dietary fibers rich in functional nutraceuticals (such as GP) may decrease lipid peroxidation and increase the antioxidative defense of rabbit semen.

Key words: grape pomace, lipid peroxidation, semen, rabbits.

INTRODUCTION

Undoubtedly, male rabbits are one of the founding blocks of reproductive success in intensive rabbit farming, especially when we consider that one single male can influence the fertility and prolificacy of approximately one hundred females when artificial insemination (AI) is routinely performed as occurs on the rabbit farms. AI technology has now been applied to rabbit production for over 25 years, particularly in Europe (Theau-Clément, 2007). The quantity and quality of buck semen definitely ensures high usable doses per ejaculate and thus a reduction in the male/female ratio. In addition, kindling rate and litter size would appear to be dependent on both semen quality and quantity (Brun *et al.*, 2002). However, many environmental factors affect semen parameters such as ambient temperature, age, breed, nutrition, etc. Furthermore dietary components are believed to have a significant effect on semen production and quality (Nizza *et al.*, 2000). Specific dietary recommendations for rabbit bucks are not available (De Blas and Mateos, 1998).

The generation of reactive oxygen species (ROS) is a normal physiological process in both animal and human tissue and organs including the testis. However, the overgeneration of ROS may be detrimental to sperm, and has even been associated with male infertility (Akiyama, 1999). The spermatozoa of vertebrates including rabbits display high rates of metabolic activity and are also rich in polyunsaturated fatty acids

(Castellini *et al.*, 2006). This renders them particularly susceptible to oxidation by ROS, especially under stress conditions in humans (Aitken *et al.*, 1989), domestic birds (Eid *et al.*, 2006) and also rabbits (Castellini *et al.*, 2000). ROS can modify the spermatozoon cytoskeleton and axoneme resulting in a reduction of sperm motility (De Lamirande and Gagnon, 1992) and the inhibition of sperm-oocyte fusion (Aitken *et al.*, 1989) which, in turn, leads to reduced fertility (Wishart, 1984). ROS can also attack the DNA within the sperm nucleus. Such damage to the genome may be responsible for infertility (Roberts, 1998) resulting in reduced/poorer reproductive performance and hence, significant financial losses.

Antioxidant defense against ROS appears to be heavily influenced by nutrition. The oxidative stability of rabbit semen increased in relation to dietary antioxidants such as Vitamins C and E (Castellini *et al.*, 2003; Yousef *et al.*, 2003). Moreover, antioxidants can protect against the damaging effect of leukocyte-derived ROS on sperm motility (Baker *et al.*, 1996).

On a worldwide scale, grape is one of the most widely grown fruit crops. Because of the high fiber and lignin content and the low protein content, the contribution of grape by-products to rabbit diets is limited and categorized as cheap indigestible fiber sources (Carabaño and Fraga, 1992). Studies in the functional components of grape by-products have revealed a wide range of functional ingredients. These include several flavonoids with a phenolic nature such as monomeric flavanols (catechin and epicatechin), dimeric, trimeric and polymeric procyanidins, and phenolic acids. These flavonoids have been reported to exhibit antioxidant activity *in vivo* and *in vitro* (Yilmaza and Toledo, 2004). Natural grape seed proanthocyanidin extract proved to be safe, novel, highly potent and bioavailable free radical scavenger and antioxidant possessing a broad spectrum of health benefits. It functions at the genetic level and promotes therapeutic efficacy (Bagchi *et al.*, 2000), having a protective effect on gastric mucosa (Iwasaki *et al.*, 2004). It also exerts an anti-diabetic effect by preserving pancreatic cell function (El-Alfy *et al.*, 2005), and generally protects multiple target organs from structurally diverse drug- and chemical-induced toxicity (Bagchi, *et al.*, 2002) and liver toxicity (Maheswari and Rao 2005).

In fact, after screening a large number of fibrous fruit by-products for dietary fibers with exceptional biological antioxidant capacity, grape pomace (GP) was found to be one of the most promising candidates (Fulgencio, 2003). Therefore, the objective of this study is to analyze the possible beneficial effects of dietary grape pomace on the quality characteristics, lipid peroxidation and the antioxidative status of rabbit buck semen.

MATERIALS AND METHODS

Animals and housing

The trial was carried out at the Poultry and Rabbit Experimental Farm of the Department of Poultry Production, Faculty of Agriculture, Kafrelsheikh University, Egypt. Twenty seven adult (6 months of age) New Zealand White rabbit bucks were divided into three homogeneous groups (n=9) and submitted to 3 dietary treatments. They were selected from a larger population in order to obtain a uniform body weight (2.95±0.11 kg) and sperm count (301±3.0 ×10^6/mL). A physical examination was conducted on each rabbit and those considered unhealthy (that is, those possessing an elevated temperature, ocular or nasal discharge, ear mites or infection, diarrhea, abnormal lung sounds, or major wounds) were excluded. Bucks were kept under a continuous 16 h light/8 h dark photoperiod (Theau-Clément *et al.*, 1995). The ambient temperature ranged from 20 to 27°C and relative humidity ranged from 65% to 70%. Animals were housed individually in flat-deck cages and had been trained earlier for semen collection using an artificial vagina without any pre-stimulation.

Feeding

Grape pomace was obtained from El-Ahram Henken for beverages (Ganaklise Company) at Ganaklise, El-Behera Governorate, Egypt. The grape pomace was obtained in a wet condition with moisture content from 65-70%. The pomace contains grape seed and grape skin + stalk with a ratio of 2:1. The humidity of grape pomace was reduced by air-drying to 9-10%, then it was ground by hammer mill and stored for subsequent processing. Three experimental diets were formulated to represent three dietary treatments. Animals in the first treatment (control) group were given the basal diet. Diets of the second (GP-10) and third (GP-20) treatment groups contained 10 and 20% GP, respectively. All the experimental diets were formulated in such a way to ensure they were both isonitrogenous and isocaloric in accordance with De Blas and Mateos (1998). Feed and water were offered *ad libitum* throughout the whole experiment which lasted for 10 continuous weeks. The composition and chemical analysis (AOAC, 1995) of the experimental diets are presented in Table 1. Bucks received the experimental diets for 10 continuous weeks.

Sampling and semen traits

Final body weight was measured and body weight gain was calculated. Semen samples were collected twice a week, and the samples from 10th week were subjected to physical and chemical analysis. Semen collection and handling were carried out and evaluated according to the international guidelines (IRRG, 2005).

Semen volume was determined using graded tubes. Sperm count ($\times 10^6$ sperm/mL) was estimated by a haemocytometer (IRRG, 2005). Immediately following semen collection, the sperm motility percentage was measured by taking a small droplet from each individual and placing this on a warm slide covered with a cover slide. This was then microscopically examined for sperm motility at 400× magnification using a stage warmer set at 39°C. Motility was classified as described by Melrose and Laing (1970). Semen was given an arbitrary score from 0 to 5 based on the following assessment: 0 (0%, no discernable motility); 1 (1 to 20% of sperm exhibiting slight undulating movement; mostly weak and oscillatory); 2 (20 to 40% of sperm showing undulating movement; no waves or eddies formed; a number of inactive sperm may be present); 3 (40 to 60% of sperm showing progressive motility; vigorous motion; the production of slowly moving waves and eddies); 4 (60 to 80% of sperm showing progressive motility; waves and eddies of great rapidity of both formation and movement); and 5 (80 to 100% of sperm in vigorous and progressive movement; extremely rapid formation of eddies and movement). Viability was estimated as the percentage of eosin-permeable sperm, which were regarded as dead (Lake and Stewart, 1978).

Immediately following semen collection, initial fructose (Foreman *et al.*, 1973) and the Resazurin Reduction Test (RRT, Reddy and Bordekar 1999) were measured. RRT depends on the ability of metabolically active spermatozoa to reduce the resazurin dye (blue), with maximum absorption at 615 nm, to resorufin (pink) with a maximum absorption of 580 nm. The ratio of the optical densities of reduced to oxidized form (i.e. 580 to 615 nm) can be used to evaluate the various grades of semen sample. The highest correlation of the RRT ratio was observed with sperm motility, count, morphology and viability (Reddy and Bordekar 1999). Both fructose and RRT tests were carried out using kits produced by Bio-diagnostic, EGYPT.

Semen samples were centrifuged at 2500 *g* for 20 min. The seminal plasma (supernatant) was stored at -20°C for subsequent analysis. Lipid peroxidation in seminal plasma was measured in the form of thiobarbituric acid reactive substance (TBARS) as described by Richard *et al.* (1992). TBARS, in particular malondialdehyde (MDA), is a product of the oxidative degradation of PUFA, and, as such, is used as an index of oxidative stress. Total antioxidant capacity (TAC) and the activity of the antioxidative enzyme glutathione peroxidase (GPX) in the seminal plasma were measured according to (Koracevic *et al*., 2001) and (Paglia and Valentine, 1967), respectively, using kits produced by Bio-diagnostic, Egypt.

Table 1: Ingredients and chemical composition of experimental diets.

Ingredients	Control	GP-10 (10% GP)	GP-20 (20% GP)
Berseem hay	32.0	23.0	14.5
Soybean meal (44%)	19.0	18.5	18.5
Yellow Corn	12.0	12.0	12.0
Barley	15.0	15.0	15.0
Wheat bran	18.7	19.0	16.5
Grape pomace (GP)	00.0	10.0	20.0
Vegetable oil	00.6	01.2	02.3
Limestone	01.3	00.0	00.0
Di-calcium phosphate	00.5	00.5	00.5
Methionine	00.2	00.1	00.0
Salt	00.2	00.2	00.2
Premix[1]	00.3	00.3	00.3
Anti-Fungi[2]	00.1	00.1	00.1
Anti-oxidant[3]	00.1	00.1	00.1
Chemical composition (% DM)			
Dry matter (DM)	92.05	92.93	93.08
Organic matter (OM)	90.70	92.36	92.23
Crude protein (CP)	20.39	20.31	19.87
Ether extract (EE)	4.91	4.54	5.20
Crude fibre (CF)	13.35	14.58	14.73
Nitrogen free extractive (NFE)	52.05	52.93	52.43

[1] PESTMIX (Pestar Co., China.); Each 3 kg mixture contained: Vitamin A, 12000000 IU; Vit.D_3, 2200000 IU; Vit. E, 10000 mg; Vit.K, 2000 mg; Vit.B_1, 1000 mg; Vit.B_2, 4000 mg; Vit.B_6, 1500 mg; Vit.B_{12}, 10 mg; Pantothenic Acid, 10000 mg; Niacin, 20000 mg; Biotin, 50 mg; Folic acid, 1000 mg; Choline chloride, 500 gm; Selenium, 100 mg; Manganese, 55000 mg; Zinc, 50000 mg; Iodine, 1000 mg and carrier $CaCO_3$, to 3000 g.
[2] Mycostat, Agil, England.
[3] FEEDOX®dry, IMP EXTRACO (Belgium).

Statistical Analysis

The differences among treatments were statistically analyzed with a one-way ANOVA test in a completely randomized design using Statistical Packages for the Social Sciences (SPSS®, 2001). The significant differences among treatment means were compared using Duncan's new multiple-range test. $P < 0.05$ was set as the limit of significance.

RESULTS AND DISCUSSION

The present study was performed in order to obtain preliminary information about the effects of dietary GP on semen quality, lipid peroxidation and the antioxidative status of rabbit buck seminal plasma. Within the framework of this experimental design GP had no significant effect on body weight gain (Table 2). Dietary low fermentable fibre rich on lignine has been related to a reduction of digestive disorders, being required an adequate inclusion of both soluble and insoluble fibre (Alvarez *et al*., 2007). However, high

fiber supply leads to energy dilution of the diet and the animal thus attempts to increase its feed intake to satisfy energetic needs and the feed conversion is reduced. When the dietary fiber level is very high (> 25% acid detergent fibre), the animal cannot increase its intake sufficiently to meet its energetic needs, thereby resulting in a lower growth rate (Gidenne, 2000). Zaza *et al.*, (2005) found that up to 50% of dietary GP had no effect on the body weight gain of growing rabbits.

Semen characteristics are important in determining the fertility of rabbit bucks. Non-genetic factors such as stress, nutrition, age and management are believed to influence semen characteristics and subsequently buck fertility. Data presented in Table 2 seem to indicate that dietary GP improved both semen volume and semen quality characteristics. Bucks receiving 10 or 20% GP had a higher semen volume ($P<0.05$) than the control group. The average semen volume of GP-10 and GP-20 treatments was approximately 32% above that of the control group. The same trend was observed for the sperm count. Dietary GP significantly elevated the sperm counts in GP-10 and GP-20 treatments in comparison with the control group (Table 2). Semen samples from rabbits receiving 10 or 20% GP contained a significantly lower percentage of dead sperms than the control group. Dietary GP significantly elevated sperm motility ($P<0.05$). Theses findings were correlated with the concentration of initial fructose in seminal plasma which, when compared to other treatments, was significantly elevated in the GP-10 treatment. Sperm motility parameters were increased in this study due to dietary GP. Motility is critical in enabling the sperm to ascend the female reproductive tract to the site of fertilization which is necessary if fertilization is to be achieved (Aitken, 1990). In addition, GP antioxidants may offer protection against the damaging effect of leukocyte-derived reactive oxygen species on sperm movement (Baker *et al.*, 1996). Furthermore, high quality semen should contain a high percentage of vigorous and active sperms as well as having higher glycolytic or fructolytic rates than weak immobile sperms (Mann, 1964). It could be assumed that the observed increases in sperm motility for the GP treatments could in part be attributed to the concomitant induction in semen fructose (Yousef, *et al.*, 2003). The RRT results lend support to the previous assumption (Table 2). Could be argue that the positive effects of dietary GP on both sperm count and sperm motility and the reduced percentage of dead sperm could be linked to the antioxidative properties of GP (Ahn *et al.*, 2002; Murthy *et al.*, 2002). It had been suggested that the morphology and the motility of sperm cells would be preserved by binding antioxidants to endo-peroxides (Marin-Guzman *et al.*, 2000). Recently, Eid *et al.* (2006) found that a higher antioxidant intake was associated with greater sperm numbers and motility.

Seminal plasma affects the viability and membrane integrity of the sperms. However, seminal plasma may play another potential role in sperm viability with regard to its lipid peroxidation level and antioxidant content (Castellini *et al.*, 2000). The data presented graphically in Figure 1 shows that dietary GP

Table 2: Means±standard error of effect of dietary grape pomace (GP) on body weight gain and some semen parameters of rabbit bucks.

	Control	GP-10	GP-20
Body weight gain (g/10 wks)	705±15	690±10	680±20
Semen volume (mL)	0.62±0.02[b]	0.82±0.02[a]	0.81±0.02[a]
Sperm count ($\times10^6$ sperm/mL)	303±3.7[b]	332±1.8[a]	329±3.3[a]
Dead sperm (%)	22.3±2.24[a]	13.9±1.18[b]	14.1±1.12[b]
Semen motility score	3.3±0.2[b]	4.6±0.3[a]	4.5±0.3[a]
Semen initial fructose (mg/dL)	185.2±7.7[b]	212.5±8.4[a]	200.3±6.3[a]
Resazurin Reduction Test (RRT)	2.94±0.83[b]	5.85±0.41[a]	4.11±0.52[a]

Means with different superscripts in the same row differ from each other ($P<0.05$).
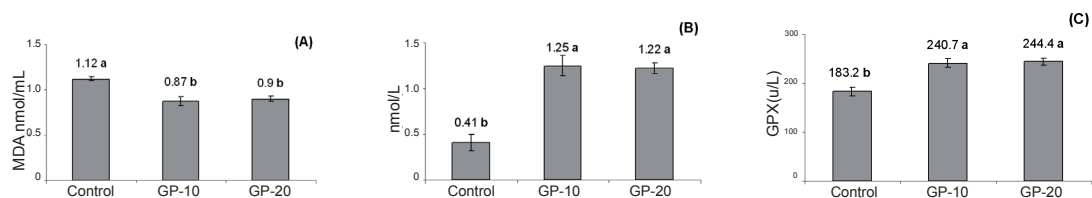
Figure 1: Effect of dietary grape pomace (GP) on TBARS (A), total antioxidant capacity (B) and glutathione peroxidase (C) in the seminal plasma of rabbit bucks. Values are expressed as means±standard error; means with different superscripts significantly differ from each other ($P<0.05$).

significantly enhanced the antioxidative properties of rabbit seminal plasma ($P<0.05$). It is noteworthy that dietary GP significantly reduced lipid peroxidation in seminal plasma as indicated by TBARS (Figure 1 A). Also, it can be observed that both total antioxidant capacity and glutathione peroxidase were significantly elevated through the using of dietary GP (Figure 1 B, C).

It is a fact that vertebrate sperm including rabbit display high rates of metabolic activity and are rich in polyunsaturated fatty acids (Castellini *et al.*, 2006), rendering them particularly susceptible to ROS-induced oxidation. For some years, lipid peroxidation has been known to be one of the major reactions leading to phospholipid loss, membrane damage, and the loss of motility in mammalian spermatozoa (Mann and Lutwak-Mann, 1981). A positive correlation exists between final TBARS and sperm viability indicating that peroxidation could be one of the causes of rabbit sperm deterioration. Our data shows that dietary GP significantly reduced the level of TBARS in the seminal plasma which may be attributed to its free radical scavenging ability as an antioxidant. This assumption was confirmed by measuring the total antioxidant capacity in the seminal plasma which was significantly elevated in response to dietary GP (Figure 1 B).

In addition to reactive oxygen species, glutathione and glutathione-related enzymes are also involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds (Knapen *et al.*, 1999). The antioxidative enzyme GPX occupies a particularly important role in the antioxidant protection of the cell by converting hydrogen peroxides into less harmful components (Olafsdottir and Reed, 1988). GPX activity was enhanced by dietary GP (Figure 1 C). These results suggested a low level of ROS in the seminal plasma. Furthermore, it has been demonstrated that the rate of lipid peroxidation in human spermatozoa was dramatically enhanced following the specific inhibition of GPX by mercaptosuccinate or using GSH oxidation, indicating that GPX plays an important role against lipid peroxidation in spermatozoa *in vitro* (Alvarez and Storey, 1989).

Plant-derived antioxidants can prevent ROS-induced damage through various means, such as interfering with the initial reactions responsible for generating ROS, scavenging of the free oxygen molecules required to begin the production of ROS or by chelating metals which expedite oxidative processes. GP phenols, such as flavonoids, are antioxidant compounds derived from plants considered to be of greater interest for dietary supplementation in organisms presenting an imbalanced redox state (Sgorlon *et al.*, 2005). The results of the current study indicate that the active biological compounds present in GP may be involved in the control of redox homeostasis in rabbit spermatozoa and seminal plasma. This assumption concurs with the findings made by Sgorlon *et al.* (2005) when studying humans.

To conclude, the inclusion of grape pomace in the diet increase some characteristics of the rabbit semen probably due to their physiological and antioxidant actions. More detailed studies regarding this particular aspect are required.

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