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POMEGRANATE PEEL AS A NATURAL ANTIOXIDANT BOOSTS BUCKS' FERTILITY UNDER EGYPTIAN SUMMER CONDITIONS

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Abstract: Exposure of male rabbits to heat stress during summer adversely affects their fertility, leading to major production losses. A total of 16 male rabbits were randomly divided into 4 experimental groups exposed to temperatures ranging from a high of 30.3 to a low of 27.3 °C. Animals from first to fourth groups were fed diets containing 0, 1.5, 3.0, or 4.5% pomegranate peel (PP) to evaluate the ability of PP feeding to enhance bucks' reproductive status. Pomegranate peel treatments significantly increased ejaculate volume by 19, 18 and 12%, increased seminal plasma fructose by 7, 18, and 24%, improved sperm motility by 28, 34 and 49%, increased sperm total output by 37, 69 and 102% and reduced dead sperm by 24, 32 and 64% with diets containing 1.5, 3.0 and 4.5% of PP compared to the heat stressed control animals. Seminal plasma total lipids, cholesterol and triglycerides increased while alkaline phosphatase decreased with PP treatments. Seminal plasma total antioxidant capacity increased to reach 126, 143 and 191% with diets containing 1.5, 3.0 and 4.5% of PP, while lipid peroxide (malondialdehyde) levels decreased significantly to reach around 54% of the heat stressed bucks' value with the three PP dietary doses used. It was concluded that supplementations of PP in the diet of bucks during summer season in Egypt can improve their semen characteristics, probably due to their antioxidant actions.

Key Words: rabbit, heat stress, pomegranate peel, sperm, antioxidant.

INTRODUCTION

In tropical and sub-tropical areas (such as Egypt), rabbits are faced with many problems related to hot climate, particularly heat stress, which induces a vast array of biological changes (Marai *et al.*, 1995). Exposure of adult male rabbits to >86 THI (temperature-humidity index) units as severe heat stress during summer adversely affects their reproductive traits and reduces their resistance to diseases. Testosterone concentration, spermatogenesis, temporary sterility, libido, ejaculate volume, motility, sperm concentration and total number of spermatozoa in an ejaculate decrease and sperm abnormalities and dead sperm increase, which can be described as "seasonal sterility" by exposure to the same factor. The drastic changes that occur in rabbits' biological functions are depression in feed intake and feed efficiency and utilisation, disturbances in metabolism of water and protein, energy and mineral balances, enzymatic reactions, hormonal secretions and blood metabolites (Marai *et al.*, 2002 ; Elnagar, 2010). The high environmental temperature not only has adverse effects on rabbits' performance but also causes an increase in oxidative stress (Lee, 2002), which can impede disease resistance and impairs antioxidant status (Sabin *et al.*, 2001).

On the other hand, global production and consumption of pomegranate has greatly expanded in recent years, together with recognition of the health-promoting potential of various components of this fruit (Aviram *et al.*, 2008). Pomegranate peel (PP) attracts attention due to its apparent wound-healing properties (Chidambara *et al.*, 2004), immunomodulatory activity (Gracious *et al.*, 2001), antibacterial activity (Navarro *et al.*, 1996) and antiatherosclerotic and antioxidative capacities (Tzulker *et al.*, 2007). Antioxidative activity has often been associated with a decreased risk of various diseases and mortality (Huxley and Neil, 2003). In rabbit bucks, positive effects of antioxidants

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supplementation on sperm motility have been described (Mangiagalli *et al.,* 2012). Whitley *et al.*, 2003 and Singh *et al.*, 2001 reported that PP is a good source of antioxidants. Li *et al.* (2006) reported that PP provides higher yields of phenolics, flavonoids and proanthocyanidins than the pulp. Flavonoid content was significantly greater in the peel than the pulp (59 *vs.* 17 mg/g), as were proanthocyanidins (11 *vs.* 5 mg/g). Also, peel extract acted more dramatically in protecting LDL against oxidation as compared to the pulp extract. Oxidation of LDL has been proposed as playing a key role in the hardening of arteries (atherosclerosis). Moreover, the most synthetic antioxidants have been restricted recently, mainly because of their possible carcinogenetic effect (Mhdavi and Salunkhe, 1995), causing liver swelling and changing liver enzyme activities (Martin and Gilbert, 1968). Reactive oxygen species (ROS) may be detrimental to sperm, and have even been associated with male infertility (Akiyama, 1999). ROS can also attack the DNA within the sperm nucleus, and such damage to the genome may be responsible for infertility (Roberts, 1998), resulting in reduced reproductive performance.

The present study aimed to obtain preliminary information about the effects of dietary PP on semen quality (ejaculate volume, concentration, total sperm output, mass activity, individual motility and dead sperm %), lipid peroxide (malondialdehyde) and the antioxidative status of heat stressed bucks' seminal plasma (expressed as total antioxidant capacity).

MATERIALS AND METHODS

Animals and housing

This study was carried out at the Rabbit Research Laboratory of the Poultry Production Department, Faculty of Agriculture, Alexandria University, during the period from August to September 2009. Sixteen V-Line rabbit bucks, with average initial live body weight 3.32±0.045 kg, were randomly distributed into 4 homogeneous treatment groups. Bucks were kept under a continuous 16 h light/8 h dark photoperiod and the ambient temperature ranged from 27.3 to 30.3°C and relative humidity ranged from 72.8 to 75.4%. Animals were housed individually in flat-deck cages and had been trained earlier for semen collection using an artificial vagina.

Feeding

Ground PP was obtained from Fathalla Gomla Market, Alexandria Governorate, Egypt. The PP was obtained in dried, ground form with moisture content of 9-10%. Four experimental diets were formulated to represent 4 dietary treatments. Bucks in the 1st treatment (heat stressed control, C) group were given the basal diet without supplementation. Diets for the 2nd (PP-1.5), 3rd (PP-3.0) and 4th (PP-4.5) treatment groups contained 1.5, 3.0 and 4.5% PP, respectively. All the experimental diets were formulated 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 8 wk experimental period. Composition and chemical analysis (AOAC, 1995) of the experimental diets are presented in Table 1.

Sampling and semen traits

Semen samples were collected biweekly using an artificial vagina and samples from the 8th week were subjected to chemical analysis. Semen collection and handling were carried out and evaluated according to international guidelines (IRRG, 2005). Ejaculated volume was measured to the nearest 0.01 mL. A weak eosin-formalin (10% formalin) solution was used for evaluation of sperm concentration by the improved Neubauer hemocytometer slide, as described by Smith and Mayer (1955). Total sperm output was calculated by multiplying semen ejaculate volume by semen concentration. Semen mass motility was given an arbitrary score from 0 to 3 based on the following assessment and the following variables were estimated: 0=No current, (0.5)=Very few slow currents, 1=Few slow currents, 1.5=Many moderate waves, 2=Many sweeping waves, 2.5=Numerous vigorous waves, 3=Numerous rapid and vigorous waves, as described by Moule (1965). Individual sperm motility was estimated at 400× magnification (Kamar, 1960). Evaluation of seminal initial fructose was carried out immediately after collection according to Mann (1948). Assessment of dead spermatozoa was performed using an eosin-aniline blue staining mixture (Shaffer and Almquist, 1948). Seminal plasma at 8th week of experiment was separated by centrifugation at 3000 rpm for 20 min and was stored at –20°C in Eppendorf tubes for further analysis of total lipid (mg/dL), cholesterol (mg/dL), LDL (mg/dL),

Table 1: Ingredients and chemical composition of pomegranate peel (PP) and experimental diets. [Control (C) and diets containing 1.5, 3.0 and 4.5% of PP (PP-1.5, PP-3.0, and PP-4.5 respectively)].

	PP	C	PP-1.5	PP-3.0	PP-4.5
Ingredient					
Yellow corn		7.5	7.5	7.5	7.5
Wheat bran		24	24	24	24
Barley		20	20	20	20
Clover hay		22	20.5	19	17.5
Soybean meal (44% CP)		23.5	23.5	23.5	23.5
Pomegranate peel		0	1.5	3	4.5
Limestone		1.15	1.15	1.15	1.15
Di-calcium phosphate		0.5	0.5	0.5	0.5
DI-Methionine		0.2	0.2	0.2	0.2
Anti-aflatoxin+Anti-coccidian		0.5	0.5	0.5	0.5
Vitamin and minerals Velamax-premix[1]		0.30	0.30	0.30	0.30
Salt		0.35	0.35	0.35	0.35
Chemical analysis					
Dry matter (DM)	91.04	92.30	92.36	92.46	92.38
Organic matter	80.53	81.25	82.12	81.93	81.91
Crude protein (CP)	10.06	20.15	20.10	20.09	20.07
Ether extract	2.40	3.74	3.69	3.51	3.54
Crude fibre	11.43	11.03	11.00	10.73	10.58
Nitrogen free extract	56.64	46.33	47.33	47.6	47.72
Total phenolic content: mg GAE/g DM	268				

[1]Each kg of vitamin and mineral mixture contained: Vit A 2 000 000 IU; E 10 mg; B1 400 mg; B2 1200 mg; B6 400 mg; B12 10 mg; D3 180000 IU; Colin chloride 240 mg; Pantothenic acid 400 mg; Niacin 1000 mg; Folic acid 1000 mg; Biotin 40 mg; Mn 1700 mg; Zn 1400 mg; Fe 15 mg; Cu 600 mg; Se 20 mg; I 40 mg and Mg 8000 mg. GAE: Gallic acid equivalents.

HDL (mg/dL), total antioxidants capacity (mM/L), lipid peroxide (malondialdehyde) (nmol/mL), triglycerides (mg/dL), alkaline phosphatase (IU/L) and AST (U/L) were determined in seminal plasma calorimetrically using commercial kits obtained from Bio-Diagnostics Ltd., Egypt according to the procedure outlined by the manufacturer.

Statistical Analysis

Data were analysed by analysis of variance using the general linear model procedure (Proc GLM; SAS Institute, 1996). For the overall means, data were classified according to 4 treatments and the mean of each treatment was used.

Interaction between time and treatment was confirmed by repeated measures using mixed model analysis. Differences among means were determined using Duncan test (Duncan, 1955).

RESULTS AND DISCUSSION

Reproductive performance

Results of this study (Table 2) demonstrated that daily consumption of PP for 8 wk caused a significant improvement in the overall ejaculate volume ($P<0.001$). Semen volume of bucks receiving 1.5, 3.0 and 4.5 % PP were 19, 18 and 12% significantly higher than those of the control, although there was no significant difference among the treated groups. Regarding interaction of treatments with time, it can be noted (Table 2) that the highest ejaculate volume was recorded with the 3% PP treatment by the 4th week, whereas the lowest was reported with control at the end of the experiment period ($P<0.001$).

Overall sperm concentration increased significantly in a dose dependent manner with PP treatments (Table 2). This is an increase of 15, 43 and 80% (PP-1.5, PP-3.0 and PP4.5, respectively) compared to the C group, respectively. However, interaction of treatment with time did not show any significant differences ($P=0.1017$).

Table 2: Mean values (±standard error) of some semen characteristics of heat stressed rabbit males fed with the experimental diets. [Control (C) and diets containing 1.5, 3.0 and 4.5% of pomegranate peel (PP) (PP-1.5, PP-3.0, and PP-4.5, respectively)].

Treatments		Ejaculate Volume (mL)	Concentration ($\times10^6$/mm^3)	Total Sperm ($\times10^6$/ejaculate)	Mass Activity	Individual Motility (%)	Dead (%)
Diet							
C		0.89±0.02^{b}	177.86±8.42^{c}	146.6±13.29^{c}	1.83±0.20^{b}	43.07±5.00^{b}	38.05±4.76^{a}
PP-1.5		1.06±0.03^{a}	204.64±9.58^{c}	220.6±12.67^{b}	2.53±0.07^{a}	55.15±2.59^{a}	28.79±2.17^{b}
PP-3.0		1.05±0.05^{a}	254.76±21.90^{b}	249.1±26.68^{b}	2.55±0.12^{a}	57.80±2.47^{a}	26.02±2.46^{b}
PP-4.5		1.00±0.02^{a}	320.34±21.00^{a}	313.0±24.83^{a}	2.73±0.06^{a}	64.21±2.15^{a}	13.84±0.96^{c}
P-value		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Diet×week							
C	Wk 2	0.96±0.03^{cd}	200.38±16.51	191.88±15.57	2.50±0.16	56.88±6.61	36.68±1.95
	Wk 4	0.90±0.05^{cd}	160.00±14.72	80.60±32.96	2.00±0.13	42.00±10.20	31.28±10.14
	Wk 6	0.91±0.04^{cd}	180.00±13.42	166.17±16.49	1.56±0.49	41.67±11.01	28.90±7.07
	Wk 8	0.80±0.05^{d}	147.50±6.29	109.75±2.93	1.31±0.50	29.29±11.15	51.99±15.54
PP-1.5	Wk 2	0.96±0.04^{cd}	168.33±16.41	160.17±11.94	2.44±0.15	63.02±5.47	22.14±1.79
	Wk 4	1.03±0.04^{bd}	183.33±25.12	183.17±21.42	2.63±0.18	59.17±3.27	24.34±3.62
	Wk 6	1.08±0.06^{bd}	212.50±15.78	230.88±24.37	2.56±0.15	55.63±3.46	28.59±4.79
	Wk 8	1.19±0.04^{ab}	240.00±11.50	283.88±15.00	2.50±0.13	45.75±5.99	38.15±3.96
PP-3.0	Wk 2	1.03±0.05^{bc}	210.00±28.75	183.67±50.97	2.43±0.23	54.70±6.03	37.75±4.10
	Wk 4	1.24±0.14^{a}	234.00±66.23	270.20±75.67	2.80±0.12	61.00±4.58	24.40±5.06
	Wk 6	0.94±0.02^{cd}	295.00±66.02	282.50±68.60	2.88±0.13	57.00±5.15	21.83±4.01
	Wk 8	0.95±0.06^{cd}	290.00±17.89	274.83±21.50	2.25±0.28	57.86±5.10	21.58±3.63
PP-4.5	Wk 2	0.96±0.05^{cd}	262.86±31.90	252.29±35.42	2.64±0.14	62.55±4.46	16.16±2.16
	Wk 4	0.94±0.05^{cd}	315.00±41.32	290.00±38.18	2.75±0.09	64.38±2.90	14.32±2.50
	Wk 6	1.08±0.03^{bd}	332.50±50.84	356.50±51.80	2.75±0.09	65.99±3.09	12.38±0.77
	Wk 8	1.03±0.04^{bdw}	378.33±33.41	356.50±72.97	2.79±0.15	63.90±7.13	12.80±1.98
P-value		0.0003	0.1017	0.3781	0.0769	0.6176	0.2165

$^{a, b, c,d}$: Different letters within a column denote significant differences between treatments ($P<0.05$).

The effects of treatments on ejaculate volume and sperm concentration were mirrored on overall of the total sperm output (Table 2). The total sperm per ejaculate in the PP-1.5, PP-3.0 and PP-4.5 supplemented groups increased significantly ($P<0.001$) to reach 137, 169 and 202%, compared to values of C group, respectively.

Sperm mass activity (Table 2) significantly ($P<0.001$) improved with PP-1.5, PP-3.0 and PP-4.5 treatments by 38, 39 and 49% compared to C, respectively. The sperm individual motility revealed similar observations. Feeding of PP in different concentrations significantly improved the sperm motility compared to the C group (128, 134 and 149%, for PP-1.5, PP-3.0 and PP-4.5 groups, respectively; $P<0.001$).

Semen samples from rabbits receiving PP-4.5 dose contained a significantly ($P=0.0001$) lower percentage of dead sperms than the control group and the other experimental groups. Percentage of dead sperms decreased in a dose dependent manner by 24, 32 and 64% compared to the heat stressed control with the three PP doses, respectively.

Seminal plasma analysis

Seminal plasma total lipids (Table 3) increased significantly ($P=0.0126$) with PP-1.5, PP-3.0 and PP-4.5 treatments to reach 115, 115, and 113% of the value with the C treatment, respectively. The increase in seminal plasma total lipids was due to the increase of both seminal plasma cholesterol and triglycerides which increased significantly ($P=0.0035$ and 0.0026, respectively) with the PP-1.5, PP-3.0 and PP-4.5 group by 32, 80, 84 and 12, 13, 50% compared to C, respectively. Seminal plasma LDL levels (Table 3) showed a non significant ($P=0.2274$) decrease with

Table 3: Mean values (±standard error) of some seminal plasma characteristics of heat stressed rabbit males fed with the experimental diets.[Control (C) and diets containing 1.5, 3.0 and 4.5% of pomegranate peel (PP) (PP-1.5, PP-3.0, and PP-4.5, respectively)].

	C	PP-1.5	PP-3.0	PP-4.5	*P*-value
Total lipid (mg/dL)	140.81±5.60^{b}	162.14±3.20^{a}	162.38±2.40^{a}	159.16±2.23^{a}	0.0126
Cholesterol (mg/dL)	38.65±1.92^{b}	51.21±2.66^{b}	69.71±3.81^{a}	71.14±8.51^{a}	0.0035
LDL (mg/dL)	13.06±0.22	12.00±0.34	12.93±0.26	12.76±0.40	0.2274
HDL (mg/dL)	9.00±0.21^{c}	12.90±0.15^{b}	13.25±0.28^{b}	14.72±0.41^{a}	0.0001
Triglycerides (mg/dL)	129.15±6.45^{b}	144.48±5.18^{b}	146.16±1.86^{b}	194.00±13.44^{a}	0.0026
Alkaline phosphatase (IU/L)	43.46±2.94	39.52±1.29	38.72±1.01	36.15±1.20	0.0891
AST (U/L)	25.04±0.50^{a}	23.63±0.87^{b}	23.60±0.63^{b}	23.52±0.91^{b}	0.0029
Fructose (mg/100 mL)	184.84±3.86^{c}	198.55±7.07^{bc}	219.01±9.29^{ab}	229.07±9.54^{a}	0.0008
pH	8.04±0.05^{a}	7.96±0.08^{a}	7.81±0.09^{ab}	7.69±0.09^{b}	0.0229
T. antioxidant capacity (mM/L)	0.69±0.12^{b}	0.87±0.07^{b}	0.99±0.07^{ab}	1.32±0.11^{a}	0.0131
Malondialdehyde (nmol/mL)	7.84±0.38^{a}	4.32±0.47^{b}	4.13±0.17^{b}	4.19±0.29^{b}	0.0001

$^{a, b, c}$: Different letters within a row denote significant differences between treatments (P<0.05).

the 3 PP doses, whereas HDL increased significantly (P<0.001) in a dose dependent manner by 43, 47, and 64% with PP-1.5, PP-3.0 and PP-4.5 groups compared to C bucks, respectively.

Seminal alkaline phosphatase showed a non significant (P=0.0891) reduction to reach 91, 89 and 83% of the C bucks' value under the PP-1.5, PP-3.0 and PP-4.5 treatments, respectively (Table 3). However, seminal AST showed a significant (P=0.0029) reduction, reaching around 94% of control value with the 3 PP treatments (Table 3)

Seminal fructose (Table 3) increased significantly (P<0.001) in a dose dependent manner by 7, 18 and 24% compared to the C bucks with the PP-1.5, PP-3.0 and PP-4.5 groups, respectively.

Seminal plasma total antioxidant capacity (Table 3) increased significantly (P=0.0131) in a dose dependent manner to reach 126, 143 and 191% of control with the PP-1.5, PP-3.0 and PP-4.5 groups, respectively. On the other hand, Lipid peroxidation (malondialdehyde) levels decreased significantly to reach about 54% of the C bucks' value (P<0.001) without significant differences between the different PP doses.

The study aimed to obtain preliminary information on the effects of dietary pomegranate peel on semen quality, lipid peroxidation and the antioxidative status of buck seminal plasma under heat stress. Results showed that the impaired reproductive status of bucks observed under heat stress can be enhanced by PP administration. The improvement in overall ejaculate volume, sperm concentration and therefore total sperm output (P<0.001) observed in this study is consistent with antioxidant effects on semen quality (Eid, 2008) when bucks were fed grape pomace as a source of antioxidant. This can be explained by the findings of Türk *et al*., (2008), who reported that treating rats with pomegranate juice resulted in increased epididymal sperm concentration, spermatogenic cell density and diameter of seminiferous tubules and germinal cell layer thickness. The enhancement observed in sperm motility could in part be attributed to the concomitant induction in semen fructose (Yousef *et al*., 2003) which was also observed in this study. In addition to the previous beneficial effects of PP on heat stressed males' reproductive status, PP treatments were able to significantly reduce dead sperm concentrations, which may be attributed to suppression of oxidative stress. Türk *et al.* (2010) suggested that ellagic acid had a protective effect against testicular and spermatozoa toxicity induced by cyclosporine A and attributed this effect to its involvement in oxidative stress suppression. Dietary PP in this study showed antioxidant capabilities evidenced by improved seminal plasma antioxidant capacity and reduced lipid peroxidation (malondialdehyde) levels (Table 3). This follows the same trend as the findings of Guo *et al.* (2008), who reported that plasma antioxidant capacity increased when subjects consumed pomegranate juice. Reducing malondialdehyde levels is evidence of reduced lipid peroxidation in seminal plasma, which was confirmed by the increase in seminal plasma total lipids with PP treatments (Table 3). This increase in seminal plasma total lipids was combined with a decrease in LDL and an increase in HDL, further enhancing semen properties. Moreover, PP treatments resulted in a decrease in seminal plasma alkaline phosphatase, which may play a role in enhancing semen

properties as high seminal alkaline phosphatase activity in the buffalo was associated with lower sperm numbers, decreased motility and live cell percentage, depressed dehydrogenase activity and a slight and non-significant decrease in fructolytic rate (Abdou *et al.*,1978). As the name suggests, alkaline phosphatase is most effective in an alkaline environment, so the reduction in pH values observed in this study with PP treatments may be attributed to the reduction observed in alkaline phosphatase (Table 3).

CONCLUSIONS

All in all, the nutritive value and antioxidant capacity of PP make it a favourable health-promoting constituent of rabbit diet, while the results of the current study indicate that the active biological compounds present in PP may be involved in homeostasis control in rabbit spermatozoa and seminal plasma. In conclusion, PP supplements in the diet of bucks during summer season in Egypt can improve their semen characteristics, probably due to antioxidant action.

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