Final version can be accessed at Animal Production Science 55:107-112.
http://dx.doi.org/10.1071/AN13110

Effect of dietary ellagic acid supplementation on semen quality parameters in chickens

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Abstract. Ellagic acid (EA), a polyphenoloic constituent in fruits and nuts has been shown to have antioxidant and antiapoptotic properties. The aim of the present study was to investigate the effect of dietary ellagic acid supplementation on semen quality parameters in chickens. Forty roosters (Dahlem Red breed) of 28 weeks age were randomly assigned to four groups and fed diets containing EA at 0, 25, 50, 75 mg/kg for eight weeks. Semen was evaluated for volume, appearance, individual motility, sperm concentration, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) dye reduction test, live, dead and abnormal sperm counts. The seminal plasma was separated and evaluated for protein, alkaline phosphatase and lipid peroxidation. At the end of the experiment, blood from individual birds was collected, serum separated and evaluated for superoxide dismutase activity, lipid peroxidation and ferric reducing ability of plasma. EA supplementation had no effect ($P>0.05$) on gross semen parameters. The seminal plasma protein and lipid peroxidation were different ($P<0.01$) between the treatment groups. The group fed EA at 75 mg/kg had high ($P<0.01$) seminal plasma lipid peroxidation and lower ($P<0.05$) serum superoxide dismutase activity. In conclusion, dietary supplementation of EA in chicken had no effect on the gross semen qualities and increases lipid peroxidation in seminal plasma.

Introduction

Sperm plasma membrane has high concentration of polyunsaturated fatty acids and the cytoplasm contains lower concentration of free radical scavenging enzymes, thus making sperm susceptible to peroxidative damage by free radicals (Agarwal *et al.* 2008). Peroxidative damage of sperm cell membrane leads to morphological defects and reduced sperm viability (Türk *et al.* 2007). Proper balance between the free radicals and antioxidants is needed to maintain optimum sperm quality. Enzymatic antioxidant system comprising of superoxide dismutase (SOD), catalase and glutathione peroxidase present in the cytoplasm and non enzymatic antioxidants such as vitamin C, vitamin E, β-carotene, carotenoids and flavonoids neutralises the effects of free radicals. Among the enzymatic antioxidants SOD is predominant in sperm (Makker *et al.* 2009). The non enzymatic antioxidants may arise through metabolism in the body such as urate, pyruvate, ubiquitol etc., or of dietary origin (vitamins C and E).

Feed supplements of plant origin rich in certain compounds which have antioxidant activity have received increasing attention (Clément *et al.* 2012). The plant derivatives have many bioactive compounds, particularly polyphenols that are known for their antioxidative capacity (Christaki 2012). Extracts of plants or fruits rich in natural compounds such as lycopene, L-carnitine, macaene and sage extract have been demonstrated to improve semen quality in livestock or poultry (Newman *et al.* 2002; Mangiagalli *et al.* 2010; Clément *et al.* 2012; Ommati *et al.* 2013). Ellagic acid (EA; 2,3,7,8-tetrahydroxy[1]-benzopyrano[5,4,3-

cde][1]benzopyran-5,10-dione) is one such natural compound rich in condensed polyphenols, which has potent antioxidant property is found in many fruits, vegetables and cereals (Landete 2011). EA has been shown to have protective effect against an anti-neoplastic drug cisplatin, by scavenging of free radicals and suppression of oxidative damage to DNA (Festa *et al.* 2001; Türk *et al.* 2008). EA or ethanol extract of pomegranate has been shown to protect testicular cells or spermatozoal damage by different toxic compounds in laboratory animals (Türk *et al.* 2010a, b; Leiva *et al.* 2011; Sönmez *et al.* 2011). The hydroxyl group in EA is known to increase antioxidant activity and protect cells from oxidative damage (Pari and Sivasankari 2008). Incorporating naturally occurring EA as a feed additive will be an option for sustainable chicken production. Increase in the industrial juice production from different fruits and berries results in nutritive rich by-products that are utilized as health promoting constituents in animal feed (Shabtay *et al.* 2008). The EA content is higher in the seeds of berries (Daniel *et al.* 1989) that eventually end up in the by-product after juice extraction. The beneficial effect of EA supplementation in diets on semen quality in chicken is limited. Establishing probable beneficial effects of EA on male fertility will increase the use of such feed additives in breeding operations. The present experiment was conducted to evaluate the effect of dietary EA supplementation on semen quality in chickens.

Materials and methods

Experimental design, animals and feeding

The experiment was carried out at the experimental poultry farm of the institute located at Hyderabad, India. Dahlem Red, a brown-tinted layer breed maintained at the Institute was used in the experiment. Forty roosters (28 weeks) were assigned at random in individual cages (38 x 40 x 60 cm) in an elevated open-sided house. A practical breeder male diet was prepared to contain 10.88 MJ/kg ME and 160 g/kg crude protein with Ca 10 g/kg (Table 1). The diet was supplemented with four (0, 25, 50, 75 mg/kg) graded concentrations of EA (HiMedia Laboratories Pvt Ltd, Mumbai, India) and a feeding trial was conducted for eight weeks. The dose of EA selected in the study was based on the values (12.5 -50 mg/kg body weight) reported in rat study (Pari and Sivasankari 2008). The body surface area (BSA) in chickens compared to rats (surface law of metabolism) and longer duration of feeding of this compound were considered in selection of the doses.The birds were fed individually with weighed quantity of feed (110g/day) and had free access to water. The average temperature and relative humidity during the start of the experiment was 21.8°C and 64% respectively and the end of the experiment was 30.6°C and 46.5% respectively.The experimental protocol was approved by the Institute Animal Ethics Committee.

Semen collection and evaluation

Semen was collected weekly by the abdominal massage method (Burrows and Quinn 1937) for eight consecutive weeks and was evaluated for different gross and biochemical parameters. Semen was collected in sterile glass funnel and diluted using high temperature diluent (NaCl 0.8g; N-Tris(hydroxymethyl)methyl-2 aminoethanesulphonic acid 1.374g; 1M NaOH 2.75ml; glucose 0.6g, in 100ml of double distilled water) (Chaudhuri and Lake 1988)

with a final pH 7.4 and osmotic pressure 382 mOsmol/kg water. Semen samples were diluted four-fold and immediately subjected to analysis.

The volume of the semen ejaculate was measured by drawing the sample in to 1 ml syringe with an accuracy of 0.02 ml. The appearance of semen was scored 1 to 5 visually as follows: 1, watery or clear semen; 2, watery semen with white streaks; 3, medium white semen; 4, thick white semen; and 5, very viscous chalky white semen (McDaniel and Craig, 1959). Sperm motility was subjectively assessed as percentage of progressively motile sperm by placing a drop of diluted semen on a clean, grease free glass slide, overlaid with a coverslip and examined under high power magnification (40×). The concentration of sperm was estimated by the method described by Taneja and Gowe (1961) using a spectrophotometer (Spectronic Genesys 5, Milton Roy Company, USA) at 540 nm of wavelength. Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test was carried out in duplicate tubes and absorbance was recorded using a colorimeter (CL 157, Elico Ltd, Hyderabad, India) at 570 nm according to Hazary *et al.* (2001).

Percentage live and dead sperm was estimated by differential staining technique using eosin–nigrosin stain (Campbell *et al.* 1953). The slides were used for estimating the percent abnormal sperm on the basis of observable abnormalities. One glass slide smear was prepared from each sample and 200 sperm were counted in each slide for calculating live, dead and abnormal sperm.

Biochemical parameters

The seminal plasma was separated by pooling the semen samples of 2 birds in each treatment (5 samples/treatment) and centrifuged twice at 5000 rpm for 10 min. Seminal plasma was then analyzed for protein by Lowry's method and alkaline phosphatase (ALP) enzyme activity was measured by the p-nitrophenol method (Bowers and McComb 1975).

Malondialdehyde (MDA), an end product of lipid peroxidation, was assessed using the thiobarbituric acid method (Hsieh *et al.* 2006). In short, 100 µl of seminal plasma was added to 0.9 ml of distilled water in a tube. Then to each tube, 0.5 ml of thiobarbituric acid reagent (0.67 g of 2-thiobarbituric acid dissolved in 100 ml of distilled water with 0.5 g NaOH and 100 ml glacial acetic acid) was mixed and heated for 1 h in a boiling water bath. Tubes were then cooled and centrifuged for 10 min at 1500 x g and the absorbance of the supernatant was recorded at 534 nm.

Serum analysis

At the end of the experiment (35 weeks of age) blood samples from brachial vein were collected from all birds and serum was separated. The serum was assessed for superoxide dismutase (SOD) activity by microtitre plate assay (Madesh and Balasubramanian 1998),

lipid peroxidation (Ohkawa *et al.* 1979) and antioxidant power by ferric reducing ability of plasma (FRAP) assay (Benzie and Strain 1996).

Statistical analysis

Percent values were arcsine transformed before analysis of data. The data of semen and seminal plasma were subjected to General Linear Model two-way repeated-measures ANOVA (SAS 9.2) and the treatment means were compared at $P<0.05$. The sources of variation were treatment, weeks and treatment*weeks. Significant values were further compared using Tukeys post hoc test. The serum parameters were analysed by one-way ANOVA and Tukeys post hoc test.

Results

The gross semen parameters were not affected by supplementing the diets with EA ($P>0.05$; Tables 2 and 3), but there was significant difference between weeks of the experiment except for abnormal sperm count. The interaction between treatment and weeks was significant only for percent abnormal sperms in the gross semen parameters. Supplementation of EA reduced ($P<0.05$) the seminal plasma protein concentration (Table 4). Lipid peroxidation in seminal plasma was significantly increased in the group fed with the highest level of EA (Table 4). The seminal plasma protein levels, alkaline phosphatase activity and lipid peroxidation were different ($P<0.05$) between weeks of the experiment (Table 4). The interaction between the treatment and weeks was found to be significant ($P<0.01$) in all the seminal plasma parameters studied.

The serum SOD activity was lower ($P<0.05$) at the highest dietary level of EA (75 mg/kg) in the diet (Table 5). The serum lipid peroxidation was numerically lower in the 75 mg/kg EA supplemented group but the difference was not statistically significant. The FRAP assay values did not differ among the treatment groups ($P>0.05$).

Discussion

The supplementation of EA did not affect the feed intake by the birds (data not shown). Earlier report indicated that EA supplementation did not have any growth depressing activity in chickens (Kratzer *et al.* 1975).

Supplementation of EA had no influence on the gross semen parameters in the present study. To the authors' knowledge there is no report in the literature indicating beneficial effects of EA in chicken or other poultry species. In laboratory animals EA supplementation ameliorated or improved the semen quality of those that have been concurrently administered chemotoxic compounds such as cisplatin and adriamycin (Türk *et al.* 2008; Çeribaşi *et al.* 2010; 2012). Cisplatin induced reduction in sperm motility was improved by EA administration at 10 mg/kg/day in rat (Türk *et al.* 2008). EA (2mg/kg) protects the sperm from damage caused by administration of cyclophosphamide (Çeribaşi *et al.* 2010) and protects from adriamycin induced testicular lipid peroxidation and apoptosis in rat (Çeribaşi *et*

al. 2012). The suggested protective mechanism of EA is by its free radical scavenging action and inhibition of correlated lipid peroxidative damage (Çeribaşi *et al.* 2012).

The seminal plasma protein was significantly reduced in the EA supplemented groups and there was no significant difference in the ALP activity observed in the present study. ALPs are a group of cell membrane metalloenzymes that catalyze the hydrolysis of phosphate esters in an alkaline environment. Alkaline phosphatase is present in all tissues and is particularly concentrated in liver, bile duct, kidney and bone. Lipid peroxidation level was significantly higher in all the groups supplemented with EA and was highest in the group fed at 75 mg/kg indicating some damage to germ cells due to oxidative stress. The significant difference between the weeks of the experiment in the semen parameters might be due to increasing ambient temperature. During the last few weeks of experiment, the environmental temperature increased due to the summer conditions.

Free radicals derived from oxygen collectively called as reactive oxygen species (ROS) are produced during use of oxygen in the cellular metabolism. Uncontrolled or higher production of this ROS leads to lipid peroxidation and oxidative stress. Cells by the antioxidative mechanisms counteract the adverse effects of ROS. This counter action is by way of antioxidant enzymes (SOD, catalase and glutathione peroxidase) and nonenzymatic defenses (vitamin C, uric acid, bilirubin, vitamin E etc.). The FRAP assay measures the nonenzymatic defenses in biological fluids and the values are related linearly to the concentration of the antioxidants present. In the present study, there is no significant effect of EA supplementation on FRAP levels. The SOD enzyme activity was significantly lower in the 75 mg/kg ellagic acid fed group. Concurrently the lipid peroxidation level, though not statistically significant, was numerically lower in this group. Thus supplementation with ellagic acid at 75 mg/kg can be considered to be beneficial in counteracting the oxidative stress in blood.

Ellagitannins or EA present in feed is not absorbed per se in mammals and is metabolized; by the flora present in the intestine to urolithins (hydroxydibenzopyran-6-one derivatives) which were then preferentially absorbed depending on their lipophilicity (Espin *et al.* 2007; González-Barrio *et al.* 2012) and excreted in urine. These urolithins have the antioxidant activity which was correlated with the number of hydroxyl groups as well as the lipophilicity of the molecules (Bialonska *et al.* 2009). The efficacy of EA and its metabolites urolithins as antioxidant depends on the degree of hydroxylation. The antioxidant capacity is higher for EA and lower in its metabolites (Landete 2011). Further EA has been shown to have lower lipophilicity and absorption than its metabolites urolithins. In contrast to the findings in mammals, it was found that urolithins are not produced by the birds feeding on ellagitannin containing foods though EA was released (González-Barrio *et al.* 2011). Beyond this, the status of EA absorption, metabolism and excretion in birds is not known. With the information available in the literature it is difficult to conclude the reasons for the results obtained in the present study and can only be speculated. Furthermore polyphenols have been shown to have antinutrient properties due to their ability to combine with different dietary components and interfering in their digestion (Butler and Rogler 1992).

Quercetin a polyphenolic compound present in many vegetables, fruits and seeds has been shown to possess anti-inflammatory, anti-carcinogenic and other beneficial properties. But quercetin has also been shown to have prooxidant property through which is produces

deleterious effects in male reproductive system of rodents such as decreasing sperm count, motility and also affects testicular histomorphology adversely (Farombi *et al.* 2012; Ranawat *et al.* 2013a). Similarly the EA and its metabolites might be acting as pro oxidant in the testis of chickens; however, this needs to be confirmed by further studies. Furthermore the distribution of quercetin and its metabolites are different among the tissues of the body, being lower in brain and concentrated higher in testis despite both having blood barriers (Ranawat *et al.* 2013b). Similar differential distributions of EA in the chicken may have produced contradictory results of reduced SOD activity in serum and increased seminal plasma lipid peroxidation.

The other possible reasons for the observed lack of effects of EA supplementation on the gross semen parameters may be due to lower dose levels applied in this study or the age of the roosters. The doses used were extrapolated from the report on rat (Pari and Sivasankari 2008) based on body surface area rule (Regan-shaw *et al.* 2007). Even after this dose calculation a lower level was used for the experiment considering the long duration of feeding and adverse effects reported for polyphenols on semen quality (Ranawat *et al.* 2013a). It was suggested that the opposing effects obtained by quercetin supplementation in rodents and human may be due to the dose and the redox state of the cell (Ranawat *et al.* 2013b). From the present experiment it was observed that the dose levels used may be lower and higher doses of supplementation should be tried to study any beneficial effects on semen in roosters. Another probable reason for lack of effect may be the age of the roosters. The age of the experimental roosters was 28 weeks at which time they must be around peak semen production. However the later duration of the experiment fell during summer period when there was steady increase in ambient temperature. This increasing temperature adversely affected the semen quality of the roosters as observed from the results. The beneficial effects of feeding EA, if any, should have been produced under these conditions but did not happen.

The results of the present study indicate that dietary supplementation of ellagic acid at the levels used in the study does not affect the gross semen parameters in roosters. Further work needs to be carried out to understand the mechanism of absorption, metabolism and excretion of ellagic acid in chicken.

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Final version can be accessed at Animal Production Science 55:107-112.
http://dx.doi.org/10.1071/AN13110

Table 1. Ingredient and nutrient composition of basal diet

Ingredient	g/kg diet
Yellow maize	565.97
Soybean meal	91.05
Sunflower meal	200.00
Deoiled rice bran	101.92
Salt	3.50
Shell grit	15.62
Di-calcium phosphate	16.22
DL-methionine	0.66
L-lysine HCl	1.01
Choline chloride, 50%	0.50
Vitamin/trace mineral premix[A]	1.25
Mycotoxin binder[B]	2.00
Antioxidant[C]	0.30
Nutrient composition[D]	
Metabolizable energy (MJ/kg)	10.88
Crude protein	160.00
Lysine	6.90
Methionine	3.50
Calcium	10.00
Available phosphorus	4.00

[A]Supplied (mg/kg diet): thiamin 1; pyridoxine, 2; cyanocobalamine, 0.01; niacin, 15; pantothenic acid, 10; a tocopherol, 10; riboflavin, 10; biotin, 0.08; menadione, 2; retinol acetate, 2.75; cholecalciferol, 0.06; choline, 650; copper, 8; iron, 45; manganese, 80; zinc, 60; selenium, 0.18.
[B]Hydrated sodium calcium alumino silicates, 800 mg/kg diet.
[C]Vitamin E 50 and selenium 0.15 mg/kg diet.
[D]Panda *et al.* 2012

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Table 2. Effect of dietary ellagic acid supplementation on semen parameters in Dahlem Red roosters. Values given are means[A].

Weeks	Ejaculate volume (ml)					Appearance (Score 1-5)					Sperm concentration (billions/ml)					MTT dye reduction (nm formazan/min /million sperms)				
	0	25	50	75	SEM[B]	0	25	50	75	SEM	0	25	50	75	SEM	0	25	50	75	SEM
	mg/kg					mg/kg					mg/kg					mg/kg				
1	0.58	0.60	0.60	0.73	0.034	3.5	3.6	3.8	3.8	0.10	5.8	5.4	5.6	5.5	0.15	13.6	12.0	13.3	15.1	0.70
2	0.53	0.55	0.47	0.62	0.031	3.7	3.4	3.5	4.2	0.11	6.1	5.6	5.4	6.1	0.16	14.6	13.6	13.6	14.8	0.50
3	0.55	0.53	0.45	0.64	0.041	3.9	3.7	3.5	3.7	0.13	6.2	5.2	4.8	5.9	0.18	17.0	18.5	16.5	17.8	0.65
4	0.60	0.61	0.52	0.70	0.047	3.5	3.6	3.6	3.7	0.11	6.0	5.5	5.8	6.0	0.17	13.1	11.5	14.0	12.0	0.70
5	0.53	0.50	0.45	0.59	0.040	3.9	3.7	3.8	4.1	0.13	6.1	5.9	5.8	6.1	0.21	11.4	13.0	15.3	13.0	0.83
6	0.50	0.53	0.45	0.63	0.035	3.8	3.8	3.7	4.1	0.11	5.8	5.7	5.2	6.1	0.19	12.1	10.6	13.0	12.3	1.04
7	0.48	0.51	0.44	0.59	0.031	3.4	4.2	3.8	4.0	0.14	5.4	5.6	5.1	6.1	0.27	14.0	14.1	13.6	15.0	0.67
8	0.48	0.48	0.46	0.59	0.045	3.3	3.4	3.6	3.4	0.16	4.7	4.9	4.7	5.1	0.31	12.5	12.0	14.0	11.0	0.83
	P-*value*																			
Trt	0.3852					0.6142					0.4024					0.5924				
Weeks	0.0004					0.0074					<.0001					<.0001				
Trt X Weeks	0.9974					0.1743					0.2112					0.3352				

[A]Each mean derived from ten observations
[B]Pooled standard error of mean

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Table 3. Effect of dietary ellagic acid supplementation on semen parameters in Dahlem Red roosters. Values given are means[A].

Weeks	Sperm motility (%)					Live sperms (%)					Dead sperms (%)					Abnormal sperms (%)				
	0	25	50	75	SEM[B]	0	25	50	75	SEM	0	25	50	75	SEM	0	25	50	75	SEM
	mg/kg					mg/kg					mg/kg					mg/kg				
1	47	40	48	49	3	74.4	72.1	70.5	72.9	3.5	25.6	27.9	29.5	27.1	3.5	3.6	3.2	1.1	2.4	0.3
2	49	56	54	50	2	88.5	86.0	81.0	82.3	1.9	11.5	14.0	19.3	17.7	1.9	3.7	2.8	1.2	2.8	0.4
3	56	53	48	52	2	89.0	86.0	75.5	80.2	1.9	11.1	14.1	24.5	20.0	1.9	7.3	4.4	3.7	2.3	0.8
4	49	48	49	43	2	91.4	86.4	89.3	89.5	1.2	8.6	13.5	10.7	10.5	1.2	4.9	3.9	3.6	1.8	0.7
5	40	37	42	37	2	87.5	86.5	82.5	89.0	1.3	12.5	13.5	17.5	11.3	1.3	6.2	4.8	3.5	2.2	0.8
6	42	43	37	41	2	85.8	79.8	84.2	87.1	1.1	14.1	20.2	15.8	12.9	1.1	4.4	5.4	3.2	2.2	0.8
7	37	31	41	38	2	87.5	84.0	82.4	82.9	1.3	12.5	16.1	17.6	17.1	1.3	7.7	5.5	2.2	1.4	1.2
8	23	24	29	23	2	69.6	73.1	72.7	79.4	3.2	20.4	26.9	16.2	20.6	1.8	2.3	3.9	2.4	1.4	0.5
	P-*value*																			
Trt	0.9627					0.6758					0.4145					0.0604				
Weeks	<.0001					<.0001					0.0002					0.0586				
Trt X Weeks	0.4426					0.3208					0.1095					0.009				

[A]Each mean derived from ten observations
[B]Pooled standard error of mean

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Table 4. Effect of dietary ellagic acid supplementation on seminal plasma parameters in Dahlem Red roosters. Values given are means[A].

Weeks	Protein (g/dL)					Alkaline phosphatase (U/L)					Lipid peroxidation (moles of MDA/g Protein)				
	0	25	50	75	SEM[B]	0	25	50	75	SEM	0	25	50	75	SEM
	mg/kg					mg/kg					mg/kg				
1	1.17	0.85	0.81	0.99	0.04	53.45	36.01	33.2	53.25	3.73	0.90	1.12	1.23	1.44	0.07
2	0.95	0.71	1.00	0.92	0.06	43.53	29.69	57.47	58.18	4.39	0.98	1.12	1.27	1.43	0.06
3	1.16	0.73	0.66	0.81	0.06	69.81	54.96	70.31	77.83	3.87	0.85	1.11	1.31	1.41	0.09
4	1.0	0.66	0.51	0.73	0.06	82.04	60.23	61.47	102.39	5.41	1.01	1.17	0.87	1.35	0.08
5	0.81	0.60	0.90	0.90	0.05	122.43	69.47	113.71	176.22	12.14	1.07	1.09	1.16	1.44	0.05
6	1.03	0.68	0.85	0.74	0.05	83.59	75.70	94.29	63.96	9.75	0.76	1.33	1.64	1.69	0.11
7	0.98	0.72	0.75	0.80	0.04	86.01	99.80	78.62	81.77	5.84	0.86	1.30	0.96	1.32	0.09
8	0.93	0.66	0.74	0.81	0.03	93.99	90.54	67.10	86.01	6.64	0.81	0.87	0.77	1.12	0.06
						P-*value*									
Trt			<0.0001					0.062					<0.0001		
Weeks			<0.0001					<0.0001					<0.0001		
Trt X Weeks			0.0002					<0.0001					0.0015		

[A]Each mean derived from five observations
[B]Pooled standard error of mean

Final version can be accessed at Animal Production Science 55:107-112.
http://dx.doi.org/10.1071/AN13110

Table 5. Effect of dietary ellagic acid supplementation on serum antioxidant variables and lipid peroxidation level in Dahlem Red roosters. Values given are means[A].

Ellagic acid (mg/kg)	Superoxide dismutase (U/mg protein)	FRAPS (μmoles/L)	Lipid peroxidation (nmoles MDA/ml)
0	77.25[ab]	2675.8	0.87
25	78.19[ab]	2693.8	0.97
50	94.59[a]	2648.3	0.88
75	41.06[c]	2722.2	0.65
SEM[B]	6.54	67.4	0.05

[a, b, c]Means within a column with different superscripts differ significantly (P<0.05).
[A]Each mean derived from ten observations
[B] Pooled standard error of mean