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The detoxication of nitrate by two antioxidants or a probiotic, and the effects on blood and seminal plasma profiles and reproductive function of New Zealand White rabbit bucks

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Forty-two New Zealand White male rabbits were housed individually in wire cages and randomly distributed among six experimental groups of seven rabbits each, during 16 to 61 weeks of age. There were three main nitrate groups: 0 (tap water), 350 and 700 ppm. Within the 700 ppm of nitrate, there were four subgroups, in which one group was used as control group and the other three groups were supplemented with either 200 ppm of ascorbic acid (vitamin (Vit) C), 200 ppm of Vit E with 0.2 ppm of selenium (Se) and 1000 ppm of probiotic. The nitrate was supplemented as a sodium nitrate. The aim is to test the ability of Vit C and Vit E, Se and probiotic on the deleterious effects (blood and seminal plasma biochemical constituents, semen quality and productive performance) of nitrate in drinking water. Rabbits given nitrate at 700 ppm had significantly lower plasma globulin, red blood cells (RBCs), hemoglobin (Hgb), packed cell volume % (PCV%) and total antioxidant capacity (TAC) than those given the other concentrations of nitrate. Vit C, Vit E with Se and probiotic resulted in significantly ($P < 0.05$) greater Hgb, RBCs, PCV% and TAC than those of bucks given water supplemented with only 700 ppm nitrate, but the aspartate aminotransferase and alanine aminotransferase concentrations in seminal plasma were lower. Testosterone in the blood plasma and the seminal plasma was significantly ($P < 0.05$) lower in rabbits given 700 ppm nitrate than in those given other concentrations of nitrate. Vit C, Vit E with Se and the probiotic significantly increased testosterone, fertility, number of offspring and total offspring weight of rabbits sired by bucks supplemented with 700 ppm of nitrate.

Keywords: rabbits, nitrate, semen quality, reproductive performance

Implication

Worldwide, nitrate contamination presents a major problem for animal and human health and production. Concentration of nitrate has gradually increased in many countries. An increase in nitrate in ground water in many European countries was shown, and concentration above 50 mg/l was reported (Fried, 1991). Similar trend was recently reported in the United States (Burow *et al.*, 2010), China (Zhang *et al.*, 1996), New Zealand (Thomson *et al.*, 2007), India (Chaudhary *et al.*, 2010), Nigeria (Okafor and Ogbonna, 2003), Saudi Arabia (Alabdula'aly *et al.*, 2010) and Jordan (Obeidat *et al.*, 2007). In Egypt, nitrate concentration in the ground water varies widely from 66.5 to 265.8 mg/l water, and this has shown to increase because of increasing agricultural activity and linked to many human health problems (Saleh *et al.*, 1998). In addition to the increase in nitrate in ground water, there is an increase in nitrate intakes in foods, which ranged from 1 to 7410 mg/kg for carrots and spinach, respectively (Hord *et al.*, 2009). Detoxication of nitrate could improve human health and performance of farm animals. Antioxidants could play a significant role in detoxication of nitrate and reduce their negative effects on animal performance in areas of high nitrate pollution.

Introduction

Nitrate is a serious problem worldwide, and recent research indicated that nitrate in ground and well water is increasing (Manassaram *et al.*, 2006; Burow *et al.*, 2010; Cockburn *et al.*, 2010; Ward *et al.*, 2010). The use of high levels of nitrate on rabbit performance was investigated by Gilman *et al.* (1998); Bassuny *et al.* (2004); Shehata (2005); and Djekoun-Bensoltane *et al.* (2007). They showed that nitrate concentration at 600 to 700 ppm had a deleterious effect on rabbits' performance, but no attention was given to semen quality and litter traits. The adverse effect of nitrate was obvious with increasing nitrate intake in animals and humans (Shehata, 2005; Djekoun-Bensoltane *et al.*, 2007; Hord *et al.*, 2009). Nitrate pollution

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could be increased because of agro-industry (Wahaab and Badawy, 2004; Burow *et al*., 2010). Ingestion of high concentrations of nitrates (NO_3) builds up nitrite (NO_2) concentrations and the nitrite is absorbed into the animal's bloodstream. Once in the bloodstream, nitrites bind with hemoglobin (Hgb), creating methemoglobin and preventing the normal transfer of oxygen (Shehata, 2005). Depending on the amounts consumed, symptoms of nitrate poisoning may be hardly noticeable in ruminant animals whose gut microbial populations are able to convert nitrites to ammonia and ultimately to amino acids and proteins (Atef *et al*., 1991; Bassuny *et al*., 2004). However, nonruminant animals are unable to carry out this conversion (Robson, 2007). Low nitrate intake often causes lower milk production, abortion, breeding problems or symptoms that mimic a nutritional deficiency (vitamin (Vit) A and E, rickets, phosphorus or calcium imbalance). Signs of acute nitrate toxicity are a result of severe inhibition of oxygen transfer and are strikingly obvious. Symptoms include bluish color of mucous membranes, rapid and difficult breathing, a rapid pulse ($>$150 beats/min), tremors, staggering, collapse and death (Bassuny *et al*., 2004). In addition, nitrites react with secondary amines in food to form nitrosamines, many of which are carcinogenic in experimental animals and exert other toxic effects (Shehata, 2005).

Increasing body reserves of antioxidants may help animals cope with the production of free radicals (oxidants) by eliminating the harmful effect of nitrate (Roth, 2000) and inhibiting the oxidation reactions (Botsoglou *et al*., 2004). In the literature, an adverse effect of nitrate in animal performance was observed at 600 to 700 ppm. In the literature, the efficacy of Vit C on detoxication of nitrate and mercury was reported (Bassuny *et al*., 2004; Shehata, 2005; Yasmina and Abdennour, 2008). In addition, Umar *et al.* (2010) demonstrated the antioxidant effect of Vit E and C. The basis for using the probiotic is to determine its ability to convert nitrite to ammonia and ultimately to amino acids and proteins (Atef *et al*., 1991; Robson, 2007; Martarelli *et al*., 2011).

Vitamins such as α-tocopherol is a natural antioxidant that protects cellular membranes against oxidative damage (Morrissey *et al*., 1994; Chow and Hong, 2002). Ascorbic acid (Vit C) is an electron donor and reducing agent, and this property accounts for all its known functions. As an electron donor, Vit C is a potent water-soluble antioxidant in many experiments *in vitro* (Attia *et al*., 2009 and 2011). In addition, Vit C can reduce regenerates of α-tocopherol from its oxidation form (Reed, 1992). Another element implicated in the degradation of hydroperoxides is selenium (Se). Se is a cofactor of glutathione peroxidase, the enzyme that catalyzes the degradation of peroxides (Castellini *et al*., 2002; Attia *et al*., 2010). Probiotics are live microorganisms that could improve gastrointestinal tract microflora and confer health benefits to the host and improve feed utilization (Rolfe, 2000; Montville and Mattews, 2005; Kannan *et al*., 2007), and have been recently shown to decrease oxidative stress and neutralizing reactive oxygen species (Martarelli *et al*., 2011).

The effect of nitrate on semen quality and reproductive performance of rabbit bucks needs further research. Hence, we aim to determine the effects of the different nitrate concentrations (0, 350 and 700 ppm) in drinking water, on blood and seminal plasma biochemical constituents, semen quality, reproductive traits and histopathology changes of testes of New Zealand White (NZW) rabbit bucks, and determine the capability of Vit C and Vit E with Se and probiotic on the deleterious effects of nitrate in drinking water.

Material and methods

Animals and dietary treatments

Forty-two, 16-week-old NZW male rabbits, having an average BW of 2180 $\pm$ 50.8 g, were distributed randomly among six treatment groups of seven rabbits each, during 16 to 61 weeks of age.

The treatments included three main groups in which nitrate concentrations were 0 (tap water), 350 and 700 ppm. Only within the 700 ppm group, there were four subgroups in which one group was used as the control group and the other three groups were supplemented with either 200 ppm of Vit C (United Company for Chemicals and Medical preparation, Cairo, Egypt) or 200 ppm of Vit E with 0.2 ppm of Se as a sodium selenite (United company for Chemicals and Medical Preparation) or 1000 ppm of a probiotic containing *Lactobacillus acidophilus*, *Saccharomyces cerevisiae*, sodium chloride, monobasic potassium phosphate, sodium bicarbonate and dextrose (Vetapharm Company, Cairo, Egypt). The nitrate was supplemented as sodium nitrate (Misr Company for Chemical and Medical Preparation, Cairo, Egypt).

The nitrate content in the tap water used was 14 ppm, whereas nitrate content in the well water was 7 ppm, and that in the agricultural drainage water was 12 ppm. The nitrate content in feed was 50 ppm. Determination of nitrate contents in feeds and water was according to Association of Official Analytical Chemists (AOAC, 2007) (method number 968.07). The experiment was approved by the committee of Department of Animal and Poultry Production, Faculty of Agriculture, Damanhour University.

Housing and management

Rabbits were individually caged in an open-system rabbitry and housed in galvanized wire cage batteries with standard dimensions. All cages were provided with a manual feeder and clean fresh water was available continuously. Rabbits were fed on commercial pellet diet and offered water *ad libitum*. Rabbits were kept under the same hygienic (health and vaccination program) and environmental (temperature (27°C), relative humidity (58%) and day length) conditions during the experimental period. Light–dark cycle was 1410 h daily.

Diet nutrient profiles

The diet was commercial pelleted composed mainly of hay barley, wheat bran, maize, soybean meal, dicalcium phosphate, sodium chloride and vitamin and mineral premix. Chemical analysis of the diets as feed basis was done according to AOAC (2007) showed that dry matter, organic

matter, crude protein, crude fiber, ether extract, nitrogen free extract and ash, whereas NDF and ADF (Van Soest and Wine, 1967) and hemicellulose contents by difference were 89.74%, 90.59%, 16.17%, 13.71%, 2.51%, 58.20%, 9.41%, 32.31%, 15.9% and 16.41%, respectively.

Collection of data

Blood hematological criteria. Hgb concentration was determined using a hemoglobinometer, according to the method by Tietz (1982). Red blood cells (RBCs) were counted on bright line hemocytometer using light microscope at 400× magnification, according to the method of Helper (1966) and Hawkey and Dennett (1989). White blood cells (WBCs) were assessed according to the method of Helper (1966) and Hawkey and Dennett (1989) using a light microscope at 100× magnification. Blood film was prepared according to the method described by Lucky (1977) to determine different leukocytes count. Ten drops from any gunwale stain stack solution, a dry, unfixed smear were added to equal amount of distilled water, then mixed and left for 1 min for staining. The dye was 'de courted' without rinsing. Diluted Giemsa's solution (10 drops of the day were added to 10 ml distilled water) was poured over the film as counter stain and left for 20 min, then rounded in water current and absolute value for each type of cells was calculated.

Packed cell volume (PCV; %) was determined according to Wintrobe (1965) by Wintrobe Hematocrit tubes after centrifugation for 20 min at 2000 × **g**. The mean cell volume (MCV), the mean cell hemoglobin (MCH) and the mean cell hemoglobin concentration (MCHC) were calculated as absolute values:

$$\text{MCV}\,(\mu\text{m}^3 \,/\, \text{red blood cell}) = \frac{\text{Hematocrit} \times 10}{\text{Number of RBCs}}$$

$$\text{MCH}\,(\mu\text{g}) = \frac{\text{Hemoglobin concentration}\ (\text{g/dl}) \times 10}{\text{Number of RBCs}}$$

Phagocytoses. Phagocytic activity (PA) was determined according to Kawahara *et al.* (1991). *Candida albicans* culture (50 μg) was added to 1 ml of citrated blood and shaken in water bath at 23°C to 25°C for 3 to 5 h, smears of the blood was then stained with Giemsa solution.

Phagocytic index (PI) was estimated by determining the proportion of macrophages, which contained intracellular yeast cell in a random count of 300 macrophages and expressed as percentage of PA. The number of phagocytized organisms was counted in the phagocytic cells and called PI (P1).

PA = Percentage of phagocytic cells containing yeast cells.

PI = Number of yeast cells phagocytes Number of phagocytic cells.

Biochemical constituents of blood and seminal plasma. At 24, 32, 48 and 56 weeks of age, three blood samples per treatment were randomly collected from marginal ear vein under vacuum in clean heparinized tubes for determination of biochemical and hematological criteria. At the same ages, seminal plasma was collected. The plasma of both fluids was obtained after centrifugation for 20 min at 2000 × **g** and the plasma was frozen at −18.0°C for analyses of biochemical constituents.

Plasma and seminal plasma total protein were measured by the methods Doumas *et al.* (1981). Plasma and seminal plasma albumin were determined according to Reinhold (1953). Plasma and seminal plasma globulin were determined by subtracting the total plasma albumin from total plasma protein, according to Coles (1974). Blood plasma total cholesterol was determined according to the method of Watson (1960). Seminal plasma urea concentration was determined according to the special kits produced by Diamond diagnostics (23 EL-Montazah St. Heliopolis, Cairo, Egypt, http://www.diamonddiagnostics.com). Seminal plasma creatinine concentration was measured using special kits delivered from N.S. BIOTEC (http://www.nsbiotec.com).

Plasma total antioxidant capacity (TAC) was determined as a mean of antioxidant status and/or indirect indicator of oxidative stress induced by nitrate. It was determined using commercial kits produced by Diamond diagnostics (23 EL-Montazah St. Heliopolis, Cairo, Egypt, http://www.diamonddiagnostics.com).

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as (U/L) in the seminal plasma were determined, according to Reitman and Frankel (1957), using commercial kits produced by Pasteur Lab (http://www.pasteurvetlab.com).

Blood and seminal plasma testosterone. Blood and seminal plasma testosterone were determined by using special kits delivered from Dia Metra Com, Foligno, Italy.

Semen quality traits. Semen was collected weekly from all bucks at 28 weeks of age and continued for 16 weeks to determine semen quality, according to Attia *et al.* (2011) and Attia and Kamel (2012). Semen collection was carried out using artificial vagina. Its internal temperature at the time of collection was kept in the range of 41°C to 44°C. Throughout the course of semen collection time, the place of collection and collector were not changed. Moreover, special attention was given to protect semen ejaculator from cold shock and direct light. The semen samples were transferred immediately after collection to laboratory where it was placed in a water bath (at 37°C) to determine spermatozoa quality.

Semen volume was measured directly by graduated collecting tubes to the nearest 0.1 ml. Percentage of progressively motile spermatozoa in a phase-contrast microscope, with warm stage, adjusted at temperature of 37°C was used to examine the motility of the spermatozoa. A small drop of raw semen and a few drops of warm sodium citrate buffer (2.9%) were deposited on a warm slide, mixed and covered with a cover slip. The advanced motility was observed at a power of 400×, and an average of five fields was obtained.

The percentage scale used was between 0 and 100 in an increment of 5%.

Estimation of the total number of spermatozoa per ml of semen was carried out by using direct count on a hemocytometer chamber, according to the procedure described by Bratton *et al.* (1956). Two subsamples from each ejaculate were diluted with 0.9% NaCl. Few drops of 20% formalin solution (instead of boric acid in Bratton's method) were added to the NaCl solution to toxicate spermatozoa to avoid its movement during counting. Semen subsamples were diluted at a rate of 1 : 200 times. Five minutes were allowed before counting to allow spermatozoa to be settled down on the hemocytometer slide. Sperm concentration per ml semen was calculated from the dilution rate of the subsample, and the mean sperm count/mm^3 of the hemocytometer. pH value for semen samples was measured by using pH paper.

Duplicated stained smears from each freshly collected ejaculate were prepared to determine the numbers of live, dead and abnormal spermatozoa and their percentage. Eosin–nigrosin stain was used to differentiate the dead and live spermatozoa, according to Blom (1950). A total of 200 sperm cells were examined randomly for each smear using oil immerimmer sead lens. Normal live sperm exclude the eosin stain and appear white in color, whereas dead sperm take up eosin and appear pinkish in color because of the loss of membrane integrity. Normal sperm have an oval head with a long tail. Abnormal sperm have head, midpiece or tail defects, such as a large or misshapen head or a crooked or double tail.

The total number of spermatozoa/ejaculate (sperm output) was calculated by multiplying semen ejaculate volume and sperm concentration.

Reproductive traits. At 32 weeks of age, reproductive efficiencies of bucks such as fertility, number of offspring, total of offspring weight, number of weaned offspring, total weight of weaned offspring, kid weight and livability were evaluated using four bucks per treatment. Bucks were met with five NZW untreated female per treatment.

Histopathology of testes. Tissue specimens ($n = 3$ per treatments) were collected from the slaughtered bucks and fixed in 10% neutral buffered formalin solution for at least 24 h. After fixation, specimens were washed in tap water and then passed through the routine paraffin embedding technique (dehydration in ascending grades of the ethyl alcohol, clearing in a series of xylene and then passed through a series of melted paraffin wax, embedded and put in paraffin blocks). Later on, the paraffin blocks were subjected for microtome to prepare paraffin sections of 3- to 5-μm thickness, which were stained with Mayer's hematoxylin and eosin (Culling, 1983), and then examined under a microscope.

Statistical analysis

Data were statistically analyzed using GLM procedure of the statistical analysis system of SAS Institute (2002). The main effect was nitrate concentration and the effect Vit C, Vit E with Se and probiotics within the high concentration of nitrate. A *P*-value of $\leqslant 0.05$ significance of Student–Newman–Keuls test was used for testing mean differences among the experimental groups. Before analysis, all the percentages were subjected to logarithmic transformation to normalize data distribution.

Results

Blood hematology

Table 1 showed the effects of different treatments on hematological constituents of blood. There was dose–response-negative effect of only 700 ppm of nitrate on RBCs, Hgb, PCV and MCHC, but the effect was linear and positive for MCV. The effect on MCH was positive only at 700 ppm of nitrate. The RBCs, Hgb and PCV were lower by 50.6%, 19.1% and 31.2%, respectively, of only group given700 ppm nitrate than those of the control group. Differences between the control group and given water contained 350 ppm nitrate were not significant.

Table 1 *Effects of different nitrate concentrations in drinking water and the supplementation of Vit C (200 ppm) or Vit E with Se (200 ppm + 0.2 mg) or probiotic (1000 ppm) on blood hematology of NZW rabbit bucks (mean ± s.d.)*

Nitrate (ppm)	Supplement/l water	WBCs (10^3)	RBCs (10^6)	Hgb (g/dl)	PCV (%)	MCV (micron^3/RBC)	MCH (μg)	MCHC (%)
Main effects of nitrate								
0	0	1.67 ± 0.16	7.96 ± 1.59^a	9.33 ± 1.10^a	37.8 ± 8.9^a	47.9 ± 5.2^c	11.8 ± 1.3^b	24.6 ± 4.9^a
350	0	1.71 ± 0.07	6.53 ± 0.42^a	8.81 ± 0.10^a	38.5 ± 9.9^a	59.2 ± 5.2^b	13.5 ± 0.4^b	22.9 ± 4.9^b
700	0	2.10 ± 0.21	3.94 ± 0.55^b	6.42 ± 1.00^b	30.6 ± 4.8^b	78.5 ± 2.8^a	16.4 ± 0.8^a	20.9 ± 2.9^b
P-value		ns	0.03	0.02	0.01	0.01	0.01	0.03
Interaction between nitrate level and type of supplement								
700	0	2.10 ± 0.21	3.94 ± 0.55^b	6.42 ± 1.00^b	30.6 ± 4.8^b	78.5 ± 2.8^a	16.41 ± 0.81^a	20.9 ± 2.9^b
700	Vit C	1.82 ± 0.15	7.94 ± 0.35^a	9.01 ± 0.58^a	36.6 ± 4.4^a	46.3 ± 2.4^c	11.42 ± 0.62^b	24.6 ± 2.8^a
700	Vit E with Se	1.92 ± 0.19	7.44 ± 0.49^a	8.92 ± 0.82^a	38.1 ± 5.9^a	51.5 ± 3.1^b	12.13 ± 0.95^b	23.4 ± 3.5^a
700	Probiotic	1.91 ± 0.15	7.53 ± 0.49^a	9.00 ± 1.10^a	37.7 ± 6.5^a	50.3 ± 3.4^b	12.01 ± 0.86^b	23.9 ± 3.8^a
P-value		ns	0.03	0.01	0.01	0.01	0.01	0.03

Vit = vitamin; Se = selenium; NZW = New Zealand White; WBCs = white blood cells; RBCs = red blood cells; Hgb = hemoglobin; PCV = packed cell volume; MCV = mean cell volume; MCH = mean cell hemoglobin; MCHC = mean cell hemoglobin concentration; ns = not significant.
$n = 12$ observations per treatment.
[a,b,c]Means within a column under similar treatment with different letters are significantly different ($P \leqslant 0.05$).

Addition of Vit C or Vit E with Se or probiotic significantly improved RBCs, Hgb, PCV, MCV, MCH and MCHC than those supplemented with only 700 ppm nitrate, and Vit C was the most potent agent for improving MCV.

WBCs, types of WBCs, PA and PI

Nitrate concentration up to 700 ppm and supplementation of Vit C or Vit E with Se or probiotic had no effect on WBCs (Table 1) and differential WBCs, nor a significant difference on the percentage of heterophil, heterophil/lymphocyte ratio, PA and PI as immune indices (Table 2).

Biochemical constituents of blood plasma

Table 3 summarized biochemical constituents of blood plasma of NZW bucks. There was a dose–response-negative effect of nitrate concentration at only 700 ppm on plasma globulin, TAC and testosterone, but differences in plasma total protein and albumin concentrations were not significant.

Globulin of probiotic-supplemented group was significantly greater than the 700 ppm nitrate basal group. TAC and testosterone were similarly recovered because of supplementation of Vit C, E with Se and probiotic.

Seminal plasma

Table 4 shows the effects of different treatments on seminal plasma biochemical constituents and testosterone. There was a dose–response-negative effect of nitrate concentration at only 700 ppm on seminal plasma total protein, albumin and testosterone concentrations, but seminal plasma urea, creatinine concentrations, AST and ALT activity increased. Vit C or Vit E with Se or probiotic similarly overcomes the negative effect of high concentration of nitrate (700 ppm).

Semen quality

The results of semen quality of the different treatments are shown in Tables 5 and 6. There was a dose–response-negative effect of nitrate concentration at only 700 ppm on most of the semen quality traits. The results showed that semen color, semen viscosity, live sperm (%), advanced motility (%), sperm concentration and total sperm output and total live sperm were significantly lower than the control and 350 ppm nitrate-supplemented group, but other semen quality characteristics were not affected.

Reproductive traits

Table 7 demonstrates the effects of different treatments on reproductive traits of NZW bucks. A significant negative effect of 700 ppm nitrate concentration on most of the criteria of reproductive traits, but number of weaned offspring, the total weight of weaned offspring, kid's weight at 28 days of age and livability at 28 days of age were not affected. The supplementation of Vit C or Vit E with Se or probiotic induced a significant greater fertility (%), number of offspring and total offspring weight than 700 ppm nitrate basal group; however, Vit C induced the greatest improvement in only fertility.

Table 2 *Effects of different nitrate concentrations in drinking water and the supplementation of Vit C (200 ppm) or Vit E with Se (200ppm+0.2 mg) or probiotic (1000 ppm) on differential WBC counts, phagocyte activity and phagocyte index of NZW rabbit bucks (mean ± s.d.)*

Nitrate (ppm)	Supplement/l water	Lymphocyte (10^3)	Heterophil (10^3)	Heterophil : lymphocyte ratio	Monocyte (10^3)	Basophil (10^3)	Eosinophil (10^3)	Phagocytic activity	Phagocytic index
Main effects of nitrate									
0	0	47.3 ± 3.9	33.6 ± 4.8	72.3 ± 15.3	1.67 ± 0.53	8.15 ± 1.14	9.62 ± 0.52	20.7 ± 0.58	1.91 ± 0.15
350	0	45.5 ± 2.5	36.6 ± 2.3	81.4 ± 8.6	1.41 ± 0.50	7.73 ± 0.90	8.92 ± 1.04	21.5 ± 2.12	1.91 ± 0.14
700	0	46.5 ± 3.3	34.8 ± 3.5	75.6 ± 12.7	1.45 ± 0.52	7.91 ± 0.90	9.31 ± 0.78	21.0 ± 1.00	2.00 ± 0.05
P-value		ns	ns	ns	ns	ns	ns	ns	ns
Interaction between nitrate level and type of supplement									
700	0	46.5 ± 3.3	34.8 ± 3.5	75.6 ± 12.7	1.45 ± 0.52	7.91 ± 0.90	9.31 ± 0.78	21.0 ± 1.00	2.00 ± 0.05
700	Vit C	47.0 ± 2.5	34.7 ± 3.6	74.4 ± 11.4	1.65 ± 0.53	7.65 ± 1.22	9.00 ± 0.89	21.3 ± 1.53	2.10 ± 0.20
700	Vit E with Se	46.2 ± 2.9	35.4 ± 2.7	76.8 ± 10.9	1.54 ± 0.53	7.94 ± 0.99	9.21 ± 0.80	20.3 ± 0.96	2.10 ± 0.05
700	Probiotic	47.3 ± 2.7	33.3 ± 2.7	70.7 ± 9.5	1.74 ± 0.49	8.65 ± 0.99	9.11 ± 0.79	20.3 ± 0.58	2.10 ± 0.06
P-value		ns	ns	ns	ns	ns	ns	ns	ns

Vit = vitamin; Se = selenium; WBC = white blood cell; NZW = New Zealand White; ns = not Significant.
n = 12 observations per treatment.

Table 3 *Effects of different nitrate concentrations in drinking water and the supplementation of Vit C (200 ppm) or Vit E with Se (200 ppm + 0.2 mg) or probiotic (1000 ppm) on biochemical constituents and testosterone of blood plasma of NZW rabbit bucks (mean±s.d.)*

		Biochemical constituents of blood plasma					
Nitrate (ppm)	Supplement /l water	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Cholesterol (g/dl)	Total antioxidant capacity (Mm/l)	Testosterone (ng/ml)
Main effects of nitrate							
0	0	6.82 ± 1.74	4.62 ± 1.26	2.22 ± 1.54[a]	36.2 ± 19.9	1.24 ± 0.14[a]	7.89 ± 0.96[a]
350	0	6.81 ± 1.98	4.61 ± 0.87	2.21 ± 1.15[a]	39.1 ± 25.1	1.09 ± 0.11[a]	6.95 ± 1.55[a]
700	0	6.33 ± 2.39	4.53 ± 1.49	1.88 ± 0.78[b]	38.4 ± 17.3	0.55 ± 0.26[b]	4.06 ± 0.82[b]
P-value		ns	ns	0.01	ns	0.001	0.004
Interaction between nitrate level and type of supplement							
700	0	6.33 ± 2.39	4.53 ± 1.49	1.88 ± 0.78[b]	38.4 ± 17.3	0.55 ± 0.26[b]	4.06 ± 0.82[b]
700	Vit C	6.52 ± 1.89	4.34 ± 0.77	2.22 ± 1.61[ab]	37.6 ± 22.6	1.63 ± 0.28[a]	9.50 ± 0.89[a]
700	Vit E with Se	6.52 ± 1.78	4.21 ± 0.86	2.31 ± 1.33[ab]	37.1 ± 11.2	1.61 ± 0.28[a]	8.18 ± 1.34[a]
700	Probiotic	6.93 ± 1.66	4.03 ± 1.66	2.99 ± 0.71[a]	37.4 ± 21.3	1.63 ± 0.30[a]	9.60 ± 0.73[a]
P-value		ns	ns	0.02	ns	0.0001	0.0001

Vit = vitamin; Se = selenium; NZW = New Zealand White; ns = not significant.
$n = 12$ observations per treatment.
[a,b]Means within a column under similar treatment with different letters are significantly different ($P \leqslant 0.05$).

Table 4 *Effect of different nitrate concentrations in drinking water and the supplementation of Vit C (200 ppm) or Vit E with Se (200 ppm + 0.2 mg) or probiotic (1000 ppm) on biochemical constituents of seminal plasma of NZW rabbit bucks (mean ± s.d.)*

		Seminal plasma							
Nitrate (ppm)	Supplement /l water	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Urea (g/dl)	Creatinine (g/dl)	AST (U/L)	ALT (U/L)	Testosterone (ng/ml)
Main effects of nitrate									
0	0	4.50 ± 0.56[a]	3.33 ± 0.47[a]	1.17 ± 0.22	41.2 ± 2.9[b]	0.94 ± 0.00[b]	20.1 ± 1.4[b]	21.4 ± 3.1[b]	5.10 ± 0.88[a]
350	0	4.17 ± 0.40[a]	3.02 ± 0.47[a]	1.15 ± 0.18	42.6 ± 3.3[b]	1.07 ± 0.09[b]	26.9 ± 2.9[b]	25.4 ± 4.2[b]	5.04 ± 0.74[a]
700	0	2.88 ± 0.58[b]	1.76 ± 0.47[b]	1.12 ± 0.34	46.7 ± 2.5[a]	1.39 ± 0.09[a]	37.6 ± 4.3[a]	37.7 ± 4.8[a]	3.20 ± 0.68[b]
P-value		0.0028	0.0020	ns	0.001	0.0028	0.0001	0.0005	0.0001
Interaction between nitrate level and type of supplement									
700	0	2.88 ± 0.58[b]	1.76 ± 0.47[b]	1.12 ± 0.34	46.7 ± 2.5[a]	1.39 ± 0.09[a]	37.6 ± 4.3[a]	37.7 ± 4.8[a]	3.20 ± 0.68[b]
700	Vit C	5.18 ± 1.14[a]	4.07 ± 1.19[a]	1.11 ± 0.17	40.9 ± 1.9[b]	1.06 ± 0.11[b]	26.1 ± 4.9[b]	24.2 ± 4.9[b]	6.47 ± 1.41[a]
700	Vit E with Se	6.27 ± 1.79[a]	5.24 ± 1.96[a]	1.03 ± 0.31	40.5 ± 1.5[b]	1.00 ± 0.11[b]	25.2 ± 3.3[b]	21.1 ± 3.7[b]	7.29 ± 1.68[a]
700	Probiotic	7.46 ± 1.60[a]	6.27 ± 1.35[a]	1.19 ± 0.38	40.8 ± 0.7[b]	0.94 ± 0.08[b]	20.9 ± 3.1[b]	23.2 ± 3.0[b]	7.80 ± 1.32[a]
P-value		0.0001	0.0001	ns	0.0001	0.0001	0.0001	0.0001	0.0001

Vit = vitamin; Se = selenium; NZW = New Zealand White; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ns = not significant.
$n = 10$ per treatment.
[a,b]Means within a column under similar treatment with different letters are significantly different ($P \leqslant 0.05$).

Histopathologic examination of the testes

The testes of control rabbit bucks revealed normal histology with presence of normal spermatozoal contents inside the lumina of the seminiferous tubules filled with spermatogenesis (Figure 1). The testes in case of administration with 350 ppm nitrate in drinking water showed moderate changes of less spermatogenesis and vacuolation in the seminiferous tubules (Figure 2). The testes in some cases of administration with 700 ppm nitrate in drinking water showed severe changes of damaged and necrotic seminiferous tubules widely separated with edema (Figure 3), whereas in other cases severe degenerative changes with central necrosis in the seminiferous tubules appeared lined with only one layer of degenerated spermatogonial cells (Figure 4). The reaction was more prominent in the two data points at 5 and 10 months of age. The supplementation with Vit C, Vit E and Se or probiotic had a beneficial effect on reducing the severity of the abnormal testicular changes induced by administration with 700 ppm nitrate. The testes in case of supplementation of Vit C or Vit E and Se showed moderate changes of less spermatogenesis in the seminiferous tubules (Figure 5), whereas the testes in case of supplementation of probiotics showed nearly normal seminiferous tubules (Figure 6).

Discussion

Nitrate effects

Nitrate up to 350 ppm had no harmful effect on RBCs, Hgb and the PCV, but 700 ppm did, and this concurred with adaptive changes in MCV, MCH and MCHC. These are in

Table 5 *Effects of different nitrate concentrations in drinking water and the supplementation of Vit C (200 ppm) or Vit E with Se (200 ppm + 0.2 mg) or probiotic (1000 ppm) on age at first ejaculate and semen characteristics of NZW rabbit bucks (mean ± s.d.)*

			Semen characteristics‡					
Nitrate (ppm)	Supplement /l water	Age at first ejaculate (days)†	pH	Sperm concentration (10^7)	Total sperm out put (10^9)	Total live sperm (10^9)	Total dead sperm (10^9)	Total abnormal sperm (10^9)
Main effects of nitrate								
0	0	160 ± 11^{b}	7.83 ± 0.25	28.8 ± 6.0^{a}	28.5 ± 8.8^{a}	19.7 ± 8.0^{a}	1.11 ± 0.55^{b}	1.32 ± 0.72^{b}
350	0	160 ± 11^{b}	7.81 ± 0.25	25.8 ± 9.4^{a}	24.3 ± 7.9^{a}	21.7 ± 7.3^{a}	1.33 ± 0.49^{b}	1.33 ± 0.53^{b}
700	0	178 ± 18^{a}	8.11 ± 0.22	17.2 ± 5.6^{b}	13.9 ± 5.1^{b}	11.8 ± 4.4^{b}	1.92 ± 0.35^{a}	1.71 ± 0.56^{a}
P-value		0.01	ns	0.03	0.008	0.002	0.006	0.01
Interaction between nitrate level and type of supplement								
700	0	178 ± 18^{a}	8.11 ± 0.22	17.2 ± 5.6^{b}	13.9 ± 5.1^{b}	11.8 ± 4.4^{b}	1.92 ± 0.35^{a}	1.71 ± 0.56^{a}
700	Vit C	158 ± 11^{b}	8.01 ± 0.04	32.3 ± 10.6^{a}	27.3 ± 11.1^{a}	24.7 ± 10.4^{a}	1.23 ± 0.33^{b}	1.33 ± 0.64^{b}
700	Vit E with Se	154 ± 12^{b}	8.11 ± 1.97	26.2 ± 10.6^{a}	23.1 ± 9.4^{a}	20.8 ± 8.4^{a}	1.13 ± 0.70^{b}	1.25 ± 0.76^{b}
700	Probiotic	155 ± 10^{b}	8.13 ± 0.42	29.3 ± 11.1^{a}	23.9 ± 11.3^{a}	21.1 ± 9.6^{a}	1.32 ± 0.84^{b}	1.26 ± 1.04^{b}
P-value		0.005	ns	0.0009	0.01	0.003	0.006	0.002

Vit = vitamin; Se = selenium; NZW = New Zealand White; ns = not significant.
†$n = 7$ animals per treatment.
‡$n = 70$ ejaculates per treatment.
[a,b]Means within a column under similar treatment with different letters are significantly different ($P \leqslant 0.05$).

Table 6 *Effects of different nitrate concentrations in drinking water and the supplementation of Vit C (200 ppm) or Vit E with Se (200 ppm + 0.2 mg) or probiotic (1000 ppm) on semen characteristics of NZW rabbit bucks (mean ± s.d.)*

		Semen characteristics†						
Nitrate (ppm)	Supplement /l water	Volume (ml)	Colour	Viscosity	Live (%)	Dead (%)	Abnormal (%)	Advanced motility (%)
Main effects of nitrate								
0	0	0.712 ± 0.341^{b}	2.51 ± 0.68^{a}	1.93 ± 0.71^{a}	90.0 ± 6.1^{a}	4.00 ± 2.05^{b}	6.00 ± 2.05^{b}	70.3 ± 3.7^{a}
350	0	0.611 ± 0.283^{b}	2.44 ± 0.55^{a}	1.55 ± 0.70^{a}	87.7 ± 3.4^{a}	5.51 ± 2.05^{b}	6.81 ± 1.76^{b}	69.5 ± 6.1^{a}
700	0	0.912 ± 0.354^{a}	1.74 ± 0.68^{b}	1.21 ± 0.78^{b}	80.6 ± 4.2^{b}	9.92 ± 3.15^{a}	9.53 ± 1.82^{a}	61.6 ± 7.9^{b}
P-value		0.02	0.007	0.03	0.0001	0.0001	0.0001	0.0001
Interaction between nitrate level and type of supplement								
700	0	0.912 ± 0.354^{a}	1.74 ± 0.68^{b}	1.21 ± 0.78^{a}	80.6 ± 4.2^{b}	9.92 ± 3.15^{a}	9.53 ± 1.82^{a}	61.6 ± 7.9^{c}
700	Vit C	0.712 ± 0.220^{b}	2.43 ± 0.55^{a}	1.82 ± 0.77^{a}	90.3 ± 2.4^{a}	3.94 ± 1.39^{b}	5.85 ± 1.56^{b}	70.9 ± 6.5^{b}
700	Vit E with Se	0.723 ± 0.315^{b}	2.66 ± 0.62^{a}	1.85 ± 0.72^{a}	90.4 ± 3.0^{a}	3.95 ± 1.97^{b}	5.73 ± 1.70^{b}	73.8 ± 7.2^{b}
700	Probiotic	0.612 ± 0.345^{b}	2.54 ± 0.66^{a}	2.00 ± 0.72^{a}	90.6 ± 2.9^{a}	4.00 ± 1.73^{b}	5.47 ± 1.65^{b}	76.8 ± 5.8^{a}
P-value		0.008	0.02	0.03	0.0001	0.0001	0.0001	0.0001

Vit = vitamin; Se = selenium; NZW = New Zealand White.
†$n = 70$ ejaculates per treatment.
[a,b,c]Means within a column under similar treatment with different letters are significantly different ($P \leqslant 0.05$).

agreement with those obtained by Kammerer and Siliart (1993), Mahboob *et al.* (2001) and Bassuny *et al.* (2004). The negative effect of 700 ppm nitrate on hematological traits could be explained, first, by an increase in the activity of the endothelial heme oxygenase by nitric oxide, which degrades heme to carbon monoxide and biliverdin (Foresti *et al.*, 1997). Second, by the formation of peroxynitrite, which reduces the production of the rabbit tissue factor, a primary initiator of physiological blood coagulation (Nielsen and Crow, 2004). Nitric oxide produced in the lung causes injury to pulmonary cells (Gow *et al.*, 1998). However, Shehata (2005) found that addition of 729 mg nitrate/l of drinking water of growing NZW female rabbits significantly decreased RBCs and Hgb. In addition, Abd El-Hamid (2006) found a decrease in the Hgb concentration in quails drinking waste water, which could be attributed to excessive amounts of nitrites that oxidize the iron in Hgb to methemoglobin and reduce the oxygen-carrying capacity of the blood (National Research Council (NRC), 1974; Hairston, 1995). High nitrate intakes can cause methemoglobinemia (blue baby syndrome) in infants, which reduces the ability to the blood to carry oxygen (Bassuny *et al.*, 2004).

Similar to the present results, Bassuny *et al.* (2004) and Shehata (2005) found that nitrate addition at 729 ppm significantly decreased total protein, albumin and globulin. The decrease in plasma total protein, albumin, globulin and TAC may be due to formation of nitric oxide or peroxynitrite, which oxidizes proteins and lipoproteins (Gow *et al.*, 1998;

Table 7 *Effects of different nitrate concentrations in drinking water and the supplementation of Vit C (200 ppm) or Vit E with Se (200 ppm + 0.2 mg) or probiotic (1000 ppm) on reproductive traits of NZW rabbit bucks (mean ± s.d.)*

Nitrate (ppm)	Supplement /l water	Fertility[†]	Number of offspring (*n*)[‡]	Total offspring weight (g)	Number of weaned offspring (*n*)[††]	Total weight of weaned offspring (*n*)	Kit weight (g)	Survival rate (%)
Main effects of nitrate								
0	0	74.5 ± 18.0[a]	8.51 ± 2.25[a]	558 ± 145[a]	7.51 ± 1.93	3200 ± 724	498 ± 104	91.1 ± 8.6
350	0	66.8 ± 27.4[a]	7.12 ± 2.08[a]	399 ± 147[b]	6.62 ± 2.46	2677 ± 599	404 ± 154	90.4 ± 17.9
700	0	48.2 ± 10.4[b]	6.53 ± 2.79[b]	340 ± 84[b]	5.64 ± 2.66	2289 ± 728	408 ± 99	85.3 ± 28.0
P-value		0.03	0.02	0.01	ns	ns	ns	ns
Interaction between nitrate level and supplement								
700	0	48.2 ± 10.4[c]	6.53 ± 2.79[b]	340 ± 84[b]	5.64 ± 2.66	2289 ± 728	408 ± 99	85.3 ± 28.0
700	Vit C	92.7 ± 10.1[a]	8.44 ± 1.57[a]	463 ± 84[a]	7.53 ± 2.18	3359 ± 10361	461 ± 82	93.2 ± 22.6
700	Vit E with Se	88.2 ± 15.5[b]	8.33 ± 2.33[a]	499 ± 144[a]	7.94 ± 2.09	2790 ± 584	480 ± 97	92.4 ± 7.2
700	Probiotic	88.1 ± 15.0[b]	8.21 ± 1.62[a]	556 ± 71[a]	7.54 ± 2.64	2733 ± 996	499 ± 25	91.5 ± 27.0
P-value		0.0001	0.01	0.0006	ns	ns	ns	ns

Vit = vitamin; Se = selenium; NZW = New Zealand White; ns = not significant.
[†]Number of observations of fertility = 20 per treatment.
[‡]Number of offspring ranged from 130 to 170 per treatment.
[††]Number of weaned offspring ranged from 112 to 158 per treatment.
[a,b,c]Means within a column under similar treatment with different letters are significantly different ($P \leq 0.05$).

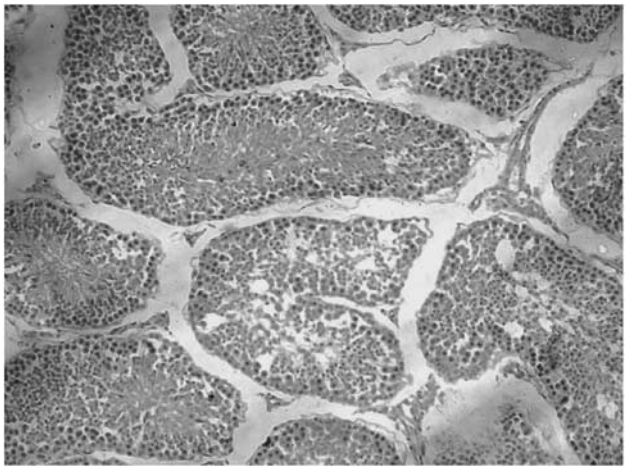

Figure 1 Testis of a rabbit in control group showing normal seminiferous tubules filled with several stages of spermatogenesis (H and E, ×100).

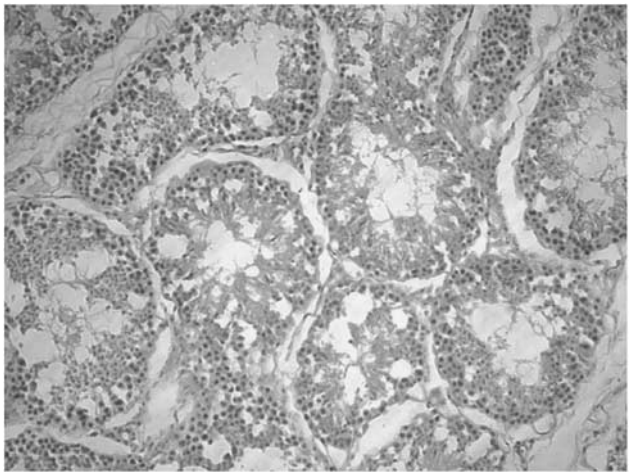

Figure 2 Testis of a rabbit administered 350 ppm nitrate in drinking water showing moderate changes of less spermatogenesis and vacuolations in seminiferous tubules (H and E, ×100).

Guzik *et al.*, 2000). The decrease in globulin caused by nitrate at 700 ppm may indicate an immunodepressive response (Atef *et al.*, 1991). However, the present findings indicated that WBCs and their fractions, PA and PI, were not significantly affected by nitrate concentration up to 700 ppm, and thus antioxidants did not show any effect.

The changes in blood profiles indicate that 700 ppm nitrate-induced hypercholesterolemia indicates vascular disease and endothelial dysfunction caused by nitric oxide and superoxide (Guzik *et al.*, 2000). Furthermore, the same dose of nitrate negatively affected seminal plasma total protein, albumin and testosterone while increasing seminal plasma urea, creatinine, AST and ALT. The change in blood and seminal plasma testosterone concentration is coincided with the alternations observed in the testes histopathology, which could explain its toxic effects of nitrate on semen quality, fertility and offspring performance. Nitrate at only 700 ppm

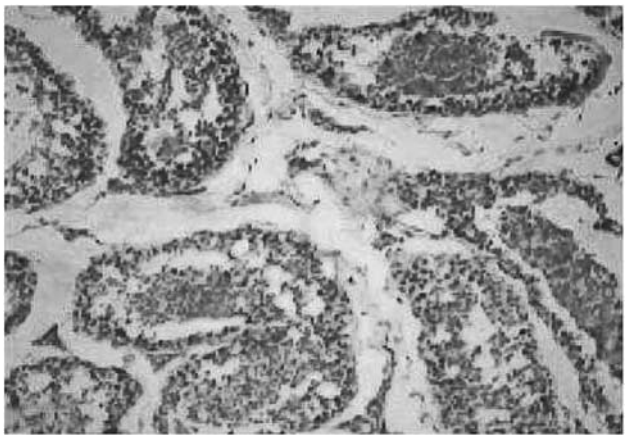

Figure 3 Testis of a rabbit administered 700 ppm nitrate in drinking water showing severe changes of damaged and necrotic seminiferous tubules widely separated with edema (H and E, ×100).

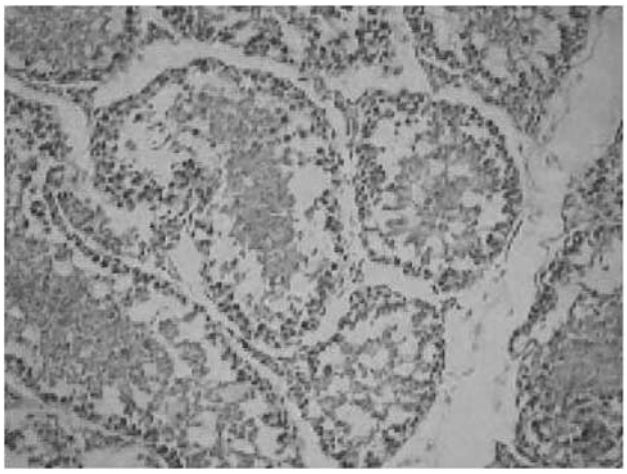

Figure 4 Testis of a rabbit administered 700 ppm nitrate in drinking water showing severe degenerative changes of central necrosis in the seminiferous tubules that lined with only one layer of degenerated spermatogonial cells (H and E, ×100).

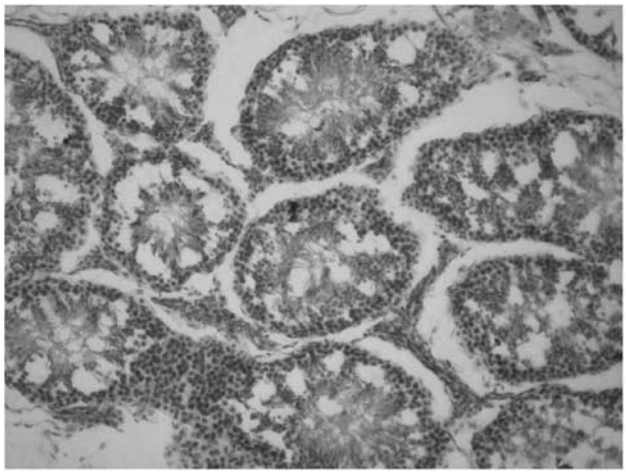

Figure 5 Testis of a rabbit administered 700 ppm nitrate and 200 mg Vit C in drinking water showing moderate changes of less spermatogenesis in the seminiferous tubules (H and E, ×100).

impaired age at first ejaculation, whereas the increase in ejaculate volume could be explained by the decrease in semen viscosity, as semen tended to be watery. The marked decrease in fertility (35.3%), number of offspring (23.3%) and total weight of offspring (39.1%) could be explained by the toxic effect of nitrate on sperm vitality. Similarly, Brun *et al.* (2002) found that the mass motility (number of motile sperm per ejaculate) significantly influenced the fertility rate, and this agreed with the decrease in motility observed in bucks supplemented with 700 ppm nitrate. In addition, Edwards and Guillette (2007) reported that increasing nitrate concentration up to 5 mg/l decreased total sperm counts per spermatozeugmatum.

Effect of antioxidants and probiotic

The Vit C or Vit E with Se as an antioxidant and probiotic as an enhancer of the gut function similarly improved TAC,

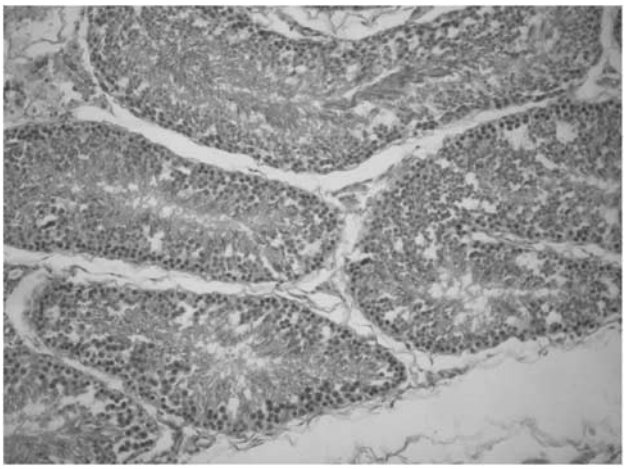

Figure 6 Testis of a rabbit administered 700 ppm nitrate and 1 g probiotics in drinking water showing nearly normal seminiferous tubules (H and E, ×100).

RBCs, Hgb and PCV of NZW bucks; in addition, probiotic improved blood plasma globulin. Similar results were reported by Hirneth and Classen (1984), who observed that Vit C inhibited the production of plasma nitric oxide and methemoglobin in female rats fed a diet containing 5% $NaNO_3$. The improvement caused by Vit C may be because of the stimulation of the immune system, which results in an increase in the corticosterone (Rama Rao *et al.*, 2002) and reduced endogenous formation of *N*-nitrosodimethylamine and *N*-nitrosopiperidine from nitrate (Vermeer *et al.*, 1999). Umar *et al.* (2010) also reported similar results when Vit C or Vit E was supplemented to rabbits. In addition, Castellini *et al.* (2003) and Yasmina and Abdennour (2008) concluded that Vit C protected testicular tissues and sperms from mercury intoxication and inhibited the oxidative stress increased glutathione concentration and improved quality of fresh and stored rabbits' semen.

In addition to the improvements in blood plasma biochemical indices, Vit C or Vit E with Se or probiotic supplementation also overcame the negative effect of 700 ppm of nitrate on seminal plasma total protein, albumin, urea, creatinine, testosterone, AST and ALT activity. These results are in agreement with the works of Castellini *et al.* (2000 and 2002). The protective effect of Vit E against the adverse effect of nitrate/nitrite could be attributed to its ability to reduce peroxynitrite (ONOO–) formation, whereas Se exerts its protective influences through seleno-enzymes/compounds, which reduce ONOO– formed (Chow and Hong, 2002). This could explain the improvement in semen quality and fertility of Vit C or Vit E with Se or probiotic-supplemented group compared with the control group. These novel findings indicated that Vit C and Vit E with Se as an antioxidant or probiotic as gut function enhancer eliminated the negative effect of nitrate on most of the semen quality and reproductive traits (fertility, number of offspring and total offspring weight), and this concurred with increasing plasma TAC, which indicated an increase in body antioxidant reserve and/or a decrease in

oxidative stress. The positive effect of these agents included an increase in Hgb, blood plasma globulin, RBCs and PCV% and increasing testosterone (Castellini *et al.*, 2000; El-Kelawy, 2003). In addition, Brun *et al.* (2002) found that the mass motility (number of motile sperm per ejaculate) significantly influenced the conception rate and this agree with the increase in advanced motility observed of bucks supplemented with Vit C, E with Se and probiotic. It should be mentioned that the potentiality of the different agents used was similar; however, fertility of Vit C was superior to the other agents and this could be attributed to its effects as an antistress and an antioxidant. Similar to the present findings, Martarelli *et al.* (2011) reported that probiotic strains (1 : 1 L. rhamnosus IMC 501 and L. paracasei IMC 502; $\sim 10^9$ cells/day) and probiotic supplementation increased plasma antioxidant levels, thus neutralizing reactive oxygen species. The two strains, L. rhamnosus IMC 501® and L. paracasei IMC 502®, exert strong antioxidant activity.

In addition, supplementation of Vit C or Vit E with Se and probiotic had a beneficial effect on reducing the severity of the changes within the somniferous tubules and improve testicular picture, as observed in histological study. Similarly, Masukawa and Iwata (1979) showed that selenite decreases nitrite-induced mortality in a dose-dependent manner, and suggested that the protective effect is because of its action in reducing methemoglobin formed by nitrite. These earlier findings indicate a critical role of dietary Vit E and Se in preventing the adverse cellular effects of nitrite. The available information, concerning the interrelationship among nitrite, Vit C, E, Se or probiotic on eliminating the free radicals NO, superoxide and ONOO, has provided a better insight of the protective effects of Vit C, E and Se or probiotic against nitrite toxicity observed (Masukawa and Iwata, 1979). The positive effect of probiotic could be attributed to the change in gut microbiota toward beneficial ones, which could utilize nitrate for growth and thus detoxication of nitrate.

Conclusion

In conclusion, 350 ppm of nitrate had no harmful effect on reproductive and physiological traits of bucks and histopathological changes of testes, although increasing nitrate concentration to 700 ppm negatively affects the aforementioned criteria. Furthermore, supplementation of Vit C, Vit E with Se or probiotic was equally effective for relief the negative effects of 700 ppm of nitrate offering protective agents for rabbits producers in area with high nitrate contamination.

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