

Original Research Paper

# The Effect of Nitrate on some Biochemical Parameters of Rabbits and Ameliorate its Effect by using Vitamin E and Rosemary (*Rosmarinus Officinalis*)

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**Abstract:** This study was conducted to investigate the effect of nitrate in the feed of the rabbits on the total protein, albumin, globulin, cholesterol, triglyceride and urea in serum and to study ameliorate nitrate toxicity by using vitamin E and rosemary. Twenty-eight adult New Zealand rabbits were divided randomly into four groups (7 rabbits in each group), Group 1 (G<sub>1</sub>): Fed basal ration as a control group. Group 2 (G<sub>2</sub>): Fed ration supplemented with nitrate (168 mg/kg B.W./day), Group 3 (G<sub>3</sub>): Fed ration supplemented with nitrate (168 mg/kg B.W./day) plus vitamin E (100 mg/kg B.W.). Group 4 (G<sub>4</sub>): Fed ration supplemented with nitrate (168 mg/kg B.W./day) plus rosemary (500 mg/kg B.W.). The results showed a significant ( $p \leq 0.05$ ) decrease in total protein and globulin in the group that fed nitrate (G<sub>2</sub>) compared with the control (G<sub>1</sub>) and groups fed a mixture of vitamin E and rosemary with nitrate (G<sub>3</sub> and G<sub>4</sub>). The cholesterol, triglyceride and blood urea concentration showed a significant increase ( $p \leq 0.05$ ) in the group that fed nitrate (G<sub>2</sub>) compared with the control group (G<sub>1</sub>), whereas the groups that fed vitamin E and rosemary with nitrate (G<sub>3</sub> and G<sub>4</sub>) showed a significant ( $p \leq 0.05$ ) decreased in the parameters measured compared with the nitrate fed rabbits (G<sub>2</sub>). The results illustrated above revealed a remarkable impact of nitrate supplementation causing a significant decrement in protein concentrations along with considerable rise in the cholesterol, triglyceride and blood urea levels which was partially neutralized by vitamin E and rosemary.

**Keywords:** Nitrate, Rabbit, Rosemary, Vitamin E, Biochemical

## Introduction

Nitrate poisoning is recorded in many studies accomplished in multiple animal species as well as humans (Jan *et al.*, 2009). *N*-nitroso and nitrite that form when nitrite binds to another component before or after ingestion are toxic and leading to severe pathologies in the body (Speijers and Vanden-Brandt, 2004). The exposure to nitrate-nitrogen is strongly associated with many diseases, such as methemoglobinemia (blue baby syndrome), gastrointestinal cancer, thyroid disorders, reproductive problems and diabetes (Olesen *et al.*, 2004). Researchers noticed that nitrate could restrict iodine uptake by the thyroid causing thyroid hypertrophy, which is responsible for several endocrine and hormonal functions in the body (Van Maanen *et al.*, 1994). In addition, another research group had shown a positive correlation between the incidence of dependent

diabetes and nitrate concentrations in the potable water (Parslow *et al.*, 1997). Several clinical studies predicted hepatorenal and/or hepatotoxicity in humans linked to nitrate intake (Parada *et al.*, 2009). Nitrites and nitrates are of abundant concern to humans and animals due to their carcinogenic, mutagenic and teratogenic activities (Krishnamoorthy and Sangeetha, 2008). Antioxidant substances are known to neutralize toxicity and this is why they are widely used. In the present study, a mixture of vitamin E and rosemary were used to ameliorate nitrate toxicity. Vitamin E is a very important feed supplement as an effective liposoluble antioxidant (Al-Attar, 2011).

Vitamin E is a potent natural biological antioxidant, which plays very important role in consolidating of the cellular membranes due to their activity as a scavenging of free radicals (Warren *et al.*, 2000). Natural antioxidants are necessary for general health because they deactivate harmful free radicals resulted from various stress conditions

(Hassan and Awad, 2007). Rosemary is aromatic herb, which is commonly used as flavoring food, also has been widely used in traditional medicine, rosemary has been used as a stimulant and mild analgesic and it effective herbs for treating headaches, poor circulation, inflammation (Raskovic *et al.*, 2014). It is a plant with a powerful antioxidant activity (Raskovic *et al.*, 2014). It contains some antioxidant components that had been shown to provide a defending role against oxidizing agents generated by oxidative stress, which include carnosol, carnosic acid, ursolic acid, rosmarinic acid (Raskovic *et al.*, 2014) and caffeic acid thus effectively participate in lipid per oxidation (Matkowski, 2006). Lo *et al.* (2002) reported that carnosol, a natural polyphenol component of rosemary leaves, illustrated effective anti-oxidative activity against free radicals.

## Materials and Methods

Twenty-eight adult New Zealand female rabbits aged 10-14 months and approximately 1800-2200 grams weight. These rabbits were maintained under suitable environmental conditions, 20-25°C, 12 h light and 12 h dark throughout the experiment. Groups (7 rabbits in each group) were housed indoors inside well separated cages (2×3 meters) containing hard-wood chips as bedding. The rabbits were cared for a month prior to the experiment for adaptation and to estimate the average amount of daily food consumption rate. Rabbits were divided randomly into four groups: Group 1 (G<sub>1</sub>): Fed basal ration (ration I, Table 1) as a control group. Group 2 (G<sub>2</sub>): Fed ration supplemented with potassium nitrate (KNO<sub>3</sub>) (168 mg/kg B.W./day) (ration II, Table 1) according to Al-Rawi (2014) and named as nitrate treated group, Group 3 (G<sub>3</sub>): Given ration supplemented with a mixture of potassium nitrate (168 mg/kg B.W./day) plus vitamin E (100 mg/kg B.W.) (ration III, Table 1) according to Alqayim and Asis (2013). Group 4 (G<sub>4</sub>): Fed ration complemented with potassium nitrate (168 mg/kg B.W./day) plus rosemary (500 mg/kg B.W.) (ration IV, Table 1) according to Andon *et al.* (2008) who determined the lethal dose 50 (LD50) which is greater than 2,000 mg kg<sup>-1</sup> body weight. Through the experiment,

blood samples were collected from heart at 0, eight, twelve and sixteen weeks intervals. Blood samples were centrifuged for 10 min at 6000 rpm. Serum specimens were stored at -20°C until analysis. Total protein, albumin, globulin, cholesterol, triglyceride and urea concentrations in serum were estimated using commercial kinetic diagnostic kits with spectrophotometric method (Biolabo kit) according to Tietz (1999).

## Statistical Analysis

The data were presented as Mean and Standard Error (Mean±SE) and subjected to statistical analysis using Two-way analysis of variance (ANOVA). In addition, post hoc test was used to find out the Least Significant Differences (LSD) between different means (p≤0.05). Statistical Package for the Social Sciences Program (SPSS) version 21 package was used for this purpose (Snedecor and Cochran, 1989).

## Results

There was a negative impact of nitrate supplementation on the total protein concentration in nitrate fed rabbits (G<sub>2</sub>), which demonstrated a significant (p≤0.05) decrease compared with (G<sub>1</sub>) and groups that fed mixture of nitrate plus vitamin E (G<sub>3</sub>) and nitrate plus rosemary (G<sub>4</sub>) during last period of experiment (16th week) (Table 2). Albumin concentration revealed slight fluctuations in all groups, but it was lowest in (G<sub>2</sub>) (Table 3). Globulin levels significantly declined (p≤0.05) in (G<sub>2</sub>), towards the end of the experiment specifically at 12<sup>th</sup> and 16<sup>th</sup> weeks of experiment (Table 4). Serum cholesterol and triglyceride levels soared significantly (p≤0.05) in (G<sub>2</sub>) in comparison with the other groups (G<sub>1</sub>, G<sub>3</sub> and G<sub>4</sub>) (Table 5 and 6). Interestingly, the effect of nitrate on the blood urea was depicted in Table 7 which clearly reveals its significant sharp rise (p≤0.05) in (G<sub>2</sub>) during the late stages of experiment 12th and 16th weeks compared with (G<sub>1</sub>). Finally, the data reported a significant (p≤0.05) increased blood urea levels within groups that fed nitrate (G<sub>2</sub>) in 16th week compared with the values registered at the start (0 time).

Table 1. Different nutritional rations fed to different rabbit groups

Ration component	Ration I (%)	Ration II (%)	Ration III (%)	Ration IV (%)
Corn	31.54	31	30.8	30
Barley	15	15	15	15
Soya	10	10	10	10
Bran	30	30	30	30
Hay	10	10	10	10
Animal protein	2.46	2.46	2.46	2.46
Calcium	0.7	0.7	0.7	0.7
NaCl	0.3	0.3	0.3	0.3
KNO <sub>3</sub>	-----	0.54	0.54	0.54
Vitamin E	-----	-----	0.2	-----
Rosemary	-----	-----	-----	1

Individual feed intake = 100±5 g

Convert factor of KNO<sub>3</sub> to NO<sub>3</sub> is 1.61

Table 2. The effect of nitrate and vitamin E or rosemary on the serum total protein (g/dl)

Groups time (week)	G <sub>1</sub> Control	G <sub>2</sub> Nitrate	G <sub>3</sub> Nitrate+Vit. E	G <sub>4</sub> Nitrate+Rosemary
0	6.94±0.21 Aa	7.07±0.08 Aa	6.98±0.18 Aa	6.99±0.22 Aa
8th	6.81±0.17 Aa	6.89±0.07 Aab	6.96±0.20 Aa	7.05±0.25 Aa
12th	6.92±0.12 Aa	6.49±0.13 Bb	7.08±0.08 Aa	6.98±0.20 Aa
16th	7.10±0.13 Aa	6.42±0.22 Bb	7.21±0.09 Aa	7.23±0.8 Aa

The different capital letters refer to a significant difference between different groups at (p<0.05)

The different small letters refer to a significant difference between different times at (p<0.05)

Table 3. The effect of nitrate and vitamin E or rosemary on the serum albumin (g/dl)

Groups time (week)	G <sub>1</sub> Control	G <sub>2</sub> Nitrate	G <sub>3</sub> Nitrate+Vit. E	G <sub>4</sub> Nitrate+Rosemary
0	3.88±0.17	3.52±0.21	3.69±0.11	3.63±0.09
8th	3.67±0.09	3.49±0.10	3.57±0.09	3.60±0.18
12th	3.97±0.16	3.42±0.16	3.47±0.08	3.34±0.23
16th	3.84±0.06	3.54±0.13	3.65±0.15	3.56±0.19

LSD = 0.35

Table 4. The effect of nitrate and vitamin E or rosemary on the serum globulin (g/dl)

Groups time (week)	G <sub>1</sub> Control	G <sub>2</sub> Nitrate	G <sub>3</sub> Nitrate+Vit. E	G <sub>4</sub> Nitrate+Rosemary
0	3.05±0.08 Aa	3.35±0.26 Aa	3.29±0.10 Aa	3.35±0.22 Aa
8th	3.13±0.21 Aa	3.40±0.16 Aa	3.18±0.17 Aa	3.34±0.20 Aa
12th	3.14±0.07 ABa	3.06±0.27 Bb	3.60±0.09 Aa	3.64±0.30 Aa
16th	3.22±0.17 ABa	2.87±0.12 Bb	3.55±0.13 Aa	3.66±0.24 Aa

The different capital letters refer to a significant difference between different groups at (p<0.05)

The different small letters refer to a significant difference between different times at (p<0.05)

Table 5. The effect of nitrate and vitamin E or rosemary on the serum cholesterol (mg/dl)

Groups time (week)	G <sub>1</sub> Control	G <sub>2</sub> Nitrate	G <sub>3</sub> Nitrate+Vit. E	G <sub>4</sub> Nitrate+Rosemary
0	55.36±8.25 Aa	52.74±4.72 Ab	56.18±3.12 Aa	57.88±6.85 Aa
8 <sup>th</sup>	62.84±7.24 Aa	56.82±7.36 Ab	55.38±4.16 Aa	55.92±3.97 Aa
12 <sup>th</sup>	58.17±8.25 Aa	64.53±5.11 Aab	55.07±6.64 Aa	57.54±10.94 Aa
16 <sup>th</sup>	59.91±6.01 Ba	72.81±8.68 Aa	58.22±5.62 Ba	56.14±8.06 Ba

The different capital letters refer to a significant difference between different groups at (p<0.05)

The different small letters refer to a significant difference between different times at (p<0.05)

Table 6. The effect of nitrate and vitamin E or rosemary on the triglyceride (mg/dl)

Groups time (week)	G <sub>1</sub> Control	G <sub>2</sub> Nitrate	G <sub>3</sub> Nitrate+Vit. E	G <sub>4</sub> Nitrate+Rosemary
0	82.77±7.86 Aa	76.28±8.12 Ac	79.46±4.74 Aa	81.75±6.20 Aa
8th	84.14±11.32 Aa	89.67±6.94 Abc	81.03±5.38 Aa	81.21±10.29 Aa
12th	83.33±8.16 ABa	99.55±11.50 Ab	80.68±7.94 Ba	76.36±7.81 Ba
16th	82.19±3.85 Ba	118.60±9.14 Aa	77.94±7.30 Ba	72.12±9.51 Ba

The different capital letters refer to a significant difference between different groups at (p<0.05)

The different small letters refer to a significant difference between different times at (p<0.05)

Table 7. The effect of nitrate and vitamin E or rosemary on the blood urea (mg/dl)

Groups time (week)	G <sub>1</sub> Control	G <sub>2</sub> Nitrate	G <sub>3</sub> Nitrate+Vit. E	G <sub>4</sub> Nitrate+Rosemary
0	45.12±4.90 Aa	45.97±5.57 Ab	46.23±5.40 Aa	47.01±11.84 Aa
8th	48.85±3.68 Aa	57.45±2.19 Ab	50.70±3.96 Aa	49.41±3.26 Aa
12th	46.05±3.92 Ba	58.24±4.96 Aa	49.94±5.38 ABa	52.04±5.83 ABa
16th	45.37±3.07 Ba	59.75±3.24 Aa	51.70±4.12 ABa	54.30±4.74 ABa

The different capital letters refer to a significant difference between different groups at (p<0.05)

The different small letters refer to a significant difference between different times at (p<0.05)

## Discussion

Nitrate supplementation has negatively influenced total protein concentrations (Table 2). This could be attributed to nitrate toxicity as many studies referred to this notion in humans and animals; therefore, nitrate classified as an oxidative agent (Atyabi *et al.*, 2012). In line with above, excessive nitrate levels could harmfully manipulate food conversion efficiency and mediate endocrinological disturbances (Hansen *et al.*, 2009), thus it leads to an enormous reduction of nutrient digestibility and eventually loss of body weight (Bassuny *et al.*, 2014). In addition, Jahreis *et al.* (1991) reported that nitrate-induced thyroid hormones deficiency could minimize body weight by inhibiting food intake through its impact on the nerve cells that could result in deprived appetite. Added to above, thyroid hormones (mainly thyroxin) deficiency inhibits the synthesis of Growth Hormone Releasing Factor (GHRF) in the hypothalamus and leads to growth retard symptoms, thus all these factors may interfere with the metabolic pathways causing sharp drop in the total protein concentrations. The paradox effect of vitamin E and/or rosemary which is the antitoxic activities could be implicated to the antioxidant function, rosemary is known as a biologically active phytochemical antioxidant as it contains phenolic compounds such as carnosol, carnosic acid, isorosmanol, rosmanol, rosmadial, 7-methyl-epirosmanol and caffeic acid (Aruoma *et al.*, 1992).

Decreased albumin and globulin levels in (G<sub>2</sub>) can be attributed to the same reasons mentioned above in addition to endocrine malfunctions and disorders as many studies highlighted that concept (Hansen *et al.*, 2009). This may interpret why nitrate considered as a stress agent. The risk of nitrate toxicity mainly belongs to formation of reactive oxygen species, free radicals as well as other toxic transient compounds such as, peroxy nitrite hydrogen peroxide and superoxide anion, which interrupt the equilibrium between oxidants and antioxidant, causing what so called oxidative stress (Ahsan *et al.*, 2003), which results in an increase in cortisol hormone secretion (from adrenal gland) under oxidative stress, that leads to suppress immunity and

reduces globulin concentrations. Furthermore, excess nitrite significantly harness B lymphocytes proliferation, thus interfere with antibody production (Suketa, 2002). The findings in this study are in line with EL-Tahan *et al.* (2010) who support the antagonistic effect on nitrate on the immune system. On the other hands, vitamin E and rosemary ameliorate the nitrate effect which are obviously observed in (G<sub>3</sub>) and (G<sub>4</sub>), by improving the globulin level compared with control group. This indicates the immunostimulatory activity of vitamin E and rosemary (Shokrollahi *et al.*, 2015). Vitamin E is one of the most frequent studied nutrients presenting immunomodulation properties (Silva *et al.*, 2011). Also, these results are consistent with Al-Sheyab *et al.* (2012). Vitamin E is a natural antioxidant targeting cell membranes plus it enhances phagocytic activity of macrophages (Konjufca *et al.*, 2004). Rosemary activity was described by Shokrollahi *et al.* (2015) who used three concentrations (100, 200, 400 mg/kg/day) for 42 days showing remarkably increased globulin concentration in serum of goat kids which are nearly parallel to what I have found. I noted that nitrate caused an elevation of lipid profile in the serum, this might be due to impairment of liver function summarized by inhibition of enzymes responsible for conversion of cholesterol into bile acids. Sachar and Raina (2014) observed histological changes represented by hepatic severe degeneration to necrosis as a response to nitrate toxicity. I agreed with Azeez *et al.* (2011) who reported that considerable rise in the concentration of creatinine and cholesterol in rats serum that fed nitrate. In contrast, the results revealed an obvious reduction in the toxic effect resulted from the nitrate administration by rosemary and vitamin E. This is consistent with many studies that interprets the antitoxic effect of rosemary and its extracts which are attributed to its antioxidant activity. Zeng and Wang (2001) mentioned that rosemary inhibited lipid per oxidation via its active components such as rosmanol, carnosol and epirosmanol phenolic. In addition, rosemary plays a protective role against hyperglycemia and hypercholesterolemia-induced by oxidative stress, thus keeps balanced blood stream (Labban *et al.*, 2014). Sufficient vitamin E

supplementation is recommended to protect from generation of toxic agents and free radicals damage. In addition, vitamin E effectively prevents lipid peroxidation (Evstigneeva *et al.*, 1998).

Urea concentrations declined in (G<sub>2</sub>) Table 7, this was because the metabolic dysfunction, that is to say, the major portion of the ingested nitrate is excreted through urine as a crude nitrate, urea or ammonia, while fecal excretion is negligible thus this process participate negatively in urea synthesis (WHO, 1985). In the same way, nitrate has nephrotoxic effect, which agreed with Shour *et al.* (1999) who reported that drenching rabbits with 25, 75, 150 and 300 mg NaNO<sub>3</sub>/kg b.w. for 30 days resulted in higher serum urea levels at 1.45, 1.78, 1.70 and 1.61 rates greater than the control level. A mixture of vitamin E and rosemary decreased the nitrate toxic effect, might be by protecting renal tissue because vitamin E was reported as an excellent antioxidant that protects cells and tissues from harmful lipoperoxidation damage (Yu, 1994). Rosemary contains polyphenolic flavonoids compounds such as rosmaridiphenol, rosmadial, carnosol, carnosic and rosmarinic acid (Borrás-Linares *et al.*, 2014), these active compounds act to inhibit carnosic and rosmarinic acid in leukemia, prostate cancer, breast cancer, pulmonary, hepatic and renal cells (Yesil-Celiktas *et al.*, 2010).

## Conclusion

To sum up, the prolonged exposure of rabbits to nitrate causes significant drop in the total protein and globulin concentrations and increases cholesterol, triglyceride and urea in the serum. Toxic levels of nitrate can be resolved by supplementing vitamin E and rosemary to the diet which is strongly recommended to combat the toxic effect of nitrate.

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## Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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