EVALUATION OF THE PROTECTIVE EFFECT OFASCORBIC ACIDAND/OR THYYMOQUINONE ON NITRATE TOXICITY IN RABBITS

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ABSTRACT:
Background: *Risk of nitrate toxicity among animal and human. The objective of this work isdesigned to study the capacity of thymoquinone and/or ascorbic acidfor detoxification of drinking water nitrate in growing New Zealand White rabbits.*
Methods: *In this experiment, Hematological and serobiochemical parameters as well as histopathological examination of liver and kidney were estimated in 5 assigned groups as follows, the 1st group served as a control, the 2nd group was orally received sodium nitrate 1gm/L drinking waterfor 8 weeks. The 3rdgroup was orally received sodium nitrate 1gm/L drinking water and treated with ascorbic acid 300mg/L drinking water for 8 weeks.The 4thgroup was orally received sodium nitrate 1gm/L drinking waterfor 8 weeks and treated with thymoquinone(10mg/kg/day) via the intraperitoneal route for7 successive days. The 5th group was orally received sodium nitrate 1gm/L drinking water and treated with ascorbic acid 200mg/L drinking waterfor 8 weeksalong withthymoquinone(10mg/kg/day) via the intraperitoneal routefor 7 days.*
Results:*The leukocyte count and red blood cells were decreased significantly (P<0.05) in the nitrate treated group compared to the control and other treated groups. Total protein and albumin concentrations were decreased significantly (P<0.05) inthe nitrated treated group compared to the control and other treated groups.The activity of AST, ALT as well as Cholesterol and glucoseconcentrations were significantly increased (P<0.05) in the nitrate treatment group compared to thecontrol and other treated groups. The creatinine and blood urea nitrogen (BUN) were significantly increased (P<0.05) in the nitrate treatment group compared tothecontrol and other treated groups. The supplementation of thymoquinone at the rate of 10mg/kg/day and/or ascorbic acid at the rate of 300mg/Litre lead to an improvement of the blood and biochemical parameters, and also ameliorate hepatic and renal damage.****Conclusion:****The present study suggeststhe possible beneficial effects ofthymoquinoneeither alone or withascorbic against sodium nitrate toxicity via an antioxidant mechanism.*

Key words: Ascorbic acid, thymoquinone, nitrate, histopathology. Liver, kidney.

INTRODUCTION

The most common causes of high nitrate levels in water include shallow wells contaminated with surface water, water containing animal wastes, and surface runoff from heavy rain after fertilization with ammonium nitrate [1]. Water containing more than 500 ppm NO_3 is potentially toxic [2].In the body, nitrate is reduced to nitrite andthe absorption of nitrite leads to methaemoglobinaemiawhich cannot transport oxygen to bodytissues [3]. Exposure to nitrites has been reported to have an adverse effect on animals and humanhealth [4].Nitrate toxicity has been observed commonly in ruminants [5]. A high level of nitrate can lead to a change in blood constituents [6]. Vitamin C has potentials to scavenge free radicals and protect cells from oxidative damage[7]. Vitamin C have also been proved to have a hepatoprotective effect against nitrite toxicity [8].Thymoquinone (TQ)isthe major constituent of the volatile oil from Nigella sativaseeds. Its valuable effects are related to its anti-oxidant, anti-infective, anti-tumor, anti-inflammatory properties [9].Previous studies have demonstrated that TQ has protective effect against oxidative injury induced bya variety of free radical generating agents [10,11,12]. As far as we know there are no documented reports onthe protective effectsof TQ on nitrate toxicity. Therefore,the present study is designed to investigate whether oral supplementationofthymoquinoneand/orascorbic acid could ameliorate or protect against nitrate toxicity.

MATERIALS AND METHODS

Animals:
A total of 40 growing New Zealand White rabbits were obtained fromthe Animal Care Unit of College of Veterinary Medicine and Animal Resources, King Faisal University (KFU). The rabbits were assigned to fivegroups (8 rabbits/group) at 8 weeks of age. Animals were housed under good ventilation with free access to food and water.

Chemicals:
Sodium nitrate (BDH)-Chemicals Ltd Poole England, L-Ascorbic acid- Techno-Pharmhem-Haryana-India,thymoquinone-99%-Sigma-Aldrich Chemical Company, St. Louis, MO, 63103 USA, which dissolved in dimethylsulphoxide (DMSO) as 14 mg /ml and then diluted in PBS as required.

Experimental design:
The 1st group (n=8) served as a control, the 2nd group (n=8) was orally received sodium nitrate 1gm/L drinking waterfor 8 weeks. The 3rdgroup (n=8) was orally received sodium nitrate 1gm/L drinking water and treated with ascorbic acid

300mg/L drinking waterfor 8 weeks.The 4th group (n=8) was orally received sodium nitrate 1gm/L drinking waterfor 8 weeks and treated with thymoquinone(10mg/kg/day) via the intraperitoneal route for 7 successive days. The 5th group (n=8) was orally received sodium nitrate 1gm/L drinking water and treated with ascorbic acid 200mg/L drinking waterfor 8 weeksalong withthymoquinone(10mg/kg/day) viathe intraperitoneal routefor 7 days.

Biochemistry and Hematology:

The rabbits were observed daily for clinical signs. Before necropsy, the rabbits were euthanized with sodium pentobarbital (300mg/kg, i.p.) according to Animal Welfare Advisory Committee (AWAC).Blood samples were collected via CardiacPuncture in EDTA tubes forhematologicalanalysis (WBC, RBC, Hb, PCV, MCV, MCH and MCHC) using a coulter counter electronic analyzer (Vet Scan 5 HM-ABAXIS-USA). Serum samples were collected in plain tubes, then separated and keptin -80°c untilanalyzed fortotal protein, albumin, cholesterol, glucose, ALT, AST, BUN and creatinine usinga biochemical blood analyzer (ELLIPSE, E0217, Italy, 2003).

Gross pathology and histopathology:

Necropsy was performedat the end of an experiment and samples of liver, kidney and lung were collected at 10% neutral buffered formalin for histopathology. Samples fixed in formalin were embedded within paraffin in 48 hours and processed routinely for hemotoxylin and eosin staining (HE) [13].

Statistical analysis:

Data were analyzed by the General Linear Model (GLM) procedure (SAS, Institute, Inc, 2002). The least Square Mean (LSM) + standard errors for each group were calculated and tested for significance usingthe "t" test [14].

RESULTS

Clinical signs, blood and biochemical parameters:

No mortality was recorded during the whole experiment. At 4 weeks, lethargy and diarrhea were observed in the nitrate treated groupwhich can persist until the end of the experiment. These findings were not observed in control and other treated groups. The leukocyte count and red blood cells were decreased significantly($P<0.05$) in the nitrate treated group compared to control and other treated group (Table 1). Total protein and albumin concentrations were significantly decreased($P<0.05$)in the nitrated treated groupcompared to control and other treated groups (Table 2).The activity of AST, ALT as well as cholesterol and glucose concentrations were significantly increased ($P<0.05$) in the nitrate treatment group compared to a control and other treated groups (table 2). The creatinine and blood urea nitrogen (BUN) were significantly increased ($P<0.05$) in the nitrate treatment group compared tothe control and other treated groups(Table 2).

Gross and histopathology findings:

Gross findings revealed enlarged,pale livers and mild gastroenteritis in the nitrate treated group, however, in other treated groups, these organs were more or less quite similar to the control group.

The liver sections of rabbits intoxicated with the nitrite showed disturbed lobular architecture and severe degenerative changes characterized by swelling and vacular appearance of hepatocytes with pyknotic nuclei (Fig.1a). The vast majority of hepatocytes had significant scattered single necrotic cells (apoptoticcells). These necrotic cells were frequent in the periportal areas, but they occurred to a lesser extent in mid zonal areas and were absent in centrilobular areas. Severe degrees of inflammatory reaction manifested with minute foci of macrophages and lymphocytes was noticed everywhere. The livers intoxicated with nitrate and treated with ascorbic acidshowed a mild degree of degenerative changes of some hepatocytes manifested with vacuolar cytoplasm (Fig.1b). However, The most hepatocytes appeared recovered and have an almost normal architecture. The livers intoxicated with nitrate and treated with thymoquinone revealed almost recovered and healthy hepatocytes, except for occasional infiltration ofmononuclear cells in the portal areas(Fig.1c).The livers intoxicated with nitrate and treated with ascorbic acid and/ or thymoquinone revealed recovered and healthy hepatocytes, which have an almost normal architecture(Fig.1d). Kidney of the rabbitsintoxicated with the nitrate showed moderate tubular degeneration, predominantly of the distal tubules, manifested with cellular swelling and fine granular appearance of the cytoplasm. Vacuolar degeneration was also noted, but the severe degree characterized by desquamation of cells in almost all distal tubules. The most common lesion was interstitial nephritis manifested by focal aggregations of mononuclear cell between the tubules (Fig.2a). The kidneysintoxicated with nitrate and treated with ascorbic acidshowed a mild degree of vascular degeneration of some tubules with cellular casts in some distal tubules (Fig.2b).The kidneysintoxicated with nitrate and treated with thymoquinoneshowed only a mild degree of vacuolar degeneration of some tubules(Fig.2c). Apart from, very mild degree of tubular cellular degeneration in rabbits intoxicated with nitrateand treated with ascorbic acidand /or thymoquinone,the majority of convoluted tubules as well as the glomeruliappeared more or less quite similar to the control rabbits (Fig.2d).

DISCUSSION

Nitrate is a health hazard because of its conversion to nitrite. Once ingested,theconversion of nitrateto nitrite takes place in the saliva and in the gastrointestinal tract of animals and human.The present study indicates that excess nitrate in drinking water leads to gastrointestinal disturbance. These signs might be due to the irritant effects of nitrite on the mucous membranes of the gastrointestinal tract [15].The leukocyte count decreasedsignificantly in the nitrate treated group compared to control and other treated groups. Furthermore, the number of red blood cells decreasedsignificantly in the nitrate treated group compared to control and other treated groups. This finding might be

due to the effect of nitrite on the Ca, Mg and ATPs activity of the cell membrane of RBC3.Dehydration due to diarrhea might be another factor for this decrease in RBC. Hemoglobindecreased significantly in the nitrate treated groupcompared to control compared and other treated groups. These results might be due to the decrease number of RBC or due to the conversion of nitrate to nitrite ions which can convert ferrous ions of hemoglobin into ferric form (methaemoglobin) resulting in tissue anoxia, which may be responsible for renal and hepatic damage[16].Total protein and albumin concentrations were significantly decreased in the nitrated treated group. The decrease of total protein and albumin concentrations could be attributed to impair liver and kidney functions[17,18] or might be due tothe formation of nitric oxide or peroxynitrite, which oxidizes proteins and lipoproteins [19]. The activity of AST and ALT weresignificantly increased in the nitrate treatment group.These results indicate hepatic damage [20]. Cholesterol concentrations were significantly increased inthe nitrate treatment group. These findings might be due to the endothelial damage of blood vessels by nitric oxide and superoxide [19].The creatinine and blood urea nitrogen (BUN) were significantly increased in the nitrate treatment group. These findings could be attributed to the renal damage. The addition of ascorbic acid to nitrateamendedtissues changes,blood andbiochemical parameters as well.These results indicated that ascorbic acid can reduce methaemoglobin [21] ormay augment the function of endogenous free radical scavengers and decreases the adverse effect of nitrates on body cells [7]. The addition of thymquinone either alone or along with ascorbic acid to nitratealso ameliorate tissues and blood changes.It has been shown that thymoquinone (TQ) works as a scavenger of various reactive oxygen species, including superoxide radical anion and hydroxyl radicals10,22.

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Table 1: Blood parameters in control group, nitrate group, nitrate and ascorbic acid group, nitrate and TQ group, and nitrate, ascorbic acid and TQ group.

	Control group	**Nitrate treated group**	Nitrate and ascorbic acid	Nitrate and TQ group	Nitrate, ascorbic acid and TQ
WBC 10^3/ml	6.20±0.15 ^{a}	3.00±0.26 ^{b}	5.56±0.14 ^{c}	5.15±0.12 ^{d}	5.75±0.06 ^{e}
LYM %	52.95±2.10 ^{a}	43.23±0.27^{b}	50.10±0.16 ^{c}	49.74±0.40 ^{c}	50.37±0.47 ^{c}
MON %	26.22±0.21 ^{a}	20.15±0.11 ^{b}	24.88±0.26 ^{c}	24.70±0.33^{cd}	25.12±0.13^{ce}
NEU %	19.24±0.07 ^{a}	13.32±0.13 ^{b}	17.32±0.15 ^{c}	17.98±0.08 ^{d}	18.06±0.09 ^{d}
RBC 10^6/ml	6.91±0.08 ^{a}	3.47±0.19 ^{b}	5.97±0.07 ^{c}	6.06±0.13 ^{c}	6.13±0.12 ^{c}
Hb g\dl	12.70±0.09 ^{a}	8.75±0.45 ^{b}	10.32±.39 ^{c}	10.70±0.26 ^{d}	11.18±0.17 ^{e}
PCV %	39.51±0.24 ^{a}	30.27±0.41 ^{b}	37.17±0.23 ^{c}	38.04±0.09 ^{d}	38.12±0.07 ^{d}
MCV fl	68.22±0.06 ^{a}	58.02±0.27 ^{b}	66.22±0.12 ^{c}	66.82±0.09 ^{d}	67.10±0.14 ^{e}
MCH pg	22.14±0.04 ^{a}	15.74±0.22 ^{b}	20.86±0.18 ^{c}	20.93±0.10^{ce}	21.03±0.11^{de}
MCHC g/dl	32.25±0.27 ^{a}	23.90±0.17 ^{b}	30.87±0.11 ^{c}	31.01±0.11^{ce}	31.14±0.08^{de}

a-e different letters between treatments are significant ($p<0.05$).

Table 2: Biochemical biomarkers in control group, nitrate group, nitrate and ascorbic acid group, nitrate and TQ group, and nitrate, ascorbic acid and TQ group.

	Control group	**Nitrate treated group**	Nitrate and ascorbic acid	Nitrate and TQ group	Nitrate, ascorbic acid and TQ
Albumin (g/dL)	5.56±0.21 ^{a}	2.10±0.07 ^{b}	4.85±0.10 ^{c}	4.96±0.08 ^{ce}	5.09±0.14 ^{de}
T protein (g/dL)	7.61±0.06 ^{a}	3.15±0.13 ^{c}	6.38±0.21 ^{c}	6.30±0.15 ^{c}	6.40±0.16 ^{c}
AST (IU/L)	30.18±0.07 ^{a}	40.41±0.30 ^{b}	31.35±0.23 ^{c}	31.94±0.12 ^{d}	32.12±0.12 ^{d}
ALT (IU/L)	50.17±0.08 ^{a}	63.52±0.38 ^{b}	51.37±0.33 ^{c}	51.95±0.21 ^{d}	52.15±0.11 ^{d}
Cholesterol mg/dL)	40.35±0.34 ^{a}	74.07±0.18 ^{b}	41.90±0.18 ^{c}	42.52±0.32 ^{d}	41.04±0.52 ^{e}
Creatinine (mg/dL)	0.81±0.02 ^{a}	3.33±0.27 ^{b}	0.98±0.04 ^{cd}	0.99±0.04 ^{cd}	0.88±0.04 ^{ad}
BUN	25.19±0.08 ^{a}	49.68±0.13 ^{b}	26.59±0.34 ^{c}	26.33±0.19 ^{d}	25.99±0.10 ^{e}
Glucose	137.2±0.04 ^{a}	256±0.66 ^{b}	139.3±0.49 ^{c}	139.1±0.26 ^{c}	138.2±0.81 ^{d}

a-e different letters between treatments are significant ($p<0.05$).

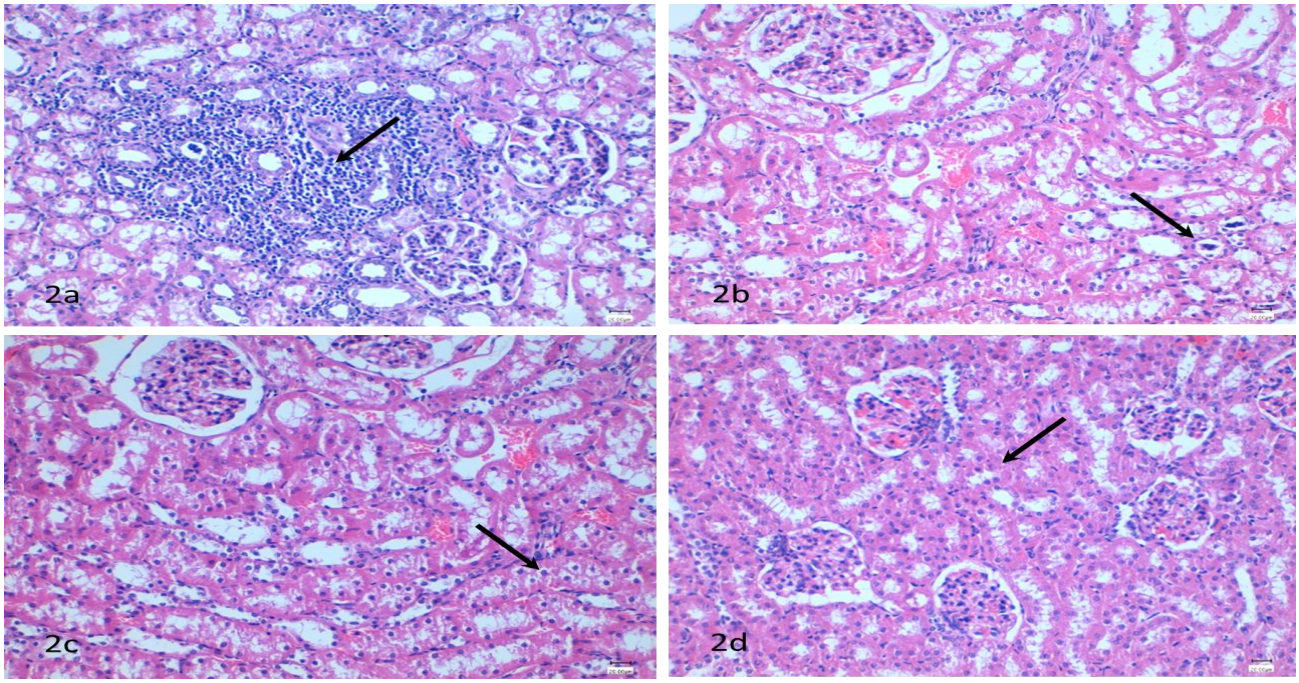

Fig. 1a: Liver of the nitrate group showing sever vacuolar degeneration with pyknotic nuclei (arrow). HE bar 20 µm.Fig. 1b: Liver of the nitrate group treated with ascorbic acid showing vacuolar hepatocytes (arrow). HE bar 20 µm. Fig. 1c: Liver of the nitrate group treated withthymoquinone showing slight mononuclear infiltration in portal area (arrow). HE bar 20 µm. Fig. 1d: Liver of the nitrate group treated with ascorbic acid and /or thymoquinone showing recovered and heathy hepatocytes (arrow). HE bar 20 µm.

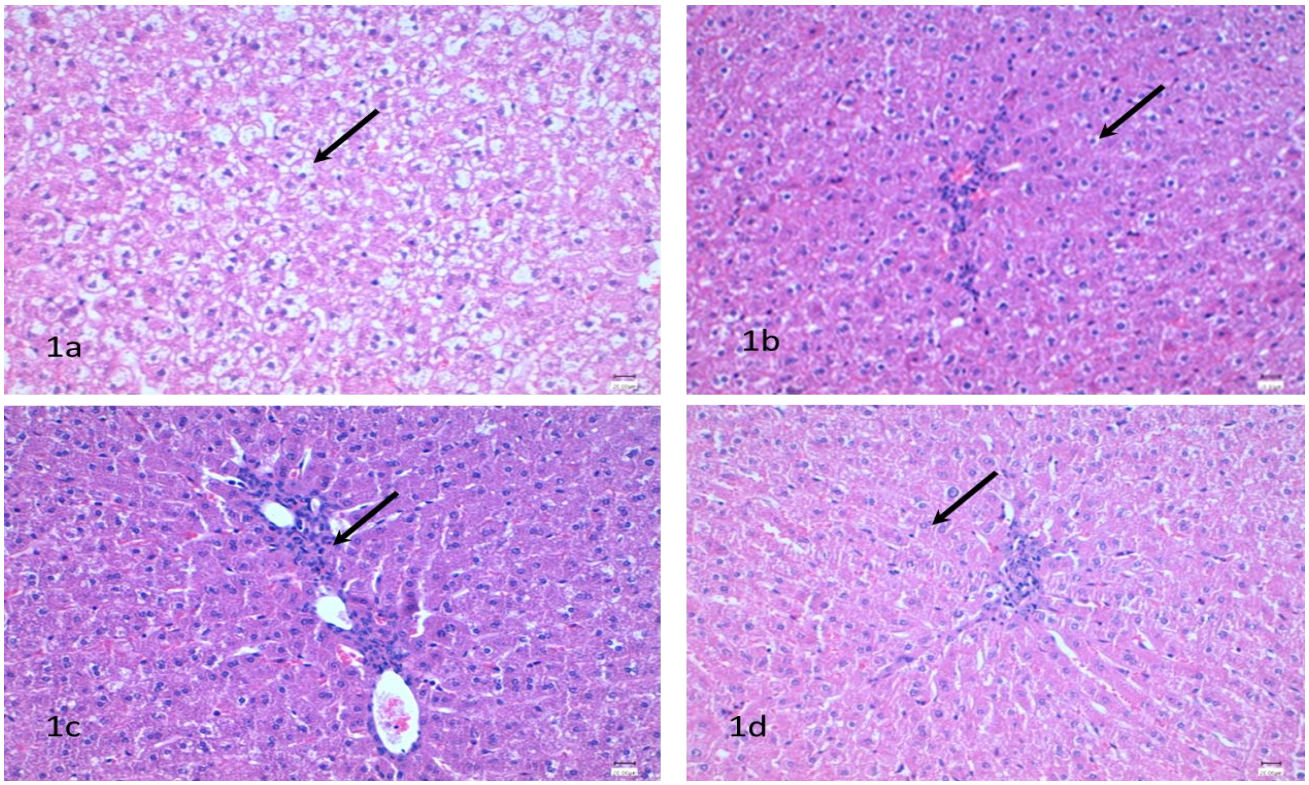

Fig. 2a: Kidney of the nitrate group showing multiple foci of mononuclear cells beween glomeruli (arrow). HE bar 20 µm.Fig.2b: Kidney of the nitrate group treated with ascorbic acid showing cellular casts in some tubules (arrow). HE bar 20 µm. Fig. 2c: Kidney of the nitrate group treated with thymoquinone showing a mild degree of vacular degeneration of some tubules (arrow).

HE bar 20 µm. Fig.2d:Kidneyof the nitrate group treated with ascorbic acid and /or thymoquinone showing heathy renal tubules (arrow) and normal glomeruli . (HE bar 20 µm.

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