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Effect of Flavonoids Extracted from Black Cumin (*Nigella sativa*) and Vitamin E in Ameliorating Hepatic Damage Induced by Sodium Nitrate in adult male rats

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Summary

This study was designed to investigate the effect of sodium nitrate as oxidant agent on hepatic function of adult male rats, as well as the possible protective role of vitamin E and flavonoid extracted from *Nigella Sativa* seeds against the deleterious effects of sodium nitrate. Forty adult male rats were randomly divided in to 4 equal groups and treated daily for 84 days as follows: Animals in the first group were received normal saline, serving as control (group C), rats of the second group (T1) were intubated orally sodium nitrate 30mg/kg. B.W.; animals in T2 group were intubated orally vit. E 40mg/Kg B.W. in addition to sodium nitrate, while rats in the fourth group (T3) were intubated orally 50mg/kg B.W of flavonoids was extracted from *Nigella Sativa* seeds with sodium nitrate. Blood samples were collected at 0, 21, 42, 63 and 84 days of experiment to study the following parameters: Serum alanine aminotransferase (ALT) and alkaline phosphtase activity (ALP), serum billirubin, as well as hemoglobin concentration. The result revealed that oral intubation of 30mg/kg. B.W of sodium nitrate (T1 group) for 84 days caused hepatic damage manifested by significant increase ($p<0.05$) in serum ALT and ALP activities, bilirubin concentration and depression in hemoglobin concentration. On other hand, the protective role of vitamin E and flavonoids extracted from *Nigella Sativa* was clarified in groupsT2 and T3, including correction of hepatic damage manifested by significant ($p<0.05$) depression in ALT and ALP activities and bilirubin concentration as well as significant ($p<0.05$) elevation in hemoglobin concentration. In conclusion, the results of this study confirm the protective role of vitamin E and flavonoids of *Nigella sativa* seed against hepatic dysfunction caused by sodium nitrate manifested by structural and functional changes.

Key word: Sodium nitrate, Black cumin, Vitamin E, liver function, Hemoglobin.

تاثير فلافونويدات الحبه السوداء وفيتامين E في تحسين وظيفة الكبد في ذكور الجرذان البالغة المعاملة بنترات الصوديوم

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الخلاصة

صممت هذه الدراسة لمعرفة تاثير نترات الصوديوم كعامل مؤكسد على وظيفة الكبد في ذكور الجرذان البالغه وكذلك البحث عن الدور الوقائي لفيتامين E و فلافونويدات الحبه السوداء في القليل من التاثيرات الضارة لنترات الصوديوم . تم أستخدام (40) من ذكور الجرذان البالغة قُسّمت عشوائيا الى اربعة مجاميع متساوية وعوملت يوميا كالتالي لمدة84 يوم:أعطيت المجموعة الأولى محلول الملح الفسلجي وعدت كمجموعة سيطره (Group C) ،في حين جرعت حيوانات المجموعة الثانية نترات الصوديوم فمويا بتركيز 30 ملغم/كغم (Group T1)، اما حيوانات المجموعة الثالثة فقد جرعت فمويا فيتامين E بتركيز 40 ملغم \كغم من وزن الجسم بالآضافة الى نترات الصوديوم (Group T2) في حين جرعت حيوانات المجموعه الرابعه فمويا فلافونيدات الحبة السوداء بتركيز 50 ملغم /كغم من وزن الجسم (Group T3) بالآضافة الى نترات الصوديوم . تم جمع عينات الدم في الفترات 0 و21 و42 و63 و84 يوم من التجربة ,وتم سحب الدم بطريقة الوخز القلبي لغرض دراسة المعايير التالية : قياس فعالية الأنزيم الناقل للأمين (ALT) و فعالية انزيم الفوسفاتيز القاعدي(ALP)في مصل الدم، وقياس تركيز البليروبين و هيموكلوبين الدم. اظهرت نتائج هذه الدراسة ان اعطاء

الحيوانات نترات الصوديوم بتركيز 30 ملغم\كغم لمدة 84 يوماً قد تسبب في حدوث خلل وظيفي في الكبد تمثل بحدوث زيادة معنوية في فعالية انزيمي ALT و ALP في مصل الدم وارتفاع معنوي في البليروبين في الأيام 0 و21 و42 و63 و84 من التجربة، بالاضافة الى انخفاض معنوي تركيز هيموكلوبين الدم مقارنة مع مجموعة السيطره (Group C). كما بينت النتائج ان التجريع الفموي لفيتامين (E) و فلافونويد الحبه السوداء اضافة الى نترات الصوديوم في المجموعه الثالثه والرابعه على التوالي ادى تحسين في وظيفة الكبد والتي تمثلت بحصول انخفاض معنوي في فعاليةALT و ALP. وحصول انخفاض معنوي في تراكيز البليروبين بالاضافة الى الزياده المعنويه في تركيز الهيموكلوبين. لقد اكدت نتائج هذه الدراسة الدور الوقائي لفيتامين E وفلافونويدات حبة السوداء ضد الخلل الكبدي المحدث بنترات الصوديوم تمثلت بتغيرات وظيفية ونسجية.

Introduction

Nitrate and nitrite are naturally occurring ions that are part of the nitrogen cycle and are ubiquitous in the environment. Nitrate salts are used widely as inorganic fertilizers, and are also used in explosives, as oxidizing agents in the chemical industry. Nitrite salts have been also used as food preservatives, especially to cure meats (1,2). The major source of nitrate in the human body is through intake of food and water (3). Dietary nitrate intake is considerable and many vegetables are particularly rich in this anion(4) green leafy vegetables such as lettuce or spinach (4,5). Drinking water may contain variable amounts of nitrates which accounts for up to 21% of total nitrates intake in a typical human diet (5, 6). Several proteins have been shown to be capable of facilitating nitrite bioactivation by reducing it to NO, including hemoglobin (7), myoglobin (8), xanthine oxidoreductase (9), nitric-oxide synthase (10), and cytochrome c oxidase (11).

The first adverse effects of nitrate were reported by Comly in1945 after observing cyanosis in infants in Iowa (12). Since then there has been concern of possible health effects related to high nitrate consumption, including some forms of cancer (13), thyroid disorders (14) and reproductive effects (15,6). Nitrates in drinking water may react in the gastrointestinal tract synthesizing the powerful hepatotoxic and carcinogenic dimethyl nitrosamine (16).Antioxidants are found in varying amounts in foods such as vegetables, fruits, grain cereals, eggs, meat, legumes and nuts (17). Many medicinal plants contain substantial amounts of antioxidants other than vitamin C, vitamin E and flavonoid, carotenoids (18). Anioxidants have been reported to prevent oxidative damage caused by free radical and may prevent the occurrence of disease, cancer and aging. It can interfere with the the oxidation process by reacting with free radicals, chelating, catalytic metals and also by acting as oxygen scavengers (19) and thus can be utilized to scavenge the excessive free radicals generated from human body (20) with no side effects and economic viability . In accordance with this, the present study was focused on investigation the effect of sodium nitrate as oxidant agent on hepatic function of adult male rats, as well as the possible preventive role of vitamin E and flavonoid extracted from *Nigella Sativa* seeds against the deleterious effects of sodium nitrate.

Materials and Methods

Forty male Albino Wistar rats (200-250 gm) were used in this investigation .Their ages ranged between (2.5-3) months. Animals in all stages of the experiment housed in plastic cages in conditioned room (22-25 $^{\circ}$C) in the animal house of department of physiology and pharmacology at the College of Veterinary Medicine -University of Baghdad for the period from December 2010 to March 2011with providing daily light of twelve hours (7.00 to 19.00). They were left for ten days for adaptation with the experimental conditions. Animals had free access to water and standard pellet diet along the experiment.

Forty adult Albino male rats were randomly divided into four groups (10 rat/ group) and were handled as follows: Group C: Animals in this group will be administered normal saline and served as control. ; Group T1: Animals in this group will be administered sodium nitrate 30mg/kg.B.W by gavage needle (21) .Group T2: Animals in this group will be administered sodium nitrate 30mg/kg.B.W plus 40mg/kg.B.W vit E by gavage needle; Group T3: Animals in this group will be administered sodium nitrate 30mg/kg.B.W by gavage needle plus 50mg/kg B.W of flavonoids was extracted from *Nigella sativa* according to the method of

Harborn, (22) . Blood samples were collected at 0, 21,42 ,63 and 84 days of the experiment .Blood was drawn by cardiac puncture technique for measuring the following parameters:Hb (23), serum ALT and ALP activities enzymatically using enzyme kit (BioMerieux, spain),Serum Bilirubin concentration (Biolao, spain). Data were shown as the mean ± SE .Statistical analysis of data was performed on the basis of Two-Way Analysis of Variance (ANOVA) using a significant level of ($P<0.05$). The least significant differences (LSD) was used to identify significant differences (24).

Results

Hemoglobin (Hb) concentration

Table (1) showed a general trend for the hemoglobin value to decrease significantly ($P<0.05$) after 12 weeks in sodium nitrate treated group (30mg/kg.B.w) as compared to the control group and other treated groups (T2and T3) . A significant decrease ($P<0.05$) in mean value of hemoglobin concentration were detected after 21days of experiment in groups T2,and T3comparing to control . Within the time, Significant ($P<0.05$) depression in the mean value of hemoglobin concentration was observed in sodium nitrate treated group (T1) at 21,42,63 and 84 days comparing to pretreated period .

Table (1):Effect of sodium nitrate , vitamin E and flavonoids extracted from *Nigella Sativa* on hemoglobin concenteration(g/dL) in male rats.

Groups / Days	C (control)	T1 (30mg NaNO3)	T2 (30mg NaNO3+ 40mg VitE	T3 (30mg NaNO3+ 50mg flavonoids)
Zero	15.00±0.04 A a	14.90±0.03 A a	15.02±0.05 A a	15.30±0.12 A a
21	15.00±0.32 A a	13.41±0.30 B b	14.27±0.15 C b	14.19±0.30 C b
42	14.85±0.17 A a	11.52±0.31 B c	14.76±0.32 A ab	14.67±0.22 A b
63	15.04±0.18 A a	9.45±0.34 B d	14.93±0.29 A a	14.14±0.27 C b
84	14.55±0.16 A a	8.37±0.02 B e	16.39±0.20 C c	16.19±0.04 C c

Values are expressed as mean ± SE. n=10rats/group. Capital letters denote differences between groups, P<0.05 vs. control. . Small letters denote differences within group, P< 0.05 vs. pretreated period .

Alanine aminotransferase (ALT):-

The result of table (2) indicated that intubations of sodium nitrate (groupT1) caused significant elevation ($P<0.05$) in serum ALT activity at 21,42.63and 84 days of experiment as compared to control ,T2 and T3 groups. The mean values of serum ALT activity in T1,T2,T3and control at the end of the experiment were (162.80±2.69), (57.20±0.66) , (59.20±0.66) and (58.40±2.31), respectively.

The data pertaining to mean value of serum ALT indicated that rats received sodium nitrate concurrently with vitamin E (group T2) and sodium nitrate concurrently with flavonoids (50mg) extracted from *Nigella sativa* (group T3) caused significant decrease ($P<0.05$) in this parameter at day 21,42 and 63days of the experiment comparing to group T1 .It seems that vitamin E and flavonoids normalize the value of ALT in T2 and T3 groups with that of control.

Table (2): Effect of sodium nitrate and vitamin E and flavonoids extracted from *Nigella Sativa* on serum alanine aminotransferase ALT (IU/L) activity in adult male rats.

Groups Days	C (control)	T1 (30mg NaNO3)	T2 (30mg NaNO3+ 40mg VitE.)	T3 (30mg NaNO3+ 50mg flavonoids
Zero	59.00±1.67 A a	58.80±1.80 A a	57.80±1.20 A a	59.00±0.83 A a
21	58.80±1.46 A a	94.20±2.31 B b	79.00±3.49 C b	83.80±1.46 D b
42	60.60±2.11 A a	107.60±4.20 B c	75.80±1.52 C b	80.00±1.44 C b
63	58.86±0.34 A a	143.00±1.30 B d	65.00±0.24 C c	67.56±0.34 C c
84	58.40±2.31 A a	162.80±2.69 B e	57.20±0.66 A a	59.20±0.66 A a

Values are expressed as mean ± SE. n=10rats/group. Capital letters denote differences between groups, P<0.05 vs. control. Small letters denote differences within group, P< 0.05 vs. pretreated period.

Serum Alkaline Phosphatase (ALP) Activity

The results in table (3) showed a significant increase (P<0.05) in serum ALP activity in T1,T2and T3 treated group at the 21,42,63and 84days of experiment as compared to control . On the other hand, lack of significant differences (P>0.05) were observed in T2 and T3 along the treated period as compared to each other .Within the time the activity of serum ALP in three treated groups T1,T2 and T3increased significantly (P>0.05) compared to the values at day zero.

Table (3): Effect of sodium nitrate, vitamin E and flavonoids extracted from *Nigella Sativa* on serum alkaline phosphatase activity (IU/L) in adult male rats.

Groups Days	C (control)	T1 (30mg NaNO3)	T2 (30mg NaNO3+ 40mg VitE.	T3 (30mg NaNO3+ 50mg flavonoids)
Zero	247.80±11.36 A a	249.40± 0.74 A a	248.20±0.37 A a	249.30±0.30 A a
21	247.00±11.88 A a	583.20±19.56 B b	462.40±13.98 C b	484.60±10.98 C b
42	246.81±5.48 A a	631.80±13.54 B c	452.60±12.77 C b	469.40±18.38 C b
63	246.60±1.36 A a	777.56±7.06 B d	377.00±1.41 C c	398.80±1.42 C c
84	246.40± 0.97 A a	987.60±37.28 B e	283.00±0.89 C d	295.60±4.83 C d

Values are expressed as mean ± SE. n=10rats/group. Capital letters denote differences between groups, P<0.05 vs. control. Small letters denote differences within group, P< 0.05 vs. pretreated period.

Serum bilirubin concentration

A significant increase (P<0.05) in serum billirubin concentration were detected in group T1(received sodium nitrate)at 21,42,63 and 84 days of treatment compared to control and other treated groups. The highest increment in this parameter were detected at the end of experiment (day84) in sodium nitrate treated group comparing to T2 and T3group . On the other hand , oral intubations of vitamin E or flavonoids extracted from *Nigella sativa* in combination with sodium nitrate caused significant depression (P<0.05) in mean value of serum billirubin compared to T1treated group(table-4). Within the time it seems that vitamin E intubations concurrently with sodium nitrate caused normalized billirubin value with that pretreatment period at day 84 compared to the pretreatment period.

Table (4): Effect of sodium nitrate, vitamin E and flavonoids extracted from *Nigella Sativa* on serum bilirubin concentration (mg/dl) in male rats.

Groups Days	C	T1 (30mg+NaNO3)	T2 (30mg NaNO3+ 40mg VitE.)	T3 (30mg NaNO3+ 50mg flavonoids)
Zero	0.17±0.007 A a	0.17±0.004 A a	0.17±0.003 A a	0.16±0.003 A a
21	0.17±0.009 A a	0.40±0.03 B b	0.32±0.008 C b	0.33±0.018 C b
42	0.16±0.009 A a	0.44±0.01 B c	0.28±0.008 C c	0.30±0.016 C c
63	0.16±0.005 A a	0.47±0.007 B d	0.23±0.006 C d	0.22±0.005 C d
84	0.16±0.003 A a	0.56±0.025 B e	0.18±0.004 C a	0.19±0.003 C e

Values are expressed as mean ± SE. n=10rats/group. Capital letters denote differences between groups, P<0.05 vs. control. Small letters denote differences within group, P< 0.05 vs. pretreated period

Discussion

Hemoglobin (Hb) concentration

Regarding the effect of nitrous compounds on blood picture and immune response, $NaNO_2$ administration induced reduction in hematological parameters as RBCs count, hematocrit and Hb concentration (25). A significant decrease in lymphocyte percentages as well as IgG was also observed (26). The recognition that nitrite is a NO donor has led to the proposal that nitrite accumulation may cause excess production of NO that disturbs NO homeostasis and contributes to nitrite toxicity (27).The main toxic action of nitrate is the result of the conversion of oxygen-carrying pigment to forms that are incapable of carrying oxygen (28). Excessive consumption of nitrite and nitrate also has been implicated as a cause of other health problems (29). Nitrite methemoglobinemia is potent process for free radical generation. Nitrite can stimulate oxidation of ferrous ions in oxyhemoglobin to form MetHb and superoxide –anion radicals. This xenobiotic is a ready source of nitric oxide which reacts rapidly with superoxide to form highly reactive peroxinitrite ($ONOO^{--}$) (30) .Methemoglobinemia may lead to tissue protein oxidative radical formation, inflammatory cascades, and CNS injury (31,32). From these facts we can conclude that methemoglobinemia occur after nitrate intubation may lead to suppression of Hb. Vitamin E, was observed to repair the genotoxicity and improves the hematological and biochemical changes (33).Its protective role against the effects of nitrate/nitrite exposure has been attributed to its ability to decrease the formation of peroxynitrite and ROS production (16,34), and increased GSH levels in RBCs, indicating a decrease in their oxidative status .It is also important in prolonging the span life of RBCs and and ameliorate immunological response. (35). When vitamin is administered with cadmium chloride, the rate of abnormal dissociation of oxyHb to MetHb is diminished (36). Besides, the presence of vitamin E ameliorate the interactions favorite folding such as hydrophobic forces and metal ion protein interaction (37), this lead to unfolded Hb (36) and elevation in Hb concentration. Antioxidant were found to reduce oxidative radical induced reactions and have protective effect on stabilization of metabolic process in erythrocytes that prevent the development of oxidative and hypoxia (38). The beneficial effect of flavonoids against oxidative damage of hemoglobin caused by hydroperoxides could be due to the ability of flavonoids to scavenge ferryl hemoglobin. (39) *Nigella sativa* has appreciable antioxidant and free radical scavenger properties (40) by which it could maintain cellular and tissue integrity with restoring Hb level.

Serum Alanine aminotransferase and Alkaline Phosphatase Activity

Liver enzymes (ALT and ALP) are normally found in circulation in small amounts because of hepatic growth and repair. They are good indicators for liver state that increased when hepatocytes are damaged and they suffer from exacerbation and remissions irresponsive to liver condition (41) . On the other hand, ALP belongs to a group of enzymes catalyze the hydrolysis of phosphomonoesters at alkaline pH. ALP present in cell surface in most human tissues. The highest concentration is found in the intestine, liver, bone, spleen and kidney (42).The specific location of the enzyme within both sinusoidal and bile canalicular membranes accounts for the more predominant elevations in certain disorders (43) .

The observed elevation in the activity of liver enzymes in NaNO2 treated group is in agreement with the previous studies of (44,45) .Such elevation in the activity of these enzymes could be attributed to the toxic effect of nitroso-compounds formed in the acidic environment of the stomach in causing severe hepatic injury and necrosis (46). Hepatic injuries mainly depend on two mechanisms: the massive production of deleterious reactive oxygen species (ROS) and the activation of resident macrophages (Kupffer cells) resulting in the release of pro-inflammatory cytokines (TNFα, IL-1, IL-8)(47) .The activation of inducible nitric oxide synthase (iNOS) from hepatocytes and macrophages which leads to increased production of nitric oxide (°NO) may also contribute to these alterations with overproduction of ROS, cytokines and proteases leading to damage to both parenchymal and non-parenchymal cells, finally resulting in liver dysfunction (48) and elevation of liver enzymes.

The development of liver injury by administration of nitrosamine compounds was associated with a significant increase in liver lipid peroxidation content (increasing TBARS), oxidative stress and altering the antioxidant status in several tissues (44,45,49,50).A decrease in the concentration of non-enzymatic antioxidant (GSH) in this study document the previous statement and indicating that such decline in GSH may participated in liver injury and proceeding depletion in serum AST and ALT activity. Antioxidant nature of vitamin E in the management of hepatotoxicity due to oxidative stress was reported by previous researches(51, 52, 53). Vitamin E has already been shown to decrease cellular injury(52) and improve liver function (55) .

Although Yildiz and his coworkers (56) recorded the efficacy of *Nigella sativa* (*N. sativa*) in relieving the deleterious effects of ischemia reperfusion injury in the liver, the exact mechanism of action of anti ischemic activities is not clear. Flavonoids including those of *Nigella sativa*, are powerful antioxidants against free radicals and are described as free-radical scavengers (57). This activity is attributed to their hydrogen-donating ability (58) . Flavonoids are capable of modulating the activity of enzymes and affect the behavior of many cell systems and exerting beneficial effects on body (59)They terminate chain radical reaction by donating hydrogen atom to a peroxy radical as thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain (60) leading to suppression of oxidative stress maintaining liver function and its enzyme activity. Accordingly, the antioxidant activity of flavoniods and vitamin E as documented in this study by elevation in serum GSH concentration may be participative in this issue.

Total Serum Bilirubin (TSB) concentration

The increase in total bilirubin level in the serum of the sodium nitrate treated rats in the present study could be attributed to the increase in the rate of red blood corpuscles destruction and/or damage of the liver tissue (60). Moreover, hyperbilirubinaemia is a result of severe haemolysis, impaired secretion of bilirubin or cholestasis (61). The deformability of destructed RBCs and increased RBCs haemolysis belong to increased production of FRSr duo to any cause (62) has been documented. RBCs membrane is very susceptible to free radical mediated peroxidation with formation of lipid peroxide and eventually haemolysis (63). Accordingly, excessive production of ROS in the presence of NO can lead to the formation of reactive nitrogen species such as peroxynitrite radicals which cause cell injury and death (64). On conclusion the resultant liver injury and haemolysis after sodium nitrate intubation may lead to increased bilirubin concentration . The result revealed that oral administration of

flavonoids extracted from *Negilla sativa* or vitamin E caused a significant depression in serum bilirubin concentration as compared to T_1.Vitamin E is non-enzymatic membrane antioxidant and it is less specific in reaction with the free oxygen than enzymatic antioxidants but it is more universal and therefore, it can play a preventive role in the free radical reaction (65). It has been found that parenteral administration of vitamin E decreased lipid peroxidation in RBCs membrane in chronic anemic patients (66).

The antioxidant properties of *N. sativa* has been well documented previously (67, 68, 69), therefore its administration protects hepatic tissue from deleterious effects of toxic metals such as lead, and attenuates hepatic lipid peroxidation following exposure to chemicals such as carbon tetrachloride.(70, 71).Accordingly, the antioxidant activity of both vitamin E and flavoniods restore cellular integrity with maintaining liver function including maintaining bilirubin concentration.

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