Original Articles

Effects of Intake of Nitrate, Nitrite and Isosorbid Dinitrate (ISDN) on Plasma Nitrate/Nitrite Contents in Rabbits

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Background: The objective of the present study was to demonstrate the changes in plasma levels of nitrate and nitrite in rabbits after oral (intragastric) administration of inorganic nitrate (KNO_3), inorganic nitrite ($NaNO_2$) and organic nitrate (isosorbide dinitrate, ISDN).

Material and Methods: Twenty eight rabbits were divided into four groups (7 in each group) and various doses of these solutions were given via intragastric route. Group-I (control) was given 6 ml distilled water. Group-II (nitrate) was given 500 mg nitrate, group-III (nitrite) 50 mg nitrite and group-IV 20 mg ISDN per kg body weight per day. The experiment was conducted for 12 weeks and blood samples were taken at regular intervals. Plasma nitrate and nitrite levels were determined as stable metabolites of nitric oxide (NO) formation.

Results: Results indicate 28.2±3.3 µmoles/dL nitrate and 21.24±2.8 µmoles/dL nitrite, a nitrite/nitrate ratio of 0.75 in all 28 rabbits at zero-day. After 1-day of nitrate intake, an 8.1-fold increase in nitrate content (from 29.3±3.32 to 238.5±48.9 µmoles/dL, $p<0.05$) and 2.3-fold increase in nitrite content (from 20.67±2.88 to 48.75±6.36 µmoles/dL) in group-II animals was seen. No significant change was observed in their levels in group-III and IV animals during this period. On 2-day, plasma nitrate content decreased to 165.4±18.3 µmoles/dL and nitrite contents dropped to normal in group-II animals. After 1-week, nitrate content in group-II also decreased to normal values. However, nitrite content in group-III animals increased 3-fold (from 18.43±3.06 to 57±4.96 µmoles/dL) during 1-week with no increase in nitrate content. No sudden rise or fall in contents was observed for 12-weeks in all the groups.

Conclusion: The results demonstrate that the plasma nitrate and nitrite contents are not changed significantly in all four groups in 12-weeks duration (except the initial rise in week-1) and animals may possess some adaptive metabolic mechanisms to normalize these levels.

Key words: Nitrate, Nitrite, Nitric oxide, Rabbits

Introduction

Nitric oxide is an important mediator in nervous, immune and cardiovascular systems. Its involvement has been demonstrated in normal as well as pathophysiological states.[1,2] NO is synthesized by a family of three isoenzymes called nitric oxide synthases (NOS).[3] Various molecules act as NO donors which include nitrate, nitrite, isosorbide dinitrate (ISDN), L-arginine, nitroglycerin etc. Nitrates and nitrites are ingested with food and water whilst their endogenous production by intestinal and oral microbial flora is also reported.[4] It has been estimated that 60-70% of the total oral intake of nitrate is excreted in urine in the first 24 hours and remaining is stored in various tissues or re-enters at various points in the gastrointestinal tract.[5] About 25% of ingested nitrate is excreted in saliva where it is partially reduced to nitrite by oral micro flora. Nitrite can react with hemoglobin to form methemoglobin[7] or secondary and tertiary amines to form N-nitroso compounds which are involved in the aetiology of some types of cancers.[8] This nitrite is also a source of NO.[9] L-arginine, another source of NO, is immediately converted into various stable products like nitrite and nitrate.[10] Because of the gaseous nature, determination of NO itself is difficult and the estimations of plasma nitrate and nitrite are used as a measure of the formation of NO radical.[11]

To study the toxic effects of nitrate, Mascher and Marth (1993) gave calcium nitrate to mice over a dose of zero (control), 100 and 1000 mg nitrate in drinking water over a period of 18 months. Liver and kidney functions showed no statistical differences with the control group and significant differences were found between individual groups in their body weights, urea and ammonium levels.[12] Similarly, in another study, rats were given sodium nitrite at 100 mg/kg body weight in drinking water daily during their entire life span over three generations and no evidence of chronic toxicity, carcinogenicity or teratogenicity was observed.[13] Mulsch et al (1995) treated rabbits with

Mg/kg), ISDN (10 mg/kg) etc. NO formation was higher in organs like liver, kidney, heart, lungs, spleen and low levels were detected in blood vessels. ISDN and NTG formed NO preferentially in the mesenteric bed.[14]

These individual studies in different animals and different laboratories partially demonstrate the plasma nitrate and nitrite levels and some associated biochemical or pathological parameters during the studied period. A comparative study for the determination of plasma nitrate/nitrite levels with oral intake (by passing intragastric tube) of inorganic nitrate, inorganic nitrite and organic nitrate has not been carried out earlier. This paper demonstrates the changes in plasma nitrate and nitrite contents in rabbits in response to these NO donors during a 12-weeks duration.

Material and Methods

Twenty eight rabbits, 4 males, one in each group and 24 females, 6 in each group (0.85-1.520 kg with a mean of 1.55 kg/rabbit) of local race were purchased from the local market and were kept in the animal house in controlled conditions (23°C; 12h light and 12h dark cycle).

The animals received standard rabbit diet (dry food) and water ad libitum. Before the start of experiment, animals were acclimatized for one week. Water and food given to animals was lacking in nitrate and nitrite.

Rabbits were divided into four groups (7 in each group). Group-I (control) was given orally 6 ml distilled water/kg body weight/day. Group-II (nitrate) was given 500 mg nitrate (KNO_3), group III (nitrite) 50 mg nitrite ($NaNO_2$) and group-IV 20 mg organic nitrate (ISDN) per kg body weight per day. The experiment was conducted for 12 weeks and blood samples from internal jugular vein were taken at zero-day and after 1, 2 dayand then 1 to 12 weeks. Rabbits were weighed before taking blood samples to observe changes in their weights. Plasma was obtained from heparinized blood and stored at -20°C until used for analyses.

Plasma nitrate levels were determined by standardized Cd-Zn reduction column method.[15,16] Briefly, Zn pellets (10g) were mixed with 100-200 ml saturated solution of cadmium sulphate for 2-3 hours. Cadmium grains deposited on Zn pellets as sponges were separated, washed with distilled water (100 ml), washed twice with 0.5N HCl (100 ml x 2) for 30 minutes each followed by washing with water (100 ml x 2) and finally with elution buffer (50 mg ammonium chloride and 20 g sodium chloride in 1L water; pH 9.6-9.7 was adjusted with ammonium hydroxide). Cd-Zn grains were packed upto 1-1.5 ml in a 5ml disposable syringe column. 100 µl plasma was loaded onto the column.

Elution was carried out with elution buffer, collecting 3 ml eluate in each of the 3 test tubes. 2 ml coupling reagent (1% Sulphanilamide in 3M HCl and 0.02% N-ethylenediamine HCl in water in 1:1 ratio) was added to each of the test tube. Absorbance was read at 540 nm after 20 minutes. Nitrite concentration was measured from the standard curve. Results were expressed in terms of µmoles/dL plasma.

For the regeneration of columns, pre-used columns were placed overnight in 0.5N HCl. The next day, columns were washed with water, 0.5N HCl, water, and buffer solution, respectively, as mentioned earlier. These columns proved as efficient as the fresh columns (80-95% efficiency).

For the determination of plasma nitrite levels, 200 µl plasma was added to distilled water to make volume of 3ml followed by the addition of 2ml coupling reagent. After 20 minutes nitrite contents were measured at 540 nm.

In some sets of experiments where nitrite levels were below the detection limit, 10 nmoles nitrite was added to 100 l plasma as an external standard and assay performed as mentioned above. Statistical analysis was carried out by SPSS package using ANOVA test.

Results

1. Control Group

In control group-I rabbits (n=7), mean plasma nitrate and nitrite contents on zero-day were 24.43±1.38 and 19.43±2.19µmoles/dL respectively. With statistically insignificant changes ($p>0.05$) on 12-week, nitrate and nitrite contents were 21.33±2.85 and 18.17±3.12 µmoles/dL respectively, exhibiting 46% nitrite contents **(Fig- 1 & 2)**. These results show that the given diet had no effect or contribution in the total nitrate+nitrite (NOx) contents **(Fig-3)** in plasma and nitrite/nitrate ratio remained consistent, that is, 0.88 and 0.87 **(Fig- 4)**.

2. Effects of Potassium Nitrate

In group-II, plasma nitrate and nitrite contents on zero-day were 29.33±3.32 and 20.67±2.88 µmoles/dL, respectively. After day-1 of oral intake of first dose of first dose of nitrate, nitrate and nitrite

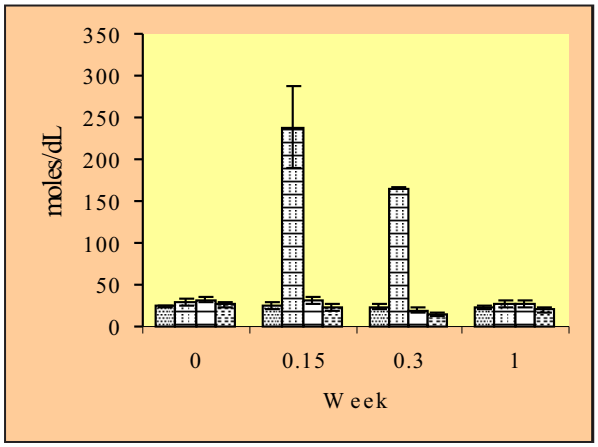

Fig-1: Changes in plasma nitrate contents.

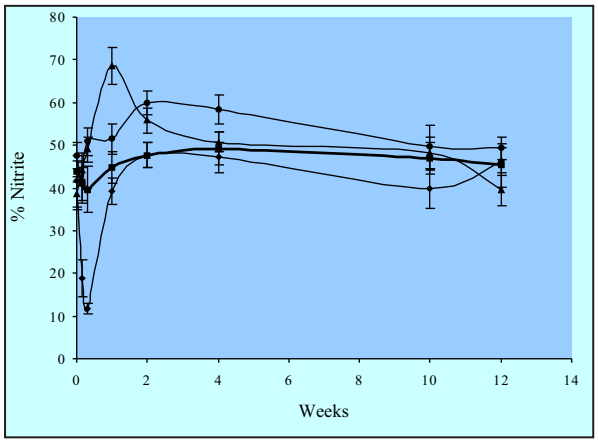

Fig-2: Changes in percent nitrite levels in plasma. Percent nitrite indicates the percent nitrite in the total nitrate+nitrite contents (Nox).

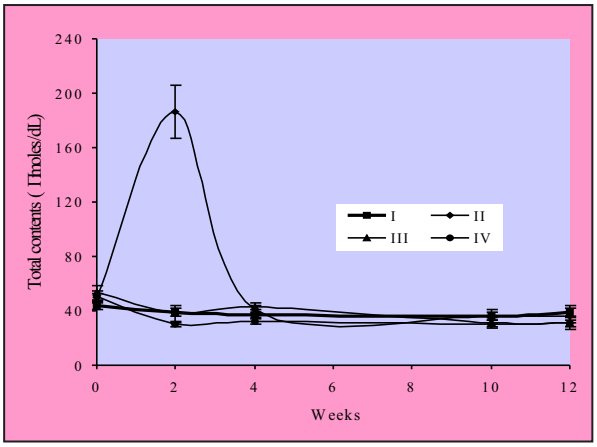

Fig-3: Changes in plasma total nitrate and nitrite contents (Nox).

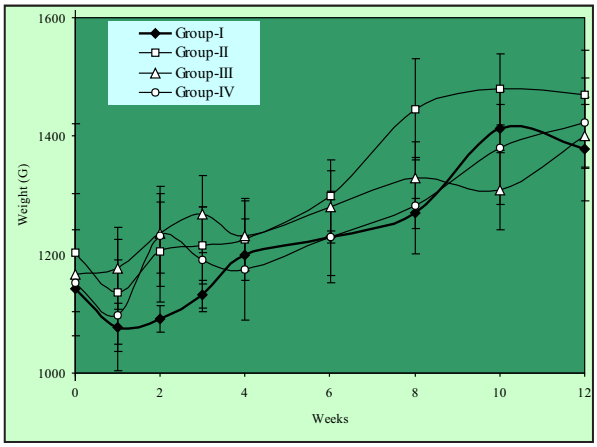

Fig-4: Nitrate/nitrite ratio in plasma.

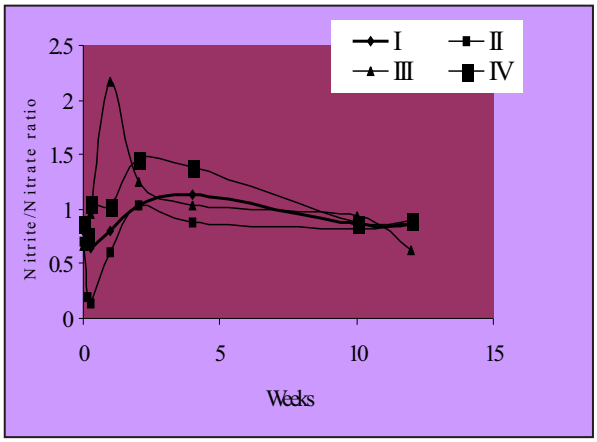

Fig-5: Change in body weight of rabbits during the studied period of 12- weeks Values are mean±s.e.m. (n=7)Rabbits were given distilled water (group-I), potassium nitrate (group-II), sodium nitrite (group-III) and ISDN (group-IV) by passing through intragastric tube. Blood was drawn at specified time and plasma was used for the determination of nitrate and nitrite. ANOVA was applied to the data. Values (means.e.m.) are significantly different when *p<0.05.

contents jumped upto 238.5±48.9 and 48.75±6.36 µmoles/dL, respectively (n=4, p<0.01), with just 18.88% nitrite contents (a nitrite/nitrate ratio of 0.20, p<0.05) (Figure 1 & 2). This indicated an 8.1-fold increase in nitrate and 2.3-fold increase in nitrite contents in these animals. On day-2, nitrate contents decreased to 165.4±18.3 µmoles/dL (p<0.01) and nitrite contents dropped to normal values (21.2±2.65µmoles/dL). No significant changes in the nitrate/nitrite contents were observed in these animals till week-12 (p>0.05). These results indicate that oral intake of potassium nitrate brings about

Sudden increase in plasma nitrate and nitrite contents which reach basal values soon after a week.

3. Effects of Sodium Nitrite

Plasma nitrate and nitrite contents in group-III animals on zero day were 32.17±2.76 and 21.17±2.80 μmoles/dL (n=7), respectively. These values indicated 38.75% plasma nitrite contents (a nitrite/nitrate ratio of 0.658). After day-1 of oral intake of sodium nitrite, nitrate and nitrite contents remained unchanged though %nitrite increased from 38.75 to 44.21% ($p>0.05$). On day-2, a decrease in nitrate contents from 32±4.44 to 19.43±3.61 μmoles/dL ($p<0.05$) was observed associated with a slight decrease in nitrite contents (Figure 1 & 2). NOx value (Figure 3) decreased from 56.3±4.81 to 37.86±6.46 μmoles/dL ($p<0.05$). A nitrite/nitrate ratio (Figure 4) of 0.948 ($p>0.05$) was observed. These results indicate that after 48 hours, nitrate and nitrite contents are almost 50% in plasma though there was a decrease in total contents. When these values were monitored after 1-week, nitrate and nitrite contents were 26.29±3.96 and 57±4.96 μmoles/dL ($p<0.05$), respectively, with a nitrite content of 68.6% and nitrite/nitrate ratio of 2.168 ($p<0.05$). In further weeks, a slow decrease in total contents as well as in nitrite contents was observed and a nitrite/nitrate ratio was found 1.248 (2-week), 1.03 (4-week), 0.938 (10-week) and 0.637 (12-week). NOx contents reached less than the basal values.

4. Effects of Isosorbide Dinitrate

In group-IV animals, basal nitrate and nitrite contents were 26.86±3.27 and 23.71±1.92 μmoles/dL, (n=7) with nitrite/nitrate ratio of 0.883. After 1-day, little decrease in NOx (from 50.57±4.3 to 43.29±5.38 μmoles/dL) was observed with nitrite/nitrate ratio of 0.757 ($p>0.05$) (Figure 3 & 4). On 2-day, this ratio increased to 1.049 ($p<0.05$) and remained at this higher level till week-4 with insignificant changes in total nitrate and nitrite contents. However, on 10-week and 12-week, basal values of nitrite/nitrate ratio of 0.87-0.89 was reached. These results indicate that ISDN action is delayed till 2-day or 1-week and lasts longer (upto 4-week) compared to other NO donors. Further, there were no recorded variations in plasma nitrate and nitrite contents.

5. Change in Body Weight of Rabbits

Change in body weight of animals was monitored each week for 12-weeks and results are shown in Figure 5. A consistent increase in weight of animals is depicted in all four groups; group-II animals had comparatively a higher weight than the rest of the animals.

Discussion

Plasma nitrate and nitrite contents determinations are used for the synthesis of NO.[11] Some workers prefer to measure nitrate as the major metabolite of NO in blood,[17] others report only nitrite contents in blood.[18] The present paper reports the changes in plasma nitrate, nitrite, nitrate+nitrite (NOx), %nitrite of total and nitrite/nitrate ratio.

Plasma samples were kept at -20°C and used within a year for determination since it is already documented that nitrate and nitrite are stable in plasma for 1-year.[11] Cd-Zn column method was standardized for the reduction of nitrate into nitrite and determination by Griess reaction with or without added nitrite as an external marker where the plasma nitrite levels were below the detection limit. Using human plasma, with or without added nitrate as an external marker, the column showed 80-95% efficiency even after regeneration (data not included), 87% nitrate and nitrite recovery is also documented.[11] In other sets of experiments, plasma samples were deproteinized with 2% sulphosalicylic acid but no effect on the estimation of nitrate or nitrite was found (data not shown). Therefore, all values were taken from deproteinized plasma.

In the present studies, nitrate and nitrite contents have been expressed in terms of nitrate, nitrite, NOx, %nitrite and nitrite/nitrate ratio. The presence of plasma nitrate and nitrite (24.43±1.38 and 19.43±2.19 μmoles/dL, respectively) in control group exhibits NOx of 43.86±3 μmoles/dL or 43.80% nitrite contents. This makes nitrite/nitrate ratio of 0.795. On day-1 of oral intake of nitrate, significant changes in nitrate, nitrite and NOx contents have been seen in group II animals compared with other groups (all values are $p<0.05$). On day-2, nitrate and NOx contents were significantly different ($p<0.05$) in group II animals. This initial increase in nitrate/nitrite plasma pool indicates excess of NO formed during the said period. This study coincides with the findings of Cortas and Wakid (1991)[19] wherein they gave 470 μmoles/kg sodium nitrate (equivalent to 292 mg nitrate) orally to animals and peak levels of plasma nitrate (183 μmoles/dL) reached in 40 minutes. Plasma nitrite contents were not detected in their experiments. In the present study, animals were given 500 mg nitrate/kg body weight and nitrate levels reached 238.5±48.9 μmoles/dL in 24 hours. Initial

Been reported after oral intake of ammonium nitrate or intravenous sodium nitrate.[20]

In group II, these changes disappeared after a week but appeared in nitrite-fed animals, group III, wherein nitrite and NOx contents increased significantly ($p<0.05$) compared with the other groups including control. These differences persisted in group III on week-2 ($p<0.05$). In group IV, the increase in nitrate contents was delayed till week-4, when the nitrate and total NOx contents were higher than the other groups ($p<0.05$) while nitrite contents remained indifferent ($p>0.05$). No further statistically different readings were recorded till week-12 which indicates that ISDN intake causes little change in the plasma nitrate, nitrite or NOx contents.

Moshage et al (1995)[11] found no relationship in the plasma nitrite or nitrate contents as measured by %nitrite of NOx which varied from 3.9-88% in plasma samples. They found that in whole blood, >95% nitrite is converted into nitrite within an hour and suggested that nitrite determination alone was meaningless. The present study reveals that the NOx contents and amount of nitrate are good indicators and results should be expressed in terms of these configures.

In the previous studies by Guy (1998),[13] rats were allowed to drink nitrite containing water for the entire life and no carcinogenic effects were observed. This study did not tell how much nitrite was taken in by the animals. In the present study, nitrate or nitrites were given to rabbits according to their body weight via intragastric tube. The present study therefore reveals the effects of these chemicals in animals kept in the similar conditions in a more quantitative way, given the same food and the environment with different NO donors. Results reveal that changes in plasma nitrate and nitrite contents occur till the first week, later the levels of these anions remain consistently constant. This suggests that some form of NO tolerance mechanism is operative to maintain total nitrate and nitrite contents in plasma. However, it does not indicate that how much nitrate or nitrite is present in tissues and how much damage has been done to the body systems/organs when their plasma levels reach peak levels.

Conclusion

Plasma nitrate and nitrite levels did not change significantly in all four groups in 12-weeks duration (except the initial rise in week-1) probably because the animals possess some adaptive metabolic mechanisms which normalize these levels.

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Medical News

Changes in HbA1-C Units

International changes have been announced for the units for reporting HbA1c results. There was an agreement that it was necessary for glycated haemoglobin to be standardized to the IFCC Reference Measurement procedure but there was a concern regarding the introduction of a dual reporting system including new units with which patients and clinicians were unfamiliar. It was felt that there was a real risk that patient control would deteriorate due to lack of understanding, particularly with an unheralded change to IFCC units. It was agreed that dual reporting would be necessary for a considerable period until both unitary systems were understood. However, this should only occur after extensive educational programmes had been performed. Moreover, it must be ensured that these programmes will reach all the appropriate health-care professionals as well as patients.For a period of about two years, results will be reported in both old and new units.

Comparative Values

DCCT- HbA1c (%)	IFCC-HbA1C (mmol/mol)
6.0	42
6.5	48
7.0	53
7.5	59
8.0	64
9.0	75