African Journal of Biotechnology Vol. 5 (9), pp. 749-754, 2 May 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

Effects of acute and sub-chronic ammonium nitrate exposure on rat liver and blood tissues

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Accepted 6 March, 2006

Use of fertilizers like ammonium nitrate (NH_4NO_3) for agricultural purposes has increasingly contaminated the ecosystem with nitrate and/or nitrites. Nitrite is a toxic substance that can cause multiple physiological effects if allowed to build up to high concentrations in animals such as methemoglobinemia. This work is concerned with the study of short term (3 days intoxication) and mid-term (over 21 days) NH_4NO_3 exposure to wistar rats at the dose of 250 mg/Kg. Under these conditions, some hematological and biochemical parameters were affected. Methemoglobinemia, increase in serum nitrates as well as a hepatic cytotoxicity indicated by an increase in bilirubin and transaminases levels were observed.

Key words: Ammonium toxicity, Blood tissues, Methemoglobin, Bilirubin, Rat liver, Fertilizers.

INTRODUCTION

Mineral fertilisers are intended to correct soil deficiencies in organic nutrients, especially nitrogen. But excessive use of mineral fertilisers like ammonium nitrate (NH_4NO_3) subsequently leads to ecosystem pollution by the accumulation of nitrates in vegetables and fodder as well as the contamination of underground water that are subsequently found in drinking water (Addiscott, 1988; Fan and steinberg, 1996; Bouwer, 2000; Testud, 2005). Nitrates/nitrites are likely to exert harmful effects on the respiratory function as a consequence of acute intoxication while being responsible for methemoglobinemia (Mansouri and Perry, 1987; Kammerer, 1994; Jensen, 2005).

Nitrates, in case of their oral absorption, are reabsorbed rather quickly in intestines and over 80% are released in a mass in the urine. The high speed of absorption is due to the shortness of the ion nitrate radius. Nitrites are formed in the buccal cavity by bacterial reduction of about 20% of the nitrates ingested which escaped from elimination, circulating between the buccal cavity and the digestive system (Hageinstein, 1989; L'Hirondel, 1998).

It has been shown that nitrite treatments in intact RBC causes a noticeable oxidation of oxyhemoglobin to methemoglobin by radical generation along with a decrease in glutathione level in the intracellular medium associated with membrane lipid peroxidation. The oxidative reactivity induced by nitrite alters cell ionic flux (Batina, 1990). This leads to osmotic fragility of the erythrocytary membrane as well as a perturbation of membrane transport that tends to hemolysis. Oxidative damage and precipitation of the globin portion of hemoglobin into large aggregates result in the formation of Heinz bodies that can bind to and alter membranes. Membrane structure also is altered by the oxidation of sulfhydryl groups and by lipid peroxidation (Harvey, 1997). Oxidative injury to erythrocyte membranes may result in pyknocytes formation (Fischer et al., 1985; Fischer, 1986).

Nitrate transformation into *N*-nitroso compounds in the stomach in the presence of secondary amines makes them still more dangerous. Although secondary amines represent the essential constituents of human food, risk of cancer can only be reduced if the nitrate or even nitrite levels in food remains as low as possible (Knekt et al., 1999; Apfelbaum, 2001).

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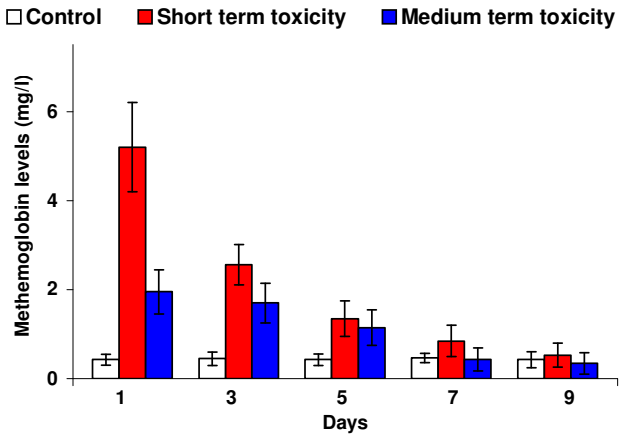

Figure 1. Methemoglobin levels at the end of short (3 days) and medium (21 days) term exposure to NH_4NO_3 (mean ± SD, n = 5).

This work deals with acute and sub-chronic toxicity of NH_4NO_3 on male rats at a dose of 250 mg/kg and the evaluation of short and average term effects on blood and liver, known compulsory routes of all xenobiotics, by using several biochemical and enzymatic analyses.

MATERIAL AND METHODS

Rats

Adult male wistar rats, weighing approximately 200 ± 25 g each (Pasteur Institute of Algiers, Algeria), were acclimatised to laboratory conditions for 15 days.

Chemicals

Pure ammonium nitrate produced in form of pills was obtained from a local factory (ASMIDAL: National company of plant health products). This chemical fertiliser was dissolved in distilled water at a concentration of 100 g/l.

Treatment of rats

100 rats are separated into 2 experimental batches containing 5 groups each. In each group (of 10 rats), 5 rats were used in the different treatments and the other 5 rats serve as control. The first batch is exposed by means of gastric probe to distilled water containing NH_4NO_3 of 250 mg/Kg for 3 days (short term exposure). The second batch receives the same treatment as above for 21 days (medium term exposure). The control groups of each batch undergo the same treatment with only distilled water. The rats were sacrificed on the first, third, fifth, seventh and ninth day after the end of the treatment (Guastadisegni et al., 1989). The blood is collected into two tubes: one tube is heparinised for the sake of methemoglobin determination while the other is kept dry so it can be used for analysis of serum nitrate, transaminase activity and reduced glutathione levels. The spleen is weighed before the histological study.

Biochemical assays

Methemoglobin assay was carried out according to Evelyne and Malloy (1938) method following to Burgat-sacaze et al. (1981) modification and based on the measurement of absorption at 635 nm of a dilution of blood before and after total transformation of haemoglobin into methemoglobin and cyanmethemoglobin. Serum nitrates level was measured according to Kallel and Safta (1984) method based on nitrites copulation in the presence of alphanaphtylamine and sialic acid and production of a red complex compound at 520 nm. This reaction could be total when nitrates are transformed into nitrites in presence of cadmium. Bilirubin and transaminases levels were made automatically by Technicon (Automate108 A00902, USA).

Hematological measurements

The confection of blood smear was performed on the basis of spreading it on a blade, the colouring was curried out according to Hemateck (2000), and the observation was done by optic microscopy.

Statistics

Results are represented as the means ± SD. Student t-test was used to compare between groups at P = 0.005.

RESULTS

Effect of NH_4NO_3 on methemoglobin (metHB)

Methemoglobinemia is the accumulation of metHb above steady levels. It can be induced by the ingestion of nitrates (in well water, some foods, and fertilizers). Nitrates are converted by intestinal and oral flora to nitrites, which can lead to the formation of metHb. Therefore it was reasonable to determine the effect of NH_4NO_3 on metHb levels in each group of rats. 24 h after the end of the treatment by NH_4NO_3, the metHB level is extremely high ($P < 0.05$). Three days after the treatment, the methomoglobin level decreases by approximately 50% compared to that observed after 24 h (Figure 1). On the 5th day after the treatment, the methomoglobin tends

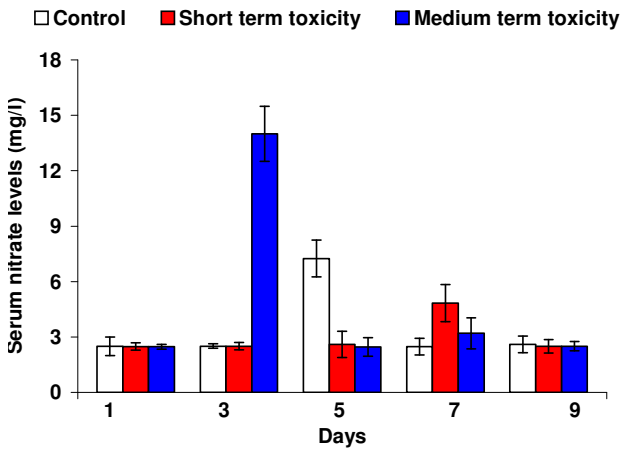

Figure 2. Serum nitrate levels at the end of short (3 days) and medium (21 days) term exposure to NH_4NO_3 (mean ± SD, n = 5).

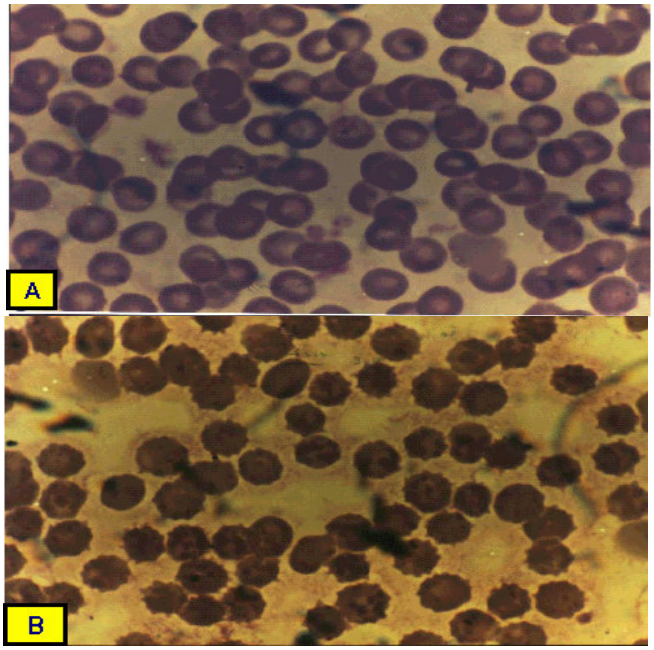

Figure 3. Optical microscopy of the blood smear in a control rat (A) and in a rat treated with NH_4NO_3 at 250mg/kg for 21 days and sacrificed 24 hours after stopping the treatment (B). (Magnification x 40).

to go back to the normal value and towards the controls' value which remains constant. In rats treated over 21 days, we observe that 24 h after the end of the treatment, the metHB levels in the treated rats is significantly higher than that of the control ($P< 0.05$). Yet, this decreases and becomes equivalent to that of the control rats seven days after the end of the treatment.

Effect of NH_4NO_3 on serum nitrates

Figure 2 show an increase in serum nitrates after NH_4NO_3 exposure. 24 h after the end of the treatment, the concentration of nitrates in serum was approximately six fold higher in 3 days treated rats than that in control ones, which does not exceed 2.55 ± 0.15g/l ($P< 0.05$). Five days later, we notice that serum nitrates levells of the treated rats are equal to those of the control rats which remain constant. 24 h after the end treatment in 21 days treated rats, the serum nitrate level is 4.85 ± 1.00 mg/l. This decreases with time to reach control rats level towards the 5th day after the end of the treatment.

Effect of NH_4NO_3 on bilirubin

Bilirubin stems from the degradation of meme of hemoglobin of the red blood cells and the increase in its amount may be hemolytic or due to hepato-cellular lesions. Therefore, it was reasonable to determine the effect of NH_4NO_3 on this parameter. From Table 1 the global bilirubin average level is sensitive to NH_4NO_3 treatments. The short term exposure resulted in a significant increase in bilirubin levels in treated animals ($P<0.05$). The bilirubin level decreases on the third day after the end of treatment and on the 9th day after the end of the treatment, both levels of the treated and control rats become equivalent.

Meanwhile, the bilirubin level in the rats treated over 21 days is also much higher than that of control rats after 24 h. 3 days after the end of the treatment, the bilirubin rate decreases till the 9^{th} day when both treated and control levels become nearly the same.

Effect of NH_4NO_3 on glutamic pyruvic transaminases

The glutamic pyruvic transaminases (GPT) level in the control is 71.50 ± 4.50 U/l, whereas in the short-term treated rats it is approximately two times higher ($P<0.05$) after 24 h (Table 1). However, this level decreases and on the 9th day after the end of the treatment, it reaches a value which is not very far from that of the control rats.

After 21 days of treatment, the GPT rate is 125.14 ± 6.36 U/l. This rate decreases approximately to that of the control at the 9^{th} day after stopping the treatment.

Effect of NH_4NO_3 on glutamic oxalo-acetic transaminase

Glutamic oxalo-acetic transaminase (GOT) average rate in the control rats is lower than in short term treated rats. This level decreases and reaches a value not very far from that of the control rats. In medium term exposure, the GOT average level in treated rats is higher than in the control ($P<0.05$). This also decreases with time and reaches a value close to that of the control rats on the 9th day after the end of the treatment.

Hematological results

Blood smear observation with optic microscope allows visualizing red blood cells morphology. We observe that NH_4NO_3 treatment provokes in all RBC well visible membrane modifications in both short and medium term treatments. Figure 3B represents a blood smear in rats treated with NH_4NO_3 for 21 days at 250 mg/kg per day.

The smear reveals a certain toxicity of NH_4NO_3 on RBC. This toxicity is indicated by ill formation of RBC membrane that loses its regular shape and becomes pyknotic. These pyknocytes tend to disappear with time. In fact, the RBC of the treated rats on the 9^{th} day after stopping the treatment become morphologically identical to those of control (Figure 3A).

Table 1. Biochemical parameters after stopping NH_4NO_3 treatments in short 3 days) and medium (21 days) term exposure to NH_4NO_3 (mean ± SD, n = 5).

Days after treatments	Biochemical parameters (µ/l)								
	Bilirubin			GPT			GOT		
	Control	STT	MTT	Control	STT	MTT	Control	STT	MTT
1st	2.6 ± 0.25	5.50 ± 0.15*	4.10 ± 0.35*	7.501 ± 4.50	134.00 ± 6.25*	125.14 ± 6.35*	125.14 ± 6.35*	215.10 ± 9.00*	206.25 ± 8.27*
3rd	2.43 ± 0.23	4.55 ± 0.16*	3.55 ± 0.28*	69.50 ± 3.75	115.18 ± 7.00*	118.32 ± 7.00*	118.32 ± 7.00*	196.75 ± 6.45*	196.34 ± 6.93*
5th	2.56 ± 0.10	3.70 ± 0.20*	3.00 ± 0.35	72.27 ± 5.00	94.52 ± 5.75*	100.84 ± 5.75*	100.84 ± 5.75*	189.33 ± 6.38*	189.05 ± 7.25*
7th	2.45 ± 0.15	3.15 ± 0.46	2.60 ± 0.25	70.43 ± 4.25	83.65 ± 5.80	80.57 ± 5.80	80.57 ± 5.80	180.52 ± 7.20	180.55 ± 8.33
9th	2.50 ± 0.17	2.90 ± 0.12	2.55 ± 0.17	76.50 ± 5.25	72.00 ± 6.00	72.00 ± 6.00	72.00 ± 6.00	175.60 ± 8.00	175.00 ± 6.00

*P< 0.005, when compared with controls.
STT: Short term treatment (3 days).
MTT: Medium term treatment (21 days).

Spleen

The weights of the spleen before and after stopping the treatments were measured. NH_4NO_3 treatment enhanced in a significant manner the weights, but this also diminishes like the other studied parameters.

DISCUSSION

In this work, we have confirmed the methemoglobinising effect of NH_4NO_3 on the rats' red blood cells. These compound react on haemoglobin to form the metHB. Nitrite induced haemoglobin oxidation is one of the principal factors of nitrite toxicity and one of the ways of its metabolism in an organism (Titov and Petrenko, 2004). The oxidation of hemoglobin by nitrite has been recognised as an autocatalytic reaction (Tomoda et al., 1981):

$$Hb(O_2)_4 + 2NO_2^- + H_2O \longrightarrow Hb(O_2)_4 + 2NO_2^- + H_2O$$

Our results are in perfect agreement with those of Jensen (2005) who showed that nitrite transport into pig erythrocytes caused a significant increase in metHb levels with time. metHb rose to significantly higher values in oxygenated than in deoxygenated RBCs. The increase in metHb was largest in the beginning, and values subsequently approached quasi steady levels at the end of the experiment.

In the course of this reaction, oxygenated free radicals are produced and could induce a peroxidation of the unsaturated fatty acids of phospholipids. Thus, it appears like an osmotic brittleness of the erythocyte membrane as well as a disturbance of membrane transport which leads to the hemolysis (Bowdler et al., 1984; Bogard et al., 1986; Batina, 1988, 1990).

In addition to methemoglobinemia, NH_4NO_3 induces the appearance of huge quantities of nitrates in the serum. The nitrates contained in the product after ingestion, is converted into nitrous acid, then into nitrites in the gastro-intestinal tract. The nitrite resulting from this reduction is spread in the RBC, where it acts by oxidising haemoglobin. Consequently, after this reaction, the nitrite is in its turn oxidised into nitrate and thus the reappearance in the serum of great quantities of newly formed nitrates is observed with only traces of nitrite, because the latter is in perpetual reduction into nitrates during its fixing process on haemoglobin (Osterloh, 1984).

From the experimentation carried out for 21 days, the animals developed capacities of adaptation. The parameters observed did not undergo major variations in spite of a significant absorption of NH_4NO_3. Our results are similar to those of previous experiments (Batina, 1988; Csallany and Ayaz, 1978). The appearance of nitrites produced by nitrate reduction generates a cascade of physiological phenomena which affect, in most cases, the blood tissue as well as other organs involved in the haematopoiesis such as the spleen (splenomegaly) and the liver where most of the metabolisation of xenobiotics takes place. After 24 h, the weight increases of almost 70% higher in short term exposure (data not shown). Since the spleen is a haematopoietic organ, it is then easy to make a parallelism between the increase of weight of this organ and the hemolysis observed under the action of both nitrates and nitrites. Also, the bilirubin amounts is 2 at 3 times higher in treated rats compared with control ones. Transaminases levels also confirm hepatic toxicity.

Modifications of RBC shapes on the smear confirm this toxicity and the plasma membrane presents ill formations. The interaction of nitrite with the cell membrane drastically modified erythrocytes membranes (Zavodnik et al., 1999). It has been shown that nitrite-induced methemoglobinemia in throut RBC leads to an inhibition of red blood cell sodium/proton exchanger. Thus the membrane potential changes after RBC exposure to nitrite may reflect the alterations of the activity of membrane ionic exchangers. Nitrite in the protonated form can transport protons through the membrane changing the ion distribution between the inside and the outside of the cell. Nitrite anion accumulation in the erythrocytes can also influence increase membrane potential changes. The decrease in membrane fluidity may result from the changes of the membrane structural state due to the oxidative process in the membrane provoking hemolysis (Batina, 1988). The present results show that erythrocytes exposed to NH_4NO_3 suffer membrane damage.

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