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EFFECT OF HIGH AMMONIA LEVEL ON STRESS-INDUCED HEMATOLOGICAL CHANGES IN RABBITS: PREVENTIVE EFFECT OF PYRIDOXINE

M. DYAVOLOVA, I. YANCHEV, D. GUDEV and P. MONEVA
Institute of Animal Science, BG - 2232 Kostinbrod, Bulgaria

Abstract

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The object of the present experiment was to evaluate the effect of air ammonia on some hematological parameters and rabbit response to short-term psychological stress as well as to test the effect of supplemental pyridoxine.

Eighteen New Zealand rabbits at the average age of 3.6 months were divided into 3 groups as follows: Control group – reared under low air ammonia levels (6.79±2.55 ppm) and two experimental groups- reared under higher air ammonia levels (21.98±7.84 ppm). The second experimental group was given supplemental pyridoxine (200 mg/l) throughout the 10 day long experimental period.

Arterial blood samples were taken at the start (1^{-st} day) and at the end of the experiment (10^{-th} day) as well as before and following exposure to psychological stress. Stress was induced on day 10 by 1 minute "dog barking" PC record in a triple 110 dB playback. The following parameters were evaluated: total erythrocyte and leukocyte counts, hematocrit, peripheral blood leukocyte distribution, rectal temperature, respiratory rate and air ammonia concentration. Ammonia augmented leukocyte counts ($P<0.05$) and hematocrit levels ($P<0.05$) while at the same time it thwarted the expected rise in heterophil to lymphocyte ratio in response to stress. Pyridoxine prevented ammonia-provoked increase in total leukocyte numbers ($P<0.05$) and hematocrit levels ($P<0.05$) in response to stress.

Pyridoxine caused a decrease in respiratory rate ($P<0.05$) and an increase in rectal temperature ($P<0.05$) as compared to the unsupplemented rabbits. The results are interpreted to suggest that nitric oxide mediates the effects of both ammonia and glucocorticoids on leukocyte subpopulations, which ultimately may compromise the effect of glucocorticods on heterophil to lymphocyte ratio.

*Key words***:** rabbits, air ammonia, stress, pyridoxine

Introduction

Ammonia is a gas that is highly irritating, colorless and very soluble. It is absorbed in the superior part of the breathing path where it inflicts pathological and pathohistological changes (Close et al., 1980). It has also been reported that ammonia can reduce the capturing of oxygen by the hemoglobin due to its impact on the blood pH (Olanrevaju et al., 2008).

Elevated concentrations of ammonia induce the formation of free radicals in astrocytes and this process is associated with the synthesis of glutamine. Some authors propose that astrocyte-derived free radicals may be responsible for some of the pathophysiological changes associated with hyperammonemic conditions (Murthy et al., 2001). Also, the activities of glutathione peroxidase, superoxide dismutase, and catalase were decreased in brain of rats injected with ammonia (Kosenko et al., 1997) thus indicating that ammonia induces oxidative stress in the brain.

Acute ammonia intoxication also increases the formation of NO, another free radical that contributes to ammonia toxicity (Abel Lajtha et al., 2009)

The present knowledge about the effect of air ammonia on rabbit's welfare and growth performance is highly insufficient.Studies on ammonia-induced changes in hematological indexes are conducted mainly on pigs and poultry (Curtis et al., 1975; Drummond et al. 1976; Wathes et al., 2004).There is

E-mail: pv_moneva@abv.bg

little information about food supplements that antagonize the toxic effect of ammonia in rabbits.

Vitamin B6 is part of the family of water soluble substances and is a complex formed from three distinct, chemically different compounds: pyridoxine, pyridoxal and pyridoxamine. These are the dietary precursors of the active coenzyme forms, which are pyridoxal phosphate and pyridoxamine phosphate. Practically all the compounds identified as neurotransmitters in the brain are synthesized and/or metabolized with the aid of pyridoxal phosphate. These include dopamine, norepinephrine, serotonin, tyramine, tryptamine, taurine, histamine, γ-aminobutyric acid (GABA) (Travell et al., 1998).

Pyridoxine has been reported to stimulate the production of hemoglobin (Cartwright et al., 1944). Rabbits are known to excrete copious ammount of ammonia via urine. Consequently, we set ourselves the target of investigating the effect of ammonia on some hematological indexes. We also studied the possibility to alleviate the adverse effect of ammonia through the supplementation of pyridoxine.

Material and Methods

The experiment comprised 18 New Zealand White rabbits (*Oryctolagus cuniculus*) at the age of 3, 6 months, divided into three groups (control, unsupplemented and pyridoxine supplemented),consisting of 6 rabbits each. Rabbits in the control group were reared under low air ammonia levels (6.79±2.55 ppm) and the two experimental groups – under high air ammonia levels (21.98±7.84 ppm) of naturally occuring ammonia in the air throughout the experimental period. Seven days before the start of the experiment control rabbits were moved to a room where the levels of ammonia were low. The second experimental group was given supplemental pyridoxine (200 mg/l) throughout the 10 days long experimental period. Pyridoxine was produced by Rhône-Poulenc, France. Rabbits were reared in an enclosed building under spring conditions with variable natural temperatures within the range of 9°C to 11°C. The outside temperature ranged from 8.5°C to 12°C. Rabbits were housed individually in wire-floor cages, provided with feeders and automated drinkers – feed and drinking water were supplied *ad libitum*, except for the pyridoxine supplemented rabbits, which were given supplemental pyridoxine added to the drinking water.

Indoor air temperature and humidity were recorded during the experiment by the psychrometer method. Arterial blood samples were taken from all groups at the start (1^{-st} day) and the end of the experiment (10^{-th} day). Breathing rate was measured each day by counting the rate of flank movement. Stress was induced by 1 minute "dog barking" PC record in a triple 110 dB playback. Blood samples were taken before and 25 minutes following the start of the stress episode. Rectal temperature was measured by digital thermometer at the beginning of the experimental period and immediately after the exposure to stress. Total erythrocyte and leukocyte counts were determined by manual haemacytometer chamber count. Haematocrit was measured by the microhaematocrit method. Peripheral blood leukocytes were counted on smears, that were prepared immediately after blood sampling. The smears were stained using May-Grunvald and Gisma stains (Lucas and Jambos, 1961). Four hundred leukocytes including neutrophils, eosinophils, basophils, lymphocytes and monocytes were counted microscopically on a slide.Air ammonia was recorded via AeroQual S200 Monitor, equipted with ammonia sensor head (0-100±0.1 ppm).The results of one factor statistical analysis are expressed as means ±S.E.M. and were analyzed by ANOVA.

Results and Discussion

Hematocrit levels in the experimental rabbits, which were not given supplemental pyridoxine, tended to be higher than in the other two groups before exposure to stress (Figure 1). It increased further after exposure to stress and was significantly higher ($P<0.05$) as compared to control and pyridoxine supplemented rabbits. Similarly, exposure of chickens to atmospheric ammonia (0.25 and 50 ppm) has been reported to increase both hemoglobin and hematocrit on d 35 (Olanrewaju et al., 2008). The authors attributed the observed increase of hematocrit to an increase in erythropoiesis as a compensatory reaction to the lack of oxygen in the tissues. However, in our study erythrocyte concentrations in unsupplemented rabbits following exposure to stress were not increased (Fig-

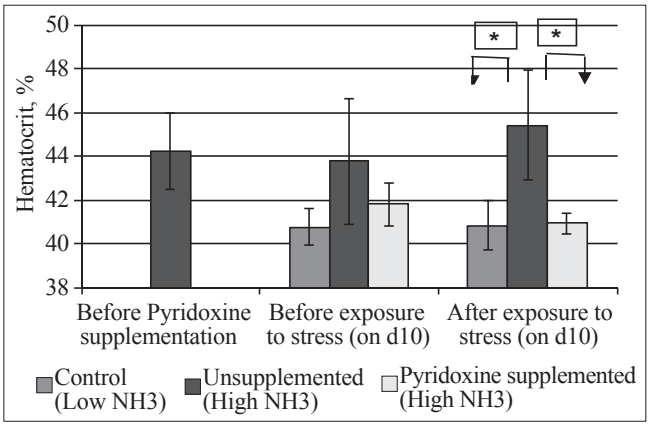

Fig. 1. Effect of supplemental Pyrodoxine (200 mg/L) on ammonia - induced change in hematocrit level after stress
* $P<0.05$, significantly different from unsupplemented rabbits after stress

ure 2). On the contrary, erythrocyte concentrations declined slightly (P>0.05). This data suggests that the increased level of hematocrit was most probably due to ammonia-induced change in erythrocyte volume rather than to increase in erythrocyte concentrations. Roller (1967) reported sustained water loss by erythrocytes concurrent with increase in packed cell volume in cattle given solutions of urea and water directly into the rumen. The observed change in erythrocyte content of water in their experiment was accompanied by a decrease in the concentration of Na and increase of K within the erythrocyte. It is well known that astrocytes are definitely the most vulnerable cells to ammonia toxicity. Exposure of cultured astrocytes to ammonia results in an increase of free radicals production (Jayakumar et al., 2006). Besides, oxidative stress alters plasma membrane redox system (transfer of electrons from intracellular substrates to extracellular electron acceptors) in red blood cells (Pandey and Rizvi, 2011). In addition, ammonia-induced oxidative stress inflicts tissue damage (Subash and Subramanian, 2008). Oxidative stress increases hemoglobin oxidation, membrane proteins and membrane lipids oxidation in red blood cells. These data give further support to the assumption that the increased hematocrit level in the pyridoxine unsupplemented rabbits (Figure 1) may be due to change in erythrocyte membrane permeability. Hematocrit level after exposure to stress could also be influenced by NO-induced deformability of erythrocytes (Bor-Kucukatay et al., 2003) since ammonia stimulates NO production (Monfort et al., 2002) thus increasing the exposure of red blood cells to external NO in addition to the NO synthesized within erythrocytes. The very fact that the increase of hematocrit level in unsupplemented rabbits did not reach level of significance before exposure to stress suggests that some individuals were able to compensate for ammonia-induced decrease in oxygen-binding capacity of the erythrocytes. Consequently, the increase of hematocrit in unsupplemented rabbits following exposure of rabbits to psychological stress (Figure 1) appears to be an adaptive response to the increased oxygen demand caused by the increased metabolism. The enumerated metabolic effects of ammonia were most probably mediated by glutamine, since the rate of ammonia uptake from the plasma and glutamine release by skeletal muscle had been found to be increased in patients with hyperammonemia (Bessman and Bradley, 1955; Canola and Ruderman, 1976). Besides, glutamine is implicated in the ammonia – detoxifying mechanism (Leke et al., 2011) and serves as a carrier of ammonia from cytoplasm to mitochondria where it interferes with mitochondria function giving rise of excessive production of free radicals (Bai et al., 2001; Albrecht and Nirenberg, 2006). Pyridoxine supplemented rabbits had significantly lower hematocrit level as compared to that in unsupplemented rabbits after being exposed to stress (Figure 2). The lower hematocrit level in pyridoxine supplemented rabbits following exposure to physiological stress may be due either to beneficial effect of pyridoxine on erythrocyte membrane function or to its regulatory role in Na^+/K^+ ATP- ase and cellular volume (Nadiger et al., 1984). In addition, pyridoxine lowers free radicals in vascular endothelial cells (Nanfouz et al., 2009) and human blood (Lesgards et al., 2005). Our view is further supported by the finding that ammonia-induced oxidative stress increases extracellular glutamate levels (Tossman et al., 1987) via its inhibitory effect on glutamate-aspartate transporter mRNA synthesis (Zhow and Norenberg, 1999) thus preventing the interconvertion of glutamic acid to glutamine which is known to be a very important aminoacid in preventing ammonia intoxication (Albrecht and Nirenberg, 2006). Besides, oxidative stress decreases erythrocyte glu-

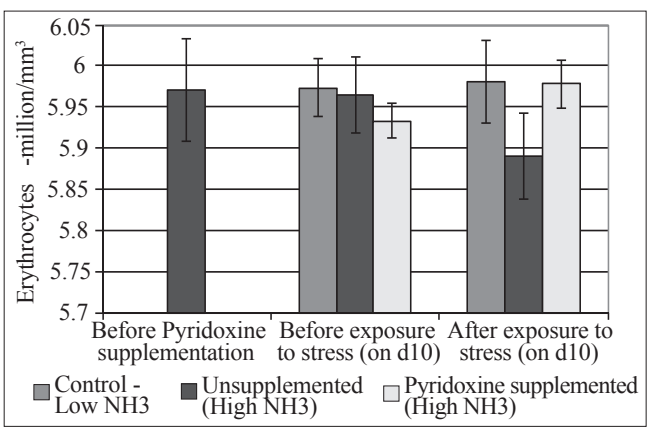

Fig. 2. Erythrocyte concentrations before and after short-term stress in Pyridoxine supplemented and unsupplemented rabbits reared under low and high ammonia levels

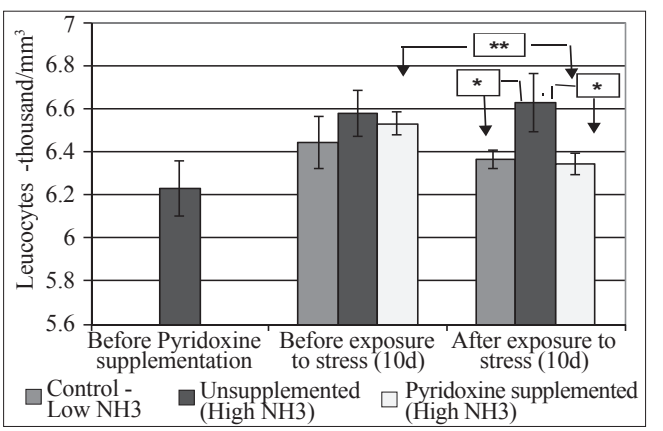

Fig. 3. Leucocytes concentrations before and after short-term stress in Pyridoxine supplemented and unsupplemented rabbits reared under low and high ammonia levels

tathione and glutamine levels thus contributing to alterations in the erythrocyte redox environment (Morris et al., 2008). Vitamin B6 deficiency has been proven to cease urea formation from L-glutamic acid (Berezov, 1953).Therefore, the observed effect of supplemental pyridoxine on hematocrit level was probably due to its ability to lower free radicals and restore the interconvertion of glutamic acid to glutamine.

The higher level of atmospheric ammonia did not influence white blood cell count (WBC) in both experimental groups on d 10 (Figure 3). However, exposure to psychological stress resulted in significant increase of WBC in unsupplemented rabbits relative to control and pyridoxine supplemented rabbits. These results suggest that the increased strain associated with maintenance of leucocyte number within the normal range under the conditions of our study (fluctuating ammonia levels) could has caused a decline in the capacity of compensatory mechanism to counter loads of adaptive mental stress (dog barking). Consequently, it could be assumed that ammonia was the main culprit for the observed increase of WBC in unsupplemented rabbits after exposure to stress. Prolonged exposure of pigs to atmospheric ammonia (0, 35 and 50 ppm) has been reported to increase WBC, absolute number of lymphocytes, serum cortisol and haptoglobin (von Borel et al., 2007). These data are not in agreement with the results of another study with the same experimental design where exposure of pigs to atmospheric ammonia 0, 25, 50 and 100 ppm) did not modify WBC differential leukocyte percentages and plasma corticosterone level (Guston et al., 1994). Ammonia-induced increase in WBC could be attributed to impaired interconvertion of glutamic acid to glutamine as judged by the reported sessation of urea formation from glutamic acid in rats deprived from vitamin B6 (Berezov, 1953) and the unchanged WBC in vitamin B6 supplemented rabbits under the conditions of our study. Lymphocyte percentages (Figure 5) showed a trend toward decline after exposure to stress but the rate of decline in unsupplemented rabbits was less pronounced relative to the other two groups (P>0.05). Exposure to stress did not cause any change in heterophil percentages (Figure 4). Our preliminary investigations on the time course of heterophil and lymphocyte dynamics in response to 10 minutes long handling stress showed that the greatest change takes place within 1-2 h after cessation of the handling in rabbits. It is known that stress caused by various noxious stimuli is accompanied by change of WBC (Tronson et al., 1997). Consequently, if we assume that the increase of WBC in unsupplemented rabbits (Figure 3) was provoked by the exposure to mental stress then we should expect higher heterophil percentages relative to those in the other groups. On the contrary, heterophil percentages in unsupplemented rabbits after exposure to stress were lower (P>0.05) than in control and pyridoxine supplemented rabbits (Figure 4). Similarly, exposure of pigs to 0, 35 and 50 ppm of atmospheric ammonia has been reported to elicit an increase in WBC, cortisol, haptoglobin, heterophil numbers and a decrease in lymphocyte numbers thus indicating that ammonia is stress-evoking agent. Stress is widely accepted to reduce lymphocyte numbers and increase heterophil numbers (Dhabher et al., 1995). On the contrary, exposure of pigs to higher ammonia concentrations (0, 25, 50 and 100 ppm) is reported to have no effect on plasma corticosterone level, differential leukocyte percentage and WBC (Guston et al., 1994). The observed discrepancy could be reconciled with the specific stimulatory effect of ammonia on nitric oxide (NO) synthesis, which in turn reduces the activity of glutamine synthetase (Monfort et al., 2002). Glucocorticoid hormones, cortisol and corticosterone are known to cause an increase in heterophil to lympho-

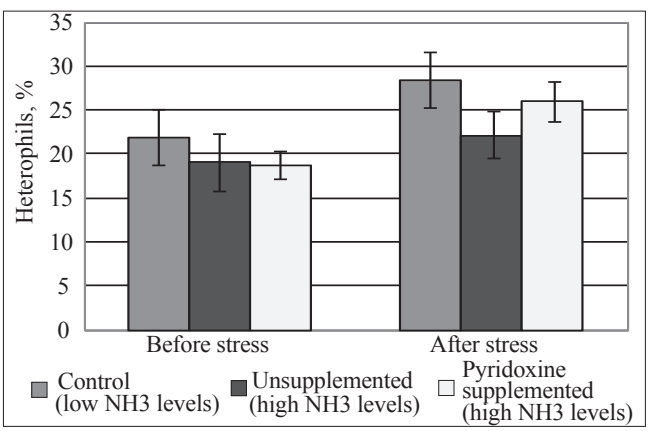

Fig. 4. Heterophil percentage before and after short-term stress in rabbits reared under low and high ammonia levels in the air

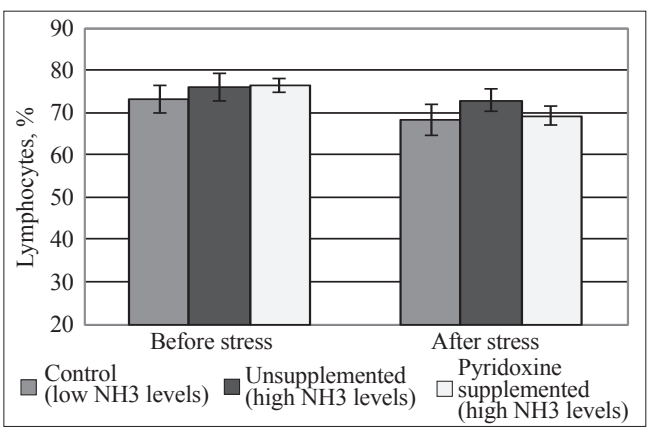

Fig. 5. Lymphocyte percentage before and after short-term stress in rabbits reared under low and high ammonia levels in the air

cyte ratio during stress (Dhabhar et al., 1995), but the underlying mechanism of their effect on leukocyte subpopulations distribution is not elucidated.

We hypothesize that glucocorticoids exert their effect on leucocyte distribution by suppressing NO synthesis (Korhonen et al, 2002) which in turn causes a dramatic increase in the number of heterophils and a moderate decrease in the number of circulating lymphocytes (Geffner et al., 1995). Ammonia unlike glucocorticoids stimulates NO production (Swamy et al., 2005) and therefore could compromise stress-induced increase in heterophil to lymphocyte ratio. Besides, adrenal zona fasciculate steroidogenesis is negatively regulated by endogenous NO (Adams et al., 1992). Also, both NO and CO are powerful inhibitors to the release of corticotrophin releasing hormone and vasopressin from the rat hypothalamus (Grossman, 2003) Our hypothesis explains at least partly the observed hemathological changes in the current experiment as well in the experiments with pigs (Guston et al., 1994; von Borel et al., 2007) and chickens (Olanrevaju et al., 2009) exposed to high atmospheric ammonia concentrations, where the classical stress response of hematological indices and cortisol were modified by the exposure to high atmospheric ammonia.

Respiratory rate in control rabbits fluctuated throughout the trial and tended to be lower (P>0.05) relative to that in unsupplemented rabbits (Figure 6). The observed fluctuation of respiratory rate may be related to ammonia-induced change in blood pH (Olanrevaju et al., 2008). Respiratory compensation has been reported to correlate with change in pH (Roller, 1997). The pH of the blood is maintained within a very narrow range during exposure to ammonia and fluctuates along with partial pressure of CO_2, O_2, hematocrit and hemoglobin (Olanrevaju et al., 2008). These changes are

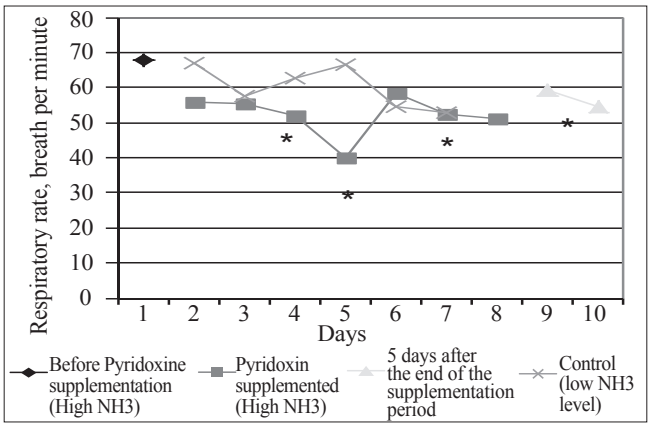

Fig. 6. .Effect of supplemental Pyridoxine (200 mg/L) on respiratory rate in rabbits reared under high and low ammonia levels in air

*P<0.05, significantly different from the mean value before vitamin B6 supplementation

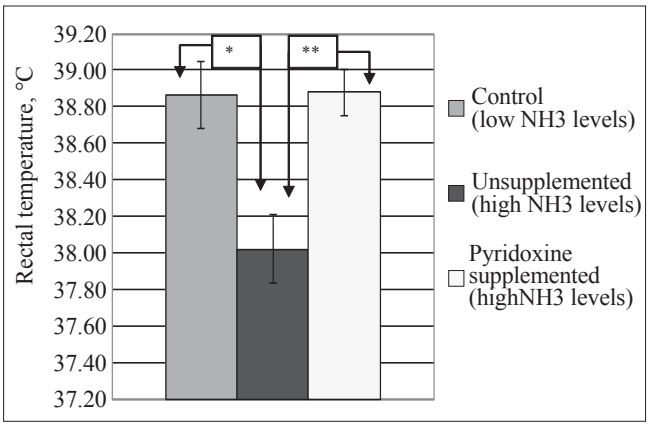

Fig. 8. Rectal temperature immediately after stress in Pyridoxine supplemented and unsupplemented reared under high ammonia levels in the air

*P<0.05 ,**P<0.01

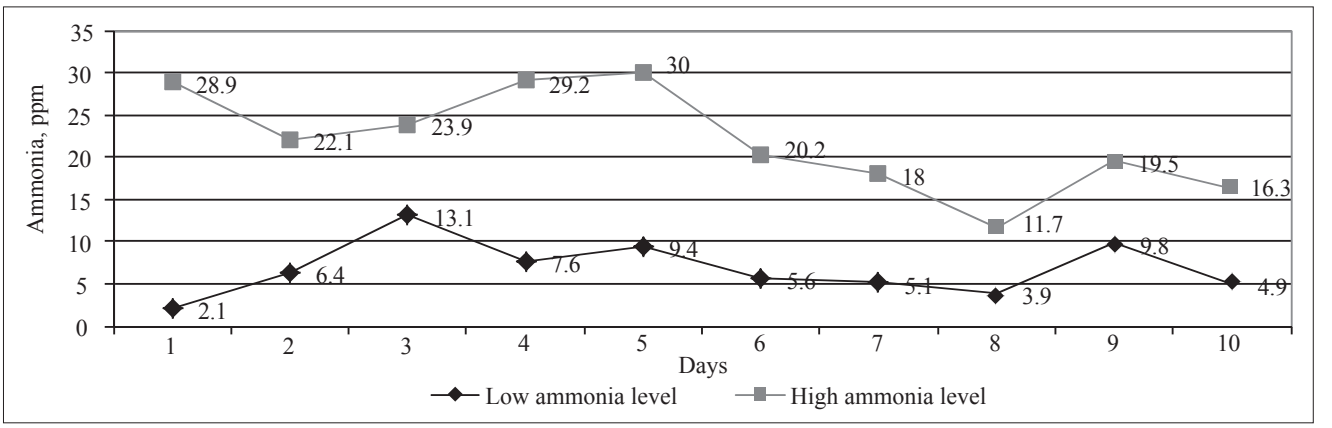

Fig. 7. Indoor ammonia dynamics

not surprising since acid-base distributions may be a consequence of polypnea or panting, leading to hyperventilation and elimination of CO_2 (Olanrevaju et al., 2008). Respiratory rate in pyridoxine supplemented rabbits declined sharply by 5 d after the start of supplementation and coincided with a surge in ammonia concentration at that time (Figure 7). The observed effect of pyridoxine could be ascribed to its beneficial effect of erythrocyte membrane $Na^+ K^+$ ATPase activity (Nadiger et al., 1984) which ultimately may lead to improvement of the oxygen-carrying capacity of the blood. This explanation is consistent with the lowered hematocrit level in pyridoxine-supplemented rabbits relative to that in unsupplemented rabbits.

Rectal temperature in the unsupplemented rabbits was significantly lower as compared to control and pyridoxine supplemented rabbits after exposure to stress (Figure 8). This data is consistent with the reported mild hypothermia in cattle subjected to ammonia poisoning (Antonelli et al., 2004) suggesting that ammonia effects thermal homeostasis.

Conclusion

High ammonia concentrations elevated WBC and hematocrit but decreased rectal temperature after exposure to psychological stress. These effects of ammonia were prevented by supplemental pyridoxine.

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