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## **Heme-induced biomarkers associated with red meat promotion of colon cancer are not modulated by the enterosalivary cycle of nitrite**

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**Key words:** Colorectal cancer – Animal models – Biomarkers - Red and Processed meat – Heme iron – Nitrosation - Apparent Total N-nitroso Compounds – Lipid peroxidation - Enterosalivary cycle - Nitrite.

### **Abstract:**

Red and processed meat consumption is associated with the risk of colorectal cancer. Three hypotheses are proposed to explain this association, via heme/alcal, heterocyclic amines or N-nitroso compounds. Rats have often been used to study these hypotheses, but the lack of enterosalivary cycle of nitrate in rats casts doubt on the relevance of this animal model to predict nitroso- and heme-associated human colon carcinogenesis. The present study was thus designed to clarify whether a nitrite intake that mimics the enterosalivary cycle can modulate heme-induced nitrosation and fat peroxidation. This study shows that, in contrast with the starting hypothesis, salivary nitrite did not change the effect of hemoglobin on biochemical markers linked to colon carcinogenesis, notably lipid peroxidation and cytotoxic activity in the colon of rat. However, ingested sodium nitrite increased fecal nitroso-compounds level, but their fecal concentration and their nature (iron-nitrosyl) would not be associated with an increased risk of cancer. The rat model could thus be relevant to study the effect of red meat on colon carcinogenesis in spite of the lack of nitrite recycling in rat's saliva.

### **Introduction:**

Epidemiological and animal studies show that high intake of red and processed meat can increase the risk of developing colon cancer (1, 2). The mechanism is not fully elucidated yet, but three major hypotheses can explain the risk: (i) well-done and charred meat contains carcinogens like heterocyclic amines and polycyclic aromatic hydrocarbons, (ii) heme iron in red meat can promote cancer by enhancing fat peroxidation and N-nitrosation, (iii) nitrate and nitrite added to processed meat may yield carcinogenic N-nitrosocompounds (NOCs) (3). In its 2011 report, the World Cancer Research Fund suggests that the relative importance of these three hypotheses compounds on CRC burden could be roughly equivalent, according to adenoma studies (4, 5) and to a large recent American cohort CRC study (6). However, heterocyclic amines might not be major players in CRC risk, as: (i) consumption of chicken is

a major contributor to intake of heterocyclic amines, but is not associated with the risk (7); and (ii) doses of heterocyclic amines that induce cancer in animals are 1,000 to 100,000 times higher than the dose ingested by humans (8).

Our experimental studies in chemically initiated rats support the role of heme iron as a major promoter of colon carcinogenesis, via fat peroxidation and genotoxic and cytotoxic alkenals production in the gut (9-11). Other publications show that potentially carcinogenic NOCs can be formed in food and/or endogenously by nitrosation of amine or amides in the presence of nitrite (12, 13). Endogenous nitrosation is estimated to account for 45% to 75% of total exposure to NOCs (14), and it is clearly enhanced by dietary heme intake in human volunteers (15, 16). Nitrite entering the gastrointestinal tract may come from three major sources: (i) processed meat contains nitrite, used as a curing agent for its antimicrobial, flavoring and coloring properties (13), (ii) drinking water and vegetables may contain nitrate (17), and (iii) human saliva contains nitrate and nitrite due to the enterosalivary cycle of nitrate (18, 19). Saliva is a significant source of nitrate in humans and in dogs, where the enterosalivary cycle increases the exposure to NOCs (20).

In contrast, the enterosalivary circulation of ingested nitrate in rats is not similar to that in humans, and saliva of rats does not contain significant concentration of nitrate or nitrite (21). The lack of nitrate in rat's saliva casts doubt on the relevance of this animal model to predict NO-associated human colon carcinogenesis (22). The present study was thus designed to clarify whether a nitrite intake that mimics the enterosalivary cycle can modulate heme-induced nitrosation or fat peroxidation.

On the one hand, dietary nitrate and nitrite can increase N-nitrosation and the risk of colorectal cancer. On the other hand, nitrite can inhibit oxidation in foods, and thus modulate the heme-induced production of toxic alkenals. We considered two hypotheses (i) Nitrate in human saliva might enhance carcinogenesis promotion by heme iron via the endogenous formation of NOCs. The cancer risk associated with red meat intake would thus be underestimated by the rat model, since rats have no nitrate in saliva. (ii) Nitrate in human saliva might reduce carcinogenesis promotion by heme iron via the reduction of fat peroxidation and alkenals production. The cancer risk associated with red meat intake would thus be overestimated by the rat model.

The present study made use of fecal and urinary markers that are associated with heme promotion of carcinogenesis in rats, and that have been used to screen meat promotion in this model (11). The biomarkers were thiobarbituric acid reactive substances (TBARS), cytotoxic activity and apparent total NOCs (ATNC) in fecal water, and mercapturic acid conjugate of 1,4-dihydroxynonenane (DHN-MA) in urine. This study shows that the addition of sodium nitrite to drinking water, at a level that mimics salivary nitrite, did not significantly modify heme-induced biomarkers of colon cancer promotion, notably lipid peroxidation and cytotoxic activity in colon of rat. We thus suggest that rats can be used as a suitable model to explore the mechanisms underlying the association between colorectal carcinogenesis and red meat intake.

## MATERIALS AND METHODS

### Animals and Diets

Two independent studies were performed (A and B), using 4-week-old male F344 rats from Charles River French laboratories. Rats were housed two per cage under standard laboratory conditions: room temperature, 22°C, and light-dark cycle (12h/12h). After a 48-hour acclimation period, forty and forty-two rats were randomly allocated to four groups of ten rats (except one group of 12 rats in study B). Animals in all groups were fed with an AIN-76 powdered diet prepared and formulated by UPAE (INRA, Jouy-en-Josas, France). Dibasic calcium phosphate was included at a low concentration of 2.7g/kg and safflower oil at 5%. The diet was stored at -20°C in the dark before use and replaced three times a week in rat's feeders.

A 2x2 design was used in both studies, the factors being heme and nitrite as shown in table 1. Freeze-dried hemoglobin (Sigma Co, St. Quentin, France) was added to the diets at levels that modulate fecal biomarkers and promote carcinogenesis in rats: 0.63% and 1% in studies A and B respectively. Study A was designed to test the effect of a high dose of sodium nitrite in drinking water (1 g/l), to see if a supra-physiological concentration may modulate the chosen biomarkers. Study B was designed to estimate the impact of nitrite and nitrate at a concentration mimicking the human salivary (0.17 and 0.23 g/l respectively). Control rats received a diet with no heme, and water without nitrite. Food and drinking water were supplied *ad libitum*.

The 48-hour intake of food and water was recorded for each cage of two rats at the start, middle and end of each study of 100d. Body weights were monitored weekly for the 8 first weeks, and then every two weeks until the end of the study. Rats were terminated by CO<sub>2</sub> asphyxiation 100 days after the start of the experimental diets.

### Fecal and urinary assays

#### *Fecal water preparation*

Fecal pellets were collected quantitatively under each cage from two rats for 24h. Preparation of fecal water was done by adding 1ml of distilled water to 0.3g of dried feces. Samples were then incubated at 37°C for one hour, stirring thoroughly every 20min, followed by centrifugation at 20.000g for 15min. Fecal water which is the aqueous phase (supernatant, modified from Lapré) was collected and conserved at -20 °C until its further use (23).

#### *TBARS assay of fecal water.*

TBARS were quantified in fecal water according to Ohkawa (24), to determine lipid peroxidative processes in the lumen.

Fecal water was diluted 10-fold in distilled water. Subsequently, 100 µL of this solution was mixed with 100 µL of 8.1% SDS and 1000 µL solution of 0.8 % TBA in 10% acetic acid. For background correction, TBA was omitted from the assay. TBARS were extracted, after heating for 75 min at 95°C, with 1mL of *n*-butanol. The absorbance of this extract was measured at 532 nm. The amount of TBARS was calculated as malondialdehyde equivalents using 1, 1, 3, 3,-tetramethoxypropane as standard.

#### *Determination of heme in fecal water*

Heme of fecal water was assayed by fluorescence according to Van den Berg (25). Briefly, 50 µL of fecal water was diluted in 250 µL of a 5:1 mix of 2-propanol and HCl (1 M). Samples were homogenized, then centrifuged at 10 000g for 10 min. Samples were mixed with 1mL of

glacial acetic acid. Subsequently, a mixture of 50  $\mu\text{L}$  of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.12 M) freshly prepared and HCL (4.5 M) were added. After mixing, the samples were immediately incubated at 60 °C for 30 min. 2 mL of 2-propanol/water (v/v) were added and agitated before measurement.

#### ***Cytotoxicity assay of fecal water***

Cytotoxicity of fecal water was quantified on CMT93 cell line according to Bonneson et al., (10, 26). Briefly, a cancerous mouse colonic epithelial cell line, CMT93 (ECAC), was seeded in 96-well microtitre plates ( $1.6 \times 10^4$  cells well in 200  $\mu\text{L}$  medium) and treated for 24h with fecal water sample diluted at 10 % (v/v) in the culture medium without fetal bovine serum. Cytotoxicity of each fecal water was quantified by the MTT 3-(4, 5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide test.

#### ***Fecal NOC analysis***

NOCs were analyzed as done previously in human stools, with an Ecomedics CLD 88 Exhalyzer (Ecomedics, Duernten, Switzerland) (27). Fecal water samples were made of three 500 $\mu\text{L}$  tubes, coming from ten rats. 100  $\mu\text{L}$  of fecal water were mixed with 500  $\mu\text{L}$  of a 5% (wt/vol) sulfanil amide solution to remove nitrite and 100 samples were injected into a purge vessel kept at 60°C and filled with a standard tri-iodide reagent (38 mg  $\text{I}_2$  was added to a solution of 108 mg KI in 1 ml water; to this mixture, 13.5 ml glacial acetic acid was added) to determine total NOC. To determine mercury(II) stable compounds, 100  $\mu\text{L}$  10 mM aqueous  $\text{HgCl}_2$  was added prior to analysis; to determine mercury(II) and ferricyanide stable compounds, 100  $\mu\text{L}$  each of 10 mM aqueous  $\text{HgCl}_2$  and 10 mM aqueous  $\text{K}_3\text{Fe}(\text{CN})_6$  solution were added prior to analysis. RSNO were determined as the difference between total NOC and mercury(II) stable NOC; FeNO was determined as difference between mercury(II) stable NOC and mercury(II) and  $\text{K}_3\text{Fe}(\text{CN})_6$  stable compounds. Other NOC were determined as mercury (II) and  $\text{K}_3\text{Fe}(\text{CN})_6$  stable compounds. The values are the concentration (in  $\mu\text{M}$ ), measured in 100  $\mu\text{L}$  of your sample. ATNC are Apparent Total Nitroso Compounds, RS-NO nitroso thiols and Fe-NO nitrosyl heme. The difference between ATNC and RS-NO+FeNO are other types of nitroso compounds such as N-Nitroso or O-Nitroso compounds.

#### ***Urinary 1,4-dihydroxynonane mercapturic acid assay***

24-hour urine samples were collected under each metabolic cage of each rat for 1 d, leading to ten samples per group for both experiments on day 60 of the experimental diet, and were frozen at -20°C until analysis. DHN-MA assay was done by competitive enzyme immunoassay as previously described (28), using DHN-MA linked acetylcholinesterase enzyme. Each urine sample was assayed in duplicate.

#### ***Statistical analysis***

Results were analyzed using Systat 12 software for Windows, and all data were reported as mean  $\pm$  SD. Data were analysed by one-way ANOVA. If a significant difference was found between the groups ( $p < 0.05$ ), then pairwise comparisons were made with Fisher's least significant difference test.

## RESULTS:

### Body weight and food intake

The addition of hemoglobin to diet and of sodium nitrite to drinking water did not cause significant differences in food intake or body weight between groups of rats (data not shown).

### Fecal and urinary biomarkers

#### *Fecal heme, TBARS*

Rats were fed diets containing no heme (control groups), and 0.36 and 0.57  $\mu\text{mol}$  heme/g diet (studies A and B respectively). As expected, heme concentration in fecal water depended directly on dietary concentrations (Table 1). Heme induces the formation of peroxy radicals in fat, and fat peroxidation was thus measured in fecal water by the TBARS assay. In both studies, the heme diets increased TBARS 2-fold in fecal waters compared with the control diet (Table 1).

As shown in Table 1, heme-fed rats given water with a high concentration of nitrite, unrepresentative of salivary concentration, excreted slightly less TBARS than heme-fed rats given pure water (-25%,  $p=0.003$ , study A). In contrast, no difference in fecal TBARS was seen between heme-fed rats given water with 0.4g sodium nitrite per liter, mimicking the concentration in saliva (study B, Table 1).

#### *Cytotoxicity of fecal water*

To test whether the heme-induced cytotoxicity was modified by nitrite added to drinking water, cytotoxicity of fecal water was measured by using MTT assay on CMT93 cells. Figure 1 panel A shows that fecal water from heme-fed rats was highly cytotoxic compared to fecal water from controls ( $p<0.001$ ). The addition of nitrite to drinking water did not change significantly the cytotoxicity of fecal water.

#### *Fecal NOC*

To test whether the heme-induced NOC level in feces was modified by nitrite added to drinking water, ATNC was measured in fecal water. Figure 1 panel B shows that hemoglobin alone induced an increase of fecal ATNC in group HO. But adding sodium nitrite into drinking water of the hemoglobin-fed group increased 3-fold fecal ATNC level. The characterization of ATNC in HO and HN groups showed that the increase of ATNC was entirely due to iron nitrosyl. Figure 1 panel B also shows also that sodium nitrite alone (group TN) induced a strong increase of fecal ATNC, but not in the form of iron nitrosyl.

#### *Urinary DHN-MA excretion*

As shown in table 1, urinary DHN-MA excretion was twice the control value in rats given a 0.63% hemoglobin diet (study A), and six times the control value in rats given a 1% hemoglobin diet (study B) (both  $P<0.001$ ). In contrast, no difference in DHN-MA excretion was observed between hemoglobin-fed rats and nitrite-hemoglobin-fed rats in both studies.

## DISCUSSION:

Relevance of the animal model is a key point in nutrition and cancer studies, and seems tricky to study nitrite toxicity. Indeed, a major difference between fate of nitrite in humans and in rats is that humans recycle nitrite in saliva but rats do not. One may argue that, due to this difference, rat is not a well-fitted model to test the effect of red and processed meat on colon carcinogenesis. However, the present data reveal that this difference does not preclude the use of the rat model to study the effect of meat on carcinogenesis promotion.

In the present article we made two hypotheses. One of them stated that nitrate in human saliva might reduce carcinogenesis promotion by heme iron via the reduction of fat peroxidation and alkenals production. The cancer risk associated with red meat intake would thus be overestimated by the rat model. If this were the case, the addition of sodium nitrite to drinking water, mimicking nitrate in human saliva, would reverse peroxidation of fat in the gut of the rat. We show here that this is not true, since swallowed nitrite at a concentration similar to human salivary level did not change the effect of hemoglobin on biochemical markers linked to promotion of colon carcinogenesis by heme. Indeed, luminal fat peroxidation was not modified in rat treated by 0.17 g/l sodium nitrite plus 0.23 g/l sodium nitrate (table 1, study B). However, a slight but significant reduction in heme-induced fecal TBARs (-25%) was observed in rats given water with 1g/l sodium nitrite, a concentration higher than what is found in human saliva (table 1, study A). Indeed, the human salivary nitrite concentration ranges from 60 to 500 $\mu$ M, but it may peak up to 1.8mM following a high nitrate meal according to Pannala (29). In the stomach, saliva mixes with food, drink, and gastric fluid and can be diluted by a factor of 10 to 40. In the present study, 1g/l sodium nitrite would correspond to 14mM in drinking water. Thus nitrite concentration in the stomach of rats given 1g sodium nitrite per liter of drinking water was 10 to 100 times higher than concentration in the stomach of human beings. The results also show that lipid peroxidation measured by fecal water TBARs and heme concentration were correlated (study A and B, Pearson  $r = 0.73$  and  $0.89$ , respectively, both  $p < 0.002$ ), but no correlation was found with nitrite level in drinking water, which supports the low importance of salivary nitrite on the modulation of this biomarker.

We used fecal water TBARs, a measure of endogenously formed secondary lipoperoxydation products, as a proxy for fat peroxidation in the gut. These same products can be found in the diet, particularly in heme-iron and polyunsaturated fat rich food (30) and formed during digestion in the upper tract. We thus chose to measure urinary DHN-MA, to estimate the effect of nitrite on heme-induced peroxidation within the whole digestive tract. DHN-MA is the major urinary metabolite of 4-hydroxy-nonenal (4-HNE) and has previously shown to be a specific and noninvasive biomarker of lipid peroxidation process (31), and its excretion is strongly associated with heme intake in rats and humans (28). As expected, dietary hemoglobin increased urinary DHN-MA, but nitrite added to drinking water had no effect on urinary DHN-MA and did not counteract the pro-oxidant effect of hemoglobin (Table 1).

Fecal water cytotoxicity is associated with heme-induced carcinogenesis (11), this is why the impact of sodium nitrite on cytotoxicity was measured in the present study (Figure 1, A). As expected, heme intake was associated to increased cytotoxicity of fecal water against CMT93 cells, as in previous studies (9-11). This increased cytotoxicity can be due to heme-induced peroxidation, and more precisely to alkenals such as 4-HNE (32). Here, the lack of effect of sodium nitrite on two biomarkers of peroxidation was associated with a lack of effect on hemoglobin-induced cytotoxicity (Figure 1, A).

We thus conclude that nitrite added to drinking water at a concentration found in saliva had no effect on heme-induced peroxidation or on biomarkers associated with heme-induced promotion of colon cancer. The above cited hypothesis that the rat model would overestimate the risk of red meat intake is not supported by present data, and carcinogen-initiated rats can be used as a model to study the effect of red meat.

However, another risk associated to the lack of enterosalivary cycle could be an underestimation of N-nitrosation in the rat model. If this was the case, we proposed that the addition of sodium nitrite to drinking water, mimicking nitrate in human saliva, would increase N-nitrosation in the colon of the rat. This increased nitrosation would enhance heme iron and red meat promotion of colon carcinogenesis in rats.

Red meat consumption is associated with an increase of luminal NOCs in humans (15, 33), but not in beef-fed rats (34). Cured meat brings heme iron and nitrite together to the digestive tract, and would yield more endogenous NOCs than fresh red meat (35-37). Santarelli et al. showed that cured meat promotes preneoplastic lesions only when it contains nitrite and yields NOCs in the rats' gut (38). In contrast Parnaud et al. did not detect any promoting effect of a bacon-based diet on preneoplastic lesions in carcinogen-initiated rats (34, 39). A major difference between these two studies is the calcium level in rats' diet, and the fecal NOCs concentration in rats' feces. A low calcium diet resulted in a very high NOCs level in feces of cured meat-fed rat (1800  $\mu\text{mol/g}$  of fresh feces) (38). In contrast, a high calcium diet resulted in a much lower NOCs level in feces of cured meat-fed rats (20  $\mu\text{mol/g}$ ), although this level was higher than in feces from no-meat diet-fed control rats (4  $\mu\text{mol/g}$ ) (34). This difference between two studies ran in our laboratory could be due to the chelating of heme by dietary calcium (11, 40). According to Kuhnle and to Hogg, most of fecal ATNC produced in the gut after heme ingestion is nitrosyl heme (41, 42). We suppose that dietary calcium can precipitate nitrosyl heme, explaining the low fecal NOCs concentrations found in Parnaud's study, and explaining the lack of carcinogenesis promotion by bacon (34). In the present study, the addition of sodium nitrite to drinking water of hemoglobin-fed rats induced a 3-fold increase in the fecal ATNC level. However, the ATNC concentration in feces was, according to Parnaud's results, not able to promote colon carcinogenesis in rats (34). Furthermore, Figure 1 panel B shows that most of the difference between HO and HN groups' ATNC is due to iron nitrosyl. Nitrosyl heme is the main source of iron nitrosyl (41), which is consistent with the strong NO-scavenging activity of hemoglobin (43). Hogg even suggested that the NO sequestration by iron nitrosyl would protect the mucosa by reducing the formation of DNA alkylating N-nitrosated compounds, and helping the excretion of the NO- radical (42). We thus suggest that the increased level of fecal ATNC due to sodium nitrite added to drinking water of heme-fed rats would not promote colon carcinogenesis because (i) ATNC concentration is too low (34) and (ii) iron nitrosyl is not carcinogenic (42).

In summary, the results show that, in contrast with our starting hypothesis, salivary nitrite did not change the effect of hemoglobin on biochemical markers linked to colon carcinogenesis, notably lipid peroxidation and cytotoxic activity in colon of rat. However, sodium nitrite in drinking water increased the level of ATNC, but level and nature of ATNC do not seem to increase carcinogenesis. This animal model could thus be relevant to study the effect of red meat on colon carcinogenesis in spite of the lack of nitrite recycling in rats' saliva.



**Table 1:** Effect of dietary hemoglobin on fecal and urinary biomarkers of fat peroxidation.

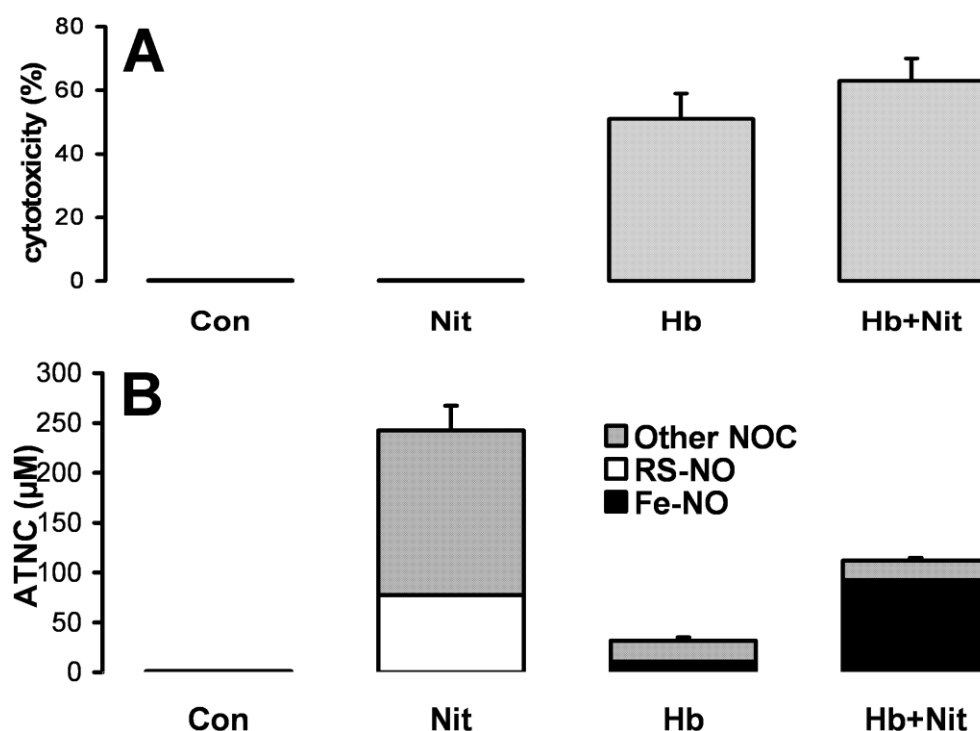
Study	Dietary group	No. of rats	Hemo-globin in diet g/100g	Na NO <sub>2</sub> in water g/l	Na NO <sub>3</sub> in water g/l	Fecal heme $\mu$ M	TBARS in fecal water (MDA eq. $\mu$ mol/l)	Urinary DHN-MA (ng/24h)
A	Con	10	0	0	0	0.0 $\pm$ 0.0	47 $\pm$ 12	87 $\pm$ 12
A	Nit	10	0	1	0	0.0 $\pm$ 0.0	45 $\pm$ 19	107 $\pm$ 22
A	Hb	10	0.63	0	0	197 $\pm$ 25 <sup>a</sup>	111 $\pm$ 23 <sup>a</sup>	219 $\pm$ 47 <sup>a</sup>
A	Hb+Nit	10	0.63	1	0	218 $\pm$ 23 <sup>a</sup>	85 $\pm$ 6 <sup>a,b</sup>	251 $\pm$ 47 <sup>a</sup>
B	Con	12	0	0	0	8 $\pm$ 9	53 $\pm$ 4	133 $\pm$ 36
B	Nit	10	0	0.17	0.23	11 $\pm$ 13	64 $\pm$ 9	133 $\pm$ 33
B	Hb	10	1	0	0	235 $\pm$ 70 <sup>a</sup>	124 $\pm$ 16 <sup>a</sup>	843 $\pm$ 224 <sup>a</sup>
B	Hb+Nit	10	1	0.17	0.23	246 $\pm$ 92 <sup>a</sup>	114 $\pm$ 26 <sup>a</sup>	802 $\pm$ 386 <sup>a</sup>

Data are mean  $\pm$  standard deviation. B. Con = control; Nit = nitrate; Hb = hemoglobin

TBARS in fecal water were measured at day 22 in study A, and day 85 in study

<sup>a</sup> significantly different from Con group,  $p < 0.05$  by Fisher's Least-Significant-Difference test

<sup>b</sup> significantly different from Hb group,  $p < 0.05$  by Fisher's Least-Significant-Difference test



**Figure1:** Fecal Cytotoxicity (A) and fecal Apparent Total Nitroso Compounds (B) in rats given experimental diets with 1% hemoglobin (groups HO and HN), and drinking water with sodium nitrate and nitrite (0.17 g/l Na NO<sub>2</sub> and 0.23 g/l Na NO<sub>3</sub> given to both groups TN and HN), for 100d. Control group TO was given no hemoglobin or nitrite. Details: see Materials and Methods section.

**Panel A** - Cytotoxicity was assessed with MTT assay. Data are mean  $\pm$  SD (n = 5).

\* significantly different from the same condition without heme.

**Panel B** - N-nitroso compounds were assessed as ATNC and are characterized in Fe-No and RS-NO in pools of fecal samples from ten rats (no SD).

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