Bull. Environ. Contam. Toxicol. (1998) 61:800-806

Bulletin of Environmental Contamination and Toxicology

Nitrite-Induced Cell Proliferation and Polyamine Synthesis in the Small Intestine of Mice

I. P. Grudzinski, F. C. P. Law

Environmental Toxicology Program, Department of Biological Sciences, Simon Fraser University, Burnaby, BC V5A 1S6, Canada

Received: 19 June 1998/Accepted: 30 October 1998

Acute nitrite intoxication of animals is manifested primarily as methemoglobinemia, anoxia, and secondary effects of vasodilation (Chow et al. 1980, Druckery et al. 1963). Nitrite-treated rats also show a decrease in the absorption of D-xylose in the intestinal mucosa (Grudzinski and Szymanski 1991a, 1991b). Other nitrite-induced toxic effects may include abdominal pain, diarrhea, and atrophied intestinal villi (Grudzinski et al. 1991, Kaemmerer 1965, Roediger and Radcliffe 1988). Recently, sodium nitrite has been shown to increase apoptotic cell death in the intestinal crypts of mice (Grudzinski and Law 1998).

Evidence exists that polyamines putrescine, spermidine, and spermine play an important role in both cellular proliferation and differentiation (Hebby 1982). Furthemore, numerous studies indicate that ornithine decarboxylase, which forms putrescine by decarboxylation of ornithine, is implicated in neoplastic growth (Higuchi and Wang 1995). Since nitrite has been found to decrease crypt survival in the small intestine of mice (Grudzinski and Jednorog 1995), the mechanism(s) of nitrite-induced toxicities probably is related to the renewal processes of the intestinal mucosa (Patel et al. 1996).

The aim of this study was to examine the dose-response relationships between sodium nitrite and the proliferation processes of the small intestinal mucosa. The possibility that nitrite-induced toxicity might be related to the biosynthesis of polyamines in the small intestine also was investigated.

MATERIALS AND METHODS

Sixty male B6C3F1 mice aged about 10-12 weeks were quarantined for at least 2 weeks in a room with a 12/12 hr light-darkness cycle, 22 ± 2 °C and 40-70 % air humidity, They were fed a normal diet (Altromin® pellet) and provided water *ad libitum.* The mice were divided into two groups containing 30 mice per group (10 mice per cage). The mice were treated *per os* with a solution of sodium nitrite (5 mg $NaNO_2$/kg bw) in saline daily for 10 days, 20 days, or 30 days (group 1). At the same time, group 2 received saline only and served as control. At the conclusion of a 10-day, 20-day, or 30-day pretreatment with nitrite or saline, the mice were killed by cervical dislocation, and the small intestines were removed and used in the following bioassays:

Correspondence to: F. C. P. Law

(a) *In vivo* cell proliferation. This was measured by the incorporation of bromodeoxyuridine (BrdU) into the DNA as described by Lee et al. (1993). Briefly, after a 10-day, 20-day, or 30-day nitrite or saline treatment, the animals of group 1 and 2 were given an *i.p.* injection of BrdU (2.5 mg/kg) in phosphate-buffered saline (PBS) exactly 1 hr before sacrificing. The entire intestine was removed, fixed in Carnoy's fixative for 30 min, cut into three equal portions and stored in 70 % ethanol Ten 1-cm segments of the proximal intestine were bundled together with a tape prior to paraffin embedding. Paraffin sections (3 pm) were cut perpendicular to the mucosa surface and affixed to albumin-coated slides. Each tissue section was deparaffinized with three changes of xylene and rehydrated in a graded series of ethanol from 100 % to 70 %. Endogenous peroxidase was blocked by immersing the section in 3 % H_2O_2 for 10 min, with subsequent washing in water. To detect BrdU incorporation into the DNA, the tissue section was hydrolyzed in 2 N HCl for 1.5 hr and neutralized with 0.1 M sodium tetraborate, pH 8.5. A blocking agent, normal goat serum, was placed onto the tissue sections and kept in a moist chamber for 20 min. Anti-BrdU mouse antibody (Boehringer Mannheim, Indianapolis, IN) was then placed onto the tissue section and incubated in a moist chamber for 1 hr. A linking reagent (goat anti-mouse immunoglobin serum; Sigma, St. Louis, OH) and labeling reagent (peroxidase labeled mouse immunoglobulin; Sigma, St. Louis, OH) were placed onto the tissue section and incubated in a moist chamber for 20 min subsequently. The entire antibody-antigen complex was made visible by 3,3'diaminobenzidine tetrachydrochloride (DAB) plus an imidazole reagent to which 3 % H_2O_2 had been added 5 min later. This step produced a blue-black stain. The tissue sections were counterstained with eosin, dehydrated in a graded series of ethanol and finally with xylene before mounting with Permount. Slides were scored for the number and position of the blue-black stained cells. The mean value from 50 intestinal crypts was calculated for each animal. Labeling index (%) was calculated by dividing the number of the blue-black stained cells with the total number of cells in the crypt column and multiplied by 100. Proliferation zone (%) was defined as the highest position of the blue-black stained cells in the crypt column divided by the total number of cells in the crypt column and multiplied by 100.

(b) Ornithine decarboxylase (ODC). This enzyme was assayed by a radiometric technique in which the amount of $^{14}CO_2$ liberated from DL-[l-^{14}C]-ornithine (51.3 mCi/mmol, New England Nuclear, Boston, MA) was estimated (Luk and Baylin 1983). Briefly, the intestinal mucosa collected from mice was placed in a pH 7.4 sodium/potassium phosphate buffer (0.067 M) containing 23 mM lauryl ether, 5 mM NaF, 10 µM EDTA, 2 mM dithiothreitol, and 100 µM pyridoxal phosphate. The mucosa was homogenized, sonicated, and centrifuged at 30,000 g for 25 min. An aliquot of the 30,000 g supernatant was incubated in a stoppered vial in the presence of approximately 2 pmol of DL-[l-^{14}C]-ornithine for 60 min at 37 °C. The $^{14}CO_2$ liberated from ornithine decarboxylation was trapped in a piece of filter paper impregnated with 20 µl of 2 N NaOH. The paper was suspended in a center well above the reaction mixture. The reaction was stopped by the addition of 10 % trichloroacetic acid. The $^{14}C O_2$ trapped in the filter was measured by liquid scintillation counting in Scintiverse II (Fisher Scientific, Houston, TX) with a

scintillation counter. ODC activity was expressed as pmol $^{14}CO_2$ liberated per mg mucosa per hr of incubation.

(c) Putrescine, spermidine, and spermine. The mucosal homogenate was mixed with an equal volume of perchloric acid (0.4 M) and left in ice for 1 hr. After the homogenate was centrifuged at 15,000 g for 15 min, the supernatant was filtered through a Millipore filter (0.22 µm). Aliquots of the filtrate were analyzed for putrescine, spermidine, and spermine as described by Simpson et al. (1982). Briefly, the putrescine, spermidine, and spermine were separated by a high-performance liquid chromatograph HPLC) using a reverse-phase, ion-pairing 5 µm C-18 column (Waters Associates, Milford, MA) with a Brownlee RP-18 guard column, The putrescine, spermidine and spermine were quantified by a fluorescence detector (with a 370 nm excitation filter and a 418 nm emission filter) after post-column derivatization with *O*-phthalaldehyde. The final conditions for HPLC analysis were a flow of 1.0 ml/min and 25 µl injection volume. The mobile phase of the HPLC was prepared from two different buffers. Buffer A was prepared by mixing 80 % 0.05 M acetic acid, 20 % MeOH with 10 mM octane sulfonic acid. Buffer B was a solution of 80 % 1.0 M acetic acid, 10 % MeOH, and 2 % tetrahydrofuran. The column was eluted with 100 % buffer A for 8 min. This was followed a linear gradient to 100 % buffer B in 10 min. The column was eluted with 100 % buffer B for an additional 20 min. The peaks of interest were identified and quantitated using putrescine, spermidine, and spermine standards (Sigma, St. Louis, MO) at know concentrations.

Statistical analysis, Results were analysed using the Student's t-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results are expressed as the mean ± SEM of ten animals.

RESULTS AND DISCUSSION

The intestinal crypts of nitrite-treated mice had greater labeling indices and more proliferative zones than the saline-treated mice (Fig. 1). These results are consistent with our previous findings that cell proliferation in the small intestine of mice was altered by sodium nitrite treatments (Grudzinski and Jednorog 1995, Grudzinski and Law 1997). An increased labeling index and expanded proliferation zone in the crypt column suggest a rapid cell proliferation in the small intestine (Gordon at al. 1992, Potten and Loeffler 1990), which may increase the risk of developing cancer in the intestine (Higuchi and Wang 1995). It should be noted that nitrite has been shown to induce pathological changes in the gastrointestinal tract by increasing apoptotic cell death and other gastric and/or colon effects such as necrosis and diarrhea (Grudzinski and Law 1998, Roediger et al. 1986, Ruddell et al. 1976).

In the present studies, sodium nitrite increased the activity of ornithine decarboxylase in the intestine of mice after a 10-day, 20-day, and 30-day treatment (Fig. 2). The increase in ODC activity also was accompanied by increases in putrescine, spermidine, and spermine levels (Figs. 2, 3). Moreover, the labeling index was found to correlate highly with ODC, putrescine and spermidine levels

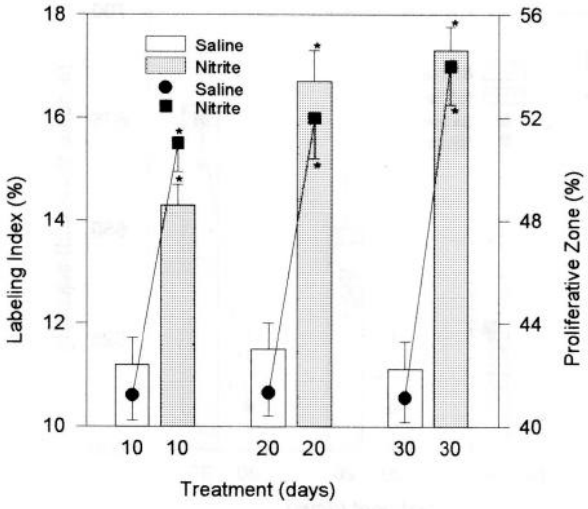

Figure 1. Effect of sodium nitrite on intestinal cell proliferation in the small intestinal mucosa of mice. Bars (labeling index) and points (proliferative zone) are means ± SEM of ten mice. Asterisk denotes statistically significant difference compared with saline-treated mice by Student's t-test ($p<0.05$).

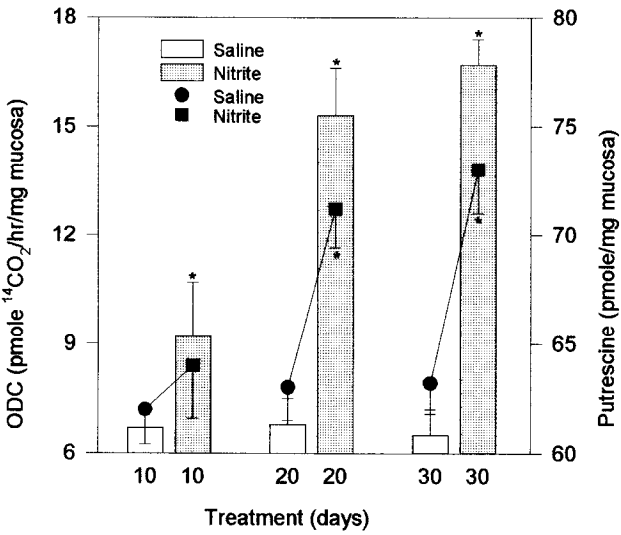

Figure 2. Effect of sodium nitrite on ODC activity and putrescine level in the small intestinal mucosa of mice. See figure 1 for comments,

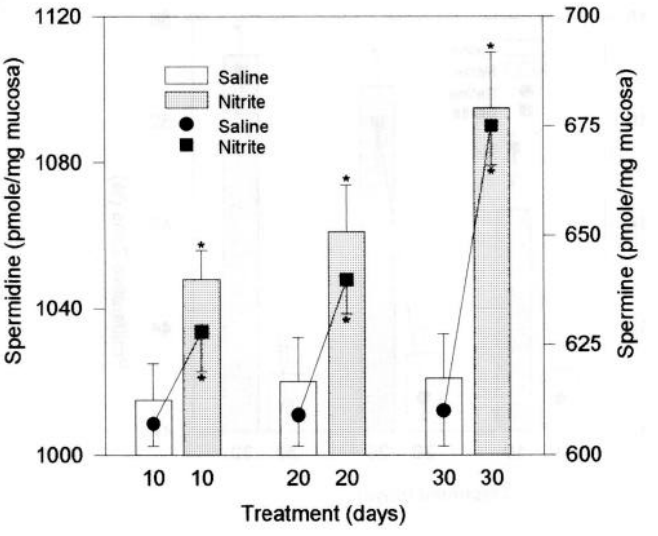

Figure 3. Effect of sodium nitrite on spermidine and spermine levels in the small intestinal mucosa of mice. See figure 1 for comments,

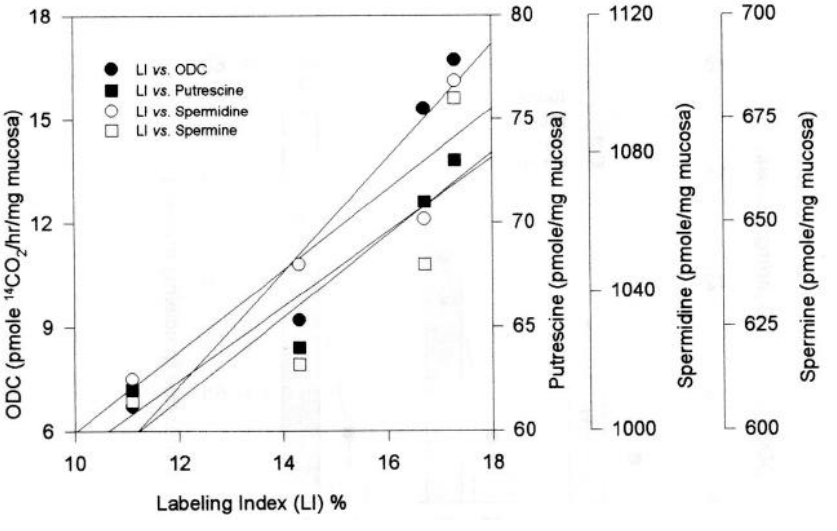

Figure 4. The curvilinear fits for the mucosal crypt cell proliferation (Labeling Index %) *versus* mucosal (a) ODC activity, (b) putrescine, (c) spermidine, and (d) spermine levels after 10 days, 20 days, or 30 days pretreatment with sodium nitrite (5 mg/kg bw).

(Fig. 4). These results clearly show that polyamines (putrescine, spermidine, and spermine) are enhanced in the proliferation process. The number of labeled cells per crypt column also was consistent with the levels of polyamine in the intestinal mucosa of the nitrite-pretreated mice. Therefore, the highest nitrite effects were found at 30 days treatment for both the intestinal crypts and the mucosa (Figs. 2, 3). Results of the present studies suggest a cumulative effect(s) of nitrite in the mice after subchronic treatments, the so-called "functional cumulation of nitrite" which appears to describe the toxic action of this chemical better than other reported toxic effects (Grudzinski and Szymanski 1991b).

Since the proliferation process in the intestinal crypts can be stimulated by ornithine decarboxylase (Hebby 1982; Luk and Baylin 1983), we hypothesize that the polyamine signaling pathway probably is involved in the nitrite-induced toxicities. Further studies are required to elucidate the mechanism(s) of nitrite-mediated ornithine decarboxylase induction in the intestine of mice. However, it is possible that cyclic nucleotides (cAMP and cGMP) are involved in the regulation of polyamine synthesis and the toxicity induced by nitrite (Grudzinski et al. 1998a). An alternate explanation for the nitrite-induced toxicity is the activation of ODC by protein kinase C since nitrite has been shown to increase serine/threonine kinase activity in the crypts (Grudzinski et al. 1998b).

In summary, sodium nitrite has been shown to increase cell proliferation in the crypts of the intestinal mucosa. The mucosal polyamines putrescine, spermidine, and spermine of mice probably play an essential role in the regulation of cell proliferation,

Acknowledgments. The work described in this paper was supported in part by a grant to F.C.P. Law from the Canadian Network of Toxicology Centres.

REFERENCES

Chow CK, Chen CJ, Gairola C (1980) Effect of nitrate and nitrite in drinking water on rats. Toxicol Lett 6: 199-203

Druckery H, Steinhoff F, Beuthner H, Schneider M, Klarner P (1963) Screening of nitrite for chronic toxicity in rats, Arzneim Forsch 13: 320-323

Gordon JF, Schmidt GH, Roth KA (1992) Studies of intestinal stem cells using normal, chimeric, and transgenic mice. FASEB J 6: 3039-3050

Grudzinski JP, Szymanski A (1991a) The effect of acute poisoning with potassium nitrate and sodium nitrite on the processes of intestinal absorption of D-xylose in rats. Arch Environ Contam Toxicol 21: 453-461

Grudzinski JP, Szymanski A (1991b) The effect of subchronic poisoning with potassium nitrate and sodium nitrite on the processes of intestinal absorption of D-xylose in rats. Arch Environ Contam Toxicol 21: 447-452

Grudzinski JP, Szymanski A, Chomiczewski K (1991) The effect of exercise associated with subchronic poisoning with potassium nitrate and sodium nitrite on the processes of intestinal absorption of D-xylose in rats, Arch Environ Contam Toxicol 21: 462-467

Grudzinski IP, Jednorog S (1995) The effect of γ-irradiation associated with acute poisoning with potassium nitrate, sodium nitrite and N-nitroso compounds on crypt survival processes in B6C3F1 mice intestine. Proc X Congress of the Polish Society of Radiation Research, 6-7 April, Warsaw, Poland. p. 68

Grudzinski IP, Law FCP (1997) Nitrite mediated T lymphocyte responses in the intestinal immune system of mice infected with Trichinella spiralis nematode. Arch Environ Contam Toxicol 32: 462-466

Grudzinski IP, Law FCP (1998) Induction of cell death in the intestinal crypt of mice following oral administration of nitrate and nitrite. Bull Environ Contam Toxicol 60: 185-193

Grudzinski IP, Szymanski A, Law FCP (1998a) Effects of sodium nitrite pretreatment on polyamine synthesis in the small intestinal mucosa of mice. Proc XVII Congress of the Polish Pharmaceutical Society, 12-13 Sep., Cracow, Poland, p 122.

Grudzinski IP, Szymanski A, Law FCP (1998b) Protein kinase activities in the small intestinal mucosa of mice treated with sodium nitrite. Proc XVII Congress of the Polish Pharmaceutical Society, 12-13 Sep., Cracow, Poland, p 123.

Hebby O (1981) Role of polyamines in the control of cell proliferation and differentiation, Differentiation 19:1-20

Higuchi CM, Wang W (1995) Comodulation of cellular polyamines and proliferation: Biomarker application to colorectal mucosa. J Cell Biochem 57: 256-261

Kaemmerer K (1965) Nitrate and nitrite poisoning of geese by mineral fertilizers. Arch Geflugelk 29: 161-174

Lee DYK, Chapkin RS, Lupton JR (1993) Dietary fat and fiber modulate colonic cell proliferation in an interactive site-specific manner. Nutr Cancer 20: 107-118

Luk DE, Baylin SP (1983) Polyamines and intestinal growth - increased polyamine biosynthesis after jejunectomy. Am J Physio1 245: G656-G660

Patel HR, Tait IS, Evans GS, Campbell FC (1996) Influence of cell interactions in a novel model of postnatal mucosal regeneration. Gut 38: 679-686

Potten CP, Loeffler M (1990) Stem cells: attributes, cycles, spirals, pitfalls, and uncertainties. Lessons for and from the crypt. Development 110: 1001-1020

Roediger WEW, Lawson MJ, Nance SH, Radcliffe BC (1986) Detectable colonic nitrite levels in inflammatory bowel disease - mucosal or bacterial malfunction. Digestion 35: 199-204

Roediger WEW, Radcliffe BC (1988) Role of nitrite and nitrate as a redox couple in the rat colon. Implication for diarrheal conditions, Gastroenterology 94: 915-922

Ruddell WSJ, Bone ES, Hill MJ, Blendis LM, Walters CL (1976) Gastric-juice nitrite. A risk factor for cancer in the hypochlorhydric stomach. Lancet 2: 1037-1039

Simpson RC, Mohammed HY, Veening H (1982) Reverse-phase ion-pairing liquid chromatographic separation and fluorimetric detection of polyamines. J Liquid Chromat 5: 245-246