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Induction of Cell Death in the Intestinal Crypt of Mice Following Oral Administration of Nitrate and Nitrite

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Nitrate and nitrite have been shown to alter homeostasis of epithelial cell membranes by reacting with thiol (oxidation) and amino groups (nitroso compounds formation) or heme iron (redox) of the small intestine (Grudzinski 1990a,b; Grudzinski 1991a,b; Grudzinski and Szymanski 1991a,b; Grudzinski, Szymanski and Chomiczewski 1991a,b; Grudzinski and Law 1997). Nitrate and nitrite also are capable of decreasing the number of clonogenic cells, which initiate mucosal cell regeneration following injuries by chemical agents (Grudzinski 1996). In addition, nitrite has been reported to alter the proliferation processes of the small intestine epithelium (Grudzinski and Jednorog 1995). A preliminary study has shown that nitrate or nitrite is capable of causing programmed cell death (apoptosis) in the intestinal crypt (Grudzinski 1996). Although the mechanism of apoptosis of intestinal crypt cells has not been filly elucidated, the stem cells are probably involved in the initiation of intestinal toxicity (Grudzinski 1996).

The purpose of the present study was to establish a quantitative relationship between potassium nitrate and sodium nitrite exposures and the incidence of apoptosis in intestinal crypt cells of mice. The possibility that apoptosis may alter the ratio of live/dead crypt in the intestine of mice was also investigated.

MATERIALS AND METHODS

Male B6C3Fl mice 10-12 weeks old were used in our studies. Animals were acclimatized for at least 2 weeks at 22 ± 2 °C room temperature, 40-70% air humidity, and 12/12 h light-darkness cycle. Animals were fed a normal diet (Altromin® pellets) and water *ad libitum* throughout the experiment.

Potassium nitrate (KNO_3) or sodium nitrite ($NaNO_2$) was dissolved in distilled water. These solutions were administered separately to mice by oral intubation (0.5 ml/20 g) in an acute dose: KNO_3 (50, 100, 200, or 400 mg/kg bw), and $NaNO_2$ (5, 10, 20 or 40 mg/kg bw). Groups of 4 or more animals were killed by cervical dislocation at various post-dosing times (1, 2, 3, 4, 5, 6, 8, 10, and 12 h). The small intestines were removed from the mice and prepared for histological examination.

In a separate experiment, potassium nitrate (50, 100, 200, or 400 mg/kg bw) or sodium nitrite (5, 10, 20 or 40 mg/kg bw) was administered *per os* to mice (0.5 ml/20 g bw) once

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daily for 3 or 90 days. Normal saline was used as a control. Groups of 4 or more animals were killed by cervical dislocation at 2, 4, and 6 h post-dosing, and the small intestines were removed and prepared for histological examination.

The terminal deoxynucleotide transferase labeling technique (TDR) was employed to detect apoptotic cells with DNA possessing a free 3'-OH end fragment (Gavrieli et al. 1992; Merritt et al. 1995). Briefly, intestine samples were fixed in Camoy's fixative for 30 min before the procedure. Paraffi sections of the small intestine were dewaxed and processed through an ethanol series before washing with phosphate-buffered saline (PBS). The sections were permeated with proteinase K (15 mg in PBS) and then washed in PBS. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide solutions for 15 min, followed by washing in PBS. Two drops of the equilibration buffer were applied to each specimen for 15 min at room temperature. The terminal dexynucleotide transferase (TdT) mixture was prepared as described by Gavrieli et al. (1992) and it was diluted 1:5 with distilled water (Merritt et al. 1995). The slides were incubated with or without the enzyme mixture for 1 h at 32°C before the reaction was terminated by washing the section for 10 min in the "stop wash" solution. Digoxygenin-labelled slides were treated with antidigoxigenin-peroxidase for 30 min at room temperature. The sections were washed in PBS and developed with 0.05% diaminobenzide and 0.03% hydrogen peroxide in PBS for 5 min, followed by washing with a weak haematoxylin counterstain. Scoring was restricted to good longitudinal sections of the crypt in which the base of the crypt was aligned with all the other crypt bases and there was evidence of the crypt lumen being present. Twenty good longitudinal sections were selected per animal. These were divided down the long axis into two half crypts which were scored separately; 50 such half crypt sections (i.e. crypt columns) per mouse or a minimum of 200 per group were scored. Starting at the base of the crypt columnm the cells were numbered up each side and data were recored using a microcomputer on a cell position by cell position basis. The location of each apoptotic event was then recorded in terms of its position, When several apoptotic fragments were seen to be clustered about a particular cell position they were recorded as one apoptotic cell. The results are presented as plots of the frequency of apoptotic events at each position in the crypt for nitrate and nitrite doses and post-treatment times. These frequency distributions can be described by a number of statistical parameters such as the peak value and the overall average apoptotic index. These parameters are described in deatail elsewhere (Ijiri and Potten 1987). In order to assess the viability of crypts, the microcolony technique described by Withers and Elkind (1970) was adopted. After killing the animals, the entire intestine was fixed in Camoy's fixative for 30 min, stored in 70 % ethanol and cut into three equal portions. Ten 1 cm segments of distal intestine were bundled together in tape prior to paraffin embedding. They were sectioned at 3 pm and stained with H&E. The approximate objective criteria of a regenerating crypt were 10 or more cells, each with a prominent nucleus and little cytoplasm, lying close together with a crowded appearance. These cell clusters were basophilic. Non-viable crypts contained no cells, and were sparsely populated by enlarged cells with prominent eosinophylic cytoplasm. The surviving fraction (SF) of the crypt was plotted semi-logarithmically against nitrate or nitrite doses. An average of 124±3 crypts around the circumference of the control mice was assumed.

RESULTS AND DISCUSSION

The ability of a single acute potassium nitrate or sodium nitrite dose to induce cell death in the small intestine was studied by examining the apoptotic profile at the base of the

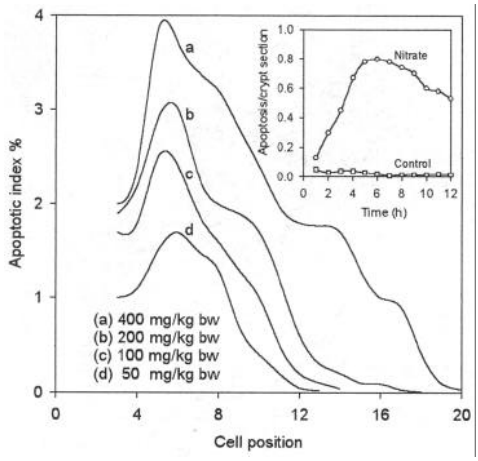

Figure 1. The smoothed frequency polygons for the apoptotic index at different cell positions of the intestinal crypt. The mice were treated with a single oral dose of potassium nitrate (0, 50, 100, 200, or 400 mg/kg bw). The insert panel shows the number of apoptosis for the entire crypt section plotted against time after treating mice with a single dose of potassium nitrate (400 mg/kg bw). The control values are shown by the horizontal line in the insert

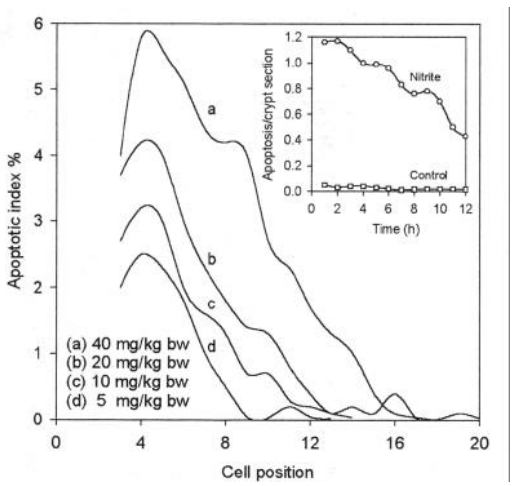

Figure 2. The smoothed frequency polygons for the apoptotic index at different cell positions of the intestinal crypt, The mice were treated with a single dose of sodium nitrite (0, 5, 10, 20, or 40 mg/kg bw). The insert panel shows the number of apoptosis for the entire crypt section plotted against time after treating mice with a single dose of sodium nitrite (40 mg/kg bw). The control values are shown by the horizontal line in the insert.

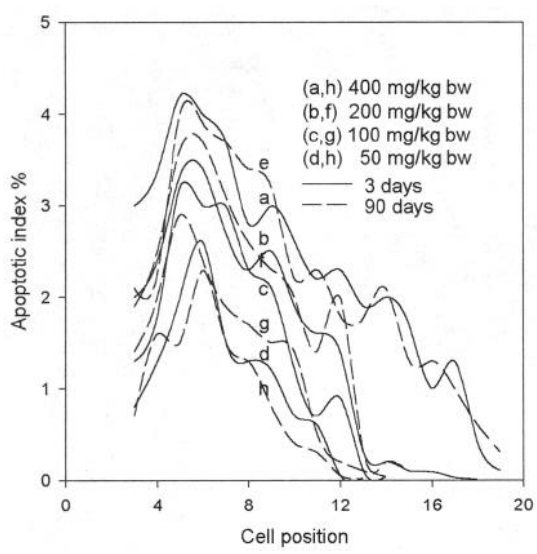

Figure 3. The smoothed frequency polygons for the apoptotic index at different cell positions of the intestinal crypt. The mice were treated with potasium nitrate (50, 100, 200, or 400 mg/kg bw) for 3 or 90 days

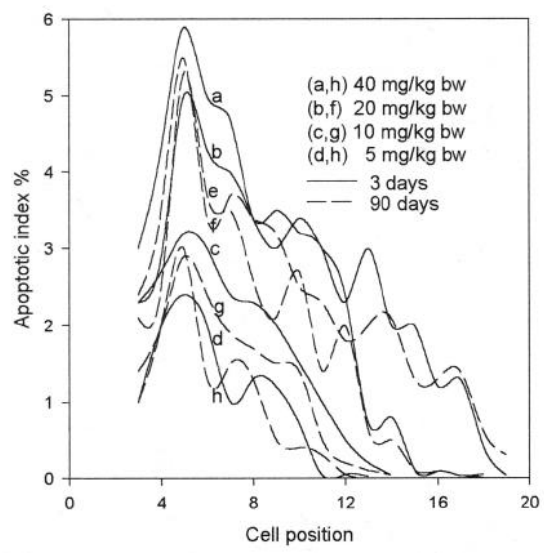

Figure 4. The smoothed frequency polygons for the apoptotic index at different cell positions of the intestinal crypt. The mice were treated with sodium nitrite (5, 10, 20, or 40 mg/kg bw) for 3 or 90 days.

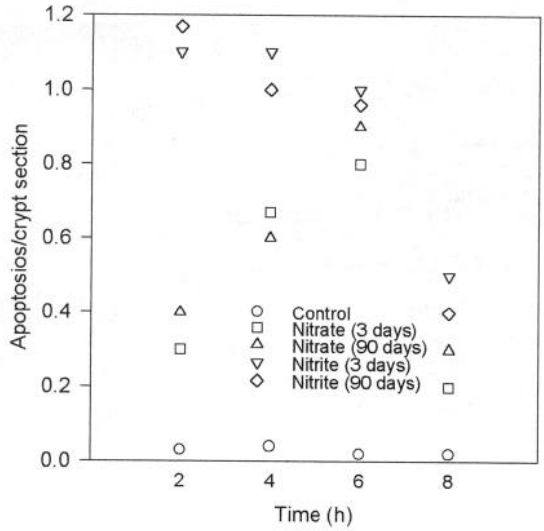

Figure 5. The number of apoptosis for the entire crypt section plotted against time after treating mice with sodium nitrite (40 mg/kg bw) or potassium nitrate (400 mg/kg bw) for 3 or 90 days.

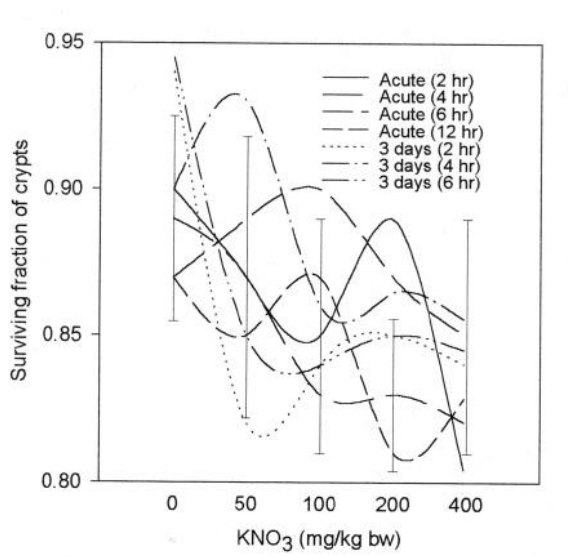

Figure 6. Crypt surviving fractions as a functon of potassium nitrate doses and time. The mice were treated with potassium nitrate in a single acute dose or a daily dose for 3 days.

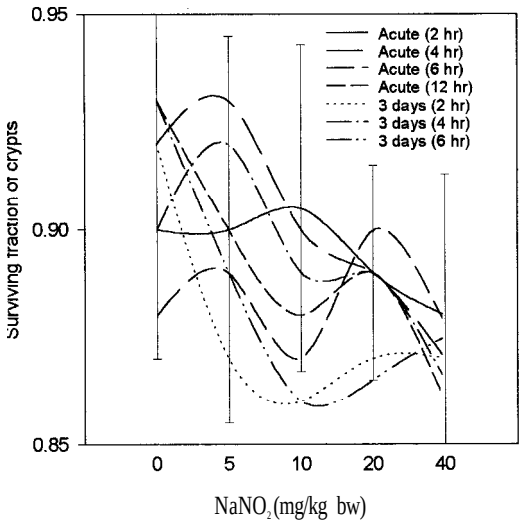

Figure 7. Crypt surviving fractions as a function of sodium nitrite doses and time. The mice were treated with sodium nitrite in a single acute dose or a daily dose for 3 days

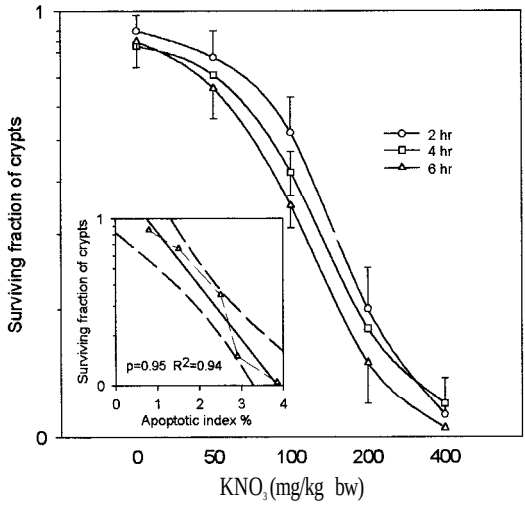

Figure 8. Crypt surviving fractions as a function of potassium nitrate doses and time. The insert panel shows the multiple regression of the apoptotic index plotted against the survival fraction of crypt section after exposing mice to potassium nitrate for 90 days. The dashed lines are 95 % confidence levels for the linear regression line and describe the range within which the regression line values will fall a percentage of the time after repeated measurements.

intestinal crypt (Figs. 1,2). In general, the highest incidence of apoptotic cell death occurred at the 5-6th (KNO_3) and 4th ($NaNO_2$) cell positions from the base of the crypt. In addition to the difference in the anatomical position of the maximal incidence of cell death, nitrate and nitrite differed in the post-dosing time when the highest incidence of cell death occurred. The highest incidence of cell death occurred at 6 h and 2 h after nitrate and nitrite treatment, respectively (Figs. 1,2). The apoptosis incidence profile of the intestinal crypts after the mice were treated by nitrate or nitrite daily for 3 and 90 days was very similar to the profile of an acute dose (Figs. 3,4,5). The highest incidence of intestinal apoptosis also occurred in the 5-6 th position at 6 h post-dosing after treating mice with nitrate for 3 or 90 days and in the 4 th position at 2 h post-dosing after treating mice with nitrite. Results of the present studies are consistent with the findings that many cytotoxic agents are able to induce apoptotic cell death in murine intestinal crypts (Ijiri and Potten 1983; 1987; Potten 1992) and that the spatial distribution of the dead cells differs among the agents (Potten 1992). Thus, each agent may selectively target a location of the crypt (Ijiri and Potten 1983). For example, direct-acting agents such as N-nitroso-N-methylurea, N-nitroso-N-ethylurea, isopropyl-methane sulphate, bleomycin, and adriamycin have been found to cause the highest incidence of cell death at cell position 4-6 in the crypt (Ijiri and Potten 1987, Potten et al. 1992a). On the other hand, compounds such as actinomycin D, cyclophosphamide, and cyclohexamide have been shown to produce the maximal cell killing at cell position 6 and 8. Another group of drugs including hydroxyurea, nitrogen mustard, methotrexate, vincristin and 5-fluorouracil have been reported to act on the cells at 8-11 position of the crypt (Ijiri and Potten 1987).

When mice were treated with nitrate or nitrite in a single acute dose or a daily dose for 3 days, the SF of the intestinal crypt was unchanged (Figs. 6,7). However, when mice were treated by nitrate or nitrite daily for 90 days, the SF of the crypt decreased with increasing nitrate or nitrite doses (Figs. 8,9). These results are consistent with those reported by Moore (1975; 1984a,b; 1986). The correlation between stem cell death and the SF of the crypt was high (R^2=0.94 for nitrate and R^2=0.91 for nitrite; see inserts of Figs. 8,9), and it clearly shows that nitrate or nitrite induces stem cell death in the small intestine. In general, cell death has been viewed as a part of the intestinal crypt regeneration processes (Hall et al 1994; Potten 1990, 1992; Potten et al. 1992b, 1994). Since intestinal cells are regenerated along a hierarchial cell lineage of the crypt (Potten and Loeffler 1990), a small number of stem cells would determine the rate of crypt regeneration following chemical injuries (Hendry et al. 1992). Results of the present study suggest that nitrate and nitrite exert their toxicities by killing cells in the early cell hierarchies (cell position 4-6 in the crypt) of the small intestine and ultimately change crypt survival by selectively killing some of or most of the functional (steady-state) stem cells,

In summary, nitrate and nitrite are capable of inducing programmed cell death in the small intestine. They also can change the surviving fraction of the intestinal crypt. However, additional studies are required to elucidate the biochemical mechanism(s) of nitrate or nitrite-induced injuries in the intestinal epithelium.

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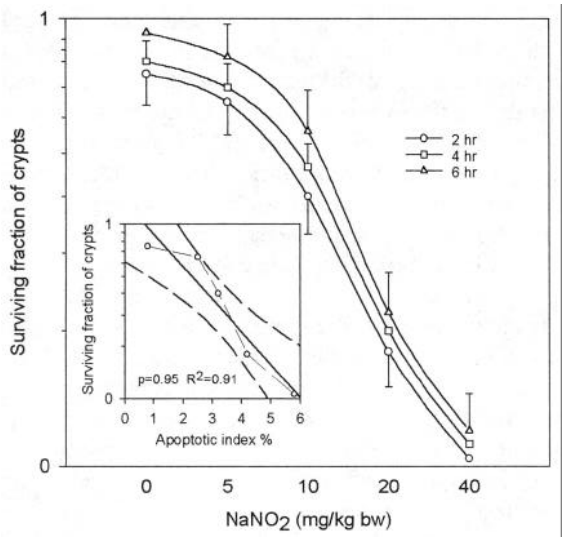

Figure 9. Crypt surviving fractions as a function of sodium nitrite doses and time. The insert panel shows the multiple regression of the apoptotic index plotted against the survival fraction of crypt section after exposing mice to sodium nitrite for 90 days. See Figure 8 legend for comments.

REFERENCES

Gavrieli Y, Sherman Y, Ben-Sasson A (1992) Identification of programmed cell cell death in situ via specific labelling of nuclear DNA fragmentation, J Cell Biol 119: 493-501

Grudzinski IP (1990a) Selected hygienic-feeding problems of nitrates and nitrites. Lek Wojsk 66: 213-214.

Grudzinski IP (1990b) Nitrosoamines and nitrosoamides - carcinogens in food. Lek Wojsk 66: 225-228

Grudzinski IP (1991a) Selected topics of the toxic influences of nitrates and nitrites on living organisms. Lek Wojsk 67: 750-754.

Grudzinski IP (1991b) Studies on the mechanism of the toxic action of sodium nitrite on intestinal absorption in rats. Arch Environ Contam Toxicol 21: 475-479.

Grudzinski IP, Szymanski A (1991a) 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 IP, Szymanski A (199lb) 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 IP, Szymanski A, Chomiczewski K (1991a) 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 2 1: 462-467.

Grudzinski IP, Szymanski A, Chomiczewski K (1991b) The effect of exercise associated with acute poisoning with potassium nitrate and sodium nitrite on the processes of intestinal absorption of D-xylose in rats. Arch Environ Contam Toxicol 21: 468-474.

Grudzinski IP, Jednorog S (1995) The effect of γ-irradiation associated with acute poisoning of potassium nitrate, sodium nitrite, and N-nitroso compounds on crypts survival processes in B6C3Fl mice intestine. Proc X Congress of the Polish Society of Radiation Research, 6-7 April, 1995, Warsaw, Poland. p. 68

Grudzinski IP, Law F.C.P (1997) Nitrite mediated T lymphocyte responses in the intestinal immune system of mice infected with Trichinella spiralis nematode. Arch Environ Contam Toxicol 33: in press

Grudzinski IP (1996) Target cells for the cytotoxic effects of sodium nitrite and potassium nitrate in the murine intestine. J Physiol Pharmacol 47: 95

Hall PA, Coates PJ, Ansari B, Hopwood D (1994) Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. J Cell Sci 107: 3565-3577

Hendry JH, Roberts SA, Potten CS (1992) The clonogen content of murine intestinal crypts: Dependence on radiation dose used in its determination. Rad Res 132: 115-119

Ijiri K, Potten CS (1983) Response of intestinal cells of differing topografical and hierarchical status to ten cytotoxic drugs and five source of radiation. Br J Cancer 47: 175-185

Ijiri K, Potten CS (1987) Further studies on the response of intestinal crypt cells of different hierarchical status to eighteen different cytotoxic agents. Br J Cancer 55: 113-123

Merrit AJ, Potten CS, Watson AJM, Loh DY, Nakayama KI, Nakyama K, Hickman JA (1995) Differential expression of bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. J Cell Sci 108: 2261-2271

Moore JV (1975) Ablation of murine jejunal crypts by alkylating agents. Br J Cancer 39: 175-181.

Moore JV (1984a) Death of intestinal crypts and of their constituent cells after treatment by chemotherapeutic drugs. Br J Cancer 49: 25-32.

Moore JV (1984b) Examination of the correction of first-week mortality with the gastrointestinal syndrome following chemotherapy. Cancer Treat Rep 68 : 1005 - 10 13,

Moore JV (1986) The gastrointestinal syndrome after chemotherapy: Inferences from mouse survival time and from histologically - and clonogenically-defined cell death in intestinal crypts. Br J Cancer 53: 16-19.

Potten CS (1990) A comprehensive study of the radiobiological response of the murine (BCFl) small intestine. Int J Radiat Biol 6: 925-937.

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

Potten CS (1992) The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. Cancer Metast Rev 11: 179-195

Potten CS, Li YQ, O'Connor PJ, Winton DJ (1992a) A possible explanation for the differential cancer incidence in the intestine, based on distribution of the cytotoxic effects of carcinogens in the murine large bowel. Carcinogenesis 13: 2305-2312

Potten CS, Kellett M, Roberts S, Rew D, Wilson G (1992b) The in vivo measurement of proliferation in normal human colorectal mucosa using bromodeoxyuridine. Gut 33 : 7l-78

Potten CS, Merritt A, Faranda A (1994) Characterization of radiation induced apoptosis in the small intestine and its biological implications. Int J Radiat Bio1 65 : 71-78.

Withers HR, Elkind MM (1970) Microcolony survival assay for cells of mouse intestinal mucosa exposed to radiation, Int J Radiat Biol 17: 261-267.