

RESEARCH COMMUNICATION

Zinc and Zinc Related Enzymes in Precancerous and Cancerous Tissue in the Colon of Dimethyl Hydrazine Treated Rats

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Abstract

Trace element zinc deficiency or excess is implicated in the development or progression of some cancers. The exact role of zinc in the etiology of colon cancer is unclear. To cast light on this question, an experimental model of colon carcinogenesis was applied here. Six week old rats were given sub cutaneous injections of DMH (30 mg/kg body weight) twice a week for three months and sacrificed after 4 months (precancer model) and 6 months (cancer model). Plasma zinc levels showed a significant decrease ($p < 0.05$) at 4 months and a greater significant decrease at 6 months ($p < 0.01$) as compared with controls. In the large intestine there was a significant decrease in tissue zinc levels ($p < 0.005$) and in CuZnSOD, and alkaline phosphatase activity ($p < 0.05$) in the pre-cancerous model and a greater significant decrease in tissue zinc ($p < 0.0001$), and in CuZnSOD and alkaline phosphatase activity ($p < 0.001$), in the carcinoma model. The tissue zinc levels showed a significant decrease in the small intestine and stomach ($p < 0.005$) and in liver ($p < 0.05$) in the cancer model. 87% of the rats in the precancer group and 92% rats in the cancer group showed histological evidence of precancerous lesions and carcinomas respectively in the colon mucosa. This study suggests that the decrease in plasma zinc, tissue zinc and activity of zinc related enzymes are associated with the development of preneoplastic lesions and these biochemical parameters further decrease with progression to carcinoma in the colon.

Keywords: Colon cancer - zinc - DMH - CuZnSOD - alkaline phosphatase - precancerous

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Introduction

Cancer of the large bowel has been the subject of intensive investigations for many years and its incidence has been found to vary with geographic area and socioeconomic level (Fabricant & Broitman, 1990). Colorectal cancer is one of the most common malignancies in industrialized countries and one of the most common causes of cancer-related death. From the distribution of large bowel cancer in various parts of the world and data dealing with migrant populations, it is apparent that environmental factors, rather than genetic and social factors, play a significant role in the etiology of colon cancer in man (Haenszel et al., 1973).

The trace element zinc has been demonstrated to be essential for the growth, development and differentiation of all types of life, including microorganisms, plants and animals (Vallee, 1986). Zinc plays a major role in protein synthesis and has an important function in gene expression; the involvement in gene expression is both structural and an enzymatic role. These proteins require zinc for their conformation and DNA binding abilities (Berg, 1990; Choo et al., 1997). The zinc atoms are an integral, firmly bound part of the proteins molecule and are often involved in the active site. They also contribute to the conformation

and structural stability of many metalloenzymes. Decrease in zinc metalloenzymes activity along with decrease in zinc varies with different zinc enzymes and is dependent on the tissue, enzyme turnover rate, and affinity of the enzyme for zinc. (Rhodes & Klug, 1993)

The plasma and tissue zinc levels in various diseases have been the subject of a multitude of investigations and possible involvement of zinc has been recognized in many cancerous conditions. The role of zinc in carcinoma development has been the subject of debate and reports of zinc values in the biological fluids from cancer patients are often conflicting and contradictory. Hence the mechanism by which plasma zinc and tissue zinc decrease in various cancerous tissues is still obscure (Ehud et al., 1983).

Colorectal cancer, which is a common cause of cancer related deaths, is often due to persistent oxidative stress leading to DNA damage. Oxidative stress caused by reactive oxygen species, (ROS) results in damage to cellular structure, and this has frequently been implicated in the initiation and promotion phases of carcinogenesis. Levels of ROS are controlled by the antioxidant defense system. Several components of this system are micronutrients (e.g. vitamins C and E) or enzymes produced by specific endogenous pathways. (e.g. CuZn and Mn superoxide dismutase) (Evans & Halliwell, 2001)

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; Vertuani et al., 2004). Super oxide dismutases (SOD) are metalloenzymes that play a vital role in the protection of aerobic cells against oxygen toxicity (Cindy & Yi Feng, 1999). Tissue CuZnSOD activity is reduced in a number of malignancies. Some tumors have less CuZn SOD in comparison with the more metabolically active tissues (Grigolo et al., 1998).

The E.coli alkaline phosphatase (ALP) was first recognized as a zinc metalloenzyme in 1962. It is a marker enzyme of microvillus membranes (MVM) in the intestine and exists both in the soluble and membrane bound forms (Katica et al., 2006). Intestinal ALP has been shown to decrease in patients with gastric or colonic cancer (Sotaro et al., 2006). Studies in tissue homogenates indicates that the activity of alkaline phosphatase in tissues decrease markedly when a zinc deficient state is induced in experimental animals as compared with pair fed controls (Reinhold & Kfoury, 1969).

Development of colon cancer is a multistep process involving a series of pathological alterations ranging from discrete microscopic lesions like aberrant crypt foci (ACF), also known as preneoplastic lesions to malignant tumors (Bird, 1995). It is believed that colon cancer, like the majority of cancers, evolves from precursor lesions also known as adenomatous polyps (Elizabeth, 1988; Farber & Cameron, 1980). To identify the possible precursor lesions of adenomatous polyps of colon cancer, several investigators have studied the development of colon cancer in rodents treated with chemical carcinogens. Carcinogens are administered to experimental animals like rats and mice, to study the effect of certain diets and preventive agents on tumor induction (Day & Morson, 1978; Richards, 1977).

The genotoxic chemical 1, 2-DMH (symmetrical dimethyl hydrazine) is one of the agents most frequently used in experimental models of colon carcinogenesis. It is a complete carcinogen that induces the initiation and promotion steps of carcinogenesis yielding macroscopically visible neoplasms (Park 1997; Sequeira, 2000). Regardless of the mode of administration, DMH specifically induces tumors within the descending colon and the histopathology is similar to that observed for human sporadic colon tumors (Rogers & Nauss, 1985). Numerous studies have been carried out to investigate the protective effect of various chemicals, drugs and food items on the induction and development of DMH induced colon tumors in rats, (Anisimov & Popovich, 1997; Cameron et al., 1997; Furkawa, 1997) but so far, to the best of our knowledge no study has shown the relationship between changes in tissue zinc levels and zinc related enzymes in DMH induced colon carcinogenesis. Thus the DMH induced colon cancer model in rats is a good tool to investigate the relationship of zinc and zinc related enzymes to colon cancer. In a study reported by Davis et al, [1994] using the DMH model for induction of carcinoma, tumors were found after the administration of DMH at a dosage of 30mg/kg twice a week for 5 months. Hence we used a similar dose of 30mg/kg DMH twice a week for 3 months and sacrificed rats at the fourth month in order to induce the development of precancerous lesions

in the colon. Since we found an association between tissue zinc levels and zinc related enzymes such as SOD and alkaline phosphatase in the colon in precancerous stage we decided to further set up a colon carcinoma model by injecting rats with DMH (30mg/kg body wt.) twice a week for 4 months and sacrificing them at 6 months.

The aim of this study was to investigate whether the biochemical changes in the colon with respect to tissue zinc levels and zinc related enzymes such as CuZnSOD and Alkaline phosphatase are associated with the histological changes that take place in the colonic mucosa from normal to precancerous stage and then to the cancerous stage.

Materials and Methods

Animals: Thirty two adult Wistar rats (100-120g) at six weeks of age, obtained from the Institutional animal house were housed in polypropylene plastic cages, in an animal holding room under controlled conditions with $25 \pm 2^\circ\text{C}$, $50 \pm 10\%$ humidity, and 12 hour light-dark cycles. The rats were allowed water and food ad libitum, observed daily and weighed weekly. This study was approved by the Animal Experimentation Ethics Committee of our Institution.

Chemicals: Dimethyl hydrazine, stock Zn standard (1002 mg/ml), bovine serum albumin, Triton X100, bathocuproindisulfonate sodium salt, MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), Xanthine, and Xanthine Oxidase, 0.5 M Tris-HCl buffer (pH -9.0), 5nM MgCL₂, 1M NaOH, 5mM p-nitrophenyl phosphate, p-nitrophenol standard, were purchased from the Sigma Chemical Company, India. Deionized water was used for all purposes. All other reagents were of analytical grade and were purchased from Indian companies.

Experimental design and induction of colonic tumors.

Thirty two rats were randomly assigned to two groups, Group A (Precancerous) and Group B (cancerous). All groups were fed the same diet and maintained as described. Group A was further subdivided into control group (n=6) and experimental group (n=8), which received subcutaneous dose of saline or 30mg/kg body weight DMH dissolved in saline respectively twice a week for 3 months and were euthanized at 4 months.

Group B was further subdivided into control group (n=6) and experimental group (n=12), which received a subcutaneous dose of saline or 30mg/kg body weight DMH dissolved in saline respectively twice a week for 4 months and were euthanized at 6 months. All groups were euthanized by chloroform inhalation. Blood was collected at the time of sacrifice for estimation for plasma zinc.

Tissue preparation-histopathology, measurement of tissue zinc, CuZnSOD, Alkaline phosphatase, plasma zinc: all rats were examined grossly at necropsy. The entire intestine from the stomach to anus was removed and the large intestine was isolated. It was slit length wise, washed in saline and the mucosal surface examined for gross pathology. Any lesions detected were measured, location noted and dissected. A portion was taken for

histological examination after fixation in 10% w/v formaldehyde overnight and was routinely processed as per standard methods, embedded in paraffin, sectioned at 5µm, stained with hematoxylin and eosin for evaluation by light microscopy. At autopsy, a section of the colon of the rats of both the groups A and B and a section of the stomach, small intestine and liver of the rats of group B were harvested, washed with ice cold saline and stored at -20 °C until analysis for tissue zinc estimation as described [Kahnke, 1966].

The mucosa of the stomach, small intestine, and large intestine were used for cytosolic homogenate preparation using phosphate buffered saline pH 7.4. CuZnSOD activity (units/mg/protein) in the homogenate was measured by MTT reduction as described [Kim, 2000]. Activity of alkaline phosphatase (µmoll/min/mg protein) was assayed as described [Dorai, 1977].

Plasma zinc was estimated as described [Rosner & Garfien, 1968]. Zinc determination was carried out using Perkin Elmer AAS model-100. The concentration of tissue zinc is reported in µg/g dry weight tissue, with an appropriate blank being used for correction of zinc content in the acids used for digestion.

Ethanediol-stabilised QC serum, prepared in our laboratory, was run along with each batch of plasma zinc estimations. The small intestine from a control rat was cut into many pieces, and processed for tissue zinc with every batch of samples. Similarly the mucosa of the small intestine and large intestine of control rat was aliquoted into storage tubes and included with each batch for the estimation of CuZnSOD and Alkaline phosphatase activity respectively.

Statistical Analysis

Data are expressed as Mean ± SD. Differences between groups were analyzed using Non-Parametric test -Mann Whitney U test and Kruskal Wallis test. A difference was considered statistically significant when the probability associated with it was less than 0.05 (p<0.05).

Results

A) DMH induced precancer model

In the DMH induced precancerous model, colonic precancerous stage was established at 4 months in animals that were treated with DMH. The colon of control group that received vehicle saline was grossly normal. Precancerous stage of the colon was observed upon histological examination in 87.5% animals (7 out of 8) that were treated with DMH and sacrificed after 4 months. Histological examination of the large intestine of rats, showed proliferated glands lined by cells with hyperchromatic pseudo stratified nuclei and cytoplasmic -mucin depletion consistent with dysplasia (Figure 1).

Plasma zinc levels in the DMH treated rats at four months showed a significant decrease (mean 23%) as compared with the control group (93 ±9 vs 121±4.5µg/dl, p<0.01). As shown in Fig 2 (A), the mean tissue zinc levels (wet weight) in the large intestine showed a significant decrease as compared with controls (mean

46%, (p<0.005), the dry weight analysis also showed a similar decrease (Figure 2B). CuZnSOD activity in the mucosal homogenate of the large intestine showed a significant decrease (mean 24%, p<0.05), as compared with controls (Figure 2C). The alkaline phosphatase activity in the mucosal homogenate in the large intestine was significantly lower (mean 27.3%, p<0.05) as compared with controls (Figure 2D).

B) DMH induced cancerous model

In the DMH induced carcinoma model, the colon of the control group of rats that received saline was grossly

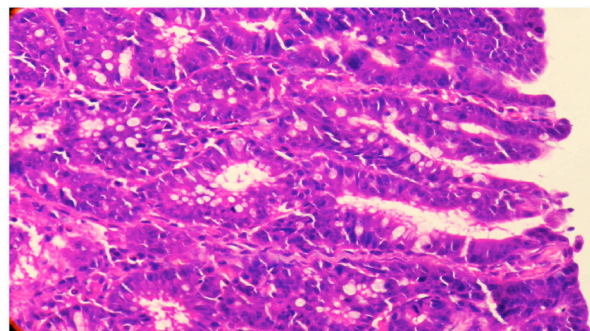


Figure 1. Light Microscopy of Large Intestine of DMH Treated Rats at 4 Months Showing Precancerous Stage, Colonic Mucosa with Closely Packed Glands Lined by Epithelial Cells Exhibiting Dysplasia. Magnification 40 xs

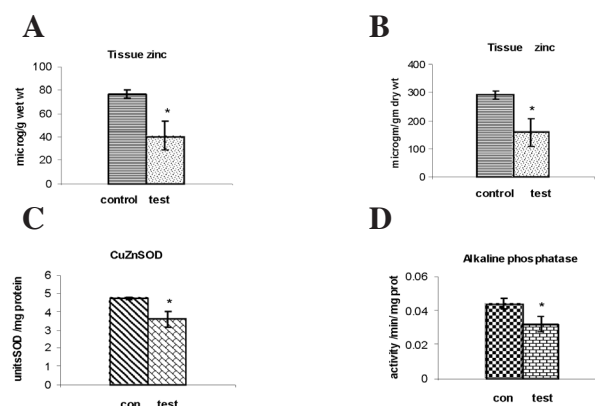


Figure 2. Large Intestine Tissue Zinc Levels. A) wet wt. (B) dry wt. (C) CuZnSOD (D) alkaline phosphatase activity in large intestine in DMH treated rats at 4 months. (Precancerous stage). Values are represented as mean ± SD. *p <0.05 when compared to control.



Figure 3. Large Intestine of Rat Showing Large Tumor Growth in the Colon (Colon of Rat Injected with DMH at 6 months)

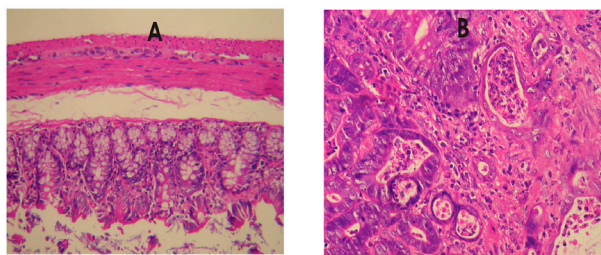


Figure 4. Light Microscopy of the Colon in DMH Treated Rats at 6 Months. A) Normal colonic tissue, magnification 40 xs, B) Colonic mucosa with infiltrating adenocarcinoma composed of irregular glands lined by malignant columnar epithelial cells infiltrating the stroma. Magnification 40 xs

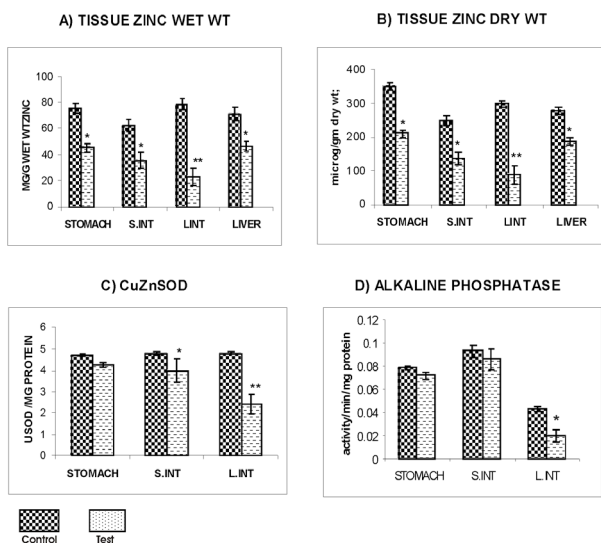


Figure 5. Zinc Status in Various Organs. (A) wet wt., (B) dry wt., (C) CuZnSOD (D) Alkaline phosphatase activity in large intestine, small intestine and stomach (body) in DMH treated rats as compared to controls- at 6 months. Values represent mean \pm SD from 6 rats, * $p<0.05$, ** $p<0.001$ when compared to control.

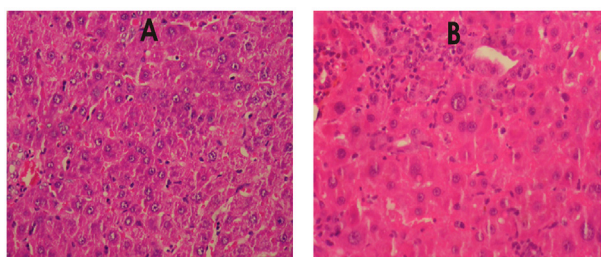


Figure 6. Light Microscopy of Liver. A) normal liver tissue of rat treated with saline, B) Liver of DMH treated rat at 6 months showing liver with scattered hepatocytes, mild nuclear enlargement and hyperchromasia.

normal and showed normal morphology (Figure 4A) Out of the 12 experimental rats that received DMH for four months and sacrificed at 6 months, 11 rats (91.6%) showed tumors in the large intestine. Macroscopical examination of the resected colon of the 11 rats showed tumors varying between diameters of 2mm to 1.5 cm (Fig. 3). Out of the 11 rats which showed macroscopically large tumors, 4 rats (30%) showed tumors in the ascending colon and the remaining 7 rats (60%) showed tumors in the descending colon. Three of these rats showed multiple tumors in the colon (2 to 4 small size tumors). Colon carcinoma was

established histologically (Fig 4.B).

The mean plasma zinc levels in the DMH induced carcinoma model was significantly lower (mean 33.4%, $p<0.01$) as compared with control group treated with saline. (81 ± 11 vs. 122 ± 4.3 $\mu\text{g/dl}$). As shown in Figure 5A, tissue zinc levels (wet weight) in the large intestine showed a significant decrease as compared with the control group (mean 72%, $p<0.001$). Dry weight analysis showed a similar % mean decrease in tissue zinc levels (Fig 5B). CuZnSOD activity in the mucosal homogenate of the large intestine shows a mean 45% decrease, which was statistically significant as compared with controls ($p<0.001$), (Fig 5C). A significant decrease in alkaline phosphatase activity in the mucosal homogenate of the large intestine was observed (51% mean) as compared with controls ($p<0.001$) (Fig 5D).

In the small intestine and in the body of the stomach, a significant decrease in the tissue zinc levels (dry weight, mean 44%, $p<0.01$, and mean 42%, $p<0.01$ respectively) was observed as compared with controls (Fig 5B). As shown in Fig 5C, the CuZnSOD activity in the small intestine showed a significant decrease (mean 19%, $p<0.05$) as compared with controls, where as in the stomach the CuZn SOD activity remained unaltered. The Alkaline phosphatase activity in the stomach and in the small intestine remained unaltered (Fig 5D). The liver showed a significant decrease in tissue zinc levels ($P<0.05$, mean 34%) as compared with controls. (Figure 5 A, B). CuZnSOD and alkaline phosphatase activity were not measured in the liver. Histological examination of the liver showed scattered hepatocytes, mild nuclear enlargement and hyperchromasia (Figure 6).

Quality control

Quality control (QC) values for tissue zinc, plasma zinc and tissue SOD are presented below. QC values for plasma zinc showed a CV of 2.7%, for tissue zinc a CV of 3.75%, for CuZnSOD activity and alkaline phosphatase activity a CV of 1.2 % and 3.6 % respectively.

Discussion

Zinc plays an important role in cellular physiology and this element functions as a specific activator of many enzymatic reactions (Hambidge, 1989). Zinc deficiency is implicated in the development and progression in some cancers (Valcovic, 1980). Initiation of mucosal changes, which progress to preneoplastic lesions, may be necessary in the triggering of the adenoma-carcinoma sequence (Sandforth, 1988). The present study, in DMH induced colon precancerous and cancerous stage in the rat model, examined the relationship between plasma zinc, colonic tissue zinc levels and the zinc requiring enzymes CuZnSOD and Alkaline phosphatase activity in the progression from normal to precancerous stage and then to the cancerous stage.

In the DMH induced precancerous model, histologically severe dysplasia was observed in the colon of majority of rats (87.5%) at 4 months. Early precancerous lesions established in the proximal and distal regions of the

colon was associated with a significant decrease in the concentration of plasma zinc, colonic tissue zinc and zinc requiring enzymes CuZnSOD and alkaline phosphatase in the DMH treated rats at 4 months as compared with controls. In the DMH induced colon carcinoma model, the results show that colon carcinoma was histologically established in the majority of rats (92%) at 6 months. In agreement with other studies we found maximum tumors in the distal colon (Corpet & Tache, 2002). Histologically differentiated DMH induced colon cancer was directly related to a greater significant decrease in the concentration of plasma zinc, colonic tissue zinc and zinc related SOD and alkaline phosphatase activities as compared to DMH induced precancerous model. The trend towards a gradual decrease in zinc concentration in the malignant tissue in our study is in agreement with the results reported earlier in cancer patients with respect to the differences in normal – malignant tissue zinc (Sadamitsu & Sadao, 1978; Brown, 1980). The exact cause of low zinc levels in the malignant tissue is not known. Neoplastic diseases have been reported to lead to changes in zinc metabolism as evidenced by alterations in plasma zinc concentration (Rao, Ganesh, 1998). Mc bean et al suggested that the diseased conditioned may lead to an overall zinc decrease in the tissues (Mc Bean, 1972).

It has been documented that significant oxidative stress, caused by free radicals, occurs in carcinoma of the intestinal mucosa. In particular, the super oxide radical ion has been postulated as the possible cause of cancer (Oberley, Buttner, 1979). As observed in previous studies, altered activities of superoxide dismutase were shown to be important in multistage carcinogenesis of both rodents and humans. They showed that tumors possessed decreased SOD activity compared to metabolically active tissues (Van Driel, 1997; Dionisi, 1975; Westman & Stefan, 1981) The results in our study illustrates that CuZnSOD activity is impaired in the colonic precancerous tissue at four months and in the colonic cancerous tissue at six months of DMH treatment. This may mean that it might have a functional role in human colon carcinogenesis. The decreased concentration of the trace element zinc found in the precancerous tissue and cancerous tissue may contribute to the differences observed in tissue CuZnSOD enzyme activities.

Intestinal enzymes associated with the striated cell border, such as intestinal alkaline phosphatase may be useful markers for malignant expression in colonocytes (Brown et al., 1980). The results in this study are consistent with the findings of studies reported earlier, in that a decrease in alkaline Phosphatase was observed in tumors (Harmenberg, 1991) indicating that these enzymes are very sensitive to zinc depletion. The decrease in alkaline phosphatase in the cancer tissue was greater than the decrease in the precancer tissue. These studies indicate modifications in the enzyme structure that could be brought about by zinc decrease (Reinhold & Kfoury, 1969). Intestinal alkaline phosphatase was found to decrease in gastric or colonic cancer with an exacerbation of its cancerous lesions as compared to higher activity in normal individuals (Katsuyuki, 1978).

Studies have shown that tumors develop in various regions of the gastrointestinal tract and small bowel when DMH was administered to rats (Zhurkov, 1996). The present study, in the DMH treated carcinoma model a significant decrease in tissue zinc levels and CuZnSOD activity was observed in various parts of the gastrointestinal tract i.e. body of stomach, and in small intestine. Trans mural changes along the wall of the gastrointestinal tract could be the cause of this change. These findings led to the hypothesis that early mucosal alterations, which are preneoplastic lesions, and which can lead on to further development of carcinogenesis might also lead to a decrease in tissue zinc concentration and zinc-requiring enzyme CuZnSOD and alkaline phosphatase activity in the large intestine and to possible changes in other parts of the gastrointestinal tract.

In this study, we also observed significant decrease in tissue zinc levels in the liver in DMH treated rats at six months. Mild histological changes leading to atypical hyperplasia and nuclear enlargement were observed. As reported in earlier studies, the presence of atypical cells in the liver suggests that besides gastrointestinal tumors, the liver may also be a site for abnormalities (Zhurkov, 1996; Inagake, 1995). The significant decrease in liver zinc in our studies could be attributed to the histological changes taking place with administration of DMH, which occurs alongside with the progression of dysplasia in the colon.

The above findings support the view that decreasing tissue zinc levels and zinc requiring enzymes SOD and alkaline phosphatase are representative of the biochemical alterations in the colonic mucosa, which may in turn reflect the cellular changes that progressed to dysplasia and frank malignancy. In our study, histologically preneoplastic lesions in the large intestine also presented progressive changes. At fourth month, in the DMH treated group, the colon exhibited hyperplastic changes, while frank malignancy was established at 6 months of DMH treatment. These may be sequentially related to the biochemical events in the large intestine in DMH treated rats. In this study, the decreased concentration of the trace element zinc found in the precancerous colon tissue and a greater decrease observed in the cancerous colonic tissue may contribute to the decreases observed in tissue CuZnSOD and alkaline phosphatase enzyme activities in the colon in the precancerous and the cancerous models.

In conclusion, this study indicates that there is a decrease in plasma zinc, tissue zinc concentration, CuZnSOD activity, and alkaline phosphatase activity which occurs alongside the changes in the colonic mucosa during precancerous and cancerous transformation. It may be finally suggested that the zinc decrease in tissue is associated with carcinogenesis which may limit the functions of zinc requiring enzymes such as CuZnSOD and alkaline phosphatase.

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References

- Anisimov P, Popovich IG (1997). Melatonin and colon carcinogenesis: inhibitory effect of melatonin on development of intestinal tumors induced by 1,2-dimethyl hydrazine in rats. *Carcinogenesis*, **18**, 1549-53.
- Berg JM (1990). Zinc finger domains: hypothesis and current knowledge. *Ann Rev Biophys Chem*, **19**, 405-21
- Bird RP (1995). Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Letters*, **93**, 55-71.
- Brown DA, Chattel KW, Chan AY, Knight B (1980). Cytosolic level and distribution of copper and zinc in pretumors from diethyl nitrosamine-exposed mice and in non-cancerous tissue from cancer patients. *Chem Biol Interact*, **32**, 13-27
- Cameron L, Hardman H (1997). The nonfermentable dietary fiber lignin alters putative colon cancer risk factors but does not protect against DMH induced colon cancer in rats. *Nutr Cancer*, **28**, 170-6.
- Choo Y, Klug A (1997). Physical basis of a protein -DNA recognition code. *Curr Opin Struct Boil*, **7**, 117-25.
- Corpet DE, Tache S (2002). Most effective colon cancer chemo preventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr and Cancer*, **43**, 1-21.
- Davis C, Feng Y (1999). Dietary copper, manganese and iron affect the formation of aberrant crypts in colon of rats administered 3,2'-dimethyl -4-aminobiphenyl. *J Nutr*, **129**, 1060-7.
- Dionisi D, Galeotti T, Terranova T, Azzi A (1975). Superoxide radicals and hydrogen peroxide formation in mitochondria from normal and neoplastic tissues. *Biochim Biophys Acta*, **403**, 292-300.
- Dorai B, Bachhawat BK (1997). Purification and properties of brain alkaline phosphatase. *J Neurochem*, **29**, 503-12.
- Davis AE, Patterson F (1994). Aspirin reduces the incidence of colonic carcinoma in the dimethylhydrazine rat animal model. *Aust. NZ J Med*, **24**, 301-3.
- Day DW, Morson BC (1978). The adenoma-carcinoma sequence, In: Morson, B.C. (ed.), *The Pathogenesis of Colorectal Cancer*, Philadelphia: W. B. Saunders Company. 58-71.
- Ehud JM, Joseph GS, Mordechai C (1983). Copper and zinc levels in normal and malignant tissues. *Cancer*, **52**, 868-72.
- Evans P, Halliwell B (2001). Micronutrients: oxidant/antioxidant status. *Br J Nutr*, **85**, 67-74.
- Fabricant M, Broitman SA (1990). The geographical distribution of cancer. *Br J Cancer*, **23**, 1-8.
- Farber E, Cameron R (1980). The sequential analysis of cancer development. *Adv Cancer Res*, **31**, 125-226.
- Fujimoto S, Misaki F, Kato K (2006). Serum alkaline phosphatase (Al-pase) isozyme in gastric and colonic cancer. *Am J Physiol-Gasterol*, **290**, 737-46.
- Furukawa K, Yamamoto I, Tanida N, Tsujiai T (1995). The effects of dietary fiber from lagenaria scineraria yugaomelon on colonic carcinogenesis in mice. *Cancer*, **15**, 1508-15.
- Grigolo B, Lisignoli G, Toneguzzi S, Mazzetti I (1998). Copper /Zinc Super oxide dismutase expression by different human osteosarcoma cell lines. *Anticancer Res*, **18**, 1175-80
- Hambidge KM (1989). Mild zinc deficiency in human subjects. In: Mills, C. (ed.), *Zinc in Human Biology*. Human Nutrition Reviews, Springer-Verlag, 281-96.
- Harmenberg U, Koham M, Koshida K (1991). Identification and Characterization of alkaline phosphatase isoenzymes in human colorectal adenocarcinomas. *Tumor Biol*, **12**, 237-48
- Haenszel W, Berg JW, Kurihara M, Locke MB (1973). Large bowel cancer in Hawaiian Japanese. *J Natl Cancer Inst*, **51**, 1765-79.
- Inagake M, Yamane T, Kitao Y, Oya K (1995). Inhibition of 1, 2 dmh induced oxidative DNA damage. *Jpn J Cancer Res*, **86**, 1106-11.
- Inutska S, Araki S (1978). Plasma copper and zinc levels in patients with malignant tumors of digestive organs. *Cancer*, **42**, 626-31.
- Katica BK, Karmen S, Matilda D, Zoran K (2006). Intestinal alkaline phosphatase activity as a molecular marker of enterotoxicity induced by single dose of 5-fluorouracil and protective role of orally administered glutamine. *Arch Oncol*, **14**, 101-5.
- Kahnke MJ (1966). Atomic absorption spectrophotometry applied to determination of zinc in formalinised human tissue. *At Absorption Newsl*, **5**, 1.
- Kim TS, Jung Y, Kim KS (2000). Molecular cloning and expression of copper and zinc containing super-oxide dismutase from Fasciola Hepatica. *Infection Immunity*, **8**, 3941-8.
- Katsuyuki Y, Sotaro F, Fumio M, Keiichi K (1978). Serum alkaline phosphatase (Al-pase) isozyme in gastric and colonic cancer. *J Gastroenterology*, **13**, 264-71.
- McBean JT, Dove JA, Smith JR, et al (1972). Zinc concentration in human tissues. *Am J Clin Nutr*, **25**, 672-6.
- McLellan EA, Bird RP (1988). Aberrant crypts: potential preneoplastic lesions in the murine colon. *Cancer Res*, **48**, 6187-92.
- Oberley LW, Buttner GR (1979). Role of super oxide dismutase in cancer. *Cancer Res*, **39**, 1141-9.
- Park HS, Goodlad RA, Wright NA (1997). The incidence of aberrant crypt foci and colonic carcinoma in dimethylhydrazine treated rats varies in a site-specific manner and depends on tumor histology. *Cancer Res*, **57**, 507-10.
- Reinhold JG, Kfoury GA (1969). Zinc dependent enzyme in zinc depleted rats. *Am J Clin Nutr*, **22**, 1250.
- Richards TC (1977). Early changes in the dynamics of crypt cell populations in mouse colon following administration of 1,2-dimethylhydrazine. *Cancer Res*, **37**, 1680-5
- Rogers AE, Nauss KM (1985). Rodent models for carcinoma of the colon. *Dig Dis Sci*, **30**, 87-102.
- Rosner F, Garfien PC (1968). Erythrocytes and plasma zinc and magnesium levels in health and disease. *J Lab Clin Med*, **72**, 213-?.
- Rao DN, Ganesh B (1998). Estiamtes of cancer incidence in India in 1991. *Indian J Cancer*, **35**, 10-18.
- Sequeira JL, Kobayasi SK, Rodrigues MM (2000). Early and late effects of wound healing on development of colon tumors in a model of colon carcinogenesis by 1,2 dimethylhydrazine in the rat. *Pathology*, **32**, 250-2.
- Sandforth F, Heimpel S, Blazer T, Gutshmidt S (1988). Characterization of stereo microscopically identified preneoplastic lesions during dimethyl hydrazine induced colonic carcinogenesis. *Eur J Clin Invest*, **6**, 655-62.
- Vallee BL, Bertini I, Gary (1986). eds. *A synopsis of zinc biology and pathology in zinc enzymes* Birkhauser Boston.
- Vertuani S, Angusti A, Manfredini S (2004). The antioxidants and pro-antioxidants network: an overview. *Curr Pharm Des* **10**, 1677-94
- Valcovic V (1980). *Analysis of Biologic material for Trace elements using XRay Spectroscopy*, Boca Raton FL: CRC Press Inc, 125-43.
- Van Driel BE, Lyon H, Hoogenraad DC, et al (1997). Expression of Cu Zn and Mn SOD in human colorectal neoplasm. *Free Radic Biol Med*, **23**, 435-44.
- Westman NG, Stefan L, Marklund S (1981). Copper zinc SOD and manganese containing SOD in human tissues and human malignant tumors. *Cancer Res*, **41**, 2962-6.
- Zhurkov VS, Sycheva LP, Salamatova O (1996). Selective inhibition of micronuclei in the rat/mouse colon and liver by 1,2-dimethylhydrazine – a seven tissue comparative study. *Mut Res*, **12**, 115-20