Protective Role of Aspirin, Vitamin C, and Zinc and their Effects on Zinc Status in the DMH-Induced Colon Carcinoma Model

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Abstract

Chemoprotection refers to the use of specific natural or synthetic chemical agents to suppress or prevent the progression to cancer. The purpose of this study is to assess the protective effect of aspirin, vitamin C or zinc in a dimethyl hydrazine (DMH) colon carcinoma model in rats and to investigate the effect of these supplements on changes associated with colonic zinc status. Rats were randomly divided into three groups, group 1 (aspirin), group 2 (vitamin C) and group 3 (zinc), each being subdivided into two groups and given subcutaneous injection of DMH (30 mg/kg body wt) twice a week for 3 months and sacrificed at 4 months (A-precancer model) and 6 months (B-cancer model). Groups 1, 2, 3 were simultaneously given aspirin, vitamin C, or zinc supplement respectively from the beginning till the end of the study. It was observed that 87.5% of rats co-treated with aspirin or vitamin C showed normal colonic histology, along with a significant decrease in colonic tissue zinc at both time points. Rats co-treated with zinc showed 100% reduction in tumor incidence with no significant change in colonic tissue zinc. Plasma zinc, colonic CuZnSOD (copper-zinc superoxide dismutase) and alkaline phosphatase activity showed no significant changes in all 3 cotreated groups. These results suggest that aspirin, vitamin C or zinc given separately, exert a chemoprotective effect against chemically induced DMH colonic preneoplastic progression and colonic carcinogenesis in rats. The inhibitory effects are associated with maintaining the colonic tissue zinc levels and zinc enzymes at near normal without significant changes.

Keywords: Colorectal cancer - zinc - DMH - CuZnSOD - alkaline phosphatase - vitamin C - aspirin - zinc supplement

Introduction

The high incidence and mortality of colon cancer make effective prevention an important public-health and economic issue. Suppression or reversal of the carcinogenic process, in the colon, with non-pharmacologic or pharmacologic agents, i.e. chemoprotection, is an area of considerable research interest and activity. In addition to demonstrating efficacy, chemo protective agents must be safe for long-term use, well accepted by patients and also cost-effective. Based on observational epidemiologic studies, it is clear that individuals who consume a diet high in vegetables and natural fibers and low in fat have a reduced risk of polyps and colon cancer. Optimal nutrient intake for the protection against cancer might be more readily achieved via food fortification or supplementation, but this requires more research.

One promising group of compounds with cancer protective activity includes Nonsteroidal anti-inflammatory drugs (NSAIDs) which display a protective effect on reducing the incidence of polyps and cancer of the large bowel. Aspirin or acetyl salicylic acid, is a drug in the salicylate family and of the class of NSAID. Recent epidemiological studies and clinical trials indicate that long term use of aspirin, could possibly decrease the incidence of certain malignancies, including colorectal, oesophageal, breast, lung and bladder cancers (Smalley and DuBois, 1997; Wang and DuBois, 2006; Bosseti et al., 2009). The best known targets of NSAIDs are cyclooxygenase (COX) enzymes, which convert arachidonic acid to prostaglandins (PGs) and thromboxane which can promote tumour growth (Wang and DuBois, 2006; Rothwell et al., 2011). Aspirin, which is often used as an analgesic, antipyretic and anti-inflammatory agent is an irreversible inhibitor of COX and is said to reduce cancer cell proliferation and induce cancer cell apoptosis (Rosenberg et al., 1995; Fosslien, 2000; Ashtorab et al., 2005; Seung and Kim, 2008; Peter et al., 2009). Aspirin which is a commonly available drug is being investigated and tried out both in experimental models as well in human volunteers. The aim being to evaluate its role in reducing the incidence of carcinoma of colon, stomach, lung, bladder (Michael, 1992; Allison et al., 2006; Mahipal et al., 2006; Enrico and Peter, 2007; Jack et al., 2009).

Another group of chemoprotective compounds are the antioxidants which are molecules that can safely interact

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with free radicals and terminate the chain reaction before vital molecules are damaged. Oxidative stress is a general term used to describe a state of damage to cells caused by free radicals. Their chief danger comes from the damage they can cause when they react with important cellular components such as DNA, or the cell membrane, thus leading to an unwanted proliferation of cells, further leading to cell dysfunction and eventually to cancer or cell death (Osmak et al., 1997; Rosa et al., 2012). Antioxidant defense mechanisms in the human body can be divided into two different categories. Firstly, a number of enzymes with antioxidant properties are synthesized in the human body. The second group of antioxidants has to be obtained from the diet, since they cannot be synthesized by humans. These include the nutrients and plant metabolites such as vitamins E and C. As an antioxidant, the primary role of vitamin C is to neutralize free radicals. In the last few years there has been an increase in information about the role of oxidative stress in causing, a number of serious diseases such as certain cancers and the potential therapeutic role of antioxidants in preventing them. Since oxidative damage has been implicated in the etiology of cancer, there is a need to ensure sufficient antioxidant supplies as a central measure in preventive medicine. Studies have linked low blood levels of the antioxidant nutrients such as vitamin C with a higher risk of cancer (Ames, 2001; Mikirova et al., 2013). Vitamin C can enhance the body’s resistance to many diseases, including infectious disorders and many types of cancers (Dorgan and Schatzkin, 1991; Riordan et al., 2005).

Zinc, an essential trace metal which is important in normal cell growth and development might aid in the prevention and treatment of cancer. The effect of zinc supplementation on the immune system strengthens the body’s defense against abnormal cell growth associated with cancer development (Somer, 1995; Jayant et al., 2013). In a few studies, human subjects who received supplemental zinc appeared to have reduced esophageal cell proliferation (Fong et al., 1998; Taccioli et al., 2009). Animal studies showed that zinc supplements given to DMH treated rats restored colonic architecture towards normal, with no apparent signs of neoplasia. The results suggested that zinc has a positive beneficial effect against chemically induced DMH colonic preneoplastic progression in rats (Dani et al., 2007). Positive results of zinc supplementation have been observed in HIV and cancer patients (Kristal et al., 1999; Patrick, 2000; Prasad et al., 2009). It was observed that replenishing zinc deficient cells with zinc, rapidly reversed DNA damage in these cells. Therefore it can be hypothesized that perhaps repair mechanisms were “turned back on” with the addition of zinc. However it is also possible that damaged cells were replaced with new undamaged cells, because zinc repletion also restored cell growth (Poories et al., 1976; Emily and Bruce, 2002).

Trace elements consist mostly of metal ions mainly acting as basic components of essential enzymatic system or protein which play major roles in the physiology of the gastrointestinal tract (Jackson, 1989; Federico et al., 2001; Schrag et al., 2005). The trace element zinc plays a major role in protein synthesis, gene transcription, enzyme activities, and cell adhesions have an important function in gene expression (Prasad, 1993, Christos et al., 2010; Jayant et al., 2013). The role of zinc in carcinoma development has been the subject of debate. Decrease in tissue zinc and zinc related enzymes- CuZnSOD and alkaline phosphatase activity have been linked to malignancy in the past. In our earlier study, we have shown that a significant zinc decrease (82%) in colonic tissue which may limit the function of zinc requiring enzymes such as CuZnSOD and alkaline phosphatase in the colonic tissue is associated with carcinogenesis (Pamela et al., 2012).

Chemoprevention or protection against colon cancer appears to be a very realistic possibility because various intermediate stages have been identified preceding the development of malignant colonic tumors. Animal cancer models have played an important role in advancing our understanding of the influence of nutrients and chemoprotective agents on carcinogenesis. Chemoprotection has the potential to be a major component of colon cancer control.

The beneficial role of Aspirin, Vitamin C or Zinc supplements as discussed above could be due to their ability to induce apoptosis or due to their antioxidant role in carcinogenesis. Hence this study has focused on agents that could be protective in a colon carcinoma model in rats and to assess whether these agents, in our DMH model would prevent alterations in mucosa histologically from normal to preneoplastic and further to neoplastic lesions. This study also focuses on whether the prevention of such histological changes in the colon is associated with the maintenance of the biochemical parameters, which is plasma zinc, colonic tissue zinc and the activity of zinc related enzymes, CuZnSOD and alkaline phosphatase. If supplements prove to reduce cancer risk, increasing intake of Aspirin, Vitamin C or zinc may be an attractive public health strategy.

Materials and Methods

Animals

Six weeks old adult Wistar rats (100-120g) obtained from the Institutional animal house were housed in polypropylene plastic cages, in an animal holding room under controlled conditions with 25±2°C, 50±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-diphenyletrazolium 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.
Aspirin 350 mg in the form of commercially available disipurin was used. (Reckitt and Colman company Ltd; India.). Vitamin C, 500mg (Roche company Ltd; India.). Zinc in the form of ZnSO4·7H2O (BDH diagnostics Ltd; India.) was used.

Experimental design
Six week old rats were randomly assigned to 3 groups: Group 1 (Effect of aspirin), Group 2 (Effect of Vitamin C) and Group 3 (Effect of Zinc supplement). Each group was further divided into 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=8) and experimental group (n=8), which received subcutaneous dose of 0.25 ml saline or 30 mg/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=8) and experimental group (n=8), which received a subcutaneous dose of 0.25 ml saline or 30 mg/kg body weight DMH dissolved in saline respectively twice a week for 4 months and were euthanized at 6 months.

Co-treatment with aspirin, vitamin C or zinc
All the animals of Group 1 (A) and (B) received daily doses of 0.5 ml acetylsalicylic acid (Aspirin) 60 mg/kg/ day by gavage, till the end of the study (Davis, 1994). All the animals of Group 2 (A) and (B) received daily doses of 0.5 ml Vitamin C 50 mg/5 g diet /day by gavage, till the end of the study (Reddy et al., 1982). All the animals of Group 3 (A) and (B) received daily doses of 0.5 ml zinc supplement 400 µg/day by gavage, till the end of the study (Fenwick et al., 1990). All rats were euthanized by chloroform inhalation. Blood was drawn by heart puncture from all the rats at the time of sacrificing the animal for estimation of plasma zinc.

Tissue preparation-histopathology, measurement of tissue zinc, CuZnSOD, Alkaline phosphatase, plasma zinc
All rats were examined grossly at necropsy. The entire portion from the stomach to anus was removed and the large intestine was isolated. The colon of the rats of the precancerous groups (A) was harvested, and slit length wise and checked for abnormalities. The stomach, small intestine, colon and liver of the rats of the cancerous groups (B) were harvested. Only the colon was slit length wise, 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 as per standard methods. 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-20ºC until analysis for tissue zinc estimation. (Kahnke, 1966; Pamela et al., 2012).

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 by (Kim et al., 2000). Activity of alkaline phosphatase (µmol/min/mg protein) was assayed as described (Dorai and Bachhawat, 1977; Satish et al., 2006).

Plasma zinc was estimated as described (Rosner and Garfien, 1968; Swaminathan et al., 1998; Pamela et al., 2012). Zinc determination was carried out using Perkin Elmer Atomic Absorption Spectrophotometer (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.

Ethanol-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
As observed in our earlier study of DMH treated precancerous and cancerous model in rat, the colonic cancerous incidence was 92%, there was an associated significant decrease in the plasma zinc (33.4%), colonic tissue zinc (72%), CuZnSOD (45%), alkaline phosphatase activity (51%) as compared to saline controls. The decrease in all the above mentioned parameters was greater in the DMH induced carcinoma group compared to the precancerous model. (Pamela et al., 2012). The results of the present study are compared to results of our earlier study in which rats were given the same DMH doses but without Aspirin, vitamin C or zinc supplement. Data shown to facilitate comparison.

DMH and co- treatment with aspirin/vitamin C/zinc in the precancerous group
In the DMH treated precancerous group, rats cotreated with Aspirin or Vitamin C showed normal histology in the colon in 7 out of 8 rats (87.5%) in both groups. Only one out of 8 rats in each group showed mild dysplasia histologically. Co-treatment with zinc maintained normal colonic morphology and histology in all 8 rats. There was a 100% reduction in the incidence of development of precancerous lesion with zinc supplementation. The control group of rats that received either saline+Aspirin, saline+vitamin C or saline+zinc showed normal colonic morphology.

There were no significant changes in the mean plasma zinc level of the DMH+Aspirin (112±8.5vs 122±4.0µg/dl), DMH+Vitamin C (112±8.5vs 120±4.0µg/dl), DMH+Zinc (112±6.7 vs. 122±3.9). Cotreatment with Aspirin or...
Vitamin C, showed relatively lesser significant decrease in colonic tissue zinc (15% and 9% respectively), whereas the DMH+Zinc group maintained colonic zinc levels at near normal. The zinc dependent enzymes CuZnSOD and Alkaline Phosphatase in all the 3 co-treated groups showed no significant changes as compared to controls (Figure 1).

**DMH and co- treatment with aspirin/vitamin C/zinc in the cancerous group**

In the cancerous group, DMH+co-treatment with Aspirin or Vitamin C maintained normal histology in the colon of 7 out of 8 rats (87.5%) in both groups (Figure 2 A, B). Only one out of the 8 rats in each group showed mild dysplasia histologically in the colonic tissue. Co-treatment with zinc in the DMH induced carcinoma group maintained normal colonic architecture with no signs of dysplasia in all 8 rats. (Figure 2 C). There was a 100% reduction in tumor incidence in colon when rats were co-treated with zinc. The colon of the control groups of rats that received saline+Aspirin, saline+vitamin C, saline+zinc were grossly normal.

The mean plasma zinc level of the DMH+Aspirin (112±8.6 vs. 124 ±9.1), DMH+ Vitamin C (112±8.5 vs 120±3.4μg/dl), DMH+ Zinc (114±6.7 vs. 123±3.9) showed no significant changes. Co-administration with Aspirin showed a relatively lesser decrease in colonic tissue zinc (mean 18%), which was statistically significant as compared to controls (240±28 vs. 291±16.4μg/g), and with vitamin C (mean 16%) as compared to controls (242±32 vs 288±5±11.8 μg/g; p<0.05). Co-treatment with zinc in the carcinoma model showed no significant changes in the tissue zinc. There was no significant

![Figure 1. Zinc Status in Large Intestine in DMH Treated Rats and Rats Co-Treated with Aspirin, Vitamin C and Zinc Supplement at 4 Months (pre -cancerous group). A) Tissue zinc levels-dry wt; B) CuZnSOD; C) Alkaline phosphatase. Values represent mean±SD from 8 rats, *p<0.05, **p<0.005 when compared to control](image1)

![Figure 2. Light Microscopy of Large Intestine of Rats Treated. A) DMH+Aspirin; B) DMH+Vitamin C; C) DMH+Zinc showing Normal Architecture (magnification 40X)](image2)

![Figure 3. Zinc Status in Large Intestine in DMH Treated Rats and Rats Co-Treated with Aspirin, Vitamin C and Zinc Supplement at 6 Months (cancerous group). A) Tissue zinc levels-dry wt; B) CuZnSOD; C) Alkaline phosphatase activity. Values represent mean±SD from 8 rats, *p<0.05, **p<0.005 when compared to control](image3)

![Figure 4. Tissue Zinc Levels in Various Organs In DMH And Various Treated Groups at 6 Months (μg/g wet wt zinc). Values represent mean±SD from 8 rats, *p<0.05, when compared to control](image4)
Chemoprotection of colorectal cancer has become essential in the modern industrialized world as cancer of the large bowel has become one of the major causes of cancer mortality. Colon cancer integrates lifestyle factors and multistep genetic alterations and without preventive intervention, a substantial part of the population is likely to develop colorectal cancer at some point during their life time. Chemoprotective approaches are worth considering for healthy persons who have a strong family history of cancer or those who are particularly susceptible for other reasons. The ideal chemoprotective agent chosen should be easy to take and have been proven to be effective and safe and nontoxic over the period of its long term use. It may be linked in part to altered metabolic activation of this carcinogen via cyclooxygenase-dependent co-oxidation (Craven and Rubertis, 1992; Sandler, 1996; Rosenberg et al., 1998; Kanwar et al., 1999; Miliaras et al., 2004). The results of our study are significant, because the co-administration of aspirin has not only reduced the incidence of dysplasia and carcinoma in DMH rat model, but this decrease had an associated effect on the maintenance of colonic zinc status. Suppression of 1, 2-DMH induced colonic carcinoma by concurrent administration of aspirin may be linked in part to altered metabolic activation of this carcinogen via cyclooxygenase-dependent co-oxidation (Craven and Rubertis, 1992; Sandler, 1996; Rosenberg et al., 1998; Kanwar et al., 2007; 2008).

The possible role of vitamin C in the prevention of malignancy has been studied over the last few decades. Studies have demonstrated that antioxidants like vitamin C can modulate several cellular signaling pathways, thus controlling gene expression and that these gene -nutrient interactions may have important therapeutic implications in cancer. Fruits and vegetables are a good source of vitamin C which may be responsible for their anticarcinogenic activity. Nevertheless, biochemical mechanisms accounting for this activity are not fully understood (Antonio et al., 2002; Goh et al., 2007).

In our study, in the precancerous and the cancerous DMH model, cotreatment with Vitamin C by gavage, at the initiation stage inhibited histological alterations in the colonic mucosa in majority of the rats, at 4 months and six months respectively. Inhibition of early precancerous lesions in the colon was associated with a lesser decrease in concentration of tissue zinc and no significant changes in plasma zinc and zinc requiring enzymes CuZnSOD and alkaline phosphatase in the large intestine in the precancerous and the cancerous model as compared with controls.

Experimental studies have reported the possible protective effect of the antioxidant vitamin C as an anticarcinogen. Our results are in agreement with the very few experimental studies in rats where Vitamin C showed its antioxidant effect in the colon with a decrease in carcinogenesis induced by using DMH (Carpenter, 1991; Williams, 2013). Vitamin C was found to reduce the risk of certain cancers, particularly those of the gastrointestinal tract in humans. (Dorgan and Schatzkin 1991; Kim et al., 2012). Anticarcinogenic activity of vitamin C is due to
its remarkable antioxidant effect (Singh and Rana, 2007).

Zinc supplements might aid in the prevention and treatment of cancer. The effect of zinc supplements on the immune system strengthens the body’s defense against abnormal cell growth associated with cancer development. (Somer, 1995; Prasad et al., 2009). Administration of zinc (in the form of zinc sulfate at a dose level of 227 mg/L in drinking water, ad libitum to DMH treated rats significantly decreased the tumor incidence, tumor size and aberrant crypt foci number with simultaneous enhancement of SOD, catalase and glutathione-S-transferase enzyme activities (Dani et al., 2007; Chadha et al., 2010).

In this study, we have shown that DMH induced colon carcinogenesis leading to zinc deficient state is essentially reduced if zinc is administered to rats at the initiation phase of treatment with DMH. Administration of zinc supplement to DMH treated rats significantly decreased the tumor incidence by 100% and maintained normal colonic histoarchitecture, with no apparent signs of neoplasia in the precancerous group as well as in the cancerous group along with maintaining the plasma zinc levels, colonic tissue zinc concentration, CuZnSOD and alkaline phosphatase enzyme activity. However, our results are in agreement with a study reported earlier, that zinc supplements in DMH treated rats greatly helped in maintaining the colonic architecture towards normal, with no apparent signs of neoplasia (Dani et al., 2007).

This study also showed that co treatment with aspirin, vitamin C or zinc in all the three experiments in this DMH carcinoma model also has a protective effect along the wall of the gastrointestinal tract and hence inhibited the decrease in tissue zinc and zinc related enzymes in the stomach and in small intestine, probably by inhibiting transmural changes along the wall of the gastrointestinal tract that would have occurred if rats were given only DMH as discussed in our earlier study (Pamela et al., 2012). These findings led to the hypothesis that early mucosal alterations which are preneoplastic lesions and which could lead on to further development of carcinogenesis are inhibited by co treatment with aspirin, vitamin C or zinc, which might thus lead to the prevention of decrease in plasma zinc, tissue zinc, and zinc containing enzymes in the large intestine and to inhibition of possible changes in the other parts of the gastrointestinal tract. In this study we also observed that there was no decrease in tissues zinc levels in the liver in DMH treated rats cotreated with aspirin, vitamin C or zinc. 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 (Zhukov et al., 1996; Jegatheeswaran et al., 2013). In our study the lesser decrease in tissue zinc can be attributed to the inhibition of histological alterations in the liver mucosa alongside with the maintenance of the colonic architecture.

These observations clearly demonstrate that these biochemical parameters may be useful to assess the therapeutic effect of any of these compounds in rats treated with DMH. The above findings support the view that inhibition of histological changes in the colon by the administration of aspirin, vitamin C or zinc may be sequentially related to the biochemical events in the large intestine in DMH treated rats.

Epidemiological, experimental, and clinical studies provide evidence that antioxidants and anti-inflammatory agents possess unique modes of action against cancer growth. Importantly, as observed in our previous DMH study, (Pamela et al., 2012) zinc deficiency is implicated in the progression of colonic carcinogenesis. It is evident from this study that co- treatment with aspirin, vitamin C or zinc supplement has a positive beneficial effect against chemically induced DMH colonic preneoplastic progression in rats, along with maintaining the colonic zinc status. Of all the three agents studied, zinc supplement resulted in a better protective effect with improvement of the biochemical parameters studied and a 100% reduction in tumor incidence.

All the three compounds tested in this study may have blocking or suppressing effects on DMH induced colon tumorigenesis when fed by gavage during the initiation phases. Although there are various studies published with regards to the protective role of synthetic or natural supplements on colon carcinoma model, this study also focuses on the inhibitory effect of the three compounds studied on the carcinoma of colon and their associated effect on the biochemical parameters as observed by us. If supplements prove to reduce cancer risk, increasing intake of micronutrients by use of vitamin supplements to food may be an attractive public health strategy. These findings suggest possible mechanisms for the protective effects of these compounds in humans. Further experiments, including pre-clinical efficacy and mechanistic studies, are warranted to fully evaluate these compounds for their cancer preventive properties and to understand their mode of action.

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