^{65}Zn kinetics as a biomarker of DMH induced colon carcinogenesis

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Abstract

Dietary factors are considered crucial for the prevention of initiating events in the multistep progression of colon carcinoma. There is substantial evidence that zinc may play a pivotal role in host defense against several malignancies, including colon cancer. The present study was conducted *to evaluate the kinetics of* ^{65}Zn utilization following experimental colon carcinogenesis in rat model. *Twenty rats were segregated* into two groups viz., untreated control and dimethylhydrazine (DMH) treated. Colon carcinogenesis was established through weekly subcutaneous injections of DMH (30mg/kg body weight) for 16 weeks. Whole body ^{65}Zn kinetics followed two compartment kinetics, with Tb_1 representing the initial fast component of the biological half-life and Tb_2, the slower component. *The present study revealed* a significant depression in the Tb_1 and Tb_2 components of ^{65}Zn in DMH treated rats. Further, DMH treatment caused a significant increase in the percent uptake values of ^{65}Zn in the colon, small intestine, kidney and blood, whereas a significant decrease was observed in the liver. Subcellular distribution revealed a significant increase in ^{65}Zn uptake in the mitochondrial and microsomal fractions following 16 weeks of DMH supplementation. *In conclusion*, the present study demonstrated a slow mobilization of ^{65}Zn during promotion of experimentally induced colon carcinogenesis and provides a physiological basis for the role of ^{65}Zn in colon tumorigenesis, which may have clinical implications in the management of colon cancer.

Introduction

Proper intake of dietary nutrients is considered crucial for the prevention of initiating events leading to the development of carcinoma. Many dietary compounds have been suggested to contribute in cancer prevention including zinc, which plays a pivotal role in host defense against the initiation and promotion of several malignancies [1-2]. Some evidences have suggested an intriguing link between zinc and cancer [3-5]. In in-vivo studies, it has been shown that zinc treatment increases resistance against tumor challenge in mice [3] and decreases the incidence of spontaneous lung tumors arising in mice [4]. Others have also found an increase in serum copper/zinc ratios in patients with cancers of the lung, breast, gastrointestinal tract and in gyneocological malignancy [5]. Studies from our lab have also advocated the inhibitory effects of zinc on the histological changes and antioxidant status in the colon of the rats during the initiation and promotion phase of experimentally induced colon carcinogenesis [6, 7]. However, further studies are warranted to assess the biokinetics on ^{65}Zn that may be vital to help understand the metabolic function of zinc in experimentally induced colon cancer.

The colon carcinogen 1,2-dimethylhydrazine (DMH) has been widely used to study chemically induced colon cancer [8]. Regardless of the mode of administration, DMH specifically induces tumors within the descending colon in animals, and the histopathology of these neoplasms is similar to that observed in human sporadic colon tumors [8, 9]. Dimethylhydrazine alkylates DNA, and the promutagenic lesions O^6-methylguanine (O^6-MeG) have been detected in DNA from various rat and mouse tissues following DMH exposure [10, 11]. Therefore, the current study was conceived to explore the biokinetics of ^{65}Zn, its distribution in various organs, as well as the sub-cellular distribution in colon of rats subjected to DMH treatment.

Materials and methods

Experimental design and animal treatment

Animal care procedures followed in the current study were approved by our University Ethical Committee on Experimental Animals for Biomedical Research.

Male rats of the Sprague-Dawley strain weighing 120-150g were procured from the central animal house of the Panjab University (Chandigarh, India). All animals were housed in polypropylene cages under hygienic conditions and

were provided with pellet diet and drinking water *ad libitum*. Animals (n=20) were segregated into two treatment groups. Animals in Group I served as normal controls and were given water and diet *ad libitum*. Rats in this group also received 1mM EDTA (Ethylenediaminetetraacetic acid)-saline subcutaneously per week, which was used as a vehicle for the treatment in DMH treated animals. Animals in Group II were given a weekly subcutaneous injection of DMH at a dose level of 30mg/kg body weight dissolved in 1mM EDTA-normal saline (pH-6.5), for a total duration of 16 weeks [12].

Color tumore analysis

After the terminal sacrifice following 16 weeks of DMH treatment, colons were excised from the rats, blotted dry, cut open longitudinally and the inner surface was examined for the visible macroscopic lesions.

Estimation of zinc

Serum zinc estimation: 0.2ml of serum was diluted to 10ml with 10mM nitric acid and was analyzed using Perkin Elmer Atomic Absorption Spectrophotometer-3100.

Colon zinc levels: The elemental analysis in colon samples were carried out by using Energy Dispersive X-Ray Fluorescence technique (EDXRF) at UGC-DAE CSR, Kolkata, India. The colonic tissues were oven dried at 70°C to a constant weight and then ground with the help of agate pestle and mortar. Dried tissue powders (100 mg) were weighed and self supporting pellets were made using a hydraulic press. The tissue pellets were then analyzed using Jordan Valley EX-3600 EDXRF spectrometer for the determination of zinc levels. The EX-3600 spectrometer was pre-calibrated with standards that permitted evaluation of elemental concentrations present in unknown samples within an accuracy of $\pm$ 5%.

Whole body biological half life of ^{65}Zn

Animals in each group were injected intra-peritoneally (i.p) with ^{65}Zn radionuclide (specific activity-576Mbq/gr, 1.85MBq initial activity), one week after the last DMH injection. A standard, having the same activity was used as reference for accounting the physical decay of radionuclide.

The NaI(Tl) scintillation counter was calibrated for optimum peak settings of ^{65}Zn. The rats were placed at a distance of 16cm, (from the top surface of the probe of the counter) for the purpose of whole body counting. The count rate variation along the horizontal plane was found to be minimal, even if the animals would accidentally move sideways. Each rat was thereafter subjected to whole body counting immediately after each injection, and the counting was continued initially daily for three days, and then it was done for every alternate day until the count rate become significantly diminished to be able to count with reliability. The standard was also recorded each time to account for physical decay of the radioisotope. For individual counting, each rat was housed in a perspex cage (25mm thick), with the following dimensions; length-18cm, width-8cm and height-6cm. The cage had several holes to allow uninterrupted air ventilation, but it restricted the free movement of the animal during counting.

The uptake of ^{65}Zn on day 1 was taken as 100%, and the percentage retention on subsequent days was calculated. The profile of specific activity versus time for each rat was plotted on the semi-log scale. The biological half-life of ^{65}Zn was determined by considering the difference between any two points where percentage count became half on the x-axis scale.

Biodistribution of ^{65}Zn and its subcellualr distribution in colon

All the rats were injected with a tracer dose of ^{65}Zn (0.37MBq) after one week of the last DMH injection. The animals were sacrificed 24h after injection by exsanguation under light ether anesthesia. Before sacrificing, blood samples were drawn at different time intervals by puncturing the ocular vein, (retro-orbital plexus) of the animals. Various organs viz., colon, small intestine, liver, kidney, rectum and spleen were removed and were placed in a test tube containing 30% potassium hydroxide for tissue digestion. On the following day, the activity of the digested tissue fractions was determined using NaI(T1) scintillation counter. The percentage uptake per unit mass of the organ was calculated with respect to a standard having the same activity.

The colonic tissues from animals in different treatment groups were homogenized in tris mannitol buffer (2mMtris + 50mMmannitol, pH 7.2), using a mechanically driven teflon fitted Potter-Elvejhem homogenizer. The crude homogenates were subjected to different centrifugation speeds and times for the separation of mitochondrial (3000xg for 15min), microsomal (100,000xg for 30min) and post-microsomal (100,000xg for 45min) fractions. The radioactivity in each organ was recorded and the percentage uptake in each fraction was calculated with respect to a standard.

Statistical analysis

The statistical significance of the data was determined using one-way analysis of variance (ANOVA) followed by Newman Keuls test. Data in various tables are presented as Mean$\pm$SD and a P value of <0.05 was considered statistically significant.

Results

The results obtained from various experiments conducted in this study are depicted in Tables 1-3.

Table 1. *Body weight and zinc levels in serum and colon of animals subjected to DMH treatment*

Groups	Weight (gm)	Serum zinc (µg/ml serum)	Tissue zinc (µg/gm tissue)
Normal Control	195 $\pm$ 23	0.114 $\pm$ 0.021	97.50 $\pm$ 8.60
DMH treated	147 $\pm$ 22^b	0.076 $\pm$ 0.012^a	82.16 $\pm$ 4.91^b

All data are expressed as Mean$\pm$S.D. ^{a}P <0.05, ^{b}P <0.01 by Newman – Keuls test when values are compared with control group.

Body weight

DMH treatment resulted in a significant decrease in the body weights (P<0.01), when compared to the normal control rats. Additionally, the daily food and water intakes were measured, and it was determined that on an average 20-30ml of water was consumed by each

animal/day. However, no significant changes in food consumption were observed among both groups of animals.

Table 2. *Biological half-lives of ^{65}Zn in whole body of DMH treated animals*

Groups	Whole Body biological half life of ^{65}Zn (days)	
	Tb_1	Tb_2
Normal Control	8.28 ± 1.07	13.46 ± 0.67
DMH treated	6.53 ± 1.51[a]	8.40 ± 1.49[b]

All data are expressed as Mean+S.D. [a]$P \leq 0.01$ and [b]$P \leq 0.001$ by Newman – Keuls test when values are compared with control group.

Table 3. *Biodistribution of ^{65}Zn in various organs of DMH treated animals (the data are expressed as percentage specific activity)*

Groups	Colon	Small Intestine	Rectum
Normal Control	1.29 ± 0.43	1.58 ± 0.37	1.25 ± 0.45
DMH treated	2.28 ± 0.80[a]	2.38 ± 0.77[a]	0.96 ± 0.09

	Liver	Kidney	Blood
Normal Control	2.09 ± 0.40	1.02 ± 0.14	0.163 ± 0.03
DMH treated	1.60 ± 0.24[a]	1.86 ± 0.43[b]	0.251 ± 0.03[a]

All data are expressed as Mean+S.D. [a]$P \leq 0.01$ and [b]$P \leq 0.001$ by Newman – Keuls test when values are compared with control group.

Macroscopic view of tumors

Figures 1-2, depict the tumor section in the colon from the rat treated with dimethylhydrazine for a treatment of 16 weeks. As can be visualized, polypoidal tumor growth is evident (Fig. 1) and adjoining mucosa also shows sign of severe inflammation (Fig. 2). Areas of reddening on the surface of the tumor represent superficial ulceration in the tumor.

Figure 1. Polypoidal tumor growth in the longitudinally opened colon from the rat treated with dimethylhydrazine for16 weeks. Adjoining mucosa normal.

Zinc levels

We first investigated the bio-availability and steady-state levels of zinc in colonic tissues following DMH treatment. A significant decrease in tissue and serum concentrations of zinc in the colon was observed following DMH treatment, when compared to the animals in the control group (Table 2).

Figure 2. Tumor bearing colon from the rat treated with dimethylhydrazine for16 weeks. Inflamed adjoining mucosa.

Two-compartment bio-kinetics for ^{65}Zn

The results from our present study revealed two compartment kinetics of ^{65}Zn as shown in Table 3. Among the two compartments, Tb_1 represented the initial-fast component of the biological half-life of the radionuclide, while Tb_2 reflected the delayed-slower component. The present study revealed a significant decrease in both the Tb_1 and Tb_2 components in DMH treated rats when compared to normal control rats.

Biodistribution and subcellular distribution of ^{65}Zn

In order to ascertain the physiological and protective role of zinc in colon, it is meaningful to decipher the local concentrations of zinc in various tissue organs in animals following various treatments. To address this issue, we next determined the ^{65}Zn concentrations in various organs of the animals. Treatment with DMH for 16 weeks caused a significant increase in the percent uptake values of ^{65}Zn in the colon, small intestine, kidney and blood, whereas a significant decrease was observed in the liver. A significant increase in ^{65}Zn uptake in the mitochondrial and microsomal fractions following 16 weeks of DMH supplementation was also observed when compared to the control group.

Discussion

Zinc kinetics in carcinomas is an unexplored field of inquiry in cancer research. The present study evaluated the kinetics of zinc utilization following colon carcinoma in an animal model of colon carcinogenesis.

The net body weight gain of the animals intoxicated with DMH was markedly lesser when compared to the normal controls. However, in this study we did not observe any significant changes in the diet consumption of animals following DMH intoxication, suggesting increased peroxidation of lipids as a consequence of oxidative stress with DMH [13].

Since dietary factors along with other lifestyle changes associates with high risk of human colon cancer [14, 15], any chemopreventative approach using micronutrients in such a scenario mandates investigation of steady-state levels of a particular element in the diseased condition. Relevant in this context, we in this study observed a significant depression of colon and serum zinc levels following DMH treatment, which is in line with some of the previous studies [16]. Similar observations were recently reported for lower zinc levels in prostate cancers, as well [17]. Additionally, lowered zinc levels in the leukocytes in a variety of neoplastic diseases have been proposed to be a potential prognostic marker for cancer detection [18]. Although the precise role of zinc in human carcinogenesis is elusive, our current observations together with the published literature for the diminished zinc levels in colon cancer suggest that zinc directly or indirectly is associated with the control/metabolism of cancerous cells.

Our observations for the biological half-life of ^{65}Zn in the whole body of animals subjected to various treatments revealed dual-compartmental kinetics for ^{65}Zn. The Tb_1 (faster component) component indicates the rapid elimination of radionuclide, presumably through urine and feces, whereas, Tb_2 (slower component) represents the incorporation of zinc in the tissues, potentially by binding to metallothionein or other enzymes [19]. Majority of the elemental zinc in the body is homeostatically distributed either in the liver or in the plasma [20], and the dynamic balance between the two pools can be affected by a number of pathophysiological processes [21]. In the present study, a significant decrease in the fast and slow component of ^{65}Zn following DMH treatment indicates the decreased capacity of the whole body to retain ^{65}Zn in the biological system. These findings may correspond to the faster excretion of zinc during carcinogenesis as a result of displacement of zinc from zinc binding ligand or increased mobilization of metal binding ligands in the systemic circulation in order to combat the oxidative stress created by metabolites of DMH. This explanation is consistent with our Energy dispersive X-ray fluorescence (EDXRF) findings where we observed decreased zinc levels in colon tissues following DMH treatment.

Liver had the maximum percentage of ^{65}Zn uptake amongst the various organs under study, followed by colon, small intestine and kidney. Others have also reported the highest concentration of ^{65}Zn in the liver [22]. A significant increase was noted in the percent uptake values of ^{65}Zn in colon, small intestine, kidney and blood following DMH treatment, when compared to the respective normal control groups, which could be due to decrease in the concentration of zinc under cancerous conditions, as evidenced by Tb_2 component of whole body biological half life and EDXRF studies. On the contrary, a significant decrease was observed in ^{65}Zn uptake in rectum and liver following DMH treatment. It is likely that DMH treatment resulted in decreased levels of metallothionein, due to its excessive utilization to combat oxidative damage [19]. Since, metallothionein has strong binding affinity for zinc, it is possible that lower levels of metallothionein may reflect decreased uptake of ^{65}Zn in these organs. In order to support this contention further, our observations for the distribution of ^{65}Zn in the various sub-cellular fractions of the rat colon are reflective of the zinc physiology within colonic cells under the different treatment conditions. Our observations for increased nuclear ^{65}Zn fraction following DMH supplementation,

reflects the increased capacity of zinc to interact and bind with various nuclear proteins.

In conclusion, the present study for the first time demonstrated a faster mobilization of ^{65}Zn following experimentally induced colon carcinogenesis and thus provides a physiological basis for the role of zinc during colon tumorigenesis.

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