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Study to elucidate molecular mechanism behind zinc chemo-preventive role during lung carcinogenesis

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Abstract. – **OBJECTIVE: The present planned to elucidate the mechanistic role zinc supplementation in the modulation of p53 post-translational acetylation, the activity of cyclooxygenase-2 (COX-2) along with other biophysical parameters during benzo(a)pyrene (BP) induced lung carcinogenesis in mice.**

MATERIALS AND METHODS: The mice were segregated into four groups viz., normal control, BP treated, BP + zinc and zinc alone treated. Lung carcinogenesis was induced by a single intra-peritoneal (IP) injection of BP (100 mg/kg body weight). Zinc was supplemented to mice at dose levels of 227 mg/kg body weight in drinking water. All the treatments were continued for 20 weeks.

RESULTS: The BP caused a significant rise in the expression of p53. On the other hand, protein expressions of acetylated (lys382)-p53 were significantly decreased, following BP treatment. Also, a significant decrease was observed in the enzyme activities of caspase 3 and caspase 9. Moreover, BP treatment brought about a significant increase in the activity of COX-2. Supplementation of zinc to BP treated mice stimulated acetylation of p53 as observed by an increase in the protein expression of acetylated (lys382)-p53. Also, the enzyme activities of caspase 3 and caspase 9 showed a significant elevation upon zinc supplementation. On the other hand, the zinc supplementation brought about moderation in the expression of enzymatic activity of COX-2 which was restored within the normal limits. Further, BP treatment recorded increased ^{3}H-thymidine uptake as well as enhanced 14C-glucose uptake and its turnover which were reduced significantly following simultaneous treatment with zinc.

CONCLUSIONS: The treatment with zinc has the potential to modulate p53 acetylation to stimulate apoptosis against experimentally induced lung carcinogenesis.

Key Words:
Acetylaytion, p53, COX-2, Zinc, Lung cancer, Benzo(a)pyrene.

Introduction

Lung cancer elicited deaths are the most prominent deaths due to cancer worldwide[1]. The latest research is being focused on dietary micronutrients as they are viewed as promising agents in cancer prevention[2-4]. Though, many dietary compounds have been suggested to contribute to the prevention of cancer, nowadays, some recent fragmentary evidence has supported the modulatory potential of zinc in carcinogenesis[5]. Dietary deficiencies in zinc have been also noticed to promote cancer development. Zowezak et al[6] observed an increase in serum copper/zinc ratios in patients with cancer of the lung, breast, gastrointestinal tract and gynecological malignancy. Jaiswal and Narayan[7] has also stated that zinc can arrest the growth of lung cancer cells.

Tumor suppressor proteins including p53, are the favourite choice of target amongst various chemopreventive agents. These tumor suppressors are in turn controlled by various regulatory molecular modifications called as post-translational modifications[8,10]. The tumor suppressor protein p53 is the molecule of interest as it contains distinct sites where post-translational modifications have been reported[9,11]. The study of these modifications at specific sites can provide new mechanistic information chemopreventive potential of zinc. Therefore, the first aim of the present work is to investigate acetylation at the lysine-382 residue of tumor suppressor p53 which is an important post-translation modification responsible apoptotic potential as well as the functional stability of p53. Further, the study of resultant molecular apoptosis response is essential to confirm efficient stimulation of p53 that was confirmed by eval-

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uation of activities of caspases in the present study. Further, the literature supports that zinc replenishment has been shown to induce apoptosis in esophageal epithelial cells, thereby providing growth inhibition for the development of esophageal cancer[10,11]. So, the literature has confirmed the efficacy of zinc as a chemo-preventive agent during cancer. However, there is a paucity of molecular mechanistic information responsible for the protective potential of zinc against carcinogenesis. So, the present report is the first to study the molecular mechanism involved behind zinc chemoprevention against lung carcinogenesis. The study is focused towards the regulation of key post-translational modification of p53 viz. acetylation and its resultant molecular effects along with related biophysical indices in an experimentally induced lung carcinogenesis model.

Materials and Methods

Animals

Male Laka mice in the weight range of 20-22 g were procured from the Central Animal House, Soochow University, China. The animals were housed in polypropylene cages under hygienic conditions in the departmental animal house. Before initiating the experiments, the animals were adapted to the laboratory conditions for a week. Necessary approvals were obtained from the Department of Ethical Committee.

Experimental Design

Mice were segregated into four treatment groups of 10 animals each. Animals in Group I acted as controls and were administered intraperitoneally corn oil (I.P), which was used as a vehicle for the benzo(a)pyrene. Group II were treated with benzo(a)pyrene in the form of a single intraperitoneal injection in corn oil at a dose level of 100 mg/kg body weight[1]. Mice in Group III were supplemented with zinc in the form of $ZnSO_4.7H_2O$ in drinking water *ad libitum* at a dose level of 227 mg/L of drinking water[12]. Animals in Group IV were given both DMH as well as zinc in a similar manner as was given to Group II and Group IV respectively. A careful record of body weight changes of control, BP and zinc treated mice was kept throughout the study. The animals were weighed at the beginning of the experiment and twice a week, and finally before sacrificing them.

Preparation of Lung Homogenate

After sacrificing the animals, the lungs were removed immediately and washed with ice-chilled saline. 10% lung homogenates were prepared in ice cold tris buffer (pH 7.4) using mechanically driven Teflon fitted Potter-Elvejhem type homogenizer for a few minutes till the total disruption of cells. Homogenates were centrifuged at 1,000 g for 10 min at 4°C. Pellets were discarded and supernatants were preserved. A portion of the above supernatant was again centrifuged at 10.000 g for 20 min to obtain post-mitochondrial fraction.

Preparation of Nuclear Extracts

20% lung homogenates were prepared in ice cold buffer A (10 mM HEPES, 50 mM NaCl, 500 mM sucrose, 1 mM EDTA, 0.2% triton X) (pH 8) using Teflon fitted Potter-Elvejhem type homogenizer for few minutes till total disruption of cells. Homogenates were centrifuged at 5000 rpm for 2 minutes at 4°C. Pellets were resuspended in 500 μL of buffer B (50 mM NaCl, 10 mM HEPES, 0.1 mM EDTA, 25% glycerol) (pH 8) followed by centrifugation at 5000 rpm for 3 minutes at 4°C. Pellets were again resuspended in 50 μL of buffer C (350 mM NaCl, 0.1 mM EDTA, 10 mM HEPES, 25% glycerol)(pH 8), containing 10 μL of protease inhibitor. The above suspensions were kept in ice for 30 minutes with constant shaking. Further, after centrifugation again at 1000 rpm at 4 degrees, pellets were discarded and the supernatants were for expression researches.

Western Transfer Analyses

To study the modulation of p53 and its post-translational modification by zinc, the protein expressions were checked in the nuclear extracts for anti p53 (1:500) and anti acetylated-p53 (1:500), after 20 weeks all treatments. Protein concentrations in these fractions were determined by Lowry et al (1951) and then subjected to electrophoretic separation on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electro-transfer to polyvinylidene fluoride (PDVF) membranes. After transfer, the membranes were washed with phosphate buffered saline (PBS) for 10 min and then placed in blocking buffer (0.1% bovine serum albumin: BSA and 0.01% tween 20 in PBS) for 1 hr with constant shaking. Then, the membranes were incubated with each of the primary polyclonal antibodies separately. The mem-

branes were sequentially washed with PBS, 0.05% tween-20 in PBS and PBS each for 5 minutes. Then, were incubated with the peroxidase-labeled secondary antibody (1:500) for 3 hrs at 4 degrees. Thereafter, the membranes were washed again as described above. The blot was developed in dark, by adding diaminobenzidine (DAB: 10 mg/15 ml PBS containing 15 μL of H_2O_2). The reaction was concluded by rinsing the membranes with double distilled water, and the membranes were air dried. The densitometric analyses of the bands were done using Image J software.

Caspase 3 assay

The enzyme activity of Caspase-3 was assayed by the 'Biovision Colorimetric Assay Kit (Ann Arbor, MI, USA).

Caspase 9 Assay

The enzyme activity of Caspase-9 was assayed by the 'Biovision Colorimetric Assay Kit.

Estimations of zinc concentrations in the lung tissues

The zinc concentrations were estimated in lung tissues of control and different treatment groups, using Perkin Elmer Atomic Absorption Spectrophotometer (AAS)-3100.

Inflammation marker enzyme

COX-2 enzyme activities were estimated by using Cayman's colorimetric assay kit.

In-vitro uptake of ^{14}C-D-Glucose and radio spirometry studies

The *in vitro* uptake studies of lungs and radio spirometry of ^{14}C-D-glucose were recorded in lungs of normal as well as treated mice following the method of Crane and Mandelstam[13].

Uptake of ^{3}H-thymidine to assess Cell Proliferation by the Method of Crane and Mandelstam

The ^{3}H-thymidine uptake was also recorded following Crane and Mandelstam[13].

Statistical Analysis

The statistical significance of the data was analyzed using one-way analysis of variance (ANOVA) followed a multiple post-hoc least significant difference test. The results are means ± SD. $p < 0.05$ was considered statistically significant.

Results

The results obtained are depicted in Tables I-V. The data from various treatment groups have been compared with the normal control animals. However, results obtained from the Zinc+BP treated group were additionally compared with that of the BP group as well.

Zinc Levels

We observed a statistically significant decrease in the elemental concentration of zinc (Table I) in the benzo(a)pyrene treated mice. However, supplementation with zinc resulted in an appreciable improvement in the trace elemental levels of Zn.

Body weight Changes and Lung weights Changes

The benzo(a)pyrene treatment resulted (Table II) in a significant decrease in the body weights when compared to the normal control rats. Supplementation with zinc improved the body weights in comparison to benzo(a)pyrene treated animals. Moreover, lung weights of benzo(a)pyrene treated animals showed a significant increase (Table II) when compared with normal controls and showed an appreciable moderation following treatments with zinc.

Lung tumor occurrence

The benzo(a)pyrene treatment resulted in tumor occurrence to the tune of 91% (Table III). Supplementation of BP treated mice with zinc individually showed a decrease in the tumor occurrence which was observed to be 46%.

Table I. Effect of zinc treatment on the levels of trace elements in lungs mice subjected to benzo(a)pyrene treatment.

Groups	Zn μg/g tissue
Normal control	18.15 ± 0.94
Benzo(a)pyrene	12.33 ± 0.39[b]
Benzo(a)pyrene + Zinc	17.97 ± 078[a]
zinc	18.54 ± 1.09[x]

Data are expressed in Mean + SD (n=10).
[a]$p \leq 0.05$, [b]$p \leq 0.01$ and [c]$p \leq 0.001$ by Least Significance Difference test when values are compared with normal control group.
[x]$p \leq 0.05$, [y]$p \leq 0.01$, [z]$p \leq 0.001$ by Least Significance Difference test when values of Groups III, and IV are compared with Group II.

Table II. Effects of zinc on body weights and lung weights of mice subjected to single dose of benzo(a)pyrene treatment.

Groups	Body Weights (g)	Lung Weights (mg)
Normal control	36.33 ± 2.42	279.00 ± 19.68
Benzo(a)pyrene	24.00 ± 3.29[c]	335.25 ± 15.00[c]
Benzo(a)pyrene + Zinc	29.40 ± 1.67[c,y]	305.00 ± 7.70[a,y]
zinc	31.00 ± 1.87[a,y]	304.25 ± 6.50[a,y]

Data are expressed in Mean + SD (n=10)
[a]$p \leq 0.05$, [b]$p \leq 0.01$ and [c]$p \leq 0.001$ by Least Significance Difference test when values are compared with normal control group
[x]$p \leq 0.05$, [y]$p \leq 0.01$, [z]$p \leq 0.001$ by Least Significance Difference test when values of Groups III, and IV are compared with Group II.

Western Transfer Analyses

After the benzo(a)pyrene treatment, we have observed a significant increase in the protein expression of p53 (Figure 1). On the other hand, a significant decrease in the acetylation of p53 was observed in the lungs of benzo(a)pyrene treated group. However, supplementation of zinc to benzo(a)pyrene treated mice resulted in significant increase in the protein expression of acetylated p53 as seen in Western transfer. Also, no significant changes were found in the protein expression of p53 in the zinc supplemented benzo(a)pyrene group.

Apoptotic Marker Enzymes

A significant decline in the enzyme activities of both caspase 3 and caspase 9 was observed (Table IV) in the lungs of mice treated with benzo(a)pyrene. Supplementation with zinc to benzo(a)pyrene brought significant improvement in the enzyme activities of both caspase 3 and caspase 9.

Table III. Effects of zinc treatment on the tumor occurrence in mice subjected to benzo(a)pyrene treatment with single dose.

Groups	No of mice examined	Tumor Incidence (%age)
Normal control	10	0
Benzo(a)pyrene	10	90
Benzo(a)pyrene + Zinc	10	45
zinc	10	0

Inflammation marker enzyme COX-2

A statistical significant increase in the enzyme activity of cyclooxygenase-2 (COX-2) (Table V) resulted in the lungs of benzo(a)pyrene treated mice. Supplementation with zinc brought down the enzyme activity of COX- 2 to within normal levels.

Radiorespirometric Study of ^{14}C Glucose Turnover and in vitro Uptake of 14C Glucose

Benzo(a)pyrene treatment resulted in a significant increase (Table VI) in both the ^{14}C glucose turnover as well as ^{14}C glucose uptake. The treatment with zinc resulted in a significant decrease in both uptake and turnover values of ^{14}C-glucose as compared to benzo(a)pyrene treated group.

Uptake of ^{3}H-thymidine to Assess Cell Proliferation

We have observeda statistically significant increase (Table VII) in the uptake of ^{3}H-thymidine in the lungs of benzo(a)pyrene treated mice. Supplementation with zinc resulted in a decrease of the ^{3}H-thymidine uptake.

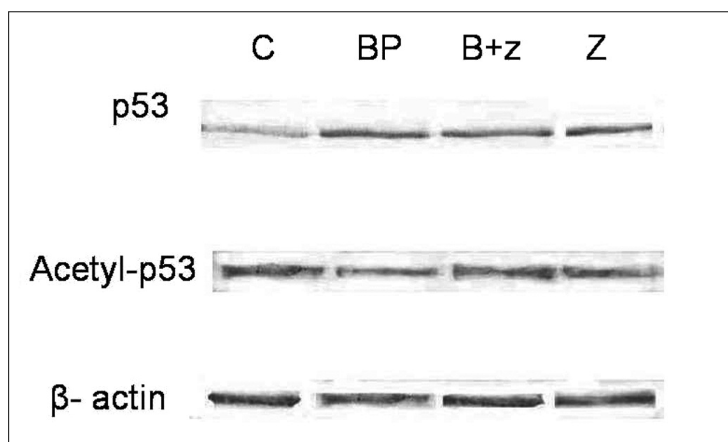

Figure 1.

Table IV. Effects of zinc treatment on the enzyme activities of caspase 3 and caspase 9 in lungs during benzo[a]pyrene induced lung carcinogenesis.

Groups	Caspase 3 [n moles of pNA formed/min/mg protein]	Caspase 9 [n moles of pNA formed/min/mg protein]
Normal control	5.31 ± 0.23	2.06 ± 0.10
Benzo[a]pyrene	3.16 ± 0.29[b]	1.32 ± 0.10[c]
Benzo[a]pyrene + zinc	5.38 ± 0.10[y]	1.98 ± 0.09[b,y]
Zinc	5.06 ± 0.63[x]	1.83 ± 0.15[c,z]

n = 10 Data are expressed in Mean + SD
[b]*p* <0.01 and [c]*p* <0.001 by Least Significance Difference test when values are compared with normal control group
[x]*p* <0.05, [y]*p* <0.01, [z]*p* <0.001 by Least Significance Difference test when values of Groups III, IV and V are compared with Group II.

Discussion

Our findings clearly indicated that the administration of zinc to the BP treated animals appreciably modulates post-translational modification viz. acetylation in p53 at lysine-382 residue, thereby regulated apoptosis via regulation of enzyme activities of apoptotic marker enzymes caspases and by the moderation of both inflammation as well as glucose metabolism.

The p53 tumor suppressor p53 is highly mutated (≥ 18,000 mutations) in many different cancers and is probably rendered inactive by a range of indirect mechanisms in most of the cancers[14]. Further, p53 is also subject to a varied assortment of post-translational modifications which in turn markedly affect the p53 target genes. The post-translational modification of interest in the present study is acetylation of p53. Acetylation has been reported to occur in the c terminal region of p53 specifically at lysine 382[15] and has been shown to enhance the DNA-binding ability of the p53 protein which subsequently helps in its tumor suppressor activity[16,17]. In addition, acetylation also contributes towards regulation of p53 stability, transcriptional activity and cellular localization[18-20]. In the present work, benzo(a)pyrene resulted in a significant decrease in the protein expression of acetylated p53 indicative of a decrease in acetylation at lysine 382. This decrease might also be responsible for the deregulation of tumor suppressor activity of p53, as the literature clearly mentions that the acetylation is a positive post-translational modification[18-20]. There are reports that showed a significant increase in the expressions of histone deacetylases (HDACs) proteins which cause deacetylation of p53 during cancer[20,21]. So, an increase in HDACs could be the reason for the decrease in the acetylation of p53. This might have resulted in a decline in apoptosis as indicated by a significant decrease in the enzyme activities of apoptosis marker enzymes caspase 3 as well as caspase 9 in lungs of benzo(a)pyrene treated mice. Supplementation with zinc resulted in a significant increase in the acetylation of p53.

The physiologic importance of zinc has been well reported as it is an integral component of a variety of enzymes, transcription factors and other functional proteins including COX-2, where it exerts specific actions over a wide variety of physiological processes[22-26]. Inflammation plays a critical role in carcinogenesis and the regulatory role played by COX-2 is well reported previously[27,28]. Also, overexpression of COX-2 leads to malignant cell proliferation and invasion of this peculiar characteristic can be exploited as a hallmark of cancer chemoprevention by COX-2 inhibitors[29]. So, the present work was planned to evaluate inflammation regulatory role of zinc during lung carcinogenesis.

Table V. Effect of zinc treatment on the activity of cyclooxygenase 2 (COX-2) in lungs of mice subjected to benzo(a)pyrene treatment.

Groups	(n moles /min/mg protein)
Normal control	23.35 ± 4.08
Benzo(a)pyrene	34.45 ± 3.25[a]
Benzo(a)pyrene + Zinc	24.44 ± 6.42[x]
zinc	23.09 ± 3.10[x]

Data are expressed in Mean + SD (n=10)
[a]*p* ≤0.05, [b]*p* ≤0.01 and [c]*p* ≤ 0.001 by Least Significance Difference test when values are compared with normal control group
[p]*p* ≤0.001 by Least Significance Difference test when values of Groups III, and IV are compared with Group II.

Table VI. Effects of zinc treatments on the uptake of 14C-glucose in lungs during benzo(a)pyrene induced lung carcinogenesis.

Groups	$^{14}CO_2$ (% age of $^{14}CO^2$ trapped as Na214CO3/min/g tissue)	^{14}C-glucose (μ moles of glucose incorporated/min/g tissue)
Normal control	1.19 ± 0.03	0.20 ± 0.02
Benzo(a)pyrene	3.01 ± 0.29^{b}	0.45 ± 0.05^{c}
Benzo(a)pyrene + Zinc	1.29 ± 0.57	0.23 ± 0.05^{b}
zinc	1.03 ± 0.10^{x}	$0.20 \pm 0.9^{a,y}$

Data are expressed in Mean + S.D (n=10)
$^{a}p \leq 0.05$, $^{b}p \leq 0.01$ and $^{c}p \leq 0.001$ by Least Significance Difference test when values are compared with normal control group
$^{x}p \leq 0.05$, $^{y}p \leq 0.01$, $^{z}p \leq 0.001$ by Least Significance Difference test when values of Groups III, & IV are compared with Group II.

We have observed a significant increase in the enzyme activity of COX-2 proving its influential aspect of the carcinogenesis process in the lungs of BP treated mice. COX-2 is the marker enzyme which is responsible for the inflammation during cancer[30]. On the other hand, a significant decrease was observed in the zinc concentrations in the same BP treated mice. These two observations collectively inferred that the elevated activity of COX 2 is owed to the decrease in the zinc levels as zinc deficiency has been reported to be associated with COX 2 stimulation during a cancerous state in earlier investigations[23]. The possible mechanism could be the creation of the precancerous state by zinc deficiency via unrestrained cell proliferation and modified gene expressions[31]. Moreover, there are reports which confirmed production of prostaglandins E2 upon stimulation of COX-2. These prostaglandins, in turn, promote cell proliferation that ultimately caused tumorigenesis, as observed by the increase in the tumor occurrence in the BP treated mice. Increased COX-2 activity meant stimulation of inflammation and was observed as a significant increase in the lung weights of BP treated mice. Furthermore, the net body weight gain of the animals subjected to benzo(a)pyrene was significantly less as compared to normal controls despite no change was found in the diet consumption of animals following BP treatment. So, the decrease in the body weights could due to the increased lipid peroxidation due to the oxidative stress.

Table VII. Effects of zinc treatment on the *in vitro* 3H-thymidine uptake in lungs during benzo(a)pyrene induced lung carcinogenesis

Groups	(% age specific activity)
Normal control	3.03 ± 0.31
Benzo(a)pyrene	4.54 ± 0.23^{b}
Benzo(a)pyrene + Zinc	3.13 ± 0.18^{a}
zinc	3.02 ± 0.10^{a}

Data are expressed in Mean + S.D (n=10)
$^{a}p \leq 0.05$, $^{b}p \leq 0.01$ and $^{c}p \leq 0.001$ by Least Significance Difference test when values are compared with normal control group
$^{x}p \leq 0.05$, $^{y}p \leq 0.01$, $^{z}p \leq 0.001$ by Least Significance Difference test when values of Groups III, & IV are compared with Group II.

Zinc supplementation to BP treated mice brought already decreased levels of zinc to near normal range. Also, maintenance of adequate zinc levels by zinc supplementation contributed towards regulation of COX-2 activity as zinc has been reported to be a strong inhibitor of COX-2[32]. So, the regulation of COX-2 by adequate zinc levels must have contributed towards a decrease in tumor occurrence in BP-treated mice. Also, the lung weights got significantly reduced due to controlled inflammation by zinc via COX 2 regulation in zinc supplemented BP treated mice. Moreover, whole body weights of mice also showed improvement after zinc supplementation which could be owed to anti-oxidative nature of zinc proved previously.

Radiorespirometric and uptake studies of ^{14}C-glucose showed a significant increase in the turnover and uptake of ^{14}C-glucose, respectively, in lung slices of benzo(a)pyrene treated mice. This increase might be the result of elevated demand of glucose by rapidly proliferating cancer cells. The rapid cell proliferation was confirmed biophysically by a significant increase in the uptake of ^{3}H thymidine in the lung slices of benzo(a)pyrene treated mice. On the other hand, supplementation with zinc caused an appreciable decrease in the both uptake and turnover of glucose in the lungs of benzo(a)pyrene treated mice proving again its anti-inflammatory as well as anti-cancerous nature. The results agree with earlier reports[33,34].

Conclusions

The present work added another facet to the accessible knowledge of trace element zinc in cancer chemoprevention research. Trace element zinc regulates acetylation of p53 at lysine 382 residue to control apoptosis, modulates COX-2 activity for regulating inflammation as well as glucose metabolism in an experimentally induced lung carcinogenesis mice model.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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