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Zinc enhances CDKN2A, pRb1 expression and regulates functional apoptosis via upregulation of p53 and p21 expression in human breast cancer MCF-7 cell

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ABSTRACT

Zinc (Zn) is an essential trace elements, its deficiency is associated with increased incidence of human breast cancer. We aimed to study the effect of Zn on human breast cancer MCF-7 cells cultured in Zn depleted and Zn adequate medium. We found increased cancer cell growth in zinc depleted condition, further Zn supplementation inhibits the viability of breast cancer MCF-7 cell cultured in Zn deficient condition and the IC_{25}, IC_{50} value for Zn is 6.2 μM, 15 μM, respectively after 48 h. Zn markedly induced apoptosis through the characteristic apoptotic morphological changes and DNA fragmentation after 48 h. In addition, Zn deficient cells significantly triggered intracellular ROS level and develop oxidative stress induced DNA damage; it was confirmed by elevated expression of CYP1A, GPX, GSK3β and TNF-α gene. Zinc depleted MCF-7 cells expressed significantly ($p \leq 0.001$) decreased levels of CDKN2A, pRb1, p53 and increased the level of mdm2 expression. Zn supplementation (IC_{50} = 15 μM), increased significantly CDKN2A, pRB1 & p53 and markedly reduced mdm2 expression; also protein expression levels of CDKN2A and pRb1 was significantly increased. In addition, intrinsic apoptotic pathway related genes such as Bax, caspase-3, 8, 9 & p21 expression was enhanced and finally induced cell apoptosis. In conclusion, physiological level of zinc is important to prevent DNA damage and MCF-7 cell proliferation via regulation of tumor suppressor gene.

1. Introduction

Dietary intake of required level of key minerals determines the genomic stability. Micronutrients affects all pathways relevant to genomic stability, including exposure to dietary carcinogens, activation and detoxification of carcinogens, DNA repair, DNA synthesis and cell apoptosis (Ames, 1998). Normal cells respond to DNA damage via activation of p53 protein, further p53 either mediates G1 phase cell cycle arrest by inducing transcription of cyclin-dependent kinase inhibitor $p21^{WAF1}$, thereby allowing time for DNA repair or activating cell death by triggering apoptosis (Bartek and Lukas, 2001). Zn deficiency increases oxidative DNA damage and induces chromosome breaks in humans (Golub et al., 1985; Hainaut and Milner, 1993). Zinc is a component of many proteins in the mitochondrial transport chain and deficiency could result in the release of oxidants (Skulachev et al., 1967; Ye et al., 2001). Thus, mitochondrial disruption may account for the source of increased oxidative stress with zinc loss. Additionally, Zn deficiency increases p53 expression in response to DNA damage, but impairs the ability of p53 to bind DNA (Ho and Ames, 2002). The p53 protein DNA binding domain is stabilized by a Zn ion, which is necessary for maintaining a functionally active conformation (Cho et al., 1994).

Under Zn deficient conditions, apo-metallothionein can chelate Zn from p53 and disrupt architecture of the DNA binding domain, allowing the protein to adopt a conformation identical to mutant forms of p53 (Meplan et al., 2000a,b) and inactivating the protein (Hainaut and Milner, 1993). The $p16^{INK4a}$/$p14^{ARF}$ locus on chromosome band 9p21 is disrupted in a wide variety of human tumors at a frequency comparable with p53 inactivation (Eun-Young et al., 2014). The common loss of this locus presumably reflects its dual coding capacity; this single genomic sequence encodes the $p14^{ARF}$ (CDKN2A) tumor suppressor and the $p16^{INK4a}$ cyclin-dependent kinase inhibitor (pRb1-retinoblastoma protein) (Rizos et al., 2003).

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The CDKN2A tumor suppressor can induce potent growth arrest or cell death in response to hyper proliferative oncogenic stimuli. Stimulated CDKN2A expression in normal cells induces G1 and G2 cell cycle arrest or apoptosis (Zindy et al., 1998; Bates et al., 1998). CDKN2A can activate the p53 tumor surveillance pathway by interacting with and inhibiting the p53-antagonist, mdm2 (Tao and Levine, 1999; Weber et al., 1999). Consequently, the loss of ARF not only diminishes the response of the p53 network to hyperproliferative signals but also reduces the duration of p53 activity in response to DNA damaging stimuli. Animals lacking CDKN2A are highly prone to tumor formation, and their embryonic fibroblasts do not senesce, continuing to cycle after DNA damage (Khan et al., 2000).

Mammary carcinogenesis remains the most common malignancy and the second leading cause of death for women (Esteller, 2008). Epidemiological studies shown that many adults may not be getting required level of zinc in their diets (Samina and Shannon, 2012). In the United States population, zinc intake was 10% less than one-half of the recommended level (Wakimoto and Block, 2001), which could put them at greater risk for DNA damage and cancer. In the present study, we aimed to explore the chemopreventive role of zinc via regulation of $p14^{ARF}$-mediated senescence of mdm2- further activation of p53 and caspases pathways in human breast cancer cell. To achieve this MCF-7 cells will be cultured in severe zinc deficient and zinc adequate media. Further, we aimed to explore the physiological level of zinc on the expression pattern of $p14^{ARF}$ and p53 pathway related gene expressions in zinc supplemented cells compared to zinc deficient MCF-7 cells.

2. Materials and method

2.1. Chemicals

Propidium iodide (PI), acridine orange (AO), ethidium bromide (EB), chelex-100 (Bio-Rad) and zinc chloride ($ZnCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), Roswell Park Memorial Institute medium (RPMI-1640) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The DeadEnd TUNEL Assay Kit was procured from Promega (Madison, WI, USA). The QuantiTect Primer Assay, Fastlane Cell cDNA Kit and QuantiFast SYBR Green PCR Kit were obtained from Qiagen, Hilden (Germany). All the other chemicals used were of molecular biology research grade.

2.2. Cell culture

The human breast cancer MCF-7 cell was generously provided by Mahatma Gandhi-Doerenkamp Center (MGDC) for Alternatives to Use of Animals in Life Science Education, Bharathidasan University, Tiruchirappalli-620024, India. MCF-7, human breast cancer cell was cultured as a monolayer with RPMI-1640 (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 μg/mL of streptomycin as antibiotics (Invitrogen) at 37° C in a humidified atmosphere of 5% CO_2, in a CO_2 incubator (Thermo Scientific, USA).

2.3. Preparation of mineral deficient medium and zinc analysis

Mineral-deficient media was prepared using a chelation method. Mineral levels were monitored by inductively coupled plasma-absorption emission spectroscopy (ICP-AES). Fetal bovine serum (FBS) was mixed with 10% chelex-100 overnight at 4 °C, after incubation FBS was filtered (Torti et al., 1998). Chelex-100 treated FBS was incubated with 40% ultrapure nitric acid (West Chester, VA) overnight (1:1 ratio). After incubation, samples were diluted with deionized water to an 8% acid solution and analyzed by ICP-AES. Further, the experimentally required concentration of zinc was added based on the protein bound Zn present in the chelex-100 treated FBS.

2.4. Measurement of cell viability

Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenases of viable cells to a purple formazon product (Mosmann, 1983). Briefly, MCF-7 cells were plated at a density of 1×10^4 cells/mL in 96-well plate containing mineral depleted medium. After overnight growth, the cells were treated with an increasing concentration of Zinc, (such as, 0, 1, 2, 4, 8, 16, 32, 64 μmol/L) and maintained for 24 h & 48 h. After incubation, 20 μL of MTT solution (5 mg/mL in phosphate-buffered saline [PBS]) were added to each well. The plates were wrapped with aluminum foil and incubated for 4 h at 37 °C. The plates were centrifuged and purple formazan product was dissolved by the addition of 100 μL of DMSO to each well. The absorbance was monitored at 570 (measurement) and 630 nm (reference) using a multiwell plate reader (Bio-Rad, CA, USA). Quadruplicate samples were run for different concentration of zinc in six independent experiments. The viability of MCF-7 cell was expressed as the percent viability of treated cells compared with the untreated control. IC_{25} & IC_{50} have been calculated based on the percentage of toxicity was calculated by using the formula:

$$\frac{\text{Mean OD of untreated cells} - \text{Mean OD of treated cells}}{\text{Mean OD of untreated cells}} \times 100$$

2.5. Experimental setup

For in vitro cytotoxicity study, MCF-7 cells were seeded in 96-well microtitre plate containing mineral-depleted media (RPMI with 10% chelex FBS) and normal mineral adequate medium (RPMI with 10% FBS).

For nuclear and morphological analysis cells will be grouped such as, Group-1: MCF-7 cells grown in normal medium, Group-2: MCF-7 cells grown in Zn depleted medium, Group 3: Zinc (IC_{50} = 15 μM) adequate medium.

For gene expression analysis, the cells were seeded in 24 well plates and cultured under Zn depleted and Zn adequate conditions (IC_{50} = 15 μM). Negative control groups were grown in the mineral depleted medium; in addition cells cultured with normal medium will be considered as positive control. Media was replaced once in 2 days.

2.6. Measurement of intracellular ROS

The production of intracellular reactive oxygen species (ROS) was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Wang and Joseph, 1999). DCFH-DA passively enters the cell were it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). Briefly, 10 mM DCFH-DA stock solution (in methanol) was diluted 500-fold in HBSS without serum or other additive to yield a 20 μM working solution. After 24 h of exposure of zinc to MCF-7 cells in the 24-well plate were washed twice with HBSS and then incubated in 2 mL working solution of DCFH-DA at 37 °C for 30 min. In addition, MCF-7 cells treated with zinc (IC_{50} & IC_{25}) along with 20 mM of *N*-acetyl cysteine, and 20 mM of *N*-acetyl cysteine (positive control) alone have been treated in separate wells. Fluorescence was then determined at 485-nm excitation and 520-nm emission using a microplate reader.