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Effect of Invitro Zinc (II) supplementation on Normal and Cancer Cell lines

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Abstract*— Zinc is the most abundant trace element which has role in genetic stability and function, present in the cell nucleus, nucleolus and chromosomes, and stabilizes the structure of DNA, RNA and ribosomes and found in many Zinc binding proteins. The purpose of this study is to access the Zinc cytotoxicity, total cellular Zinc content and total Zinquin Acid (Fluorophore) interaction with cellular Zinc in response to six different Zinc gradient medium using MTT assay, Atomic absorption spectroscopy and Fluorescence spectroscopy respectively. Statistical analysis one way ANOVA and Tukey HSD test for mean comparison was performed. Our findings are, significant differences on normal cell line survival of 108.7% at 10 µM supplemented medium was seen. Cytotoxicity was seen at 80 µM of 89.78% and 86.46% in MDA-MB-231 and U-87-MG Cell lines respectively. Significant increase in cellular influx and accumulation of Zn^{2+} ions was observed in all cell lines. Fluorescence intensity emission peak response increased with supplementation of gradient medium, which supported the hypothesis of increased cellular Zinc accumulation, λ-max shifting to shorter wavelength i.e. idea supports blue shift emission of Zinquin Acid which interacts not only with free labile Zinc ions but with Zinc bound proteins. Zinc based fluorescence probes help detection of altered expression of Zinc.

***Keywords*— MTT, Atomic Absorption Spectroscopy (AAS), Fluorescence Spectroscopy, Zinquin Acid, Zinc ions (Zn^{2+})**

I. INTRODUCTION

Zinc ion essentiality and its deficiency in humans were first recognized in the early sixties of the twentieth century [1, 2]. According to current estimation nutritional deficiency of Zinc may have affected over 2 billion subjects in the developing nations. Growth retardation, cognitive mutilation and immune disorder are among the major consequences of Zinc deficiency [3]. These effects are reversible with Zinc supplementation. Conditional deficiency of Zinc is known to have occurred in many diseases, such as chronic liver, mal-absorption syndrome, renal diseases, and excessive intake of alcohol, sickle cell disease, malignancies and other chronic conditions [3, 4]. Without Zinc cells cannot sustain life. Zinc is a cofactor for more than 300 enzymes, representing more than 50 diverse enzyme classes, and is essential for cell growth [5]. Zinc is present in the cell nucleus, nucleolus and chromosomes, and it stabilizes the structure of DNA, RNA and ribosome [6]. Zinc is involved in protein, carbohydrate, and lipid metabolism. Zinc is also involved in the control of differentiation, gene transcription, development, and growth [7]. Zinc deficiency can be harmful, causing diminutive growth and metabolic dysfunction [8]. Homeostatic mechanisms adapt Zinc absorption, circulation, cellular influx, and efflux [9] which are vital in retaining cellular function [10]. Zinc is involved in a complex multilayered procedure that involves oxidative stress and alterations in cellular signal processes controls DNA repair mechanisms and apoptosis. Increased oxidant species production has been shown in cell cultures grown in Zinc-lacking media [11]. Copper/Zinc superoxide dismutase (SOD) is a vital initial defense enzyme against reactive oxygen species and p53; an essential Zinc-containing transcription factor which plays a pivotal role in DNA damage response. Low cellular Zinc may enhance oxidative stress, weaken DNA binding activity of p53, and obstructs its role in DNA repair mechanism [12, 13]. Zinc's elementary and immense role in several cellular processes necessitates Zinc delivery to tissues and cells, availability and allocation of Zinc are firmly controlled at intracellular level [10]. These courses of action are maintained through the complex synchronization of a diverse family of transporter proteins that adjust the compartmentalization, influx, and efflux of Zinc [10]. 14 Zinc importers (SLC39/ZIP's), 10 Zinc exporters (SLC30/ZnT's) and 4 metallothioneins have been expressed in mammalian cells [14]. Temporary increase in the cytosolic free Zinc ion (Zn^{2+}) can occur through the release of Zinc from Zinc-binding proteins and are determined by changes in cellular redox potential [15], and by fluctuation of Zn^{2+} into the cytosol [5, 16, 17] through Zinc ion transporter channels. The concentration of cytosolic Zn^{2+} must be tightly controlled to prevent cell death due to Zinc insufficiency or toxicity [1]. The exact molecular method by which Zinc

deficiency impacts DNA integrity and cancer risk remain unclear. The major aim of this study was to determine cellular cytotoxicity, total Zinc present inside the cell and fluorescence interaction of Zinquin Acid with cellular Zinc ions. Our results define that the increased doses of supplementation in invitro leads to cellular Zinc accumulation at different levels and these are toxic which leads to cell death in cancer cell lines. We then identify the accumulation levels on those differentiating media and their responsiveness to the normal, breast cancer and brain cancer cell lines.

II. MATERIALS AND METHODS

A. Reagents and Cell Lines

Noninvasive MCF-7 cell line and NIH-3T3 and were obtained from Everest Biotech (Kathmandu, Nepal). Invasive MDA-MB-231 cells and U-87-MG cell line were generous gift from University of Santiago de compostella, (SPAIN). Dulbecco's modified Eagle medium (DMEM; Life Technologies, USA), fetal bovine serum (Sigma, Germany), penicillin/streptomycin (Sigma, Germany), MTT dye (Amersco,USA), Trypsin (Amresco,USA), Zinquin Acid (Sigma, Germany), and Amphotericin "B" (Sigma, Germany) was purchased.

B. Cell Culture

Cells were maintained in DMEM containing 10% fetal bovine serum, 0.5% antibiotic solution (penicillin + streptomycin + glutamine) and 0.5% anti-mycotic solution (Amphotericin "B"). Initially cell was grown in 75 cm^2 culture flask with DMEM and 5% CO_2 for 2 days. The cells were trypsinized and counted using haemocytometer. The cells were divided in different culture flasks and well plates following standard protocol [18]. Stock solution of DMEM was prepared supplementing Zinc Sulfate (100 µM) and diluted in five different gradient of 5 µM, 10 µM, 20 µM, 40 µM, 80 µM respectively and Control as 0 µM which contains approximately 4.24µM (±0.2µM)of Zinc in complete medium.

C. Micro-titration (Colorimetric) Assay

To evaluate the cytotoxic effect of Zinc sulfate on the cancer cell lines; MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5- diphenyl-2H-tetrazolium bromide) colorimetric assay was applied [19]. Cells were seeded in 96 well plates maintaining seeding density of 1000 cells per well, after 24 hours of incubation medium was removed and new Zinc gradient medium was used. After 24 and 48 hours of incubation with Zinc gradient medium cells were washed with Phosphate buffered saline twice and 10 µl of 5mg/ml MTT dye was added. It was incubated for 3.5 hours and reading was taken at 570nm with background subtraction of 630 nm band pass filter [20].

D. Atomic absorption spectroscopy(AAS)

To evaluate the effect of Zinc Sulfate on total cellular Zinc content of cancer cell lines, cells were seeded in 25 cm^2 tissue culture flask for 24 hours in incubation. Medium was removed and new Zinc gradient medium was used. After 48 hours of incubation with Zinc gradient medium cells were washed with PBS twice and trypsinized. Pellet was collected using centrifugation and counted. All samples were digested using concentrated Nitric Acid overnight and volume was made up using 0.1N Nitric Acid [21] and Flame-AAS was performed in SOLAR AA (Thermo electron).

E. Fluorescence spectroscopy

To evaluate the effect of Zinc Sulfate on free cellular and protein levels of Cancer cell lines, cells were seeded in 6 well plates maintaining seeding density, after 24 hours of incubation medium was removed and new Zinc gradient medium was supplemented. After 48 hours of incubation with Zinc gradient medium cells were washed and suspended in DPBS by gently scraping with a rubber cell scraper and pellet was collected using centrifugation. All samples were then incubated with 10µM Zinquin Acid for 30 minutes and florescence emission spectra was taken [22] using F93 fluori-spectrometer using control as DPBS with 10µM Zinquin Acid only. Emission spectra were recorded from 400 to 600 nm with an excitation wavelength of 365 nm. Raw data were fitted using area version of gauss single peak fitting function.

F. Statistical Analysis

To evaluate the statistical parameters, values were calculated in mean ± S.D. from replications, i.e. number of replications (n) and the statistical parameters were calculated. The data were analyzed using statistical software Origin Pro version 9.0. One way ANOVA was performed with single factor as Zinc treatments on six levels including control. To know the mean difference in

treatments, Tukey HSD test for mean difference comparison was done. Significant data with ($p<0.05$) were shown in asterisk considering without treatment i.e. 0µM as a test mean.

III. RESULTS AND DISCUSSIONS

In these experiments the role played by Zinc supplementation at intracellular levels, Zinc cytotoxicity, Total cellular Zinc and total free labile Zinc ions and protein bound Zinc i.e. Zinc finger proteins in NIH-3T3, MDA-MB-231, MCF-7, U-87-MG cells were addressed.

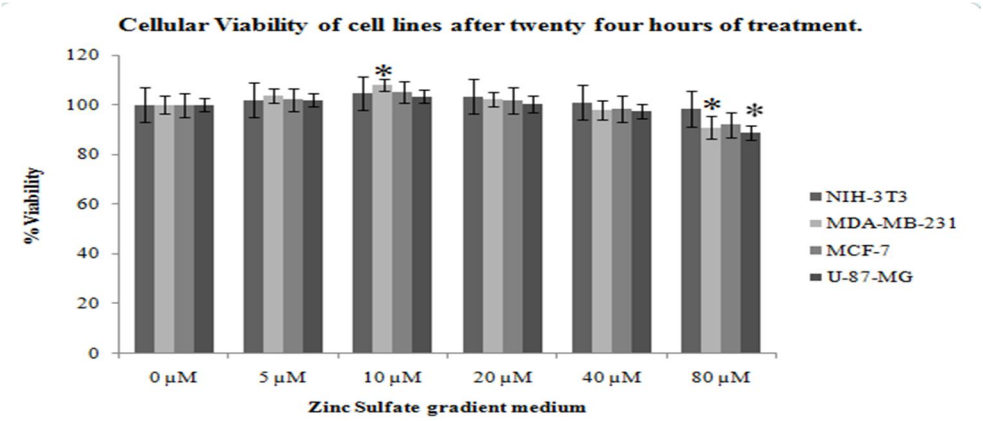

Fig 1: Cellular Cytotoxicity response of four different cell lines in Zinc sulfate supplemented gradient medium after 24 hours of incubation, n=4, bars shown on mean ±SD. Asterisks indicate significant differences of the mean from the test mean ($p<0.05$) Tukey test for pair wise mean comparison.

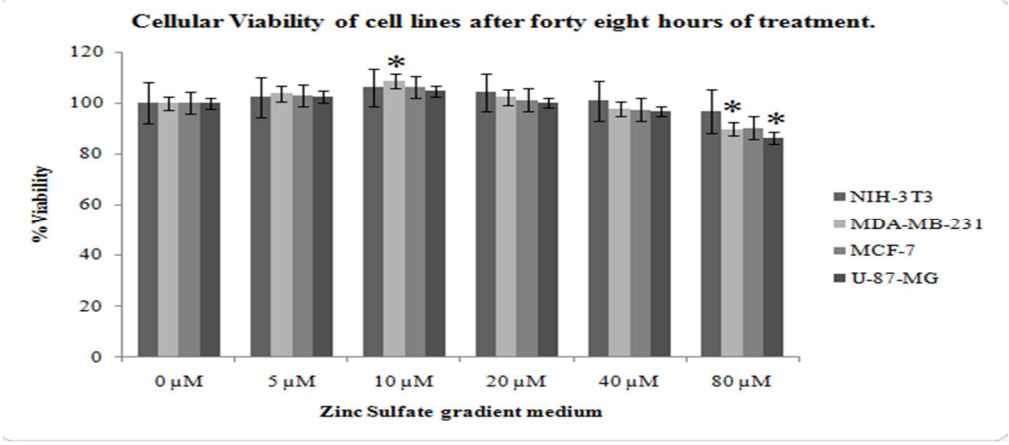

Fig 2: Cellular Cytotoxicity response of four different cell lines in Zinc sulfate supplemented gradient medium after 48 hours of incubation, n=4, bar on mean ±SD. Asterisks indicate significant differences of the mean from the test mean ($p<0.05$) Tukey test for pair wise mean comparison. Fig 1 and Fig 2 shows the survival of human MCF-7, MDA-MB-231, U-87-MG cancer cells and NIH-3T3 mouse cell after 24 and 48 hours of incubation with Zinc sulfate evaluated by MTT assay. Zinc evoked a concentration-dependent increase in the viability of the cells at lower range and decrease in the viability at higher range. At lower range of concentration cell were able to grow at higher rates [23, 24, 25]. Apart from this normal cell survived highest concentration of Zinc supplementation i.e. 80 µM, than that of cancer cells. Medium which did not contain extra supplemented Zinc sulfate was used as control (NIH-3T3-0µM as a Normal control and all others as cancer control). Viability of control was converted to 100% and others were calculated according to it. Mean percentage viability of NIH-3T3 at 10 µM was found to be of highest value of 104.72 % and 106.02%, also lowest at 80µM with value of 98.46% and 96.78% at 24 and 48 hours respectively. Cancer cell showed highest percentage viability at 10 µM of108.1%, 104.97% and 103.28% at 24 hours (Fig 1) and 108.7 %, 106.12% and 104.67% of MDA-MB-231, MCF-7 and U-87-MG at 48 hours (Fig 2) respectively. Cellular toxicity was seen at 80µM Zinc supplemented concentration at the range of 89.78%, 90.32% and 86.46% on MDA-MB-231, MCF-7 and U-87-MG respectively. The significant

difference in 10 µM concentration of MDA-MB-231 shows Zinc supports in breast cancer cell proliferation and the differences in 80 µM of MDA-MB-231 shows Zinc at high concentrations can be toxic [26]

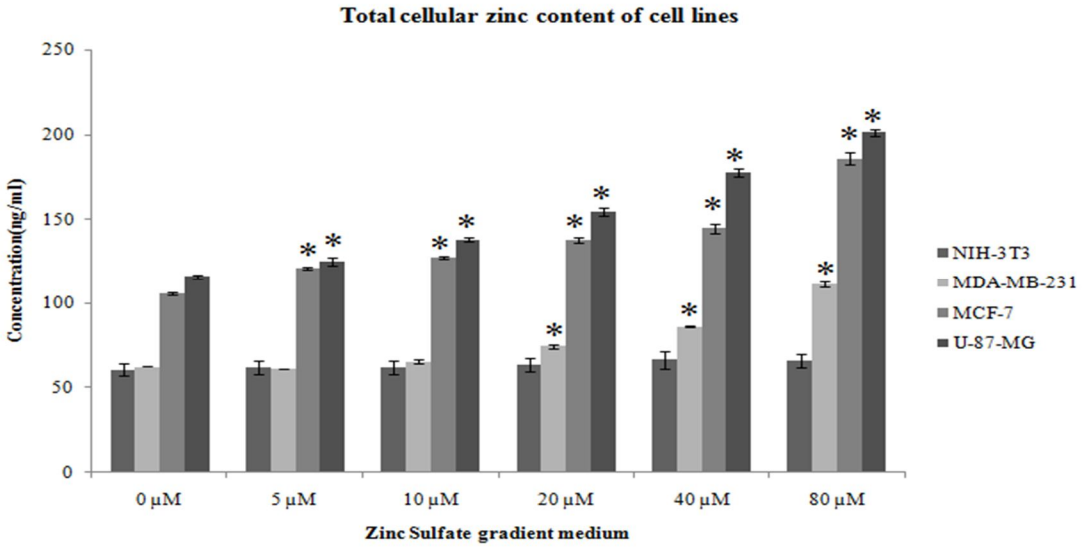

Fig 3: Total cellular Zinc content of four different cell lines in nanograms per milliliter when treated with Zinc Sulfate Gradient medium, in $1x10^6$ number of Cells, n=2, bar on mean ±SD. Asterisks indicate significant differences of the mean from the test mean ($p<0.05$) Tukey test for pair wise mean comparison.

The Results of AAS showed higher Zinc accumulation in Zinc supplemented medium on cancer than normal cell lines. Mean of NIH-3T3 was 60.78 ng/ml whereas 62.46 ng/ml 105.87 ng/ml and 115.85 ng/ml were of MDA-MB-231, MCF-7, and U-87-MG (Figure 3) respectively. The highest amount of concentration was seen on 80 µM test concentration at 66.23 ng/ml, 111.49 ng/ml, 185.86 ng/ml and 201.4 ng/ml of NIH-3T3, MDA-MB-231, MCF-7 and U-87-MG (Fig 3) respectively. There was 38.01%, 36.65% and 53.47% increased cellular accumulation in three of the cancer cell lines (Fig 3) at 40µM treatment with respect to 0µM treatment of Zinc Sulfate respectively. We found that cellular Zinc accumulation was significantly higher in cancer cell lines than in normal cell lines [27, 28, 29].

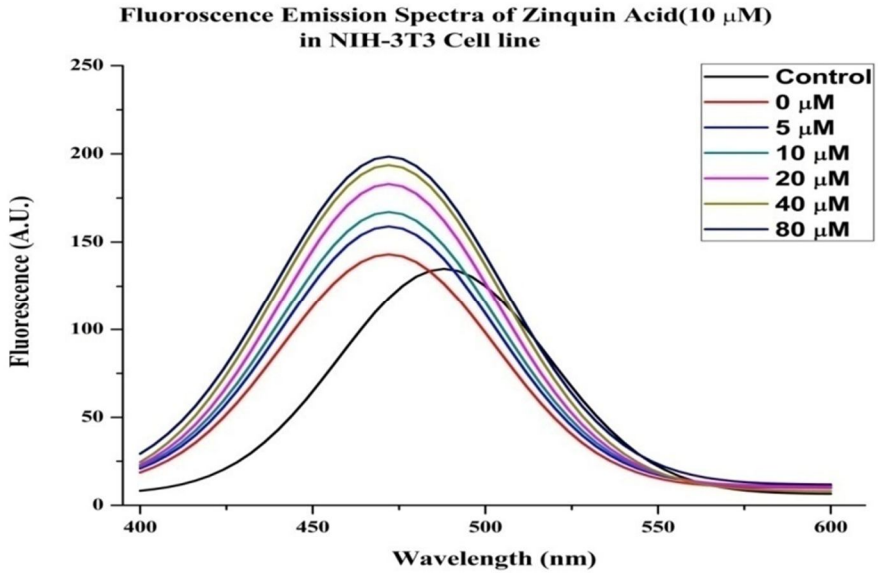

Fig 4: Fluorescence emission spectrum of NIH-3T3 cell line from 400 to 600 nm in Zinc supplemented gradient medium after 48

hours of incubation, Followed by Zinquin Acid treatment of 30 minutes, all peaks were fitted using Gaussian peak function keeping center (xc) constant, n=3.

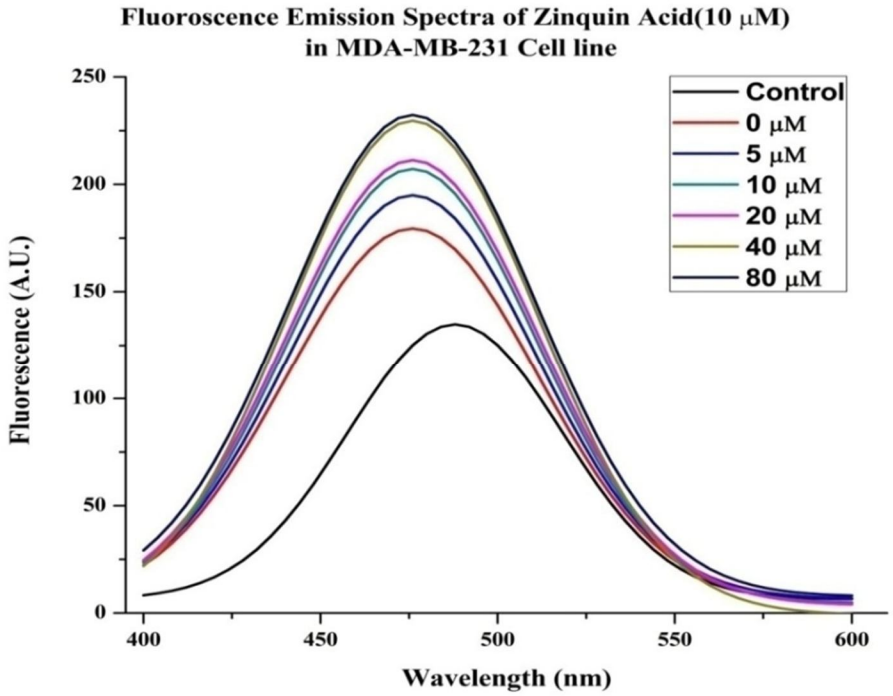

Fig 5: Fluorescence emission spectrum of MDA-MB-231 cell line from 400 to 600 nm in Zinc supplemented gradient medium after 48 hours of incubation, Followed by Zinquin Acid treatment of 30minutes , all peaks were fitted using Gaussian peak function keeping center (xc) constant, n=3.

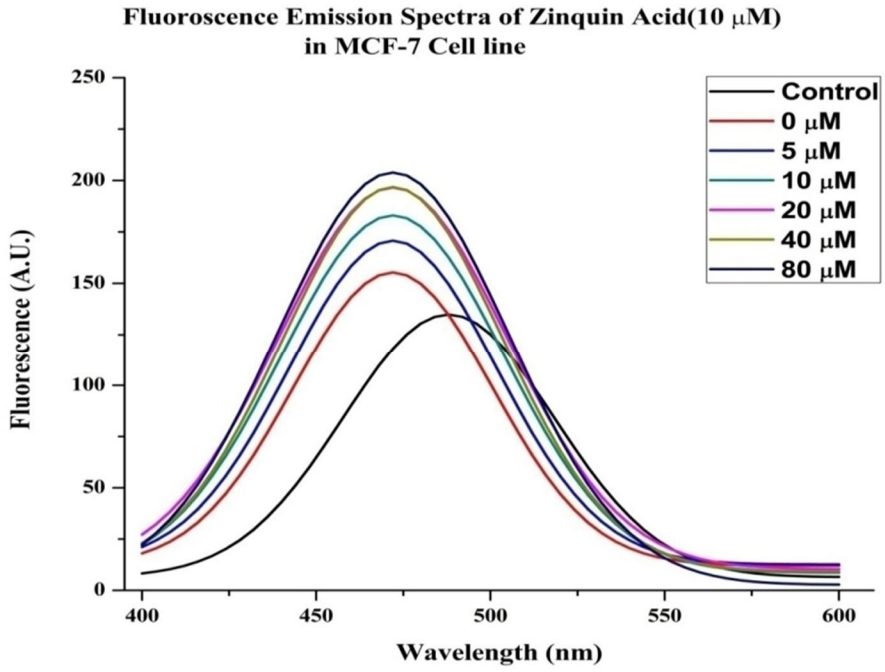

Fig 6: Fluorescence emission spectrum of MCF-7 cell line from 400 to 600 nm in Zinc supplemented gradient medium after 48

hours of incubation, Followed by Zinquin Acid treatment of 30minutes , all peaks were fitted using area version of Gaussian peak function keeping center (xc) constant, n=3.

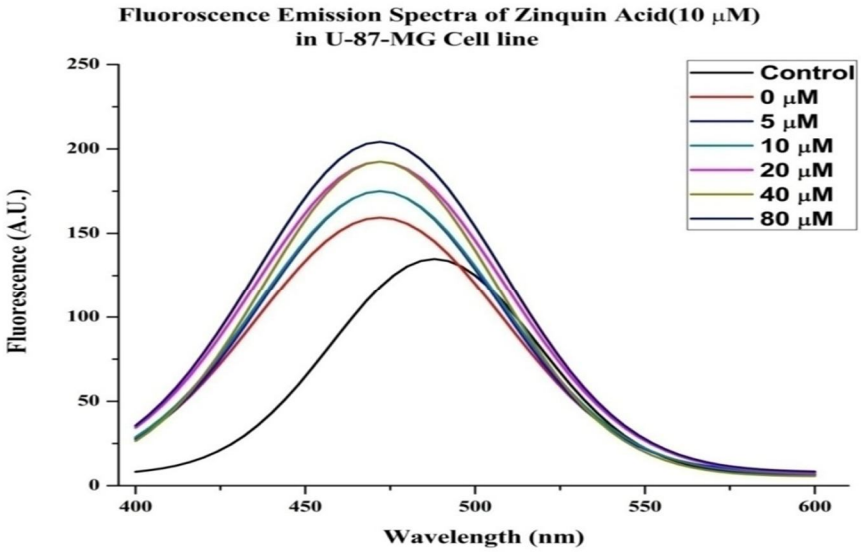

Fig 7: Fluorescence emission spectrum of U-87-MG cell line from 400 to 600 nm in Zinc supplemented gradient medium after 48 hours of incubation, followed by Zinquin Acid treatment of 30minutes , all peaks were fitted using area version of Gaussian peak function keeping center (xc) constant, n=3

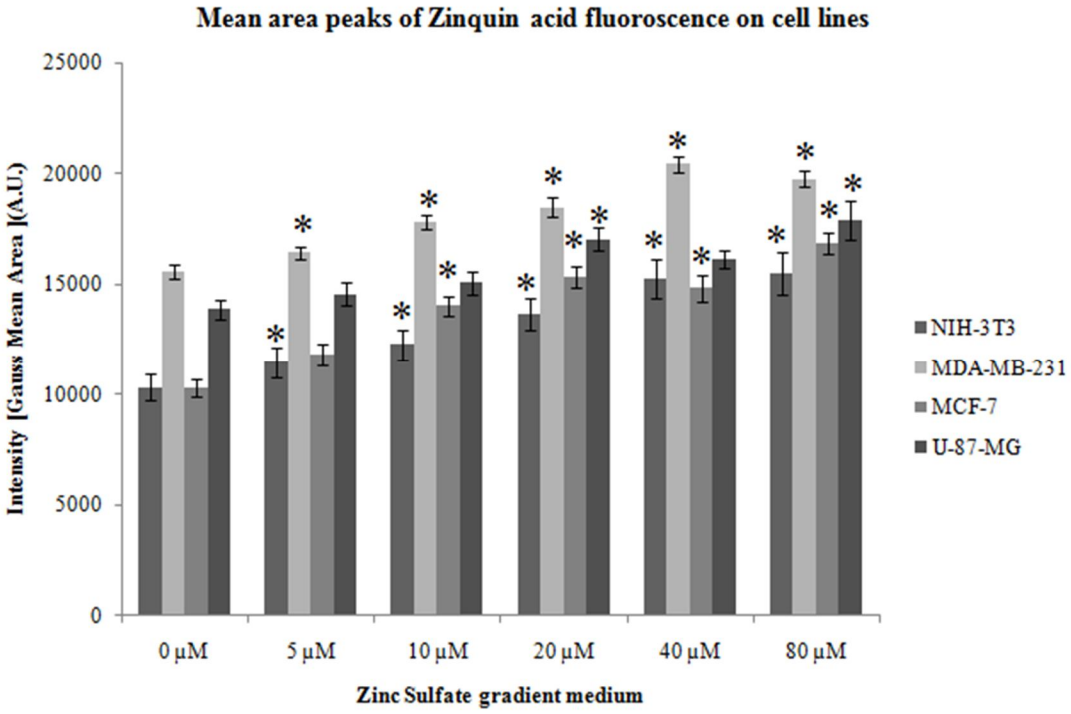

Fig 8: Gauss Mean Area of Fluorescence emission Intensity ranging 400nm to 600nm at different μM Zinc Sulfate concentration in NIH-3T3,MCF-7, MDA-MB-231 and U-87-MG, n=3, bar on mean ± SE (area). Asterisks indicate significant differences of the mean from the test mean ($p<0.05$) Tukey test for pair wise mean comparison. Fig: 4, 5, 6 and 7 depict the changes in fluorescence

intensity of the different cell lines at different concentration after exposure to Zinquin Acid. The results of fluorescence spectroscopy showed Zinc interaction increased inside the cell after gradient medium treatment. The emission spectra of control showed λ maximum at 488 nm. Whereas λ maximum of NIH-3T3, MCF-7 and U-87-MG was seen at 472nm and MDA-MB-231 was seen at 476nm. The gauss mean area emission intensity of control was calculated to be 9747.19. NIH-3T3 showed lowest emission area of 10312.18 at 0µM concentration and 15496.25 at 80µM. MDA-MB-231 showed emission area ranging from 15559.81 to 20410.46 at 40 µM concentration. MCF-7 showed emission area ranging from 10278.67 to 16818.02. U-87-MG showed emission area ranging from 13875.42 to 17877.57. The emission profiles of Zinquin acid on cancer cell lines were found higher than normal cell lines. The total fluorescence emission intensity of cells ($1x10^6$ cells/mL) suggests Zinquin senses differing amounts of Zinc ions in each system. Nevertheless, all cell lines show emission spectra that place λ-max near 470 nm. These emission spectra are different from the expected emission spectrum of free Zinc ions bound to Zinquin Acid, which displays a λ-max of 488 nm. The fluorophore Zinquin Acid was used in combination with fluorescence spectroscopy to detect Zinc ions within cultured cells. To investigate the majority of Zinquin-induced fluorescence, Zinc ion distribution and trafficking in four different cell types at various Zinc concentrations were used in this study (Fig 8) as models for Zinquin exposure. We show that with increase in Zinc Sulfate gradient in the culture medium, growth of breast cancer cell line is highly supported to some extent and cellular cytotoxicity can be seen at higher supplemented concentration. Zinc also helped in normal cell proliferation [30] in higher doses of supplementation in comparison to cancer cell lines. The cellular uptake through active membrane transport channels and accumulation of Zinc ions was also increased in all of our all test cell lines, where lower amount of Zinc supported cellular proliferation and active influx of Zinc ions into the cytoplasm and cellular components. Fluorescence intensity emission peak response improved with increase in supplementation of Zinc from gradient growth medium, which supported the hypothesis of higher cellular i.e. cytosolic and cellular components Zinc accumulation [31]. Fluorescence spectroscopy emission spectra from (400nm-600nm) supported the hypothesis of Zinquin Acid that may react with open coordination sites of Zn-proteins to form ternary adducts instead of reacting with "free" Zinc ions ultimately producing Zinc-Zinquin Acid Complex resulting in emission spectra that are blue-shifted [31,32]. Increased Tryptophan activity on Zinc bound proteins or higher degree of protonation due to free cellular Zinc ions may also be the cause of blue shifted spectroscopic observation [33].

IV. CONCLUSIONS

Zinc is an essential trace metal that is implicated in many physiological and metabolic processes. The ubiquitous nature of Zinc in physiological systems suggests that typical levels are likely to have many biological and clinical effects. The true significance of Zinc in cellular signaling is just emerging. From above result and discussion we could derive a conclusion that micro molar concentration of Zinc sulfate supplementation directly affects on cell survival, cellular toxicity and cellular Zinc accumulation. Zinc based fluorescence probes helps in detection of altered expression of Zinc at microscopic level. Invitro cell culture techniques can be improved with addition of 5 to 10 micro molar Zinc sulfate addition to cultures. Further study is recommended in separation of cytosolic and cellular component Zinc ions and study via fluorescent probes as tracking micro devices. Protein level expression of Zinc transport channels can be used for the study of regulation and function mechanisms. Tests on molecular level may reveal the minor alteration on pattern of cell growth either in Zinc deficiency or in excess.

V. CONFLICT OF INTEREST

The authors declare no conflict of interest.

VI. ACKNOWLEDGMENT

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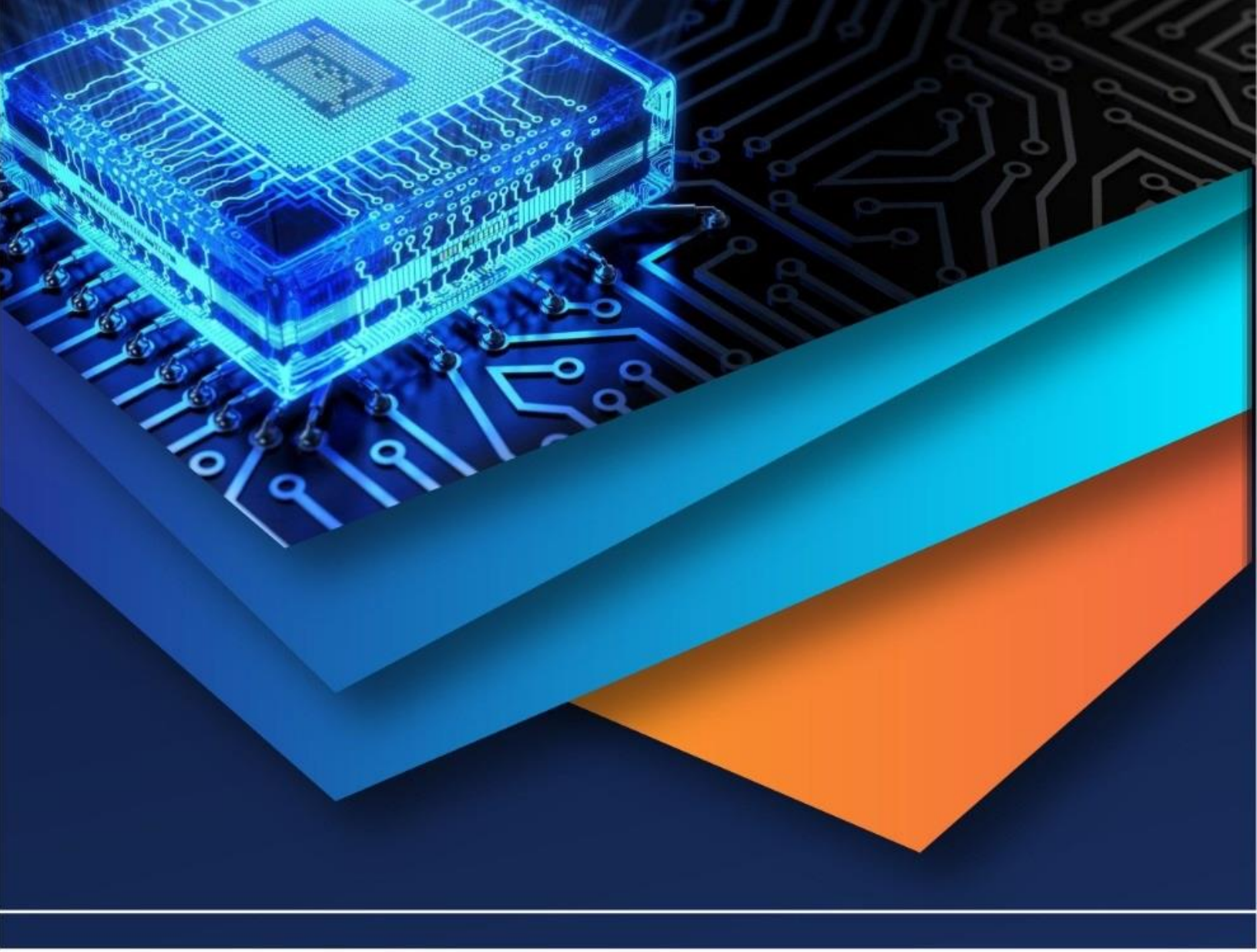

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