REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

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5. **Duration of fellowship with exact date**: 08.03.2023 to 07.06.2023

6. **Highlights of work conducted.**

   i) **Technique/expertise acquired**: Impact of environmental carcinogen arsenic on mitochondrial dynamics in bronchial epithelial cells
   ii) **Research results, including any papers**, **prepared/submitted for publication.**

Literature review

Background

The environmental metalloid arsenic is a well-known human carcinogen (1) that inflicts a myriad of health effects. Globally over 200 million people are exposed to arsenic either through contaminated groundwater or through the food chain (2). Even in India, groundwater arsenic contamination is a public health menace that has engulfed the whole of the Indo Gangetic plain (3).

Environmental arsenic has been frequently related to lung cancer (4,5). Several schools of thought have proposed varied mechanisms of arsenic-induced carcinogenicity including oxidative stress, DNA damage, DNA repair dysfunction, epigenetic modifications, signal transduction perturbations, etc. (6). However, the actual mechanism of arsenic-mediated carcinogenesis is yet to be deciphered. Emerging evidence suggests the perturbation of mitochondrial dynamics might be related to arsenic-induced health effects (7).

Natural compounds have been widely harnessed for their beneficial effects against arsenic toxicity (8,9). Among them, the principal green tea flavonoid epigallocatechin gallate (EGCG) has drawn attention due to its multimodal effects against arsenic (10,11).

With this background, the present study aimed to investigate the impact of sodium (meta) arsenite (As III) on mitochondrial homeostasis in bronchial epithelial cells. We were further interested to explore whether EGCG had any modulatory effects against As III-mediated alterations in mitochondrial dynamics, if any.

Mitochondrial homeostasis

Mitochondrial homeostasis involves the maintenance of optimal mitochondrial structure and function which is a pre-requisite for cellular metabolism and energy production. Mitochondrial homeostasis and oxidative stress are two closely intertwined cellular processes. During cellular respiration, mitochondria generate reactive oxygen species (ROS), primarily in the form of superoxide radicals, as by-products of oxidative phosphorylation alongside the electron transport chain. Under normal conditions, the production of ROS is tightly controlled by the mitochondrial antioxidant mechanism which maintains redox homeostasis and prevents oxidative stress. The imbalance in ROS generation and antioxidant defence mechanism may lead to mitochondrial dysregulation and impaired cellular metabolism, frequently encountered in the aetiology of various diseases, including neurodegenerative complications, metabolic disorders, cystic fibrosis, muscle atrophy, and cancer (12).

Importance of NEET proteins in mitochondrial dynamics

The three-dimensional protein folding pattern and specific biological functions largely depend on cofactors' binding. The iron-sulfur (Fe-S) clusters are co-factors that guide electron transfer, metabolism, gene expression and regulation, and DNA integrity. Emerging studies have evidenced a novel family of [2Fe-2S] NEET proteins involved in the regulation of iron

metabolism, redox homeostasis (13), mitochondrial morphodyamics and bioenergetics (14). These proteins have characteristic Asn-Glu-Glu-Thr (NEET) amino acid sequence at C-termini and are encoded by the *CISD1* (mitoNEET), *CISD2* [nutrient deprivation autophagy factor (NAF-1)/Miner1], and *CISD3* (Miner2/MiNT) genes (13). The other NEET isoforms like NAF1 is primarily found in the endoplasmic reticulum membrane and outer mitochondrial membrane whereas MiNT constituting two oxygen-labile [2Fe-2S] clusters per single polypeptide chain is predominantly located in the inner mitochondrial membrane. The differential oxygen sensitivities of these aforementioned Fe-S clusters guide their role in mitochondrial metabolism. Gain or loss of function of *CISD-1*/*CISD-2*/*CISD-3* governs oxidative stress and the pathophysiology of diseases like diabetes, breast cancer, and Parkinson's disease (15).

MitoNEET in cancer

MitoNEET has been evidenced to be upregulated in several malignancies including breast (16), lung (17), and oral cancer (18). High tumor mitoNEET expression has been reported to inhibit apoptosis, autophagy, intramitochondrial iron concentrations and ferroptosis thus providing advantage to tumor cells to tackle ROS-mediated oxidative burst (19). Overexpression of MiNT in triple negative breast cancer cells MDA-MB-231 and their associated xenograft, made them resistant to ROS and increased cell proliferation and tumor growth (14). In non-small cell lung cancer, mitochondrial aconitase, regulator of iron homeostasis was found downregulated which upregulated mitoNEET (20).

NAF-1 in cancer

NAF-1 localized at the interface of the endoplasmic reticulum and mitochondria, is anchored to the Ca^{2+} channel inositol 1,4,5-triphosphate receptor. It is instrumental for B cell lymphoma-2 (BCL-2)-mediated inhibition of autophagy (21) and regulation of Ca^{2+} homeostasis (22). Lack of NAF-1 separates the autophagy-promoting Beclin1 complex from BCL-2, and induces autophagy (23). MitoNEET and NAF-1 were reported to directly interact and operate as part of an 2Fe-2S cluster relay to maintain the mitochondrial iron homeostasis. This in turn inhibited apoptosis and/or autophagy and promoted proliferation of mammalian cells (24). NAF-1 overexpression in breast (25), liver (26), and cervix (27) were associated with tumorigenesis. Downregulation of NAF-1 promoted apoptosis in breast cancer *in vitro* and *in vivo* models (28). However, NAF-1 has been also proposed to interact with inhibitor of apoptosis-stimulating protein of p53 (iASPP) for activation of apoptosis in cancer cells (29).

Impact of arsenic in mitochondria

Arsenic may perturb mitochondrial homeostasis in multiple ways including generation of oxidative stress, disruption of autophagy, apoptosis, and metabolic homeostasis, induction of mitochondrial membrane damage, defective mitophagy, unfolded protein response and protein acylation, etc.(30). Arsenite may inflict mitochondrial superoxide production along with increased Ca^{+2} signaling to mediate crosstalk between mitochondria and endoplasmic reticulam (31). Sub chronic exposure of arsenic trioxide triggered the 1,2-dimethylhydrazine-induced colon carcinogenesis in Swiss albino mice through ROS and mitochondrial permeability pore opening (32).

Effect of EGCG on mitochondrial homeostasis

Several reports propose that mitochondria may play a pivotal role in the beneficial effects of EGCG. EGCG may mediate diverse mitochondrial functions associated to mitochondrial biogenesis, ATP synthesis, alterations in cell cycle, and apoptosis (33,34). Accumulation of EGCG in mitochondria, scavenging of ROS, and chelation of transition metals have been proposed as plausible mechanisms of EGCG-mediated protection against neuronal apoptosis (35). EGCG protected cisplatin-induced nephrotoxicity in mice by decreasing mitochondrial oxidative/nitrative stress, mitochondria-mediated deregulation of electron transport chain and mitochondrial antioxidant enzymes (36).

Objectives

1. To investigate the role of As III on modulation of mitochondrial dynamics mediated by mitoNEET, NAF-1 in bronchial epithelial cells, BEAS-2B.

2. To study the effect of EGCG against As III-induced mitochondrial toxicity, if any

Materials and methods:

Cell culture

The normal bronchial epithelial cells, BEAS-2B were routinely cultured in DMEM with glutamine XL (Quality Biological, Gaithersburg, MD, USA, Catalog No. 112-300-101), 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA, Catalog No. PO781-100 ml) in a humidified atmosphere of 5% CO_2 at 37°C.

Treatment

The cells were treated with wide range of As III (Toronto Research Chemicals, Toronto, Canada, Catalog No. S080868) concentrations (0.05 to 1000 µM) for varied time periods (24-72 h). Similarly, EGCG (MedChem Express, Monmouth Junction, NJ, USA, Catalog No. HY-N2228/CS-0019551) was studied for a series of concentrations ranging from 0.1-500 µM for 24 h. Subsequently As III (50/100/200 µM) and EGCG (10 µM and 50 µM) were selected for further studies.

The combination As III and EGCG were studied in two different modes as follows:

- ***i) Simultaneous treatment***: The cells were treated with both As III (50/100/200 µM) and EGCG (10/ 50 µM) for a period of 24 h.
- ***ii) Pretreatment***: The cells were treated with EGCG (10/50 µM) for 24 h, washed and treated with As III (50/100 µM) for next 24 h

Cell viability assays

Cell viability was assessed by both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [Sigma, St. Louis, MO, USA, Catalog No. M2128-1G] and cell counting kit 8 (CCK8) [abcam, Cambrige, UK, Catalog No. ab228554) assays.

For MTT assay exponentially growing cells (1×10^4) were seeded in 96-well plates. After a 24 h of growth, the cells were treated with different concentrations of As III/EGCG for varied treatment periods. Separate lanes for time-matched control, i.e., only BEAS-2B cells, and vehicle control (0.1% DMSO) were also used. The MTT solution (0.5 mg/mL) was added to each well and incubated for 4 h to react with the mitochondrial dehydrogenases of viable cells. The MTT-formazan product was dissolved in DMSO and the absorbance was measured at 570 nm in a microplate reader (BioTek EPOCH2, Agilent, Santa Clara, CA, USA) using software Gen5 2.09. The cell viability (%) was calculated by treating the O.D of control as 100%.

For CCK8 assay BEAS-2B cells were first counted, and approximately 1×10^4 cells per well were seeded in a 96-well cell culture plate (Santa Cruz, Dallas, Tx, USA, Catalog No. sc-204447). Then, after incubation at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h, the culture medium was replaced by a series of concentrations of As III/EGCG for different time periods (as mentioned under treatment). Five replicates were made for each measurement. Finally, 10 µL of CCK8 was added into each well, and OD at 450 nm was measured after 2 h using a microplate reader (BioTek EPOCH2, Agilent, Santa Clara, CA, USA) and software Gen5 2.09. The cell viability (%) was calculated by treating the O.D of control as 100%.

RNA isolation

BEAS-2B cells were counted and approximately 10^6 cells per well were seeded in 6-well culture plates (Santa Cruz, Dallas, TX, USA, Catalog No. sc-204443). Then, after incubation at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h, the culture medium was replaced by As III/EGCG/As+EGCG for 24 h. Subsequently RNA was isolated using Tri-Reagent (Zymo Research, Irvine, CA, USA, Catalog. No. R2050-1-50) and Direct-zol RNA Mini Prep kit (Zymo Research, Irvine, CA, USA, Catalog. No. R2050) according to manufacturer's protocol. The absorbance ratio of 260/280 for purity check of RNA and quantitation of RNA was done using spectrophotometer (Picodrop Ver 3.1.4, Saffron Walden, United Kingdom) and software Picodrop spectrometer application ver 3.1.4.0.

One step real-time PCR

The RNA samples of both treated and control were subjected to one step real-time PCR using primers against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [FP- 5'AACGGATTTGGTCGTATTGGGC 3', RP- 5' AGGGATGATGTTCTGGAGAGCC 3'], *CISD1* [FP- 5'TCCAGAAAGACAACCCCAAGA 3', RP- 5' GTTTTGTGTGAGCCCCATCA 3'] and *CISD2* [FP- 5' ATATTCGAGATATTTGGACCTGTTG 3', RP- 5' CTCTGGTGGTGGTTCCTGAT 3'] with Abscript II One Step Sybr Green RT-qPCR kit (ABclonal, Woburn, Ma, USA, Catalog. No. RK20404). The qRT-PCR was conducted according to manufacturer's protocol [reverse transcription 42°C, 5 min, 1 cycle; pre denaturation 95°C, 1

min, 1 cycle; circular reaction 95°C, 5s and 60°C, 30-34 s, 40 cycles and melt curve according to automatic instrument setting] using QuantStudio 6Flex (Applied Biosystems, Life Technologies, Waltham, MA, USA). All samples were run in triplicate in each experiment and the values were normalized against the levels of GAPDH mRNA. The relative level of gene expression was determined by the comparative threshold cycle (Ct) method was based on the average Ct value of the gene of interest and the internal control from the triplicate set of samples (ΔCt). Next, the ratio of ΔCt treated vs untreated samples was deduced (ΔΔCt). A log2^(-ΔΔCt) value expressed as the fold change was graphically plotted for each of the target genes.

Gene expression analysis from public domain

The Gene Expression Omnibus (GEO) dataset GSE31210 was selected for this study. The dataset was accessed and analyzed using the R programming language.
To investigate differential gene expression, the R package 'limma' (Linear Models for Microarray Analysis) was employed. The expression data from GSE31210 was loaded using the GEOquery package, and pre-processing steps, such as background correction, normalization, and log2 transformation, were performed to ensure data quality and comparability. Following data pre-processing, the 'limma' package was utilized to identify genes that exhibited significant differential expression between relevant groups or conditions. The obtained results were analyzed for statistical significance using empirical Bayes statistics. Among the differentially expressed genes, three genes, namely *CISD1* and *CISD2* were selected for further analysis based on their biological relevance to our research objectives. These genes have been implicated in various biological processes and have shown potential prognostic significance in previous studies.

Statistical analysis

The statistical significance between treated and control groups was determined using the one-way Analysis of Variance (ANOVA) followed by the Dunnett *t*-test, where the p-value was set at 0.05 to check the statistical difference between groups. Dunnett's *t*-test treats one group as a control and treats all other groups against it. All results were computed and analyzed using the SPSS statistical software package 20.0 (SPSS, Chicago, IL, USA).
The survival analysis was performed using the R package 'survival.' The gene expression data for *CISD1* and *CISD2* were extracted from the GSE31210 dataset. Simultaneously, the corresponding survival data, including the survival time and status (e.g., deceased or alive), were obtained, if available. The survival time and status data were used to create a survival object using the 'Surv' function in the 'survival' package. Subsequently, a Cox proportional hazards model was applied to assess the association between gene expression levels of *CISD1*, *CISD2*, and survival outcomes. The Cox model was fitted using the 'coxph' function from the 'survival' package. This model allowed us to estimate the hazard ratios and assess the statistical significance of each gene's impact on survival. A p-value threshold, typically set at 0.05, was employed to determine statistical significance.

Results:

Effect of As III on BEAS-2B cells

A logarithmic range of As III concentrations (0.1-100 µM) was investigated for the cytotoxic effect on BEAS-2B cells for different time periods (24-72h). Cell viability was checked with both MTT assay and CCK-8 assay. Arsenite exhibited a biphasic effect with proliferation at lower concentrations (1-10 µM/24 h and 1 µM/48h) and cytotoxicity at higher concentrations (Fig. 1a, b). With the increase in As III concentrations (above 10 µM) and treatment periods (48-72 h) the cytotoxic effect became more pronounced and the cell viability decreased (Fig. 1a). The IC_{50} of As III was 15.05 µM at 48h.

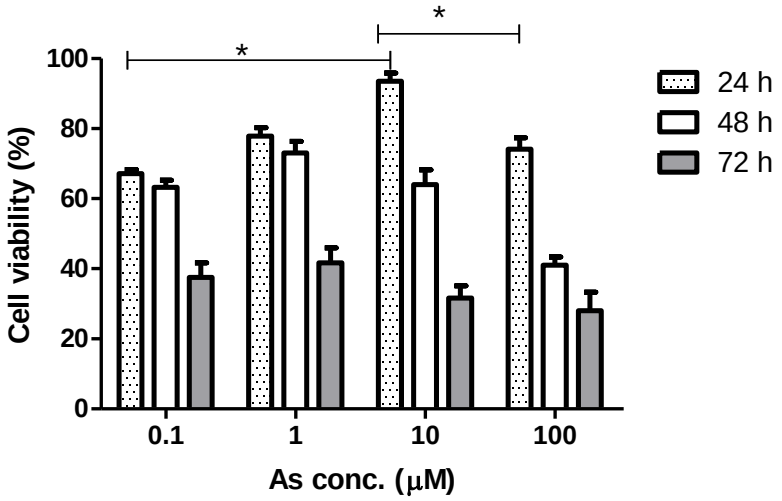

Fig.1a: Cell viability of BEAS-2B cells at varied concentrations of As III (0.1-100 µM) and different time periods (24 -72 h). (*$p<0.05$)

Since we were interested to investigate the acute effect of As III we further selected a wide dose range (0.05-1000 µM) at 24 h (Fig. 1b). As III induced cytotoxicity increased in a dose dependent manner from 50-500 µM. Since at As III (500 µM) decreased cell viability greater than 80% therefore for further combination studies As III (50, 100 and 200 µM) were selected.

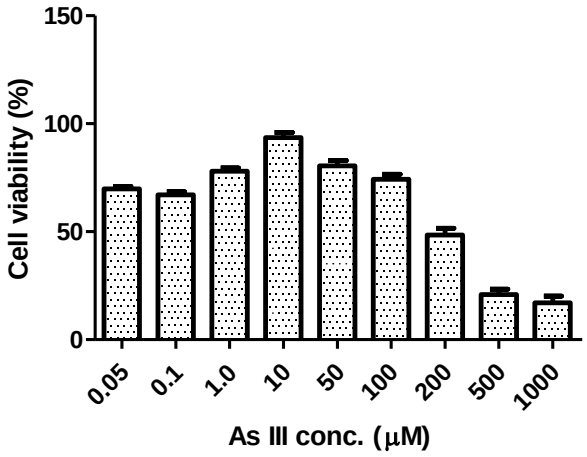

Fig. 1b: Cell viability of BEAS-2B cells at varied concentrations As III (0.05-1000 µM) for a treatment period of 24h.

Effect of EGCG on BEAS-2B cells

A wide range of EGCG concentrations (0.1-500 µM) was studied for a treatment period of 24 h to explore its cytotoxicity on BEAS-2B cells. EGCG did not induce any cytotoxicity, rather it induced cell proliferation which was evident with majority of the treatment concentrations (Fig.2). The cell viability more or less followed a plateau pattern. For further combination studies the concentrations of EGCG 10 and 50 µM were selected.

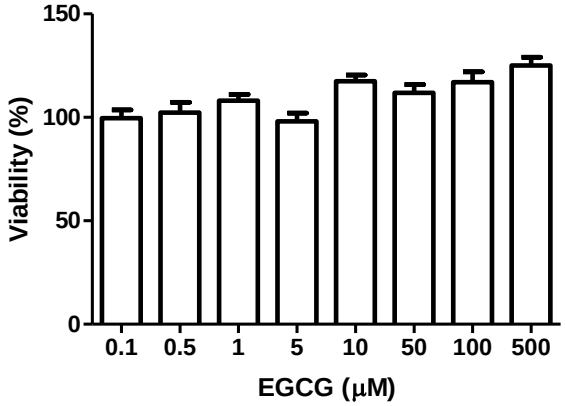

Fig.2: Viability of BEAS-2B cells with EGCG (0.01-500 µM) for a treatment period of 24 h

Protective effect of EGCG against As III-induced cytotoxicity

The simultaneous treatment of EGCG (10 and 50 µM) along with As III (50 µM) for 24 h afforded protection against As III-induced cytotoxicity. The viability of BEAS-2B cells which was reduced to 80% with As III 50 µM was significantly increased to 88% and 95% by EGCG 10 and 50 µM respectively (Fig.3). However, the cell viability which decreased to 74% by As III

(100 µM) could not be recovered with simultaneous treatment of EGCG (10 µM). Only EGCG (50 µM) was effective against As III (100 µM) and increased the cell viability to 87% (Fig.3). Therefore, further gene expression studies were done with EGCG (50 µM)). Cytotoxic insult of As III (200 µM) could not be tackled by EGCG (10 and 50 µM) (Fig. 3).

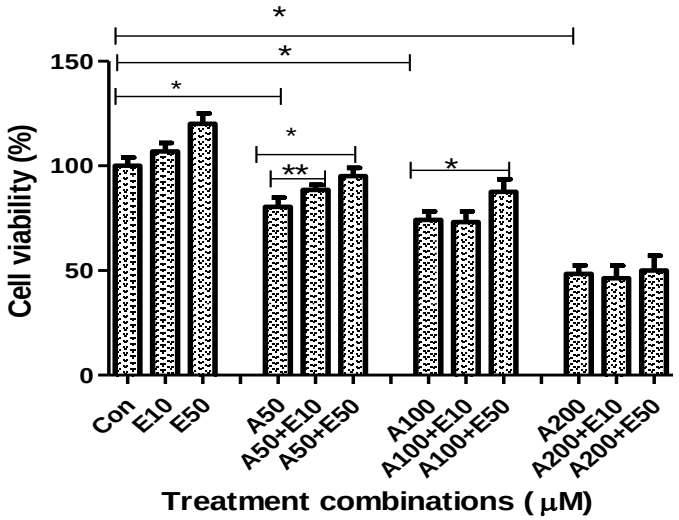

Fig.3: Effect of simultaneous treatment of EGCG (10 and 50 µM) with As III (50, 100, 200 µM) for 24 h on cell viability of BEAS-2B. [E: EGCG; A: As III, *p<0.01, **p<0.05]

Effect of pre-treatment with EGCG against As III-induced cytotoxicity in BEAS-2B cells

Pre-treatment of BEAS-2B cells with EGCG (10 and 50 µM) induced significant cell proliferation. However, EGCG pre-treatment for 24 h could not afford any beneficial effect against As III-induced cytotoxicity (Fig.4).

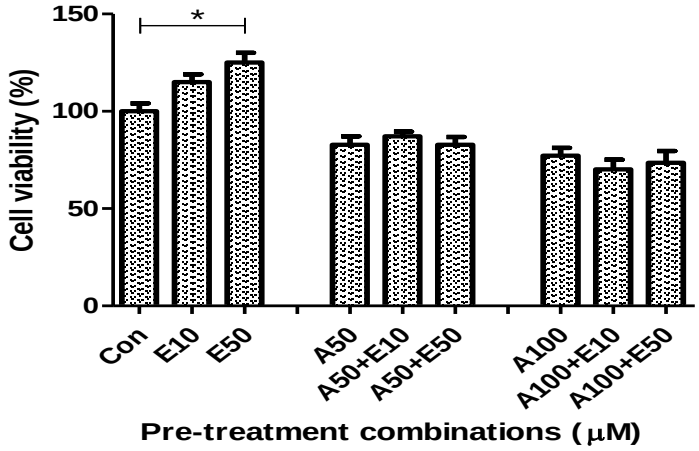

Fig.4: Effect of pre-treatment with EGCG (10 and 50 µM) against As-III (50 and 100 µM)-induced cytotoxicity in BEAS-2B cells. [E: EGCG; A: As III, *p<0.01]

Modulation of mitochondrial NEET proteins with As III and EGCG

Since we were interested to study the mitochondrial dynamics, we further investigated the effect of As III (50/100 µM), EGCG (50 µM) and As III (50/100 µM) + EGCG (50 µM) on transcript profile of *CISD1* and *CISD2*. Both *CISD1* and *CISD2* genes were upregulated in a concentration dependent manner with As III (50/100 µM). Monotherapy of BEAS-2B cells with EGCG (50 µM) too upregulated the same genes. However, the extent of activation of the *CISD1* (Fig.5a) and *CISD2* (Fig.5b) genes was much more pronounced when EGCG (50 µM) was used in combination with As III (50/100 µM).

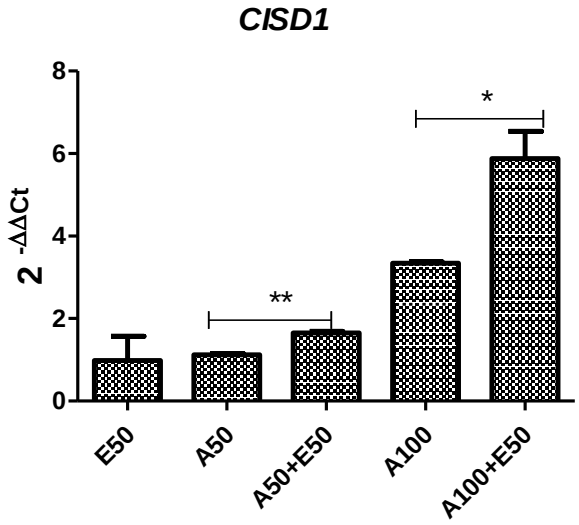

Fig. 5a: Modulation As III (50/100 µM)-induced expressions of *CISD1* by EGCG (50 µM) in BEAS-2B cells. [A: As III, E: EGCG, *p<0.001, **p<0.05]

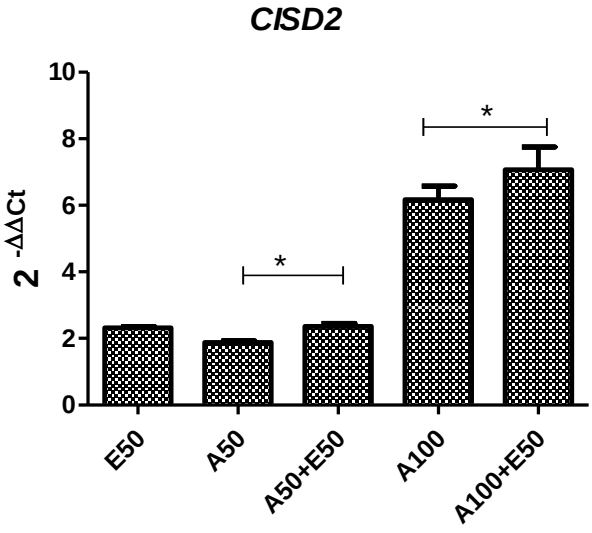

Fig. 5a: Modulation As III (50/100 µM)-induced expressions of *CISD2* by EGCG (50 µM) in BEAS-2B cells [A: As III, E: EGCG, *p<0.05]

Aberrant expression of CISD1 and CISD2 in lung adenocarcinoma

Since chronic As III exposure is frequently associated with lung cancer, we further investigated the expression profile of *CISD1* and *CISD2* genes in lung adenocarcinoma. We analyzed the dataset GSE31210 which comprised of a total of 226 tumor samples and 20 normal samples. Both *CISD1* and *CISD2* mRNA were observed over expressed in lung adenocarcinoma tissue samples compared with their adjacent normal counterparts (Figs 6a and 6b). The lung adenocarcinoma tissues with high expression of *CISD1* and *CISD2* had poor prognosis as evident from Kaplan Meier survival plots in Fig 5c and 5d respectively.

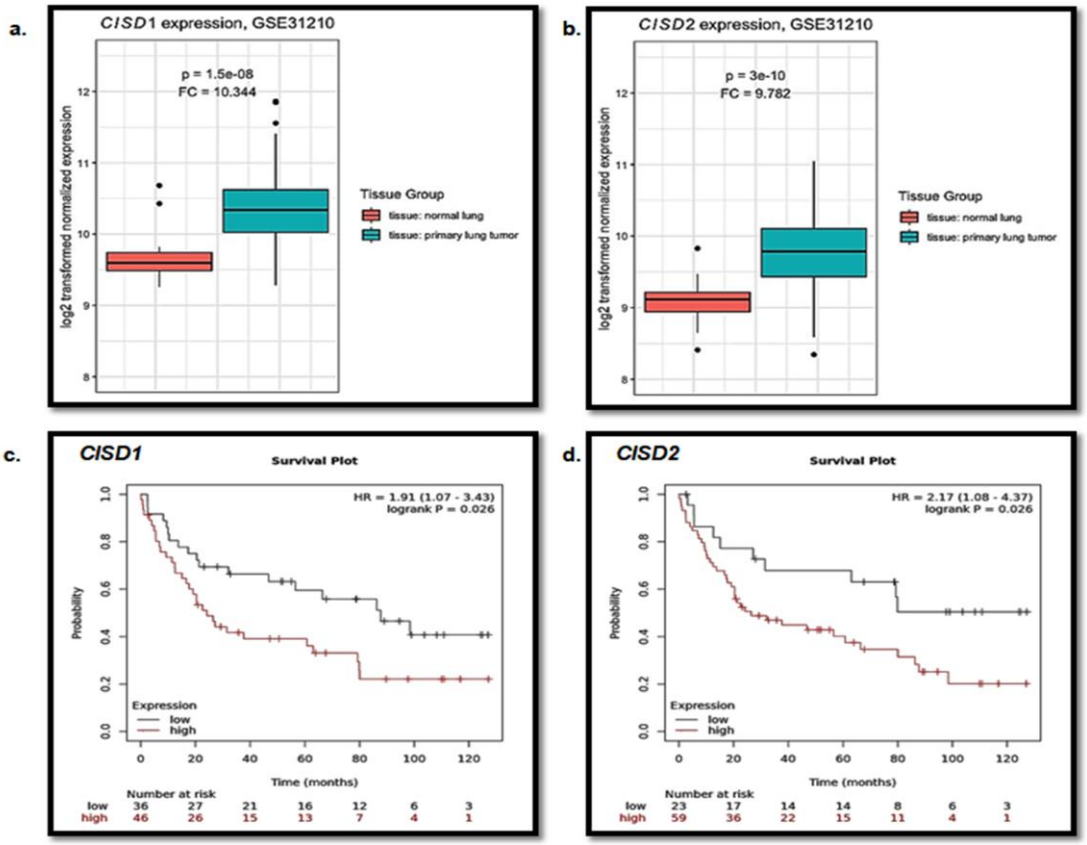

Fig. 6: Aberrant expression of *CISD1* and *CISD2* in lung adenocarcinoma was associated with poor prognosis. Elevated expression of *CISD1*(a) and *CISD2* (b) in lung adenocarcinoma tissues in comparison to adjacent normal counterparts. High expression of *CISD1*(c) and *CISD2* (d) were associated with poor prognosis as evident from Kaplan Meier survival plots.

Discussion:

Arsenic is a naturally occurring metalloid which is an established human carcinogen (1). Over 200 million people in 70 different countries across the globe are exposed to arsenic (above the WHO provisional guideline of 10 µg/L) either through groundwater contamination or through

food chain (2). Even in India, the arsenic menace has engulfed the whole of the Indo Gangetic plain (3). Groundwater arsenic have been frequently associated with lung cancer (6,37). It is well established that people exposed to high arsenic levels (>100 µg/L) have increased risk of developing lung cancer (38). Few reports have shown that people are vulnerable to lung cancer risk even at low to moderate levels (10 to <100 µg/L) of arsenic exposure (5). However, the mechanism of arsenic-caused lung cancer is yet to be elucidated. Emerging evidences suggest that arsenic inflicts a major perturbation of mitochondrial dynamics by ROS generation and increased Ca^{+2} signaling (31,39). EGCG, the principal green tea polyphenol has been well documented for its protective effect against heavy metal toxicity (40). Therefore, in this study we were interested to investigate the role of As III on modulation of mitochondrial dynamics mediated by mitoNEET, NAF-1 in bronchial epithelial cells, BEAS-2B. Further we explored whether EGCG could modulate the As III-induced mitochondrial toxicity, if any.

The association of arsenic with lung cancer has been observed to be dependent on the dose and duration of exposure (2). Arsenic has been reported to induce biphasic effect - at lower concentrations it activated cell proliferation and at higher concentrations it induced cell death by manipulating the phosphoinositide-3 kinase/protein kinase B/mammalian target of Rapamycin pathway (41). In consilience with these studies, we observed that low concentrations of As III (1-10 µM) elicited proliferation whereas higher concentrations (50-1000 µM) induced death of BEAS-2B cells within 24 h. The As III-induced cytotoxicity in BEAS-2B cells followed a concentration and time dependent pattern. EGCG has been reported to protect against As III-induced genotoxicity and oxidative stress in several *in vitro* (42,43) and *in vivo* models (11,44). In concordance with these studies, we observed that EGCG (50 µM) was able to improve the viability of As III (50/100 µM) treated BEAS-2B cells. Above As III 100 µM the protective effect of EGCG was outweighed by As III-induced cytotoxicity.

Arsenic is well known inducer of oxidative stress and mitochondrial dysregulation (30). Iron accumulation due to mitochondrial dysfunction leads to oxidative damage and ROS. Oxidative stress is intertwined with activation of mitoNEET and NAF-1which are critical for maintenance of iron and ROS homeostasis (24). Studies have shown that EGCG may antagonize oxidative stress by regulating diverse mitochondrial functions associated to mitochondrial biogenesis, ATP synthesis, alterations in cell cycle, and apoptosis (33,35). However, the effect of EGCG against arsenic-induced mitochondrial toxicity is yet to be explored. In this study we have initiated to investigate the impact of EGCG against As III-induced alteration of mitochondrial NEET proteins. We observed that with high As III insult (50/100 µM) both the genes *CISD1* and *CISD2* governing mitoNEET and NAF-1 proteins respectively were activated. However, the extent of activation of *CISD1* and *CISD2* were not enough to protect the cells from As III-induced cell death which might have been due to excessive generation of ROS. EGCG when administered alone also activated the *CISD1* and *CISD2* genes which might have facilitated proliferation of BEAS-2B cells. However, the combination of EGCG (50 µM) with As III was effective in further upregulating *CISD1* and *CISD2*. This robust activation of the NEET proteins by EGCG might have tackled the acute oxidative stress created by As III which in turn improved the viability of BEAS-2B cells.

However, overexpression of the same NEET proteins in cancer cells provide a pro survival advantage to them. Elevated expression of mitoNEET protected cancer cells by inhibiting

oxidative stress and ferroptosis (45). Similarly increased expression of NAF-1 inhibited oxidative stress and apoptosis in cancer cells (25). Additionally, it has been reported that this deregulation of ROS homeostasis by upregulation of *CISD2* may lead to poor prognosis of lung adenocarcinoma (17). On a similar note, we found that the *CISD1* and *CISD2* genes were upregulated in lung adenocarcinoma tissues which ultimately led to poor prognosis. However, how this deregulation of mitochondrial NEET proteins by As III may cause malignant transformation or initiate lung tumorigenesis is yet to be deciphered.

These are only preliminary results on acute exposure of As III and its amelioration by EGCG. Further investigations targeting protein expression of mitoNEET and NAF-1, mitochondrial respiration, mitochondria ROS generation, mitochondrial mass, apoptosis, autophagy etc. are required to establish the protective effect of EGCG against As III-induced mitochondrial toxicity in pulmonary microenvironment. Moreover, impact of chronic low exposure of As III on mitochondrial dynamics in bronchial epithelial cells will be another interesting area to explore.

Significant results:

- ✓ Acute exposure of As III for 24 h showed a biphasic response on cell viability of BEAS-2B. It increased cell proliferation at lower concentrations (1-10 µM) and induced cytotoxicity at higher concentrations (50-1000 µM).
- ✓ EGCG alone did not induce any cytotoxicity, instead exhibited cell proliferation across most of the concentrations (1, 10, 50, 100 µM etc.). EGCG triggered upregulation of *CISD1* and *CISD2* genes which might have facilitated proliferation of BEAS-2B cells.
- ✓ Simultaneous treatment of EGCG (50 µM) and As III (50/100 µM) for 24 h afforded significant protection against As III-induced cytotoxicity.
- ✓ As III (50 and 100 µM) upregulated mitochondrial [2Fe-2S] cluster genes *CISD1* and *CISD2* and administration of EGCG (50 µM) along with As III was effective in further upregulating the same. Robust overexpression of *CISD1* and *CISD2* by EGCG might have counteracted As III-induced oxidative stress by maintaining iron and ROS homeostasis. This might have improved the viability of the BEAS-2B cells.
- ✓ Over expression of same NEET proteins provide survival advantage to cancer cells. Gene expression analysis from public domain database GSE31210 revealed that *CISD1* and *CISD2* genes are highly activated in lung adenocarcinoma and are associated with poor prognosis.

Future plan of work:

- The role of EGCG against As III-induced acute mitochondrial toxicity will be further delved with assays targeting mitochondrial respiration, mitochondria ROS generation, mitochondrial mass, apoptosis, autophagy etc.

- The environmental relevant concentrations of As III 0.05 µM (< WHO recommended limit of 10 µg/L), 0.25 µM (low level arsenic exposure which prevails in many Indian aquifers i.e., >10 < 50 µg/L) and 2.5 µM (>300 µg/L which is usually associated with skin lesions and skin cancer) will be investigated for toxicity with long term chronic exposure on the BEAS-2B cells. The role of As III on modulation of mitochondrial

dynamics and endoplasmic reticulum stress in bronchial epithelial cells will be studied during chronic As III exposure.

- The role of EGCG will be again explored against As III-induced chronic mitochondrial and endoplasmic reticulum stress, if any in BEAS-2B cells.

Other academic activities

Teaching: On 24th April 2023 Pharm.D. class was taken on metal toxicity encompassing lithium, iron, lead, arsenic, and mercury

ii) Proposed utilization of the experience in India:

The knowledge gained from this fellowship will be utilized for studying arsenic-induced mitochondrial toxicity and endoplasmic reticulum-stress in pulmonary and systemic microenvironment of human population who are chronically exposed to low level and high level arsenic through groundwater contamination. This will give a better insight of the mechanism of arsenic-induced lung cancer among humans and will further help to formulate proper interventional strategies with tea phytochemicals.

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