Study on the efficacy of dietary compounds to detoxify toxic metals *in vitro*

(試験管内で毒性金属を解毒するための食餌中化合物の有効性に関する研究)

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Summary

In the era of globalization, environmental pollution has become a major human health threat. Due to heavy industrial activities, aggressive agricultural practices, unplanned mining, and natural disasters are contributing negatively to the environmental system. Almost every kind of pollutants in turn reaches to the food chain and creates burden for the human health with numerous diseases manifestations. In recent years, substantial human health risks were reported due to toxic metals through consumption of contaminated food stuffs, fishes, fruits, and water. We have also reported human health risk due to drinking from the arsenic (As) contaminated groundwater and from toxic metals (cadmium (Cd), chromium (Cr), and lead (Pb)) contained cow milk in Bangladesh. Moreover, the toxic metals induced health hazards are very concerning and so far there is no proper detoxification method available for biological systems. Therefore, in this research an environmental medicine approach to detoxify toxic metals was done using molecular and *in vitro* cell biological techniques. For instance, essential trace elements such as zinc (Zn) and selenium (Se) have been used to detoxify toxic metals such as Cd and As using PC12 cells which is well known as model cell line for fundamental molecular toxicology study. In addition, to that promising dietary supplements such as α-lipoic acid and dihydrolipoic acid (ALA and DHLA) have also been investigated against the metal-induced cytotoxicity in different cell lines including PC12 cells. The research hypothesis was 'dietary compounds may have potentials to detoxify toxic metals in cellular systems'.

In Chapter 2, Zn has been applied to detoxify Cd in PC12 cells following a simultaneous exposure pattern. Cd is one of the heavy toxic metals, which is also an inducer of cellular necrosis and apoptosis. Conversely, Zn is an essential trace element known to inhibit apoptosis induced by toxicants including Cd both *in vitro* and *in vivo*. However, the mechanism of Zn-mediated protection from Cd-induced cytotoxicity is not established yet. Thus, it was aimed to investigate the effects of Zn on Cd-induced cytotoxicity and apoptosis using PC12 cells. Cell viability (Fig. 1) and DNA fragmentation (Fig. 2) assays in PC12 cells exposed to Cd and/or Zn revealed that Cd (5 and 10 μM) alone induced significant cell death, and co-exposure to Zn (5, 10, and 100 μM) for 48 h had a

protective effect. Assessment of intracellular glutathione (GSH) levels and lactate dehydrogenase (LDH) activity suggested that Cd (10 µM)-induced oxidative stress

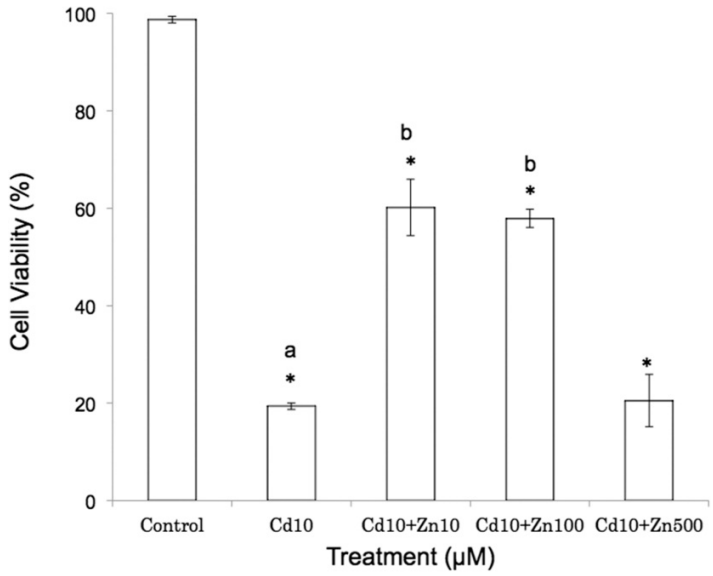

*Fig. 1 Viability of PC12 cells with and without Cd^{2+}/Cd^{2+} + Zn^{2+} treatment at different concentrations after 48-h incubation measured via trypan blue staining method. Each experiment was conducted five times independently to ensure biological reproducibility. Cells co-exposed to 10 µM Cd^{2+} and 10–500 µM Zn^{2+}. Error bars indicate the mean ± SEM (n = 5), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between [a] and [b].*

and disrupted cell membrane integrity. Addition of Zn (10 and 100 µM) reduced Cd-mediated cytotoxicity. Moreover, changes in expression of the apoptotic factors, Bax, Bcl-2, Bcl-x, and cytochrome c were measured *via* western blotting analyses (Fig. 3), and expression of caspase 9 was detected *via* reverse transcriptase polymerase chain reaction (RT-PCR). Western blots showed that Zn (10 and 100 µM) suppressed Cd-induced apoptosis (10 µM) by reducing cytochrome c release into the cytosol, and downregulating the pro-apoptotic protein, Bax. In addition, expression of caspase 9 was lower in Cd (5 µM)-treated PC12 cells when co-treated with Zn (2 and 5 µM). These findings suggest that the effective inhibition of Cd-induced apoptosis in PC12 cells by Zn might be due to suppression of mitochondrial apoptosis pathway and inhibition of Cd-induced production of ROS.

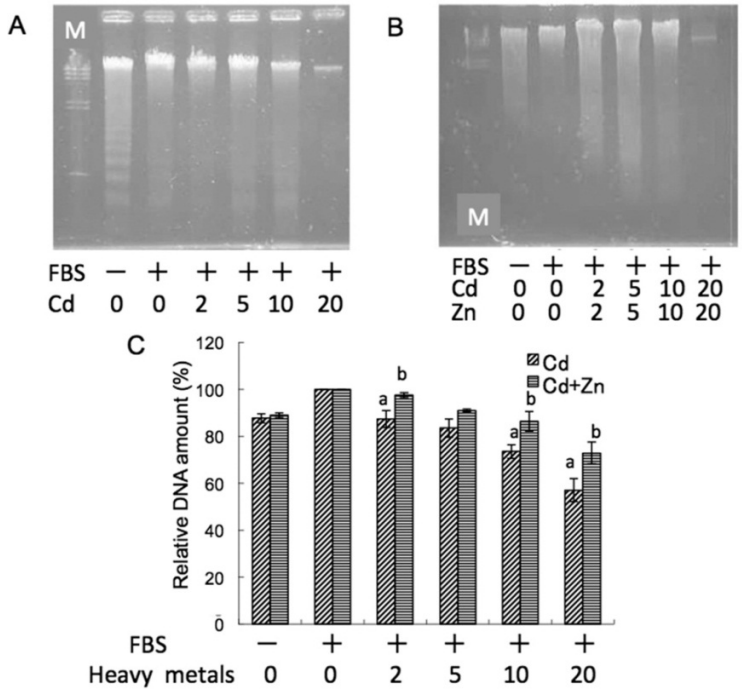

Fig. 2 Agarose gel electrophoresis of genomic DNA extracted from PC12 cells cultured in the medium with and without Cd^{2+}/Cd^{2+}+Zn^{2+} for 48 h. It was conducted four times to confirm biological reproducibility. A) DNA from the cells treated with 0-20 μM Cd^{2+}, B) DNA from the cells treated with 0-20 μM Cd^{2+} and 0-20 μM Zn^{2+}, C) the fluorescent intensity of genomic DNA extracted from PC12 cells cultured in the medium for 48 h after treatment with 0-20 μM Cd^{2+} and 0-20 μM Zn^{2+}. Lane M in the Figs. A and B means λ DNA digested with HindIII for the marker. The values are calculated following formula; (DNA/ DNA and DNA ladder) ×100. Error bars indicate mean ± S.E.M. (n=4). Significant difference between a and b is observed (P<0.05).

After Zn and Cd experiments, another combination of two metalloids (As and Se) was investigated. As is a well known toxicant responsible for human diseases, on the other hand, Se is an essential trace element with significant chemopreventive effects, anticancer potentials and antioxidant properties. Although previous studies have reported antagonism/synergism between As and Se in biological systems, the biomolecular mechanism is still inconclusive. Therefore, we hypothesized that co-exposure of Se with As may have suppressive effects on As-induced cytotoxicity in PC12 cells. Upon Se co-exposure with As increases cell viability, and suppresses As-induced oxidative stress (Fig. 4 and Fig. 5). As-induced DNA fragmentation was also reduced by co-exposure of Se in PC12

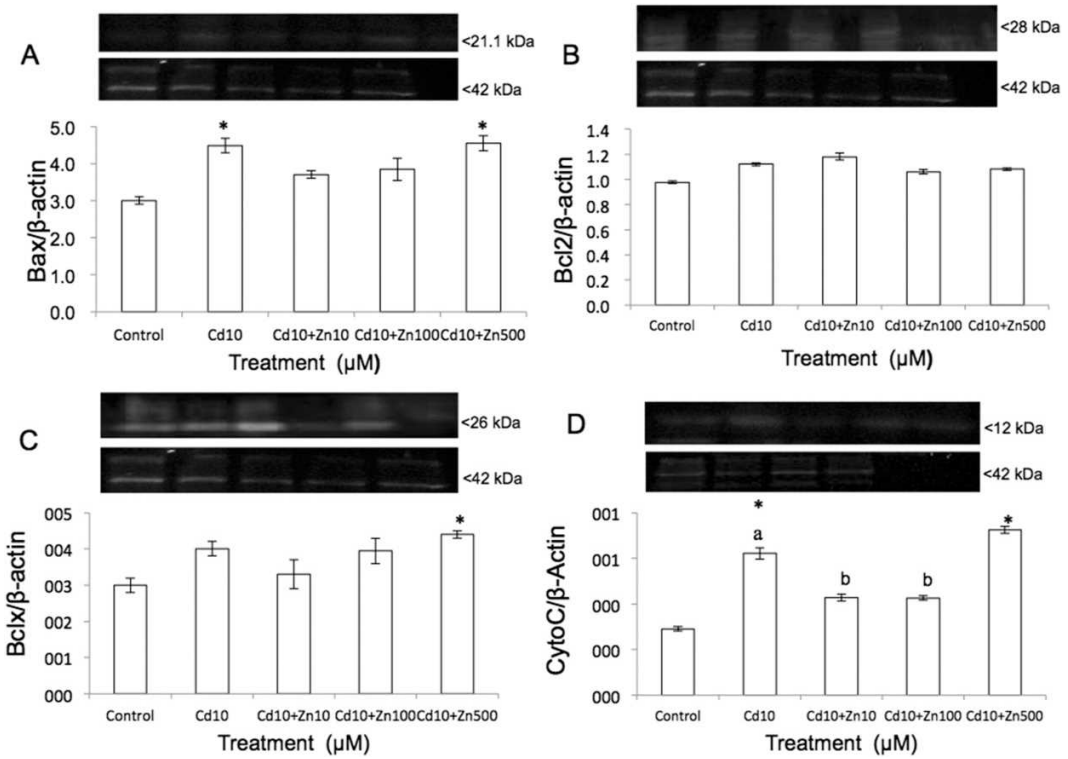

*Fig. 3 Western blot analyses for the relative contents of A; Bax, B; Bcl2, C; Bclx(L) and D; cytochrome c 48 h after treatment with and without Cd^{2+}/Cd^{2+}+Zn^{2+} treatment. Each experiment was conducted three times separately to ensure reproducibility. Immunostaining bands are present above the bar diagram for respective protein. M means Error bars indicate the mean ± SEM (n = 3), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.*

cells. Furthermore, the western blotting analyses revealed that simultaneous exposure of both metals significantly inhibited autophagic death which may further suppressed apoptosis through positively regulation of key proteins; p-mTOR, p-Akt, p-Foxo1A, p62, and expression of ubiquitin, Bax, Bcl2, NFκB, and caspase 3, although those are negatively regulated by As (Fig. 6 and Fig. 7). In addition, RT-PCR analysis confirmed the involvement of caspase cascade in cell death process induced by As, and subsequent inhibition by co-exposure of Se with As. The cellular accumulation study of As in presence/absence of Se *via* inductively coupled plasma mass spectrometry confirmed that Se effectively retarded the uptake of As in PC12 cells (Fig. 8). Finally, these findings imply that Se is capable to modulate As-induced intrinsic apoptosis pathway via enhancement of mTOR/Akt

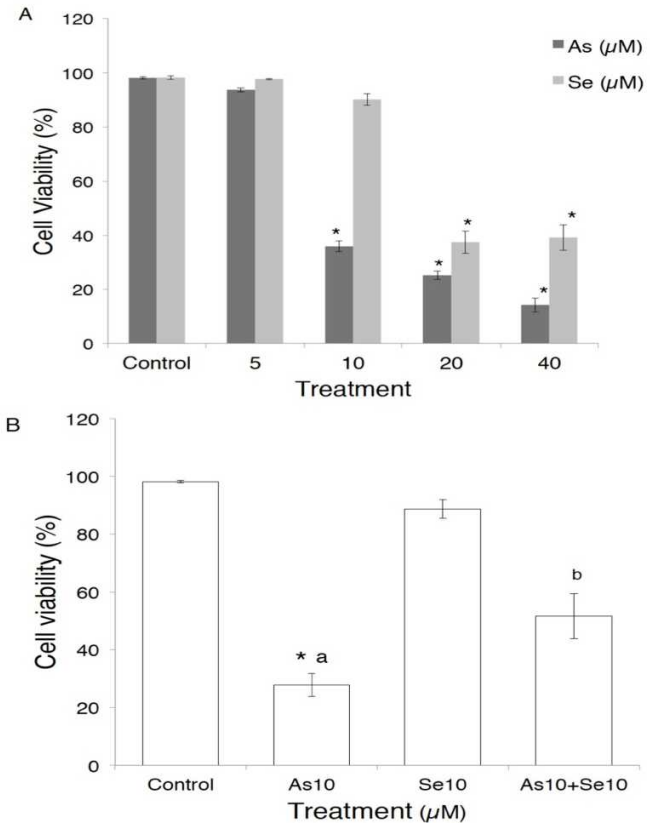

*Fig. 4 Viability of PC12 cells using trypan blue exclusion method. (A) PC12 cells treated with different concentrations of $As^{3+}$and $Se^{4+}$for 48 h. (B) PC12 cells treated withAs^{3+} (10 µM) and Se^{4+} (10 µM) separately or combined for 48 h. Each experiment was conducted 6 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=6), asterisk * indicates significance at p<0.05 compared to the control group, and [a], [b] denotes significance at p<0.05 between groups.*

autophagy signaling pathway through employing antioxidant potentials and through inhibiting the cellular uptake of As in PC12 cells (Fig. 9). So, it can be concluded that homeostatic level of Se is dramatically essential to maintain the ionic balance in the body. In a situation where As exposure is prominent Se might be crucial to reduce the negative impacts of As in human health.

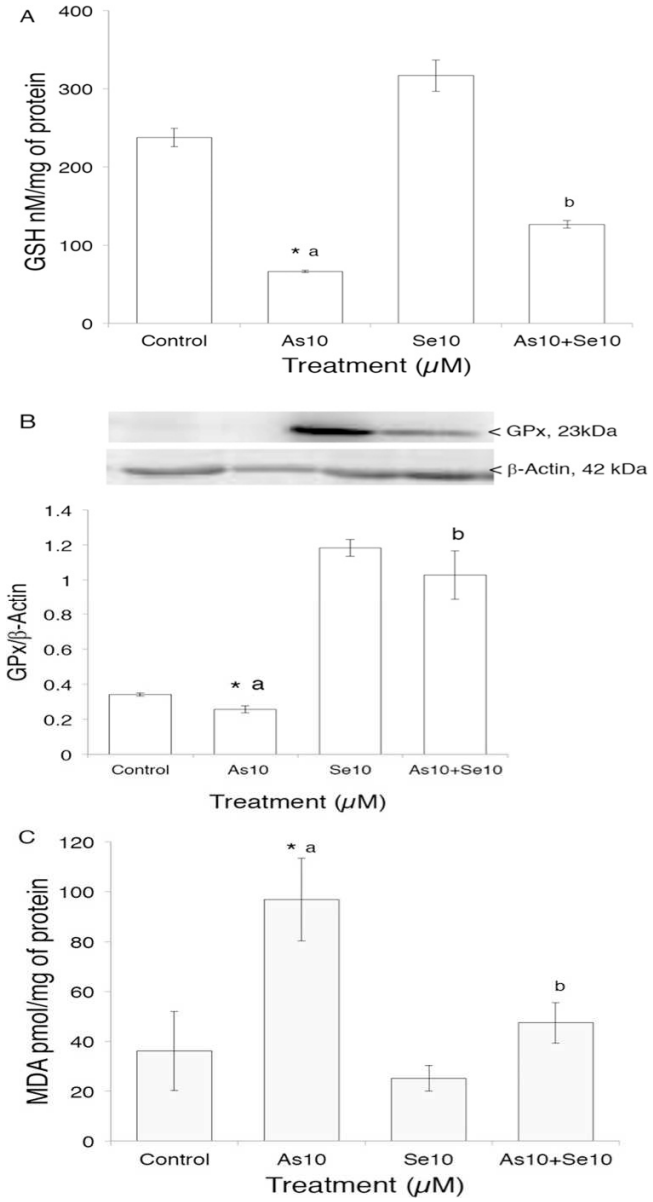

Fig. 5 Oxidative stress marker in PC12 cells after treatment with arsenic and selenium. (A) Intracellular glutathione (GSH) level in PC12 cells after exposure of As^{3+} and/Se^{4+} for 48 h via DTNB assay. (B) Glutathione peroxidase (GPx1) assay after treatment with As^{3+} and/Se^{4+} for 48 h through western blot analysis. (C) Lipid peroxidation level in PC12 cells after treatment with/without As^{3+} and/Se^{4+} for 48 h via malondialdehyde (MDA) assay. Each experiment was repeated 4 times to ensure biological reproducibility. Error bars indicate mean ± SEM (n=4), indicates significance at p<0.05 compared to control group and$^{a, b}$ indicate significance at p<0.05 between treated groups.*

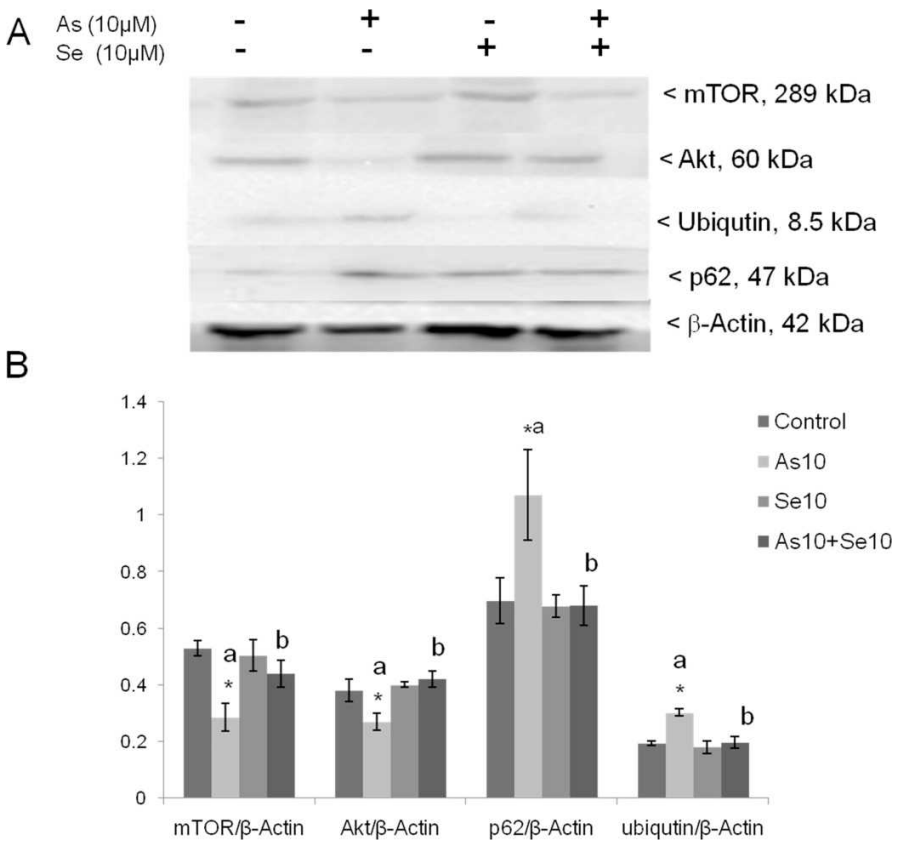

*Fig. 6 Western blot analysis for key proteins related to autophagy in PC12 cells after being exposed to As^{3+} and/ Se^{4+} for 48 h. (A)Immunoblotting for treatment with and/ without As^{3+}/Se^{4+}. This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. (B) Relative density of each protein band to β-Actin, error bars indicate mean ± SEM (n=3), * denotes significance at p<0.05 compared to control group; $^{a, b}$indicates significance at p<0.05 among treatment groups.*

Likewise, we have investigated the protective role of ALA and DHLA against As, Cd and Pb stress in PC12 and Caco-2 cells. We found that both ALA and DHLA have significant cytoprotective effects against As, Cd and Pb toxicities in PC12 as well as Caco-2 cells. Cell viability of both cells was improved upon combined exposure of ALA-metals/DHLA-metals

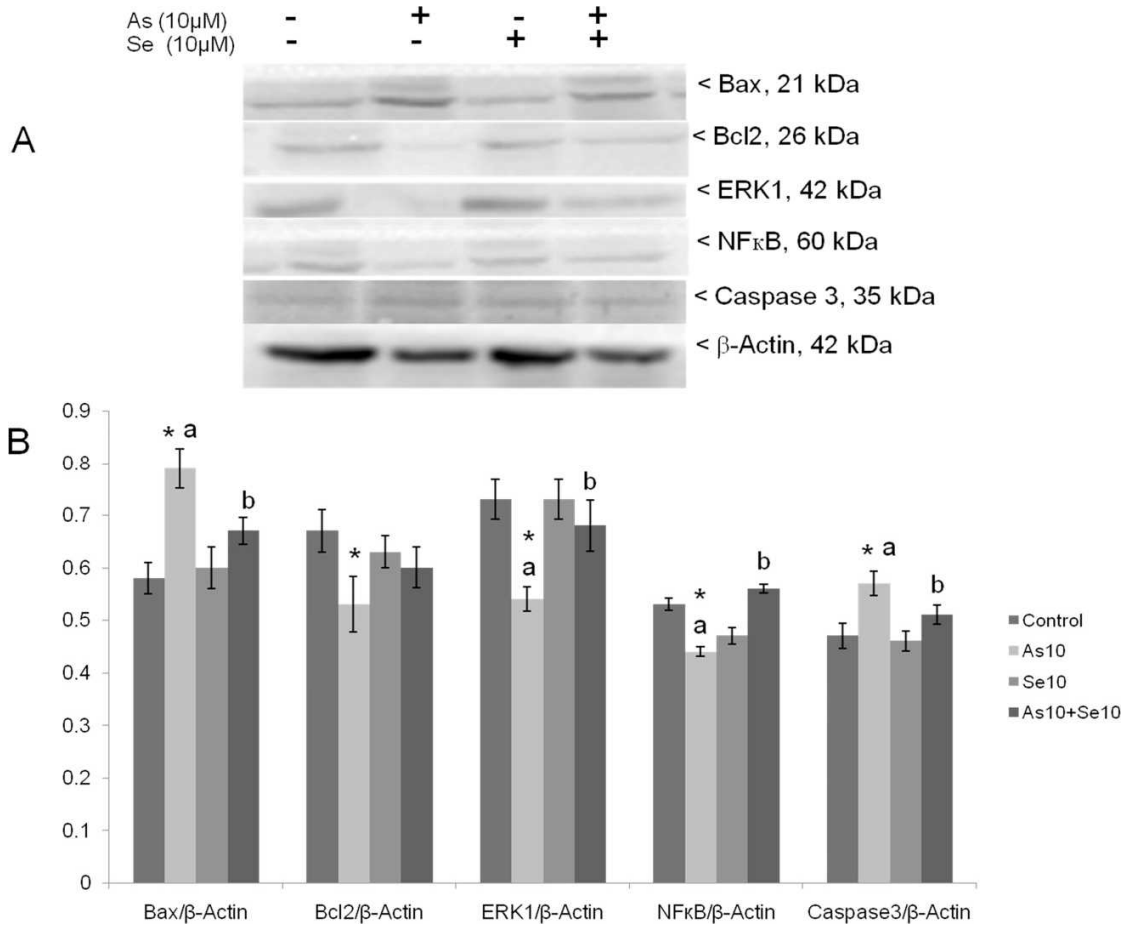

*Fig. 7 Western blot analysis for some apoptosis related proteins in PC12 cells after being exposed to As^{3+} and/ Se^{4+} for 48 h. (A)Immunoblotting for treatment with and/ without As^{3+}/Se^{4+}. This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. (B) Relative density of each protein band to β-Actin, error bars indicate mean ± SEM (n=3), * denotes significance at p<0.05 compared to control group; [a,b]indicates significance at p<0.05 between treatment groups.*

than the only metal-treated group. ALA showed cytoprotection at 250 μM concentration where as DHLA showed a similar effect at 50 μM. Boost up of the intracellular GSH level could be one of the mechanisms of the cytoprotection. We found a significant increase of GSH level in the co-treated group which was significantly lowered by the metal-treated group alone. Subsequently, DHLA (50 μM) showed potentials for the protection of DNA from possible damage caused by the metal exposure in PC12 cells. Significant damage of DNA was observed upon treatment with As, Cd and Pb. However, co-treatment with DHLA and metals lowered the damage intensity as visualized by agarose gel electrophoresis.

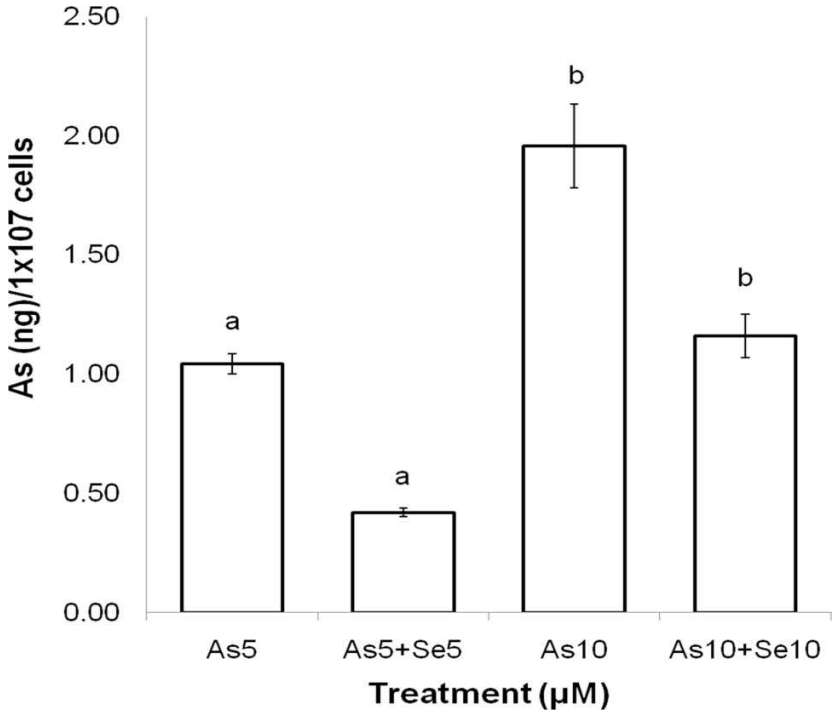

Fig. 8 Effects of selenium on PC12 cell uptake of arsenic via ICP-MS analysis. PC12 cells exposed to different concentrations of As^{3+} and Se^{4+} for 48 h. This experiment was carried out three times for reproducibility. Error bars indicate the mean ± SEM (n = 3), $^{a, b}$ denotes significance at p< 0.05 between groups.

Western blotting analysis reveals an upregulation of survival factor mTOR, Akt and downregulation of death marker Bax, cleaved PARP was induced by ALA in both PC12 and Caco-2 cells to protect the cells from As and Cd induced toxicity. ALA also induced Nrf2 upregulation thus to boost up the GSH defense to protect cells from oxidative damages. Similarly, DHLA also upregulated the expression of mTOR and Akt in PC12 cells in co-treated group with metals.

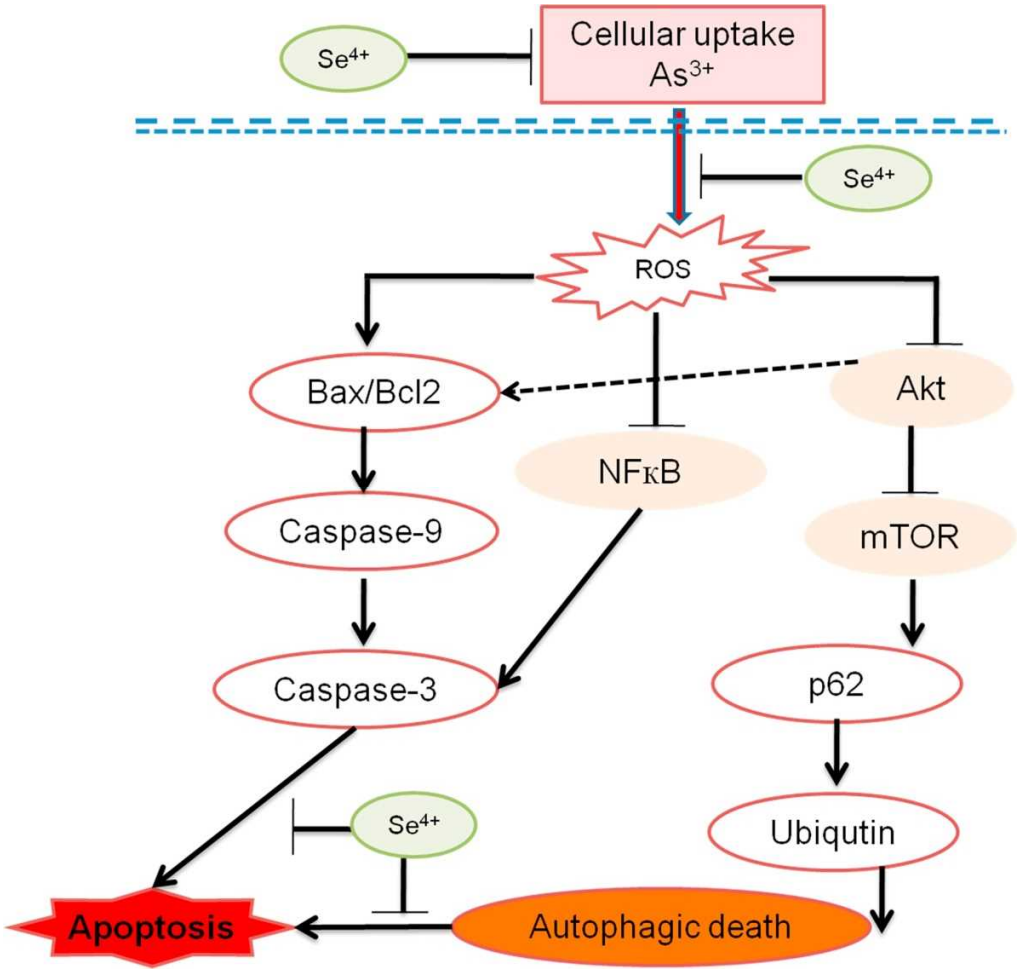

Fig. 9 A schematic diagram for potential molecular mechanism/s in PC12 cells upon arsenic and selenium co-exposure.

In conclusion, chronic ingestion of trace levels of toxic metals such as As, Cd, and Pb may possess serious health hazards. And deficiency of essential trace elements could aggravate the deleterious effects. However, proper dietary intake of essential trace elements and dietary supplements may reduce the toxic effects of metals. This study suggests beneficial role of Zn, Se and ALA/DHLA against toxic metals *in vitro*, and recommended for further investigation using animal model.

Reference articles:

1. **Md. Mostafizur Rahman,** Junki Ukiana, RU Lopez, MT Sikder, Takeshi Saito, Masaaki Kurasaki (2017). Cytotoxic effects of cadmium and zinc co-exposure in PC12 cells and the underlying mechanism. Chemico-Biological Interactions 269, 41-49. http://dx.doi.org/10.1016/j.cbi.2017.04.003

2. **Md. Mostafizur Rahman,** Rachael A. Uson-Lopez, MT Sikder, Gongxun Tan, Toshiyuki Hosokawa, Takeshi Saito, Masaaki Kurasaki (2018). Ameliorative effects of selenium on arsenic-induced cytotoxicity in PC12 cells via modulating autophagy/apoptosis. Chemosphere 196, 453-466. http://dx.doi.org/10.1016/j.chemosphere.2017.12.149