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Ebselen Mitigates Methylmercury-Induced Nephrotoxicity in NRK52E Cells

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Abstract

Mercury, a toxic environmental pollutant, is readily available in the biogeochemical cycle and poses serious health risks even at trace levels due to its tendency to bioaccumulate. Among its forms, methylmercury (MeHg) is the most toxic, capable of crossing both the placental barrier and blood-brain barrier. This study investigates the cellular toxicity of MeHg on normal rat kidney epithelial cells (NRK52E) and evaluates the protective potential of Ebselen, an organic selenium compound with antioxidant and metal-chelating properties. NRK52E cells were exposed to 0.5 µM, 1 µM, 5 µM and 10 µM concentrations of MeHg, both alone and in combination with 10 µM Ebselen. Multiple assays including MTT for cell viability, wound healing for cell migration, DCFDA for reactive oxygen species (ROS), real-time PCR for gene expression, and cell cycle analysis were performed to assess the extent of toxicity and protection. MeHg exposure led to a significant increase in ROS levels, accompanied by dysregulation of key oxidative stress and cell cycle-related genes such as cMYC, HIF-1α, VEGF, and MMP9. These molecular changes were associated with disrupted cell behaviour, impaired wound healing, and altered cell cycle progression, all indicating cellular toxicity. However, co-treatment with Ebselen significantly reversed these effects. ROS levels were reduced, gene expression patterns normalized, and cell cycle distribution improved. Ebselen's ability to restore cellular homeostasis demonstrates its strong protective effect against MeHg-induced nephrotoxicity. This study highlights the potential of Ebselen as a therapeutic agent for mitigating mercury-induced kidney cell damage. It emphasizes the importance of early detection of nephrotoxic effects and timely antioxidant intervention to prevent long-term renal impairment caused by environmental toxins like MeHg.

Keywords: Methylmercury, MTT assay, DCFDA-ROS, Ebselen, MMP9.

Introduction

Mercury (Hg), a globally prevalent environmental pollutant, poses significant risks to human health, particularly in its organic form, methylmercury (MeHg). Commonly found in contaminated fish and aquatic ecosystems, MeHg is well recognized for its neurotoxicity, but growing evidence has highlighted its deleterious effects on non-neuronal organs, including the kidneys, which play a vital role in mercury detoxification and excretion[1,2]. The renal proximal tubular epithelium is particularly susceptible to MeHg accumulation and injury due to its high mitochondrial content and active resorptive functions[3]. Upon cellular uptake, MeHg binds to sulfhydryl groups on proteins and glutathione, disrupting redox homeostasis, mitochondrial function, and triggering excessive reactive oxygen species (ROS) generation[4]. This cascade culminates in oxidative stress, inflammation, cytoskeletal disorganization, apoptosis, and necrosis[5]. In renal cells, this oxidative burden is linked to upregulation of stress-inducible genes like HIF-1α, VEGF, and MMP9, all of which contribute to tissue remodeling, angiogenesis, and hypoxia-induced responses[6].

With the limitations of conventional chelators (e.g., DMSA and DMPS) in mitigating cellular oxidative damage and their adverse side effects, there is increasing interest in identifying redox-active small molecules that provide organ protection through antioxidant and anti-inflammatory mechanisms[7]. Ebselen, a selenium-containing organic compound, mimics glutathione peroxidase (GPx) activity and possesses potent antioxidant, anti-inflammatory, and cytoprotective properties[8]. Previous studies have demonstrated that Ebselen can attenuate oxidative damage in models of neurodegeneration, ischemia-reperfusion injury, and drug-induced nephrotoxicity[9,10]. However, its role in ameliorating MeHg-induced renal cytotoxicity remains inadequately explored.

In this context, we employed NRK52E renal epithelial cells as a model system to investigate the protective potential of Ebselen against MeHg-induced nephrotoxicity.

Material and Methods

Cell Culture and Growth Analysis (Cell imaging): NRK52E renal epithelial cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic solution to ensure optimal growth conditions. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For experimental analysis, cells were divided into three treatment groups: (i)

Control (untreated), (ii) MeHg-treated group exposed to 1 µM methylmercury (MeHg), determined from preliminary dose-response analysis, and (iii) Ebselen co-treated group where Ebselen and MeHg were administered simultaneously.

Kidney Toxicity Panel (Molecular Analysis): To assess renal-specific cellular injury, cell culture supernatants were collected after experimental treatments and analyzed using the Bio-Rad Kidney Toxicity Multiplex Immunoassay Panel, which utilizes Luminex xMAP technology for simultaneous detection of multiple protein biomarkers. The panel included markers such as Kidney Injury Molecule-1 (KIM-1), Clusterin, Monocyte Chemoattractant Protein-1 (MCP-1), and Osteopontin, all of which are associated with epithelial injury, inflammation, and cellular stress in renal tissue.

MTT Cell Cytotoxicity Assay: The MTT assay was conducted to evaluate cellular viability and metabolic activity following different treatments. After a 48-hour exposure to MeHg and/or Ebselen, cells were incubated with MTT reagent at a final concentration of 0.5 mg/mL for four hours. During this period, metabolically active cells reduced the yellow MTT to insoluble purple formazan crystals via mitochondrial dehydrogenase activity. The formazan was then solubilized using dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader.

Calcein-AM Cell Membrane Integrity Assay: To determine live-cell membrane integrity, Calcein-AM staining was performed. NRK52E cells were incubated with 2 µM Calcein-AM, a non-fluorescent, cell-permeable dye that becomes fluorescent upon cleavage by intracellular esterases in viable cells, producing green-fluorescent calcein. Following dye incubation, fluorescence intensity was quantified using a microplate reader.

DCFDA-Based ROS Detection Assay: To measure intracellular reactive oxygen species (ROS), cells were subjected to the DCFDA assay. Cells were first incubated with 10 µM DCFDA (2',7'-dichlorofluorescin diacetate) for 30 minutes, allowing the dye to diffuse into the cells. Once inside, DCFDA was deacetylated by esterases and then oxidized by ROS to produce highly fluorescent dichlorofluorescein (DCF). The fluorescent signal was measured at excitation/emission wavelengths of 485/535 nm using a fluorescence plate reader.

Quantitative Real-Time PCR Analysis of Stress-Responsive Genes: Quantitative real-time PCR (qRT-PCR) was used to examine changes in gene expression related to oxidative stress and cellular damage. Total RNA was extracted from treated cells using TRIzol reagent, and complementary DNA (cDNA) was synthesized via reverse transcription using a commercially available kit. The cDNA was subjected to amplification using gene-specific primers and SYBR Green on a qPCR platform. Target genes included c-Myc, MMP9, VEGF, and HIF-1α. Gene expression was normalized to the housekeeping gene GAPDH, and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Phalloidin Staining and Cell Imaging: Phalloidin staining was conducted to visualize the organization of the actin cytoskeleton and to assess structural integrity of cells post-treatment. Cells were first fixed with 4% paraformaldehyde to preserve cellular architecture, then permeabilized using 0.1% Triton X-100 to allow entry of the stain. Fluorescently labeled phalloidin, which binds specifically to filamentous actin (F-actin), was applied to highlight the cytoskeletal network. Optionally, nuclei were counterstained with DAPI for nuclear visualization. Fluorescent microscopy was used to observe and capture high-resolution images.

Results and Discussion

NRK52E cells show mercury toxicity in microscopic images; cells treated with Ebselen are maintained like control group: In this study, NRK52E cells were cultured in DMEM-F12 medium and exposed to escalating concentrations of methylmercury (MeHg) to evaluate its cytotoxic effects. Visual assessments revealed that untreated control cells retained a healthy morphology with intact, confluent monolayers, while MeHg-treated groups displayed progressive structural deterioration, including cellular shrinkage, rounding, and detachment—hallmarks of dose-dependent cytotoxicity. In contrast, groups co-treated with Ebselen, a known antioxidant and seleno-organic compound, demonstrated significant preservation of cellular architecture, closely resembling control cells. This suggests that Ebselen effectively counteracts MeHg-induced oxidative stress and toxicity (Figure-1). The findings underscore the damaging effects of MeHg on renal epithelial cells and highlight Ebselen's potential as a cytoprotective agent, likely through its role in scavenging reactive oxygen species and mitigating mercury-mediated intracellular disruptions.

Mercury exposure caused cytotoxicity through ROS production, necrosis and cellular damage: Methylmercury (MeHg) exposure caused pronounced cellular damage in NRK52E cells, including altered morphology and loss of adhesion, in a dose-dependent manner. The MTT Cell Viability assay, which measures absorbance, showed a clear dose-dependent toxic effect from MeHg (Figure-2). The absorbance was highest in the control and 0.5 µM MeHg groups and decreased significantly at 1 µM and 5 µM MeHg concentrations, indicating a reduction in viable cells. Co-treatment with Ebselen at 50 µM, alongside 1 µM MeHg, significantly mitigated the MeHg-induced cytotoxicity as shown by an increase in absorbance, suggesting Ebselen acts as a protective agent. The Calcein-AM cell proliferation assay further supported these findings (Figure-3). Viable cells showed green fluorescence, while cells treated with MeHg demonstrated reduced fluorescence due to cytotoxicity. Co-treatment with Ebselen significantly preserved this fluorescence, indicating protection against cellular damage. MeHg toxicity leads to an increased generation of reactive oxygen species (ROS), causing oxidative stress. The DCFDA assay confirmed that cells treated

with MeHg showed a marked elevation in ROS levels (Figure-4).

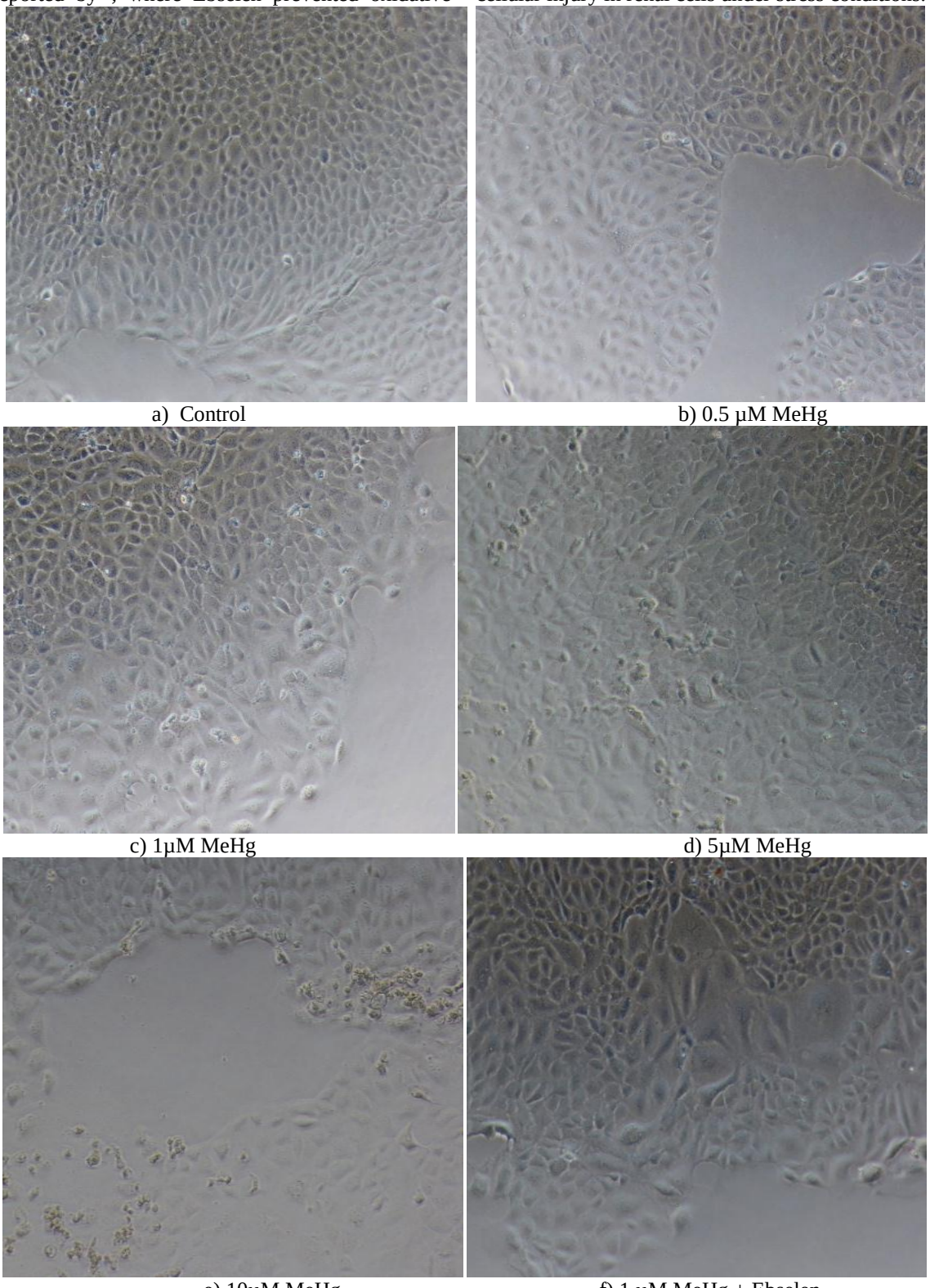
Biomarker-Based Assessment of Mercury-Induced Nephrotoxicity and the Therapeutic Potential of Ebselen: The study demonstrated that exposure to methylmercury (MeHg) leads to early, subclinical signs of kidney injury, as indicated by significant elevations in specific renal biomarkers. Utilizing the Bio-Rad Kidney Toxicity Panel, the levels of Clusterin, MCP-1 (Monocyte Chemoattractant Protein-1), KIM-1 (Kidney Injury Molecule-1), Interleukin-18 (IL-18) and Osteopontin were markedly increased in MeHg-treated cell culture supernatants (Figure-5). Clusterin reflected cellular stress and tissue damage; MCP-1 indicated inflammation; KIM-1 served as a highly sensitive marker of proximal tubular injury; and Osteopontin was associated with inflammatory and fibrotic processes. Despite these elevations, serum creatinine levels remained unchanged, suggesting that while molecular injury was evident, overt functional impairment had not yet occurred. Importantly, co-treatment with the antioxidant compound Ebselen significantly attenuated these biomarker elevations, effectively restoring levels close to those observed in control samples. This highlights Ebselen's potential to protect against MeHg-induced nephrotoxicity at an early stage, even before the development of detectable changes in kidney function.

Quantitative Assessment of Stress-Responsive Gene Expression Under Methylmercury (MeHg) Toxicity and the Protective Role of Ebselen: To investigate the molecular impact of MeHg-induced toxicity and the potential therapeutic effects of Ebselen, the mRNA expression levels of key stress-inducible genes—cMYC, MMP9, VEGF, and HIF-1α—were quantitatively analyzed using real-time PCR (Figure-6). Each of these genes plays a critical role in cellular stress responses: cMYC, a proto-oncogene, governs cell cycle progression, proliferation, and apoptosis; MMP9 encodes matrix metalloproteinase-9, which is involved in extracellular matrix degradation, tissue remodeling, and the facilitation of inflammatory cell infiltration; VEGF is a potent angiogenic factor that promotes vascular permeability and neovascularization; and HIF-1α is a master transcription factor activated under hypoxic conditions, regulating the expression of genes associated with metabolism, angiogenesis, and survival. Exposure to methylmercury significantly upregulated the expression of all four genes, reflecting a robust activation of oxidative stress, inflammatory signaling, and hypoxic adaptive pathways. The increase in gene expression correlates with intracellular accumulation of reactive oxygen species (ROS), mitochondrial dysfunction, and redox imbalance typically associated with MeHg toxicity. These molecular alterations collectively contribute to cellular injury, disruption of tissue architecture, and compromised organ function—particularly in renal tissues, where MeHg preferentially accumulates. Co-treatment with Ebselen, a synthetic organoselenium compound that mimics glutathione peroxidase activity and scavenges hydrogen peroxide and lipid hydroperoxides, resulted in a significant downregulation of these stress genes. The expression profiles in the Ebselen-treated group closely resembled those of the control group, suggesting that Ebselen effectively neutralizes MeHg-induced oxidative stress and inhibits downstream pro-inflammatory and hypoxia-related gene activation. These findings underscore the molecular pathogenesis of MeHg-induced cellular stress and validate Ebselen as a potential therapeutic agent capable of restoring redox homeostasis and preventing stress-mediated gene dysregulation. Such protective effects could have broader implications for preventing organ-specific toxicity, especially nephrotoxicity, associated with mercury exposure.

Phalloidin staining shows mercury induced cellular change which is rescued by Ebselen: Phalloidin staining was employed to investigate the cytoskeletal integrity and morphological alterations in NRK52E renal tubular epithelial cells following exposure to MeHg. Cells exposed to MeHg for 48 hours exhibited marked cytoskeletal disruption, characterized by cell rounding, contraction, and fragmentation of actin filaments—hallmarks of cytotoxic stress and compromised structural integrity, primarily driven by MeHg-induced oxidative damage. Notably, co-treatment with Ebselen significantly attenuated these effects, preserving normal cell morphology and actin organization (Figure-7). These findings indicate that MeHg impairs renal epithelial structure through ROS-mediated mechanisms targeting actin filaments, and that Ebselen confers substantial cytoprotective benefits by stabilizing cytoskeletal integrity and mitigating morphological damage.

Discussion: Methylmercury (MeHg) is a highly neurotoxic and nephrotoxic compound known for its capacity to induce severe oxidative stress and cellular dysfunction in various organ systems, particularly the kidneys[11]. The current study explored the toxicological effects of MeHg on NRK52E cells—a well-established model for renal epithelial toxicity—and evaluated the protective role of Ebselen, a seleno-organic compound with potent antioxidant properties. The findings present compelling evidence of MeHg-induced cytotoxicity, mitochondrial dysfunction, oxidative stress, cytoskeletal disorganization, and upregulation of nephrotoxicity-associated biomarkers, all of which were significantly attenuated by Ebselen co-treatment. Microscopy-based observations provided the first visible evidence of MeHg-induced cytotoxicity. Control NRK52E cells maintained a characteristic epithelial monolayer with elongated, tightly connected morphologies. However, MeHg exposure led to pronounced cytological changes including cell shrinkage, rounding, and detachment from the substrate—classic indicators of dose-dependent cytotoxic damage [12]. These structural disruptions reflect the early stages of cellular injury, possibly due to oxidative membrane damage and impaired adhesion molecule function. Conversely, cells co-treated with Ebselen retained morphology resembling that of control cells. This morphological preservation indicates that Ebselen effectively counters MeHg-induced damage, likely by stabilizing the redox environment and preserving cellular integrity. Similar findings

have been reported by[13], where Ebselen prevented oxidative cellular injury in renal cells under stress conditions.



a) Control b) 0.5 µM MeHg

c) 1µM MeHg d) 5µM MeHg

e) 10µM MeHg f) 1 µM MeHg + Ebselen

Figure-1(a-f): NRK52E cells grown in DMEM-F12 medium were exposed to varying concentrations of methylmercury (MeHg) to assess dose-dependent cytotoxic effects. Higher concentrations of MeHg caused pronounced cellular damage, including loss of

adhesion and altered morphology. Co-treatment with Ebselen significantly reduced MeHg-induced toxicity, preserving cellular integrity.

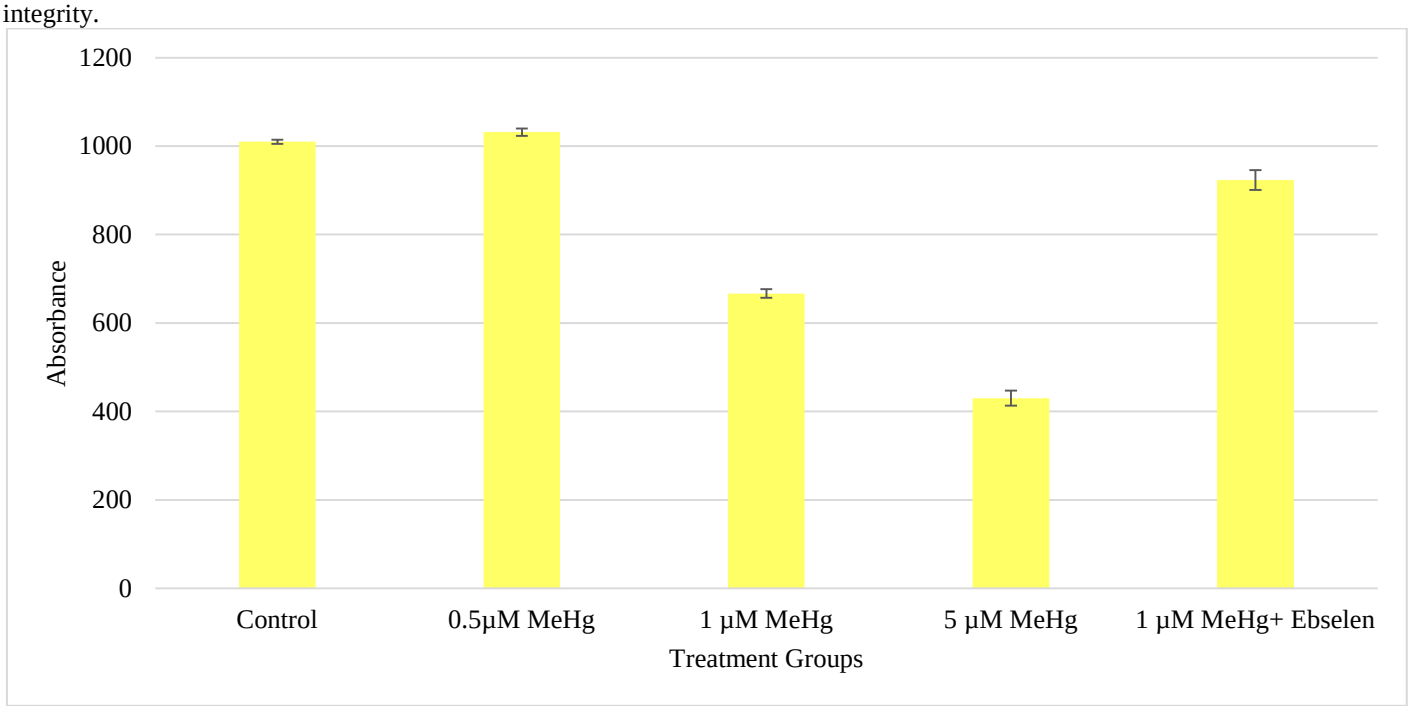
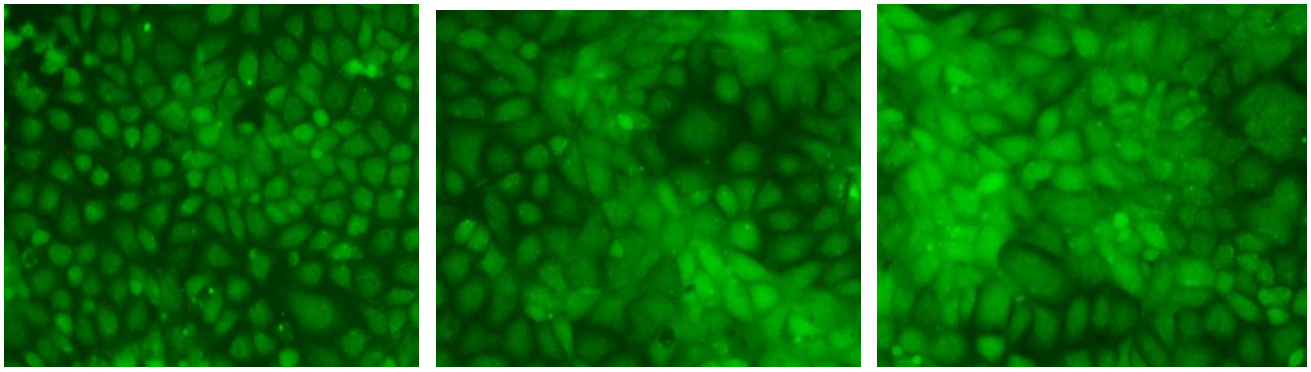
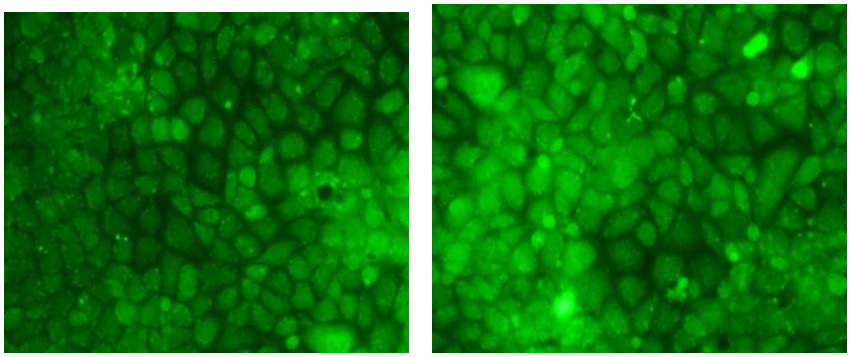


Figure-2: MTT Cell Viability Assay.



g) Control h) 0.5 µm MeHg i) 1 µm MeHg



j) 5 µm MeHg k) 1 µM MeHg + Ebselen

Figure-3: Calcein-AM is a cell-permeable dye used to assess cell viability and proliferation. Viable cells convert non-fluorescent Calcein-AM to green-fluorescent calcein via intracellular esterases. Calcein-AM assay showing fluorescence intensity correlating with cell viability and proliferation. Cells treated with MeHg demonstrate reduced fluorescence due to cytotoxicity, whereas co-treatment with Ebselen significantly preserves fluorescence, indicating protection against MeHg-induced cellular damage.

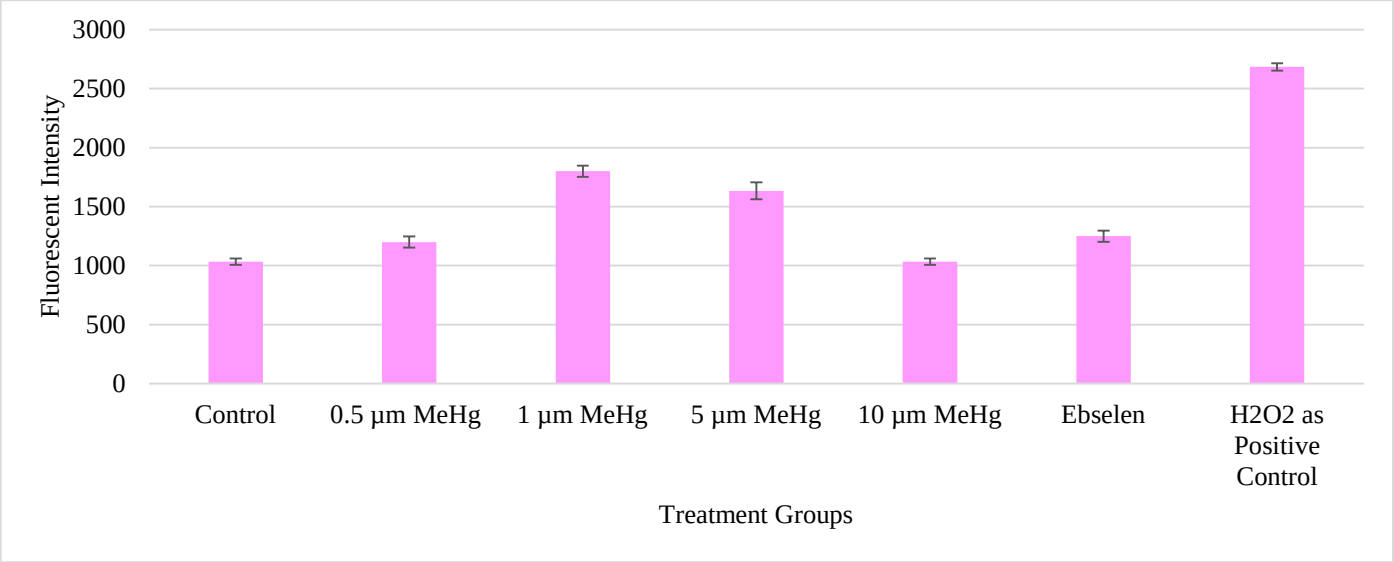


Figure-4: Reactive Oxygen Species Detection using the DCFDA Assay.

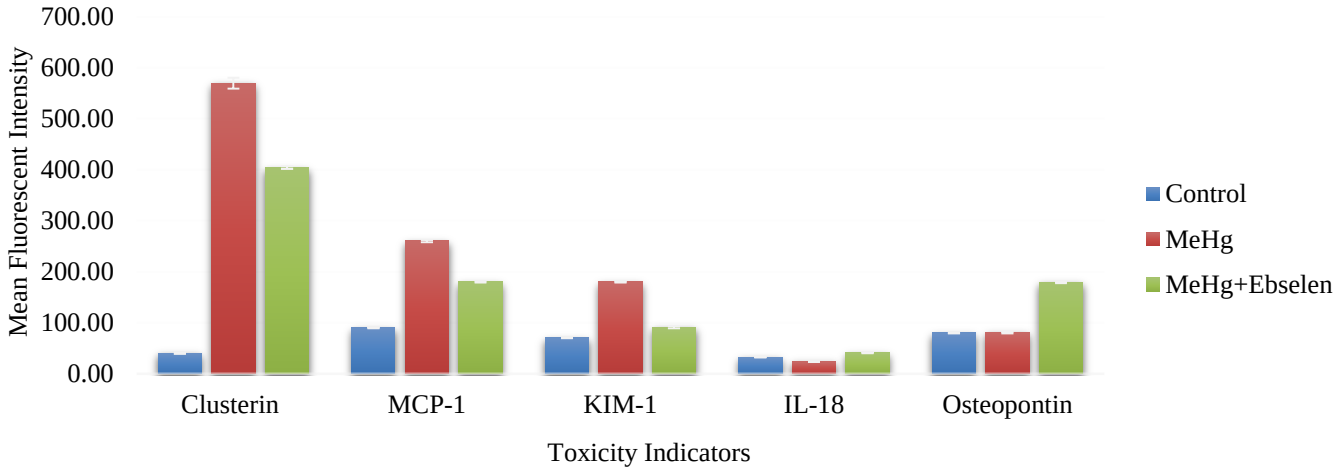


Figure-5: Kidney toxicity indicators.

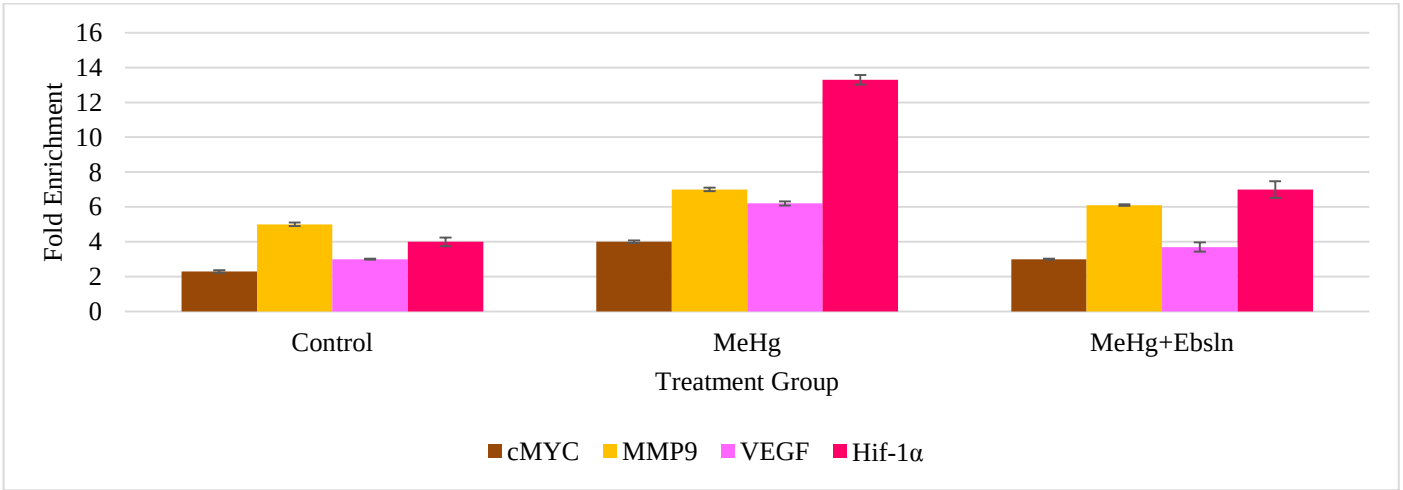
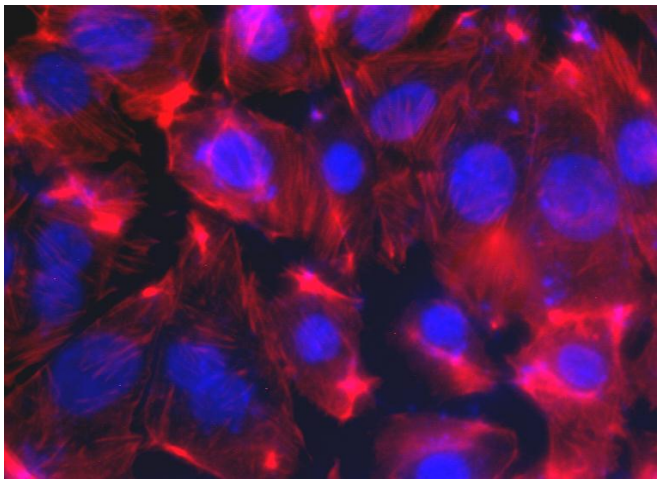
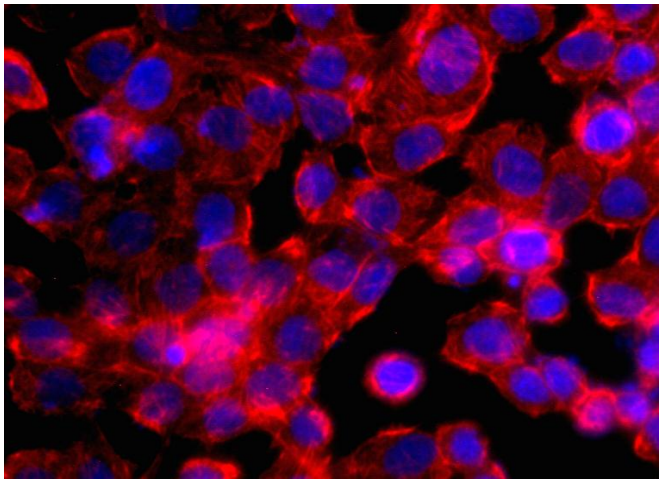


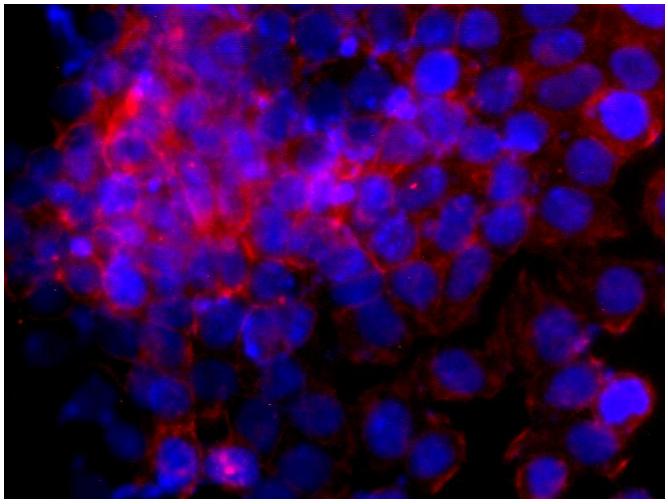
Figure-6: Quantitative Real-time PCR.



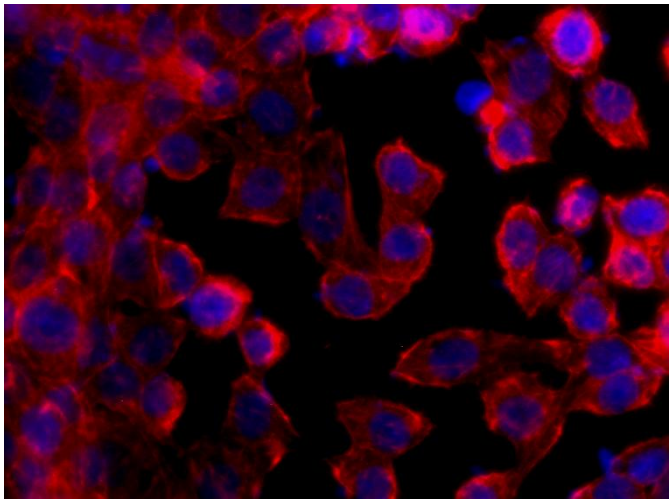
l) Control



m) 0.5 µM MeHg



n) 1 µM MeHg



o) 1 µM MeHg + Ebselen

Figure-7(a-d): Phalloidin staining of NRK52E cells after 48-hour treatment. Control cells exhibit intact act in cytoskeleton and normal morphology. Methylmercury (MeHg) treatment leads to cytoskeletal disorganization and cell rounding, indicating severe cellular damage. Co-treatment with Ebselen preserves actin structure and cellular morphology, highlighting its protective antioxidant role against MeHg-induced cytotoxicity.

The MTT assay demonstrated a clear dose-dependent decrease in cell viability with MeHg treatment, confirming its cytotoxic potential. At concentrations of 1 µM and 5 µM MeHg, cell viability significantly dropped, correlating with extensive oxidative stress and mitochondrial dysfunction[11]. Ebselen co-treatment restored cell viability, especially at 1 µM MeHg, suggesting it mitigates MeHg toxicity through its antioxidant mechanism.

The Calcein-AM assay supported these findings by showing reduced green fluorescence in MeHg-treated cells—indicating fewer viable cells. Ebselen-treated cells, however, retained fluorescence, further affirming its protective role. The DCFDA assay provided additional mechanistic insight: MeHg significantly increased intracellular ROS levels, aligning with literature indicating MeHg's ability to deplete glutathione and disrupt redox balance[14]. Ebselen, mimicking glutathione peroxidase, likely scavenges hydrogen peroxide and lipid peroxides, thereby limiting ROS-mediated cellular injury. Mitochondrial toxicity—another hallmark of MeHg exposure—was also observed. MeHg impairs mitochondrial membrane potential and inhibits ATP synthesis, disrupting energy metabolism[15]. This is partly due to MeHg's affinity for sulfhydryl groups in mitochondrial proteins, leading to enzyme inactivation and membrane permeability transition. Ebselen may prevent this by protecting mitochondrial enzymes and limiting lipid peroxidation within the organelle. Additionally, MeHg-induced calcium dysregulation may further enhance cell death, as calcium overload can trigger apoptosis or necrosis[16]. Ebselen's role in reducing oxidative stress might indirectly stabilize calcium homeostasis by protecting plasma membrane channels and buffering systems.

The Bio-Rad Kidney Toxicity Panel provided crucial molecular insights into renal epithelial injury by quantifying specific biomarkers in culture supernatants. Upon exposure to methylmercury (MeHg), there was a significant elevation in the levels of Clusterin, MCP-1, KIM-1, and Osteopontin, which are recognized as early indicators of nephrotoxicity, often preceding measurable changes in glomerular filtration rate or serum creatinine[17]. Clusterin reflects cellular stress and cytoprotective attempts, MCP-1 signals inflammatory recruitment of monocytes/macrophages, KIM-1 is a sensitive proximal tubule injury marker, and Osteopontin is associated with renal fibrosis and chronic inflammation. These elevations indicate that MeHg initiates a cascade of subclinical nephrotoxic events involving oxidative stress, inflammation, and tubular injury. Notably, Ebselen co-treatment effectively down regulated these biomarkers, suggesting its ability to mitigate or reverse early renal epithelial damage, corroborating previous rodent model studies[18]. Complementing these findings, real-time PCR analysis demonstrated that MeHg exposure significantly upregulated stress-responsive genes such as cMYC, MMP9, VEGF, and HIF-1α. cMYC is involved in proliferation and apoptosis, and its over expression under toxic stress can lead to cell cycle arrest or apoptosis; MMP9 contributes to extracellular matrix degradation and inflammatory cell infiltration; VEGF, while angiogenic, may promote vascular permeability and glomerular leakage; and HIF-1α, though a hypoxia survival factor, can exacerbate injury through maladaptive gene transcription under persistent oxidative stress[19]. The coordinated upregulation of these genes reflects MeHg-induced oxidative damage, mitochondrial dysfunction, and activation of stress-signaling pathways. Ebselen treatment normalized the expression of these genes to near-control levels, demonstrating its role in attenuating transcriptional disturbances and restoring cellular homeostasis under toxicant-induced stress conditions.

Phalloidin staining clearly revealed the structural impact of MeHg on the actin cytoskeleton. F-actin filaments, essential for maintaining cellular shape and adhesion, were fragmented and disorganized following MeHg treatment. Cytoskeletal disruption is a known downstream effect of oxidative damage, as actin is highly susceptible to redox alterations[20]. Moreover, loss of cytoskeletal integrity can lead to impaired renal tubular function and barrier disruption. Ebselen preserved actin organization, indicating that it protects cytoskeletal proteins from ROS-induced damage. This has important implications for maintaining epithelial barrier function in renal tissues during toxic exposure.

Conclusion

This study underscores the profound cytotoxic effects of methylmercury (MeHg) on renal epithelial NRK52E cells and highlights the robust nephroprotective potential of Ebselen, a selenium-containing antioxidant compound. Exposure to MeHg resulted in significant morphological and structural damage to renal cells, evidenced by cellular shrinkage, detachment, and disruption of cytoskeletal F-actin integrity. These phenotypic changes were accompanied by reduced cell viability and increased intracellular reactive oxygen species (ROS), confirming oxidative stress as a central mechanism of MeHg-induced toxicity. Additionally, MeHg exposure upregulated key nephrotoxic biomarkers such as KIM-1, Clusterin, Osteopontin, and MCP-1, indicating early renal injury. Molecular analysis further revealed upregulation of stress-responsive genes like cMYC, HIF-1α, VEGF, and MMP9, suggesting activation of apoptotic, hypoxic, angiogenic, and inflammatory pathways. Importantly, co-treatment with Ebselen significantly counteracted these deleterious effects by restoring cellular morphology, reducing ROS levels, stabilizing F-actin structure, and down regulating both nephrotoxic biomarkers and stress-related gene expression. The results suggest that Ebselen's antioxidant activity—likely via glutathione peroxidase mimicry and ROS scavenging—helps preserve mitochondrial function, prevent cytoskeletal collapse, and maintain transcriptional homeostasis under MeHg-induced oxidative stress. These findings are consistent with existing literature demonstrating Ebselen's efficacy in mitigating heavy metal toxicity, including its roles in as significant therapeutic potential in preventing or alleviating MeHg-induced renal toxicity, particularly in occupational or environmental exposure scenarios. Future in vivo studies and clinical evaluations are warranted to confirm its translational relevance and establish dosage safety profiles. Thus, Ebselen may serve as a promising candidate for inclusion in nephroprotective strategies against mercury-induced damage, offering hope for improved renal health outcomes in vulnerable populations exposed to environmental toxins.

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