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Renoprotective role of Ebselen on developmental renal toxicity following gestational Methylmercury exposure in Wistar rats

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Abstract

Methylmercury (MeHg) crosses the placental and blood-brain barriers, posing risks to neonates and adults. While its neurotoxic effects during gestation are well-documented, its impact on kidney development is less understood. This study explored the renal toxicity of gestational MeHg exposure (0.5 mg/kg and 5 mg/kg via oral gavages) from Gestational Day 4 to Post-Natal Day 1. MeHg exposure resulted in reduced body and kidney weights and elevated nephrotoxicity markers in serum and urine, indicating renal dysfunction. Histopathological analysis showed a decrease in cortical area and glomeruli count, reflecting impaired renal development. Molecular studies revealed upregulation of MMP9, a tissue injury marker, with alterations in Nephrin, Villin, and GDNF expression, essential for renal function and growth. Hypomethylation of the MMP9 promoter, confirmed by ChIP-PCR, suggested epigenetic dysregulation as a mechanism of toxicity. Ebselen, an antioxidant, demonstrated renoprotective effects by restoring cortical area and glomeruli count, normalizing nephrotoxicity markers, and reducing MMP9 expression. Ebselen's effects were linked to increased MMP9 promoter methylation, countering MeHg-induced epigenetic changes. These findings highlight Ebselen's potential to mitigate oxidative stress and epigenetic disruptions, making it a promising therapeutic agent to address developmental renal toxicity caused by gestational MeHg exposure.

Keywords: Methylmercury, Ebselen, Methylation, MMP9, GDNF.

Introduction

Mercury is one of the major environmental pollutants, occurs naturally as well as is added by anthropogenic activities. Common sources for global increase in mercury levels include gold mining, organomercury in fungicides and drugs, chlor-alkali production, volcanic eruption and fossil fuel burning. Although release of anthropogenic mercury is minimized by many nations but mercury added to environment persists for longer period and gets deposited especially in organisms such as fishes. Mercury compounds exist in three types- elemental, inorganic and organic. Organic mercury, for example, methylmercury is most toxic and commonly exposed. Human and wildlife exposure to mercury is mainly through the consumption of contaminated food and water[1,2].

Mercury compounds are detrimental to CNS and kidneys[3,4]. Organic mercury is highly lipophilic and can cross placental and blood brain barrier[5,6]. Mercury has strong affinity towards Sulphur group of proteins and hence they may interact with Sulphur containing compounds such as cysteine. Mercury causes hypoxia which induces ROS production and upregulation of several proinflammatory and angiogenic genes including c-Myc, VEGF, MMPs, which make epithelial cells to behave like myofibroblast cells[7]. Metalloproteinases dissolve component of cell-junction and free them to live as cancerous cells[8]. Once free from cell matrix, cells undergo modification and change in normal expression of genes is observed.

It is also thought to play role in epigenetic transformation of cells, particularly cells of renal tubules. Studies of the whole genome by molecular and cytogenetic methods have implicated DNA methylation in the formation of inactive chromatin. This has been confirmed by analysis of specific endogenous sequences, and has been mimicked by introducing methylated and non-methylated sequences into cells. Affecting chromatin structure, DNA methylation also represses transcription. A protein, (MeCP) which binds specifically to methylated DNA has been identified. The properties of MeCP could account for the effects of DNA methylation on both chromatin and transcription[9,10]. Methylation of DNA is involved in silencing of transposable element, genomic imprinting and X-chromosome inactivation[12]. In recent years, many studies have been done to understand how these processes affect cell's identity and functions. Of them, hydoxylation of DNA is one such process which describes demethylation mechanism of DNA. Methylation of DNA is associated with formation of inactive chromatin by repressing transcription. This is brought about by recruitment of proteins such as MECP2 and MBD to methylation site and hence preventing transcription machinery from accessing DNA[12,13]. Thus, methylation profile determines gene expression and specificity of cells of a particular tissue[14].

Our aim is to understand the effect of MeHg in kidney tubules and their correlation with 5-hydroxymethylcytosine of profibrotic and proinflammatory genes. Methylation of cytosine of CpG dinucleotide in eukaryotes plays important role in genome function and regulation[15]. Deregulation of DNA methylation is thought to be an important feature of several diseases such as cancer and fibrogenesis[16,17].

Recently, methods have been developed for the detection of 5-methylcytosine and 5-hydroxymethylcytosine in context to non methylated DNA. DNA methyltranferases (DNMTs) and Tet proteins are crucial for dynamic regulation of methylation and hydoxymethylation of DNA[18-20]. Hydroxymethylation is thought to be intermediate stage between methylated and unmethylated DNA[21]. Aberrant changes in hydroxymethylation in context to DNA methylation is the major cause of disease and early detection of such changes may provide powerful biomarkers for disease diagnosis. Understanding the role of TET proteins in dynamic changes in DNA methylation and gene expression will greatly enhance our understanding of epigenetic regulation of normal development and human diseases.

In our study, we have focused on the effects of prenatal MeHg exposure on embryonic nephrogenesis. We are assessing the key genes involved in nephrogenesis. We also assessed for the changes in epigenetic profiling of promoter of genes to better understand the mechanisms inolved. Ebselen, as a potent antioxidant were tested for the potential therapeutics against the toxic effect of MeHg.

Materials and Methods

Methylmercurychloride, protease and phosphatase inhibitors, fluoroshield with DAPI, DNAse1 and, hot start PCR master mix were bought from Sigma-Aldrich, USA. High-Capacity cDNA Reverse Transcription Kit was purchased from Applied Biosystems, USA. Primers for real-time polymerase chain reaction, methylation specific polymerase chain reaction and chromatin immunoprecipitation (ChIP) were bought from Integrated DNA Technologies, USA. Ethidium bromide and propidium iodide (PI) were purchased from Cayman Chemicals, USA. Bio-Plex Pro™ for rat cytokine assay kit was purchased from Hercules, CA, Bio-Rad Laboratories. EZ-DNA Methylation™ Kit and anti-5'-methyl cytosine (5'-MeC) antibody were purchased from Zymo Research, Orange, California. Rat kidney toxicity panel kit was bought from Randox laboratories Ltd., UK. MMP9, Podocin and beta-Actin antibodies were purchased from Abcam Inc., Waltham, USA.

Experimental Design: Pregnant female rats of the same age group were procured and maintained under standard laboratory conditions, including a 12-hour light/dark cycle and a relative humidity of 55 ± 5%, with food and water provided ad libitum. Vaginal plug-positive females were considered as gestation day (GD) 1 pregnant.

On GD2, the animals were randomly assigned to three groups: Control, 0.5 mg/kg MeHg, and 0.5 mg/kg MeHg + Ebselen. Following acclimatization, the experimental groups received daily oral gavage of either 0.5 mg/kg methylmercury (MeHg) or 0.5 mg/kg MeHg combined with Ebselen from GD4 to postnatal day (PND) 1, while the control group received normal saline containing 0.01% NaOH. Animals were sacrificed at predetermined time points—PND2, PND28, PND90, and PND148—and samples were collected for analysis. Kidney tissues were snap-frozen in liquid nitrogen and stored at –80°C for biochemical and molecular analyses, while another set of kidney samples was fixed in 4% paraformaldehyde for histopathological and immunohistochemical evaluation.

Multiplex assessment of kidney toxicity and inflammation related proteins: Bead based Multiplex Analysis of rat kidney toxicity biomarkers in urine samples was done using MAGPIX multiplex reader (Luminex, Austin, USA). Fresh samples of urine of the control and treated groups were centrifuged at ≥12000g for 10 min at 4˚C. The Bio-Plex Pro™ Rat cytokine assay kit was used to access the level of cytokine expression level. The samples were processed according to the manufacturer's instructions and protocols.

Biomarker analysis for kidney toxicity: Both urine and serum sample were tested for known biomarkers of the kidney structure and function such as Clusterin, MCP-1, Osteopontin, KIM-1 and VEGF. Fresh blood sample were taken for analysis of biomarkers. Luminex bead-based assay and Randox clinical bioanalyser were used as per manufacturer's instructions. Serum creatinine and urine biomarkers such as KIM1, Clusterin, Osteopontin and VEGF were considered for early toxicity markers of kidneys.

Quantitative real-time PCR: Total RNA was extracted using Trizol reagent. To remove the remaining DNA contamination, samples were treated with DNAse 1. High-capacity cDNA reverse transcription kit from Applied Biosystems, USA was then used to synthesize cDNA from pure RNA extracted after treatment of DNAse 1. For quantitative real-time PCR Applied Biosystems 7900 HT Fast Real-Time PCR System was loaded with equal samples of cDNA from control and MeHg treated groups. For easy detection, SYBR Green PCR Master Mix by Takara Bio., Japan was used as a fluorescent dye. NCBI website was used to design primers and then were ordered from Integrated DNA Technologies, Coralville, USA. As internal control, GADPH and Beta-actin primers were used. Threshold cycle value ($C_t$value) in duplicate reactions, were used to calculate relative gene expression in control and treated samples according to standard method[22].

Histopathology and immunohistochemistry: Kidneys of both control and mercury exposed rats were washed in 1X PBS and fixed in 4% paraformaldehyde (PFA). They were then dehydrated in ethanol gradient and embedded in paraffin wax.

Thin microtome sections were prepared, dewaxed and cleared in xylene and stained with Masson's trichrome according to manufacturer's instructions (Sigma Aldrich, USA). For immunohistochemistry, section on slides were rehydrated and processed for antigen retrieval using citrate-EDTA buffer. Antigen retrieved sections were kept in 5% BSA for 1 hour to block non-specific antigens. Sections of the samples were then incubated with primary antibody for 2 hours (120 minutes) at room temperature or at 4˚C for overnight. Sections of sample were washed and further incubated with Alexa fluor 488/594 for two hours (120 minutes)**.** Sections on slides were again washed with 1X PBS three times and then mounted in fluoroshield DAPI solution from Sigma Aldrich, USA. Samples were viewed under microscope using appropriate filters (Leica Microsystems, LB30S). Further analysis of images was done using NIH Image J software for Windows (NIH, USA).

Western blotting: Tissue samples from cortex of kidneys of control as well as MeHg treated rats were dissected and homogenized using tissue lysis buffer from Sigma-Aldrich, USA and then supplemented with Protease Inhibitor Cocktail, P8340-1 ML from Sigma-Aldrich, USA and Phosphatase Inhibitor Cocktail B, sc-45045 from Santa Cruz Biotechnology, Dallas, Texas. BCA Protein Assay Kit from Thermo Scientific™ was used to calculate amount of protein. Equal volumes of protein samples were mixed in sample loading buffer, loaded in respective wells and run for SDS-PAGE. After complete run, the bands were transferred on to nitrocellulose membrane. The membranes were incubated overnight at 4˚C with primary antibody for MMP9 and Beta actin. Membranes were incubated for 2 hours with HRP-conjugated secondary antibody and the immunoreactive proteins were detected using an enhanced chemiluminescent substrate (Invitrogen) according to the manufacturer's instructions. Relative protein expressions were quantified by densitometric analysis using NIH Image J software for Windows. Beta actin was run as housekeeping gene for protein loading control.

DNA Immunoprecipitation Assay (DIP): 5µg of genomic DNA of each sample was added in 500µL of Tris-EDTA buffer and fragmented to 500-1000 base pairs range using water bath sonicator. Sonicated samples were quantified by using Qubit high-sensitive DNA quantification kit as recommended by manufacturer. Then, 2µg of sonicated DNA were taken in 450 µL TE buffer and denatured by heating at 950 degree C for 10 minutes. Denatured DNA was immediately cooled on ice and aliquots for 50µL input, 200 µl A/G agarose beads and 200 µL A/G + antibody were made for each sample. 25 µL of 10 X IP buffer (100mM sodium phosphate pH 7.0, 1.4 M NaCl, and 0.5% 100X) was added to protein A/G agarose+ IgG, and A/G agarose+1µg anti-5- MeC antibody samples. 1µg anti-5-MeC antibody and anti-IgG were added to respective tubes and incubated overnight at 4°C. 20µL of A/G beads were then added in each sample and incubated at room temperature on a rotating wheel for 30 min.

Beads were pelleted by centrifuging at 2000g for 5 minutes and pellets were washed with 1X IP buffer. The precipitated beads bound to antibody and DNA were resuspended in 250µL PK digestion buffer (5ml 1M Tris, 2ml 0.5M EDTA and 2.5ml 20% SDS in 100ml deionized water) and incubated with proteinase K for 3 hour at 50°C. DNA was then extracted by phenol-chloroform method and stored at -20˚C. Isolated DNA were stored at -82˚C or used for quantitative real-time PCR for MMP9. ChIP primers using 7900 fast real-time PCR System (APPLIED BIOSYSTEMS, USA) with SYBR green (Takara, Japan) as a fluorescent reporter. Results were expressed as fold enrichment as compared to isotype control sample.

Statistical analysis: The mean significant difference in the experimental groups was determined using one-way analysis of variance. The data represented in the graph as mean ± Standard deviation (SD) and p value <0.05 was considered as statistically significant. Data analysis was carried out using Microsoft Office-2010 software and online Graph pad Quick Calc.

Results and Discussion

Gestational exposure of MeHg causes developmental defect in kidneys: Prenatal exposure to MeHg results in defective development of kidneys and lower number of glomeruli. This results in gradual loss of kidney function. Ebselen treatment results insignificant reduction in developmental toxicity and partially restores kidney function by reducing level of MMP9 through epigenetic changes. In utero exposure to MeHg caused overall reduction in weight both in PND2 and PND28 samples. Ebselen is an antioxidant known to reduce the toxic effects of heavy metals including mercury. Cotreatment with Ebselen brought the weight of kidneys to normal as in control groups (Figure-2).

We have found reduced number of glomeruli at PND28 but no functional or structural difference in kidney structure (fig not shown). Animals were allowed to accumulate the abnormalities because of their defective kidneys upto 148 days. Routine analysis of serum and urine biomarkers were tested but found no toxicity symptoms before PND148. Gross anatomy of kidneys showed intermixing of cortical and medullary regions but lesser intermixing were detected in MeHg+Ebselen group (Figure-2). Ebselen effectively reduced the toxic effects of MeHg during organogenesis as seen in PND2 kidneys sections. Number of glomeruli and cortical region are significantly reduced in MeHg alone group (Figure-2).

In MeHg+Ebselen treated groups effects of mercury toxicity is minimized as seen in parameters like cortical radii and glomerular number. In PND148 samples, we found shrunken glomeruli, vacuolated tubules and loss of normal morphology of kidneys. Ebselen treated group was reported with less damage (Figure-3).

Biochemical analysis shows nephrotoxic markers in kidney tissues: Several biomarkers of nephrotoxicity were found to be affected due to MeHg toxicity. Clusterin, MCP-1, KIM-1, IL-18 and Osteopontin are set of clinical biomarkers for nephrotoxicity. Urine samples of PND148 rats showed increased level of Clusterin, MCP-1 and KIM-1 molecules (Figure-4). Ebselen reduced the toxicity markers, thus, less damage to kidneys were observed. Biochemical analysis of creatinine, total protein, albumin, uric acid, calcium and urea were seen as potent markers of nephrotoxicity because of MeHg exposure. These toxicity markers are present probably due to inability of kidneys proper function as a resultant of defective development. Proteinuria and albuminuria are seen lesser in MeHg+Ebselen group. These chemicals when progressively accumulated may lead to early adult-onset kidney diseases.

Genes related to oxidative stress are dysregulated due to Mercury: Regulated expression of genes is very important for embryonic development. Nephrogenesis involves interplay of several genes for the budding and branching of nephrons. Genes such asPAX2, GDNF, MMP9, TET, DNMT, Calbindin, Villin, Podocin and Nephrin are important for proper development of kidneys. Dysregulation of these genes were caused due to MeHg toxicity which led to abnormal expression and defective development of kidneys. Over expression of MMP9 is overcome by Ebselen. qRT-PCR analysis shows threefold increase in the level of TET3, threefold increase in MMP9, fourfold increase in HIF-1α and threefold increase in VEGF expressions(Figure-7). Western blot analysis shows slight increase in levels of MMP9 and Nephrin. However, expression of Podocin and Villin were down regulated to eight-fold and fourfold respectively. In MeHg+Ebselen group expression of MMP9, VEGF, HIF-1α, and Nephrin were brought to down regulated as compared to MeHg alone group.

These molecules are stress responsive and their expressions were higher in MeHg treated group due to induction of oxidative stress. Ebselen attenuates the effect by reducing the level and controls MeHg induced toxicity. Expressions of TET3, Podocin and Villin were increased in MeHg+Ebselen group in caparison to MeHg alone. Expression of and maintenance of Podocin and Villin are important as far as structural development of kidneys are concerned.

Ebselen- a selenoprotein protects kidneys from oxidative stress and accumulation of defects: Expression of MMP9 is affected during nephrogenesis in MeHg treated group. In PND2 kidneys, western blot analysis shows fourfold increase in MMP9 expression. In immunohistochemical analysis, increased expression of MMP9 is seen at PND2 and PND148 (Figure-5). Ebselen treatment during in utero toxicity of MeHg, effectively brings down the expression of MMP9 to normal level. TET3 is overexpressed due to MeHg and is not affected by Ebselen cotreatment (Figure-6).

We further assessed the methylation of TET3 and MMP9 at their promoter regions using methylated DNA immunoprecipitation method. Methylation of TET3 was slightly increased in MeHg and MeHg+Ebselen groups. But MMP9 methylation was reduced to half in the control group (Figure-7). Ebselen treatment brought the methylation slightly above the control group. Hypomethylation in promoter region of MMP9 is due to toxic effect of MeHg and responsible factor for the sustained increase in the level of MMP9. Ebselen treatment shows the epigenetic mechanism to control MMP9 expression by increasing the methylation of promoter region. Expression and methylation of TET3 gene is further associated with the maintenance of methylation of other genes including that of MMP9.

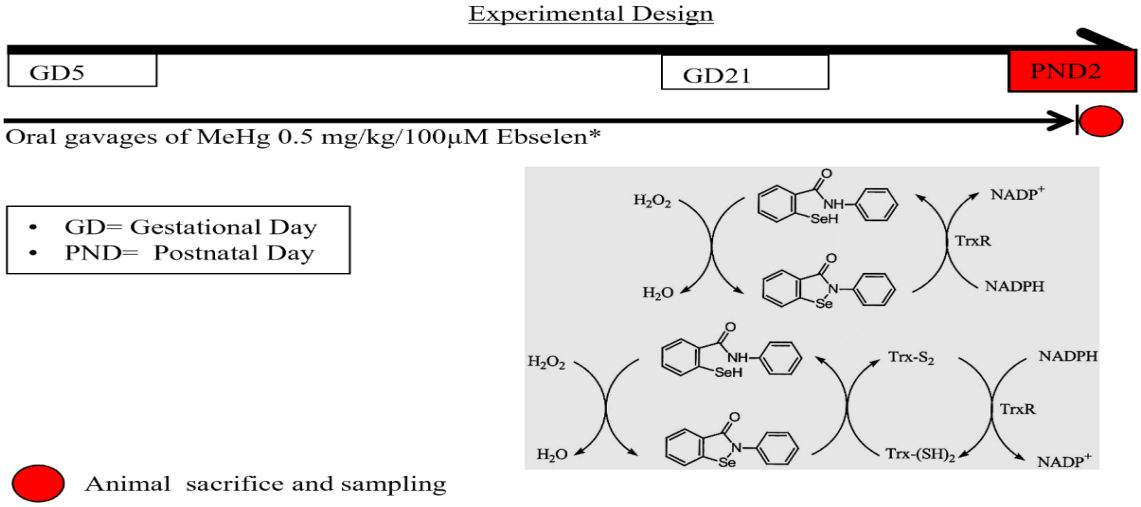
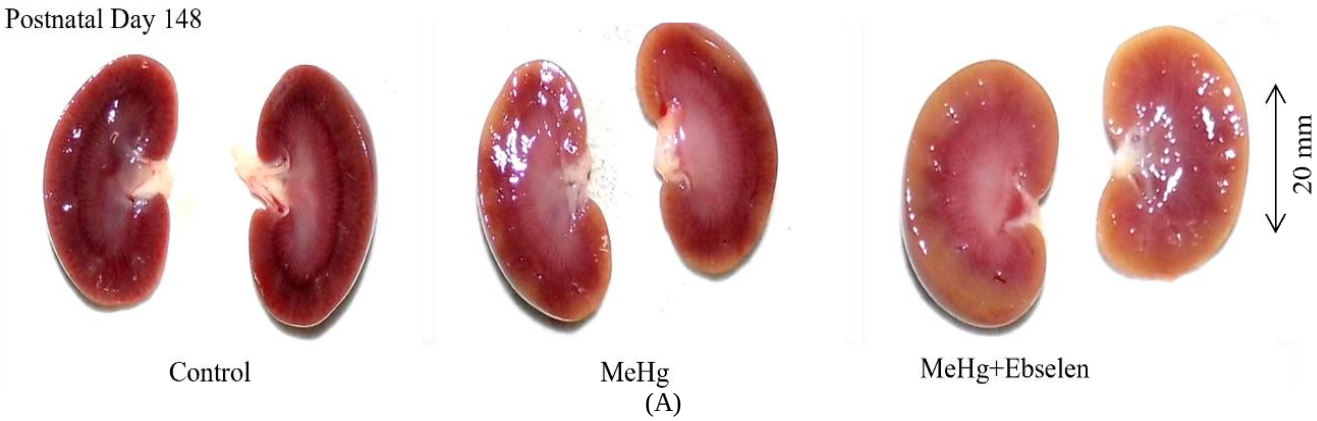


Figure-1: Treatment plan from Gestational Day 4 to Post-Natal Day 1[34].



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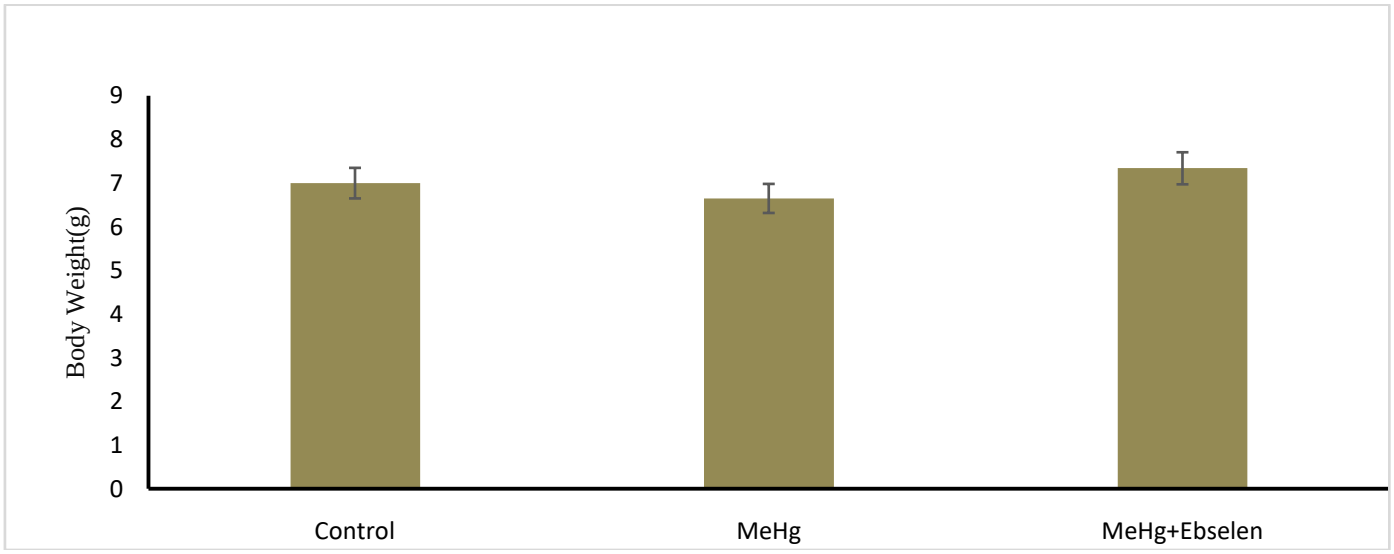

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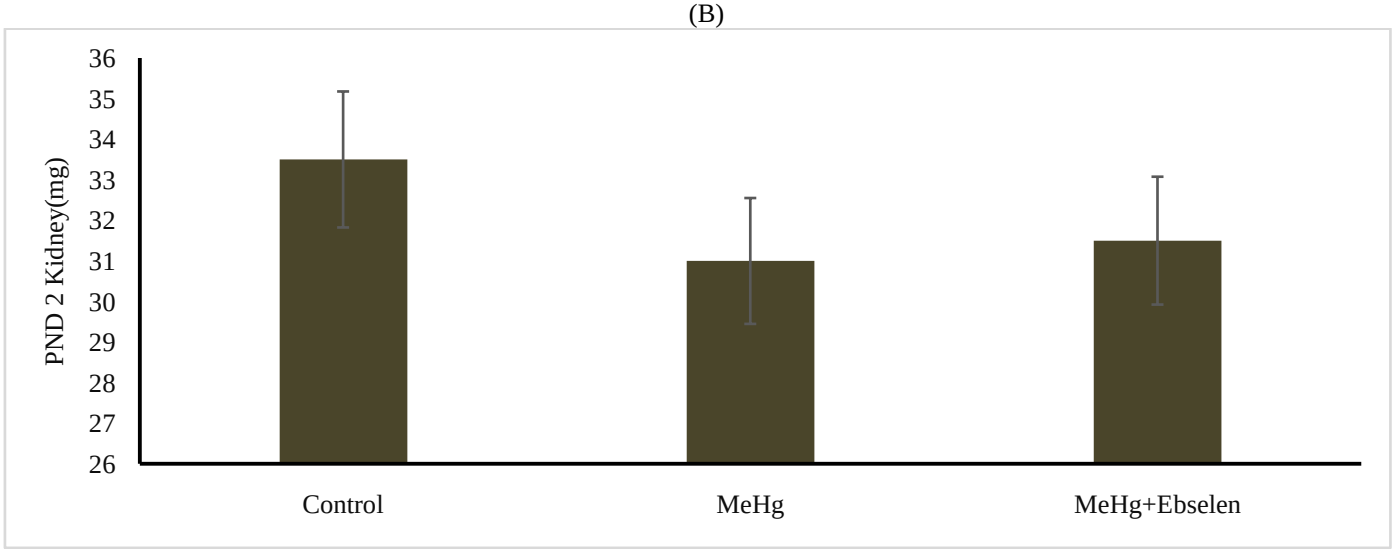
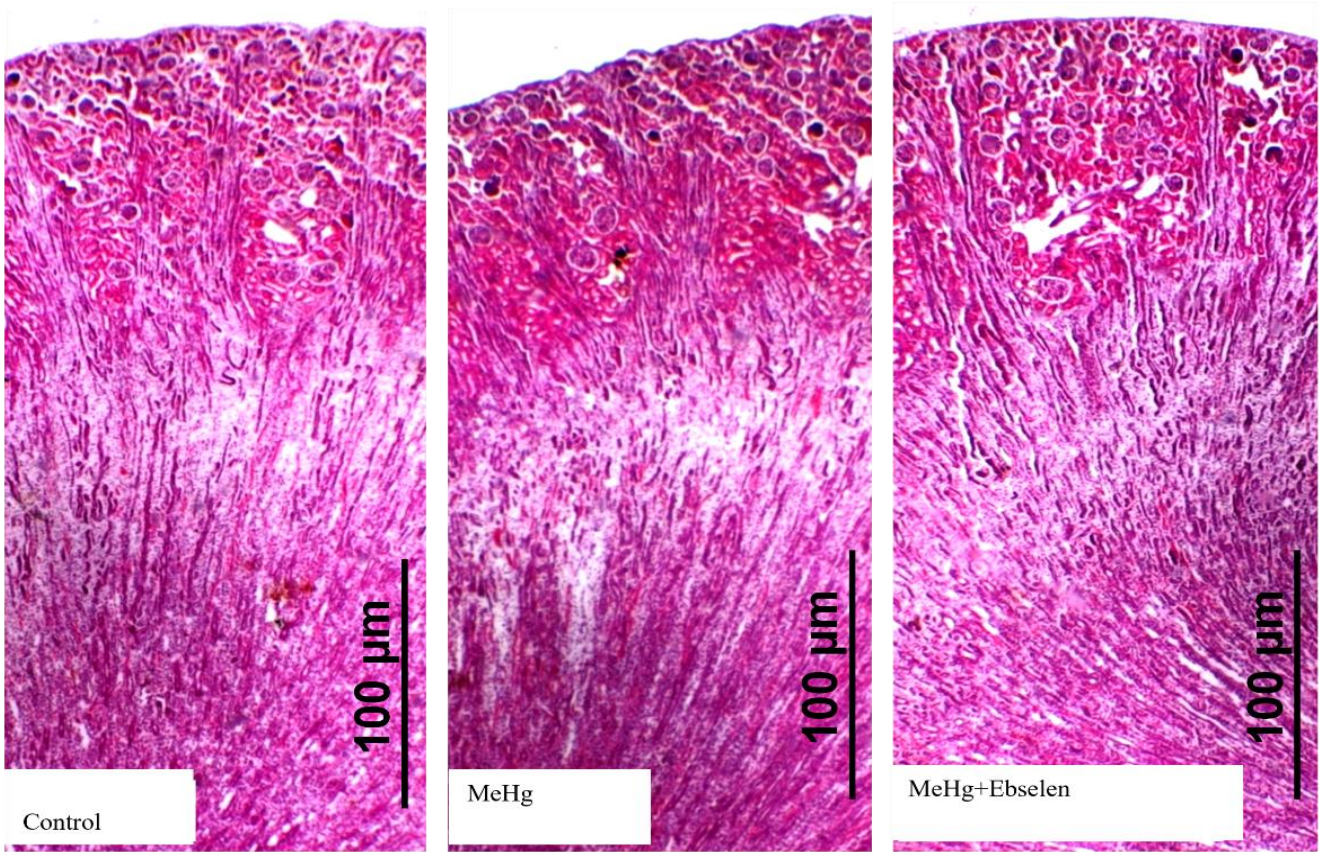
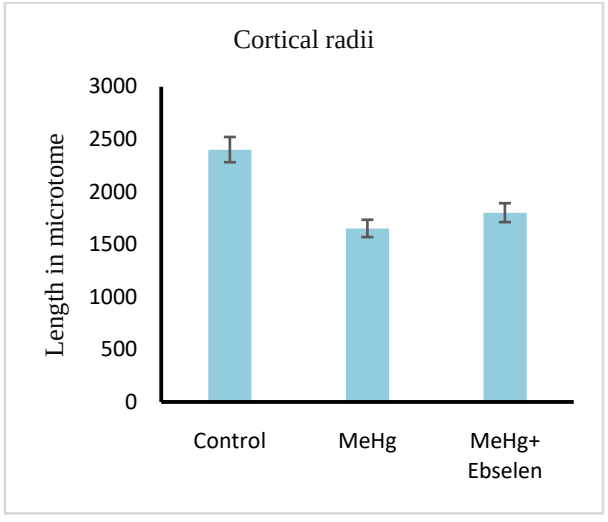


(C)

Figure-2: Mercury exposure in utero, resulted in reduced weight of kidneys which accumulates as visible structural defects in adults. Functional loss is found in adults with the structural changes. A. Structural differences in kidneys of the control group, MeHg treated group and MeHg + Ebselen group. B. Graph showing difference in body weight of the three groups. Ebselen cotreated groups show significant increase in body weight. C. Graph showing kidney weight of the three groups.

A.





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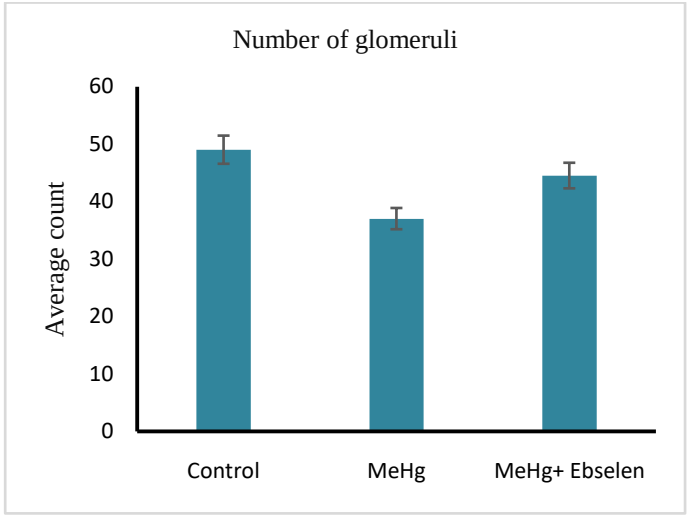


(c)

Figure-3: In prenatally MeHg exposed adults, kidneys were having reduced number of glomeruli as observed in PND148 rat samples. Intermixing of cortical and medullary regions of kidneys in MeHg treated groups. Gaps created due to loss tubular cells. Ebselen significantly restored number of glomeruli and cortical morphology. H&E stain of tissue sections shows overall damages with defective glomeruli was seen in MeHg treated groups, Ebselen reduces the tubular and glomerular defects (A): reduced cortical radii of animals treated with 0.5 ppm MeHg show lesser cortical area as compared to control group when stained with H&E (B): Graph showing reduction in length of cortical radii of the MeHg treated group & (C): Graph showing reduction in number of glomeruli in MeHg treated group whereas Ebselen cotreated groups show marked increase in number of glomeruli.

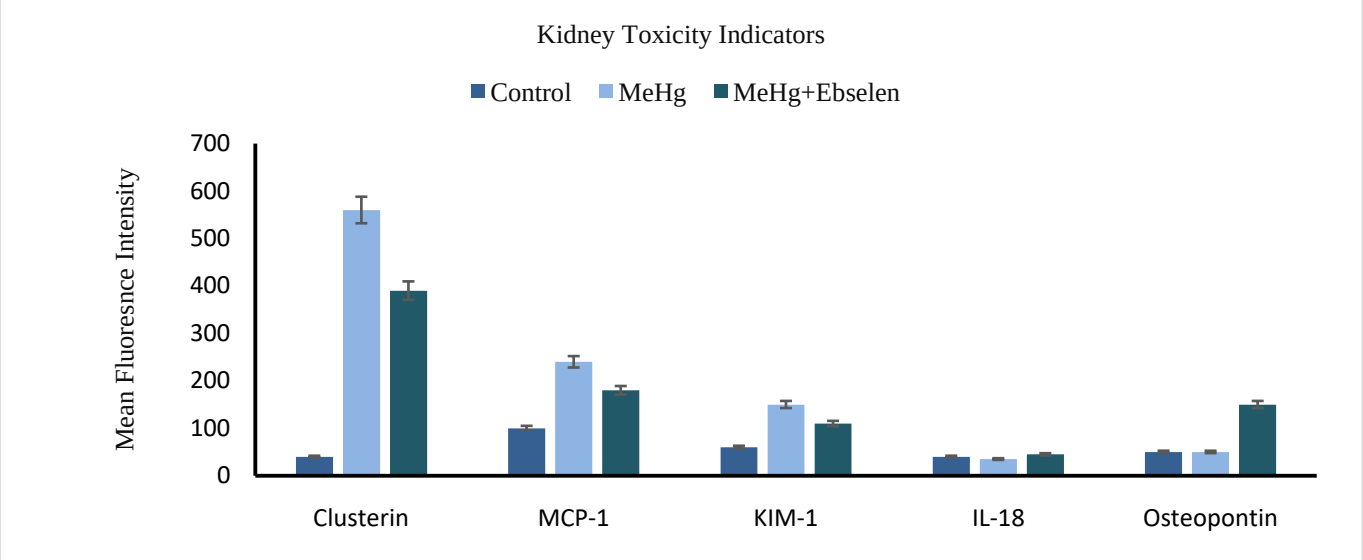


Figure-4: Analysis of urine samples for early biomarkers of kidney toxicity in PND 148 rats. Significant increase in Clusterin, MCP-1, KIM-1 and Osteopontin was seen in MeHg exposed groups, Ebselen restores near normal levels. No significant difference in serum creatinine was observed in between groups.

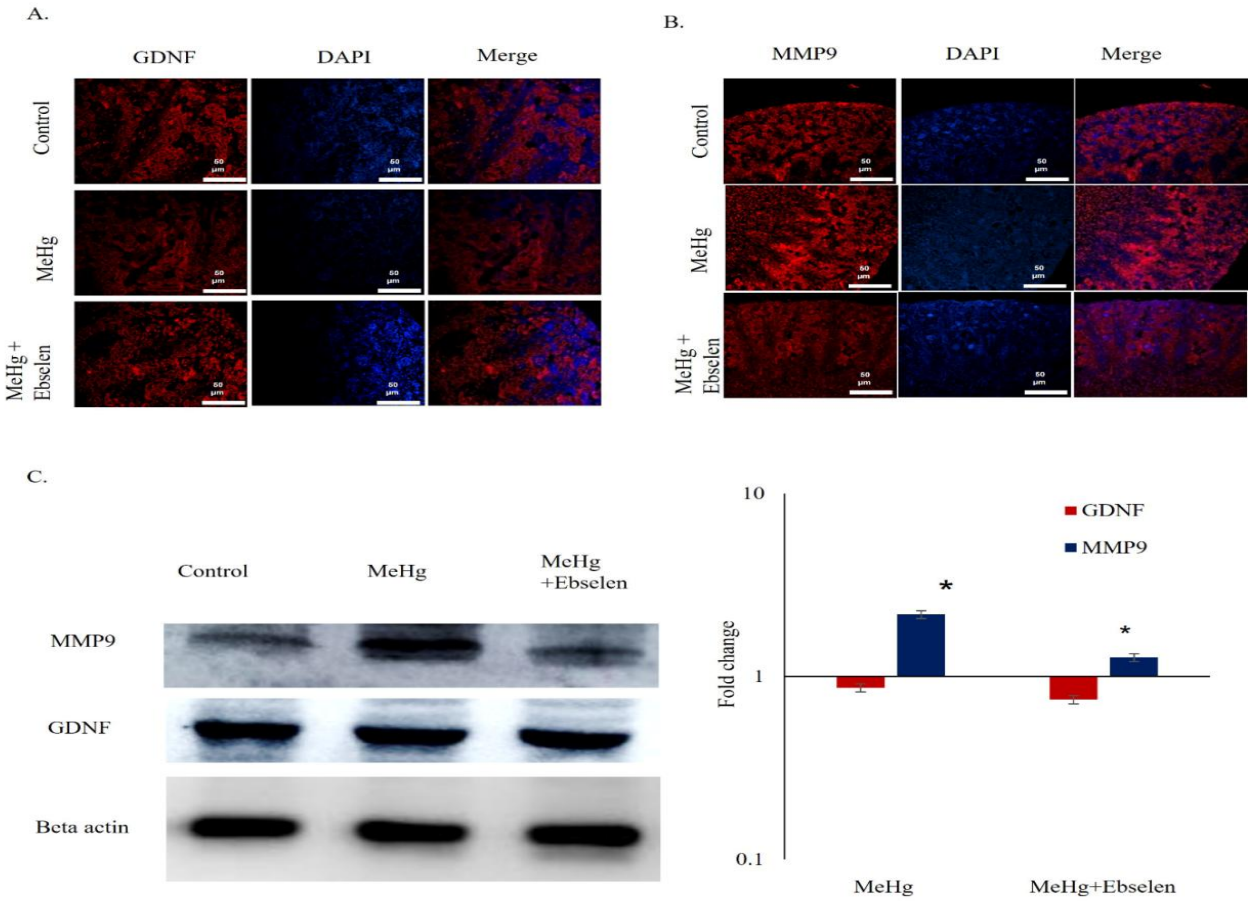
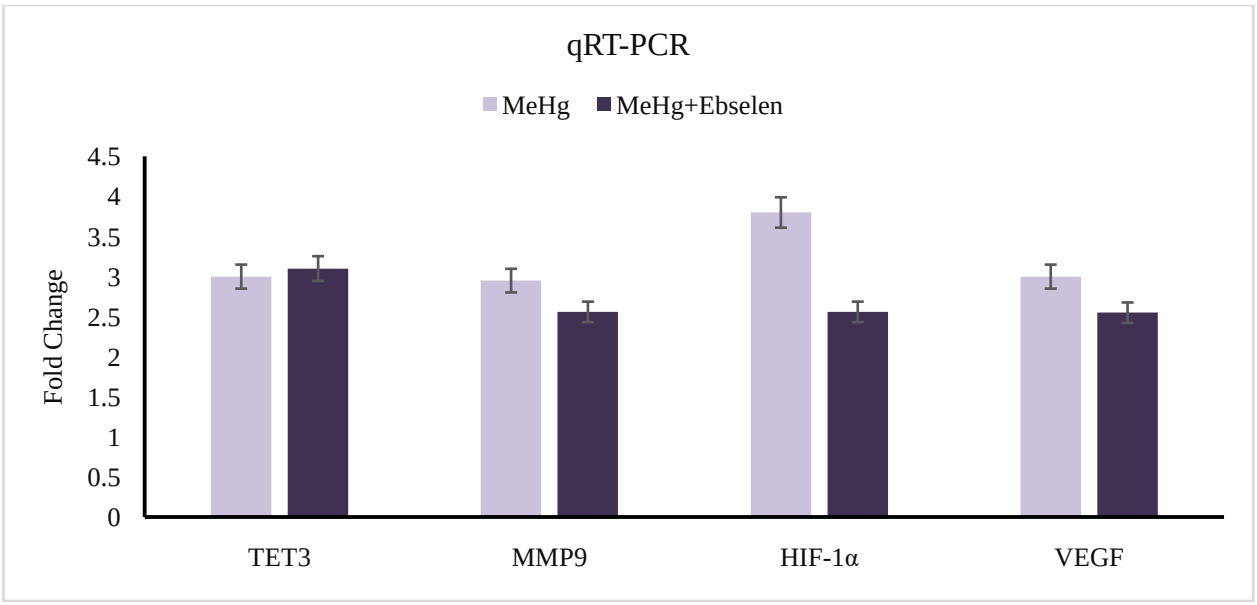


Figure-5: Low expression of GDNF and over expression of MMP9 is related with the defective kidney cortical regions of kidneys of PND2 pups (A): Less expression of GDNF in MeHg treated group. Ebselen bring the GDNF level to normal and thus the normal nephrogenesis (B): More expression of MMP9 in MeHg treated group. Ebselen bring the MMP9 level to normal (C): Western blot analysis MMP9 expression and slightly reduced level of GDNF due to MeHg and Ebselen.



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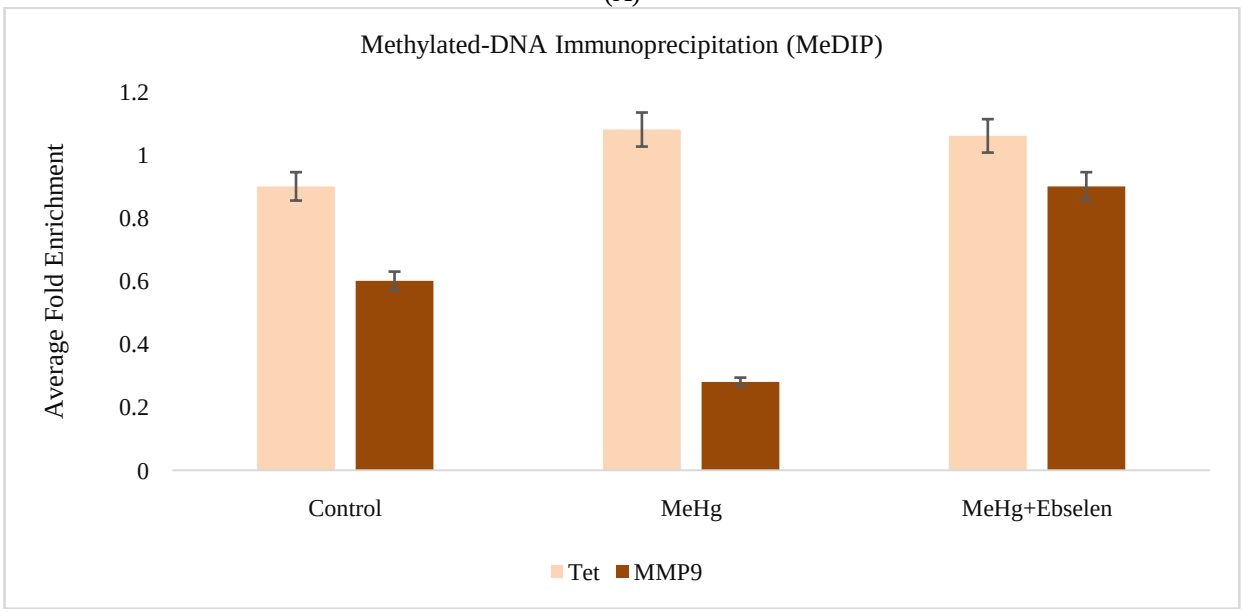

(B)

Figure-6: qRT-PCR analysis shows sustained maintenance of over expression of TET3, MMP9, HIF1-α and VEGF and seen in PND148 kidney samples. Ebselen helps in slight reduction of molecules. Methylation and Hydroxymethylation of TET and MMP9 was increased. This may be responsible for reduce level of expression in MeHg+Ebselen group.

Discussion: Oxidative stress is most common affect produced by many heavy metals including Hg. In response to this, cell follows a number of mechanisms to tackle the stress. Immediate increase in level of GSH, VEGF and HIF-1α is clear indication of hypoxia. Many compounds such as selenoproteins, vitamins are known for their potential to reduce the oxidative stress. Ebselen (2-phenyl-1,2-benzoisoselenazol-3(2H)-one) is a seleno protein compound and commonly used for its potential effects on reducing heavy metal toxicity. It mimics the function of glutathione to remove all kind of peroxides[23]. This property makes Ebselen a suitable agent for the treatment of MeHg induced renal toxicity[24]. Selenium containing compounds sequester mercury and reduce its biological availability[25]. At molecular level, MeHg causes epigenetic changes such as hypomethylation and histone modification. H3K4me3 methylations resulted in upregulation of genes due to in utero MeHg exposure[26].

Both inorganic and organic mercury reaches in higher concentration in kidneys as compared to brain. Chelating agents such as DMPS eliminate mercury effectively from the kidneys but not from brain[27].

This may be responsible for the sustained toxicity of organic mercury in brain. Calcium signaling is important in regulation and progression of nephrogenesis[28]. GDNF is important molecule during the formation of embryonic kidney. It helps in branching of the ureteric bud through GDNF/RET pathways. GDNF also promotes the continued branching of the ureteric bud. hGDNF also promotes the continued branching of the ureteric bud[29].

Expression of Nephrin, Podocin, P-cadherin, and ZO-1 and MMP9 is related to epitheliomesechymal transition which plays key role in repair of nephron during injury. Ebselen restores these genes to normal which is thought to be the probable mechanism in therapeutic properties of Ebselen against MeHg induced renal damage[30]. Podocytes are very important cells with finger like processes which makes sieve to filter urine during glomerular filtration. Loss of podocytes is clear indication on loss of kidney function due to MeHg toxicity. Ebselen maintains the numbers close to normal by combating the toxicity of MeHg. At molecular level, it was also found to increase the podocyte protein to normal as compared to MeHg group. Nephrin is another protein linked to diaphragm of podocytes. Loss of Nephrin in turn affects the functioning of the nephrons during glomerular filtration[31]. Villin is also important protein in the brush border epithelium of proximal convoluted tubule. Loss of Villin 1 in urine is a potent biomarker of kidney diseases as seen in MeHg treated groups. Ebselen prevents the loss of Villin[32]. Collectively, the loss and appearance of biomarkers in urine is a sign of end stage renal diseases and development of early adult-onset renal diseases[33].

Author Contributions

Dr. Hafizurrahman Khan (HRK) provided the animal samples and contributed to the experimental design. He also serves as the corresponding author for this article. Sameen Shafi (SS) conducted the histopathological and molecular studies. Pragati Kumari Gupta (PKG) carried out the biochemical analysis, real-time PCR, and MeDIP analysis. In recognition of their significant and equal contributions to the research work, SS and PKG share joint first authorship of this article.

Conclusion

This study shows that prenatal exposure to methylmercury (MeHg) causes serious and long-lasting damage to kidney development and function. These problems start in early life and continue into adulthood. The damage includes a lower number of glomeruli, poor structure in the kidney's cortex and medulla, vacuolated tubules, and overall defective kidney growth. MeHg exposure also raises the levels of harmful kidney markers such as clusterin, KIM-1, IL-18, and osteopontin. It causes chemical changes in the body like increased creatinine in the blood, protein in the urine, and loss of albumin, which are signs of kidney damage. At the genetic level, MeHg changes the expression of important kidney development genes. It increases genes like MMP9, VEGF, HIF-1α, and TET3, and decreases structural proteins like Podocin and Villin. These changes weaken the kidney's structure and function. MeHg also causes epigenetic changes by reducing the methylation of the MMP9 gene, leading to its high activity. However, treatment with Ebselen, an antioxidant compound, helps reduce these harmful effects. Ebselen improves kidney structure and function, brings gene activity closer to normal, and corrects the methylation of MMP9. It also lowers oxidative stress and protects kidney tissues. This shows that Ebselen can protect the kidneys in many ways—by acting as an antioxidant, reducing inflammation, and fixing epigenetic problems. In summary, this study highlights the long-term dangers of prenatal MeHg exposure and supports the use of Ebselen as a helpful treatment to reduce mercury-related kidney damage. These results offer useful information for future research and strategies in environmental health and kidney protection.

Acknowledgments

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