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Hepato Renal (Hepatorenal) Toxicity Induced ByMercuric Chloride In Male Wistar Albino Rat, *Rattus Norvegicus*(*norvegicus*)

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Abstract

This study investigated the effects of oral mercuric chloride ($HgCl_2$) treatment on sexually mature albino rats.Different doses of $HgCl_2$ (i.e., 1/4th and 1/8th of LD_{50}, or 5.25 mg/kg and 10.5 mg/kg respectively) were administered to the rats for 30 and 60 days. The results demonstrated a significant dose and duration dependant increase in serum Alanine transferase (ALT), Alkaline phosphatase (ALP), Aspartate transferase (AST), serum bilirubin, urea, uric acid, creatinine, and Blood Urea Nitrogen (BUN) concomitant with a decrease in serum protein levels. Histopathological examination revealed pronounced liver damage, characterized by sinusoidal dilation, necrosis, and fibrosis, as well as marked kidney injury, evidenced by glomerular and tubular damage. The animals also showed a significant rise in lipid peroxidation levels (MDA levels) with a decrease in reduced glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD). Immunohistochemical analysis revealed a decrease in B Cell lymphoma (Bcl-2) expression in the liver indicating apoptotic activity and an increase in Epidermal growth factor regulator (EGFR) expression in the kidney suggesting a compensatory mechanism for tissue repair and regeneration. The study underscores the need for strict regulatory measures to minimize mercury exposure and protect public health.

Keywords: Mercury toxicity; Liver and kidney function; oxidative stress; Bcl-2, EGFR

Introduction

Global environmental contamination has been primarily caused by extensive human activitiesinvolvingchemicals[1,2].Theoccurrenceofpersistentchemicals,such aspesticidesandheavymetals,insoil and water is directly associated with the declining health of non-target species, like fishes andmammals [3,4]. The global concern regarding the negative

impacts of heavy metals has risen due to therapid growth of the world's population, the expansion of industrial activities, and urban development [5].Human exposure to heavy metals leads to the development of severe health issues that deteriorate overtime, impacting major organs like the liver, heart, brain, and kidneys. Due to their non-biodegradablenature, they are present in the environment for prolonged periods, posing long-term health risks[6]. Thepathophysiology of hepatorenal mercury toxicity involves an overproduction of free radicals and animbalanceintheantioxidantdefensesystem.Asaconsequence,oxidativedamageoccursinbothhepatic andrenalcells[7].TheseROScanleadtovarioustypesofinjuries,includingdamagetotheliver,kidney,cardiovascular system, and inflammation-related diseases. Antioxidants can counteract or suppress thedetrimentalimpactsofreactiveoxygenspecies(ROS),thuseffectivelymanagingtheassociatedhazards.Occasionally,thetoxiceffectscaused byheavy metalscan alsointerfere withthis mechanism[6].

Mercury, a highly toxic heavy metal, is considered a major contributor to environmentalpollution [8]. It was previously employed in insecticides, metallurgy, and as a photographic fixative.Human activities, such as mining, smelting, extensive industrial and agricultural practices, burning offossil fuels, and other industrial activities, resulted in the release of mercury into the environment [9].The research conducted by Emanuelli et al. (1996)[13]revealedthatinorganicmercuryprimarilybuildsupintheliverandkidney,resultinginacute hepatorenal toxicity in organisms, as demonstrated by Tanaka-Kagawa et al. (1998) [14]. The toxicityassociated with mercury is mainly a consequence of its high affinity for sulfhydryl (SH) groups.Althoughmercurycompoundsexhibitspecificitytowardssulfhydrylgroups,theirtargetsarenonspecific due to the ubiquitous distribution of this group, as discussed by(Valko et al., 2005)[15]. Theaccumulation of mercury in a body within a region ~~contaminated by this toxic element~~ can lead tovariousdetrimentalconsequences.OnesucheffectistheexcessivegenerationofROSandanelevationinlipidperoxidationlevelswithinthecells[16].Freeradicalsandperoxidationintermediateproductscanimpair the integrity and function of biomembranes, thereby playing a role in the development ofmultiple pathological processes [17]. The presence of specific antioxidant enzymes, such as SOD, CAT,and GPx, is vital in preventing the generation of harmful free radicals and protecting cell membranesfrom oxidative damage [18]. An imbalance between oxidant and antioxidant levels, mainly due to enhanced levels of reactive oxygen species in the body, is regarded a crucial indicator of chemical stress [19].

Mercuric chloride is recognized as one of the most hazardous forms of mercury, with a primary metabolic pathway in the liver and accumulation in the kidneys. As a result, the liver and kidneys are identified as the principal organs targeted by mercury-induced damage [20]. It is associated with a range of detrimental effects on the hematological system [21], liver function [22], neurological health [7], as well as gastrointestinal and cardiovascular disorders [20].

With due consideration to the importance of the vital organs mentioned earlier, this study was done to understand and comprehend the influence of mercury on the overall functioning of both the liver and kidney biochemically, histologically and immunohistochemically.

Materials and methods

Experimental animals

The current investigation employed male Wistar rats (Rattus norvegicus) (*Rattus norvegicus*) 90 days old, with an average weight of 160-170 grams. The animals were housed in propylene cages. Their bed was prepared using paddy husk. The subjects were given commercial feed (VRK Nutritional Solutions, Sangli Maharashtra) and unlimited access to tapwater at an animal care facility, Department of Zoology,Karnatak University, Dharwad. They were maintained in a laboratory environment at 28±2°C andfolloweda12-

hourdark/lightcycle.Beforestartingourexperiment,thesesubjectswentthroughaweekofacclimatizatio nandwerehandledasperstandardguidelineslaiddownbytheCommitteeforPurposeof Control and Supervision of Experiments on Animals (CPCSEA) in New Delhi. These guidelinesregulatethetreatmentandutilizationoflaboratoryanimals.TheInstitutionalAnimalEthicsCom mittee(IAEC)ofKarnatak University in Dharwad alsoapproved theseprocedures.

Following the acclimation period, animalswere assigned randomly into five groups;each groupconsisted of six animals. These groups were named Control, Group 1, Group 2, Group 3, Group 4, andGroup 5. The animals were then subjected to two different concentrations of $HgCl_2$ ($HgCl_2$) , specifically ¼ oftheLD50 (LD $_{50}$)(10.5mg/kg)and1/8oftheLD50 (LD $_{50}$(5.25mg/kg),fortwodifferenttreatmentdurationsof30and60 days.

- Group1: Asacontrol, theanimals inthisgroup receivedasalinesolution.
- Group2wasadministeredadoseof5.25mg/kgof$HgCl_2$ ($HgCl_2$),whichisequivalentto1/8oftheLD50,for30days.
- Group3wasadministeredadoseof5.25milligramsperkilogramofbodyweightof$HgCl_2$ ($HgCl_2$),whichisequivalentto one-eighthof theLD50(thelethaldose for 50%of thepopulation), for 60days.
- Group4wasadministeredadosageof10.5milligramsperkilogram ofbodyweightof$HgCl_2$ ($HgCl_2$),whichisequivalentto one-fourth ofthe LD50, for30 days.
- Group5wasadministeredadosageof10.5milligramsperkilogram ofbodyweightof$HgCl_2$ ($HgCl_2$),whichisequivalentto one-fourth ofthe LD50(thelethal dose for 50%of thepopulation), for 60days.

Thetreatmentwasadministeredorallytononfastedratsfor30daysand60days,withadosevolumeof1 mL per 100 grams of body weight, during the morning hours from 9.00 am to 10.00 am. Every day,theanimalswereobservedforanysignsoftoxicity,bothbeforeandafterreceivingthedose.Measureme nts of body mass increase and consumption of food were documented. Animals wereweighed and euthanized 24 hours after the final dose using cervical dislocation while under anesthesia.Allthroughtheexperiments'durationanimalswereobservedatleastonceperdayforanyclinica lsignsindicatingtoxicity.Anautopsywasconductedaftertheexperiment,specificallyat30and60days.Blo odsamples were obtained and subjected to centrifugation at 4000×g for 10 minutes to extract serum.Testicular tissues were collected by separating them from surrounding tissues and weighed on anelectronicbalancetothenearestmilligram. Thetissues werethen preservedin formalin, afixative.

CollectionandPreparationofserumsamplesforliverandkidney functiontests

Animals were fasted for 10-14 hours before blood samples were collected by cardiac puncture. Bloodsampleswereputintoplaintubesandallowedtoclot,thentheserumwasisolatedthroughcentrifugati onat3000rpmfor10minutes.Thecollectedsupernatant(serum)wasanalyzedforliverandkidneyenzyme levels.

Biochemical assays

AssessmentofLiverfunctions

Theaspartateaminotransferase(AST)andalanineaminotransferase(ALT)levelsintheseparatedserumwe redeterminedaccordingto Bergmeyer and Walefield(1978) [34]method.

The levels of serum alkaline phosphatase (ALP) were measured using the MacComb and Bowers(1966)[35]method.

Totalprotein was estimated bythe protocol as outlinedby Lowry et al(1951)[36].

The spectrophotometric measurement of serum albumin and serum globulin was estimated using theprotocolpreviously describedby Doumas etal (1997) [37] and Busher (1990)[35]respectively.
The estimation of Direct bilirubin concentration in serum was carried out using the method developedby Malloy and Evelyn (1937) [39] which is based on the Van Den Bergh reaction. Estimation of
Indirectbilirubinwascarriedoutbyareactioninwhichthepresenceofmethylalcoholservestodissolvewater-
insolubleorunconjugatedbilirubin.Consequently,thismethodalsoallowsforthedeterminationoftotalbilirubinlevels, which includes both conjugated andunconjugated forms.

Assessmentofrenalfunctions

Theplasmacreatininelevelsweredeterminedutilizingadiagnostickitbasedon Jaffe's(1886)[40]method.The method for BUN measurement as described by Richard et al., (1984) [41]was followed in this study.Using a diagnostic kit, the amount of urea in plasma was estimated using the protocol developed byFawcett and Scott (1960) [42]. A diagnostic kit based on the enzymatic approach outlined by Caraway(1955)[43]was used to estimatetheamount of uricacid presentin the plasma.

Histopathologicalanalysis

Thehistopathologicalexaminationswerecarriedoutbythemethodologydescribedby Humason[50].Theisolated liver and kidney tissues were fixed in Bouin's fluid. Following fixation, the tissues weredehydrated using a series of alcohol gradients and then embedded in paraffin wax. The organs that hadbeen embedded in paraffin were sliced into ribbons that were 5 micrometers thick using a semi-automated microtome (LeicaRM 2255). For light microscopic evaluation, these sections were
stainedwithHematoxylinandEosinwhichwascapturedusinganOlympusphasecontrastmicroscope(Olympus BS51, Tokyo, Japan) equipped with a photography system (ProgResC3, Jenoptic-Germany).Theobservationswerenoted,andthefinalimageswerecarefullyreviewedtolookforanyinconsistencies.

Immunohistochemistry

Sections with a thickness of micrometers (µm) were acquired from the liver and kidney using amicrotome. Following this, the sections underwent a standard histological staining technique known aseosinandhematoxylinstain(SigmaAldrich).Toeliminateparaffin,thesectionsweretreatedwithxylene,andthendehydratedusingprogressivelyhigherconcentrationsofethanol.Theintrinsicperoxidaseactivitywassuppressedbygraduallyincreasingconcentrationsofethanol,rangingfrom3%to higher levels. Additionally, the peroxidase enzyme's activity in the sample was naturally suppressedby exposing it to a 3% hydrogen peroxide solution for a duration of 15 minutes. Subsequently, thesections were heated in a microwave pressure cooker for 20 minutes using a 0.01mol/1 citrate bufferand then allowed to cool down to room temperature. Following this, the sections of the tissues weresubjected to specific primary antibodies for a period of 30 minutes. The primary antibody used
forsectionsoftheliverwasBCelllymphoma2(Bcl2;Clone:EP36Rabbitmonoclonalantibody,Pathnsitu,Biotechnologies) at a concentration of 1:1000 for each antibody. For the kidney, the primary antibodyused was EGFR (EGFR; Clone: EP22 Rabbit monoclonal antibody, Pathnsitu Biotechnologies). Thesections were then stained with the avidin-biotin complex (ABC kit) from Lab Vision Corporation,which is based in Fremont, California, in the United States of America, using

the immunoperoxidasemethod.TheABCchromogenwasthenemployedtoidentifythebindinglocations.Phosphate-bufferedsalinewasusedforrinsingbetweeneachphase.Later,allofthesectionswereexaminedunderaMagnusphase contrast microscope (Magnus MLXi plus, India) after being stained with eosin and hematoxylinstain.Thecapturedimageswerelateranalyzedforanyinconsistencies,andthefindingswereDocumented.

Antioxidantenzymeassays

Theliverandkidneywerecutintosmallpieces,andthen10%homogenatesweremadewithanice-coldpotassiumphosphatebuffer(0.05M)withapHof7.4.Thehomogenateswerecentrifugedthehomogenatesfor15minutesat4°Cat6000rpm.Theactivitiesofglutathioneperoxidase(GPx),catalase(CAT), and superoxide dismutase (SOD), lipid peroxidation (LPO) were assessed using supernatant.ThemethodasgivenbyBuegeandAust(1978)[48]wasusedtomeasureLPOandwasexpressedasnmolMDA formed/mg protein. Using the protocol outlined by Luck (1965) [44] the activity of CAT wasdetermined and was expressed as µmoles of H_2O_2 degraded/min/mg protein. The activity of SOD andGPx was estimated by the method described by Kakkar et al (1884)[45] and Paglia and Valentine (1967)[46] and expressed as U/mgprotein.

Statisticalanalysis

Allacquireddataweredisplayedasmeanvalueswiththestandarddeviationforquantitativemeasurements. Analysis of variance (ANOVA) was done to ascertain statistical disparities amongdistinctgroups.Inthefollowingstep,aposthocTukey'stestwascarriedout.Thesignificancelevelwasset at $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$.GraphPad Prism 9 was utilizedto carry out theappropriatestatistical analyses.

Results

Effect of $HgCl_2$ on liver function parameters

The levels of liver enzymes, namely AST, ALP, ALT, and Bilirubin, exhibited a significant increasewhen compared to the control group ($P<0.05$). However, there was no significant difference ($P>0.05$)inbilirubinlevelsbetweenGroup1andGroup2,Group3andGroup4,aswellasGroup2andGroup4. Conversely, the levels total protein were significantly decreased incomparisonto the control group ($P<0.05$).

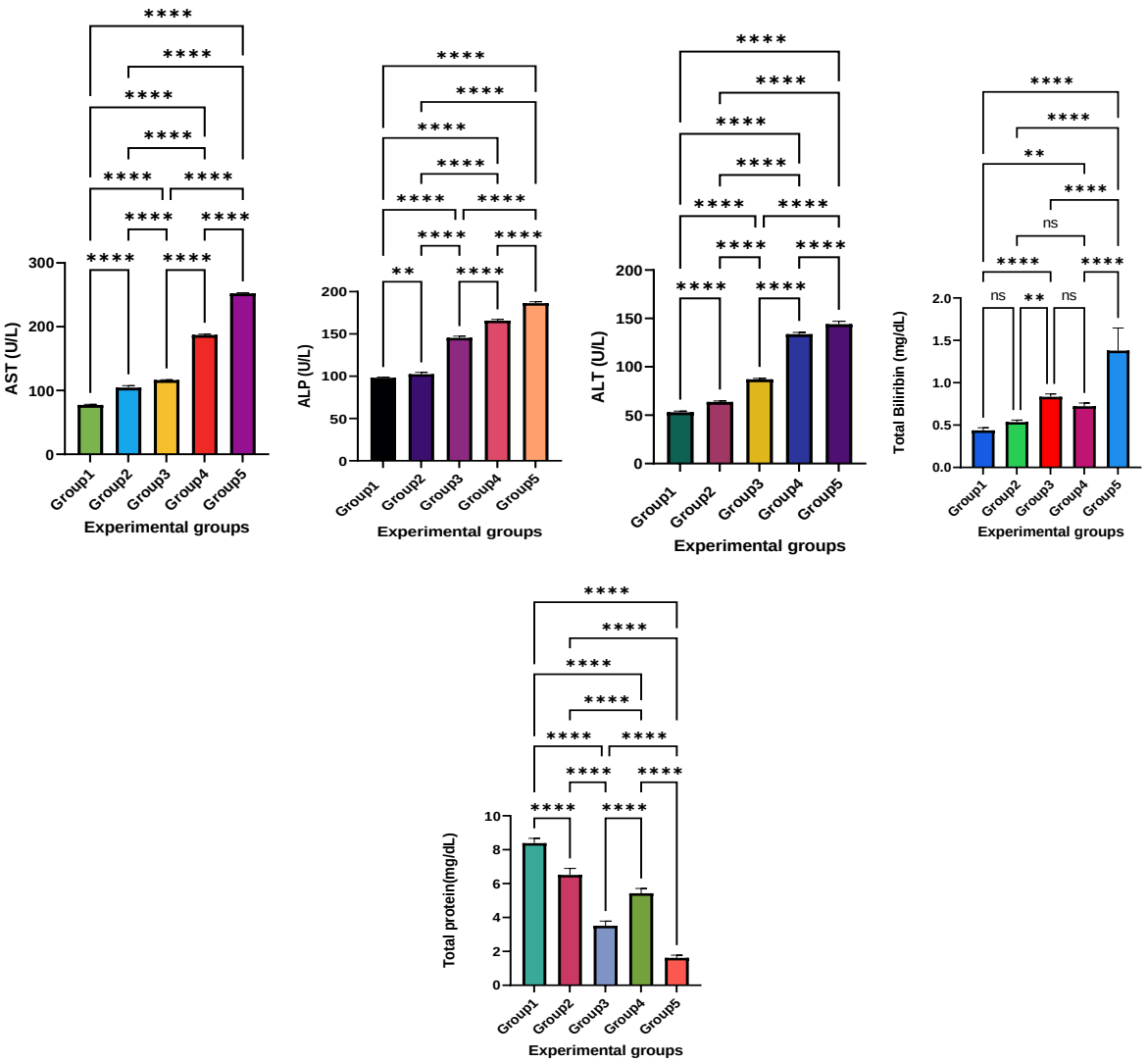


Fig 1: Effect of $HgCl_2$ on liver enzymes

The values are expressed as mean ± SD (n = 6). Asterisk (****) signifies substantial variation atP<0.0001, Asterisk (***) signifies substantial variation at P<0.001, Asterisk (**) signifies substantialvariation at P<0.01, and Asterisk (*) signifies substantial variation at P<0.05 compared betweenvariousexperimental groups.

Effect of $HgCl_2$ on kidney function parameters

Significantlyelevatedlevelsofkidneyenzymes,includingcreatinine,urea,anduricacid,wereobserved(P< 0.05)incomparisontothecontrolgroup.Conversely,therewasnostatisticallysignificantdistinction(P>0. 05)inBUNlevelsbetweenGroup1andGroup2,Group2andGroup3,orGroup1andGroup3.

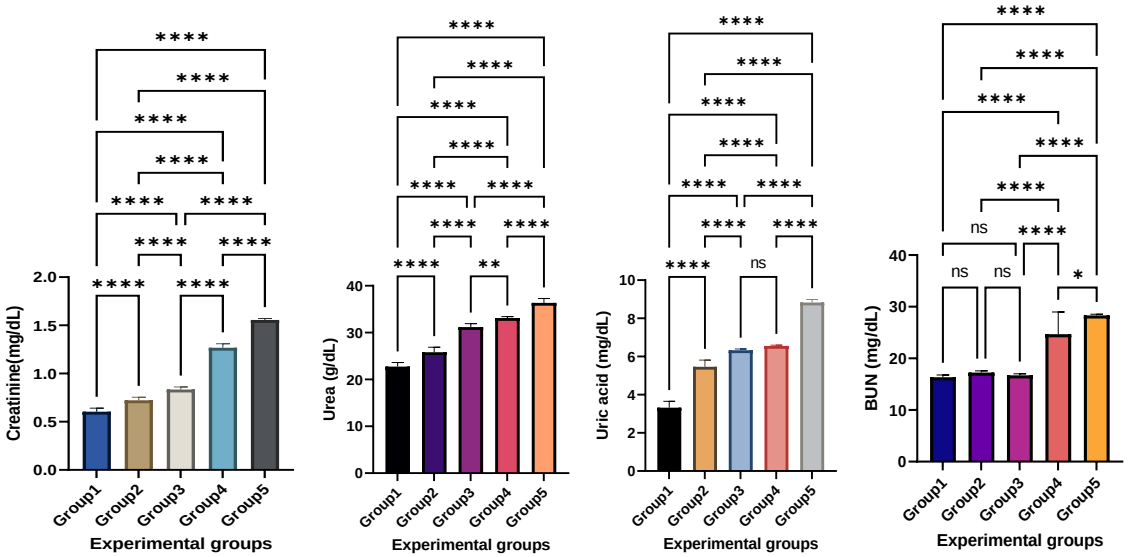


Fig2:EffectofHgCl$_2$onkidney enzymes

The values are expressed as mean ± SD (n = 6). Asterisk (****) signifies substantial variation atP<0.0001, Asterisk (***) signifies substantial variation at P<0.001, Asterisk (**) signifies substantialvariation at P<0.01, and Asterisk (*) signifies substantial variation at P<0.05 compared betweenvariousexperimental groups.

EffectofHgCl2onlevelsofantioxidantenzymesandlipid peroxidationin A)liverB)Kidney :The findingsrevealedanotable decrease (P<0.0001)intheactivityofantioxidantenzymes,suchasCatalase, SOD, and Gpx, in the low-dose treated groups (Group 2 and Group 3). Conversely, in the high-dosetreatedgroups(Groups4and5),therewasasignificantincrease(P<0.0001)intheactivityofantioxidan t enzymes compared to the low-dose groups, although it was still lower than that of the controlgroup(Group1).Ontheotherhand,theactivityofMDAwassignificantlyelevated(P<0.0001)inthelo w-dosegroups(Group3&4),whileinthehigh-dosetreatedgroups(Group4&5),MDAactivitywasnotablyreduced(P<0.0001)comparedtothelowdosegr oups,butstillsignificantlylower(P<0.0001)than thecontrol group (Group 1).

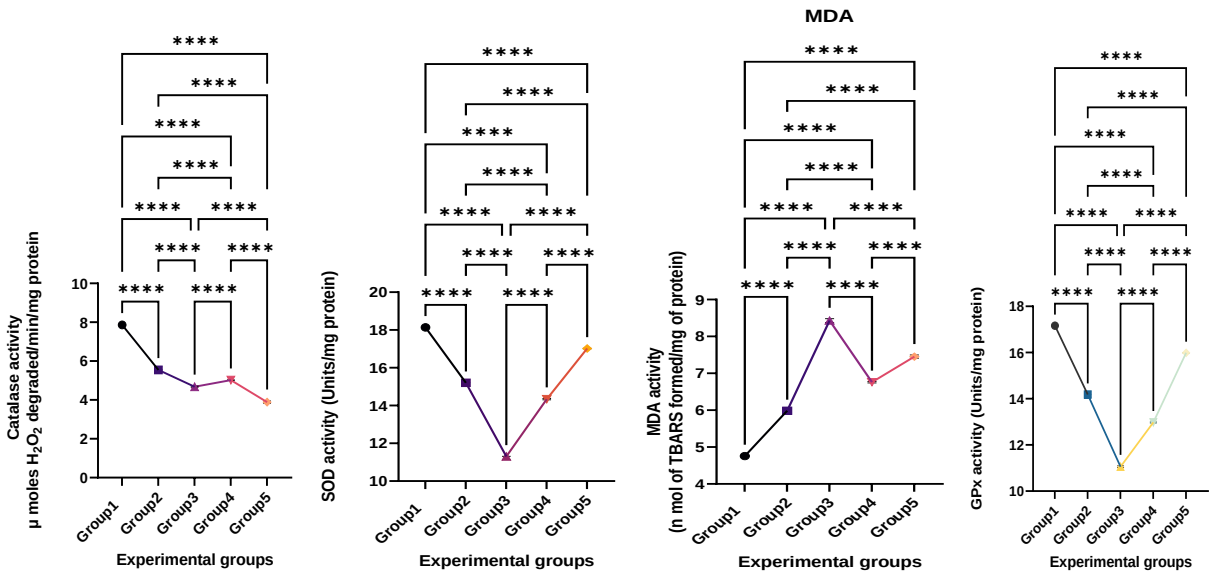


Fig 3A: Effect of HgCl$_2$ on antioxidant status of liver

The values are expressed as mean ± SD (n = 6). Asterisk (****) signifies substantial variation atP<0.0001, Asterisk (***) signifies substantial variation at P<0.001, Asterisk (**) signifies substantialvariation at P<0.01, and Asterisk (*) signifies substantial variation at P<0.05 compared betweenvariousexperimental groups.

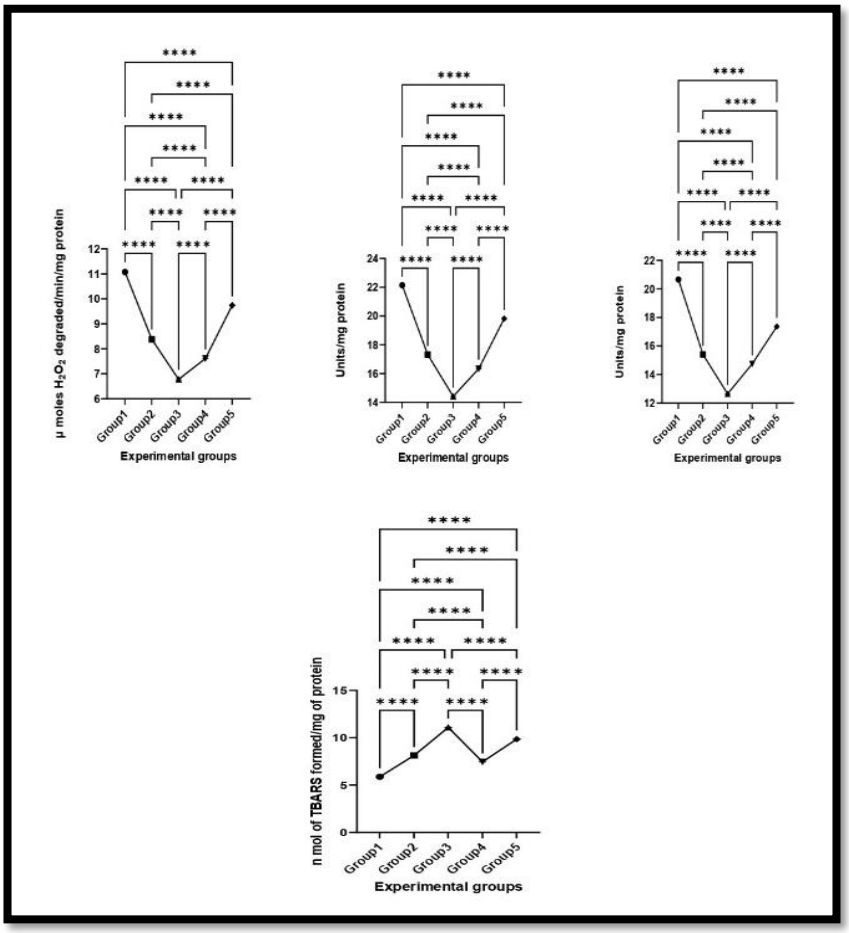


Fig 3B: Effect of $HgCl_2$ on antioxidant status of liver

The values are expressed as mean ± SD (n = 6). Asterisk (****) signifies substantial variation atP<0.0001, Asterisk (***) signifies substantial variation at P<0.001, Asterisk (**) signifies substantialvariation at P<0.01, and Asterisk (*) signifies substantial variation at P<0.05 compared betweenvariousexperimental groups.

Histopathology of liver: (Fig 3A- F) H & E sectioins of liver exhibits the histological sections of control (Fig 3A & B) and changes after different treatment periods (Fig 3B – F). Liver of control rats showed normal hepatic cells (H) (Fig 3A); Central vein (CV) with normal radiating pattern of hepatic cords (HC) (Fig 3B); Hepatic artery (HA), Portal vein (PV), Bile duct (BD) together constitutes portal traid (PT) (Fig 3A); Sinosoids (S) (Fig 3B); Group 2(Fig 3C) shows dilated sinosoids (dS), Mild vacuolations (MV); Group 3 (Fig 3D) shows clogging of Bile duct (BDC), clogging of portal vein (PVC) and damaged hepatic artery (DHA), focal necrosis (FN); Group 4 (Fig 3E) shows dilation of central vein (DCV), pyknotic nucleus in hepatocytes (PN), vacuolations (V), disorganization of normal radiating pattern of hepatic cell plates (*); Group 5 (Fig 3F) shows hypertrophy of hepatocytes (HH), loss of hepatic cord arrangement (*), foamy appearance (F).

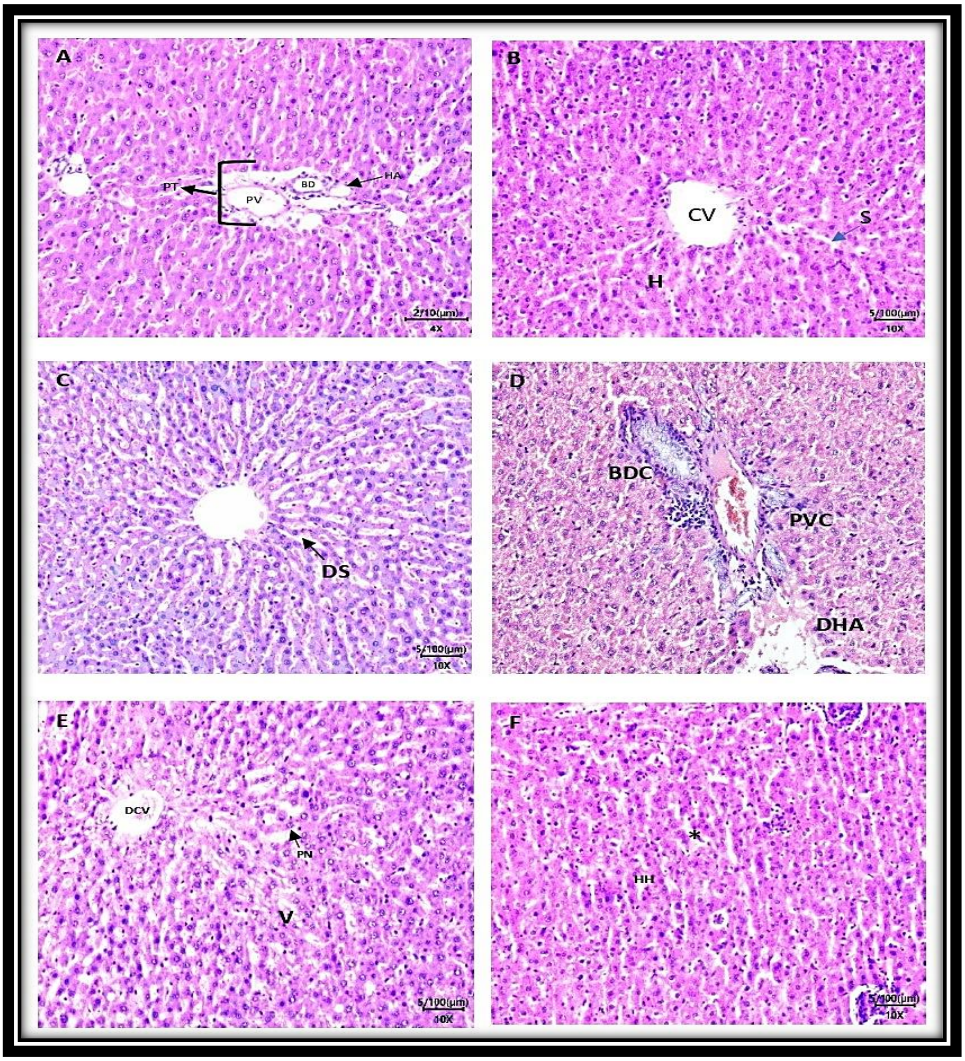


Fig 3: H & E sections of liver showshistological sections of control (Fig 3A & B) and changes after different treatment periods (Fig 3B – F). Group1 (Fig 3A &B) shows normla hepatic cells (H), central vein (CV) with normal radiating pattern of hepatic cords (HC), hepatic artery (HA), portal vein (PV), bile duct (BD) together constitutes portal triad (PT), sinosoids (S); Group 2 (Fig 3C) shows dilated sinosoids (dS), nild vacuolations (MV); Group 3 (Fig 3D) shows clogging of bile duct (BDC), clogging of portalvein (PVC) and damaged hepatic artery (DHA), focal necrosis (FN); Group 4 (Fig 3E) shows dilation of central vein (DCV), pyknotic nuclei in hepatocytes (PN), vacuolations (V), disorganization of normal radiating pattern of hepatic cell plates (*), Group 5 (Fig 3F) shows hypertrophy of hepatocytes (HH), loss of hepatic cord arrangement (*), foamy appearance (F).

Histopathology of kidney: (Fig 4A- F) H & E sections of kidney exhibits the histological sections of control (Fig 4A) shows normal renal corposcles (RC), glomerulus (G), Bowmans capsule (BC), proximal convoluted tubule (PCT), distal convoluted tubule (DCT), urinary space (US); Group 2 (Fig 4B) shows glomerular atrophy (GA), Haemmorhage (H); Group 3 (Fig 4C) shows damaged renal tubule (DRT), dilated bowmans capsule (*); Group 4 (Fig 4D) shows pyknotic nuclei (PN), damaged glomerulus (DG); Group 5 (Fig 4E & F) Haemorhage (H), damaged renal corposcle (DRC), dilated bowmans capsule (DBC), vacuolations (V)

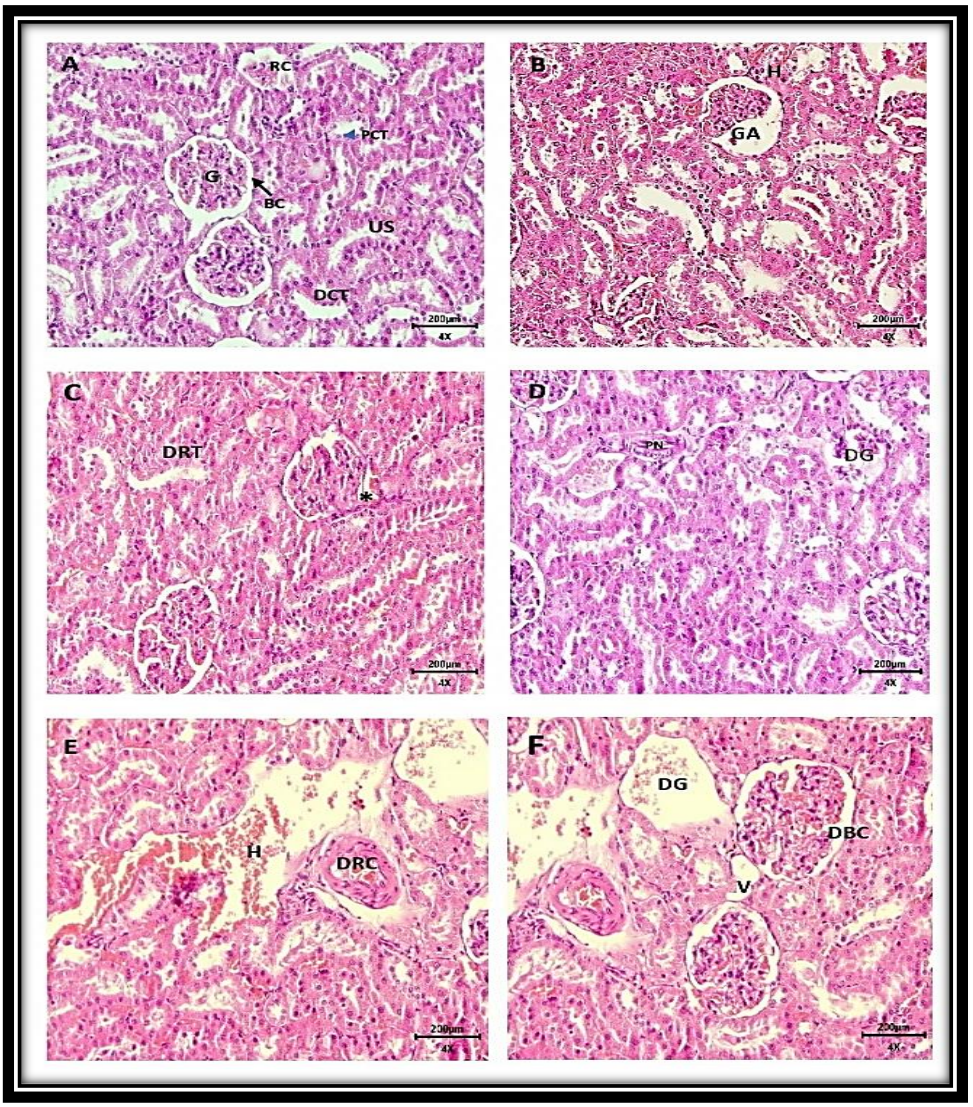
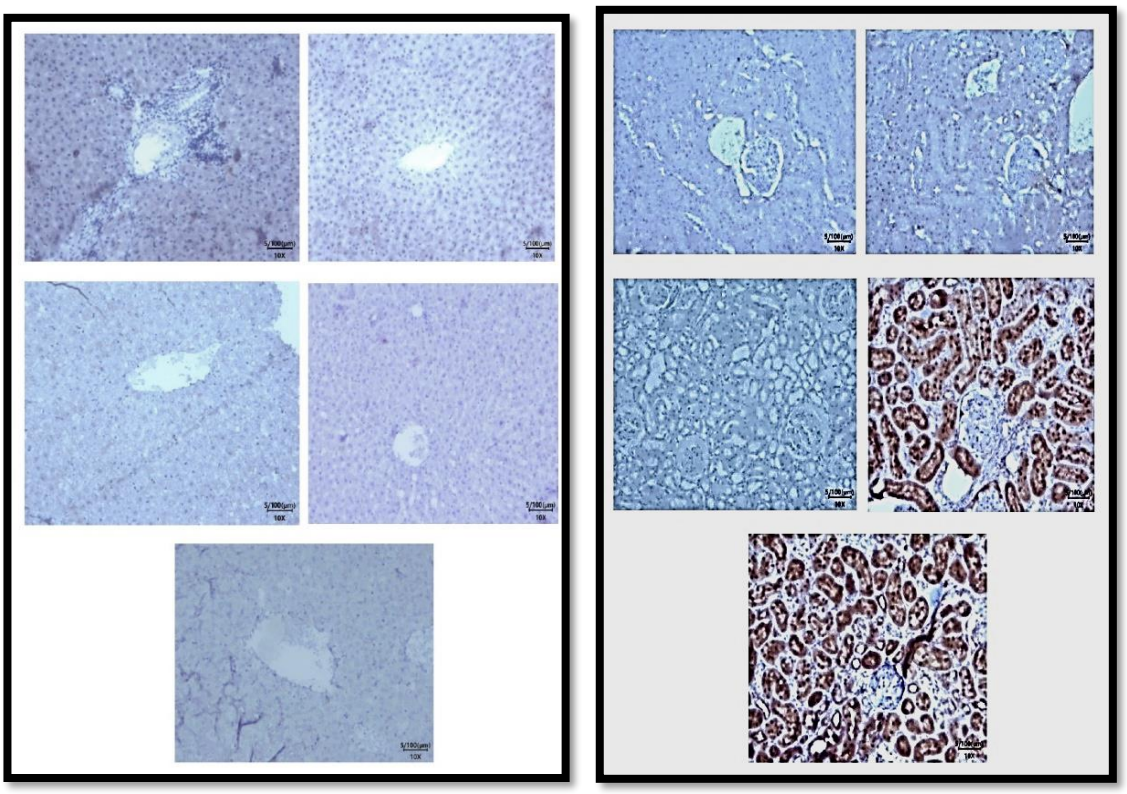


Fig 4: (A- F) H & E sections of Kidney exhibits the histological sections of control (Fig 4A) shows normal renal corposcles (RC), glomerulus (G), Bowmans capsule (BC), proximal convoluted tubule (PCT), distal convoluted tubule (DCT), urinary space (US); Group 2 (Fig 4B) shows glomerular atrophy (GA), Haemmorhage (H); Group 3 (Fig 4C) shows damaged renal tubule (DRT), dilated bowmans capsule (*); Group 4 (Fig 4D) shows pyknotic nuclei (PN), damaged glomerulus (DG); Group 5 (Fig 4E & F) Haemorhage (H), damaged renal corposcle (DRC), dilated bowmans capsule (DBC), vacuolations (V).

Immunohistochemistry

Fig 5A shows the expression of Bcl-2 in the liver of distinct experimental groups of rats. The respective antigen-antibody reaction appears to be positive as it shows the presence of granules in the cytoplasm and emerges as brown. In Fig 5a the normal distribution of anti-apoptotic protein Bcl-2 is shown, where as the expression of Bcl-2 is decreasing in dose and duration dependant manner as observed in 5b,c,d,e respectively.

Fig 5B shows the expression of EGFR protein in the kidney of distinct experimental groups of rats. The respective antigen-antibody reaction to be positive as it shows the presence of granules in the cytoplasm and emerges as brown. In Fig 6a the normal distribution of EGFR is shown, where as the expression of EGFR is increasing in dose and duration dependant manner as observed in 6b, c,d, e respectively.



A B

Fig 5: Immunohistochemical localization of Bcl-2 antigen in liver tissue(A) of rats; a) Group 1 shows good staining; b,c,d.e shows section of $HgCl_2$ rats shows less staining when compared to control. **(B) Immunohistochemical localisation of EGFR antigen in kidney tissue (B) of rats;** a) Group1 shows less staining; b,c,d,e shows section of $HgCl_2$ rats shows more staining when compared to control

Discussion

Thecurrentstudyaimstoexplorethehepatorenaltoxicitycausedby$HgCl_2$throughalterationinenzyme activities, oxidative damage, and imbalance in the antioxidant defense activity in rats, asassessedthrough biochemical, histopathological,and immunohistochemical analyses.

Liver and Kidney are crucial organs that must be taken into account during the examination ofpollutant effects, as they have a significant impact on the metabolism and detoxification of drugs.Moreover, the majority of substances absorbed in the intestine pass through the liver, where theaccumulationoftoxinsandheavymetalsmayoccur[51].Thetoxicmanifestationsofmetalsareprimarilyat tributedtooxidativestress[52].Thebody'simbalancebetweenantioxidantsandoxidantsiswhatdefinesoxid ative stress, increasing the levels of ROS including hydroxyl, superoxide, and hydrogen peroxide.TheseROS cancausedetrimental effects on various organs[53].

The administration of $HgCl_2$ causes the body to produce substances that cause oxidative stress,such as ROS. The endogenous antioxidant enzymes, including Superoxide Dismutase (SOD), Catalase(CAT), and Glutathione Peroxidase (GPx), act as the primary defense against damage caused by freeradicals [54].Superoxide anion is converted by SOD into hydrogen peroxide, which is subsequentlytransformedintowaterbyCAT[55].CATplaysanimportantroleinbalancingthegenerationofhy drogenperoxide and superoxide radicals. In this study, the inhibition of SOD and CAT activities is attributedto the excessive production of ROS. GPx, an enzyme containing selenium, is found in both the cytosolandmitochondrialmatrix[56].Itsprimaryfunctionistoreducehydrogenperoxide.Mercuricchloride

maydirectly inhibit GPx by disrupting its functional groups. Additionally, the decrease in CAT and GPxactivitiesmaybeadefensiveresponseagainstthehydrogenperoxideproducedbyHgCl2[57].Consequently, the lipid peroxidation level increases and antioxidant enzymes decrease. In the presentstudy, we found that $HgCl_2$ significantly diminishes the antioxidant enzyme activities, S0D, CAT, andGPxinliverandkidneytissues.Inthisstudy,theantioxidantenzymelevelsweresubstantiallydecreased ($P<0.0001$)inthelow-dosegroups(Group2andGroup3)thatweretreatedwith5.25mg/kgofHgCl2for 30 & 60 days respectively. However, in high-dose groups (Group4 & Group5) that were treated with10.5mg/kg of $HgCl_2$ for 30 & 60 days respectively, the levels of all mentioned antioxidants weresignificantly increased ($P<0.0001$) than low-dose groups where less than control (Fig 2). Additionally,the end product of lipid peroxidation malonaldehyde (MDA), the content was significantly increased($P<0.0001$) in low-dose treated groups (Group2 &Group3) that were treated with 5.25mg/kg of HgCl2for 30 & 60 days respectively, whereas, in high dose groups (Group4 &Group5) that were treated with10.5mg/kg of HgCl2 for 30 & 60days respectively, the MDA levels were significantly decreased($P<0.0001$)thanlowdosegroups,however,was morethanControl(Fig2).Mercury-inducedoxidativestress in liver and kidney tissues is linked to the integrity of the mitochondrial membrane's structure,reduction in mitochondrial glutathione levels, and elevation in hydrogen peroxide production by theelectron transport chain within mitochondria [58,59,60]. Raised levels of MDA and NO may arise from theenhanced generation of free radicals caused by $HgCl_2$ toxicity. This causes enhanced concentrations ofMDAandN0[61,62].Moreover,elevatedconcentrationsoffreeradicalssuchasH_2O_2mayblocktheSOD,CAT, GPx, and GR enzymes, leading to the reported reductions in these enzymes [63,64]. Furthermore,mercuryinhibitsGSHactivityduetoitsaffinityforthethoilgroupsfoundinGSHstructure[65].Ramadanetal., 2023[66], showedsimilarresults, indicating thatmercurycaused oxidativestress.

Thefollowingeventswithinthecellmaybethecauseoftheoxidativestresscausedbymetalsaltsaspreviously mentioned.

a) AFenton-type/Haber-Weissreactioninvolvingtheoxidationofferrousiron(2)byhydrogenperoxideto ferric iron (3), a hydroxyl radical, and a hydroxyl anion [67] may result in the generation of a freeradical. Furthermore, according to the Fenton and Haber-Weiss types of processes, these metal salts,such as mercuric chloride, may be directly reacting with the molecules within the cell to produceROS, or free radicals, which have the potential to damage polyunsaturated fatty acids found inbiological membranes. Hydrogen peroxide is catalyzed by glutathione peroxidase and catalase, twoselenium-containingenzymes that functiononly in the presenceofreducedGSH.

b) These metal salts have no redox potential, which reduces antioxidant defenses, particularly thoseinvolving thiols like glutathione peroxidase, which is dependent on GSH. This could lead to GSHdepletion,which would changethe cell's redox balance(GSH/GSSG).

Free radical generation is caused by [51] Hg toxicity in various tissues, primarily the kidney and liver,accordingtoFloraetal.(2008)[52].Theyproposedthatakeymechanismforthehepatorenaltoxicitycausedbymercurywasreactive oxygenmetabolites.Theanimalsinthecurrentstudythatwereintoxicatedwithmercury showed several signs of oxidative stress, including decreased antioxidant enzymes in the liverand kidney tissues and an increase in lipid peroxidation. As a result, oxidative stress may play a role intheorgandysfunctioncausedbymercury.Priorresearchhasindicatedelevatedlevelsofreactiveoxygenspecies (ROS) following mercury exposure. ROS then targets every component of the cell, which alsoincludes lipidsin the membrane, and produces LPO[69].

Our findings were in agreement with Madhura et al. (2007)[70] and Kumar et al. (2002) [71], Whorevealed that $HgCl_2$ ($HgCl_2$)caused oxidative damage to the cells by generating free radicals. This increasedproductionoffreeradicalsmayresultindamagetothecellmembrane,whichwoulddeactivatemembraneNa+-K+ ATPase thus permitting calcium entry into the cell. A persistent rise in intracellular calciumgenerates free radicals, which further inhibit Na+-K+ ATPase, resulting in lipid peroxidation and aweakenedstate of antioxidant defense.

Phase I and Phase II reactions are involved in the hepatic biotransformation. Phase I includes oxidative,reductive, hydroxylation, and demethylation pathways, mostly through the endoplasmic reticulum'scytochromeP-450enzymesystem,theliver'smostsignificantfamilyofmetabolizingenzymes.Additionally,theNADPH-dependentmixed-functionoxidasesystemandflavin-containingmonooxygenasesfoundintheendoplasmicreticulumareresponsiblefortheoxidationofsulfur compounds and amines. Certain intermediates produced by phase I reactions are converted to nontoxicforms by phase II reactions. Phase II reactions create more readily excretable, water-soluble metabolitesby conjugating substances with hydrophilic moieties, such as amino acids, glucuronide, or sulfate.Another phase II reaction involves glutathione, in which glutathione-S-transferase [72] can covalently bindto toxic intermediates. These reactionsarethereforetypicallythought of asdetoxification pathways.Butthisstagemayalsoresultinthecreationofunstableprecursorstoreactivespecies,whichcanbeharmfulto theliver[73,74].

The intermediates of the citric acid cycle have been linked by AST and ALT, two significantclasses of enzymes that link the metabolism of carbohydrates and amino acids. It is believed that thetransaminaseenzymesserveasindicatorsofliverinjury[75].Sincealkalinephosphate(ALP)ismembrane-bound, changes to it may impact membrane permeability and result in disruptions to thebody's metabolite transport process. In the present study the levels of AST, ALT, ALP, and bilirubinactivity increased with mercury intoxication in a dose and duration-dependent manner (Fig 1A). Thesefindings could be the consequence of membrane disruption or hepatocellular necrosis, which releasesthese enzymes into the bloodstream [76]. Moreover, a notable decrease in hepatic cell protein synthesis(Fig 1A) may be the cause of the substantial decrease in serum albumin and globulin, reflecting thehepatic dysfunction brought on by mercury treatment [76]. The histological alterations seen in the liverwere disruption of the typical cell plate radiating patterns, hepatocyte enlargement, vacuolization,cloggingofthe bileductandportalvein,andbilenecrosis (Fig3).Al-

Salehetal. and Agarwalet al. [77,78]reportedsimilarhistopathologicalalterationsintheliverfollowingmercuryexposure.Lipidperoxidation causesthereactiveradicalstoassaultthecellmembrane,causingittobecomeunstableanddisintegrate[79]. Mercuricchloride-inducedenhancedlipidperoxidationthatproducesfreeradicalscouldbe the cause of the histopathological alterations. These modifications also align with our biochemicalanalysis.
Heavy metals have been linked to changes in kidney function [80,81,82,55,] and particularly mercury hasbeen linked to negative effects on renal parameters [78,83,84]. Kidney function can be assessed usingparameterssuchasBUN,Uricacid,andcreatininelevels,whichareimportantmarkers[85].Inthepresent study,thelevelsofalltheabove-mentionedrenalenzymesareincreasedindoseandduration-dependentmanner (Fig 1B). Blood urea nitrogen is a significant metabolic product of protein metabolism andstudiesshowthatitcausesglomerulardamage[83].Groupstreatedwithmercuricchloridehadsignificantly higher levels of blood urea nitrogen, uric acid, and creatinine, increased synthesis of thearginaseenzyme,whichisinvolvedinthemanufactureofurea,and/orincreasedproteincatabolismare connected to raised blood urea nitrogen levels [82]. The kidneys eliminate creatinine, which is an endmetabolic result of muscle catabolism [83]. The glomerular filtration mechanism is necessary for theexcretion of creatinine. The increased level of creatinine indicates injury to the tubular and glomerularfiltering systems[86,87,88].Theprimarymetabolicproductofpurine nucleotidesis uricacid [89].Onerenalprognostic factor is an elevated amount of uric acid. Nonetheless, elevated levels of serum uric acidcouldbearesultofthebody'smetabolicreactiontoincreasedendogenousoxygenspeciesproduction [90]. According to earlier research, heavy metals have been linked to elevated BUN, uric acid, and $urea_{levels}$82,91, and 55.

The immunohistochemistry findings showed that the amount and length of therapy both affected theexpression of Bcl-2 (Fig. 5A). This finding, together with the earlier research by Yuan et al., 2014 [92],demonstratesthatdamagetothemitochondriacausedbyelevatedexpressionofapoptoticmitochondrialproteins,likethoseintheBaxfamily,maybelinkedtomercury-inducedcelldeath.Apoptoticprocessesare initiated by the simultaneous activation of caspase-3 brought on by the change in Bax expression.Thishinderstherele aseoftheantiapoptoticproteinBcl-2fromthemitochondrialmembrane[93].Thecellapoptotic pathway [94] can be regulated by the interaction between the pro-apoptotic protein Bax and theanti-apoptoticproteinBcl-2.Furthermore,oneofthemostimportantapoptoticpathwaysisthemitochondrial(intrinsic)pathway,whichisindicatedbyincreasedcytochromeClevelsinthecytoplasmdue to a disrupted Bax/Bcl-2 equilibrium [95]. Enhanced cytochrome c levels trigger the activation ofcaspase-3, which promotes apoptosis [96]. Apoptosis is triggered by caspase-3, which becomes moreactive due to an increase in intrinsic pathways. According to recent research, HgCl2 ($HgCl_2$)decreased Bcl-2levels and increased caspase-3 and Bax in the liver and kidney, hence having an apoptotic effect [97,98].Our results supported this, demonstrating that exposure to HgCl2 ($HgCl_2$)led to the death of liver and kidneycellsby overactivating caspase-3 and Baxwhile inhibiting the Bcl-2 protein.
Theepidermalgrowthfactorreceptor(EGFR)isaplasmatransmembraneglycoproteinthatisexpressedbyvariouscells.EGFRinitiatesitsintrinsictyrosineactivityuponligandbinding[99].Celldifferentiation,migration,proliferation,angiogenesis,apoptosis,proteinrelease,and/oroncogenesiswereallregulatedbytheEGFRsignalingpathway[99,100].Ithasalsobeendocumentedtobeinvolvedinhepatocellularcancer,cirrhosis,and liverregenerationafterbothacuteandchronicliverinjury[101].Itiswellestablishedthat activated EGFR

upregulates the expression of genes linked to inflammation, such as COX-2, viathe EGFR-Ras-MAPKs-AP-1-COX-2 caspase[102] pathway. It's interesting to note that mice lacking COX-2hadworseliverregeneration[103].Ontheotherhand,theconnectionbetweenHgCl2exposureandEGFR expression remains unknown. According to the current investigation, the HgCl2-treated groupexhibitsgreaterimmunopositivityforEGFRinadose-andduration-dependentwayascomparedtothecontrol group (Fig 5B)[97].otherresearchers reported similarfindings.

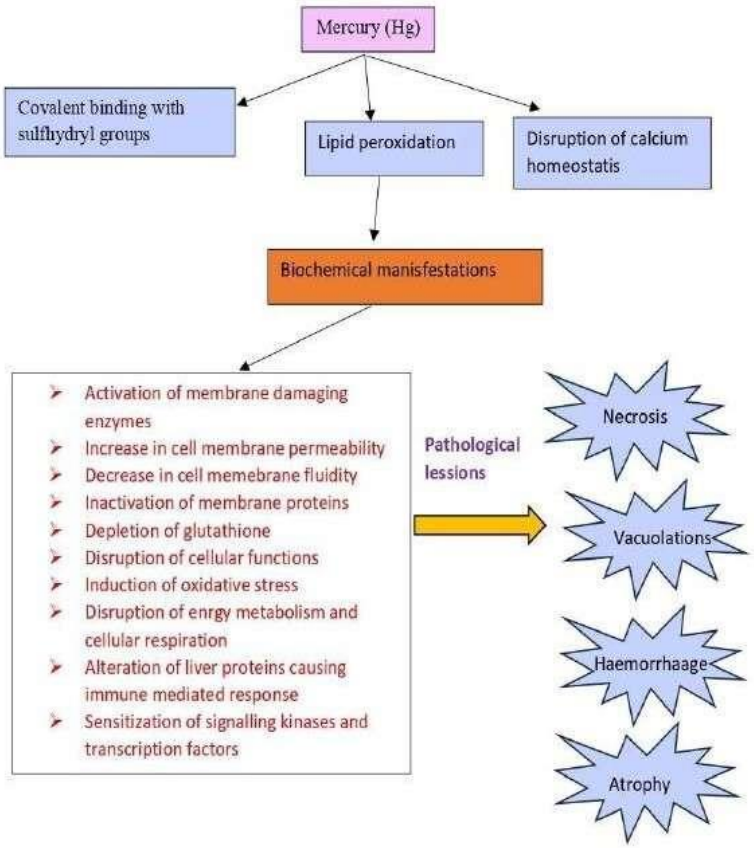


Fig6:Proposedmechanismoftoxicityinduced byHgCl2in liverandkidney

Conclusion

This study showed that long-term exposure to mercury at both low and high concentrations damagedliverandkidneytissuethroughoxidativestress.Theresultsofthisstudyunderscoretheharmfulef fectsof mercury and the significance of putting policies in place to lessen the toxicity that it causes.

Thedevelopmentofdetoxificationmethodstoenhancegeneralhealthwillbeaidedbyadeeperunderstandi ng of the molecular mechanisms behind the toxicokinetics of mercury toxicity in livingbeings.

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ConflictsofInterest

Theauthorsdeclarethatthey havenoConflict of Interest.

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GuidelinesforEthical Approval

Theexperimentsconductedinthisstudyadheredtotheguidelinessetbytheinstitutionalanimalethicscommittee (IAEC) (KUD/Zoo/007/IAEC/23). The study's experimental animals were treated withmeticulouscarebytheguidelinesestablishedbytheCommitteeforControlandSupervisionofExperimentson Animals (CPCSEA) in New Delhi,India.

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