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Original Article

Protective effect of resveratrol in mercuric chloride induced neurotoxicity in rats

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ABSTRACT

Mercuric Chloride (HgCl2) is one of the most toxic heavy metal which induces oxidative stress in the body. And also it acts as a neurotoxin. Mercury contamination in drinking water and inhalation appears to be serious and causes metabolic, functional and structural damages in the brain. This study aims to evaluate the protective effect of Resveratrol in mercuric chloride-induced neurotoxicity. Neurotoxicity was induced by drinking water containing 1.29mg/kg of mercuric chloride (HgCl2) for 21 days. Neurotoxicity induced by mercuric chloride was assessed by behavioral, biochemical and Histopathological studies. There is decrease in muscle strength by rota rod, locomotor activity by actophotometer, number of entries into closed and open arms by elevated plus maze test, time spent in central squares by open field test in mercuric chloride treated group compared to normal control. There is increase in antioxidant parameters like malondialdehyde (MDA) and decrease in catalase (CAT), glutathione peroxidase (GP_x), AChE activity and reduced glutathione in mercuric chloride treated group when compared to control group. Administration of Resveratrol significantly ($P<0.05$) attenuated the behavioral, biochemical and Histopathological changes. Resveratrol is generally available in all the daily consumed grape fruits and vegetables. Finally we conclude that mercuric chloride intoxication in water can be detoxified by Resveratrol which is naturally present in daily consumed food.

Key Words: Neurotoxicity, Resveratrol, Mercuric chloride, Antioxidant, Oxidative stress.

INTRODUCTION

Mercury is one of the wide spread environmental and industrial pollutant, which cause severe physiological and bioenzymological alteration in the tissues of both animal and men [**1]**. It is known that mercury promotes the formation of reactive oxygen species (ROS) such as hydrogen peroxide. The cellular mechanisms by which mercury compounds exert their neurotoxicity were obtained from in vitro studies [**2]** proposed that the mechanism of mercury toxicity could be via binding to thiol groups. Mercury compounds can inactivate a number of enzymes by blocking the functional sites binding to -SH groups, which are part of the catalytic or binding domains. Mercury treatment induced the dramatic increase in reactive oxygen species accumulating in rat brain cell cultures, leading to increased lipid peroxidation, protein degradation, and finally cell death [**2].**

$HgCl_2$ causes oxidative damage [**3]** in normal cells. Mercuric Chloride ($HgCl_2$) is one of the most toxic heavy metals which induces oxidative stress in the body. Normally $HgCl_2$ has been widely used to study the hemodynamic changes, functional alteration and tissue damages in animals [**4].** HgCl2- induced oxidative damage is generally attributed to the formation of highly reactive hydroxyl radical (OH•), the stimulator of lipid peroxidation and the source of cell membrane damage as a neurotoxin.

$HgCl_2$ highly affected the cells of brain tissue in animals results in neurotoxicity [**5].** $HgCl_2$ is one of the most toxic forms of mercury because it easily forms Organo mercury complexes with proteins, leading to functional and structural alterations in many organs, such as central nervous system [**6]**. The most serious effect of heavy metal poisoning is damage to the central nervous system.

Resveratrol (3, 5, 4´- tri hydroxyl stilbene) is a Poly Phenolic Phytoalexin. It is a stilbenoid a derivative of stilbene. Resveratrol, an active ingredient of red wine extracts, has been shown to exhibit neuroprotective effect [**7]**. Reported activities of Resveratrol are anti-oxidant [8], anti-Parkinsonism

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activity [9], anti-diabetic [10], anti-mutagenic [11], anti-colorectal cancer [12], anti-aging agent [13], anti-inflammatory agent [14], anti-bacterial agent [15], antiviral agent [16] and as antioxidant in hyperlipidemia [18]. It is also used to treat neuro degenerative disorders because of its appreciable free radical-scavenging and anti-oxidant capacities [19]. Resveratrol works as a scavenger of reactive oxidative species (ROS) by donating hydrogen atoms to peroxy radicals, superoxide anions, and singlet oxygen and hydroxyl radicals [20].

As there was no study which has investigated the biochemical and molecular role of Resveratrol against neurotoxicity induced by mercuric chloride, this study will do so by exploring the behavioral, biochemical and molecular changes induced by mercuric chloride in the brain of rats. In addition, the antioxidant effects of Resveratrol on neurotoxicity induced by $AlCl_3$ will be investigated.

MATERIALS AND METHODS

Animals: Healthy Wistar albino rats weighing 250-300g, obtained from National Institute of Nutrition, Hyderabad, were used for the present study. The animals were housed in standard polypropylene cages and maintained at an ambient temperature with natural day-and-night cycles (12:12 h light and dark cycles). Standard pelletized feed and tap water were provided *ad libitum*. All procedures were conducted as per guidelines of the committee for the purpose of control and supervision of experimental animals. All the pharmacological experimental protocols were approved (MRCP/CPCSEA/IAEC/2014-15/MPCOL/07).
Animals were grouped, one to five per cage and were allowed a one-week habituation period to the animal room before testing.

Chemicals: Resveratrol was purchased from Zenith laboratories Pvt ltd, Bangalore. Thiobarbituric acid, reduced glutathione and all other chemicals were procured from Sigma Aldrich Ltd. Mercuric chloride was purchased from Finar chemicals ltd, Ahmedabad

Experimental design: Animals were assigned to five groups of 6 rats each. Group I served as control and animals were provided Normal saline solution. Group II animals received (low dose) $HgCl_2$ (1.29mg/kg Body weight) orally. Group III animals were administered with standard drug Vitamin C along with $HgCl_2$. Group IV received low dose of Resveratrol (25mg/kg) along with $HgCl_2$. Group V was administered with high dose of Resveratrol (50mg/kg) along with $HgCl_2$.

Group I: (Normal control) animals were given standard diet and normal saline for 21 days.
Group II: mercuric chloride (HgCl2) 1.29mg/kg oral for 21 days
Group III: Vitamin C 100mg/kg orally + $HgCl_2$ (1.29mg/kg) oral for 21 days
Group IV: Resveratrol 25mg/kg i.p + $HgCl_2$ (1.29mg/kg) oral for 21 days
Group V: Resveratrol 50mg/kg i.p + $HgCl_2$ (1.29mg/kg) for 21 days by oral route.

Total weight of the diet was kept constant throughout the experimental period. The total experimental duration was 21 days. On the 21st day behavioural studies were conducted. On 22nd day the rats were sacrificed by cervical dislocation. Animals were randomly divided for the biochemical studies, Neurochemical studies and histopathological studies. On 22nd day of experiment one set of rats were sacrificed, whole brain was dissected out and was separated. Tissue was washed with 0.9% saline, weighed and was homogenized in sodium phosphate buffer (0.1M, pH 7.4) using REMI tissue homogenizer. Homogenization procedure was performed as quickly as possible under standardized conditions. The homogenates were centrifuged at 10,000 rpm speed for 20min at 4^0c in cooling centrifuge. Obtained supernatant liquid is separated and stored at -20^0c for biochemical studies. The homogenate was used for various biochemical estimations like, lipid peroxidation (LPO), reduced glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT) and neurochemical parameter i.e, Acetyl cholinesterase. Brain were removed in another set of animals, and stored in 10% buffere d formalin at room temperature for histopathological studies.

Behavioural parameters:
Rota rod method: The Rota rod test is a classic method to assess motor coordination. Balance and motor learning in mice. In the rota rod test, a mouse is placed on a rotating rod. The speed of rotation is gradually increased and the rat's ability to remain on the rotating rod is recorded. The mice were trained few days prior to the experiment and the "fall of time" for each rat within the 5 min time period was recorded [21].

Locomotor activity by Actophotometer: Locomotor activity is an index of alertness or wakefulness of mental activity. The equipment was switched on and stabilized for some time and the accurate working of the photo cells was checked. Each animal from their respective groups were placed in the chamber individually for 5min. After 30 minutes all the animals were rested for activity and the No .of beams crossed of each animal was tabulated [22]**.**

Elevated plus maze: Elevated plus maze is the simplest apparatus to study anxiolytic response of almost all types of anti-anxiety agents. Exposure of animals to novel maze alleys evokes an approach avoidance conflict which is stronger in open arm as compared to enclosed arm. Rodents have aversion for high and open space and prefer enclosed arm and therefore spend greater amount of time in enclosed arm when animals entered to open arm they freeze become immobile defecate and shown fear like moments. Elevated plus maze consists of two open arms (50×10) and two closed arms (50×10×30cm) with an open roof and is elevated to a height of 55 cm. and the No .of entries into arms of each rats was tabulated [23].

Open field method: The open field test, which provides simultaneous measures of locomotion, exploration and anxiety, was used for this study. The open field is a400×400×300mm area with thin black stripes painted across the floor, dividing in to quadratic blocks. Animal was placed in the center of area and an observer quantified the spontaneous ambulatory locomotion of each animal for 5min during this period, the No .of squares crossed and time spent in central squares were measured [24].

Estimation of Antioxidant parameters:

Tissue homogenate: The brain of each of the sacrificed rats was removed, weighed and individually homogenized in ice cold phosphate buffer solution (0.1 M, pH 7.4) to give a 10 % (w/v) brain homogenate, which was centrifuged at 10,000 rpm for 20 min[21]. The supernatant was used for the estimation of anti-oxidant parameters like MDA, Catalase, GSH, GP_X, and neurochemical parameter AChE.

Malondialdehyde: MDA level were assessed according to the method of Niehaus and Samuelsson [25]. The level of MDA in the supernatant was determined spectrophotometrically by measuring thiobarbituric acid- reactive substances with a maximum absorbance at 532 nm. Briefly, 0.5mL of the sample was mixed with 3mL of 1% phosphoric acid and 1mL of 0.6% TBA solution. The mixture was heated in a boiling water bath for 45 min and cooled to the room temperature. Then, 4mL of n-butanol was added, and mixture was vortexed and centrifuged at 3000×g for 10 min. The absorbance of butanol phase (supernatant) was measured at 532 nm. Tissue MDA content was expressed as nmol/mg protein.

Catalase: Catalase activity will be measured by the method of Aebi [26]. A 5% tissue homogenate was prepared with 0.1M phosphate buffer (pH 7.4) with 1% triton X-100. The catalase activity was measured by calculating the rate of degradation of H_2O_2, the substrate of the enzyme. The enzyme activity was expressed as µmole H_2O_2 utilized per minute per mg of protein.

Reduced glutathione (GSH): The GSH content in brain was estimated by the method of Ellman as modified according to Davila et al [27]. In this assay equal volume of 5% heart tissue homogenate was treated with 20% TCA containing 1 mM EDTA to allow precipitation of proteins. The centrifugate was then treated with freshly prepared Ellman's reagent (DTNB in 1% sodium citrate). The reading was taken in a spectrophotometer at 412 nm. Tissue glutathione level was calculated from the standard curve generated using aliquots of solution having known concentration of GSH.

Glutathione Peroxide (GPx): Enzymatic method of Wandel et al. [28] was employed for estimating glutathione peroxidase parameter in tissues. The principle is based on catalytic reduction of hydro peroxides. Including H_2O_2 by GSH and functions to protect the cell from oxidative damage. The oxidation of NADPH to $NADP^+$ is accompanied by a decreased as nmol of NADPH oxidized per minute mg of protein. The enzyme activity at 37°C was determined by measuring the disappearance of NADPH at 366nm and was expressed as nmol of NADPH oxidized per min per mg of protein. Reaction mixture was prepared by adding 500µl buffer to 100µl sample which is equivalent to 40µg protein. Absorbance was read in UV visible spectrophotometer (perkin emlar lambda 25) at 366nm for 3min with addition of 100µl H_2O_2 and calculated the difference/min to find the value of standard graph.

Neurochemical parameter-Acetyl cholinesterase (AChE): The method of AChE activity estimation is popularly known as Ellman's method named after George Ellman who developed this method in 1961 [29]. The esterase activity is measured by providing an artificial substrate, acetylthiocholine (ATC). Thiocholine released because of the cleavage of ATC by AChE is allowed to react with the -SH reagent 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which is reduced to thionitrobenzoic acid, a yellow coloured anion with an absorption maxima at 412nm. The extinction coefficient of the thionitrobenzoic acid is 1.36 × 104/molar/centimetre. The concentration of thionitrobenzoic acid detected using a UV spectrophotometer is then taken as a direct estimate of the AChE activity.

Histopathological Examination: The Brain of rats of all groups was removed immediately and fixed in 10% buffered formalin. The tissue was

processed and sections were cut. The slides were prepared and stained with haematoxylin and eosin and examined under high power microscope (40X) and photomicrographs were taken. Histopathology of the control brain tissue showed normal morphological appearances, where in mercuric chloride treated group showed Vacoular degeneration noticed in cerebral hemispheres only in periphery region of brain was observed.

Statistical analysis: The experimental results were expressed as the mean ± SEM and were evaluated by using one way analysis of variance followed by Dunnett's multiple comparison, using graph pad prism version 6.0.

RESULTS

Effect of Resveratrol on Behavioural parameters in mercuric chloride induced neurotoxicity: Mercuric chloride exposure produced significant decrease ($P<0.01$) in muscle strength in rota rod test, locomotor activity in actophotometer test, number of entries into arms in elevated plus maze test, time spent in central squares in open field test in mercuric chloride treated group when compared to normal group. Resveratrol administration along with mercuric chloride exposure showed significant ($p<0.05$, $p<0.01$) increase in muscle strength in rota rod test, locomotor activity in actophotometer test, number .of entries in to arms in elevated plus maze test, time spent in central squares in open field test, when compared to mercuric chloride induced group(Table 1).

Effect of Resveratrol on anti-oxidant parameters in mercuric chloride induced neurotoxicity: There is a significant ($p<0.01$) increase in MDA, there is significant ($p<0.01$) decrease in Catalase, GPx, and GSH levels in mercuric chloride treated group when compared to normal control group. Treatment with Resveratrol at different doses there is a significant($p<0.05$) decrease in MDA, increase in Catalase, GPx and GSH levels, when compared to mercuric chloride induced group(Table 2)

Effect of Resveratrol on neuro chemical parameter in mercuric chloride induced neurotoxicity: There is a significant ($p<0.01$) decrease in AChE in mercuric chloride treated group when compared to normal control group. Resveratrol administration along with mercuric chloride exposure showed significant ($p<0.01$, $p<0.001$) increase in AChE, when compared to mercuric chloride induced group (Figure 1).

Effect of Resveratrol on rat's brain histopathology: This result demonstrated that mercuric chloride shows vacoular degeneration in cerebral hemispheres of peripheral region when compared to normal control group. Administration of VitaminC, Resveratrol 25mg/kg, and Resveratrol 50mg/kg completely suppressed vacoular degeneration and brought them to normal architecture when compared to mercuric chloride (Figure 2)

DISCUSSION

Mercuric chloride is one of the most potent thiol binding agents. The most serious effect of mercury and its compounds is damage to the central nervous system. And they are known to accumulate in various parts of brain and central nervous system in animals**.** The central nerves and peripheral nerves were damaged directly by mercury and its compound [**30].** The Neurotoxic effect of mercuric chloride on the brain may be exhibited by metabolic perturbations at the sub cellular level. The brain is the target organ of mercuric chloride. Increased dose of inorganic mercury causes severe neurological damages in animal [**31].**

Mercuric chloride exposure produced significant decrease in muscle strength in rota rod test, locomotor activity in actophotometer test, number of entries into open and closed arms in elevated plus maze test, time spent in central squares in open field test in mercuric chloride treated group when compared to the normal control group. Resveratrol administration along with mercuric chloride exposure showed significant increase in muscle strength in Rota rod test, Locomotor activity in Actophotometer test, No .of entries in to arms in elevated plus maze test, time spent in central squares in open field test in varying doses of Resveratrol (25mg/kg, 50mg/kg) along with mercuric chloride group when compared to mercuric chloride treated group.

Toxicity with mercury is associated with oxidative stress in which mercury induces the formation of free radicals including ROS and RNS, and alters the antioxidant capacity of the cells [**32].** Lipid peroxidation (LPO) is a chain reaction which is initiated molecular rearrangement and easily reacts with oxygen molecule to give a per-oxy radical (R-OO•). Per-oxy radical could attach a hydrogen atom from another lipid molecule and continue the chain reaction finally yield the lipid hydro peroxide (R-OOH) and combine with the hydrogen atom that it abstracts to give lipid peroxide [**33].** Lipid peroxidation is highly destructive process and induces a plethora of alteration in the structure and function of cellular membrane, which could lead to

cell injury. The significant increase in lipid peroxidation level in mercuric chloride intoxicated rat could lead to the damage of plasma membrane of the respective tissue which is causing oxidative stress induced by mercuric chloride. The increase in the levels of LPO indicates enhanced lipid peroxidation leading to brain tissue injury and failure of the antioxidant defense mechanism to prevent the formation of excess free radicals [**34].**

The reduced glutathione functions as free radical scavenger. Glutathione helps in removing toxic peroxides by a reaction catalyzed by glutathione peroxidase [**35].** GPx helps in clearing the toxic intermediate hydrogen peroxide which is formed in the cells. Both GSH and GPx work together in the intoxicated cell to remove the toxicant and free radicals simultaneously. Glutathione peroxides alone are not enough to remove the free radicals from the living system with the help of other scavenging enzymes it completes the process, other scavenging enzyme is Catalase (CAT) **[36].** Catalase is an antioxidant enzyme which destroyes H_2O_2, that can form a highly reactive radical in the presence of iron as catalyst. CAT is involved in the detoxification of hydrogen peroxide in intoxicated cells**.**

There is a significant increase in MDA, and decrease in Catalase, GPx, GSH levels in mercuric chloride treated group when compared to normal control group. Treatment with Resveratrol at different doses there is a significant decrease in MDA, and increase in Catalase, GPx GSH levels, when compared to mercuric chloride treated group. This result suggests that the Resveratrol can reduce the reactive oxygen free radicals and also improve the anti-oxidant enzymes activities in the mercury intoxicated brain tissue**.**

Acetylcholine (ACh), the neurotransmitter secreted by cholinergic postganglionic neurons, allows for transmission of nerve impulses across the synapse. Acetylcholinesterase (AChE) plays an important role in neurotransmission in both vertebrates and invertebrates, being responsible for the hydrolysis of acetylcholine into choline and acetic acid at the cholinergic synapses and neuromuscular junctions. Acetyl cholinesterase is found in high concentration in the nervous system. Acetyl cholinesterase is an important regulatory enzyme that controls the transmission of nerve impulses across cholinergic synapses by hydrolyzing the excitatory transmitter acetylcholine.

Mercury and its compounds mainly affect the central nervous system due to the inhibition of AChE enzyme system by disrupting the nervous activity through the accumulation of acetylcholine at nerve endings in the central and peripheral nervous system during the toxicants exposure. The inhibitory effect on AChE activity indicates that toxicants might interfere in vital processes like energy metabolism of nerve cell. AChE inhibition not only induces the brain cell injury and also inhibits the AChE synthesis and also disrupts the smooth transmission of the nerve impulses across the synapses causing neurosis, tremor and depression in the respiratory centers**.** The present investigation also confirms the brain damage which is caused by mercury exposure as observed in brain histopathology and this effect was attenuated by the administration of Resveratrol. In the present study, administration of Resveratrol on mercury intoxicated brain tissue plays a vital role to detoxify the mercury toxicity**.**

CONCLUSION

Mercuric chloride proved to be neuro toxic and caused vacoular degeneration in cerebral hemisphere of brain in peripheral region and administration of Resveratrol reduces the resulting damage probably due to its ability to neutralize or scavenge the free radicals that are generated by mercuric chloride. Finally we conclude that mercuric chloride intoxication in water can be detoxified by Resveratrol which is naturally present in daily consumed food.

Table 1: Effect of Resveratrol on behavioural Parameters in $HgCl_2$ induced neurotoxicity.

Treated groups	Muscle strength (No. of falls in 5 min)	Elevated plus maze (No. of entries into closed arms)	Elevated plus maze (No .of entries into open arms)	Actophotometer (locomotor activity score in 5 min)	Open field test (time spent in central squares in min)
Normal control	26.78±0.85	43.05±0.6	30±0.4	44±0.4	5±0.04
$HgCl_2$ induced	40.50±0.64##	27.5±1.19##	45±0.1##	20.25±0.6##	1.5±0.16##
$HgCl_2$+Standard Vitamin C	23±1.82**	35.5±0.6**	30±0.7**	35.25±0.85***	3.8±0.1***
$HgCl_2$+Resveratol 25mg/kg	34.5±0.5**	31±0.707*	38±0.5*	30.65±0.25*	3±0.17**
HgCl2+Resveratrol 50mg/kg	24±0.7**	36±0.92**	29±0.8***	36.25±0.85***	4.1±0.10*

All values are expressed as Mean ±SEM, n=6. ##p<0.01 when compared to normal control group, **p<0.01, *p<0.05 when compared to $Hgcl_2$ treated group, by using one way ANOVA followed by DUNNETT's multiple comparison test.

Table 2: Effect of Resveratrol on anti-oxidant parameters in $HgCl_2$ induced neurotoxicity.

Treatment Group	MDA(nmol/mg tissue)	GSH (µmol/mg tissue)	Catalase (µmol/mg tissue)	GP_X (nmol/mg tissue)
Control	0.7903±0.02271	24.18±1.161	67.24±2.010	8.302±0.0266
$HgCl_2$ Induced	$1.625±0.02715^{\#\#}$	$12.33±0.3453^{\#\#}$	$49.82±0.3821^{\#\#}$	$5.61±0.450^{\#\#}$
Standard (vitamin C)	$0.6845±0.1815^{**}$	$18.16±0.1545^{***}$	$57.36±0.8503^{**}$	$7.896±0.0415^{***}$
Resveratrol (25mg/kg)	$0.2782±0.0299^{**}$	$14.82±0.1715^{*}$	$46.76±0.9424^{*}$	$6.016±0.0382^{**}$
Resveratrol (50mg/kg)	$0.1265±0.0169^{**}$	$16.81±0.1851^{***}$	$58.34±0.7815^{**}$	$7.594±0.0993^{*}$

All values are expressed as Mean ±SEM, n=6 ##p<0.01 when compared to normal Control group, ***p<0.001 , ** p<0.01, * p<0.05 compared with $Hgcl_2$ treated group, by using one way ANOVA followed by DUNNETT's multiple comparison test.

Figure1: Effect of Resveratrol on AChE in $HgCl_2$ induced neurotoxicity

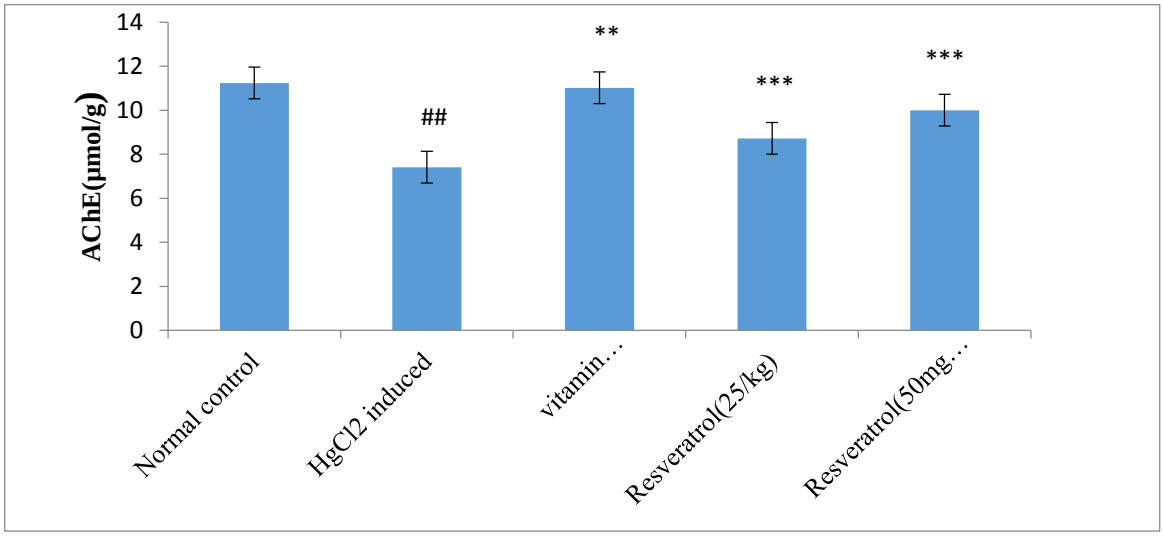
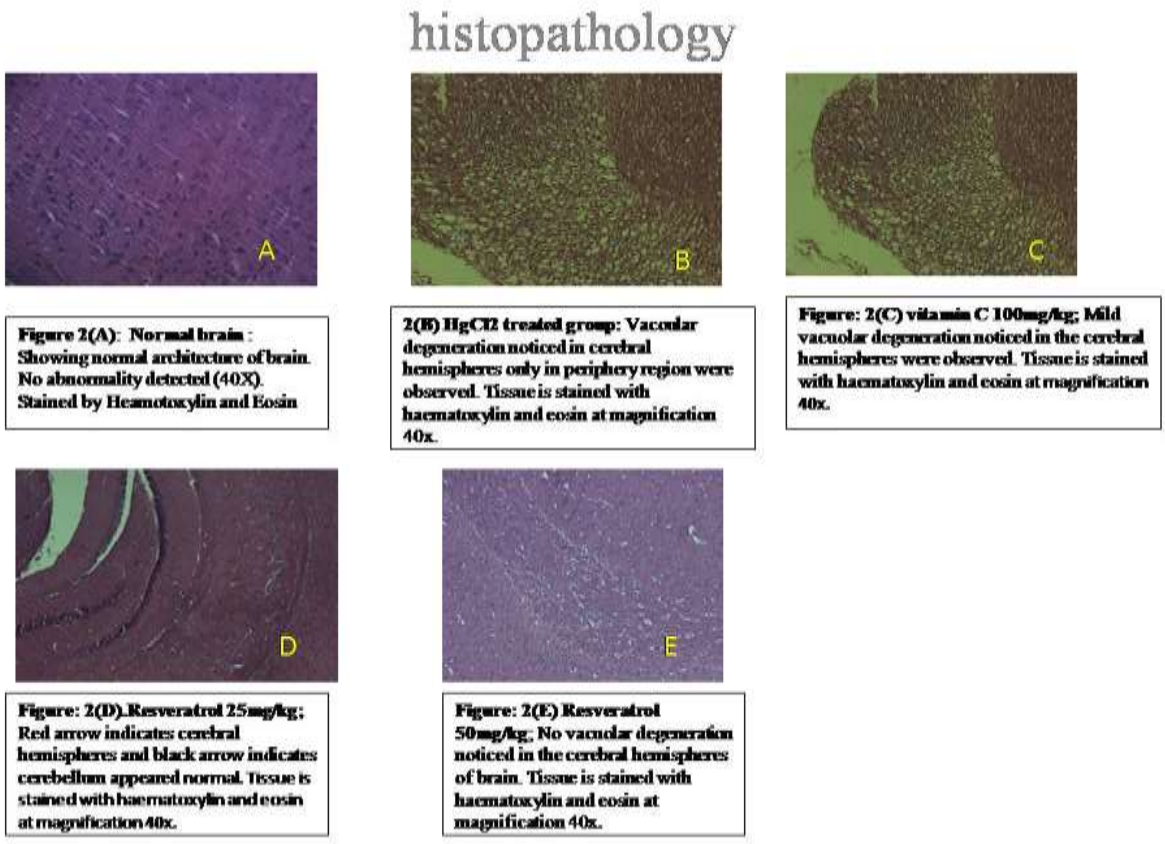


Figure1: Levels of AChE (nmol/g tissue) in control, $HgCl_2$, VitaminC, Resveratrol 25mg/kg and Resveratrol 50mg/kg administered rats. Data are expressed as mean ± SEM n=6. *** p<0.001; when compared to control group **p<0.01 when compared to mercuric chloride measured by ANOVA followed by Dunnett's test.

Figure2: Effect of mercuric chloride and Resveratrol on histopathology



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