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# Neuroprotective Potential of Vitamin B-12 Against Cadmium–Induced Neuroinflammation Mediated Memory Dysfunctions in Adult Albino Mice

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**ABSTRACT**

Cadmium induces neurotoxicity in the brain and results in increased enzymatic functions, accumulation of Amyloid beta plaque and Neuo Fibrillary Tangles (NFTs) causing neurodegenerative diseases. The purpose of the present work was to investigate the anti-inflammatory or anti-oxidative potential of vitamin$B_{12}$ against Cd -induced memory impairment and mechanism of signaling pathway of vitamin-B12 protection in Cd-induced neuroinflammatory in mice model. Male albino mice of BALB/C trait of age 7 to 8 weeks and weighing about (30-32±3g) were used as model animal in the study. All mice were divided into three groups. Control Group (CG) treated with 0.9% saline (1mL/Kg), Cd $Cl_2$ administered Group (CD) (1mg/kg) and Cd +Vitamin administered group (CV) B-12 (500µg/kg). Behavior tests like Morris Water Maze (MWM) and Y-Maze (YM) tests were conducted on the experimental mice to study the neuro protective potential of Vitamin-$B_{12}$ against $CdCl_2$ induced memory dysfunctions. After completing the above procedures, mice were sacrificed, and the brains of each group were collected for protein quantification step and western blotting. The Immuno blots of Iba-1, NF-kB, TNF-α, IL-1β, BACE1 and Aβ proteins in the brain homogenates of CD and CV mice confirm *the* anti-neuroinflammatory potential of vitamin-B12 against Cadmium –induced Neuroinflammation. Escape latency of CV group mice receiving Methylcobalamine was less and showed better performance in MWM test. The results of the Y-maze test also showed that the spontaneous percentage alternation of CD group mice was less than CV and CG group of mice. Immuno blot of p-Akt confirm the signaling pathways of vitamin-B12 protection in Cd-induced neuroinflammatory mediated memory-impairment in the model mice. Our findings suggest that Vitamin-$B_{12}$ could be a potent candidate drug for treating cognitive dysfunctions due to its anti-inflammatory and anti-oxidative potential. Our study also shows that Vitamin-$B_{12}$ activates p-Akt signaling pathway along with decreasing the NF-Kb, TNF-α and IL-1β to protect the brain of mice against $CdCl_2$ neurotoxicity.

**Key words**: Neuro inflammation, Cadmium, Amyloid beta, mice

## INTRODUCTION

Cadmium is a neurotoxic element which enters our ecosystem through different ways either commercially or naturally (Skipper *et al.*, 2016; Khan *et al.*, 2019). Its toxicity and environmental fate are like Pb and Hg. Cd can disrupt neuronal functions by crossing the Blood Brain Barrier (BBB) and changing the production and release of neurotransmitters by blocking calcium influx into nerve terminals. Cd also decreases the activities of

Glutathione Peroxidase (GPX), Catalases (CAT), and Oxide Dismutase (SOD) in the cell and increases the activity of free radicals. The result is increased peroxidation of lipid and ultimate disruption of cellular membrane via apoptosis (Batool*, et al*., 2019; Andrade *et al.,* 2017). Neurodegenerations like Alzheimer, Parkinson's diseases, amyotrophic lateral sclerosis, and multiple scleroses are linked with the neurotoxicity of Cd. Cd can also disrupt DNA repair mechanisms by Reactive Oxygen Species (ROS). Memory dysfunctions and mood swings have also been reported in transgenic mice upon exposure to Cadmium toxin (Rahman *et al.,* 2020). Heavy toxic metals induce neurotoxicity in brains which result in increased enzymatic functions, ROS; accumulation of Amyloid beta plaque and Neuo Fibrillary Tangles (NFTs) causing neurodegenerative diseases and Alzheimer like pathology (Lee *et al.,* 2018). To date very few medications including Cholinesterase antagonist like Galantamine, Rivastigmine Tacrine, Memantine, Donepezil, *N*-Methyl-D-Aspartate Receptor Inhibitors (ARI) are prescribed for the treatment of neurodegenerative diseases but are very expensive and not free from side effects (Huang *et al.,* 2020).

Vitamin E and C are also potent antioxidants that can limit per oxidation of lipid in the brain cells and can improve brain illness by clearing the Amyloid beta debris from the brain (Mielech *et al.,* 2020; Ibrahim *et al***.,** 2017). Several studies have reported the potential role of Vitamin-$B_{12}$ to be a natural remedy for the axonal growth of neuronal cells due to its superoxide scavenging capabilities (Lai *et al.,* 2018). The Vitamin-B family is shown to stabilize intellectual mutilations and decrease the risk of Dementia induced by Cd through peroxidation of lipids Reactive Oxygen Species (ROS) in the brain (Wolffenbuttel *et al.,* 2019). The purpose of the present work was to investigate the anti-inflammatory, Anti-oxidative, behavior improving potential and signaling pathways of vitamin-B12 protection against Cd -induced memory impairment in mice model.

## MATERIALS AND METHODS

Total nine male albino mice of BALB/C trait of age 7 to 8 weeks and weighing about (30-32±3g) were acquired from VRI (Veterinary Research Peshawar) and were kept in Bio-Base China cage in a breeding room of 12/12-hour light and dark cycle with proper supply of food and water under the supervision of an expert laboratory attendant. All mice were divided into three groups. Each group includes three mice. Control Group (CG) treated with 0.9% saline (1mL/kg); Cd Cl2 administered Group (CD) (1mg/kg) and Cd + Vitamin administered group (CV) B-12 (500µg/kg).

### Administration of Neurodegenerative (CdCl2) and Neuroprotective agents (Vitamin B12)

CD group was treated with Cd Cl2 for three weeks on alternate days while CV group was treated with Vitamin $B_{12}$ and toxin CdCl2 for the next two weeks intraperitonealy.

### Behavioral test

A behavior test was conducted on the experimental mice to study the neuro protective potential of Vitamin-$B_{12}$ against CdCl2 induced memory dysfunctions.

### Morris Water Maze (MWM) Test

MWM was performed on the test animal to study spatial and learning memory. In order to find out the compound efficacy there is an approach that must be helpful for knowing about cognition performance and behavior monitoring domains. This y-maze was designed from wooden material having three arms named as A, B and C disposed at an angle of 120˚ from each other. Mice and rodents always investigate a new arm (wooden maze) excitingly rather moving back to previously visited arm of y-maze. For recording the data animals were placed over the wooden maze at its middle point and allowed free movements to explore new area of entries. For recording data this assembly was set under camera for tracking up mice entries in maze. When during time for the task was finished then analyzed the data with the help of video for each mice group session. In order to measure the percentage alternation a multiple number of entries and triads were noted for three consecutive locations in arms (Shah *et al.,* 2015).

$$\% \text{ Spontaneous Alternations} = \frac{\text{Number of Spontaneous Alternations}}{\text{Total number of arms entries -2}} \times 100$$

### Y-Maze (YM) Test

To study spatial and learning memory a special navigational task was performed by mice. Mice were placed in a large circular tank of 100cm in diameter and 40 cm in height filled with water at 23±1.Mice were first allowed for behavior training first for three consecutive days. After training mice were then again subjected for behavior test for recording data to measure their spatial learning and memory. It was done by hiding the platform for

specific quadrant below water surface especially 1cm. This step was escaping latency and time was recorded for 1 min for each group. If the mice failed to reach that quadrant in required time, then placed the mice on platform manually for 10 seconds to recognize the location. After collection of data rest day was given to them and then probe test were performed by completely removing the platform and then mice were allowed to locate the same position of platform for each event. During this stay time was noted in a specific targeted quadrant (Miedel *et al.,* 2017).

**Mice Sacrifice**

After completing the above procedures, mice were sacrificed. The brains of each group were collected in PBS (Phosphate buffer saline) solution and were homogenized by adding 140µL tissue protein extract (T-PER) solution136. Brain samples were centrifuged for 25 minutes at 14000 rmps and the supernatants were collected for protein quantification step.

**Optical density (OD) or Protein quantification step**

Protein in the supernatant of each sample was quantified by UV- spectrophotometer at 595nm. OD was noted.

**Western Blot Analysis for protein**

This technique was used using antibodies as a detector with the help of SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis) (Kavak *et al.,* 2010).

## RESULTS AND DISCUSSIONS

**The anti-neuroinflammatory potential of vitamin-B12 against Cadmium–induced Neuroinflammation**

**Down regulation of activated Microglial cells.**

The $CdCl_2$ induced activation of microglial cells in adult albino mice for three weeks increased the expression of protein Iba-1 in the brain as shown in the figures (1A) and (1B). When Methylcobalamine (Vit B12) was administered for the next two weeks, it significantly reduced the expression of Iba-1 protein by deactivating the Microglial cells (Figures 1)· (Khan *et al.,* 2019).

**Inhibition of neuroinflammation**

CdCl2 induced downstream signaling of NF-k [141] like IL-1β and TNF-α (Figures 2). Administration of VitaminB12 significantly inhibited not only NF-kβ but also IL-1β and TNF-α respectively (Figures 2).

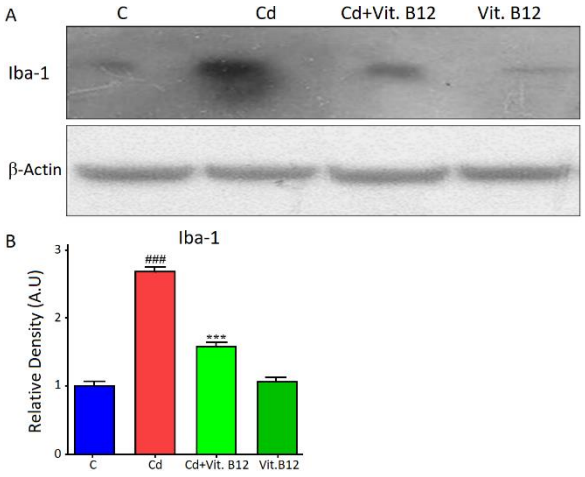


Figure 1. The Immuno blot (A) histogram (B) of Iba-1 in the brain homogenates ofCD and CV mice.

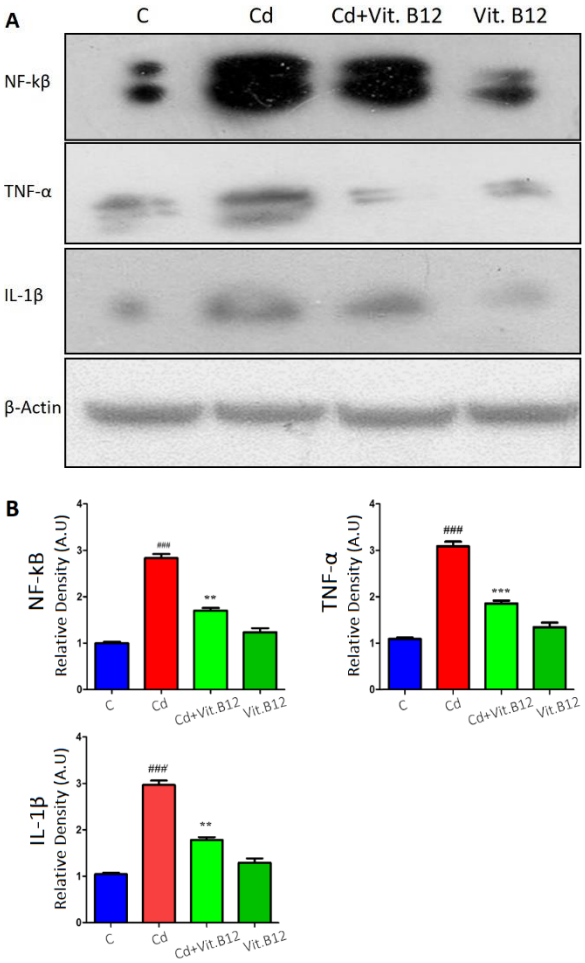


Figure 2. The the Immuno blots (A) and histogram (B) of NF-kB, TNF-α and IL-1β proteins in the brain homogenates of CD and CV mice.

**Inhibition of BACE-1 expression and reduction of β-Amyloid production**

Production of Amyloid beta (aβ) in the brain of CD group was triggered upon administration of CdCl2 for three weeks by increasing production of β-Secretase (BACE-1) and lead to the onset of Alzheimer disease (Meleleo *et al.,* 2020). Administration of Vitamin B12 in CV group significantly reduced β-secretase and Aβ expression (Figures 3 and 4).

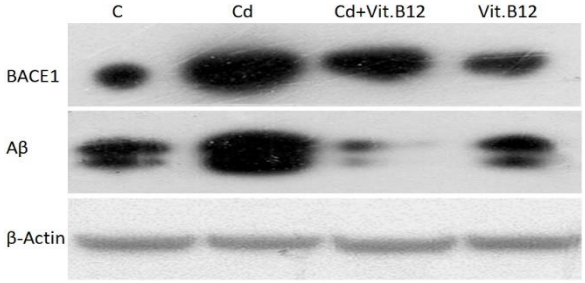


Figure 3. The Immuno blots of BACE1 and Aβ proteins in the brain homogenates CD and CV mice.

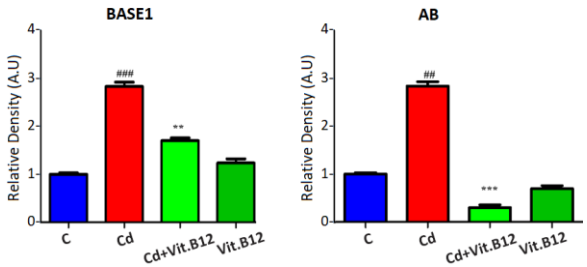


Figure 4. The histograms of BACE1 and Aβ proteins in the brain homogenates CD and CV mice.

**Behavior improving abilities of vitamin-B12**

Both MWM and YM tests were conducted to study the behavior improving abilities of vitamin-B12 in Cd induced memory and behavior impairment in mice (Zang *et al.,* 2019). The results of MWM showed that on day first the escape latency of mice in CD group was higher than the mice in in the (control group) CG group, suggesting their failure to find the submerged platform in 1 minute. Escape latency of CV group mice receiving Vit B12 was less and showed better performance in MWM test (Figure 5).

**Signaling pathways of vitamin-B12 protection in Cd-induced neuroinflammatory mediated memory-impairment**

Western Blot analysis was carried out to find the signaling pathway of vitamin-B12 to prevent Cd induced neuroinflammatory mediated memory-impairment. The results show that vitamin-B12 increases the phosphorylation of p-Akt to protect the brain of the mice from $CdCl_2$ toxin (Figures 6).

The purpose of the present work was to investigate the possible neuroprotective role of Vit. B-12 as a natural remedy in Cadmium –induced AD mice model. Neuro inflammatory pathways were triggered in the study animals (CD) by the administration of $CdCl_2$ (1mg/kg) daily for 3 weeks. Administration of CdCl2 increased the expressions of NF-κB (Nuclear Factor Kappa B), TNF-α (Tumor Necrosis Factor alpha), IL-1β (Interleukin 1 beta) and Amyloid beta by up regulating BACE-1 protein and hence impaired the memory in the adult mice (CD). When the mice in the CD group were administered Vit. B-12 (500 µg/kg) for the next 2 weeks, it not only improved memory dysfunctions, but also reduced the Cd induced neuroinflammation in the affected mice. The enhanced expression of Ionized Calcium Binding Adaptor molecule 1(Iba-) was found in hippocampal and cortical regions of mice brain of CV group as compared to the CD group in our Western blot analysis. Methylcobalamin showed its potent antioxidant capacity in neuronal cells of the target mice (Zang *et al.,* 2019; Agnihotri *et al.,* 2015). As NF-κB is actively involved in cellular inflammation, differentiation and survival of the cells as described earlier (Lai, *et al.,* 2017). NF-κB pathway can be stimulated even by a trace amount of CdCl2. The probable mechanism involves the breaking of NF-κB p65 subunit from IκBα and its translocation to the nucleus from the cytoplasmic side (Dai, *et al.,* 2016). Heavy metals are found to be transcriptomic factors for the induction of inflammation. Our findings suggest that induction of CdCl2 in mice brought out an inflammatory response by the excessive release of pro-inflammatory cytokines (TNF-α and IL-1β) in cortical cells and administration of Methylcobalamine decreased their levels in the cortical cells (Olszowski *et al.,* 2012; Almeer *et al.,* 2019). Researchers are trying to verify the exact mechanistic pathway of Cd induced neurodegenration. Earlier studies reported amyloidogenic pathway about pathogenesis of Alzheimer which overproduces amyloid beta Aβ on exposure to heavy metals like Cd. Our study also supports the above hypothesis that Cd mutates gene in BACE-1 (Beta Secretase Enzyme) which leads to the overproduction of amyloid beta (Aβ) protein in the experimental animal's brain.

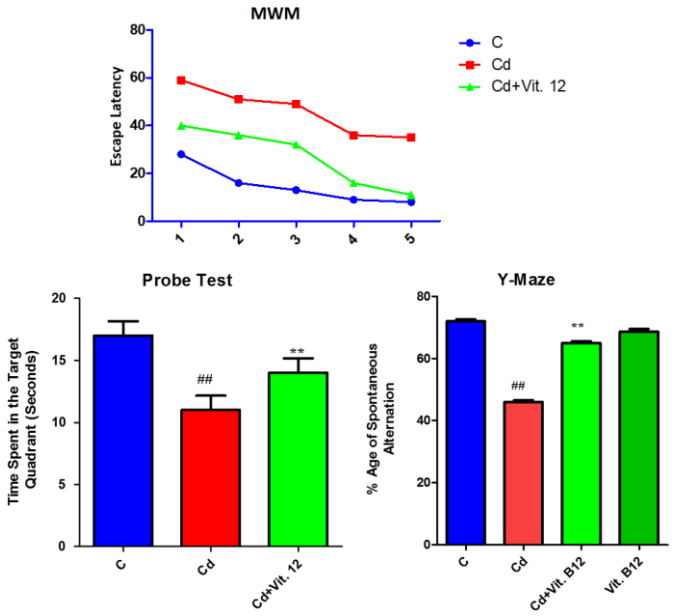


Figure 5. Escape latency graph (A), the submerged plate form was removed on the sixth day and the time spent by each group was noted in the target quadrant. It was observed that CG group spent more time than CD group (Probe test); Probe test (B), results of Y-maze test also showed that the spontaneous percentage alternation of CD group mice was less than CV and CG group of mice (C) and Y-maze test(C).

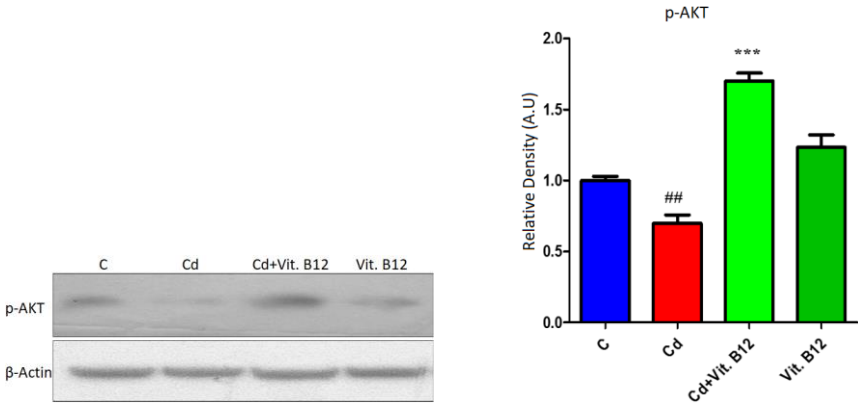


Figure 6. Immuno blot (A) and histogram (B) of p-Akt in the brain homogenates of CD and CV mice.

The overproduction of beta secretases-1 and Amyloid beta plaque damages the brain cells and causes memory impairment. Methylcobalamine treatmen t of the Cd treated animal showed positive results through western blot of protein expressions (Meleleo *et al.,* 2020). Thus, Methylcobalamine could be a potent candidate that can reduce Alzheimer like pathology.

Cd is well known neurotoxic metal and its prolonged exposure may lead toward memory deficit and behavioral disturbances. The behavioral changes and memory deficit associated with its neurotoxicity in rats were dependent on inflammation and oxidative stress by the suppression of SIRT1 and over activation of PAPR1.Kaempferol was found to have a neuroprotective effect in animal models in the same study (Tezotto *et al.,* 2012). Both MWM and YM tests were conducted to study the behavior improving abilities of vitamin-B12 in Cd induced memory and behavior impairment in mice (Zang *et al.,* 2015). The results of MWM showed that on day first the escape latency of mice in CD group was higher than the mice in in the (control group) CG group mice suggesting their failure to find the submerged platform in 1min. While the CV group

mice receiving Methylcobalamine showed less escape latency and good performance in MWM test. The submerged plate form was removed on the sixth day and the time spent by each group was noted in the target quadrant. It was observed that CG group spent more time than CD group.

The results of the Y-maze test also showed that the spontaneous percentage alternation of CD group mice was less than CV and CG groups of mice. The findings of the current study suggest that administration of Methylcobalamine improves memory and behavior in animal models (Shah *et al.,* 2017). P13K/pAkt is considered as the central pathway for cell survival and as an important therapeutic target for drug treatment for different kind of diseases including neurodegenerative diseases (Lee *et al.,* 2017). Some studies suggest that induction of $A\beta_{1-42}$ increases tau phosphorylation of p-Akt at Ser473 site of Ser/threonine kinase (p13K/Akt) . When Osmotin is administered, it reverses the process of phosphorylation on Ser473 site and activates the p-Akt signaling pathway by blocking pro –apoptotic proteins and improves memory impairment (Ali *et al.,* 2015; Nitulescu *et al.,* 2018). Cognitive dysfunctions in AD brain was restored by the long term use of EEAK(Ethanol extract of Acanthopanax Koreanum) by the activation of Akt signaling pathway through phosphorylation at Ser473 in a Korean population based study(Lee *et al.,* 2017). The other aspect of the present study was to know the exact mechanism of neuroprotection by Vitamin-$B_{12}$ in the brain of mice model. The literature study show that $CdCl_2$ deactivates PI3K activity by removing the phosphate group and activate NF-κB along with its down streaming signaling pathway in the neurons of mice brain (Kott *et al.,* 2020).

This potential connection was confirmed by our western blot results. We observed that administration of $CdCl_2$ inhibit momentarily phospho-PI3K, phosphor-Akt (Ser473) but administration of Vitamin-$B_{12}$ increases the expression of phosphorylated PI3K, Akt (Ser473) and reverses that default by phosphorylation and also activates signaling pathway of cell survival to protect mice brain from Cadmium induced Neuroinflammation that leads towards brain disease like AD pathology. The results of this study suggest that Cd can impair memory and Neuroinflammation in adult albino mice and can also deactivate p-Akt signaling pathway. The neuroprotective effect of vit B12 was also confirmed by western blot results, due to its anti-inflammatory, anti-oxidative and anti-apoptotic action, facilitated by reversing the events.

## CONCLUSION

The present study proposes a new mechanism for the protective effect of Vit B12 against CdCl2-induced neurotoxicity. Our findings suggest that Vitamin-$B_{12}$ could be a potent candidate drug for treating cognitive dysfunctions or memory impairment due to its anti-inflammatory and anti-oxidative potential. Most importantly our present study also show that Vitamin-$B_{12}$ (Methylcobalamine) activates p-Akt signaling pathway along with decreasing the NF-Kb, TNF-α and IL-1β to protect the brain of mice against $CdCl_2$ neurotoxicity.

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