Concomitant Administration of Quercetin and α -tocopherol Protects Rats from Cadmium Chloride Induced Neural Apoptosis and Cognitive Dysfunction

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Abstract

Cadmium (Cd), a highly toxic environmental pollutant, leads to a neurotoxicity and cognitive dysfunction in both animals and humans. In spite of the available in vitro evidence, there is a paucity of literature on the mechanisms by which CdCl2 induced these effects, in vivo. While numerous in vivo studies have shown a protective effect of Quercetin (QUR) against neurodegenerative disease, there is still uncertainty about the safety of such efficiency of QUR due to its low bioavailability. Therefore, trails to enhance this have shown increased brain accumulation of QUR when administered in conjugation with α-tocopherol. Consequently, this study aimed to investigate the molecular effects of individual and combined administration of QUR and α-tocopherol against CdCl2 induced apoptosis and memory loss in rats. Results of the current study revealed that CdCl2 induced neural apoptosis, spatial memory loss and disturbed brain cholinergic system in mechanisms related to increased oxidative stress, inhibition of protein phosphatase (PP2A) induced activation of ERK1/2 and JNK and activation of PTEN induced inhibition of Akt/mTOR/S6K1 signaling pathways. As compared to individual effects of QUR or α-tocopherol which were not effective to ameliorate CdCl2 induced apoptosis and cognitive dysfunction, combined administration of both drugs in both control and CdCl2 intoxicated rats could significantly enhance levels of antioxidant defense, improved cognitive dysfunction by enhancing levels of acetylcholine (Ach) and protein levels of CREB and BDNF, and decreased neural apoptosis by upregulating levels of PP2A induced inhibition of ERK1/2 and JNK signaling as well as downregulating expression of PTEN induced activation of Akt/mTOR/S6K1 signaling. In conclusion, only concomitant administration of QUR and α-tocopherol but not their individual use, provide an excellent protective formula against CdCl2 induced neurodegeneration and memory loss.

Keywords: ERK1/2; JNK; Akt/m-TOR; Quercetin; α-tocopherol; apoptosis; cognitive dysfunction

1. Introduction

Cadmium (Cd) induced neurotoxicity is characterized by neurotransmitters levels alterations (Lafuente et al., 2000; Pari and Murugavel, 2007), metabolic changes (Lafuente, and Esquifino, 1999; Lafuente et al., 2000); behavioral and memory disturbance (Wright et al., 2006; Mendez- Armenta and Rios, 2007) and neurodegeneration (Mendez-Armenta and Rios, 2007; Chen et al., 2008).

Currently, there is a growing recognition that neuronal cell death characteristic of Cd-induced neurodegeneration and its associated cognitive dysfunction are partially but highly related to increased apoptosis. In this regard, in vitro evidence using neurons cultures has shown that such effect is mediated by over-production of reactive oxygen species (ROS) induced activation of two members of mitogen-activated protein kinase (MAPK) signaling, namely, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (Erk1/2) (Chen et al. 2008, Chen et al., 2011a). Moreover, using the same in vitro model, Cd-induced-neuronal apoptosis was also partially mediated by an unexpected activation of the mammalian target of rapamycin (mTOR) signaling pathway (Chen et al. 2008, Chen et al., 2011a; Chen et al., 2011b, Chen et al., 2014). On the other hand, the flavonoid, Quercetin (QUR), is a well reported neuroprotective agent in rodents that acts by inhibiting apoptotic cell death, preventing neurotransmitter disturbance and enhancing cognitive dysfunction in various animal models of neuronal injury and neurodegenerative diseases induced experimentally (Zhang et al., 2015, Xia et al., 2015) or induced by toxic metals such as lead (HU et al., 2008), methyl mercury (Barcelos et al., 2011), tungsten (Sachdeva et al., 2015) and Cd (Unsal et al., 2015).

The neuroprotective effect of QUR has been referred to its antioxidant and anti-apoptotic effects as well as its ability to activate sirtuins (SIRT1) induced autophagy (Yokoo and Kitamura, 1997; Costa et al., 2016). QUR inhibits apoptosis by inhibiting the activities of both JNK and ERK-1 in mesangial cells, fibroblasts, and epithelial cells (Yokoo and Kitamura,

1997; Ishikawa et al., 1997; Uchida et al., 1999). In spite of this, there is still uncertainty about the safety and efficiency of QUR administration on neural function in both animals and human. This has been raised as QUR could induce cell death through the inhibition of Akt/PKB and ERK survival pathways in cultured neurons (Spencer et al., 2003).

Further criticism of the neuroprotective effect of QUR has been raised later as only very low concentrations have been detected in the brain of the animals, after oral or intraperitoneal administration (from pmol to nmol) (de-Boer et al., 2005; Ishisaka et al., 2011) which was shown to be due to the extensive intestinal and hepatic metabolization of QUR (Shirai et al., 2006). Hence, several trails to increase QUR bioavailability have confirmed accumulative concentrations in the brain of animal when administered in conjugation with α-tocopherol (Ferri et al., 2015). Of interest is also the observation that conjugated QUR is less metabolized and can enter the red blood cells where it is converted to its non-conjugated form being transported to various tissue (Fiorani et al., 2003).

However, the regulatory effect of QUR on various members of MAPKs and Akt/mTOR cell signaling in the brain of rats intoxicated with Cd is completely lacking. In addition, given the fact that co-administration of QUR with α-tocopherol enhances its blood-brain barrier (BBB) transport and the fact that QUR is still debated regarding its neural safety, this study aimed to investigate the molecular effects of administration of QUR alone or in combination with α-tocopherol on memory function, cholinergic neurotransmitters levels, and neural survival and apoptotic pathways in control rats and in rats intoxicated with cadmium chloride ($CdCl_2$). The latter aim was achieved by examining the cortical activities of ERK1/2, JNK, P38 and Akt/mTOR signaling pathways.

2. Materials and Methods

2.1 *Animals*

The study was conducted at the animal house of the College of Science, King Khalid University, Abha, Saudi Arabia, according to the guidelines for the care and use of laboratory animals set by the institution which follow the regulations of laboratory animal care and use, published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). A total of fifty-six young adult male Wistar albino rats 5 weeks old, weighing 100–105 g were used in this study. Rats were randomly divided into seven groups (n=8/group). Each group was housed in a separate cage in a constant temperature (22–24◦C) and light-controlled room on an alternating 12:12 h light-dark cycle and had free access to food and water. Rats were fed a standard commercial pellet diet and were kept for one week before beginning the experiment for acclimatization.

2.2 Experimental Protocol

Cadmium chloride $CdCl_2$ and Quercetin (QUR, Empirical Formula: C15H10O7) were purchased from Sigma-Aldrich (St. Louis, MO) and were always prepared freshly by dissolving in normal saline. Rats groups (n=8/group) were subjected to treatment as follows:

Group 1 (Control group): Rats received distilled water (5 ml/kg .b.w. and 0.1ml of coconut oil), orally.

Group 2 ($CdCl_2$ intoxicated group): Rats received $CdCl_2$ (5 mg/kg.b.w.), orally.

Group 3 (QUR treated group): Rats received QUR (15 mg/kg.b.w.), intraperitoneally.

Group 4 (α-tocopherol treated group): Rats received a dose of 120 IU α-tocopherol diluted in 0.1 ml of coconut oil, orally.

Group 5 (QUR+ α-tocopherol treated group): Rats received QUR (15mg/kg.b.w.), i.p. and received a concomitant dose of 120 IU α-tocopherol diluted in 0.1 ml of coconut oil, orally.

Group 6 (Cd+Qur treated group): Rats orally received $CdCl_2$ (5 mg/kg.b.w.) and received a concomitant dose of QUR (15 mg/kg.b.w.), intraperitoneally.

Group 7 (Cd+Qur+ α-tocopherol treated group): Rats orally received $CdCl_2$ (5 mg/kg.b.w.) and received concomitant doses of QUR (15 mg/kg.b.w.), intraperitoneally and treated with 120 IU α-tocopherol diluted in 0.1 ml of coconut oil, orally.

All treatments were carried out on daily basis for four consecutive weeks (30 days). Dose selections and routes of administrations of all drugs were based on previous studies that showed a neurotoxic effect of $CdCl_2$ at this dose (Shagirtha et al., 2011) and showed a neuroprotection effect of QUR (Unsal et al., 2013) and neurological safe dose of α-tocopherol (Guimarães et al., 2015).

2.3 Assessment of cognitive performance

Spatial learning and memory were assessed using the Morris Water Maze (MWM) as previously described by Sethi et al. (2008). MWM consists of a black 168cm diameter circular pool divided into 4 equal hypothetical quadrants and filled with water (50 cm deep, temperature 20± 2 °C. An escape circular platform was submerged at 2 cm below the surface of the water. The principle of the test is to train the rats to escape from swimming by climbing onto the visible platform and over time the rats apparently learn the spatial location of the platform from any starting position at the circumference of the pool. During the test, each rat of any group was placed inside the water tank at 4 different entry quadrant points and was given 4 trials/day (90 sec/trial) for 4 consecutive days, starting from day 31 to day 34 following the end of all treatments. Escape latency (sec) was recorded to determine changes in learning dysfunction. The animal was allowed to swim for 60 sec to find the hidden platform. If failed, then it was manually guided to the platform. At the end of each trial, each rat was allowed to remain on the platform for 30 seconds. MWM training was recorded by a web camera attached to the laboratory ceiling.

2.4 Collection of brains and homogenates preparation

After the MWM test, rats were anesthetized with sodium pentobarbital (60-70 mg/kg, i.p.) their brains were quickly removed on ice, washed with cold saline and immediately replaced in ice-cold dishes. Parts of brain tissues (100mg) were homogenized individually in either 9 volumes cold 50 mM Tris buffer (pH 7.4) or in 0.5 ml RIPA buffer (150 mM sodium chloride 1.0% NP-40 or Triton X-100 0.5% sodium deoxycholate 0.1% SDS, 50 mM Tris, pH 8.0). Protease inhibitors (Cat. No. P8340, Sigma-Aldrich, St. Louis, MO, USA) and phosphatase (PhosSTOP, Cat. No. PHOSS-RO Sigma-Aldrich, St. Louis, MO, USA) were added to these buffers according to manufacturer's instructions to prevent protein degradation and auto-phosphorylation. Supernatants collected were used from these homogenates were used for biochemical assays and western blot studies, respectively. However, other parts of the brain were stored in liquid nitrogen for further use.

2.5 Determination of oxidative stress markers in the brain homogenates

Malondialdehyde (MDA) levels as Lipid peroxidation markers were measured as levels of Thiobarbituric acid reactive substances (TBARS) using a commercial assay kit (Cat No. NWK-MDA01, NWLSS, USA). Reduced glutathione (GSH) concentrations were measured using an assay kit (Cat. No. 703002, Cayman Chemical, Ann Arbor, MI, USA). Superoxide dismutase (SOD) activities were measured using a commercial kit (Cat. No. 706002, Cayman Chemical, Ann Arbor, MI, USA). Glutathione peroxidase (GPx) activities were measured using a commercial kit (Cat. No. 703102, Cayman Chemical). All tests were done according to the manufacturer's instruction.

2.6 Assay of cholinergic markers

The cholinergic markers were measured in the brain homogenates of all rats using commercially available kits. The content of choline acetyltransferase (ChAT) was determined using ELIZA commercial available kit (Cat. No SEB929Ra, Cloud-Clone Corp. Houston-TX, USA). Colorimetric commercially available kits were used to determine the content of Acetylcholine (Ach, Cat. No. Cell Biolabs, Inc, STA-603, San Deigo, CA-USA) and the activity of Acetylcholine esterase (AChE, CAT. No. ab138871, abcam, UK). All tests were done according to the manufacturer's instruction and were run in triplicates.

2.7 Western blot analysis

Protein concentrations in the supernatants collected from homogenization of brain tissues in RIPA buffer were measured by Bradford assay and then were separated using 10% SDS-PAGE (60 µg protein/well). Membranes were incubated for 10 minutes at room temperature in blocking buffer (5% milk in 1X TBST buffer). Then, the membranes were then washed 3X with 1X TBST buffer (10 minutes each) followed by incubation with the desired primary antibody for 2 hours. After washing again with 3X with 1X TBST buffer, the membranes were incubated for another 2 hours with horseradish peroxidase-conjugated secondary antibody. All incubations with the antibodies were done in a rotator shaker and all dilution was minimized for each antibody. Bound antibodies intensities were evaluated using a Pierce-enhanced chemiluminescence (ECL) kit (Thermofisher, USA, Piscataway, NJ). Images were scanned using C-DiGit Blot Scanner (LI-COR, USA) with the supplied Image Studio DiGits software. Protein expressions were presented as relative expressions to that of β-actin.Monoclonal or polyclonal antibodies against ERK1/2 (p44-42 MAPK, Cat # number 9102, 42,44 kDa), p-ERK1/2 (p-p44-42 MAP, Thr202/Tyr204, Cat # number 9101, 42.44 kDa), p-BAD (ser 112, Cat # number 9291, 23 kDa), mTOR (Cat # number #2972, 289), phospho-mTOR (Ser2448,

Cat # 2971, 289 kDa), c-Jun (Cat # 9165, 43. 48 kDa), phospho-c-Jun (Ser63, Cat # 9261, 48 KDa), Akt (Cat #9272, 60 kDa), phospho-Akt (Thr308, Cat # 9275, 60 kDa) , and p-70S6K (Cat #9202, 70.85 kDa), pp-70S6K (Thr389, Cat # #9205, 70.85 kDa), PP5 (Cat #2289, 58 kDa) and cleaved caspase 3 (Asp175, Cat #9661, 17/19 kDa) were purchased from Cell Signaling Technology, USA). Antibodies against PP2Aα (Cat#, ab137825, 36 kDa) and p-BAD (ser 184) (Cat # ab216829, 18 kDa) were purchased from Abcam (UK). Antibodies against PTEN (Cat #, A2B1 sc-7974, 55 kDa); were purchased from Santa Cruz Biotechnology Inc., CA, USA, 36 kDa).

2.8 Statistical Analysis

Graphpad prism statistical software package (version 6) was used to perform all statistical analysis and generating figures. In Morris Water Maze (WMT), Comparisons between the rats within the same groups or within the groups were performed using one-way analysis of variance (ANOVA) with repeated measure or one-way ANOVA on rank, respectively followed by Tukey's test form multiple comparisons. All other data were analyzed using one-way ANOVA followed by Tukey's test. Differences were considered significant if $p < 0.05$. Data are presented as mean ± SD.

3. Results

3.1. Mortalitiy rate

Although, the experimental procedure started with 8 rats, 3 deaths were found in group 2 administered with $CdCl_2$ and no mortality was detected in any of the other groups during the experimental procedure. Therefore, the mortality rate due to $CdCl_2$ intoxication was 37.5%. Hence, it could be speculated that QUR or α-tocopherol administration alone or in conjugation increased survival rate to 100% in $CdCl_2$ intoxicated rats. However, the 3 dead rats in the $CdCl_2$ intoxicated group were replaced by other 3 rats to make the total number for each group is eight.

3.2. Assessment of cognitive and memory dysfunction

Assessment of cognitive dysfunction in rats was achieved by analysis of Morris water maze (MWM) (Fig. 1), measuring levels of brain cholinergic markers (Fig. 2) and protein levels of CREB and BDNF (Fig. 8), two markers related to memory and synaptic plasticity. In MWM, escape latencies (time needed to find a visible submerge platform) of 4 trails/day/rat were analyzed and compared within and between the groups over periods of consecutive 4 days performed at the end of all treatments. While individual administration of QUR or α- tocopherol to control rats has no effect on all of the above-mentioned parameters, concomitant administration of both drugs significantly shortened the escape latencies for test days 3 and 4, enhanced levels of Ach and activities AChT as well as protein levels CREB and PDNF and significantly lowered activities of AChE, in respect to control rats. On the other hand, increased average escaped latencies over all days of memory assessment with significantly decreased levels of Ach, CREB and BNDF and activities of AChT and significantly enhanced levels of ACHE were seen in $CdCl_2$ intoxicated rats received the vehicle or QUR alone as compared control rats, results that were not significantly different within or between both groups. However, Concomitant administration of QUR and α-tocopherol to $CdCl_2$ intoxicated rats significantly decrease the average escape latencies and significantly alleviated the levels of Ach, CREB, BDNF and activities of AChE, AChT to normal levels seen in the control rats, as compared to $CdCl_2$ model rats. The resulted latencies in this group of rats were not significantly different to their corresponding latencies observed in the control rats overall testing days.

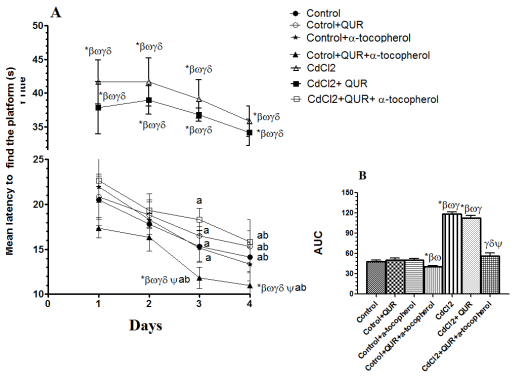

Fig. 1: Average mean latency to find hidden platform in water maze test (MWM, A) and area under the curve (B) in all groups of rats. Each data point represents the mean ±SD latency of the 4 trials/rat/day for a total of 8 rats/group. a: significantly different when compared to day 1 within the same group. b: significantly different when compared to day 2 within the same group. *: versus control, β: versus Control+QUR. ώ: versus control + α-tocopherol. : versus Control+QUR + α-tocopherol. δ: significantly different as compared to CdCl2. Ψ: Significantly different as compared to CdCl2+QUR.

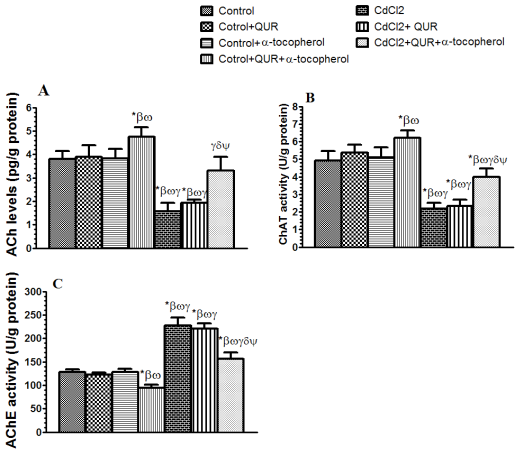

Fig. 2: Levels of Acetylcholine (Ach, A), Acetylcholine transferase (AChT, B) and Acetylcholine esterase (AChE, C) in the brain homogenates of all rat groups. Values are presented as mean ±SD of 8 rats/group. *: versus control, β: versus Control+QUR. ώ: versus Control+α-tocopherol. : versus Control+QUR+ α-tocopherol. δ: significantly different as compared to CdCl2. Ψ: Significantly different as compared to CdCl2+QUR

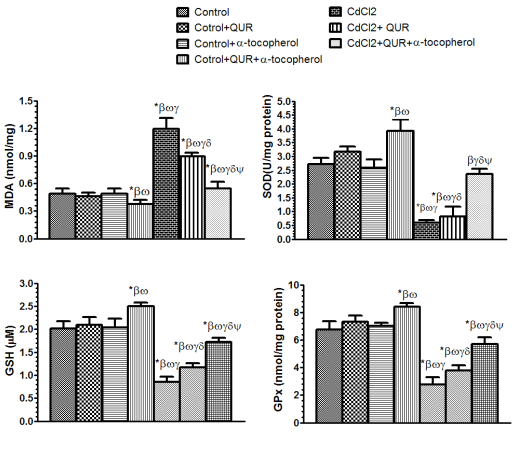

Fig. 3: Levels of Malondialdehyde (MDA) and reduced glutathione (GSH) and activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in brain homogenates of all rat groups.*: versus control, β: versus Control+QUR. ώ: versus Control+α-tocopherol. : versus Control+QUR+ α-tocopherol. δ: significantly different as compared to CdCl2. Ψ: Significantly different as compared to CdCl2+QUR.

3.3. Oxidative stress evaluation:

Biochemical analyses of oxidative stress markers are depicted in Fig. 3. Data revealed that individual administration of QUR or α-tocopherol to control rats has no effect on brain levels of MDA and GSH or activities of SOD and GPx, as compared to control rats received the vehicle. However, combined administration of QUR or α-tocopherol to control rats significantly lowered MDA levels and significantly raised GSH levels and activities of SOD and GPx in the brain of these control rats as compared to control rats received the vehicle. On the other hand, $CdCl_2$ intoxication resulted in significant decreases in the levels of GSH and activities of SOD and GPx and significantly increased levels of MDA in the brain tissue of intoxicated rats, levels that was partially but significantly improved by individual QUR therapy. However, when conjugated together, QUR and α-tocopherol resulted in further significant increases in the levels of GSH and activities of SOD and GPx and significantly lowered the MDA as compared to $CdCl_2$ model groups received the vehicle or QUR.

3.4. Effects on mitogen-activated protein kinase signal (MAPK) signaling:

Phosphorylated and total protein levels components of ERK1/2 signaling pathway including (MAPK 54/56 (ERK1/2), p-ERK1/2, BAD and p-BAD (Ser 112) and JNK signaling pathway including (JNK, p JNK, c-Jun, p-c-Jun and p- BAD (Ser128) in the brain tissue of all groups of rats were investigated (Fig. 4 and Fig. 5). Moreover, as MAPK activity is regulation by kinase and phosphatases, the levels of their upstream regulators namely protein phosphatase 2A and 5 (PP2A and 5, respectively) were also investigated (Fig. 8). While increased P-BAD (Ser 112) is known to be anti-apoptotic, enhanced level of p-BAD (Ser 128) is apoptotic. To confirm these effects levels of cleaved caspase-3 in the brain tissues were also measured (Fig. 6). Total levels of all the tested proteins were always stable in the brain tissue of all groups of rats. In the control groups of rats, solely QUR or α-tocopherol $CdCl_2$ intoxication didn't affect phosphorylation levels of all of the above-mentioned proteins neither levels of cleaved caspase-3, PP2A or PP5. $CdCl_2$ administration to rats significantly activated both ERK 1/ and JNK signaling and activated intrinsic apoptosis 2 as indicated by upregulation of p-ERK1/2, p-JNK, p-c-Jun, decreased levels of p-BAD (Ser 112) and increased levels of p-BAD (Ser128) and cleaved caspase-3, as compared to control rats. In this regards, $CdCl_2$ intoxication significantly lower levels of PP2A but not PP5 in the brain tissues of these rats.

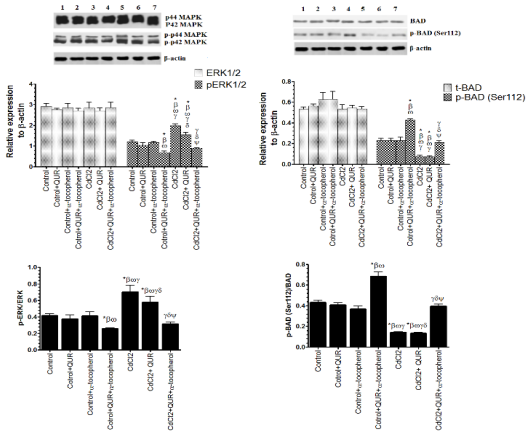

Fig. 4: Protein levels of total extracellular signal-regulated kinase 1/2 (Erk1/2), p-ERK1/2 (Thr202/Tyr204), total BAD and p-BAD (Ser 112) in the brain tissues of all rat groups as detected by western blot. *: versus control (1), β: versus Control+QUR (2). ώ: versus Control+α-tocopherol (3). : versus Control+QUR+ α-tocopherol (4). δ: significantly different as compared to CdCl2 (5). Ψ: Significantly different as compared to CdCl2+QUR. 7: CdCl2+QUR+α-tocopherol

On the other hand, However, combined administration of QUR and α-tocopherol to control rats significantly downregulated ERK1/2 and JNK signaling as indicated by the significant decreases in the levels of p-ERK1/2, p-JNK and p-c-JUN and enhanced levels of PP2A. Interestingly, associated with these changes, both drugs significantly increased levels of p-BAD (Ser 112) and significantly decreased levels of p-BAD (Ser128) and cleaved caspase 3. Such effects were also partially achieved by individual QUR administration and further significantly increased toward their normal levels in the brain of $CdCl_2$ intoxicated rats when QUR and α-tocopherol were administered in conjugation, as compared to CdCl2 intoxicated rats received the vehicle.

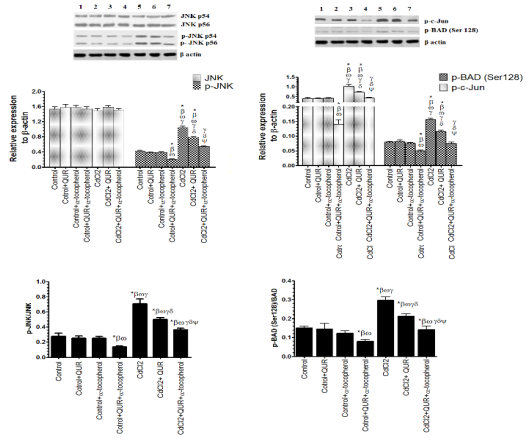

Fig. 5: Protein levels of the total c-Jun N-terminal kinase (JNK), p-JNK (Thr183/Tyr185), p-c-Jun (Ser63) and p-BAD (Ser128) in the brain tissues of all groups of rats as detected by western blot. *: versus control (1), β: versus Control+QUR (2). ώ: versus Control+α-tocopherol (3). : versus Control+QUR+ α-tocopherol (4). δ: significantly different as compared to CdCl2 (5). Ψ: Significantly different as compared to CdCl2+QUR. 7: CdCl2+QUR+α-tocopherol.

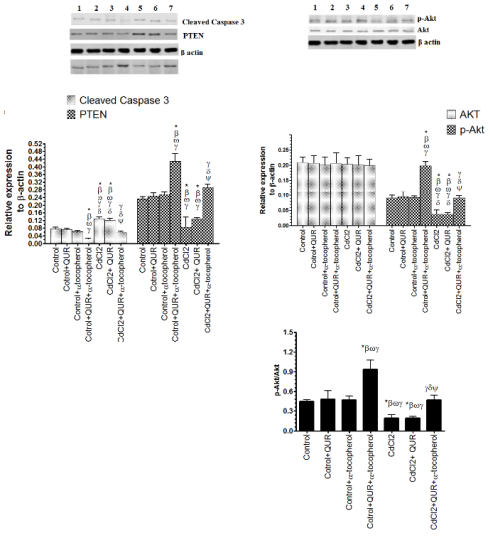

Fig. 6: Protein levels of Cleaved caspase 3, PTEN, Akt and p-Akt (Thr308) in the brain tissues of all groups of rats as detected by western blot. *: versus control (1), β: versus Control+QUR (2). ώ: versus Control+α-tocopherol (3). : versus Control+QUR+ α-tocopherol (4). δ: significantly different as compared to CdCl2 (5). Ψ: Significantly different as compared to CdCl2+QUR. 7: CdCl2+QUR+α-tocopherol

3.5. Effects on mammalian target of rapamycin (mTOR) signaling

The levels of phosphorylation and total protein levels of the signaling pathway Akt/mTOR/S6K1 and the levels of its negative regulator PTEN were studied in the brain tissue of all groups of rats (Fig. 6 and Fig. 7). Of interest, individual QUR administration tended to increase the activity of Akt but significantly increased levels of p-mTOR and it downstream targets, p-S6K1 without affecting the expression of PTEN. However, when QUR was administered concomitantly with α-tocopherol, it significantly lowered levels of PTEN and raised protein levels of p-Akt, p-mTOR and p-S6K1 as compared to all other control groups. $CdCl_2$ intoxication significantly inhibited this signaling pathway as indicated by the significant decreases in the levels of p-Akt, p-mTOR and p-S6K1 and significant increases in the levels of PTEN. On the other hand and unlike to situation in the control group, individual administration of QUR to $CdCl_2$ intoxicated rats failed to regulate expression levels of all of this protein and only significant downregulation of PTEN and

upregulation of p-Akt, p-mTOR and p-S6K1 levels were seen when it was administered in conjunction with α-tocopherol, all levels of which were not significantly different as compared to control rats.

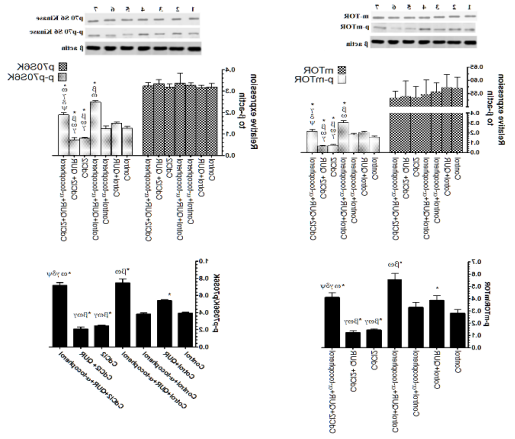

Fig. 7: Protein levels of mammalian target of rapamycin (m-TOR), p-mTOR (Ser2448), p70S6K and p-p70S6K (Thr389) in the brain tissues of all groups of rats as detected by western blot. *: versus control (1), β: versus Control+QUR (2). ώ: versus Control+α-tocopherol (3). : versus Control+QUR+ α-tocopherol (4). δ: significantly different as compared to CdCl2 (5). Ψ: Significantly different as compared to CdCl2+QUR. 7: CdCl2+QUR+α-tocopherol.

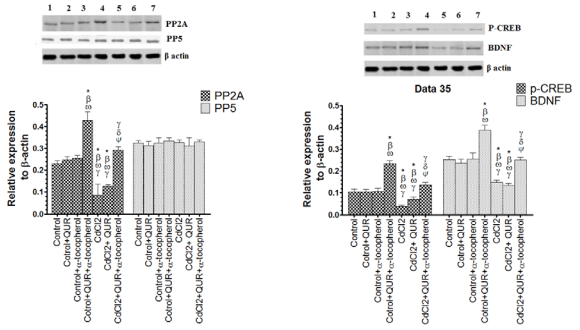

Fig. 8: Protein levels of protein phosphatase 2A (PP2A), protein phosphatase 5 (PP5), cAMP-responsive element-binding (p-CREB) and brain-derived neurotrophic factor (BDNF) in the brain tissues of all groups of rats as detected by western blot. *: versus control (1), β: versus Control+QUR (2). ώ: versus Control+α-tocopherol (3). : versus Control+QUR+ α-tocopherol (4). δ: significantly different as compared to CdCl2 (5). Ψ: Significantly different as compared to CdCl2+QUR. 7: CdCl2+QUR+α-tocopherol.

4. Discussion

The aim of the current study was to investigate the effect of QUR alone or in conjugation with administered α-tocopherol on neural apoptosis and associated cognitive dysfunction and in rats which are either control or intoxicated with $CdCl_2$, in vivo and to investigate the molecular mechanisms behind this protection. The major finding of the current study are 1) $CdCl_2$ activated neural apoptosis and induced neurotransmitters disturbance and spatial memory loss. 2) These effects were associated with enhanced oxidative stress and inhibition of protein phosphatases 2A (PP2A) induced activation of ERK1/2 and JNK as well as with activation of PTEN induced inhibiting of Akt/mTOR signaling. 3) In both control and $CdCl_2$ intoxicated rats, QUR and only when administered in conjugation with α-tocopherol, enhanced spatial memory function, and reduced markers of apoptosis by activating Akt/mTOR survival pathway and downregulated the ERK1/2 and JNK apoptotic pathways, an effect that is associated with decreased neural oxidative stress.

The mechanism by which $CdCl_2$ induces neural oxidative stress induced neural damage, apoptosis and its own enhanced permeability across the blood brain barrier (BBB) of adult brains appears to be mediated by disruption of the prooxidant/antioxidant balance. This is achieved indirectly by replacing iron and copper ion from a number of cytoplasmic and membrane proteins, activating Fenton reaction and (Casalino et al. 1997) and depletion of selenium-induced reduced glutathione (GSH) levels and GSH peroxidase (GPx) activities (Lopez et al., 2006; Chen et al., 2014). In support to this, enhanced levels of Malondialdehyde (MDA), reduced levels of GSH as well as activities of total superoxide dismutase (SOD) and GPx have been detected in the brains of rats intoxicated with $CdCl_2$. However, in control rats, QUR tended to lower MDA levels and enhance the antioxidant systems with a more profound when it was administered as a conjugate with α-tocopherol, suggesting to act as a prooxidant rather than antioxidants to stimulate the cell's own antioxidant defense mechanisms as has been previously suggested (Halliwell, 2008; Halliwell, 2013).

In comparison to its partial antioxidant ameliorative effect in $CdCl_2$-intoxicated rats when administered alone, QUR completely restored the activities of these antioxidant enzymes and GSH levels when it was administered in conjucation with α-tocopherol even look similar, the finding does not support the findings reported by Unsal et al. (2003) who have shown that QUR completely ameliorated levels of MDA and activities of SOD in the brain of rats intoxicated with $CdCl_2$. This could be explained by the expected low levels (nanomolar) of QUR in the brain tissue upon solely QUR administration (Ishisaka et al., 2011). In support, it has been reported that at least

millimolar levels of any antioxidant (i.e glutathione and vitamin C) are required to scavenge free radicals in the brain tissue (Schaffer and Halliwell, 2012).

On the other hand, cell apoptosis is a well control mechanism that includes many cell signaling pathways. Among all, MAPKs (ERK1/2, JNK and P38) and PI3K/Akt/mTOR play many roles under pathological intoxication with $CdCl_2$ (Chen et al., 2008, Chen et al., 2013). In most tissues and Erk1/2 and PI3K/Akt/mTOR were shown be survival pathways, whereas JNK and p38 signaling cascades have been shown to promote neuronal cell death (Detta et al., 1997; Davis 2000; Lei and Davis , 2003; Rockwell et al., 2004; Shimamura et al., 2000).

In the neural cortical tissues, while JNK activation phosphorylates BAD at Serine128 (Ser128) to promotes its apoptotic effect (Donovan et al., 2002), both ERK1/2 and Akt induce phosphorylation of Bad at Ser136 (Ser136) and/or Serine112 (Ser112) to inhibit apoptosis (Detta et al., 1997; Shimamura et al., 2000). However, even it is a survival signal, ROS-induced sustained activation of ERK1/2 initiates either intrinsic or extrinsic apoptosis in vitro and in vivo (Cagnol and Chambard, 2010).

Using cultured neurons, ROS induced by $CdCl_2$ activated all members of MAPK family including ERK1/2, JNK, and p38 by direct inhibition of serine/threonine protein phosphatases 2A (PP2A) and protein phosphatase 5 (PP5). In the same line, orally administered $CdCl_2$ to rats upregulated both ERK1/2 and JNK, an effect that was mediated at least by inhibition of PP2A but not PP5, as evident by the detected cellular protein levels. Interestingly, p-BAD (Ser 128) significantly increased whereas p-BAD (ser 112) significantly decreased, suggesting that both ERK1/2 and JNK activations are pro-apoptotic. However, in contrast to in vitro studies of Chen et al. (2011a; 2011b; 2014) who have shown that $CdCl_2$ activated PI3K/Akt/mTOR induced apoptosis on culture neurons by inhibiting PTEN, the current in vivo model shows that administration of $CdCl_2$ inhibited Akt/ mTOR survival pathway through activating PTEN, suggesting a decreased activity of this survival pathway.

Neither QUR nor α-tocopherol solely administration affected any protein levels involved in all these signaling pathways in both control and Cd-intoxicated rats. Interestingly, conjugation of both drugs together significantly enhanced levels of PP2A resulting in significant inhibition of both ERK1/2 and JNK signaling with a concomitant inhibition of PTEN mediated activation of Akt/mTOR/S6K1 signaling in the brains of control rats or $CdCl_2$-intoxicated rats. Associated with these effect enhanced levels of p-BAD (Ser 112) and decrease levels of pBAD (Ser 128) with a parallel decrease in cleaved caspase 3 were seen, suggesting a neurosurvival effect of QUR, only if administered with α-tocopherol under both control or $CdCl_2$-intoxication conditions.

In parallel to the enhanced oxidative stress, a decrease in cognitive dysfunction with parallel decreases in the levels of acetylcholine (Ach)and choline acyltransferase (ChAT) activity and increase in acetylcholinesterase (AChE) activity were seen in the brain tissues of $CdCl_2$-intoxicated rats. These effect were only ameliorated in the group of rats only administered QUR in conjugation with α-tocopherol.

Although previous reports have shown decreased levels of Ach in the brain of rat after $CdCl_2$ intoxication (Desi et al., 1988) and are positively correlated with enhanced oxidative stress (Ahmad et al., 2012; Xi et al., 2014; Lafuente et al., 2000; Pari and Murugavel, 2007), this study enriches the available literature and shows that $CdCl_2$ induced cognitive dysfunction is also associated with lower brain protein levels of p-CREB and its downstream target, BDNF, both of which play roles in neuronal proliferation, synapse remodelling, synaptic plasticity and memory function (Finkbeiner et al., 1997; Mizuno et al., 2002). However, large number of intracellular signal transduction pathways converge CREB and regulate CREB activity (Carlezon et al., 2005). In CNS, activation of numerous signaling pathways are known to upregulate CREB levels. These include PKB/Akt, MAPKs, and calcium-calmodulin kinase IV (CaMKIV) (Barco et al., 2003; Carlezon et al., 2005). In accordance with the available evidences, it can be conclude that CdCl2 affects the spatial memory in rats by inhibiting Akt signaling pathway and that conjugation of QUR and α-tocopherol rather than their individual doses are able to enahanc the levels of CREB and PDNF by activation of this pathway. In support, a flavonoid extracted from blueberry induced activation of CREB and BDNF expression has also been shown to lead to the activation of the PI3K/Akt/mTOR signaling pathway (Williams et al., 2008).

Despite the current evidences, this study still has some limitations. For instance, the doses of QUR used here was based on many previous studies in literature that showed a neuroprotective effect of QUR at this dose. Hence, future work should investigate the effect of QUR alone in this animal model at higher doses to compare the effects and confirm the main findings of the current study. Furthermore, results presented in

this study showed that the effect of QUR on cognitive function and biochemical alterations related to this effect in whole brain. As the hippocampus is one of the important brain structure affected in neurogenerative diseases, further investigation on these molecular pathways at the level of the hippocampai would be advantageous and will clearly add much more support to the current findings. Finally, it is highly recommended to repeat this study on other species (e.g. mice and rabbits) and on the same species of another sex or aged rats.

5. Conclusion

This study clearly demonstrates neither QUR nor α-tocopherol alone are able to rescue the neurons and enhanced special memory against CdCl2 induced neurotoxicity. However, when both drugs are administered in conjugation, are able to reduced cell apoptosis and enhance spatial memory in CdCl2 intoxicated rats by activated PP2A induced inhibition of ERK1/2 and JNK apoptotic pathways and by inhibiting PTEN induced activation of Akt/mTOR/S6K1 survival pathway.

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Conflicts of Interest

The author has no conflict of interest and the work was not supported or funded by any company.

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