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Kaempferol protects against cadmium chloride-induced hippocampal damage and memory deficits by activation of silent information regulator 1 and inhibition of poly (ADP-Ribose) polymerase-1

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Abstract:

The neuroprotective effect of Kaempferol against cadmium chloride (CdCl₂) -induced neurotoxicity is well reported. The silent information regulator 1 (SIRT1) and poly (ADP-Ribose) polymerase-1 (PARP1) are two related cellular molecules that can negatively affect the activity of each other to promote or inhibit cell survival, respectively. It is still largely unknown if the neurotoxicity of CdCl₂ or the neuroprotection of Kaempferol are mediated by modulating SIRT1 and/or PARP1 activities. In this study, we tested the hypothesis that CdCl₂-induced memory deficit and hippocampal damage are associated with downregulation/inhibition of SIRT1 and activation of PARP1, an effect that can be reversed by co-treatment with Kaempferol. Rats (n=12/group) were divided into 4 groups as control, control + Kaempferol (50 mg/kg), CdCl₂ (0.5 mg/kg), and CdCl₂ + Kaempferol. All treatments were administered orally for 30 days daily. As compared to control rats, CdCl₂ reduced rat's final body weights (21.8%) and their food intake (30%), induced oxidative stress and apoptosis in their hippocampi, and impaired their short and long-term recognition memory functions. Besides, the hippocampi of CdCl₂-treated rats had higher levels of TNF- α (197%), and IL-6 (190%) with a concomitant increase in nuclear activity and levels of NF- κ B p65 (721% & 554%). Besides, they showed reduced nuclear activity (53%) and levels (74%) of SIRT1, higher nuclear activity and levels of PARP1 (292% & 138%), increased nuclear levels of p53 (870%), and higher acetylated levels of NF- κ B p65 (513%), p53 (644%), PARP1 (696%), and FOXO-2 (149%). All these events were significantly reversed in the CdCl₂ + Kaempferol-treated rats. Of note, Kaempferol also increased levels of MnSOD (73.5%), and GSH (40%), protein levels of Bcl-2 (350%), and nuclear activity (67%) and levels (46%) of SIRT1 in the hippocampi of the control rats. In conclusion, Kaempferol ameliorates CdCl₂-induced memory deficits and hippocampal oxidative stress, inflammation, and apoptosis by increasing SIRT1 activity and inhibiting PARP1 activity.

Keywords: CdCl₂, Kaempferol, SIRT1, PARP1, Memory, Hippocampus, rats.

Running Head: Kaempferol activates SIRT1 in the hippocampi of CdCl₂-induced rats

1. Introduction

Cadmium (Cd) is one of the most neurotoxic heavy metals that leads to neurodegeneration and memory deficits and is considered a major risk factor for the development of Alzheimer's disease (AD) and Parkinson's disease (PD) (**Jiang *et al.*, 2007; Okuda *et al.*, 1997; Shati *et al.*, 2019**). The neurotoxicity of Cd ions mediated by its ability to cross the blood-brain barrier (BBB), deplete endogenous antioxidant content and induce ROS, oxidative stress, and apoptosis (**Jiang *et al.*, 2007; Okuda *et al.*, 1997; Shati *et al.*, 2019**). Currently, the available evidence is showing that Cd ions induce apoptosis in the brain and hippocampi of rodents by activating several pathways, including mTOR and P38 MAPK, and protein phosphatase 2A and 5 (**Chen *et al.*, 2011; Yan *et al.*, 2016**).

Up to date, the precise mechanism by which Cd ions induce neural cell death remains enigmatic and needs further investigation. Currently, the important role of the silent information regulator 1 (SIRT1), an (NAD⁺)-dependent protein deacetylase as an antioxidant, anti-apoptotic, and anti-inflammatory neuroprotective agent in various neurodegenerative continue to fascinate the scientist. In the majority of cells, SIRT1 can promote cell survival by stimulating the synthesis of the antioxidant enzyme and inhibiting cell inflammation and apoptosis by deacetylation of various substrates and transcription factors, including p53, nuclear factor- κ B (NF- κ B), peroxisome proliferators-activated receptor- γ (PPAR- γ) and its coactivator-1 α (PGC-1 α), and forkhead transcriptional factors (FOXO) (**Chen *et al.*, 2005; Chong *et al.*, 2012; Zhang *et al.*, 2018**).

As a neuroprotective molecule, SIRT1 promotes neuron survival, inhibits apoptosis, and enhances the synaptic plasticity, neural axon growth, and dendritic branching (**Ferrante *et al.*, 1997; Guo *et al.*, 2011; Karuppagounder *et al.*, 2009**). Also, SIRT1 reduced motor dysfunction, memory deficits, demyelination, and neural apoptosis, and slowed down the progression of dementia in several neurodegenerative disorders including AD, PD,

Huntington's disease (HD), Multiple sclerosis (MS), and Prion diseases (**reviewed in Herskovits and Guarent, 2014**). Moreover, SIRT1 protected against anxiety, depression and psychiatric disorders such as schizophrenia and bipolar disorder (**Herskovits et al., 2014; Kishi et al., 2011; Kishi et al., 2010**).

On the other hand, base excision repair (BER), mismatch repair (MMR), and nucleotide excision repair (NER) are major pathways for repairing the oxidative-stress-induced single-strand cell damage (**Mitra et al., 2001**). Poly (ADP-Ribose) polymerase-1 (PARP1) is the major enzymes that promote DNA synthesis across the lesion site and strand resection by utilizing NAD⁺ as a substrate (**Rajamohan et al., 2009**). Current accumulating data have implicated PARP1 as a key player in the mechanisms of apoptosis and memory loss in a variety of neurodegenerative disorders. In patients with AD, higher levels of PARP1 were detected in the brain, skin fibroblast, and lymphocytes (**Abeti et al., 2011**). Similar results were shown in the brains of AD transgenic mice (**Abeti et al., 2011**). Also, PARP1 levels and activity were significantly increased in the cerebrospinal fluids (CSF) and the brains of PD patients and were associated with an accumulation of α -synuclein and neural cell death (**Kam et al., 2018**). Besides, pharmacological inhibition of PARP1 prevented cell death in cortical neurons exposed to death stimuli and in rats exposed to traumatic brain injury (**Stoica et al., 2014**). In the same line, mice deficient in PARP1 showed increased hippocampal cell death and had a significant reduction in cognitive and motor performance after traumatic brain injury (**Satchell et al., 2003; Stoica et al., 2014**). At the molecular level, although PARP1 protects the DNA from oxidative damage, over-activation of PARP1 leads to cellular energy failure and apoptosis by depleting intracellular NAD⁺ levels (**Szabo et al., 1996**). Besides, PARP1 stimulate and potentiate the inflammatory response in a variety of inflammatory conditions by acting as a transcriptional coactivator of nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) (**Hassa et al., 2002**).

Of note PARP1 levels not only regulated by transcription, but also by acetylation (**Rajamohan et al., 2009**). Recently, studies have shown a bidirectional negative association between PARP1 and SIRT1. On one hand, over-activation of PARP1 can inhibit SIRT1 activity due to the over-consumption of NAD⁺. On the other hand, SIRT1 doesn't only inhibit PARP1 activity by deacetylation but also down-regulates its transcription (**Rajamohan et al., 2009**).

Unfortunately, and even explored in other neurodegenerative disorders, the roles of SIRT1 and PARP1 in Cd ions induced neural damage and cognitive deficits were never shown yet and need further investigation. Also, it seems reasonable that any drug that can stimulate SIRT1 and inhibit PARP1 can afford neuroprotection. The plant flavonoids are well reported for their neuroprotective effects against numerous neurodegenerative disorders with many thanks to their abilities to cross the BBB and their antioxidant and anti-inflammatory properties (**Kouhestani et al., 2018a**). Kaempferol, a major flavonoid glycoside of *Carthamus tinctorius* L (**Yu et al., 2013**), was studied extensively and was shown to inhibit ROS generation, cell apoptosis, inflammation, and mitochondria damage and improve cognitive function in various neurodegenerative disorders, including AD, traumatic brain injury (TBI), and dementia (**Chitturi et al., 2019a; Kouhestani et al., 2018a; Yu et al., 2013**). Recently, we have shown that Kaempferol is also able to protect the rat's against cadmium chloride (CdCl₂) -induced hippocampal damage and memory loss by increasing the levels and activity of AMPK, one of the most potent activators of SIRT1 (**El-Kott et al., 2020**). Of interest, Kaempferol protected the cardiomyocytes from ischemia-reperfusion injury by upregulation and activation of SIRT1 (**Guo et al., 2015**). In addition, Kaempferol, like many other flavonoids, can inhibit PARP1 activity and the associated inflammation and cytokine production in lipopolysaccharide-stimulated pulmonary epithelial cells (**Geraets et al., 2007**).

Given the negative crosstalk between PARP1 and SIRT1 in their important contradictory roles in the pathogenesis of neurodegeneration and memory loss, in this study, we first hypothesized that CdCl₂-induced hippocampal damage and memory dysfunction involve inhibition of SIRT1 and activation of PARP1. Besides, we also tested if Kaempferol protective effect against CdCl₂-induced memory deficits and hippocampal oxidative stress and damage are mediated by modulating the levels/activity of SIRT1 and/or PARP1.

2. Materials and methods

2.1. Animals

Adult Wistar male rats (Charles River, Strain code: 003) (120 ± 5 g) were bred, provided from, and maintained in the animal unit at King Khalid University, Kingdom of Saudi Arabia (KSA). All rats were housed under automatically well-controlled conditions (23 ± 1°C, 12/12 h, and light/ dark cycle). During the adaptation period for 1 week, all rats were fed a standard diet and had free access to drinking water all the time. All procedures used in this study were approved by the animal ethics and use committee at King Khalid University, where their regulations follow those established by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

2.2. Experimental Design

After an adaptation period, rats were randomly selected and segregated into 4 groups (n=12/group) as **1) control:** administered 2% 2-methyl cellulose (Cat. No. M0512-100G, Sigma Aldrich, St. Louis, MO) as a vehicle; **2) control + Kaempferol:** treated with Kaempferol (50 mg/kg/day) (Cat. No. 60010, Sigma Aldrich, St. Louis, MO) dissolved in 2% of 2-methyl cellulose; **3) CdCl₂:** treated with 0.2 ml of CdCl₂ (Cat. N O. 202908, Sigma Aldrich, St. Louis, MO) (0.5 mg/kg) and co-administered 2%2-methylcellulose, **4) CdCl₂ +**

Kaempferol: received a concomitant dose of CdCl₂ and Kaempferol as in the previous groups. All treatments were given as a single dose/day for consecutive 30 days daily.

2.3. Dose selection

The dose of CdCl₂ was adopted below the LD₅₀ by oral administration of 30 days. Besides, the authors have found that at this dose, CdCl₂ affects mainly the brains rather than any other organs and the main mechanism for the neural damage occurs mainly by activation of oxidative stress and mitochondria-mediated cell death-related mechanisms (Agnihotri *et al.*, 2015). The dose of Kaempferol and route of administration was based on another study that showed neuroprotection in other animal models of neurodegeneration (Alkhalidy *et al.*, 2018).

2.4. Assessment of recognition memory

Short and long-term recognition memory in all rats was assessed 48 after the last dose of treatment using the Novel Object Recognition Task (NORT) test and as previously described by Bevins *et al.*, (Bevins *et al.*, 2006). Briefly, the rats were allowed to adapt and explore the wooden apparatus for 3 times each of 10 minutes. During the training phase, each rat explored two identical objects (glass blocks). Three hours later, the rats were inserted again into the apparatus to explore one familiar and another new object (a glass block and a wooden block) (short-term recognition memory). Eighteen hours later, the rat was returned to the apparatus and explored a familiar object and a new novel object (a glass block and a plastic cube) (long-term recognition memory). To avoid the interference of the olfactory stimuli on the outcome of the test, the apparatus and the objects were always cleaned with 70% ethanol after each trial. Effective exploration time was considered valid if the rat directed toward or touches the object with its nose for a distance less than 2 cm. Turning around or sitting on the object was not considered an exploration behavior and were not included. Time to explore each object/trial was recorded and the discrimination ratio defined as time explore the target object

(familiar or novel) divided over the total time needed to explore both objects. The experiments were performed for n=12/group.

2.5. Hippocampi collection fractionation, and preparation of homogenates

After the memory testing, hippocampi were collected from all groups of rats under stereomicroscope after being anesthetized with sodium pentobarbital (60–70 mg/kg, i.p). The hippocampi were snap-frozen in liquid nitrogen and directly stored at -80°C for later biochemical and molecular studies. To prepare total hippocampal homogenates for the biochemical analysis, 20 mg of the hippocampi of all groups of rats were homogenized separately in a 100 µl of ice-cold phosphate-buffered saline (PBS, pH of 7.4) supplied with a protease inhibitor cocktail (Cat. No. P8340, Sigma-Aldrich, St. Louis, MO, USA) and supernatants were collected and stored at -80°C until the time of use. For western blotting, 20 mg of the hippocampi powder was homogenized in 200 µL RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 0.5% sodium deoxycholate, 150 mM sodium chloride, 1.0% NP-40, 0.1% SDS, and 5µl the protease inhibitor cocktail and the supernatant was isolated and stored at -80°C until the time of use. The nuclear and cytoplasmic fractions from the hippocampi of all groups of rats were prepared using a special kit (Cat No. 78835 and ThermoFisher Scientific). Once needed, protein concentrations were measured by a Bradford assay using BSA as standard, incubation time 10 min at room temperature and reading the absorbance at 595 nm (Standard Pierce Detergent Compatible Bradford Assay Kit; Cat. No 23246, ThermoFisher Scientific, MA, USA). All samples were run in duplicate and accordance with the manufacturer's instructions.

2.6. Biochemical measurements in the brain and hippocampi

Total levels of free radical (ROS & RNS) were measured in the hippocampi homogenates using a special kit that measures the levels of a highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF) (Cat. No. STA-347, OxiSelect, Cell Biolabs, Inc. CA,

USA). Hippocampal levels of reduced glutathione (GSH) and malondialdehyde (MDA), were measured using special calorimetric and ELISA kits (Cat. No. 7511-100-K, Trevigen, Gaithersburg, USA & Cat. No. ab118970, Abcam, UK) Hippocampal levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and MnSOD were measured using commercially available ELISA kits (Cat. No. CSB-E11987r, CSB-E04640r, & Cat. No. P07895, CUSABIO technology, TX, USA). All measurements were performed for n=6 rats/group (duplicate) following the manufacturer's instructions.

2.7. Biochemical measurements in the nuclear fraction:

The nuclear SIRT1 deacetylase activity was measured using a fluorometric Cyclex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (Cat. No. CY-1151V, Nagano, Japan) using 20 μ g proteins of the nuclear extract. The fluorescent signal was read using a microplate fluorescence reader (FL600Bio-Tek Instruments, Inc., Winooski, VT, USA) at excitation at 340 nm and emission at 460 nm. The nuclear PARP-1 activity was measured by the universal colorimetric assay kit (Cat. No. 4677-096-K, Gaithersburg, MD, USA) using 25 μ g proteins of the nuclear extract. The nuclear activity of NF- κ B P65 was measured using TransAM assay kit (Cat. No. 40596, Active Motif, Tokyo, Japan) and recombinant NF- κ B p65 as a standard (Cat. No. 31102, Active Motif, Tokyo, Japan) using 20 μ g proteins of the nuclear extract. For the measurements of PARP1 and NF- κ B P65 activity, the absorbance was read at 450 nm using a Spectramax microplate reader. All measurements were performed in n=6 rats/group (duplicate) per the manufacturer's instructions.

2.8. Immunoprecipitation

For Immunoprecipitation, 100 μ g of the nuclear protein was mixed with 500 μ l lysis buffer (20 mM HEPES, 1 mM DTT, 75 mM KCl, 2.5 mM MgCl₂, 0.1% NP-40, and 5 μ l the

protease inhibitor cocktail (Cat. No. P8340, Sigma-Aldrich, St. Louis, MO, USA). Then, 50% protein-A/G plus-agarose (20 μ l) and of rabbit IgG (2 μ l/2 μ g) (Santa Cruz Biotechnology) (Both prepared in the lysis buffer) were added to each sample. The whole mix was incubated at 4 °C overnight with shaking. The next day, all mixtures were centrifuged at 1000 x g, for 10 min and all supernatants were separated. For different samples, 4 μ g of PPAR-1, P53, FOXO1, PGC- α 1, NF- κ B p65 (**Table 1**) or normal rabbit IgG were added to the isolated supernatant of each sample and incubated at 4 °C overnight with shaking. Then, 50% of protein-A/G plus-agarose (30 μ l) was added to every supernatant and incubated for 1 h at 4 °C. The Protein-A/G plus-agarose beads were washed 3 times with the lysis buffer (each for 10 min). Then, all the precipitated complexes were eluted with 30 μ l of 2x Laemmli buffer, boiled for 5 min and then processed for western blotting to detect protein levels of acetylated PARP-1, FOXO-1, NF- κ B p65, and P53.

2.9. Western blotting analysis

This has been done according to our established methods (**El-Kott *et al.*, 2019**). All samples were homogenized in RIPA buffer to which protease inhibitors were added to prevent protein degradation (mentioned before). All samples were first mixed with the loading buffer and boiled for 5 minutes. All samples (40 μ g) were then separated by 8–12% SDS–polyacrylamide gel, transferred nitrocellulose membranes and then processed to be blotted with the primary antibodies (**Table 1**). Then all membranes were incubated with appropriate corresponding horseradish peroxidase (HRP) -conjugated secondary antibody. All antibodies were prepared in Tris-buffered saline-tween-20 (TBST) buffer. A Pierce ECL kit (ThermoFisher, USA, Piscataway, NJ) was used to develop the bands. Band intensities were photographed and analyzed using a C-Di Git blot scanner (LI-COR, NE, USA). The relative expression between different gels for measurement of the expression of the same protein was normalized by an internal control sample. All membranes were stripped up to 5 times. For the

detection of acetylated P53, NF- κ B p65, PARP1, and FOXO-1 in the nuclear fractions, nuclear proteins were first immune-precipitated (as mentioned above) and then followed by Western blotting analysis with an acetylated lysine antibody (Cat. No. 9441, 1:500, cell signaling).

2.10. Statistical analysis

Statistical analysis for all measured parameters was done using Graph Pad Prism statistical software package (version 8). Differences among the experimental groups were assessed by two-way ANOVA, followed by Tukey's test as post hoc. Data were presented as mean \pm SD. Values will be considered significantly different when $P < 0.05$.

3. Results

3.1. Kaempferol increase body weights of CdCl₂-treated rats, independent of food intake, and improve the histological structures of their hippocampi.

Cadmium chloride (CdCl₂) significantly reduced final body weights of rats ($P < 0.0001$) and their food intake ($P < 0.0001$) as measured, daily, over the last week of the experiment (**Figure 1 A-B**). Co-treatment with Kaempferol didn't final body weights ($P = 0.9894$) or food intake ($P = 0.9965$) in the control rats as compared to control rats administered the vehicle (**Figure 1 A-B**). However, co-treatment with Kaempferol significantly increased the final body weights of CdCl₂-treated rats ($P < 0.0001$) but didn't affect their daily food intake ($P = 0.9884$) as compared to CdCl₂-treated rats (**Figure 1 A-B**). On the other hand, CdCl₂ caused thinning of the glandular tissue of rat hippocampi as compared to control rats, whereas co-treatment with Kaempferol increased the number of cell layers (**Figure 1C I-IV**).

3.2. Kaempferol improves Short and long –term memory in CdCl₂-treated rats

As shown in **Figures 2 and 3**, CdCl₂-treated rats showed impaired memory function during the training session, as well as during testing both short and long-term memory functions as compared to control rats. During the training session, the total and individual times to explore objects A1 and A2 were not significantly altered between control and control + Kaempferol-treated rats ($P=0.9891$; $P>0.9999$; $P=0.8779$, respectively). CdCl₂-treated rats showed decreased total time to explore the 2 familiar objects, A1 and A2, ($P<0.0001$) as compared to control rats (**Figure 2A**). Also, individual times to explore objects A1 and A2 were significantly reduced ($P<0.001$) in CdCl₂-treated rats ($p<0.001$) as compared to control rats (Figure 2B). However, total and individual times to explore objects A1 and A2 were significantly increased in CdCl₂ + Kaempferol treated rats as compared to CdCl₂-treated rats ($p<0.0001$) (**Figure 2A-B**). In this test, the individual and total times needed to explore objects A1 and A2 were not significantly different between control and control + Kaempferol-treated rats ($p<0.0001$) (**Figure 2A-B**). These data suggest that the basal memory function is reduced in CdCl₂-treated rats and is improved in CdCl₂ + Kaempferol-treated rats.

To test short-term memory, the test was repeated 3 h later, but object A2 was replaced with new novel object B and the same parameters were measured in addition to the calculation of the discrimination ratio (time to explore B/total time to explore A1 and B) (**Figure 2C-E**). There was no variation in total and individual times to explore objects A1 and B, as well as object B discrimination ratio between control and control + Kaempferol-treated rats ($P=0.7804$; $P=0.9867$; $P>0.9999$; and $P=0.9411$, respectively) (**Figure 2C-E**). On the other hand, the individual and total times needed to explore objects A1 and B, as well as, the discrimination ratio of object B were significantly reduced in CdCl₂-treated rats ($p<0.0001$, $P=0.0481$; $P<0.0001$; and $P=0.013$, respectively) as compared to their values detected in the control rats, thus suggesting an impairment in their short-term memory function (**Figure 2C-E**). Of note, there was no significant difference between the time needed to explore object A1

and B in the CdCl₂-treated rats (**Figure 2D**). On the other hand, the total and individual times to explore object A1 and B as well as the discrimination ratio of object B were significantly increased ($p < 0.0001$, $P = 0.0003$, $P < 0.0001$, and $P = 0.0132$, respectively) as compared to CdCl₂-treated rats (**Figure 2C-E**), thus suggesting an improvement in their short-term memory. Then, we have tested the long-term memory 24 hours by replacing object B with a new novel object, C. Similar findings to those found in the short memory tests were also seen in this test (**Figure 3 A-C**), thus indicating an impairment in the long-term memory function in CdCl₂-treated rats which is improved after Kaempferol co-treatment.

3.3. Kaempferol inhibits oxidative stress, inflammation, and apoptosis in the hippocampi of CdCl₂ treated rats and stimulates GSH, MnSOD, Bcl-2 levels in the hippocampi of control rats

Levels of ROS/RNS ($p = 0.032$) and MDA ($p = 0.0496$) were significantly reduced, but levels of GSH ($p < 0.0001$) and MnSOD ($p < 0.0001$), as well as protein levels of MnSOD ($p < 0.0001$) and Bcl-2 ($p < 0.001$) were significantly increased in the hippocampi of control + Kaempferol-treated rats as compared to control rats (**Figure 4 A-C**, **Figure 4F** and **Figure 5 A-B**). However, there were no significant variations in the levels of TNF- α ($P = 0.9965$), and IL-6 (0.9960) and in protein levels of cleaved caspase-3 ($P > 0.9999$) and Bax ($P > 0.9999$) between the control and control + Kaempferol-treated rats (**Figure 4D-E** and **Figure 5C**). On total free radicals (ROS/RNS) ($P < 0.0001$), MDA ($P < 0.0001$), TNF- α ($P < 0.0001$), and IL-6 ($P < 0.0001$) as well protein levels of cleaved caspase-3 ($P < 0.0001$) and Bax ($P < 0.0001$) were significantly increased, whereas levels of GSH ($P < 0.0001$) and MnSOD ($P < 0.0001$), as well as, protein levels of MnSOD and Bcl-2 ($P < 0.0001$) were significantly decreased in the hippocampi of CdCl₂-treated rats as compared to control rats and were reversed ($P < 0.0001$) in

the hippocampi of CdCl₂ + Kaempferol-treated rats as compared to CdCl₂-treated rats (**Figure 4 A-F and Figure 5 A-C**).

3.4. Kaempferol inhibits CdCl₂-induced upregulation of PARP1 in CdCl₂-treated rats and upregulates SIRT1 in the hippocampi of both control and CdCl₂-treated rats

The nuclear activity (P<0.0001) and protein levels of SIRT1 (p=0.0019) was significantly increased but the nuclear activity of NF-κB p65 (P=0.9890) and PARP1 (P>0.9999), as well as protein levels of PARP1 (P>0.9999) and acetylated PAPR1 (P=0.9996), remained unchanged in the hippocampi of both controls + Kaempferol as compared to control rats (**Figure 6A-C, Figure 7A-B**). Also, the nuclear protein levels of SIRT1 were not significantly different (P=0.9859) when detected with the precipitated PARP1 as compared to control rats (Figure 7C). However, the nuclear activity (P<0.0001) and protein levels of SIRT1 (P=0.0015) were significantly decreased, but the activities of PAPR1 (P<0.0001) and NF-κB p65 (P<0.0001), as well as the total and acetylated protein levels of PARP1 (P<0.0001) were significantly increased in the hippocampi of CdCl₂-treated rats as compared to control rats (Figure 6A-C and Figure 7A-B). Also, the detected nuclear protein levels of SIRT1 were significantly increased (P<0.0001) when detected after the precipitation of the nuclear PAPRP1 in the hippocampi of CdCl₂-treated rats as compared to control rats (Figure 7C). On the other hand, the hippocampi of the CdCl₂ + Kaempferol-treated rats showed a significant increase in the nuclear activity (P<0.0001) and protein levels of SIRT1 (P=0.0029) with a concomitant decrease in the nuclear activities of PAPR1 (P<0.0001) and NF-κB p65 (P<0.0001), as well as in total protein levels of PARP1 (P<0.0001) and acetylated PARP1 (P<0.0001) as compared to CdCl₂-treated rats (Figure 6A-C and Figure 7A-B). Besides, CdCl₂ + Kaempferol-treated hippocampi showed a significant increase in the nuclear protein levels of SIRT1 (P=0.0002) when detected after precipitation of PAPP1 as compared to their corresponding levels detected in the hippocampi of CdCl₂-treated rats (Figure 7C).

3.5. Kaempferol reduces total nuclear levels of P53 and NF-κB and nuclear acetylation of NF-κB, P53, and FOXO1 in CdCl₂-treated rats.

In control rats, Kaempferol didn't affect the total or acetylated nuclear hippocampal protein levels of NF-κB p65 (P=0.9998 and P>0.9999, respectively) and p53 (P>0.9999 and P=0.9589, respectively), as well as total nuclear protein levels of FOXO-1 (P>0.9999) as compared to their levels measured in the hippocampi of control rats (**Figure 8A-C**).

However, control + Kaempferol-treated hippocampi showed a significant decrease in the nuclear levels of acetylated FOXO-1 (P=0.0051) as compared to the hippocampi of the control rats (**Figure 8C**). With no alteration in total nuclear levels of FOXO1, nuclear total protein levels of total NF-κB p65 (P<0.0001) and P53 (P<0.0001), as well as acetylated levels of NF-κB p65 (P<0.0001), P53 (P<0.0001), and FOXO1 (P<0.0001) were significantly increased in the hippocampi of CdCl₂-treated rats as compared to control rats (**Figure 8A-C**). However, while Kaempferol does not affect total protein levels of all these markers in the hippocampi of control rats, it significantly lowered total and acetylated levels of NF-κB p65 (P<0.0001) and P53 (P<0.0001), as well as the nuclear levels of acetylated FOXO-1 (P<0.0001) in the hippocampi of CdCl₂-treated rats as compared to their corresponding levels detected in the hippocampi of CdCl₂ + vehicle-treated rats (**Figure 8A-C**).

4. Discussion

The novel finding of this study is that we are the first to show that the memory deficits and hippocampal oxidative stress, inflammation, and apoptosis induced by CdCl₂ in rats are associated with downregulation/inhibition of SIRT1 and upregulation/activation of PARP1. Besides, the findings of this study confirm that the neuroprotection afforded by Kaempferol in this animal model is mediated by reversing these events (i.e. increasing the nuclear levels and activity of SIRT1 and inhibition of PARP1).

At higher doses, Cd ions can lead to systemic toxicity and death (**Agnihotri et al., 2015; Celik et al., 2005; Piotrowski et al., 1974; Rafati Rahimzadeh et al., 2017**). For this reason, in this study, we have used a daily dose of CdCl₂ (0.5 mg) for 30 days, which has been previously shown to be below the LD₅₀ dose (**Agnihotri et al., 2015**). This dose was also used by others to specifically induce brain damage through an oxidative stress-related mechanism and activation of mitochondria-mediated (intrinsic) apoptosis (**Agnihotri et al., 2015**). This has been also shown in the hippocampi of rats in this study (as discussed below). In addition to these effects, we have also seen lethargy, loss of activity, and a significant reduction in the final body weights and food intake in CdCl₂-treated rats. This is expected given the well-reported negative impact of CdCl₂ on the hypothalamic neural circuit and loss of appetite (**Amara et al., 2008; Hwang et al., 2001; Nwokocho et al., 2012**).

Furthermore, we have concomitantly found severe damage in the livers and kidneys of CdCl₂-treated rats which could further explain the contribution of these toxicities in the reduction of rats' weights (data not shown). Of note, even this data in our hand has shown neuroprotection (shown later), as well as, hepatic and renal protection in CdCl₂ + Kaempferol-treated rats, Kaempferol didn't affect rats' final body weights nor food intake in both the control or CdCl₂-treated rats. These data suggest that the neuroprotection afforded by Kaempferol is not due to the improvement of food intake and body weights but rather to further cellular and molecular events. Indeed, Kaempferol afforded protection against brain, renal and hepatic damage in several animal models (**Yu et al., 2013; Zang et al., 2017**).

Recognition memory can be identified as the ability to judge a previously encountered item as a familiar object and depends mainly on the integrity of the medial temporal lobe (**Squire et al. 2007**). Tests that evaluate the object recognition memory have become increasingly valid useful tools for investigating the neural basis of memory function in both humans and animals (**Antunes et al., 2012; Broadbent et al., 2010; Winters et al., 2008**). Within this view,

NORT (known as the visual paired-comparison task in monkey's and humans) become the most valid test to study the recognition memory after hippocampal damage in rodent, monkeys, and humans (**Broadbent et al., 2010**). The major advantage of this test is that it relies on rat's proclivity for exploring novelty and doesn't require numerous training sessions nor any reward or reinforcement to motivate the rat's behaviour (**Lueptow, 2017**). Now, it has been evident that any damage that is limited to the hippocampus is sufficient to alter the recognition memory and NORT results in the above-mentioned species (**Broadbent et al., 2010; Squire et al., 2007**). By modulating the retention interval to short or long periods, both the short and long-term recognition memory can be studied (**Lueptow, 2017; Moore et al., 2013**).

In general, several lines of evidence have shown that CdCl₂ induces memory deficits in experimental animals, mainly by inducing oxidative stress, inflammation, and apoptosis in their hippocampi, reducing hippocampal ACH levels and impairing the cholinergic function (**Gumilar et al., 2019; Pulido et al., 2019; Shati et al., 2019**). Numerous studies have shown that CdCl₂-induced reduction in the intracellular GSH stores and over-production of ROS are the major triggers for all remaining adverse effects in the brain of intoxicated rats (**Chen et al., 2011; Shati and Alfaifi, 2019**). Similar to these findings, we have also shown in this study a reduction in both the short and long-term recognition memory in the CdCl₂-treated rats. Besides, the hippocampi of CdCl₂-treated rats showed higher levels of ROS, MDA, TNF- α , and IL-6, the activity of NF- κ B p65, and protein levels of Bax, and cleaved caspase-3. They also had lower levels of GSH, MnSOD, and Bcl2. These data confirm the emerging role of oxidative stress, inflammation and apoptosis in CdCl₂-hippocampal damage and memory loss. Further support came from the histopathological section of the hippocampi of the CdCl₂-treated rats where they showed a thinning of the granular cells and layer indicating active cell death.

Interestingly, Kaempferol significantly improved the short and long-term recognition memories, significantly ameliorated all markers of the oxidative stress, inflammation, and apoptosis, and improved the histological structures of the hippocampi in the CdCl₂-treated rats. Also, Kaempferol lowered the levels of ROS and MDA and increased the levels of MnSOD, GSH, and Bcl-2 in the hippocampi of both control rats CdCl₂-treated rats, thus suggesting a potent antioxidant effect of Kaempferol. These data are similar to many other previous studies which have shown that the neuroprotection afforded by Kaempferol in TBI and dementia animal models is mediated by ROS scavenging, increasing levels of GSH and MnSOD, and stimulating the expression of other anti-apoptotic genes (**Chitturi *et al.*, 2019b**; **Kouhestani *et al.*, 2018b**). Indeed, the antioxidant potential of Kaempferol is well-reported in numerous studies. In this regard, it was shown that Kaempferol could enhance the normal cell's antioxidant ability and increase the expression of several antioxidant enzymes and GSH by regulating the expression of heme oxygenase (HO) -1 expression (**Hong *et al.*, 2009**). Besides, the ROS scavenging ability of Kaempferol was attributed to its unique hydroxyl groups, especially the hydroxyl group on the C-3 position (**Liao *et al.*, 2016**).

However, the novel finding in this study is that we're showing that CdCl₂ administration in rats is also associated with a reduction in the hippocampal activity and nuclear levels of SIRT1, inhibition of SIRT1 signaling, and concomitant activation of PARP1. However, these pathways were reversed by co-administration of Kaempferol. Such findings were never reported in the literature before. Based on these findings, our data present a novel pathway for the neurotoxic effect of CdCl₂ and suggest a new mechanism of protection by Kaempferol.

Under stress conditions, the cell response by increasing the levels and the nuclear translocation of SIRT1. As a deacetylase, SIRT1 stimulates cell survival, inhibit cell inflammation and apoptosis, and increase mitochondria biogenesis by deacetylation of numerous substrates and transcription factors, including p53, NF-κB, FOXO-1 (**Chen *et al.*,**

2005; Chong et al., 2012; Donmez, 2012; Zhang et al., 2018). However, SIRT1 requires NAD^+ as a substrate. Within this view, it was shown that the deacetylation of p53 by SIRT1 inhibits apoptosis by downregulating Bax (**Maki et al., 1997; Yi et al., 2010**). In the same way, SIRT1 interacts and deacetylates FOXO1 in the nucleus, thus preventing the stimulatory effect of FOXO-1 on gene expression of apoptotic genes, Baim and FasL (**Chong et al., 2012**). This also stimulates the synthesis of the anti-apoptotic and antioxidant genes, including Bcl-2 and Bcl-XL and MnSOD (**Chong et al., 2012**). Furthermore, SIRT1-induced deacetylation of NF- κ B can inhibit cell inflammation by downregulating the production of the inflammatory cytokines (**Chong et al., 2012**). Indeed, SIRT1 can inhibit TNF- α induced NF- κ B activation and nuclear translocation of p65 (**Kauppinen et al., 2013**).

Therefore, the significant reduction in nuclear levels and activity of SIRT1 in the hippocampi of CdCl₂-treated rats explains the significant increase in the acetylated levels of FOXO1, NF- κ B, and p53. This also explains alternative resources for the origin of oxidative stress, inflammation, and apoptosis in the hippocampi of these rats. Therefore, although CdCl₂ neurotoxicity was reported mainly to the reduction in GSH levels (**Chen et al., 2011**), our data could also suggest that the reduction in antioxidant levels, as well as inflammation and apoptosis after chronic CdCl₂ administration in the hippocampi of rats, could be consequences for the reduction in SIRT1 levels. Hence, activation of SIRT1 seems to be a golden therapeutic approach to protect against CdCl₂-induced brain and hippocampal damage.

The major question remained here is by which mechanism does CdCl₂ inhibits the activity of SIRT1. This could be explained by the concomitant significant increase in the levels and activity of PAPR1 in the hippocampi of CdCl₂-treated rats. PARP1, an NAD^+ -dependent enzyme, is one of the major DNA repair enzymes in most of the neural and non-neural cells and is usually found in the nuclei and mitochondria (**Ling et al., 2016**). Currently, it is well-established that PAPP1 can inhibit SIRT1 activity by depleting intracellular NAD^+ levels

(Rajamohan et al., 2009). Besides, PARP1 can induce cell inflammation through activating NF- κ B p65 by interacting with p300 to provide additional interaction sites for p50 on p300 **(Hassa et al., 2002).** However, PARP1 activity can be inhibited by deacetylation through SIRT1 **(Rajamohan et al., 2009).** Also, SIRT1 can inhibit the transcription of PARP1 directly **(Rajamohan et al., 2009).**

In this study, another interesting unique observation is that we also found a significant increase in the nuclear levels, acetylation, and activity of PARP1 in the hippocampi of CdCl₂-treated rats. Also, the interaction between PARP1 and SIRT1 was significantly increased in the hippocampi of these rats, thus confirming the close relationship between PARP1 and SIRT1. Hence, it could be possible that the oxidative induction of PARP1 by CdCl₂ triggered the reduction in SIRT1 through decreasing NAD⁺ levels. Besides, such an increase in PARP1 activity may participate significantly in the observed inflammatory response in the hippocampi of CdCl₂-treated rats by increasing the activation of NF- κ B. This is supported by the findings that have shown that Cd ions don't only induces ROS but also leads to DNA-protein crosslinks, chromosomal aberrations, DNA strand breaks, and sister chromatid exchange **(Rafati Rahimzadeh et al., 2017).** However, the opposite that the decrease in SIRT1 triggered the activation of PARP1 could be also correct.

On the other hand, Kaempferol significantly increases the nuclear levels and activity of SIRT1, inhibited the nuclear levels, acetylation, and activity of PARP1, and significantly decreased the acetylation of FOXO-1, p53, and NF- κ B. Interestingly, Kaempferol also increased the nuclear activity and levels of SIRT1 and concomitantly increased the deacetylation of FOXO1 and increased levels of MnSOD and GSH in the hippocampi of the control rats. Based on these data, it seems that Kaempferol inhibits CdCl₂-induced activation of PARP1 through the activation of SIRT1. Also, it could be possible that Kaempferol inhibits

the activation of PARP1 due to its ROS scavenging and antioxidant abilities. In both cases, and based on the data in control rats, it seems that the neuroprotection afforded by Kaempferol is mediated by its antioxidant and anti-inflammatory potentials which could be explained by its antioxidant and ROS scavenging abilities as well as its ability to activate SIRT1 levels and signaling.

However, despite these findings, this study still has some limitations. Most importantly, we need further molecular studies using transgenic animals or gene silencing to show the exact effect of Kaempferol on SIRT1 and PARP1. Besides, some natural factors such as rodent strains, as well as, experimental conditions such as temperature, odor, noise, and light may affect the exploration and retention intervals of the test (**Lueptow, 2017**). In this study, we have tried the best to minimize the effect of the stress and other external factors on the output of NORT. Besides, all neurological behavioral tests were conducted by a person who is unaware of the experimental condition. Therefore, the effect of these factors on behavioral analysis can't be neglected. Furthermore, in addition to the hippocampus, other studies have shown that the perirhinal cortex also plays a significant role in the recognition memory in rodents (**Aggleton et al., 2010**). This has been not tested in this study. Therefore, more studies on the effect of CdCl₂ on Kaempferol on this brain area is highly required to explain its memory protective effect.

5. Conclusion

The data of the current study are very interesting and show for the first time the important roles of SIRT1 and PARP1 in the pathogenesis of neurotoxicity and memory loss induced by CdCl₂. Also, it represents a new mechanism for the protective effect of Kaempferol against CdCl₂-induced neurotoxicity. Accordingly, the data presented here show that Kaempferol neuroprotective effects are mainly due to its antioxidant, anti-inflammatory, and anti-apoptotic effects and involves activation of SIRT1 and inhibition of PARP1. These data

encourage further translational studies in humans with neurodegenerative disorders. Besides, further studies using PARP1 and SIRT1 deficient mice are required to clarify the crosstalk between PARP1 and SIRT1 in the hippocampi of the CdCl₂-treated rats with or without Kaempferol therapy.

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Compliance with Ethical Standards:

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Conflict of Interest: The authors declare that they have no conflict of interest.

Authors' contribution:

All authors contributed equally to this work.

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Figure captions:

Figure 1: Changes in final body weight (A), daily food intake (over the last week, B), and histological findings in dentate gyrus (DG) area of the hippocampal of all groups of rats. In C: I&II were taken from control and control + Kaempferol treated rats and show the normal thickness of the glandular cells; III was taken from CdCl₂-treated rats and show severe thinning of the glandular layer with external and internal edema. Values are presented as mean \pm SD of n=12 rats/group. ***: significantly at P<0.001.

Figure 2: Assessment of Memory function test by the novel object recognition test in all groups of rats. Subfigures A&B: total and individual times needed to explore two familiar objects, object A and B during the training phase. **C-D:** total and individual times needed to explore one familiar object (A) and one novel object during the short memory tests (3h after the training session). **E:** Calculated discrimination ratio of object B during the short memory testing and defined as the time needed to explore object B over the total time needed to explore objects A and B. Values are presented as mean \pm SD of n=12 rats/group. *, **, ***: significantly at P<0.001.

Figure 3: Assessment of Memory function test by the novel object recognition test in all groups of rats. Subfigures A&B: total and individual times needed to explore two familiar objects, object A1 and C during the long-term memory testing. **C:** Calculated discrimination ratio of object C during the short memory testing and defined as the time needed to explore object B over the total time needed to explore objects A and C. Values are presented as mean \pm SD of n=12 rats/group. ***: significantly at P<0.001.

Figure 4: Total levels of reactive oxygen and nitrogen species (ROS/RNS), malondialdehyde (MDA, B) reduced glutathione (GSH, C); tumor necrosis factor α (TNF- α , D), interleukin-6 (IL-6, E) and manganese-dependent superoxide dismutase

(MnSOD, F) in the hippocampi of all groups of rats. Values are presented as mean \pm SD of n=6 rats/group. *, **, ***: significantly at P < 0.05, P < 0.1, and P < 0.001, respectively.

Figure 5: Total protein levels of manganese-dependent superoxide dismutase (MnSOD, A), Bcl-2, cleaved caspase-3 (C), and Bax (D) in the hippocampi of all groups of rats. Values are presented as mean \pm SD of n=12 rats/group. ***: significantly at P < 0.001.

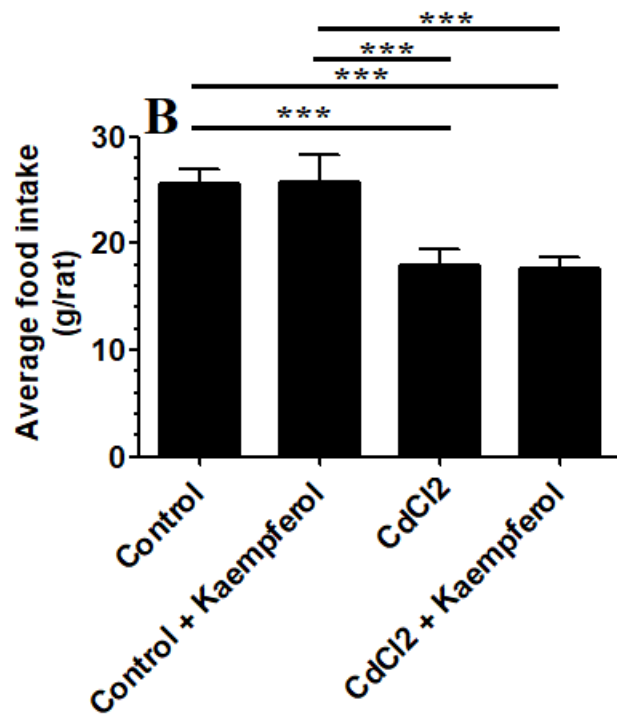
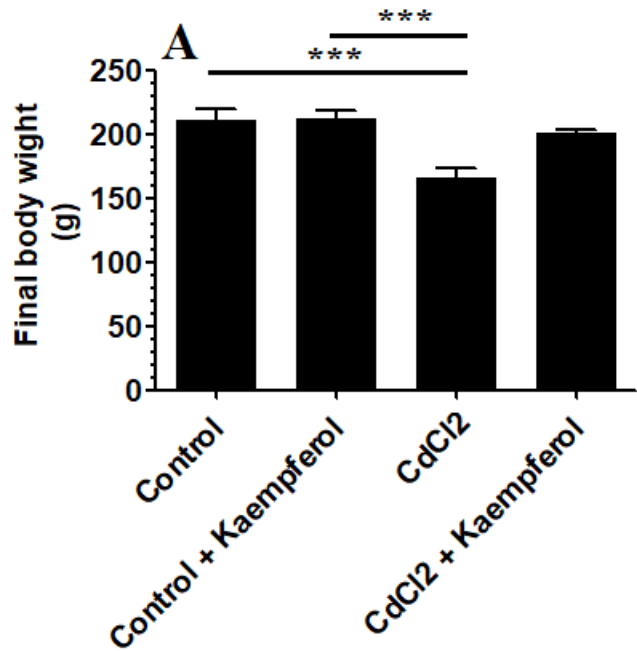
Figure 6: Activity of SIRT1, PAPP1, and NF- κ B in the hippocampi of all groups of rats. Values are presented as mean \pm SD of n=6 rats/group. **, ***: significantly at P < 0.1 and P < 0.001, respectively.

Figure 7: Total protein levels of PARP1 /Acetylated PARP1 (A), SIRT1 (B&C) in the hippocampi of all groups of rats. In C: nuclear PARP1 was first immuno-precipitated, then blotted with an antibody against SIRT1. Values are presented as mean \pm SD of n=6 rats/group. ***: significantly at P < 0.001.

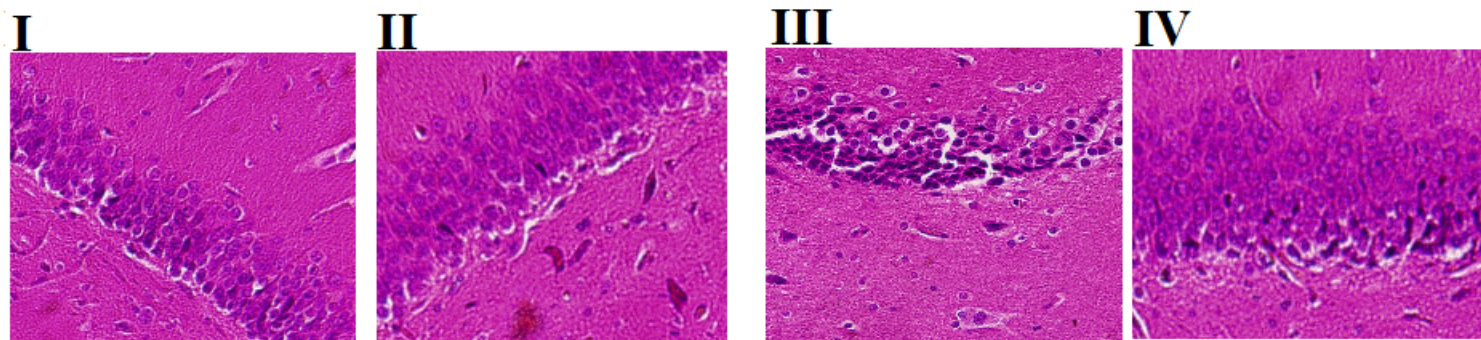
Figure 8: Nuclear Protein levels NF- κ B p65/acetylated p65 (A), P53/acetylated P53 (B) and FOXO1/Acetylated FOXO1 in the hippocampi of all groups of rats. Values are presented as mean \pm SD of n=6 rats/group. ***: significantly at P < 0.001.

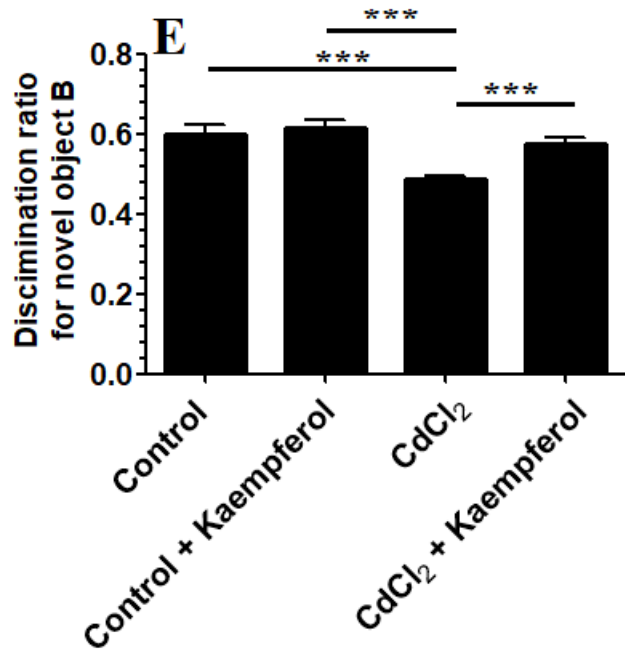
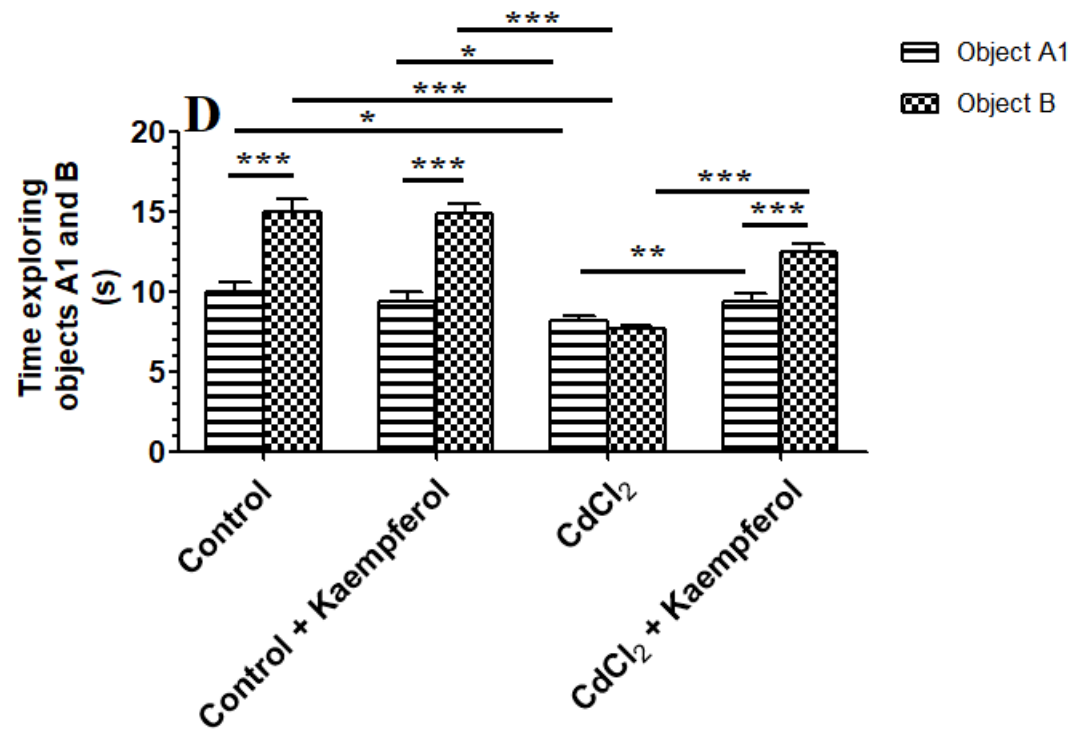
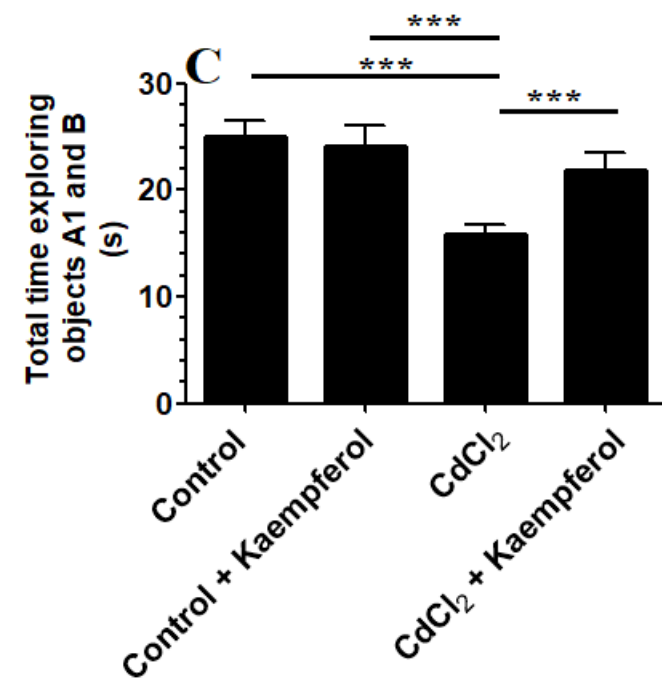
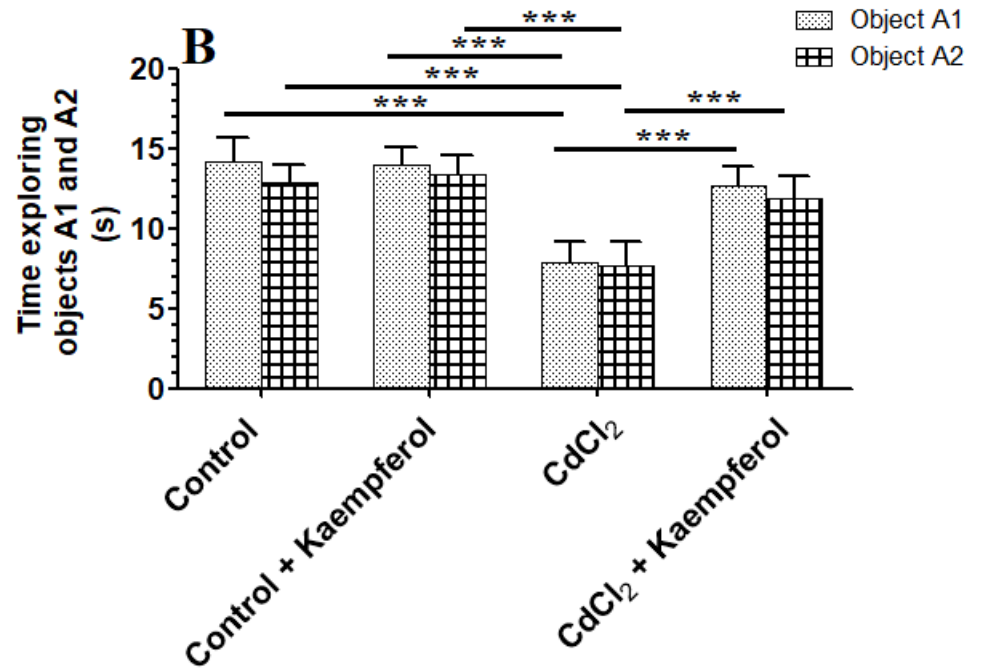
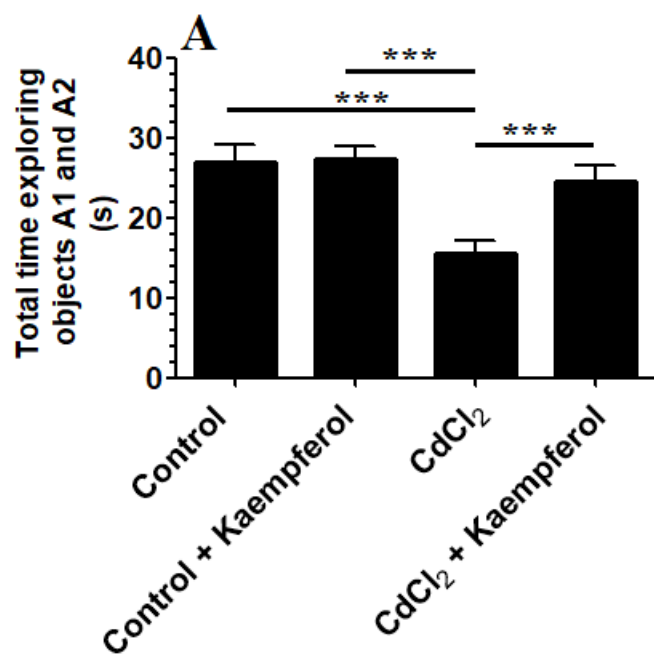
Graphical abstract: A graphical Abstract that illustrates the possible mechanisms by which cadmium chloride (CdCl₂) induces reactive oxygen species (ROS) and the neuroprotective mechanisms afforded by Kaempferol. CdCl₂ induces ROS and DNA breaks which leads to an increase the levels of P53 and PAPR1 and the activity of PARP1. Overactivation of PARP1 consumes NAD⁺ levels which is a common substrate of SIRT1. This leads to downregulation and inhibition of SIRT1. As a result, inhibition of SIRT1 increases the acetylation of P53, FOXO-1, and NF-KB. Activation of FOXO-1 decreases the synthesis of GSH, BCl-2, and other antioxidant enzymes whereas activation of P53 and the lower levels of Bcl-2 stimulate

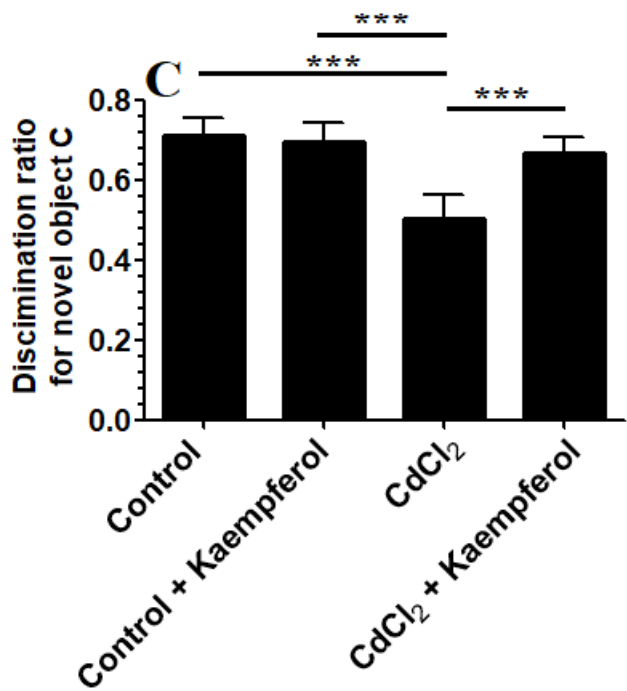
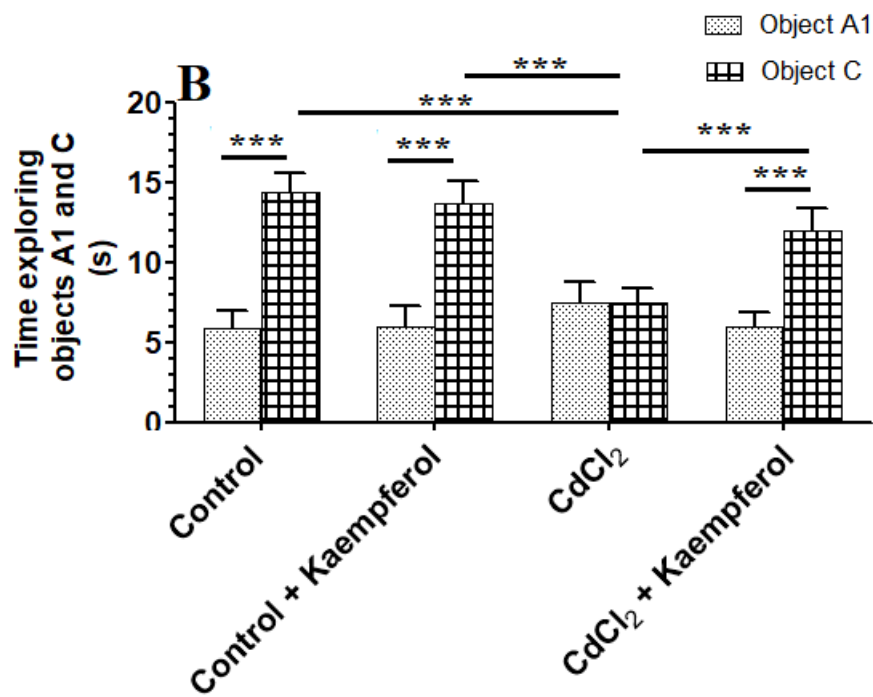
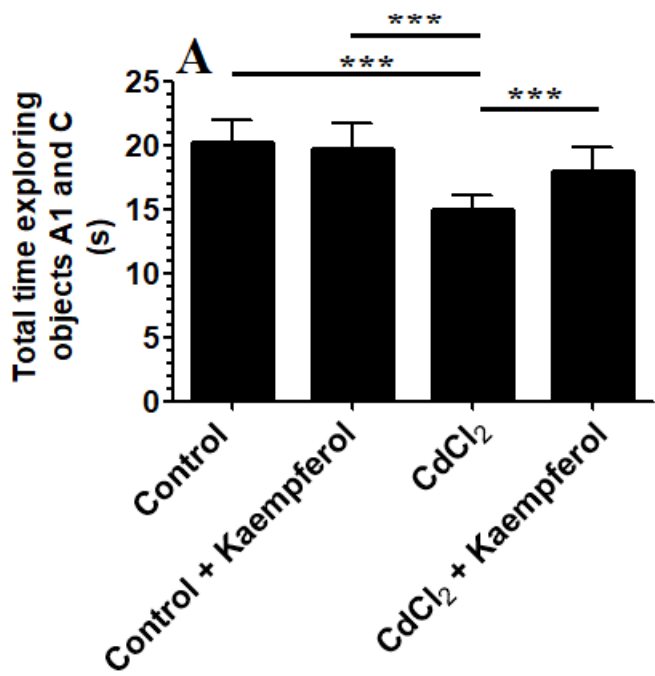
the level of Bax which translocates to the mitochondria to initiate cytochrome-c release and caspase-dependent apoptosis. Besides, the acetylation of P53 stimulates its transcriptional activity to increase the synthesis of the inflammatory markers including tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6). These, in turn, increases the production of the ROS more. Kaempferol inhibits the activation of NLRP1 and/or upregulates and activates SIRT1, which negatively affect each other, thus inhibiting all these events. Also, Kaempferol may increase levels of GSH and MnSOD independent of SIRT1.

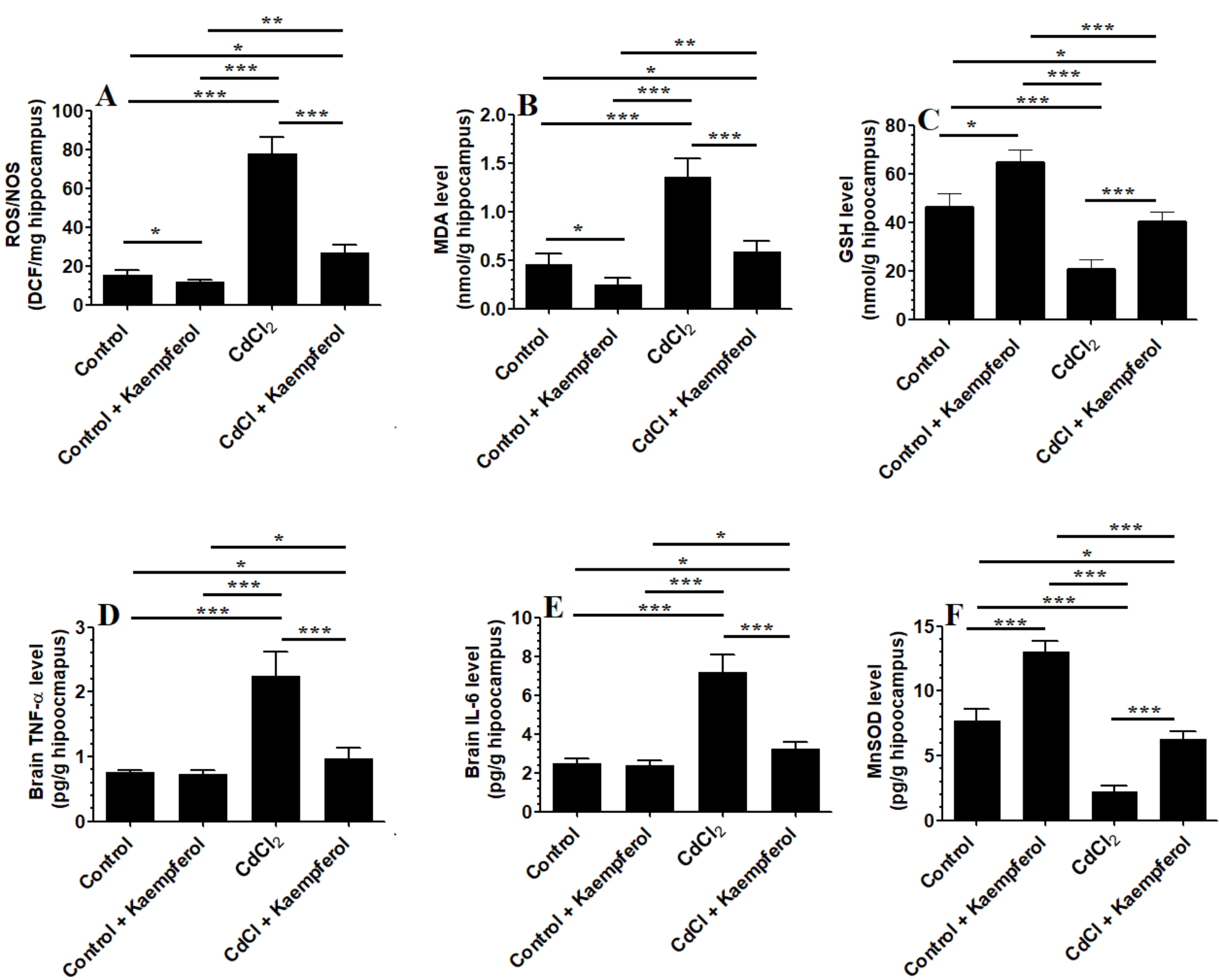


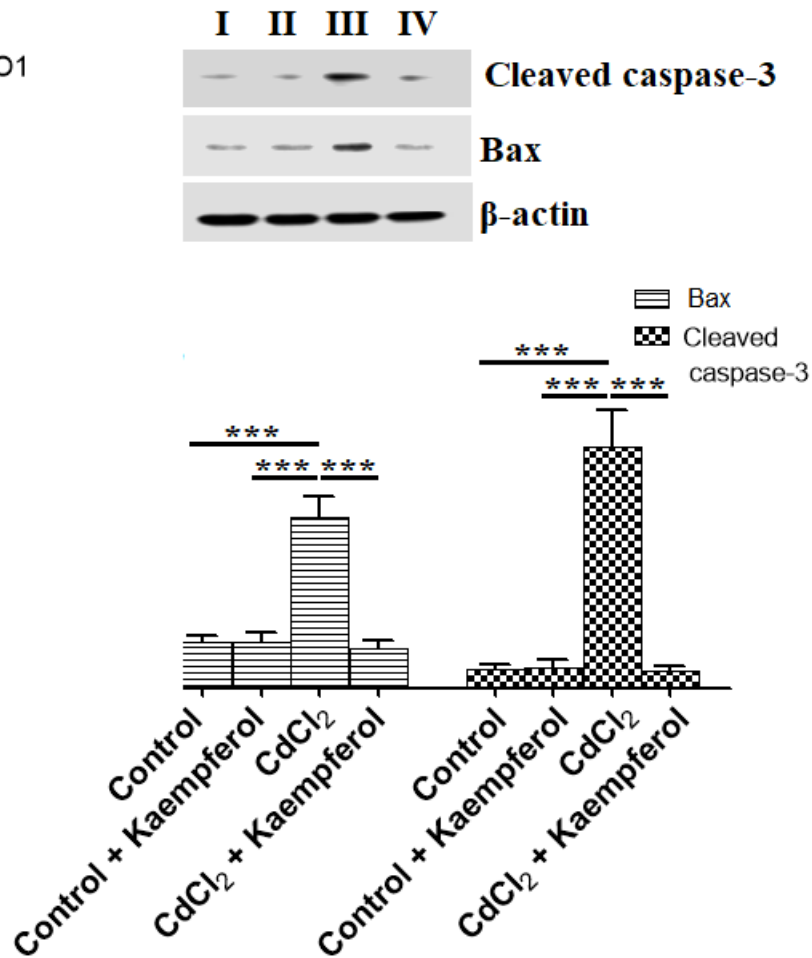
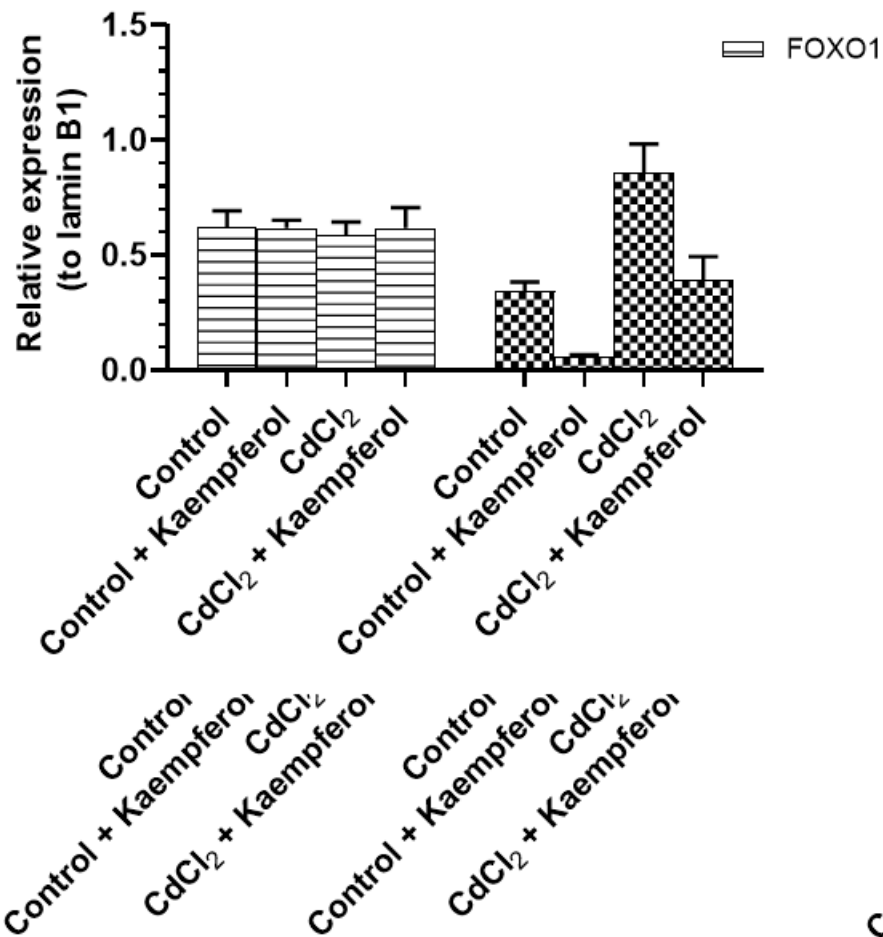
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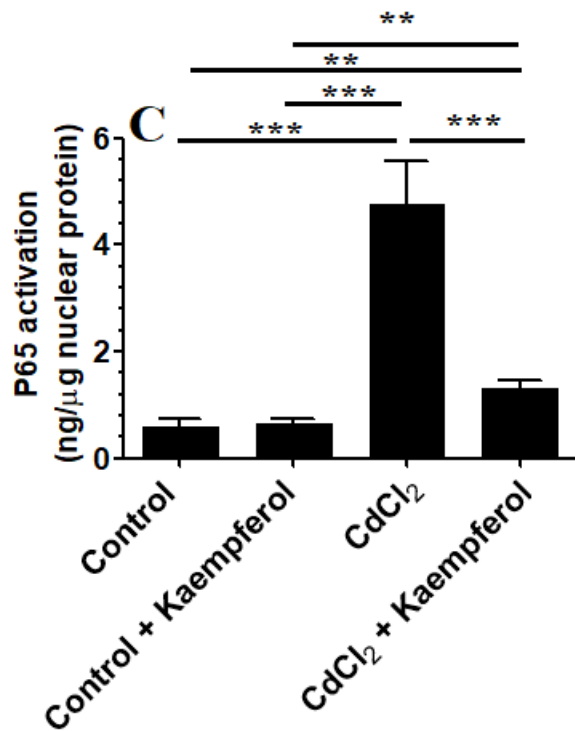
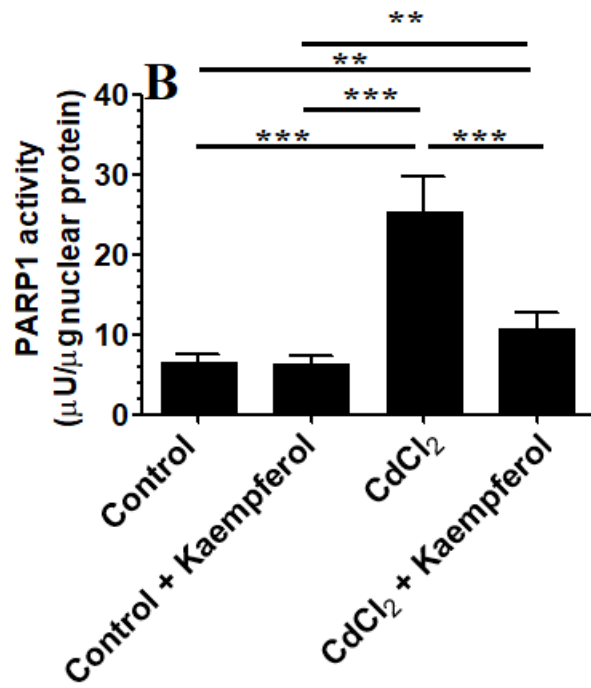
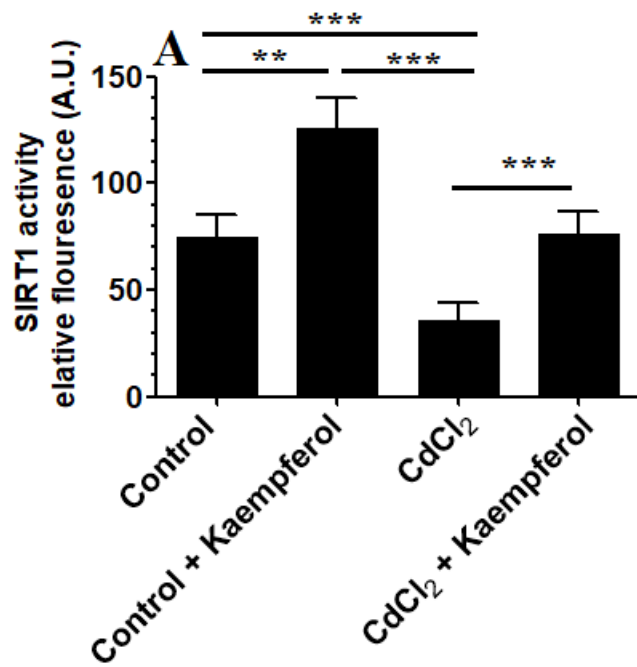


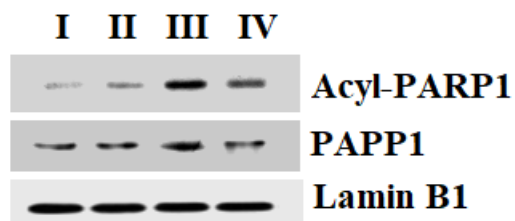




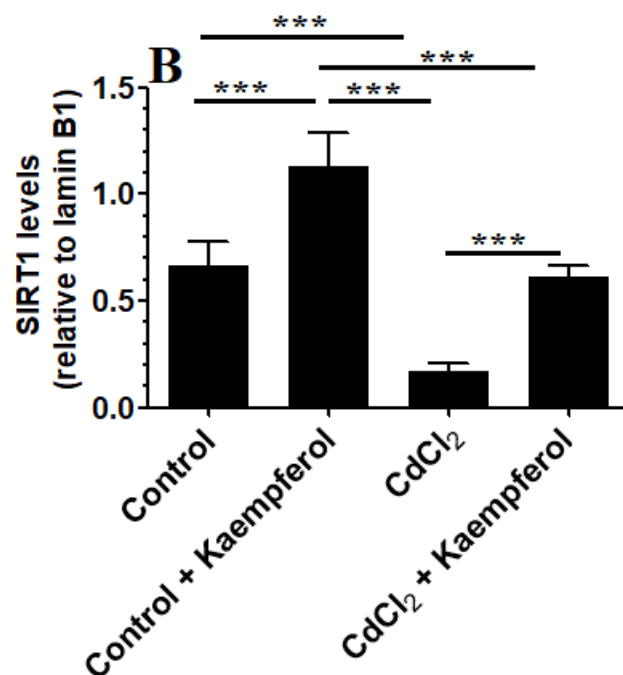
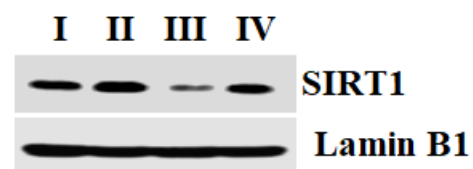
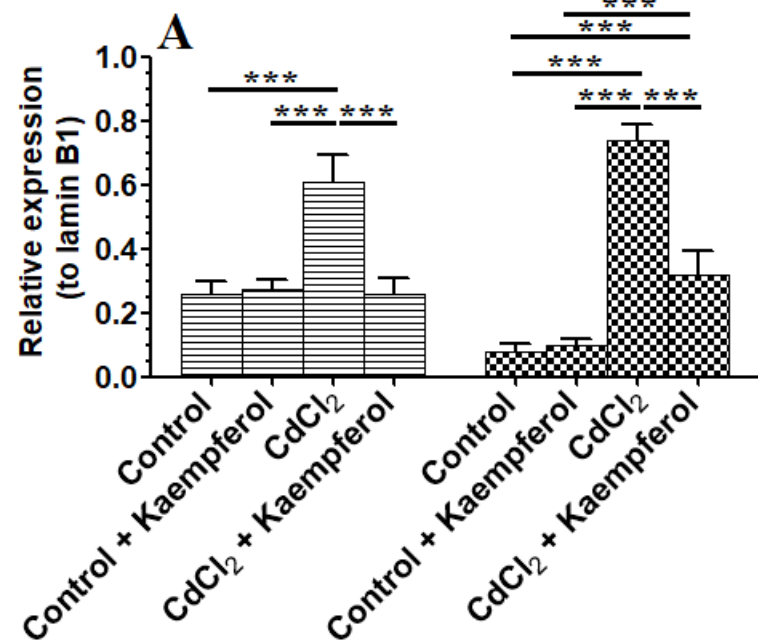




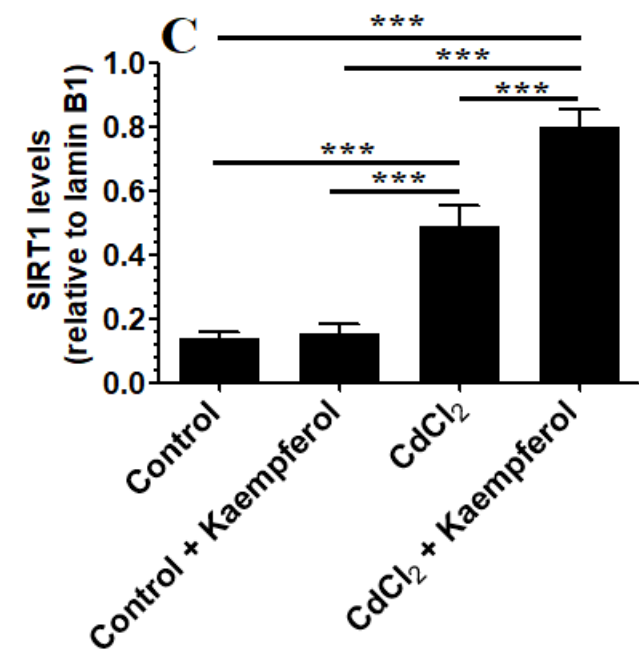
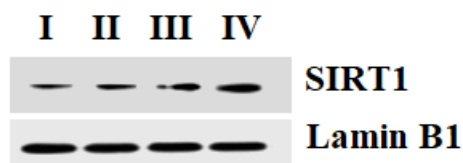




▨ PAPR1
 ▩ acetylated PARP1



IP: PARP1



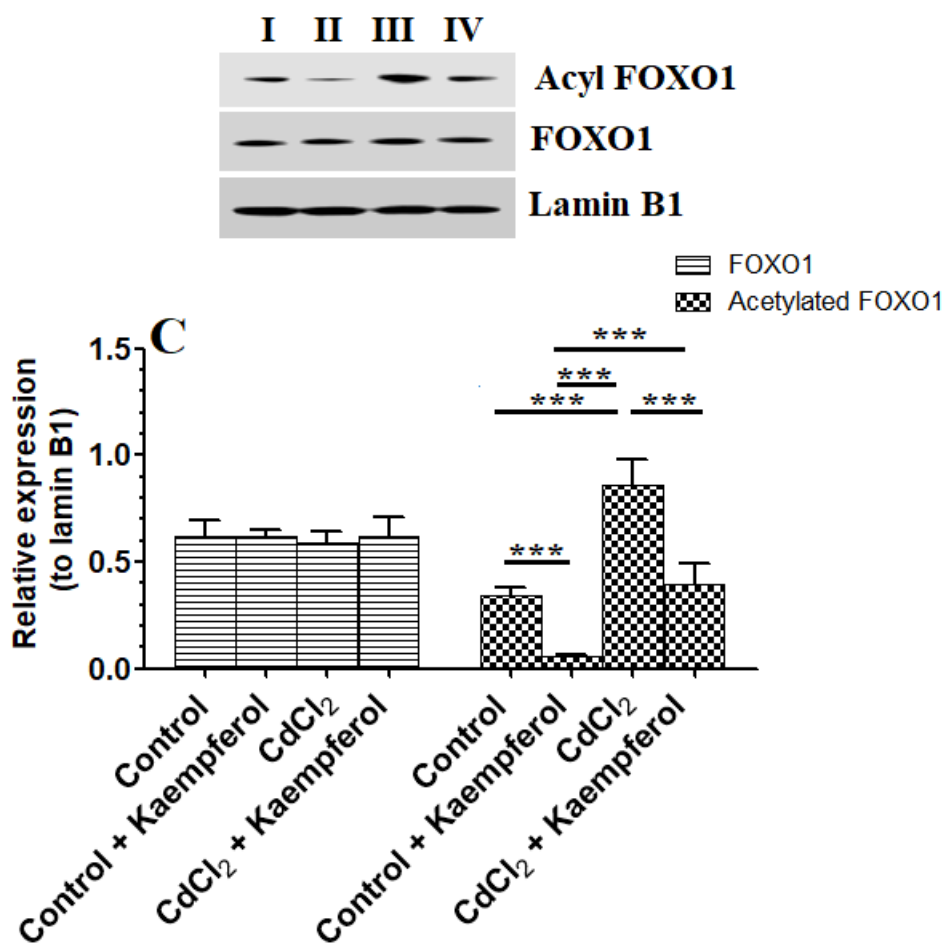
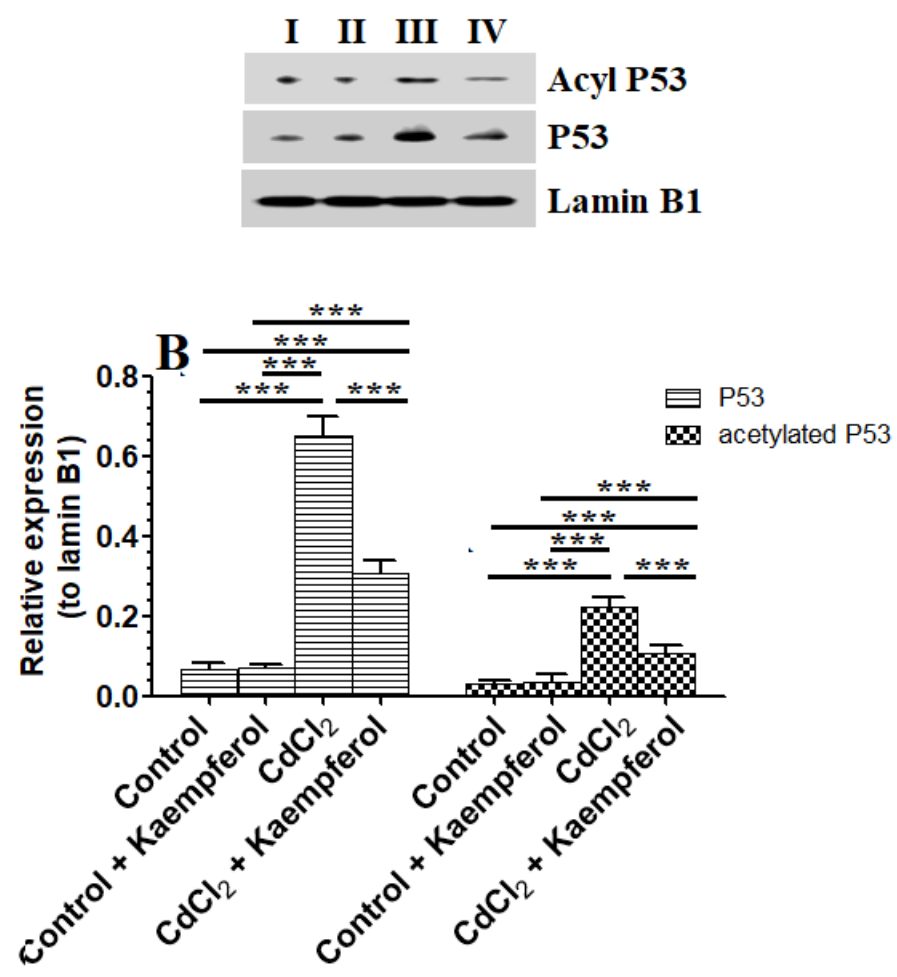
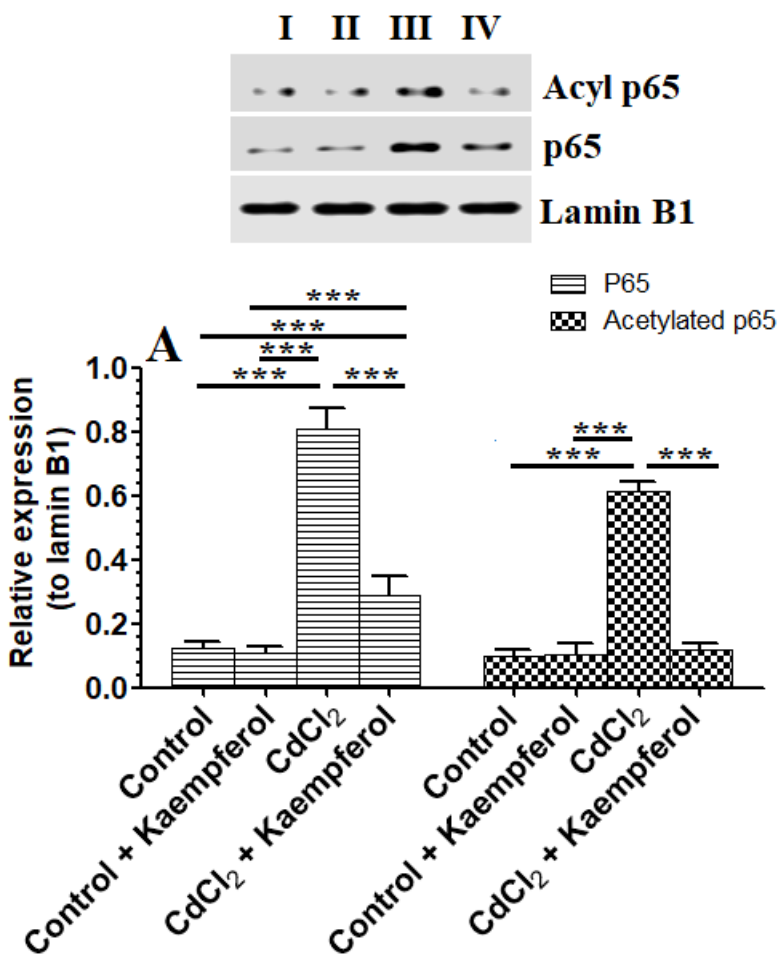


Table 1: Antibodies used in this study

Antibody	Manufacturer	Cat. No.
SIRT1	Cell Signalling Technology	Cat. No. 74465
NF-κB p65	Cell Signalling Technology	Cat. No. 3034
PARP-1	Cell Signalling Technology	Cat. No. 9452
FOXO-1	Cell Signalling Technology	Cat. No. 2880
P53	Cell Signalling Technology	Cat. No. 9282
Bax	Cell Signalling Technology	Cat. No. 2772
Bcl-2	Santa Cruz Biotechnology	Cat. No. sc,7382
Cleaved caspase-3	Cell Signalling Technology	Cat. No. 9661
β-actin	Santa Cruz Biotechnology	Cat. No. sc-47778
Lamin B1	Cell Signalling Technology	Cat. No. 12856

