



Research Article

Neuroprotective Efficacy of Cadambine Against Cadmium in Primary Brain Neonatal Rat Cells

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Abstract

Background and Objective: Cadmium is a heavy metal that accumulates with time in different tissues of the body like central nervous system, kidney, liver etc. causing toxic effects especially to the brain and has a very long biological half-life. The objective of this study was to explore the neuroprotective effect of cadambine an indole alkaloid against cadmium-induced toxicity.

Materials and Methods: Three cell lines namely N2A, SH-SY5Y and primary brain neonatal rat cells were used in this study. Cell viability and neuroprotection assays were assessed by MTT assay to determine the effect of cadmium and cadambine. Changes in the intracellular calcium levels, ROS and mitochondrial membrane potential were determined by flow cytometer. Western blotting was done to analyse the effects of cadmium and cadambine on pro-apoptotic and anti-apoptotic genes. Student's t-test was used to compare the data.

Results: In this study, it was confirmed that cadmium induced toxicity was reversed by cadambine. Cadambine increased the viability of cells treated with of cadmium induced toxicity. Furthermore, cadmium treatment led to generation of ROS, increased calcium levels, loss of MMP and increase in the expression of pro-apoptotic proteins. Interestingly, these effects was reversed by cadambine in primary brain neonatal rat cells. **Conclusion:** These findings led us to the conclusion that exposure to cadmium induces neurotoxic effects in brain and cadambine not only reversed the toxic effects of cadmium but also led to inhibition of cadmium-induced apoptosis. Based on this data, cadambine can further be investigated as potent neuroprotective agent.

Key words: Cadambine, neuroprotection, primary brain neonatal cells, cadmium, apoptosis, neurodegeneration

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Neurodegenerative diseases like Parkinson's, Alzheimer's, Huntington's etc. are characterised by progressive degeneration of neuron cells¹. Socio-economic effects of neurodegenerative diseases are devastating and are responsible for causes of disability and death. The pathogenesis of neurodegenerative diseases is complex and comprises both genetic and environmental factors, thus, they are multifactorial. Several biochemical and pathological mechanisms such as protein aggregation, oxidative stress, mitochondrial dysfunction and inflammation in brain are responsible for neurodegeneration^{2,3}. With recent major advances in understanding the neurobiology of neurodegenerative diseases, it paves the way for researchers to identify compounds capable that may have potential to inhibit or reduce the progress of deterioration in neural cells. The compounds isolated from natural sources have complex structure and in turn have diverse activities and multiple targets⁴. These natural products exert various modifying effects on signal transduction pathways owing to their antioxidant potential^{4,5}. Polyphenols are potent therapeutic agents in neurodegenerative diseases, they exert their effects via epigenetic modifications and chromatin remodelling⁶. These diverse biological and pharmacological activities make them strong candidate to combat against neurodegenerative diseases⁷⁻⁹. Some studies have shown that consumption of flavonoids decreases the rate of dementia¹⁰. In ongoing research on screening of natural compounds against neuroprotection, it was found that compound cadambine having promising neuroprotective activity. Cadambine an indole alkaloid derived from the tree *Neolamarckia cadamba*. *Neolamarckia cadamba* belongs Rubiaceae family, is found in South Asia and South China and is widely distributed due to its high economic value¹¹. *N. cadamba* Roxb. also known as Kadam tree has wide spectrum bioactivities such as antipyretic, analgesic, hypolipidemic, antidiabetic and anti-inflammatory¹². Cadambine has been reported to exhibit moderate growth inhibitory activity against *Plasmodium falciparum* (malarial parasite) cultured in human erythrocytes¹³. Cadmium (Cd) is heavy metal commonly found in industrial workplaces and is extremely toxic in nature, It is also a food contaminant and a major constituent of cigarette smoke. Cd is very toxic and can severely harm several organs, including the brain. There has been a report wherein neurotoxic effect of cadmium has been mentioned in neonatal mouse brain and young rat brain¹⁴. The focus of the present study therefore has attributed to explore the

molecular mechanisms associated with protective role of cadambine induced by cadmium in *in vitro* primary brain neonatal rat cells.

MATERIALS AND METHODS

This study was performed between August, 2016 to June, 2017 at two places:

- Cell viability and all flow cytometer assays was performed in Department of Paediatrics, Puai Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
- All Western blotting experiments were performed in Department of Biochemistry and Molecular Biology, School of Medicine, Wuhan University of Science and Technology, 430065 Wuhan, China

Reagents: All trans-retinoic acid, Phosphate buffered saline, Bradford, cadmium, Dulbecco's minimal essential medium, BSA, RIPA, MTT, DMEM, Sodium pyruvate, Streptomycin sulphate, Penicillin G sodium salt were purchased from Sigma (China, Shanghai, Huangpu, Huaihai Middle Rd, 398). H2DCFDA, Fetal bovine serum (FBS), Fluo-3AM were purchased from Invitrogen (Hongqiao Rd, XuJiaHui, Xuhui Qu, Shanghai Shi, China, 200000). PVDF membrane and Immobilon Western Chemiluminescent HRP substrate were purchased from Merck Millipore (Merck Chemicals (Shanghai) Co., Ltd). All antibodies were obtained from Santa Cruz (Santa Cruz Biotechnology, Inc. 10410 Finnell Street Dallas, Texas 75220 U.S.A). All the chemicals/reagents used in this study were of analytical grade.

Cell culture: Neuro2a mouse neuroblastoma and SH-SY5Y cells were purchased from ATCC. These cells were cultured in DMEM media that was supplemented with 10% FBS, sodium pyruvate 1 mM, 2 mM L-glutamine, penicillin 100 units mL⁻¹, streptomycin 100 µg mL⁻¹ and was grown in an incubator at 37°C with 5% CO₂ and humidity 95%. Cells were treated with retinoic acid at a concentration of 10 µM with 2% FCS for differentiation in N2a cells and 10% FCS for differentiation of SH-SY5Y cells to induce and media were changed for every 2 days. Neuritis outgrowth was observed via Olympus phase contrast microscope.

Isolation and culture of primary brain neonatal rat cells:

To carry out the primary mixed culture (neuronal/glia), 1-5 days old pups of C57 mouse strain were decapitated

and whole brain isolation was done in ice-cold L-15 media. The media contained penicillin 70 mg L⁻¹ and streptomycin (100 mg L⁻¹). The digestion of the brain tissue was done with papain (2 mg mL⁻¹) in L-15 media for 30 min at 37°C. The mixture was tapped after every 5 min. Digested brain tissue was pulverised. It was done by passing it through serological pipette already coated with serum. Centrifugation at 2500 g for 5 min at 4°C was done. Then the pellet was washed for three times. L-15 media was used to wash the pellet and then passed through 40 µm cell strainer. Cells that were obtained through above procedure were cultured for 21 days in DMEM. The media constituents were 10% foetal bovine serum (FBS), penicillin G (70 mg L⁻¹), streptomycin (100 mg L⁻¹). The cells were grown in a flask coated with poly-L-lysine (2 mg mL⁻¹). The culture media was changed after every 2 days during this period.

Neuroprotection and cell proliferation assay: Primary brain neonatal rat cells, differentiated SH-SY5Y and differentiated N2a cells were treated with cadambine and cadmium for 24 h. Cell proliferation and neuroprotection was assessed by MTT assay. Briefly, cells were seeded in 96 well plates for 24 h in an incubator at 37°C with 5% CO₂ and humidity 95%. For cell proliferation assay, cells were treated with different concentration of cadambine and co-treatment of cadmium (10 µM) and cadambine (20 µM) for neuroprotection assay for 24 h. After 24 h of treatment, MTT (2.5 mg mL⁻¹) was added in each well and the plate was incubated at 37°C for 4 h. Media was decanted and 150 µL optical density of DMSO were added into each well. Absorbance of each well was taken at 570 nm Synergy Mx multimode microplate reader (BioTek) USA.

Measurement of Intracellular calcium by flow cytometer: Fluo-3 AM fluorescent dye was used for this study to measure accumulation of Ca²⁺ ions. Primary brain neonatal rat cells were seeded for 24 h in 6 well plate and were co-treated with cadambine (20 µM) and cadmium (10 µM) for 24 h. Before 30 min of termination, Fluo-3 AM at a concentration of 5 µM were added into each well at 37°C. Cells were washed three times with PBS washing to remove unbound dye. These cells were collected and acquired through flow cytometer BD FACSCalibur BD Biosciences, San Jose, California to measure fluorescence emitted by the Fluo-3 AM when bound to Ca²⁺ ions.

Determination of ROS by flow cytometer: 2, 7 dichlorofluorescein diacetate (H2DCFDA) was used to detect reactive oxygen species (ROS). Briefly, primary brain neonatal

rat cells were grown in 6 well plate for 24 h. After 24 h, cells were treated with cadmium (10 µM) in presence and absence of cadambine (20 µM) for 24 h. Before 30 min of termination, 10 µM H2DCFDA added to the cells, incubated at 37°C. After 30 min of incubation with H2DCFDA, cells were washed three times with PBS resuspended and acquired through flow cytometer BD FACSCalibur BD Biosciences, San Jose, California.

Analysis of mitochondrial membrane potential by flow cytometer: JC-1 dye was used to measure the mitochondrial membrane potential in primary brain neonatal rat cells. Briefly, primary brain neonatal rat cells were grown in 6 well plate for 24 h and then co-treated with cadambine and cadmium for 24 h. JC-1 dye at a concentration of 5 µM added in each well for 30 min. After 30 min, cells washed and resuspended in 400 µL of PBS. The samples was analysed by flow cytometer BD FACSCalibur BD Biosciences, San Jose, California and 20,000 events captured for analysis.

Western blot: Ice cold RIPA buffer was used to lyse the treated cells (Tris-HCl 20 mM, pH 7.4, NaCl 150 mM, EDTA 5 mM, PMSF 1mM, Na₃VO₄ 1mM with 1% protease inhibitor cocktail). Protein estimation was done by Bradford method. Protein at a concentration of 60 µg were loaded to SDS-PAGE and transferred to PVDF membrane for 2 h at 100V. Skimmed milk was used to block the protein membrane for 1 h at room temperature, followed by overnight incubation of primary antibodies at 4°C. The membrane was washed three times with TBST (NaCl 150 mM, pH 8.0, Tris 50 mM, Tween-200.05%, KCl 2.6 mM) for 5 min. The protein membrane incubated with HRP conjugated secondary antibody for 1 h at room temperature, the membrane was washed again with TBST 5 min each for 15 min. Membrane bound antibodies was detected with ECL chemiluminescence kit from Amersham Biosciences according to the manufacturer's instructions and protein bands were analysed by X-ray film.

Statistical analysis: Values of three independent experiments are shown as Mean ± SD of three independent experiments. Students' t-test was performed for comparison of each experiment. Statistically significant figure was p < 0.05.

RESULTS

Cadambine protects cell death induced by cadmium in primary brain neonatal rat and neuro2a cells: To assess neuroprotective effect of cadambine by MTT assay on primary brain neonatal rat, neuro2a and SH-SY5Y cells. Cadambine at

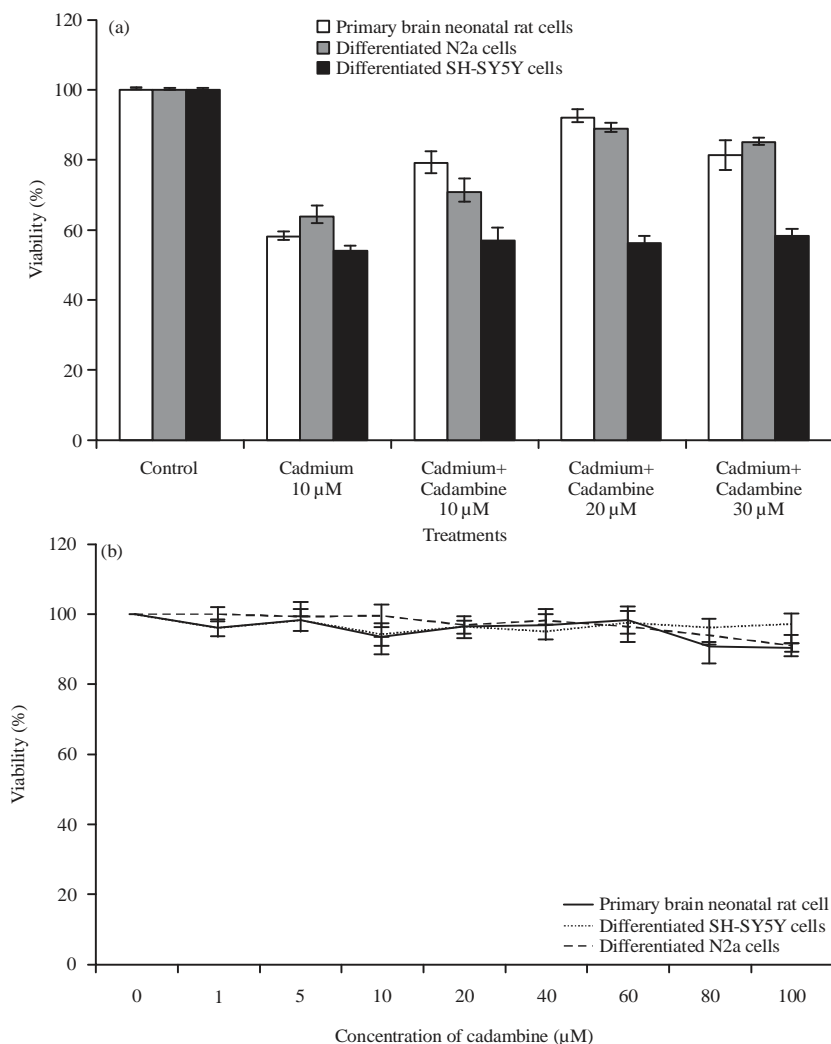


Fig. 1(a-b): Neuroprotection assessment by MTT assay, (a) Cadambine reversed toxicity induced by cadmium (10 μM) on different cells lines except SH-SY5Y cell and (b) Cadambine was non-toxic to cells upto 100 μM in different cell lines
Data represented is the mean of three different experiments performed independently

10, 20 and 30 μM was co-treated with cadmium (10 μM) for 24 h. Surprisingly the viability of cells were increased in primary brain neonatal rat cells and differentiated neuro2a cells when compared with cadmium. There was not any significant change in cell viability on SH-SY5Y when co-treated with cadambine and cadmium (Fig.1a). It was seen that beyond 30 μM concentration of cadambine, no substantial change was observed on cell viability (Data not shown). Further, it was also shown that cadambine was non-toxic at different concentrations for 48 h (Fig. 1b). Therefore, these results revealed that cadambine protects primary brain neonatal rat and neuro2a cells against cadmium toxicity.

Cadambine reduces intracellular calcium level increased by cadmium exposure: Cadmium is known to increase the

intracellular levels of calcium. Although based on neuroprotection assay, we hypothesise that cadambine can reduces intracellular calcium level in primary brain neonatal rat. Cells were co-treated with cadambine (20 μM) and cadmium (10 μM) for 24 h and evaluated on flow cytometer which clearly reveals decreases in intracellular calcium level by co-treated (cadambine (20 μM) and cadmium (10 μM)) cells compared with cadmium alone (Fig.2). Therefore these results displayed that relative fluorescence intensity was increased by cadmium alone which was attenuated by co-treated cells (Fig. 2).

Cadambine decreases reactive oxygen species levels by exposure of cadmium: Several toxic effects produced by cadmium to the primary brain neonatal cells, in which reactive

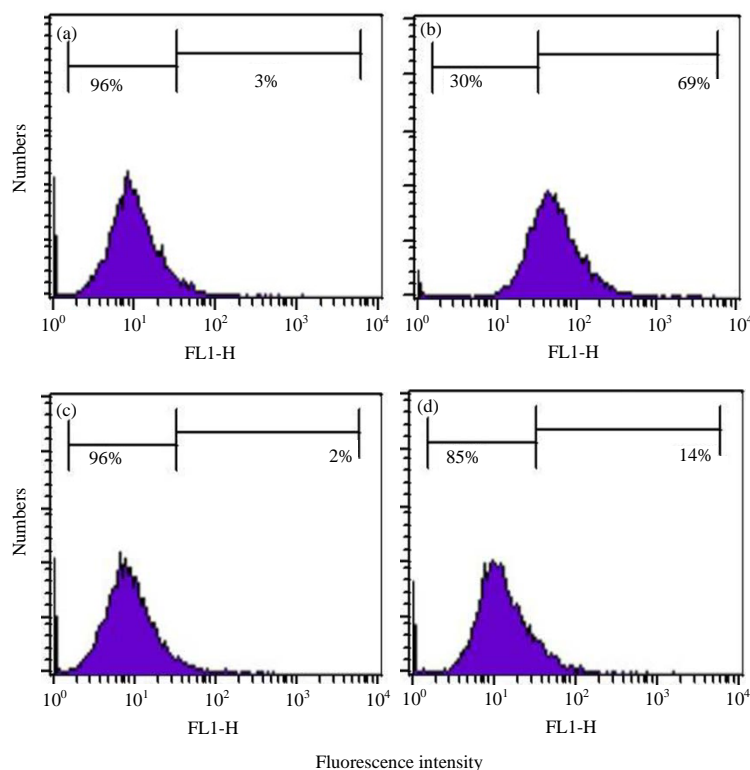


Fig. 2(a-d): Elevated intracellular calcium level inhibited by cadambine, (a) UC, (b) Cadmium (10 μ M), (c) Cadmium (20 μ M) and (d) Cadmium (10 μ M)+Cadmium (20 μ M)

Fluo-4 AM dye (5 μ M) was used to stain the cells, All the experiments were performed three times independently

oxygen species is one of the harmful toxicant, which is related to toxicity of mitochondria. Cells were treated with cadmium (10 μ M) were able to generate ROS, which was significantly attenuated by co-treatment of cells with cadambine (20 μ M) and cadmium (10 μ M) which was evaluated by flow cytometer (Fig. 3). These results clearly depict that the relative fluorescence intensity was sharply decreased in co-treated cells as compared with cadmium alone.

Increase in mitochondrial membrane potential by cadambine after cadmium exposure:

Mitochondrion known as powerhouse of the cell has a predominant function in apoptosis progression and loss of mitochondrial membrane potential is an early event that might lead to apoptosis. In this study we found that cadambine reverses the effect of cadmium, a known toxicant that induces loss of mitochondrial membrane potential. To analyse mitochondrial membrane potential, cells were treated with cadmium (10 μ M) alone and in combination with cadambine (20 μ M) using JC-1 dye, a dye that is used to detect the loss of mitochondrial membrane potential. Quenching of fluorescence observed in samples treated with cadmium alone as compared with control and

the same effect was reversed in cells co-treated with cadambine and cadmium analysed by flow cytometer. These results clearly support the fact that loss of mitochondrial function due to cadmium exposure can be reversed by cadambine (Fig. 4).

Cadambine reverses cadmium induced apoptosis: Cadmium, a heavy metal is known to induce apoptosis in neuronal cells. Expression level of p53 was significantly increased in cadmium (10 μ M) treated cells. However, co-treatment with cadambine (20 μ M) significantly reduced the expression level as observed by Western blots (Fig. 5). In addition to that, the expression level of Bcl-2 (an anti-apoptotic protein), Bid was decreased and Bax (Pro-apoptotic protein) was significantly increased in cadmium treated cells. It was also revealed that co-treatment of cadambine (20 μ M) with cadmium (10 μ M) reversed the above effect wherein there was an increase in the expression level of anti-apoptotic proteins Bcl2 and Bid and reduction in the expression of pro-apoptotic protein Bax. Cadmium (10 μ M) alone lead to PARP cleavage whereas the same phenomenon was reversed when the cells were co-treated with cadambine (Fig. 5).

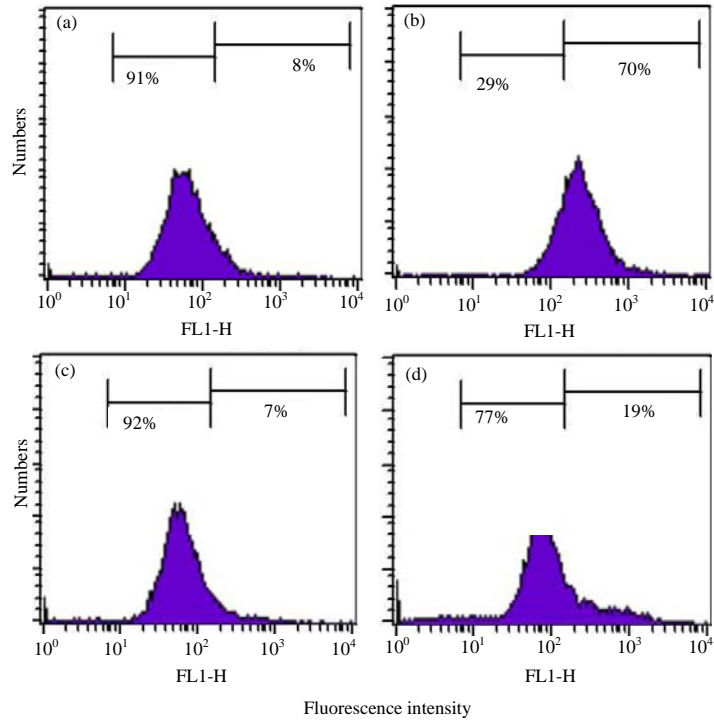


Fig. 3(a-d): Cadambine attenuates the ROS generation by cadmium, (a) UC, (b) Cadmium (10 μM), (c) Cadmium (20 μM) and (d) Cadmium (10 μM)+Cadambine (20 μM)

Data shown above is the mean of three independent experiments

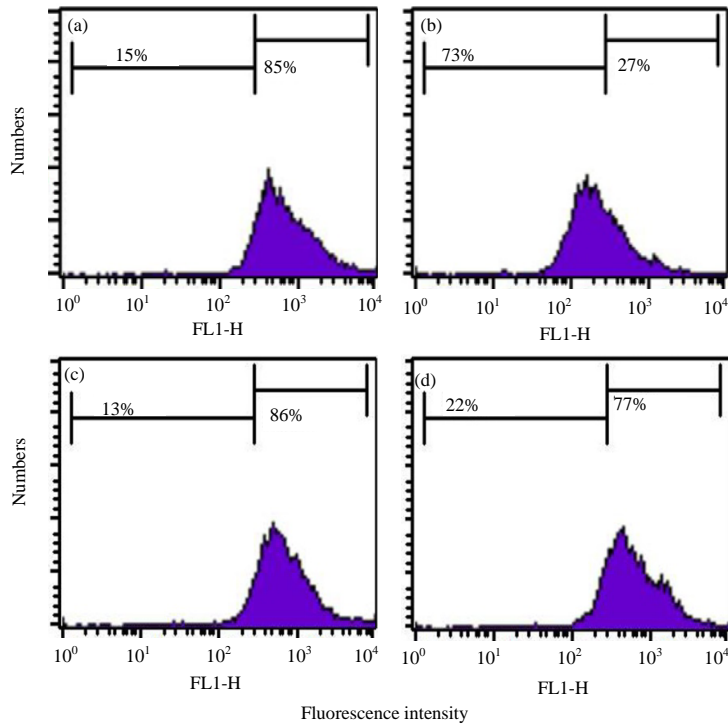


Fig. 4(a-d): Effect of cadambine on mitochondrial membrane potential, (a) UC, (b) Cadmium (10 μM), (c) Cadmium (20 μM) and (d) Cadmium (10 μM)+Cadambine (20 μM)

Data shown above is the mean of three independent experiments

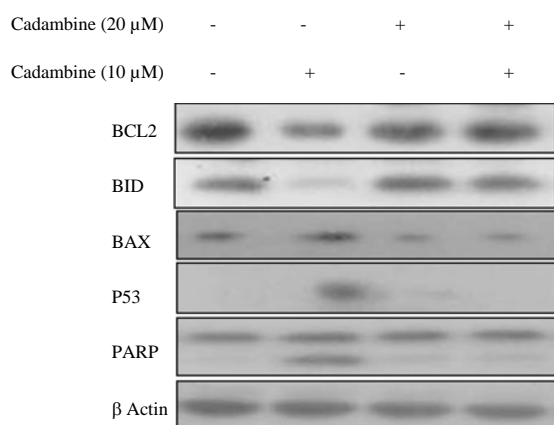


Fig. 5: Cadambine reverses cadmium induced Apoptosis

Western blot analysis of Anti apoptotic (BCL2 and BID) has significantly reduced in cadmium treated cells which was reversed by cadambine when co-treated with cadmium. Expression level of apoptotic proteins (BAX and P53) were significant increased by cadmium, which has been decreased by cadambine co-treated with cadmium. PARP cleavage was observed in Cadmium treated cells, which was reversed with co-treated cells (Cadmium and cadambine)

DISCUSSION

In neurobiology, cadmium plays a crucial role and various clinical explorations have finally led to the fact that cadmium intoxication is one of the major etiological factors responsible for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease¹⁵. In Asia and Europe, many people have exceeded exposure levels towards cadmium and for the large groups the margin is extremely narrow¹⁶. However, the main question to be answered is the impact of long term exposure towards cadmium even at low doses especially to people who are more vulnerable in a population like children, foetuses, elderly people, pregnant women etc¹⁷. Cadmium exposure is reported to induce changes in a cell biochemically and in nervous system functionally, particularly during exposure for a long period¹⁸. In this study, we also found that cadmium exposure induces toxicity in brain neonatal cells and therefore lead to decrease in cell viability¹⁹. Cadmium like other metals affects mitochondrial integrity and therefore leads to decrease in the mitochondrial membrane potential, which is an early event of apoptosis²⁰. There are also reports that bioenergetics of mitochondria, which is known to be important for production of energy, is altered in cadmium exposure that leads to generation of ROS which was observed in results and this can be one of the main reasons of cadmium induced neurotoxicity²¹. Cadmium in nature is pro-oxidant and was

seen to decrease the expression of anti-apoptotic protein Bcl2 whereas increase in the expression of pro-apoptotic protein Bax²².

Cadambine belongs to a class of polyphenols that are known to act as potent therapeutic agents in neurodegenerative diseases wherein they exert their effects via epigenetic modifications and chromatin remodelling²³. These polyphenols are known to have diverse biological and pharmacological activities that makes them strong candidate to combat against neurodegenerative diseases²⁴. Cadambine was tested against the cadmium-induced neurotoxicity in neuronal cells. Overall, these results proved that cadambine not only reversed the toxicity induced by cadmium but also led to decrease in the intracellular calcium levels, which was increased by exposing the cells to cadmium. Cadmium induced generation of reactive oxygen species, were also quenched by co-treating the cells with cadambine. Besides having anti-oxidant properties, polyphenols are known to prevent apoptosis by altering/modulating the expression of various apoptotic proteins like Bax, Bcl2 etc²⁵. In this study, it was seen that cadambine co-treatment not only restored the mitochondrial membrane potential that gets lost due to exposure to cadmium but also reverses the apoptosis induced by cadmium. It was observed that cadambine efficiently decreased the expression of Bax, a pro-apoptotic protein whereas it increased the expression of anti-apoptotic proteins like Bcl2. These results led us to the conclusion that cadambine inhibits apoptosis by altering the expression of Bax/Bcl2 proteins and by decreasing the expression of caspase3.

CONCLUSION

It was concluded that cadmium induces neurotoxic effects in brain by increasing the intracellular calcium levels, by generating ROS, decreasing the mitochondrial membrane potential and by increasing the expression of pro-apoptotic proteins. However, cadambine not only reversed the toxic effects of cadmium but also led to inhibition of cadmium-induced apoptosis by modulating the expression of Bax/Bcl2 and caspase3. Therefore, these results lead us to conclusion that if cadambine can be taken in the form of food or feed by humans can reverse the cadmium induced toxicity in neuronal cells.

SIGNIFICANCE STATEMENT

This study discovers the neuroprotective effects of cadambine that can be beneficial for people who are exposed

to heavy toxic metals on regular basis especially cadmium. We report a novel mechanism of cadambine that involves neuroprotection by decreasing ROS and calcium level and significantly increasing MMP level in primary brain neonatal rat cells. Thus the enhanced effect of MMP as well as reduction effect of calcium and ROS level decreased programmed cell death in response to cadmium treatment. Therefore, these results clearly demonstrate a neuroprotective role for cadambine against cadmium in primary brain neonatal rat cells.

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