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Relative Neuroprotective Effects of COX Inhibitors and Curcumin against Cholesterol Challenged Neurotoxicity in PC12 Cells

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ABSTRACT

This recent study was aimed to assess the relative neuroprotective effect of NSAID's and curcumin against cholesterol mediated proinflammatory release induced oxidative stress studied in *in-vitro*. PC12 cell lines were treated with cholesterol (10 & 50 µg/ml) alone or along with ibuprofen (5µg/ml), rofecoxib (6µg/ml) and curcumin (5&10µg/ml). Cells were studied for morphology, viability and proinflammatory mediators such as interleukin1β and TNF-α as markers for the assessment of neurotoxicity. Neurotoxicity effect on PC12 cell lines had shown by cholesterol treatment (50µg/ml) as evidenced by a significant decrease in cell viability, decrease in neurite growth, with increased release of interleukin (IL-1β) and TNF-α. However, the intervention of neuroprotective effect had shown by treatment with ibuprofen (5µg/ml), rofecoxib (6µg/ml) and curcumin (10µg/ml). Results of our study show that the non-selective COX inhibitor ibuprofen and curcumin have relative protective effects on PC 12 cell line from cholesterol induced neurotoxicity. Furthermore, selective COX-2 inhibitor rofecoxib had a less influencing on attenuating cholesterol induced pro-inflammatory mediator release and neurotoxicity as compared with non-selective COX inhibitor.

Keywords: Cholesterol; Neuroinflammation; Neurotoxicity; NSAID's; Curcumin; Proinflammatory mediators; Interleukin 1β; TNF-α

Introduction

The most regions of the neuronal cells in the central nervous system (CNS) are not capable to repair and regenerate when their axons or cell body are damaged. Overview of neuroinflammation considered as a one of the causative factor for a neurodegenerative condition such as Alzheimer's and Parkinsonism disorder. Neuroinflammation is most often disadvantageous, and damage neurons in the CNS and liked with the neurodegeneration[1]. Some reports have been confirmed the neurotoxic effect of hypercholesterolemia is associated with neuroinflammation and mimic Alzheimer's Diseases (AD) in the CNS[2, 3]. The onset of a neurodegenerative disorder such as Alzheimer's disease has been delayed or prevented by the use of non-steroidal anti-inflammatory drugs[4]. The long-term use of NSAIDs may attenuate the onset of AD progress caused by the release of pro-inflammatory mediators from activated microglia through the early stage of Aβ deposition. Advanced stage of Aβ deposition in turn release of pro-inflammatory mediators from the chronic activated microglial cells not effectively reversed by NSAIDs[5]. Certainly, epidemiologic studies have also reported that the anti-inflammatory drugs users less likely affected for risk of developing AD[6]. The plentiful huge potential investigations using herbal or natural products have been made to observe their anti-inflammatory effects to improve neuroinflammation related neurodegeneration in AD[7]. Alzheimer's transgenic mouse model of amyloid pathology induced neuroinflammation attenuated by various doses of curcumin has been reported[8]. Furthermore, multiple molecular mechanisms of actions such as β-amyloid plaques clearance, inhibit neuronal degradation, metal-chelation, anti-inflammatory, antioxidant and delay microglial activation possess by curcumin may improve the memory in patient with AD recently[9]. Neuroprotective effect of curcumin with non-selective and selective COX inhibitors against cholesterol mediated neuroinflammation in *in-vitro* has been investigated in this study.

Methods

Drugs and chemicals

Curcumin was obtained from Sabinsa Corporation, USA. Ibuprofen and rofecoxib were purchased from

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Abbott Pharmaceuticals and Shasun Pharmaceuticals, India, respectively. Carboxymethylcellulose, Cholesterol, Sodium pyrophosphate, n-butanol, Ammonium sulphate, Bovine serum albumin, Periodic acid and Arsenic oxide were purchased from Hi-Media. Glucose diagnostic kit was purchased from Merck.

Cell Culture

PC12 cell lines were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's Medium containing 10% of fetal bovine serum, 5% of horse serum, 1% of glutamine and 1% of Penicillin. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Cells (1×10^5 cells/ml) were incubated with various concentrations of cholesterol (25&50µg/ml) alone or co incubated with various concentrations of curcumin (5&10 µg/ml), ibuprofen (5µg/ml) and rofecoxib (6µg/ml) for 24 hrs. Morphological assessment was done by observing the neuronal cells under inverted tissue culture microscope (Olympus IX 70, Japan).

Assessment of cell viability

Viability of cells was determined by the MTT assay. Exponentially growing cells (1×10^5 cells/ml) were plated in 96-well plates and treated with either DMSO as a control group or two different concentrations of cholesterol (10 & 50 µg/ml) as a positive control. The reaction results in the reduction of MTT by mitochondrial dehydrogenases of viable cells to form a purple coloured formazan product. The formazan product was dissolved in DMSO and the amount was estimated by measuring absorbance at 570 nm in an ELISA plate reader (Bio-Rad).

Nitrate assay

Nitrites concentrations in the cell supernatants were determined as free nitrites by using Greiss reagent[10]. 100 µl of cell supernatant were mixed with equal volume of Griess reagent, and the optical density was determined at 540nm in an ELISA reader.

Lactate Dehydrogenase (LDH)

Cell supernatants were assayed for LDH using a LDH kit (Ecoline). 20µl supernatant was mixed with 1ml of 0.1M Tris buffer pH 8.9 containing 50 mM lactate and measuring the absorbance change at 360 nm every minute for 3 min as a measure of NADH consumption[11]. The results are expressed as U/L of LDH activity.

Estimation of Cholesterol

Determination of cholesterol was used by cholesterol estimations kits (Ecoline). The supernatant was removed and 1ml of reagent was added. The colour intensity was measured in a UV-VIS Spectrophotometer at 540 nm[12].

Cytokines Determination

The cellular debris free cell culture supernatants were assayed in triplicate for IL1β and TNF-α with specific Quantikine ELISA kits according to the manufacturer's instruction, using micro plate reader[13].

Statistical analysis

Data are expressed as Mean ± SEM and are analysed using Graph Pad Prism version 5.0, USA. One way ANOVA followed by Bonferroni's Multiple Comparison Test. The level of significance $P<0.05$ was considered as significant.

Results

MTT Dye Assay

A significant neuronal loss observed in cell cultures treated with cholesterol (50 µg/ml). This result reflects that cholesterol may have influences on mitochondrial respiratory dehydrogenases enzymes and PC12 cell proliferation. Curcumin (10 µg/ml) and ibuprofen had protective ($p <$ 001) effect. Whereas, curcumin (5µg/ml) and rofecoxib also significantly protected the cells challenged with cholesterol (Figure 1).

LDH Assay

Cells treated with cholesterol led to a dose-dependent increase in cell death (Figure 2). This was evident by higher LDH levels. The neuronal cells losses were protected significantly ($p < 001$) with curcumin (10 µg/ml) treatment as compared with the ibuprofen and rofecoxib.

Nitrite Assay

The release of nitrites was found to be significant ($p<$ *0.001*) with cholesterol 50µg/ml as compared to normal cell culture. Co-incubation of the neuronal cells with curcumin 10 µg/ml, ibuprofen and rofecoxib attenuated the cholesterol induced effects on nitrite levels (Figure 3).

Cytokines

Cholesterol (50µg/ml) significantly ($p< 0.001$) increased the release of IL-1β and TNF-α. This high cholesterol incubation induced IL-1β and TNF-α release significantly ($p<$ *0.001*) attenuated by co-incubation of curcumin 10 µg/ml and ibuprofen (Figure 4&5).

Cholesterol levels in cell supernatants

Co-incubation of cultures with curcumin 5µg & 10µg/ml dose dependently decrease the cholesterol levels (Figure 6). There was a significant ($p < 0.001$) decreased in cholesterol levels in the supernatant in cultures treated with ibuprofen and rofecoxib.

Discussions

In this recent finding, we established the relative protective effect of selective and non-selective COX inhibitor and curcumin against the neuroinflammation induced by cholesterol. The results of the present *in-vitro* study emphasize the release of IL-β1 and TNF-α and nitrosative stress mediates the inflammatory effect of cholesterol on PC12 cell lines. This is our established results that the cytokines and nitrite release occur in PC12 cell lines significantly when cholesterol incubated for 48 hrs[14]. Increased in free cholesterol level in the cell supernatants

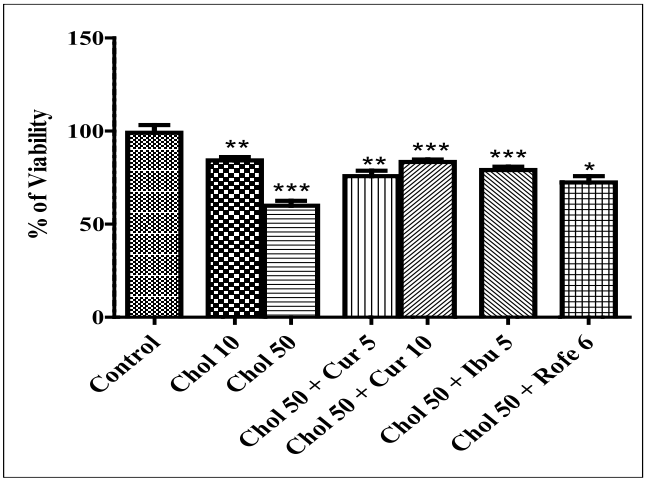

Fig.1: Effects of curcumin and NSAIDs on MTT assay in cholesterol incubated PC12 cells. Values are expressed as mean ±S.E. *** p<0.001 treatments vs control.

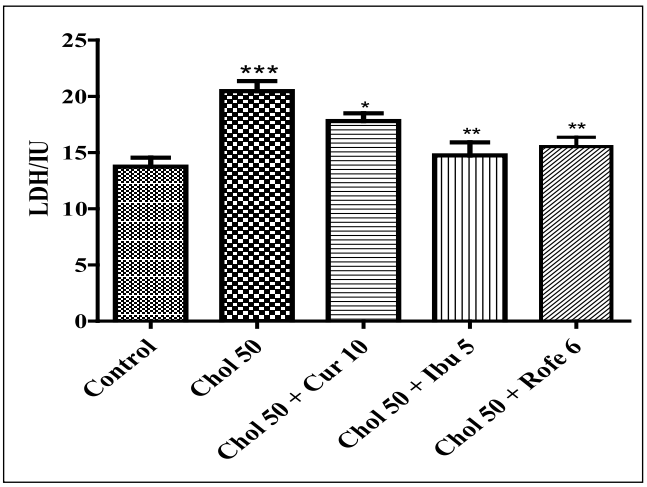

Fig.2: Effects of curcumin and NSAIDs on LDH release in cholesterol incubated PC12 cells. Values are expressed as mean ±S.E. *** p<0.001 treatments vs control.

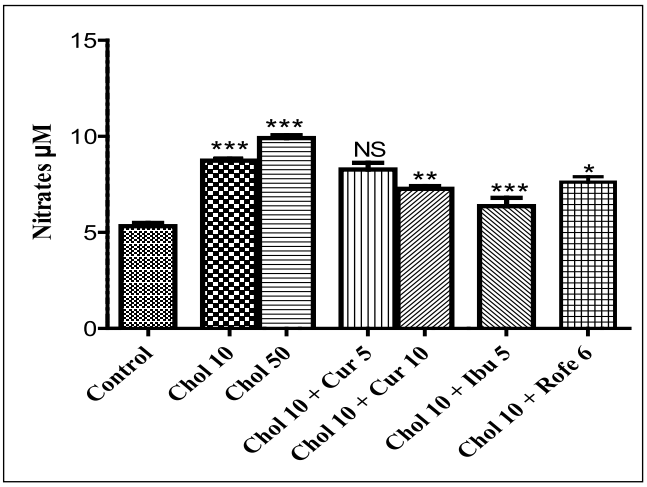

Fig.3: Effects of curcumin and NSAIDs on nitrites levels in cholesterol incubated PC12 cells. Values are expressed as mean ±S.E. *** p<0.001 treatments vs control.

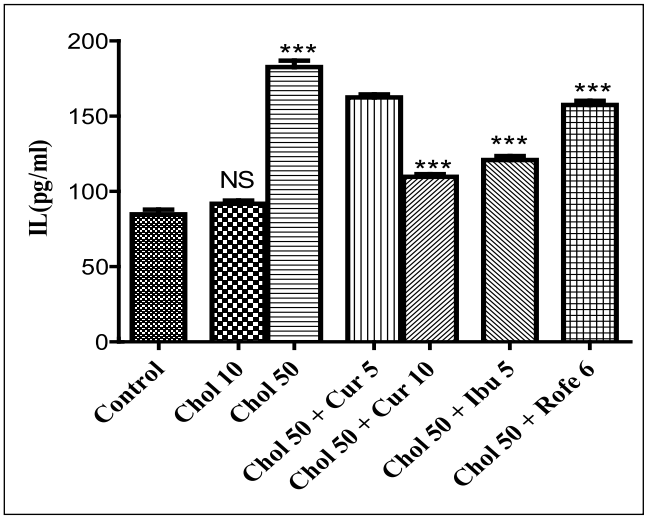

Fig.4: Effects of curcumin and NSAIDs on interleukin-1β release in cholesterol incubated PC12 cells during 24 hours. Values are expressed as mean ±S.E. ***p<0.001 treatments vs control.

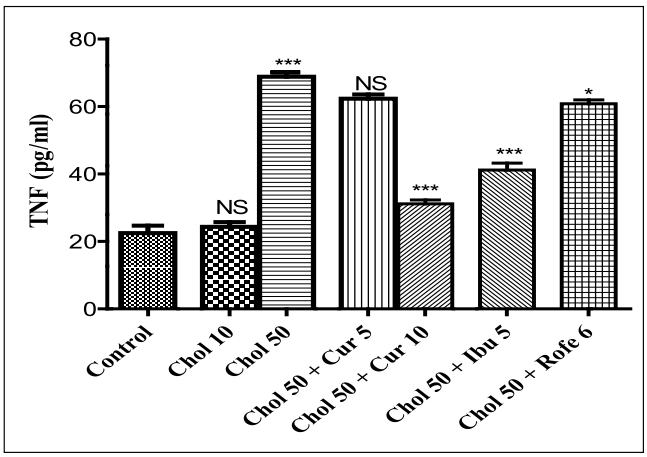

Fig.5: Effects of curcumin and NSAIDs on TNF-α release in cholesterol incubated PC12 cells during 24 hours. Values are expressed as mean ±S.E. ***p<0.001 treatments vs control.

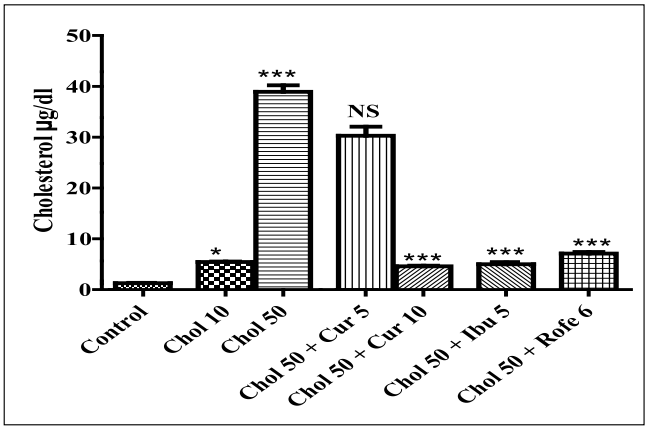

Fig.6: Effects of curcumin and NSAIDs on culture cholesterol levels in cholesterol incubated PC12 cells during 24 hours. Values are expressed as mean ±S.E. ***p<0.001 treatments vs control.

may associate with this effect. There is a clear relation and scientific evidence among nitrate stress and inflammatory pathway leads to pro-inflammatory mediator release in an experimental *in-vitro* model of neurotoxicity using PC12 cell lines. *In-vivo, a* reproductive senescent female rat model of high-fat diet lead to increased proinflammatory cytokines release was reported. However, there are many research report shown that the impairment of anti-oxidants, mitochondrial dysfunction and NF-k activation occur by free cholesterol treatment [15, 16].

The cholesterol induced proinflammatory release was attenuated significantly by ibuprofen, a non-selective COX inhibitor and curcumin in PC12 cells. This finding suggests that the cholesterol as such can trigger cytokine gene expression in PC 12 cell lines and that curcumin, possibly through its cytokine inhibition property. The various pharmacological activities of curcumin including inhibition of cytokines gene expression are at least partially mediated by inhibition of GSK-3 β[17]. We have confirmed previously that the *per se* curcumin inhibit the GSK-3β, is protein kinase family enzyme in *in-silico* which is involved in gene expression for cytokines[18]. In this investigation we used selective and non-selective COX inhibitor (5µg/ml and 6µg/ml respectively) used to shown any cytokine inhibition property. Non-selective inhibitor had very significant inhibitory effect against TNFα release as compared to curcumin. This clearly indicates the direct effects and influence of NSAIDs against the cholesterol induced neuroinflammation and neurodegeneration in PC 12 cell lines. It has been shown that cholesterol mediated oxidative stress, increased the expression of inducible nitric oxide synthase (iNOS) produces excessive levels of nitric oxide in the activated microglia lead to a disruption of neuronal mitochondrial electron transport chain function[19, 20]. However, LDH assay shown that the selective and non-selective inhibitor had significant effect compare to curcumin as evidenced by inhibiting nitrosative/nitrative stress in our experimental study. Hence, the percentage of cell viability of curcumin (10µg/ml) and ibuprofen (5µg/ml) have been significantly increased as compared with rofecoxib.

Conclusion

Results of our study show that the non-selective COX inhibitor ibuprofen and curcumin have relative protective effects on PC 12 cell line from cholesterol induced neurotoxicity. Furthermore, non-selective COX inhibitor had significant influencing on attenuating cholesterol induced pro-inflammatory mediator release and neurotoxicity as compared with selective COX-2 inhibitor.

Competing interests

None

Authors Contributions

ME: Carried out the whole research work; RM: Investigator for cell culture work; RRT: Investigator for animal study. All authors read and approved the manuscript.

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