Effect of excessive cholesterol and lipopolysaccharide on cerebellar neuronal cells in *in vitro* and protective role of anti-inflammatory drugs

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The present work was carried out to elucidate the role of NSAIDs, PPARγ agonist and HMG CoA inhibitor on cholesterol and lipopolysaccharide (LPS) induced neurodegeneration. The cerebellar neuronal cells were exposed to cholesterol (10 and 50 µg/ml), LPS (1 ng/ml) or both. Neuroprotective effect of ibuprofen, rofecoxib, simvastatin and pioglitazone was assessed by measuring the neuronal loss, MTT dye assay, nitric oxide, LDH and lipid peroxide measurement. The results indicated that incubation of cholesterol and LPS showed less synaptic connections, neurite outgrowth and cell shrinkage as compared to normal cerebellar cells. Significantly decreased survival cells count along with increased LDH, lipid peroxide and nitrite levels were observed in the cells that confirmed neurodegeneration with cholesterol and LPS challenge. In comparison to individual toxins (LPS or cholesterol), combination of LPS and cholesterol produced more deleterious effect indicated synergistic effect of toxins. Interestingly, in comparison to LPS, cholesterol produced significantly low level of nitrites, LDH and lipid peroxides which indicated excessive cholesterol might not influence radical generation directly and might be a secondary effect. Among the drugs studied, NSAIDs showed better effect indicated inflammatory mediator response played vital role in cholesterol and LPS induced neurodegeneration. Simvastatin demonstrated moderate neuroprotective effect. It could be concluded that excessive cholesterol might produce cell death and led to release of nitrites and other cytokines. NSAIDs had better neuroprotective activity than simvastatin that produced moderate effect.

**Keywords**: Cerebellar cells, Cholesterol, Lipopolysaccharide, NSAIDs, Pioglitazone, S-methyl isothiourea

Cholesterol is an essential component of cell membrane and precursor for many hormones. Excessive cholesterol and its altered metabolic state are linked to many central nervous system diseases like Alzheimer’s disease, Parkinson’s diseases, stroke etc. Also it attributes to amyloid beta 42 (Aβ 42) deposition, Lewis body and tau fibrils formation1-3. Rabbits fed with cholesterol rich diet have shown a tendency to accumulate Aβ- 42 in brain4. Recent report has shown reduced Aβ formation in cultured hippocampal neurons treated with lovastatin. The results have been correlated with reduced amyloid precursor protein (APP) levels and beta-secretase activities5. However, there are contradictory reports available on cholesterol induced Aβ 42 deposition6.

Neuro-inflammation is one of the causes in precipitating neurodegeneration. It is well known that excessive cholesterol can lead to loss of cell membrane fluidity and activation of inflammatory mediators. Bosco and coworkers1 have demonstrated that cholesterol metabolites are cytotoxic and play role in generation of reactive oxygen species (ROS) and enable alpha synuclein aggregation. Further, it has been shown that chronic exposure to cholesterol up-regulates the expression of interleukin-6 and caspase-1 in knock out and wild type mice brains7. From the reports, it can be hypothesized that cholesterol induced neurodegeneration can also mediate through formation of toxic metabolites (antigen) or by inflammatory mediators. Therefore, an attempt has been made in the present study to elucidate whether the exposure of neuronal cell to excessive cholesterol can lead to neurodegeneration? If so, what category of drugs can protect the neuronal cells?

Effect of drugs, such as, HMG-CoA reductase inhibitor: simvastatin, PPARγ agonist: pioglitazone, and NSAIDs: ibuprofen and rofecoxib were studied on exogenous cholesterol and LPS induced...
neurotoxicity in cerebellar neuronal cells. Along with cholesterol, neuronal cells were incubated with LPS at low concentration (1 ng/ml) which may aggravate the release of inflammatory mediators. Neuroprotective effect of the drugs was assessed by measuring cell viability, morphological and biochemical changes of the neuronal cells. Effect of aforementioned drugs was compared with iNOS inhibitor, S-methyl isothiourea.

Materials and Methods

Drugs and Chemicals—Trypsin, deoxyribonuclease I, poly L-lysine, fetal calf serum, cytosine arabinoside, lipo polysaccharide, S-methyl isothiourea were purchased from Sigma Aldrich USA. (3-(4,5 dimethyl thiazole-2yl)-2,5 diphenyl tetrazolium bromide) MTT dye, cholesterol and cyclodextrin were purchased from Hi- Media India. The drugs simvastatin (Ranabxy, New Delhi, India), pioglitazone (USV, Mumbai, India), ibuprofen (Abbott Pharmaceuticals, India) and rofecoxib (Shasun Pharmaceuticals, India) were purchased. All other chemicals and reagents used were extra pure chemicals.

Cell culture—Cerebellar neuronal cell cultures were prepared from cerebellum of 2 days old neonatal Wistar pups. Cerebellum was dissected in isotonic buffer with trypsin (4 mg/ml) and deoxyribonuclease I (0.4 mg/ml). Cells were placed at a density of 2.5 × 10^5 cells/cm² on poly L-lysine coated dishes in conditioning medium. The culture medium was supplemented with fetal calf serum (5%) and incubated at 37°C with CO₂ (5%) and air (95%). After plating for 48 h, cultures were treated with cytosine arabinoside (5 μM) for 24 h. Cultures were exposed to toxicants, cholesterol cyclodextrin complex (10 and 50 μg/ml) or LPS (1 ng/ml or both (10 μg/ml cholesterol cyclodextrin complex and 1 ng/ml LPS) for 4 h, followed by incubating the cells with normal medium. Simvastatin, pioglitazone, ibuprofen, rofecoxib and S- methyl isothiourea 10 μM each were added in the culture medium to assess the neuroprotection. On the basis of preliminary study, dose concentration (10 μM) of drugs was considered for the present study. Vehicle controls were treated with DMSO (0.1%). Guidelines of “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed throughout the study.

Assessment of neurotoxicity—Two different methods were employed to assess the neurotoxicity of cholesterol and LPS. (3-(4, 5 dimethyl thiazole-2yl) -2, 5 diphenyl tetrazolium bromide) dye (MTT) reduction assay reflecting mitochondrial succinate dehydrogenase enzyme activity was used. Secondly, residual cells were counted using Miotic inverted microscope with image analyser. The neurons were assessed according to the morphological criteria; neurons with intact neurite and a smooth, round soma were considered viable, whereas those with degenerated neurite and an irregular soma considered non-viable.

Measurement of cholesterol—Neuronal culture was incubated with 10 μg/ml of cholesterol. The internalization or uptake of cholesterol by neuronal cells was evaluated by measuring residual cholesterol in the medium using Ecoline kits. Values have been expressed as μg cholesterol/ml of cell supernatant.

Antioxidant measurement—Cell supernatants were assayed for LDH using a diagnostic kit. The results are expressed as U/L of LDH activity. Nitrates concentrations were determined as nitrites by using Greiss reagent. Ohkawa et al. method was used to estimate total amount of lipid peroxidation product (Thiobarbituric acid reacting substances) in the homogenate.

Statistical analysis—Data were analysed with one way ANOVA followed by post hoc comparison with Tukey-Kramer test. The probability level at 95% was considered as statistical significance.

Results

Neuronal count—Cholesterol and LPS treatment resulted in neuronal death as observed by significantly decreased formazan dye formation. In comparison to individual toxicant exposure, increased cell death was observed in cells exposed with cholesterol (10μg/ml) and LPS (1ng/ml) [F(9,50)=8.650, P<0.001]. Neurotoxicity induced with cholesterol and LPS was partly attenuated by ibuprofen, rofecoxib and pioglitazone and simvastatin. Among the treatments, HMG-CoA inhibitor, simvastatin, did not protect the neuronal cells. S-methyl isothiourea showed better neuroprotective effect against the toxicants (Table 1).

Morphological analysis—The morphology of neurons treated with cholesterol and LPS were
examined by light microscopy. In control cultures (Fig. 1A) neurons had extended neurite and smooth round cell bodies. There were markedly fewer cells and extensive debris was seen with characteristic cell bodies shrunk and neurite loss in cholesterol and LPS treated cultures at 48 hr (Fig. 1B-D). However, cholesterol (10 μg/ml) and LPS (1 ng/ml) induced degeneration of neuronal cells was more pronounced than the individual treatment, which indicated synergistic effect of these toxicants (Fig. 1E). The drugs ibuprofen (Fig. 1F), rofecoxib (Fig. 1G), pioglitazone (Fig. 1H), attenuated the cholesterol and LPS induced neurodegeneration. However, simvastatin (Fig. 1I) had marginal neuroprotective effect as evidenced with loss of cell structure. Cells treated with S-methyl isothiourea showed better protective effect in comparison to other treatments.

**Antioxidant evaluation**

**LDH**—Incubation of cell cultures with cholesterol led to a dose-dependent increase in LDH level. Similarly LPS (1 ng/ml) significantly elevated LDH levels. In comparison to individual toxicant effect, cholesterol (10 μg/ml) and LPS (1 ng/ml) in combination significantly increased LDH level \[F (9,50)=213.3, P<0.001\]. NSAIDs ibuprofen and rofecoxib, PPARγ agonist-pioglitazone significantly attenuated cholesterol and LPS induced neurodegeneration. However, simvastatin (Fig. 1I) had marginal neuroprotective effect as evidenced with loss of cell structure. Cells treated with S-methyl isothiourea showed better protective effect in comparison to other treatments.

**Nitrites**—Incubation of the cerebellar cells with cholesterol (10 and 50 μg/ml) led to release of nitrites. Four fold increase in nitrite level was observed with LPS (1 ng/ml) challenge. Incubation of cultures with cholesterol and LPS in combination led to further significant increase in nitrite levels, which was higher than the individual toxicant effect \[F (9,50)=247.0, P<0.001\]. Treatment with ibuprofen, rofecoxib and pioglitazone prevented cholesterol and LPS induced nitrite elevation. HMG-CoA inhibitor, simvastatin, significantly decreased nitrite release. However, NSAIDs and pioglitazone decreased more nitrite level than simvastatin. S-methyl isothiourea significantly attenuated the release of nitrites due to cholesterol and LPS challenge. Among the drugs tested, ibuprofen, rofecoxib and treatment were comparable with S-methyl isothiourea (Table 1).

**Lipid peroxides**—Cerebellar cells grown along with cholesterol (10 and 50 μg/ml) did not provoke any effect on TBARS levels. Significant increase in TBARS level was observed with LPS treatment. In comparison to individual toxicant, TBARS level was higher with cholesterol and LPS treatment in combination \[F (9,50)=247.0, P<0.001\]. Treatment of the culture with ibuprofen, rofecoxib, pioglitazone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage viability</th>
<th>LDH (U/L)</th>
<th>Nitrites (μM)</th>
<th>TBAR (pmol/1 × 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>97.78±6.48</td>
<td>28.00±1.15</td>
<td>6.89±0.35</td>
<td>82.50±1.38</td>
</tr>
<tr>
<td>Cholesterol (10 μg/ml)</td>
<td>75.17±6.54</td>
<td>40.03±1.15</td>
<td>6.87±0.41</td>
<td>92.6±7.2</td>
</tr>
<tr>
<td>Cholesterol (50 μg/ml)</td>
<td>62.17±7.05</td>
<td>56.0±1.15</td>
<td>6.90±0.42</td>
<td>100.4±5.3</td>
</tr>
<tr>
<td>LPS (1 ng/ml)</td>
<td>55.8±5.48</td>
<td>75.3±1.45</td>
<td>23.6±0.71</td>
<td>483.5±12.3</td>
</tr>
<tr>
<td>Cholesterol (10 μg/ml) + LPS (1 ng/ml)</td>
<td>36.5±4.23</td>
<td>94.0±0.57</td>
<td>29.22±0.31</td>
<td>587.4±16.2</td>
</tr>
<tr>
<td>Cholesterol (10 μg/ml) + LPS (1 ng/ml) + Ibuprofen 10 μM</td>
<td>53.79±6.37</td>
<td>70.0±1.97</td>
<td>8.62±0.53</td>
<td>223.4±6.3</td>
</tr>
<tr>
<td>Cholesterol (10 μg/ml) + LPS (1 ng/ml) + Pioglitazone 10 μM</td>
<td>48.27±5.31</td>
<td>73.0±0.88</td>
<td>10.36±0.84</td>
<td>267±3.43</td>
</tr>
<tr>
<td>Cholesterol (10 μg/ml) + LPS (1 ng/ml) + Rofecoxib 10 μM</td>
<td>51.63±6.77</td>
<td>68.0±1.37</td>
<td>8.44±0.46</td>
<td>211±3.67</td>
</tr>
<tr>
<td>Cholesterol (10 μg/ml) + LPS (1 ng/ml) + Simvastatin 10 μM</td>
<td>40.0±6.37</td>
<td>90.3±1.17</td>
<td>26.3±0.3</td>
<td>288±5.67</td>
</tr>
<tr>
<td>Cholesterol (10 μg/ml) + LPS (1 ng/ml) + S-Methyl Isothiourea 10 μM</td>
<td>64.13±5.52</td>
<td>43.0±2.33</td>
<td>7.712±0.25</td>
<td>209±6.34</td>
</tr>
</tbody>
</table>

* and ** indicate statistical significance in comparison to normal cells at P < 0.05; P < 0.01 P < 0.001, respectively. * and *** indicates statistical significance in comparison to cholesterol (10 μg/ml) + LPS (1 ng/ml) at P < 0.05 and P < 0.001, respectively.
and simvastatin significantly attenuated the increased TBAR level. Significant decrease in TBAR level was observed with S-methyl isothiourea treated cells (Table 1).

**Cholesterol measurement**—Cholesterol level in cell supernatants was measured to assess the utilization of cholesterol by neuronal cells. After 24 hr of cholesterol (10 μg/ml) treatment, a marked decrease (5.6 μg/ml) in the supernatant cholesterol level was observed that indicated the utilization of cholesterol by neuronal cells. Co-incubation of cultures with LPS (1 ng/ml) did not influence the cholesterol utilization (5.8 μg/ml). Among the drugs ibuprofen (5.3 μg/ml), rofecoxib (6.0 μg/ml) and S-methyl isothiourea (5.4 μg/ml) did not alter cholesterol uptake by neuronal cells. In comparison to vehicle treated neuronal cells there was insignificant increase in cholesterol level of cultures treated with pioglitazone (6.3 μg/ml) and simvastatin (6.2 μg/ml).

**Discussion**

Cerebellar cells grown with cholesterol (10 and 50 μg/ml) and LPS (1ng/ml) produced neurodegeneration as indicated by altered morphology, decreased formazon dye formation, increased nitrites and TBAR levels. The cells also showed less synaptic connections, neurite out growth and cell shrinkage as compared to normal cells. Treatment of cholesterol and LPS in combination produced more pronounced degeneration in all the parameters studied indicated synergistic effect of neurotoxins in induction of degeneration. The present study provided evidence that excessive cholesterol might lead to apoptosis and cell death. Earlier reports

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**Fig. 1**—(A) Normal cerebellar neuronal cell culture showing cell bodies, dendrites and axon. Neurodegeneration as observed with loss of cell morphology after treatment with (B) cholesterol (10 μg/ml); (C) cholesterol (50 μg/ml); (D) LPS (1 ng/ml) and (E) cholesterol (10 μg/ml) and LPS (1 ng/ml) in combination.
have indicated that excessive cholesterol can precipitate AD\(^3\). However, in clinical reports, no clear evidence on cholesterol with dementia and degeneration has been reported\(^13\).

LPS, an endotoxin widely used to study neurodegenerative condition like multiple sclerosis, produced significant neuronal loss in the present study. The present model was undertaken to study the role of excessive cholesterol in endogenous degeneration. Cholesterol is an important lipid that control membrane fluidity in neurons. Excessive cholesterol can produce rigidity and loss of membrane fluidity resulting in cell destruction and leading to formation of cell debris. The formed debris can act as antigen and trigger inflammatory response or gliosis. Hence, we simulated similar \textit{in vitro} model by treatment of cholesterol and LPS, where LPS acted as antigen and had synergistic effect along with cholesterol.

It is observed that in comparison to LPS, cholesterol incubation in neuronal cells produced significantly low level of nitrites and LDH level which indicated excessive cholesterol might not influence radical generation directly and it might be a secondary effect due to cell death. It is, further, confirmed with low level of lipid peroxides in
cholesterol treated cells. Cholesterol has been taken by the neuronal cells as evident from the reduction in cholesterol in the growth medium and all the study drugs did not influence the cholesterol uptake. Insignificant elevation in cholesterol level with pioglitazone treatment indicates that it can inhibit the absorption of cholesterol inside the cells. Hence, it can be stated that excessive cholesterol may produce cell rigidity, which is the main component of lipid rafts involved in cell signalling that alters protein expression and inflammatory response. Earlier study carried out with ApoE knock out and wild type mice exhibits increased expression of inflammatory mediators involved in neurodegeneration with hypercholesterol diet which supports our present findings.

On the other hand LPS administration produced approximately 4 fold increase in LDH, nitrite and TBAR levels indicated that LPS might have direct effect on defence mechanism. LPS activates iNOS leading to release of NO. Activation of iNOS and subsequent NO release was maximum when neuronal cells were incubated with a combination of cholesterol and LPS. Similarly, LDH and TBAR levels were also found to be higher than individual toxicant effect. Hence, it was predicted that the observed deleterious effect in neuronal cultures might be in part due to excessive cholesterol and LPS could act through different mechanism in inducement of apoptosis and or both could act complementary to each other.

Among the drugs studied NSAIDs had shown better effect indicated inflammatory mediator response played vital role in cholesterol and LPS induced neurodegeneration. The selective and non-selective COX inhibitors have been shown to produce anti-inflammatory response in various models which supports our present findings. These drugs mediate their neuroprotective action either through nuclear receptor peroxisome activated proliferation receptors or cellular targets. Pioglitazone, a PPARγ ligand, down-regulates the MAPK activity, thereby, inhibits iNOS expression and NO activity. S-methyl isothiourea, a specific iNOS inhibitor, effectively protected the cells. Recent epidemiological survey has shown that NSAIDs can be useful in prevention of CNS diseases and can act as neuroprotective agents. Hence, at this juncture it can be stated that cholesterol and LPS incubation could lead to altered cytokine expression, thereby, aggravating the inflammatory response.

Simvastatin demonstrated moderate neuroprotective effect against cholesterol and LPS challenge. Previous studies have reported that the pre-treatment with statins confers protection against amyloid β peptides, and LPS challenge, but all statins do not protect against all neurotoxic insults. Further, HMG CoA reductase inhibitors have shown inhibitory activity against LPS induced production of cytokines and NO in astrocytes, microglia and macrophages in in vitro. It has been suggested that use of statins may be advantageous to improve brain oxygen supply in AD patients. The primary action of statins in humans with AD may reduce inflammation rather than decrease Aβ load. Apart from lipid lowering properties statins exhibits multiple functions to modulate intracellular signalling pathways, inhibit inflammation, suppress the production of ROS and modulate T cell activity.

The present study revealed that the cholesterol mediated neurodegeneration might be due to loss of cell structure and inflammatory response. The inconclusive clinical reports on correlation between cholesterol, dementia and AD and failure of statins in controlling cholesterol induced degeneration reflected that cholesterol neurodegeneration might be occurred mechanism through inflammation rather than other pathways. It was concluded that excessive cholesterol might have led to cell death and release of nitrites and other cytokines. NSAIDs showed better neuroprotective activity than simvastatin treatment that resulted to moderate effect.

References


