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Cyclooxygenase inhibitors: a novel direction for Alzheimer's management

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Abstract:
Research in Alzheimer's disease (AD) currently includes various cellular, molecular, genetic, clinical and therapeutic approaches. The cytopathological significance of oxidative damage has been studied in neurons of AD patients. Many epidemiological studies suggest that use of non-steroidal anti-inflammatory drugs (NSAIDs) delay or slow the clinical expression of AD, and anti-oxidant properties of NSAIDs have also been previously described. Therefore, in this study we examined the role of various cyclooxygenase (COX)-1 and COX-2 inhibitors (NSAIDs) in a rat model of aluminum-induced oxidative stress to mimic AD-like conditions. We found that the animals receiving aluminum treatment for one month (4.2 mg/kg, *ip*) had highly elevated levels of reactive oxygen species (expressed as malondialdehyde – MDA). Moreover, treatment with the COX-2 inhibitor, rofecoxib (0.83 mg/kg, *po*), was able to significantly reduce this oxidative stress ($p < 0.05$ when compared to aluminum treatment alone on MDA levels). But, non-specific COX inhibitors (flurbiprofen, 0.83 mg/kg twice a day *po* and ibuprofen, 100 mg/kg, *po*), did not protect again oxidative stress. Thus, in agreement with earlier epidemiological studies, we propose that COX-2 specific NSAIDs may be beneficial in AD management. Further experimental work towards identifying the most efficacious COX-2 inhibitors, as well as the mechanism of action and the optimal dosage regimen should be executed.

Key words:
cyclooxygenase inhibitors, rofecoxib, flurbiprofen, ibuprofen, Alzheimer's disease, NSAIDs

Abbreviations: AD – Alzheimer's disease, COX – cyclooxygenase, HBSS – Hank's balanced salt solution, *ip* – intraperitoneally, LPO – lipid peroxidation, MDA – malondialdehyde, NSAIDs – non-steroidal anti-inflammatory durgs, *po* – per oral dose, RBC – red blood cell, SOD – superoxide dismutase, TBA – thiobarbituric acid

Introduction

Alzheimer's disease (AD), the most common cause of dementia associated with neurodegeneration in the elderly, is characterized clinically by progressive memory loss and other cognitive impairments [32]. Research in AD is rapidly expanding and currently includes various cellular, molecular, genetic, clinical and therapeutic research approaches [10].

The prevalence of AD increases exponentially with age [7]. The free radical hypothesis of aging states that the aging process is associated with multisystem failure due to oxidative damage caused by an imbalance between reactive oxygen species production and antioxidant defenses [5]. The cytopathological significance of oxidative damage is supported by findings of upregulation of anti-oxidant enzymes like heme oxygenase-1 and superoxide dismutase in the neurons of AD patients [23, 27, 33, 36]. Reactive oxygen is

a ubiquitous byproduct of both oxidative phosphorylation and the myriad of oxidases necessary to support aerobic metabolism. In AD, there are a number of additional contributory sources that are thought to play important roles in oxidative stress, such as increased neuronal iron in an active redox state, increased nitric oxide (NO) synthesis in microglia, and abnormalities in the mitochondrial genome. Furthermore, lipid peroxidation, a hallmark of oxidative tissue injury, has been found to be elevated in the AD brain [37]. Hence it is thought that oxidative stress may be an underlying mechanism in AD, and agents that prevent oxidative damage may be particularly efficacious in the treatment of AD [35].

Free radical-mediated lipid peroxidation has been shown to activate cyclooxygenase (COX)-2 [4]. Furthermore, the two step oxygenase and peroxidase action of COX leading to the formation of a reactive oxygen species and prostaglandin H_2 (PGH_2) has been described in detail [25]. However, aggregated synthetic Aβ1-40 peptides have been shown to induce COX-2 expression in SH-SY5Y neuroblastoma cells, and Aβ1-40 has been shown to stimulate COX-2 oxygenase and peroxidase activity in a cell free system [24]. These findings are further supported by evidence showing that increased basal levels of oxidative stress significantly increases Aβ neurotoxicity in hippocampal neurons *in vitro* [26]. Therefore, based on epidemiological studies and experimental work, it is hypothesized that COX-2 plays an important role in neurodegeneration in AD [25].

Many epidemiological studies suggest that use of non-steroidal anti-inflammatory drugs (NSAIDs) delay or slow the clinical expression of AD [2, 13, 16, 30]. Furthermore, new evidence that COX is involved in neurodegeneration [17, 20, 24, 25] and the development of selective COX inhibitors has led to renewed interest in the therapeutic activity of NSAIDs in AD [1, 30, 38].

A number of environmental factors have been advanced as possible contributory factors of AD, including aluminum [18]. The possible mechanisms of aluminum-induced neurotoxicity may include cell damage *via* free radical production [39]. Moreover, aluminum has been shown to be associated with both plaques and tangles in AD. Various investigations have suggested that AD is more common in areas where the aluminum content in water supplies is the highest [18]. Not surprisingly, aluminum induced oxidative stress in rat is a commonly utilized animal model to mimic human AD-like symptoms [12, 14, 21].

However, most of the studies conducted so far have been limited to clinical surveys, prospective studies and autopsy-based research. Therefore, there is a need for *in vivo* experimental data demonstrating the role of NSAIDs in management of AD-related oxidative damage in the brain. The current study aimed to examine the effect of different COX inhibitors on aluminum induced oxidative stress in the *in vivo* rat model.

Materials and Methods

Animals

Sprague Dawley rats (n = 72) of either sex, with an average weight of 209.17 ± 7.66 g were used in this study. Animals were bred in the animal colony at the B. V. Patel PERD, Center, Ahmedabad, from the original stock obtained from the National Institute of Nutrition in Hyderabad. The animals were housed in temperature controlled rooms (27 ± 1°C), with 10% air exchange with a relative humidity of 60 ± 5% and with a light : dark regimen of 14:10 h. Amrut certified rodent diet (Maharashtra Chakan Oil Mills Ltd.) and tap water (boiled water cooled to room temperature) were provided *ad libitum* to the experimental animals. Body weight, food consumption and changes in physical or behavioral pattern were monitored daily for the experimental animals.

The experimental protocol was approved by the Institutional Animal House Ethics Committee, constituted by the Ministry of Social Justice and Empowerment, Government of India, prior to the initiation of the experiment.

Chemicals

Aluminum chloride ($AlCl_3$) was obtained from SD Fine Chemicals Ltd. (Mumbai, India). Rofecoxib used for this study was synthesized in the Medicinal Chemistry Department of our Institute. Flurbiprofen and ibuprofen were obtained as gift samples from Boots Pharmaceuticals Ltd. (Mumbai, India).

Dosage regimen

Animals were divided into the following treatment groups: 1. Vehicle control group (normal saline used as vehicle for all the drugs as well as for aluminum); 2. Disease control group: aluminium chloride (4.2 mg/kg, *ip,* daily for 28 days); 3. Test group 1: rofecoxib (0.83 mg/kg, *po*) / flurbiprofen (0.83 mg/kg twice per day, *po*) / ibuprofen (100 mg/kg, *po*); 4. Test group 2: aluminium chloride (4.2 mg/kg, *ip*) + rofecoxib (0.83 mg/kg, *po*) / flurbiprofen (0.83 mg/kg twice a day, *po*) / ibuprofen 100 mg/kg *po*).

In vivo treatment

Animals in all the groups were treated for 28 days as per the above treatments (n = 6 per group). Animals were euthanatized on day 29 and the intact brain was removed. The brain was washed with saline to clean traces of blood and divided into the two hemispheres. The two hemispheres were processed further for lipid peroxidation (LPO) estimation and superoxide dismutase (SOD) activity, respectively.

Estimation of lipid peroxidation

One half of the brain was weighed and placed in 5 ml of Hank's balanced salt solution (HBSS, pH 7.4) and homogenized at 3,500 rpm using a Polytron homogenizer (Kinematica, Switzerland) (3 cycles of 30 s each). The homogenate was then centrifuged at 3,500 rpm (500 × g) for 10 min. The pellet was resuspended in 0.1 ml of HBSS that was then used for estimation of lipid peroxidation.

LPO was measured in terms of MDA:TBA reaction as reported by Ohkawa et al. [22]. The reaction mixture contained 0.1 ml of tissue homogenate (as described above), 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with 1 M NaOH) and 1.5 ml of 0.8% aqueous solution of TBA. The reaction mixture was made up to 4 ml with the addition of 0.7 ml of double distilled water and heated at 95°C for 1 h, in a water bath. After cooling, 1 ml of double distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added, and the mixture was shaken vigorously on a vortex mixer for 5 min. This mixture was centrifuged at 3,000 rpm for 7 min, the upper organic layer was separated, and the amount of MDA formed in this layer was measured at 532 nm using an ultraviolet/visible spectrophotometer (Systronics, India). MDA has an extinction coefficient of 1.45×10^{-5}/min/cm. Appropriate controls were used at different steps during this estimation.

Assay for superoxide dismutase activity

The other half of the brain was placed in 4 ml of chilled 50 mM Tris buffer (pH 8.2, with 2 mM EDTA) and homogenized at 13,000 rpm (3 cycles of 30 s each) using a Polytron homogenizer. The homogenate was treated with 1 ml of 0.1% Triton X 100 (v/v) for 20 min at 4°C and then centrifuged at 15,000 rpm at 4°C for 30 min using a Sorval high-speed centrifuge (Sorval, USA) with a fixed angle rotor (SS34). The supernatant was used for the SOD activity assay according to the method of Marklund and Marklund [15]. All calculations were made as per gram fresh weight.

Circulating superoxide dismutase levels

On day 29 of treatment, blood was collected from the rats by cardiac puncture in heparinized disposable syringes. The blood was then centrifuged at 4,000 rpm for 7 min at 4°C, and the plasma was separated and stored at –70°C until further assay for superoxide dismutase activity as detailed above. The remaining red blood cells (RBCs) were then subjected for extraction of superoxide dismutase

Extraction of superoxide dismutase from RBCs

Heparinized blood was centrifuged at 2,500 rpm for 30 min at 4°C and the plasma was carefully separated. Five milliliters of normal saline (0.9%) were added to the erythrocyte pellet, mixed, and the resuspension was transferred to 15 ml centrifuge tubes. After the erythrocytes were washed three times with saline as above, they were diluted with 4 ml double distilled water in order to lyse them. One milliliter of ethyl alcohol and 0.6 ml of chloroform were added to separate the hemoglobin. The tubes were shaken vigorously for 15 min and centrifuged at 2,500 rpm for 10 min at 4°C. The water-ethanol layer was aspirated and diluted with 0.7 ml of double distilled water. SOD activity was measured in the hydro-alcoholic layer as described above.

Statistical analysis

All results are presented as the mean ± SEM. Statistical analyses were performed using a one way ANOVA followed by Dunnet's test, and $p < 0.05$ was considered significant.

Results

The experimental animals showed no significant change in body weight, food consumption, physical appearance or behavioral pattern during the entire period of the study.

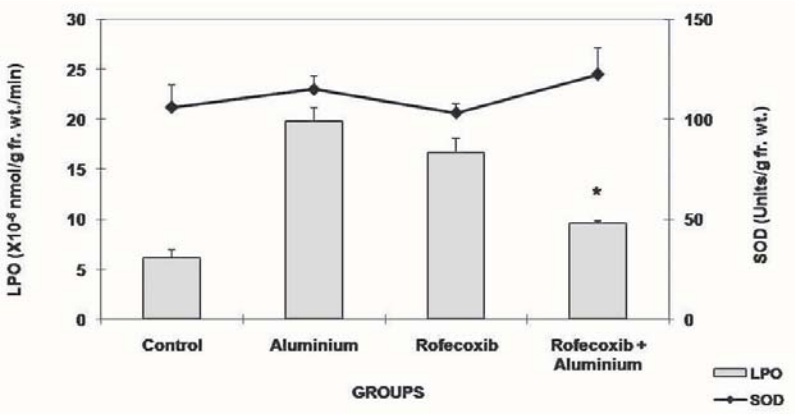

Fig. 1. *In vivo* changes in lipid peroxidation (LPO) (expressed as MDA levels) and superoxide dismutase (SOD) activity in different groups of rat brain homogenate. A significant decrease in the aluminum and rofecoxib treated group (* $p < 0.05$) was found when compared to the aluminum treatment alone. However, no significant changes were found in SOD activity in any of the groups

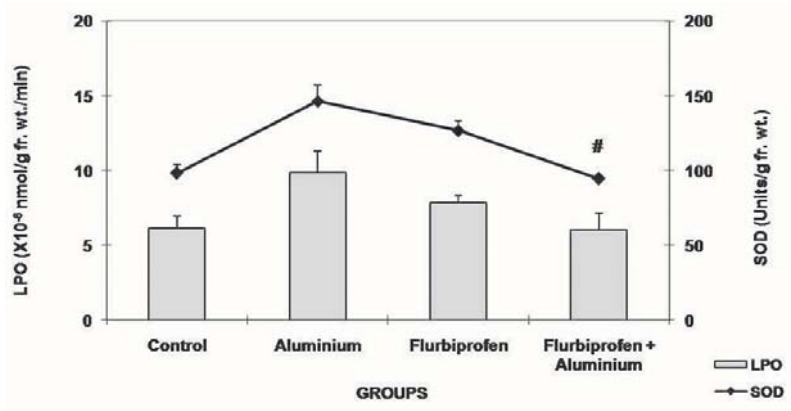

Fig. 2. *In vivo* changes in lipid peroxidation (LPO) (expressed as MDA levels) and superoxide dismutase (SOD) activity in different groups of rat brain homogenate. No significant changes were found in MDA levels in any of the groups. However, a significant decrease in SOD activity (# $p < 0.05$) was observed in flurbiprofen plus aluminum treated group when compared to aluminum alone treated group

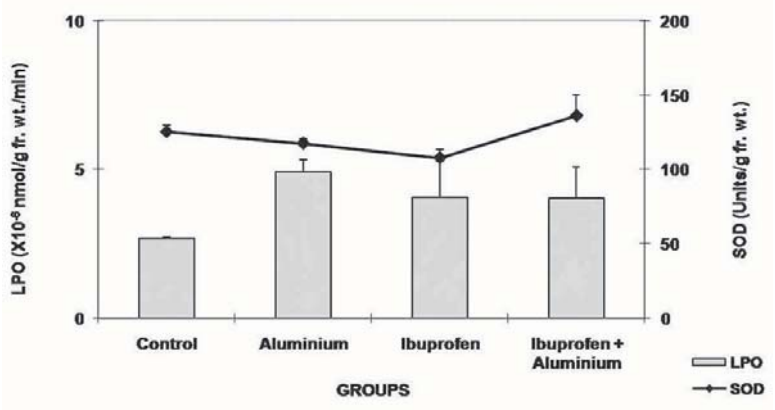

Fig. 3. *In vivo* changes in the lipid peroxidation (LPO) (expressed as MDA levels) and superoxide dismutase (SOD) activity in different groups of rat brain homogenate. No significant changes in MDA levels or SOD activity were found in any of the groups

Figure 1 shows the changes in the lipid peroxidation and SOD activity *in vivo* in each group of rat treated with vehicle, aluminum, rofecoxib and aluminum with rofecoxib. Aluminum treatment significantly increased malonaldehyde (MDA) levels in the rat brains. Furthermore, a significant decrease in MDA level was found in the aluminum and rofecoxib treated animals when compared to the aluminum only treated group ($p < 0.05$). However, no significant change in SOD activity was found in any of the groups. Thus, rofecoxib treatment served as a potent free radical lowering agent in the rat brain.

No significant changes in MDA levels were found in animals treated with flurbiprofen alone or when co-administered with aluminum (Fig. 2). However, a significant decrease in SOD activity ($p < 0.05$) was found in the flurbiprofen plus aluminum treated group when compared to aluminum treatment alone.

Ibuprofen treatment failed to protect against LPO and did not interfere with SOD activity (Fig. 3). Similarly, circulating SOD levels did not differ between any of the experimental groups (data not presented).

Discussion

The free radical hypothesis of aging states that tissue damage from reactive oxygen species may underlie multisystem failure [5], and these mechanisms may also occur in the progression of AD. Targets of reactive oxygen species include activation of COX-1 and -2, which are blocked by NSAIDs. Furthermore, daily doses of NSAIDs have been shown to increase circulating levels of antioxidants, which combat reactive oxygen species, in rheumatoid arthritis [8, 19]. Therefore, in this study we examined the effect of NSAIDs acting on both COX-1 and COX-2 to maximize functional anti-oxidant properties, in a rat model of AD.

Rofecoxib is a NSAID that exhibits anti-inflammatory, analgesic and antipyretic activities in animal models. The mechanism of action of rofecoxib is believed to include inhibition of prostaglandin synthesis *via* selective inhibition of COX-2. At therapeutic concentrations in humans, rofecoxib does not inhibit the COX-1 isoenzyme. In 2000 and 2001, Merck and Co., Inc. conducted several studies of rofecoxib (Vioxx) aimed at determining whether the drug slows the onset of AD [31, 34]. In 2002, the Food and Drug Administration approved Vioxx use for this purpose and also revised Vioxx prescription information to reflect the cardiovascular protection and data from long-term, placebo controlled studies in elderly patients with AD, which did not show increased cardiovascular risk [28]. Further trials in AD patients indicated that selective COX-2 inhibition by rofecoxib did not slow the progression of AD in patients with advanced dementia [29]. These findings are inconsistent with previous findings suggesting a protective role of COX-2 inhibition against AD [1, 3, 17, 19, 20]. Hence, no clear picture regarding the protective role of rofecoxib in AD has been attained so far. In this study, we found that rofecoxib may reduce the free radical load in the rat brain with chronic administration. From these data, we may conclude that COX-2 does indeed play a role in the oxidative stress in AD that may be controlled using selective COX-2 inhibitors like rofecoxib.

Flurbiprofen is a NSAID that also possesses analgesic, and antipyretic properties. It is a potent prostaglandin synthesis inhibitor, and this property may be involved in its anti-inflammatory effect [6]. Several studies are currently underway to study the activity of the R stereoisomer of flurbiprofen in AD. This enantiomer does not inhibit COX activity but has been shown to potently reduce levels of beta amyloid resulting in rescue of learning dysfunction in transgenic AD model in mice [9]. Therefore, in this study we asked whether the racemate of flurbiprofen was also able to reduce oxidative stress in the AD brain. Our results indicate that flurbiprofen may interact with the anti-oxidant system in the AD model rat brain to disrupt the normal oxy-radical : anti-oxidant balance in the brain. Thus, flurbiprofen may not play a direct role by reducing oxy-radicals in the brain during AD but may instead exert its effect *via* an interaction with the anti-oxidant system.

Ibuprofen is also known to possess analgesic and antipyretic activities and is used to relieve the pain, inflammation, swelling, and stiffness caused by certain types of arthritis. It is used to reduce fever, headaches, muscle aches, menstrual pain, backache or pain from surgery or dental work. The mechanism of action of ibuprofen, like that of other NSAIDs, is not completely understood, but may be related to prostaglandin synthetase inhibition. Ibuprofen was used in this study based on previous findings in mouse model of AD wherein chronic administration of the drug was able to control interleukin-1β and glial fibrillary acidic

protein levels as well as number and total area of β-amyloid deposits [11]. However, in the current study a protective role of ibuprofen in terms of controlling free radical load was not found.

Several epidemiological studies have been conducted to examine the protective role of NSAIDS in AD, and these studies have suggested that COX-2 plays an important role in AD neurodegeneration [17, 20, 25]. In earlier experiments, we provided preliminary evidence regarding the role of a COX-2 specific NSAID, meloxicam, in AD management [18]. The current experiments provide concrete evidence in a rat model of AD to support the hypothesis that COX-2 inhibition alone is an important target for reducing AD-related oxidative stress. We recommend that further experimental work be carried out in order to assess which of the COX-2 inhibitors is most suitable for AD treatment, to determine the optimum dosage regimen for its usage, and to reveal the mechanism of action of COX-2 inhibitors in AD amelioration.

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