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Protective effect of non-selective and selective COX-2-inhibitors in acute immobilization stress-induced behavioral and biochemical alterations

Beenta Kumari, Anil Kumar, Ashish Dhir

Pharmacology Division, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh-160014, India

Correspondence: Anil Kumar, e-mail: kumaruips@yahoo.com

Abstract:
Acute stress has been known to produce several behavioral, neurochemical and biochemical alterations. Cyclooxygenase (COX) enzymes are involved in pathogenesis of several brain disorders including Alzheimer disease, epilepsy, depression, in addition to pain and inflammation. In the present study, we examined the role of non-selective (naproxen) and selective (rofecoxib, valdecoxib) COX-2 inhibitors against acute immobilization stress-induced behavioral alterations and oxidative damage in mice. Mice were subjected to acute immobilization stress for a period of 6 h. Naproxen (7 and 14 mg/kg, *ip*), rofecoxib (5 and 10 mg/kg, *ip*) or valdecoxib (5 and 10 mg/kg, *ip*) were administered 30 min before acute stress. Six-our immobilization stress significantly caused anxiety-like behavior, memory deficit and impaired motor activity as well as oxidative damage (raised lipid peroxidation, nitrite activity, depletion of reduced glutathione and catalase activity) as compared to naive animals placed on sawdust ($p < 0.05$). Pretreatment with naproxen (7 and 14 mg/kg, *ip*), rofecoxib (5 and 10 mg/kg, *ip*) and valdecoxib (5 and 10 mg/kg, *ip*) significantly improved locomotor activity, antianxiety effect, memory retention (memory deficit) and attenuated oxidative damage (lowering of raised malondialdehyde, nitrite activity, restoration of reduced glutathione and catalase activity as compared to immobilization stress group ($p < 0.05$). Results suggest the neuroprotective and antioxidant effect of both non-selective and selective COX-2 inhibitors.

Key words:
cyclooxygenase, immobilization stress, naproxen, rofecoxib, valdecoxib

Abbreviations: AD – Alzheimer disease, CMC – carboxymethyl-cellulose, COX – cyclooxygenase, DTNB – dithiobisnitrobenzoic acid, HPA – hypothalamic-pituitary-adrenal axis, MDA – malondialdehyde, NO – nitric oxide, NSAIDs – Non steroidal antiinflammatory drugs, PGs – prostaglandins, ROS – reactive oxygen species, TL – transfer latency

Introduction

Cyclooxygenase (COX) is a rate-limiting enzyme in the metabolism of arachidonic acid to prostanoids. It is constitutively expressed in the neuronal tissues and speculated to be involved in various neurodegenerative disorders, such as Alzheimer disease, depression, Huntington's disease, besides being implicated in pain and inflammation [1–3]. COX exists mainly in two distinct isoforms (COX-1 and COX-2). However, expression of the third isoform COX-3, a splice variant of COX-1, has recently been recognized in the brain (cerebral cortex) followed by heart [6]. COX-3 produces prostaglandin-derived metabolites, which have inherent anti-inflammatory properties.

COX-1 is constitutively expressed in nearly all brain tissues including neurons, microglia, astrocytes

and act as a housekeeper of cellular functions. COX-1 isoform is associated with tissue homeostasis and catalyzes identical reactions that lead to some pro-inflammatory product at the site of inflammation [3, 48]. In contrast, COX-2, an inducible isoform, increases its response in several pathological inflammatory conditions including neuroinflammation. COX-2 is constitutively expressed in brain, but is up-regulated by cytokines, mitogenes, growth factors and bacterial lipopolysaccharides [8, 28]. Although, COX-2 expression is undetectable in most of the neurons in the central nervous system. The COX-2, inducible isoform is also up-regulated in pathological conditions, such as seizures, ischemia, or some degenerative diseases [28, 29, 40, 59].

Study also indicated that 2–6 h of immobilization stress caused an enhancement of COX-2 protein expression in cortex and hippocampus [29–31]. Recently, neuroprotective effects of COX-2 inhibitors have been demonstrated in various CNS-related disorders [2, 8, 11, 21, 52]. However, cellular cascade and mechanism regulating stress-related effects are not well understood. Various studies suggest that a number of inflammatory processes including cytokines are involved in the pathogenesis of several neurodegenerative disorders [6]. Restraint stress has also been demonstrated to increase inducible isoform of NO synthase expression in rat brain, and its inhibition protects against stress-induced cell damage [40–42].

Based on the above, the present study was undertaken to evaluate the role of non-selective and selective COX-2 inhibitors against immobilization stress-induced behavioral alterations and oxidative damage in mice.

Materials and Methods

Animals

Albino (Laca) mice weighing between 22–30 g bred in Central Animal House (CAH) facility of the Panjab University, Chandigarh, were used. The animals were housed under standard laboratory conditions and maintained on natural light and dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. Each group consists of minimum of five animals. All the experiments were carried out between 9.00 and 15.00 h. The experimental protocols were approved by Institutional Animal Ethics Committee (IAEC) and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals.

Immobilization stress

Animals were immobilized for 6-h by taping all the four limbs on board by putting them on their backs using zinc oxide hospital tape [8, 30]. Release was affected by unraveling the tape after moistening with acetone. In unstressed group, the mice were handled without any stress.

Behavioral assessments

Various behavioral parameters were assessed after the 6-h acute immobilization stress in mice.

Measurement of locomotor activity

Animal was kept in actophotometer for the first 3 min and then locomotor activity was recorded using actophotometer for a period of 5 min. The apparatus was placed in a darkened, light-sound attenuated and ventilated testing room. Each animal was observed over a period of 5 min in a square (30 cm) closed arena equipped with infrared light sensitive photocells using digital photoactometer and values expressed as counts per 5 min [45].

Measurement of anxiety (mirror chamber test)

The mirror chamber consisted of a wooden chamber having a mirror cube enclosed within it. The container box measured 40 × 40 × 30.5 cm. Each animal was placed at the distal corner of the mirror chamber at the beginning of the test. During the 5 min test session, the following parameters were recorded (i) latency to enter the mirror chamber (ii) the number of entries in mirror chamber and (iii) the total time spent in mirror chamber. An anxiogenic response was defined as decreased number of entries and time spent in the mirror chamber [22].

Measurement of memory

Elevated plus maze was used to evaluate spatial long-term memory [17, 45]. Briefly, the apparatus consisted

of two open arms (16 × 5 cm) and two closed arms (16 × 5 ×12 cm). The arm extended from a central platform (5 × 5 cm). The mice were placed individually at the end of one of the open arms facing away from the central platform and the time it took to move from the open arm to either of the closed arm (TL) was recorded. TL was the time that elapsed between the time the animal was placed in the open arm and the time when it fully entered (all the four paws in) the closed arm. On the first day, the mice were allowed to explore the plus maze for 20 s after the measurement of TL. The mice were returned to their home cages after the first trial. Retention was examined 24 h after the 1st day trial. Each animal was again placed into the maze and transfer latency was recorded. Percentage retention of memory was calculated from the basal reading. The data are expressed as % deficit in memory and calculated as:

$$\frac{\text{Final transfer latency} - \text{Initial transfer latency}}{\text{Initial transfer latency}} \times 100$$

Biochemical parameters

Animals were sacrificed within few minutes by decapitation immediately after behavioral assessments of 10 min duration. The brains were removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1 M phosphate buffer (pH = 7.4). The post-nuclear fraction was obtained by centrifugation of the homogenate at 12000 × g for 20 min at 4°C.

Lipid peroxidation assay

The quantitative measurement of lipid peroxidation in the whole brain was measured according to the method of Wills [58]. The amount of MDA formed was measured by the reaction with thiobarbituric acid at 532 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as nanomoles of MDA per milligram protein using the molar extinction coefficient of chromophore ($1.56 \times 10\ M^{-1}\ cm^{-1}$).

Estimation of reduced glutathione

Reduced glutathione in the brain was estimated according to the method of Ellman [10]. A 1.0 ml of homogenate was precipitated with 1.0 ml of 4% sulfosalicylic acid by keeping the mixture at 4°C for 1 h and the samples were immediately centrifuged at 1200 × g for 15 min at 4°C. The assay mixture contained 0.1 ml of supernatant, 2.7 ml of phosphate buffer pH 8.0 and 0.2 ml of 0.01 M dithiobisnitrobenzoic acid (DTNB). The yellow color developed was read immediately at 412 nm using Perkin Elmer lambda 20 spectrophotometer. The results were expressed as nanomole GSH per milligram protein.

Catalase estimation

Catalase activity was assayed by the method of Luck [26], wherein the breakdown of hydrogen peroxide (H_2O_2) is measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2 phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and the change in absorbance was recorded at 240 nm. The results were expressed as micromole H_2O_2 decomposed per milligram of protein/min.

Nitrite estimation

The accumulation of nitrite in the supernatant, an indicator of the production of NO, was determined with a colorimetric assay with Greiss reagent [0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid] [13]. Equal volumes of supernatant and Greiss reagent were mixed, the mixture was incubated for 10 min at room temperature and the absorbance was measured at 540 nm using Shimadzu Spectrophotometer. The concentration of nitrite in the supernatant was determined from a standard curve and expressed as percentage of stress group.

Protein estimation

The protein content was measured according to the method of Lowry [25] using bovine serum albumin as a standard.

Drugs and treatment

The following drugs were used in the present study: naproxen (7 mg/kg and 14 mg/kg, *ip*), rofecoxib (5 mg/kg and 10 mg/kg, *ip*) and valdecoxib (5 mg/kg and 10 mg/kg, *ip*). Drugs were suspended in 0.25% CMC and administered *ip* 30 min before the animals were subjected to acute immobilization stress.

Statistical analysis

All the values are expressed as the mean ± SEM. The data were analyzed by using analysis of variance (ANOVA) followed by Tukey's test. $p < 0.05$ was considered statistically significant.

Results

Measurement of behavioral parameters (locomotor activity, memory and anxiety)

Six-hour acute immobilization caused significant locomotor activity impairment (as shown by decreased ambulatory movements), poor memory retention and anxiety-like behavior (increased latency to enter the mirror chamber, decreased number of entries and time spent in the mirror chamber) as compared to naive animals (animals placed on sawdust) ($p < 0.05$). Pretreatment with naproxen (7 mg/kg and 14 mg/kg, *ip*), rofecoxib (5 mg/kg and 10 mg/kg, *ip*) and valdecoxib (5 mg/kg and 10 mg/kg, *ip*) significantly improved ambulatory movement (Fig. 1), percent memory retention (Fig. 2) and anti-anxiety effect (Tab. 1) as compared to stress group (immobilized stressed) ($p < 0.05$).

Tab. 1. Effect of rofecoxib, valdecoxib or naproxen on anxiety in mirror chamber test

Drug treatment (mg/kg)	Latency to enter mirror chamber (mean ± SEM)	No. of entries in mirror chamber (mean ± SEM)	Time spent in mirror chamber (mean ± SEM)
Naive	63.6 ± 1.45	4.6 ± 0.65	67.8 ± 6.40
Stress	178.2 ± 27.13[a]	1.65 ± 0.50[a]	20.8 ± 3.60[a]
Rof (5)	44.0 ± 2.90[b]	5.0 ± 0.87[b]	41.2 ± 3.07[b]
Rof (10)	24.0 ± 2.04[b,c]	4.8 ± 0.577[b]	55.8 ± 4.53[b,c]
Val (5)	61.8 ± 5.93[b]	3.8 ± 0.66[b]	48.6 ± 2.59[b]
Val (10)	38.6 ± 5.51[b,d]	4.4 ± 0.67[b]	53.0 ± 4.39[b,d]
Nap (7)	62.4 ± 3.51[b]	4.6 ± 0.67[b]	58.8 ± 3.47[b]
Nap (14)	59.6 ± 5.17[b]	5.6 ± 0.577[b]	64.6 ± 4.25[b]

Values are expressed as the mean ± SEM. [a] $p < 0.05$ as compared to naive, [b] $p < 0.05$ as compared to stress, [c] $p < 0.05$ as compared to Rof (5), [d] $p < 0.05$ as compared to Val (5) (one-way ANOVA followed by Tukey's test)

Measurement of biochemical parameters (lipid peroxidation, reduced glutathione levels, brain nitrite levels and catalase activity)

Six-hour acute immobilization stress produced significant oxidative damage as shown by a significant increase in whole brain MDA, nitrite activity and depletion of reduced glutathione and catalase activity as compared to naive (non-stressed animals placed on sawdust) animals ($p < 0.05$). Pretreatment with naproxen (7 mg/kg and 14 mg/kg, *ip*), or rofecoxib (5 mg/kg and 10 mg/kg, *ip*) or valdecoxib (5 mg/kg and 10 mg/kg, *ip*) significantly attenuated MDA (Fig. 3), nitrite activity (Fig. 5) and restored depleted reduced glutathione level (Fig. 4) and catalase activity (Fig. 6) as compared to stress group (stressed animals) ($p < 0.05$).

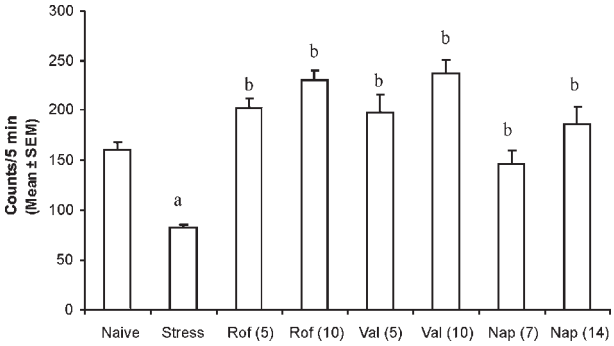

Fig. 1. Effect of rofecoxib, valdecoxib or naproxen on locomotor activity in acute immobilization stress. [a] $p < 0.05$ as compared to naive, [b] $p < 0.05$ as compared to stress group (one-way ANOVA followed by Tukey's test)

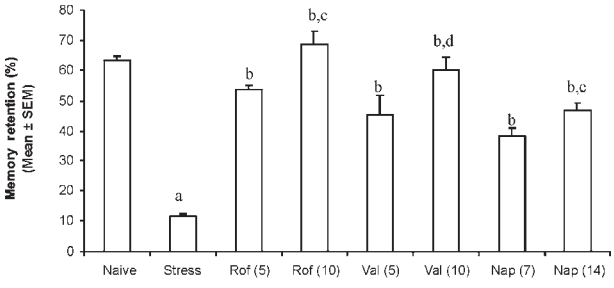

Fig. 2. Effect of rofecoxib, valdecoxib or naproxen on memory retention in acute immobilization stress. Values are expressed as the mean ± SEM. [a] $p < 0.05$ as compared to naive, [b] $p < 0.05$ as compared to stress, [c] $p < 0.05$ as compared to Rof (5), [d] $p < 0.05$ as compared to Val (5), [d] $p < 0.05$ as compared to Nap (7) (one-way ANOVA followed by Tukey's test)

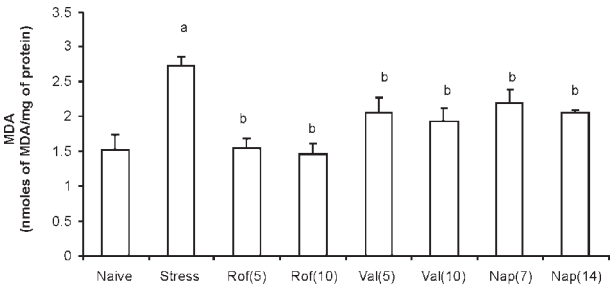

Fig. 3. Effect of rofecoxib, valdecoxib and or naproxen on brain lipid peroxidation. [a] $p < 0.05$ as compared to naive, [b] $p < 0.05$ as compared to stress group (one-way ANOVA followed by Tukey's test)

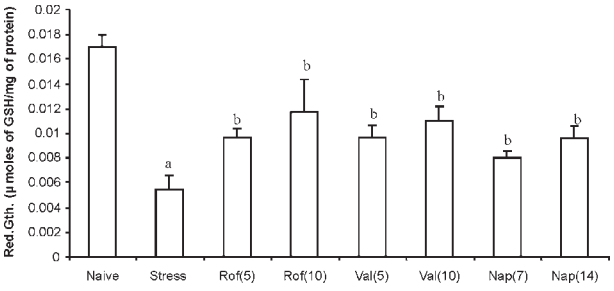

Fig. 4. Effect of rofecoxib, valdecoxib and or naproxen on reduced glutathione level. [a] $p < 0.05$ as compared to naive, [b] $p < 0.05$ as compared to stress group (one-way ANOVA followed by Tukey's test)

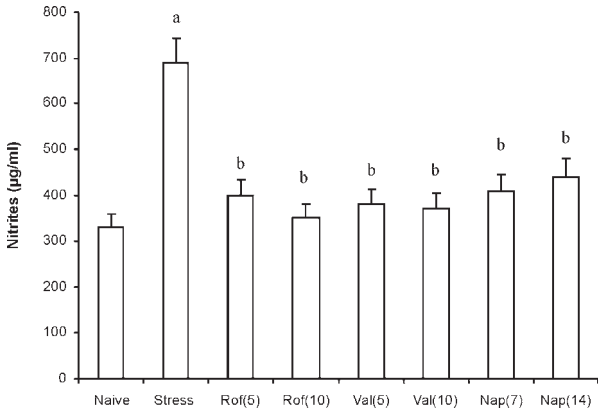

Fig. 5. Effect of rofecoxib, valdecoxib and or naproxen on brain nitrite levels. [a] $p < 0.05$ as compared to naive, [b] $p < 0.05$ as compared to stress group (one-way ANOVA followed by Tukey's test)

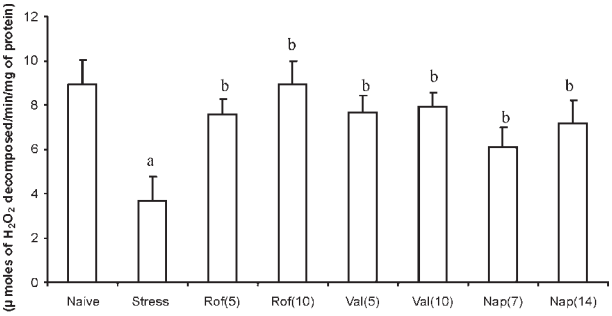

Fig. 6. Effect of rofecoxib, valdecoxib or naproxen on catalase activity. [a] $p < 0.05$ as compared to naive, [b] $p < 0.05$ as compared to stress group (one-way ANOVA followed by Tukey's test)

Discussion

The present study suggests that both non-selective and selective COX-2-inhibitors have neuroprotective effect against acute immobilization stress-induced behavioral alterations and oxidative damage in mice. The study further indicated that both non-selective and selective COX-2 inhibitors could be used to manage stress and related conditions.

The most striking distinctions between COX-1 and COX-2 are differential regulation of their expression and their tissue distribution [49]. However, this distinction is not entirely accurate, since COX-2 can be induced and up-regulated under certain conditions and COX-1 has been consistently shown to be expressed in brain [16, 18]. Prostaglandins are now recognized as important inflammatory mediators in neural tissues and brain functioning. COX-2 is expressed under basal conditions in the neuronal cells. Yamagata et al. [59] provided detailed description of cyclooxygenase isoenzymes in the brain. However, COX-1 has also been observed in neurons. Breder et al. [3] and other authors [35] reported COX-1 immunoreactivity in pyramidal and granular cells of hippocampus, cortex, and hypothalamic nuclei. It has been seen that COX-2 expression was triggered during hypoxic stress [47] and laminar shear stress [56]. In contrast, COX-2 expression was strongly repressed by glucocorticoids. Constitutive COX-2 immunoreactivity and COX-2 mRNA expression have been detected in neurons, especially in the forebrains [59]. The expression of COX-2 in the brain is relatively high in neonates [20, 43] since brain of neonates has a higher level of

cerebral PGs than that of adults. Besides, COX-2 has been proposed to play a key role in the final stages of development and brain modeling, when COX-2 becomes active in a manner that coincides with imprinting of environmental influences [19]. Nevertheless, several observations demonstrated the complex relationship between COX-1 and COX-2. Supporting the recent study of Goyal et al. [12], in the present study 6-h acute immobilization stress significantly impaired locomotor activity and anxiety-like behavior in animals. Marked behavioral changes might be due to alterations in the brain regions controlling motor activity and anxiety-like behavior. Impaired motor activity could be due to stress-induced depression [37]. It is lucid that oxidative stress plays a role in the pathogenesis of motor activity. Hyperactivity of CNS has also been strongly implicated in the pathophysiology of anxiety. Immobilization stress has also been reported to induce 2–3-fold higher rise of plasma cortisol level [9]. Increased cortisol level has been linked with anxiety-like behavior and painful response in humans [4, 14]. Centrally administered corticotropin-releasing factor (CRF) has numerous actions including mediation of anxiety, feeding and stimulation of sympathetic nervous system [32, 38, 51]. Acute stress has been reported to influence behavioral activity such as motor activity, anxiety-like effect and depression [8, 12, 22, 36, 37, 39, 50]. In the present study, acute immobilization stress caused poor retention of memory, which is further improved by pretreatment of both non-selective and selective cyclooxygenase (COX-2) inhibitors. Study suggested that COX-2 plays a role in the selective loss of neural connections but not in their formation [20, 37]. Recent studies have shown that COX-2 potentiated brain parenchymal amyloid plague formation leading to Alzheimer's disease, thus supporting a therapeutic potential for NSAIDs in the treatment of neurological disease [5, 46, 53, 57]. It has been well documented that high level of stress, fear commonly causes memory loss and disturbs cognition [27, 41]. Exposure to stress causes subsequent impairment of hippocampus-dependent forms of memory in both humans and animals [36, 41]. Further, hippocampus also regulates stress response and inhibits HPA response to stress. Acute stress can impair short-term memory, but repeated stress causes the atrophy of dendrites of pyramidal neurons in the CA_3 region of the hippocampus through a mechanism involving both glucocorticoids and excitatory amino acid neurotransmitters released during and after stress [36]. Stress has also been reported to alter brain acetylcholinesterase activity and thus to be involved in cognitive dysfunction [7].

In the present study, administration of clinically relevant dose of naproxen, rofecoxib and valdecoxib produced remarkably neuroprotective effect in mice against restraint stress. Pretreatment with naproxen, rofecoxib and valdecoxib significantly reversed impaired locomotor activity. Jain et al. [18] also reported that COX inhibitor reversed immobility period against lipopolysaccharide administration [18]. Besides, beneficial role of COX inhibitors in various neurodegenerative disorders have also been suggested [34]. One study reported that PGE_2 levels were higher in the substantia nigra of the Parkinson's disease patients [34]. Furthermore, peroxidase activity of COX has been shown to catalyze the oxidation of dopamine to reactive dopamine quionone, which causes damage to dopaminergic neurons [15, 55]. Improvement in locomotor activity by COX inhibitors represents a viable therapeutic option for parkinsonian-like patients.

In the present study, naproxen, rofecoxib and valdecoxib significantly improved memory retention, suggesting their role in stress-induced memory dysfunction. Documentary benefits of NSAIDs in AD, aged-related memory disorders further confirm present observations [28, 46]. Rogers et al. [46] reported that AD patient treated for 6 months with indomethacin, non-selective COX inhibitor showed an improvement in cognitive function tests. Jain et al. [18] also demonstrated that naproxen could ameliorate CNS parenchymal cell death and edema formation mediated by excessive activation of neuronal NMDA receptors *in vivo* with no adverse effect. Jain et al. [18] study suggested that naproxen could be a promising candidate for the treatment of neurological diseases associated with over-activation of NMDA receptors [50]. Pretreatment with naproxen, rofecoxib and valdecoxib significantly attenuated anxiety like effect in animals, suggesting their beneficial role in anxiety states. However, the exact role of COX isoenzymes and their expression in anxiety and related states remains to be defined. It seems that both selective and non-selective COX isoenzymes have neuromodulatory action.

Oxidative stress has been implicated in the pathophysiology of many neurological disorders, such as AD, Huntington's disease, etc. [24, 44]. Oxidative stress can cause cellular damage and neurodegeneration by inducing the reactive oxygen species (ROS)

that oxidize vital cellular components such as lipids, proteins and DNA [33]. In the present study, 6-h immobilization stress significantly increased lipid peroxidation, nitrite activity and depleted reduced glutathione and catalase activity in stressed mice brains, suggesting immobilization-caused oxidative damage. Stress has also been known to increase oxidants and impair antioxidant defenses [35, 38]. Moreover, COX-2 leads to release of inflammatory PGs, such as PGE_2 that account for the accumulation of oxidative mediators in stressed brain. COX-isoforms also lead to the formation of hydroxyl free radicals and form peroxynitrite free radicals due to peroxidase activity. Therefore, inhibiting COX isoenzymes has been proposed to inhibit induction and accumulation of oxidants. One source of hydroxyl radicals is the peroxynitrite, which is generated by the spontaneous reaction of $O_2^{-\bullet}$ and NO. Expression of inducible nitric oxide synthase and COX-2 enzymes increases in response to acute stress [30, 32, 50] and production of oxygen and nitrogen free radicals causes oxidation of cellular components in the brain [45].

In the present study, pretreatment with both naproxen, rofecoxib and valdecoxib COX inhibitors reduced lipid peroxidation, nitrite levels and restored the depleted reduced glutathione and catalase activity, suggesting their neuroprotective role against immobilization stress. However, there are few inadequate reports exploring the protective effect of COX-inhibitors in restraint stress and related conditions. Further, the discovery of selective COX-2 inhibitors has improved our understanding on COX and its biology. Therefore, exact cellular events in their neuroprotection are still to be explored.

In conclusion, the present study suggests the neuroprotective and antioxidant effect of both non-selective and selective COX-2-inhibitors against acute immobilization stress-induced behavioral alterations and oxidative damage in mice. The present study further provides hope that these non-selective and selective cyclooxygenase enzyme (COX-2) inhibitors could be developed as potential remedies for the management of stress and related conditions.

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