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Vitamin C Attenuate Neurological Changes in the Medial Prefrontal Cortex of Juvenile Mice Exposed to Diclofenac Sodium

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ABSTRACT

Diclofenac sodium is one of the commonly used therapeutic non-steroidal anti-inflammatory drugs; notwithstanding, diverse adverse effects are clearly described. In humans, vitamin C is an essential nutrient that is ubiquitously a water-soluble electron donor with biological characteristics. To a greater extent, it has been widely recognized not only as an antioxidant but also as a specific co-factor in patho-enzymatic processes and reactions. This study investigated the effect of vitamin C on the medial prefrontal cortex (mPFC) of juvenile mice exposed to Diclofenac sodium. Thirty juvenile mice were randomly assigned into 5 experimental groups; control, saline-treated, vitamin C treated, Diclofenac sodium treated, and vitamin C + Diclofenac sodium. Histochemical, immunohistochemical, stereological, and quantitative neurochemical studies were respectively, employed to assess the effect of vitamin C on Diclofenac sodium-associated neurological damage. Results showed that the histoarchitectural profile of the mPFC was well preserved in the control, saline, vitamin C, and vitamin C + Diclofenac sodium treated groups compared with the Diclofenac sodium treated group. Exposure to Diclofenac sodium during elicited significant glutamate level reduction in the mPFC. Furthermore, co-administration of vitamin C + Diclofenac sodium significantly decreased ($p<0.05$) glutamate level compared with the Diclofenac sodium-treated group. It could be concluded from this study that vitamin C conferred neuroprotective effect on the mPFC of the juvenile mice exposed to Diclofenac sodium.

Key words: *Brain; Cell count; Drug abuse; Neuronal dysfunction; Neurotransmission; Central nervous system*

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used in household for the relief of aches and pain, congestion of blood vessels or inflammation, and fever or pyrexia (Bures et al. 2002; Campbell et al. 2017). As with other NSAIDs, Diclofenac sodium [sodium-(O-((2,6-dichlorophenyl)-amino)-phenyl)-acetate], has potent anti-inflammatory, analgesic, and antipyretic effects. However, Diclofenac sodium may cause some adverse cerebral and cerebellar effects such as convulsion, disorientation, hallucination, and loss of consciousness (Kudo et al. 2003). Studies have shown that Diclofenac sodium is a potent anti-inflammatory, analgesic, and antipyretic (Siu et al.

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2000; Kudo et al. 2003; Chang et al. 2005). It is widely used for the alleviation of pain, fever, and inflammation associated with arthritis, rheumatoid arthritis, osteoarthritis, acute gout, dysmenorrhoea, menorrhagia, and postoperatively after some surgery (Siu et al. 2000). Treatment with Diclofenac sodium may be accompanied by adverse effects such as serious upper gastrointestinal bleeding, platelet dysfunction, convulsions, and cardiovascular hazard. However, the neurological effect(s) of Diclofenac sodium on the central nervous system (CNS) of juvenile subjects remain unclear (Kudo et al. 2003).

Diclofenac sodium prevents the cell cycle at the Go/G1 phase, thereby inducing cytotoxicity and cell death (Chang et al. 2005). Diclofenac sodium not only inhibits the proliferation of neural stem cells but also suppresses their differentiation into neurons (Kudo et al. 2003). Although these side effects have been widely reported (Yasmeen et al. 2007), little is known about the effect of NSAIDs on the development of the central nervous system or the peripheral nervous system. Ragbetli et al. (2007) previously reported a toxic effect of Diclofenac sodium on the sciatic nerve, pyramidal and granular cells of the cerebellum, and Purkinje cells of male rats but not females, following prenatal exposure. Although the side effects of Diclofenac sodium on several tissues and organs have been mentioned, information on the effects of Diclofenac sodium is scarce regarding its effect on the mPFC during juvenile life.

Exposure to various levels of toxicants during this developmental period can make the animals susceptible and vulnerable to adult exposure to different toxic substances. Several pharmacological substances suggest gender differences for neurotoxic (deleterious) effects (Torres-Rojas and Jones 2018).

On the other hand, vitamin C (ascorbic acid) has several different functions in humans and other mammals. In addition to its well-known role as an antioxidant, the vitamin serves as a co-factor in several important enzymatic reactions, including synthesis of catecholamines, carnitine, cholesterol, amino acids, and certain peptide hormones (Akbari et al. 2016; Animoku et al. 2018). Of course, its best-known function is to facilitate the hydroxylation of proline and lysine residues in collagen, allowing proper intracellular folding of pro-collagen for export and deposition as mature collagen (Padayatty and Levine 2016).

Ascorbate has also been shown to assist other prolyl and lysyl hydroxylase in the hydroxylation of hypoxia-inducible factor 1α (HIF-1α). HIF-1α is a transcription factor responsible for the other cellular response to low oxygen conditions through the activation of genes controlling diverse cellular pathways including glycolysis, iron transport, angiogenesis, and cell survival (Lee et al. 2020). In all of its known functions, ascorbate serves as one-electron donor, generating the ascorbate free radical (AFR). The AFR is reduced back to ascorbate within the cells by NADH and NADPH-dependent reductase that has a high affinity for the low concentrations of the radicals generated.

This study was undertaken to examine the effects of vitamin C against Diclofenac sodium-induced neurotoxicity in the mPFC of juvenile BALB/c female mice.

MATERIALS AND METHODS

Statement on Ethics

The use of animals and the methods employed in this study were approved by the Health Research Ethics Committee (UNIOSUN/CoHS-2021-0003) of the College of Health Sciences, Osun State University, Osogbo, Osun State, Nigeria, with strict adherence to the guidelines of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (Gettayacamin and Retnam 2017).

Animal Husbandry, Groupings, and Treatments

Timed pregnant BALB/c mice were purchased from Newton-Maxwell Farms (Ede, Osun State, Nigeria): Pups were littered after 21 days of gestation. Three weeks after delivery, the genders of the pups were determined by an animal scientist (blind to the itinerary of the study). Thirty female mice were weaned from the dam and were randomly distributed into 5 groups (n=6). The groups were designated as control (not exposed to any protocol); saline-treated mice received a daily dose of 0.1 mL/kg of normal saline (vehicle); vitamin C treated mice received a daily dose of 15 mg/kg of vitamin C; Diclofenac sodium treated mice received a daily dose of 10 mg/kg Diclofenac sodium. In the vitamin C + Diclofenac sodium co-treated, the mice in this group were exposed to a decoction of 15 mg/kg of vitamin C and 3.6 mg/kg of Diclofenac sodium. The animals in the respective experimental groups were subcutaneously exposed to the treatment paradigm on daily basis for 14 consecutive days.

Animal Sacrifice

Twenty-four hours after the administration of the last respective doses (i.e. 15th day), five mice in each of the groups were rapidly decapitated (Kook et al. 2014) and processed for glutamate quantification, Nissl stain and immunohistochemical demonstration of astrocytes in the mPFC. A mouse brain atlas (Paxinos and Franklin 2004) was used to establish the identity of the mPFC (anterior cingulate cortex; bregma +1.2 mm to +0.6 mm); the mPFC were dissected into two halves (i.e. right and left halves). Halves (right) of the mPFC were fixed in a freshly prepared 10% neutral-buffered formalin, and histologically processed for Nissl staining to demonstrate the overall histomorphology of the neuronal cells, and glial fibrillary acidic protein (GFAP) to demonstrate astrocytic activation in the mPFC.

Biochemical Study

Preparation of Tissue Homogenate and Glutamate Estimation

The preparation of the mPFC homogenates was done according to the method of Biala et al. (2018). Briefly, the left halves of the mPFC were rinsed in ice-cold phosphate-buffered saline, homogenized in cold 10% Tris buffer (pH 7.4) and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected for spectrophotometric evaluation of the level of glutamate using HITACHI 2800 apparatus and microplate reader (EPOCH).

Glutamate levels were quantified in the mPFC tissue homogenate using the enzymatic method (Kazi and Oommen 2014). The method is based on the dehydrogenation of glutamate by glutamate dehydrogenase coupled to NAD+ and the amount of NADH formed was estimated spectrophotometrically at 340 nm. Glutamate levels were estimated from standards (10-50 nm) run in parallel and expressed as nmoles glutamate/mg protein.

Microscopic Studies

Demonstration of Nissl Substance

Paraffin-embedded sections (5 µm) of the mPFC were mounted on glass slides (Menzel Gläser). The sections were deparaffinized, rinsed in water, stained for 15 min in 0.1% cresyl violet, and rinsed in an acetate buffer (pH 4.0). The sections were then respectively differentiated in 96% ethanol for 30 sec, dehydrated in absolute ethanol, cleared in two changes of xylene, and mounted with dibutylphthalate polystyrene xylene (DPX). In this study, viable neuronal cells were typically depicted by a light purple cytoplasm with a conspicuous nucleus, while dead neuronal cells were portrayed by dark purple-stained cytoplasm and by diminishing nucleus.

Immunohistochemistry for Light Microscopy

Thin sections of 5 µm thickness were obtained from routine paraffin-embedded mPFC. After deparaffinization, the sections were subjected to heat-mediated antigen retrieval in citrate buffer solution (pH 6.0). Endogenous peroxidase blocking was done with 0.3% hydrogen peroxide. Sections of the mPFC were then incubated overnight at 4°C in rabbit anti-GFAP (ThermoFisher, USA; #16825-1-AP) at 1:1,000. Secondary antibody incubation was done in ImmPRESS® horseradish peroxidase (HRP) anti-rabbit IgG (peroxidase) polymer reagent, made in a horse (Vector® #MP- 7401). 3, 3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector® #SK-4100) was used for colour development. The sections were counterstained in freshly prepared 0.1% cresyl violet and subsequently treated in 1% acid alcohol to reduce the counter-stain intensity.

Cell Count

Cresyl violet stained and GFAP immunolabelled sections were respectively, observed under a light microscope and photomicrographs were obtained at 400x magnification. Ten high power fields from each group were randomly selected for cell quantification and analysis was performed with Image J software (NIH, USA). The quantification of viable and dead cells was performed using the method of Mohammed et al. (2014). Astrocytic immunoreactivity was quantified by counting positive expressed cells using the cell counter tool of Image J software as previously described by Akingbade et al. (2021).

Data Analysis

Statistical analyses of data obtained in this study were performed using Graph-Pad Prism 3.0 software (Graph-pad software, San Diego, CA). One-way ANOVA and Sidak's test for a series of independent post hoc pair-wise comparisons between groups were carried out. The results were expressed as mean ± SD. Differences between data sets were considered as significant when $p < 0.05$.

RESULTS

Glutamate Level in the mPFC

In Figure 1, the glutamate levels in the prefrontal cortices of the experimental juvenile mice are presented. Subcutaneous injection of saline or vitamin C showed significantly different ($p<0.05$) glutamate levels compared with the control. Furthermore, the subcutaneous administration of Diclofenac sodium significantly increased ($p<0.01$) glutamate level compared with the control, saline, and the vitamin C treated groups respectively. On the other hand, the co-administration of vitamin C + Diclofenac sodium significantly

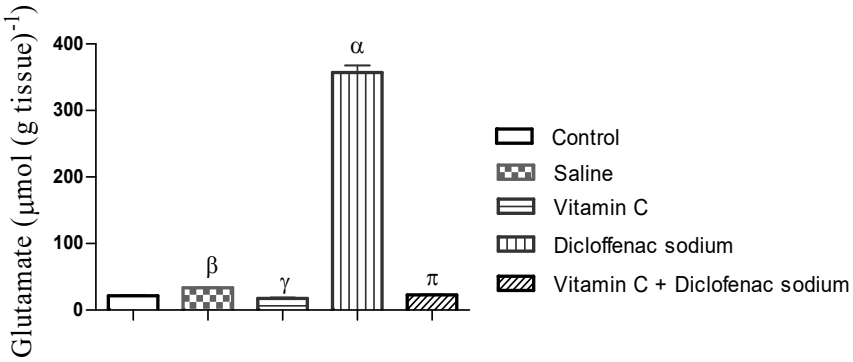

Fig. 1: Level of glutamate in the mPFC homogenate of the mice. Data are presented as the mean ± SEM. One way ANOVA, post hoc Sidak's multiple comparison test. α, β, γ, and π values are significantly different at $p<0.05$

decreased ($p<0.05$) the glutamate level compared with the Diclofenac group, but was not significantly different from the vitamin C group.

cytoplasm and peri-nuclear Nissl deposits, pyknotic neurons, and neurons with a ruptured membrane. The neuroprotective effect of vitamin C was observed

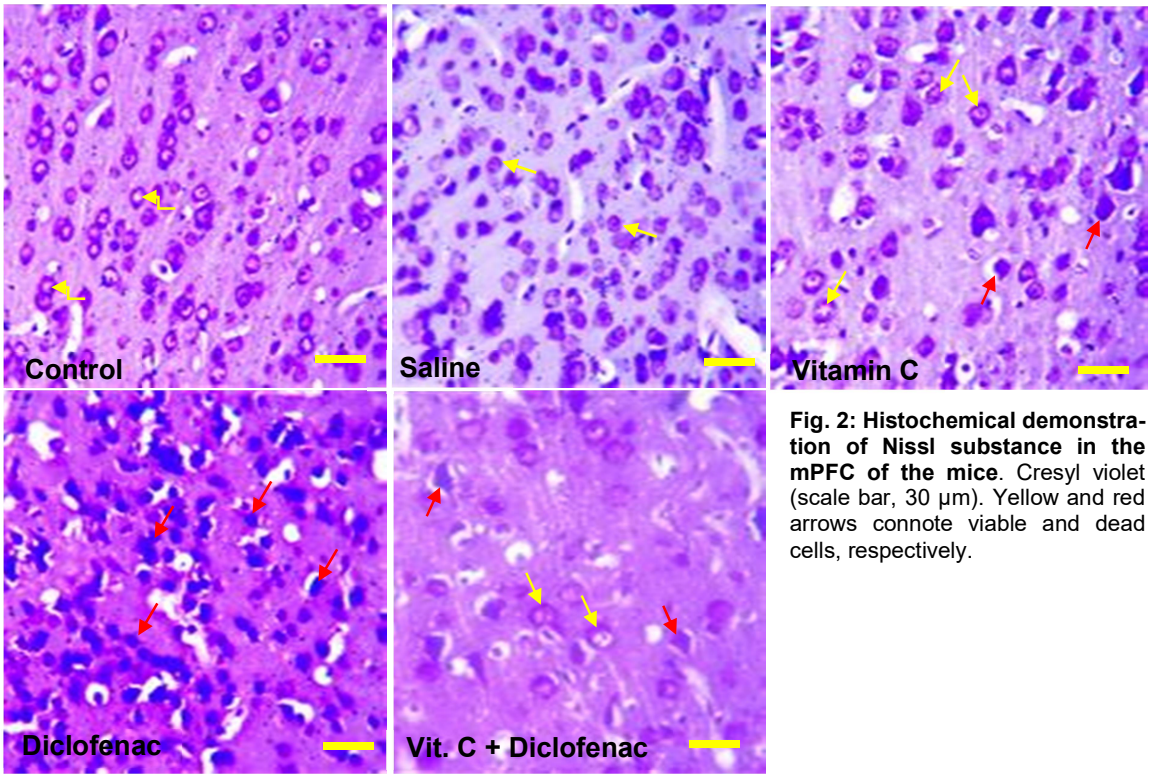

Fig. 2: Histochemical demonstration of Nissl substance in the mPFC of the mice. Cresyl violet (scale bar, 30 µm). Yellow and red arrows connote viable and dead cells, respectively.

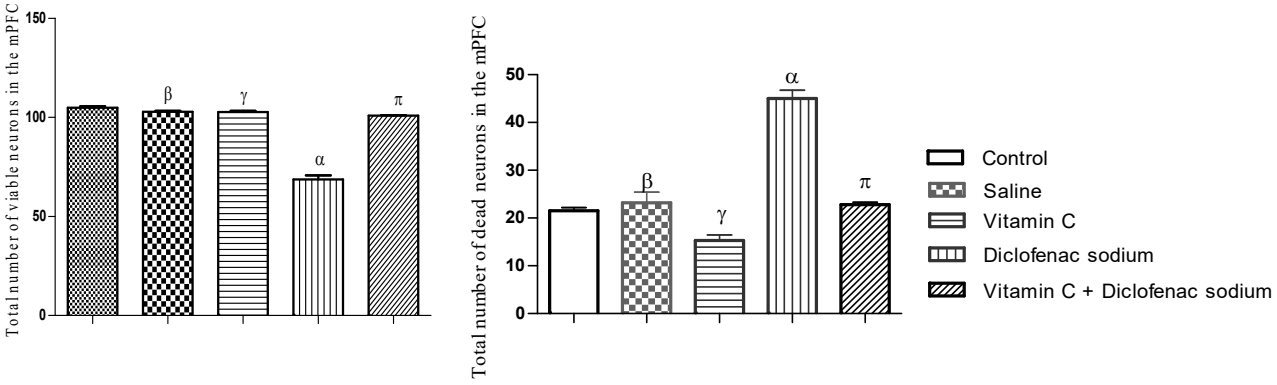

Fig. 3: Quantification of the total number of viable and dead neurons in the mPFC of the mice. Data are presented as the mean ± SEM. Oneway ANOVA, post hoc Sidak's multiple comparison test. α, β, γ, and π values are significantly different at $p<0.05$

Histochemistry of Nissl Substance in the mPFC

Histochemical demonstration of Nissl substance in the mPFC with cresyl violet stain revealed that the control group neurons appeared normal with prominent basophilic cytoplasm. A similarly normal histological feature was observed in the saline-treated group. The Nissl substance in the mPFC of the mice in the vitamin C treated group was well preserved. There were observable changes in the distribution of Nissl substance in the Diclofenac-sodium `treated group. These include; chromatolysis, fragmented

in the Diclofenac-sodium + vitamin C treated group. The histological features were preserved compared with the Diclofenac-sodium treated group (Fig. 2).

Upon quantification of viable and dead neurons in the mPFC of the experimental mice (Fig. 3), the number of viable cells was not significantly different between the control and the saline, vitamin C and vitamin C + Diclofenac sodium co-treated groups, but significantly more ($p<0.001$) compared with the Diclofenac sodium treated group. There was no significant difference between the saline group compared with the vitamin

C and vitamin C + Diclofenac sodium co-treated treated groups, but significantly more ($p<0.001$) compared with the Diclofenac sodium treated group. The number of viable cells was significantly more ($p<0.001$) in the vitamin C treated group compared with the Diclofenac sodium treated group, but not significantly different from the vitamin C + Diclofenac sodium co-treated group; The number of viable cells was significantly less ($p<0.001$) in the Diclofenac sodium treated group compared with the vitamin C + Diclofenac sodium co-treated group.

Immunoreactivity of GFAP in the mPFC

Immunohistochemical demonstration of astrocytes in the mPFC of the mice using GFAP is presented in Figure 4. In the control and Diclofenac-sodium + vitamin C treated groups, astrocyte processes were not overlapping, and many of the astrocytes did not express detectable GFAP. In the vehicle and vitamin C treated groups, there was moderately reactive astrogliosis; most of the astrocytes had slight expression of GFAP though without noticeable overlapping of astrocytic processes or proliferation. In the Diclofenac-sodium treated group, there were abundant

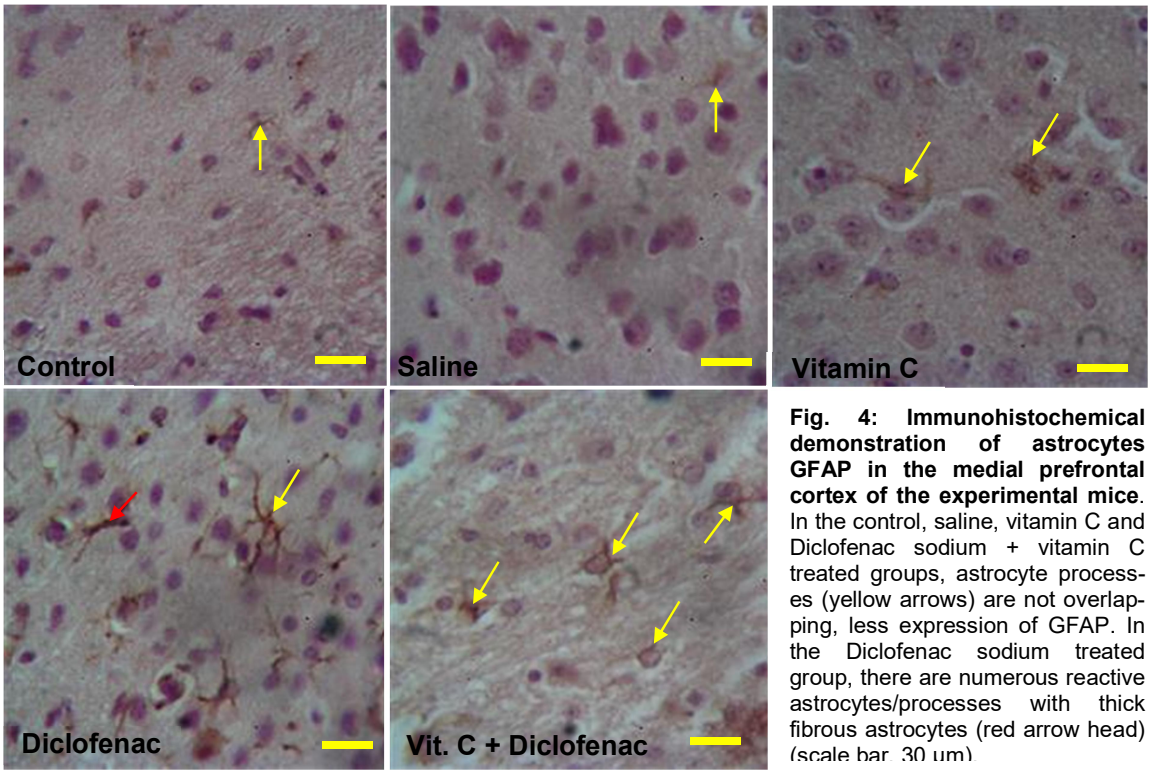

Fig. 4: Immunohistochemical demonstration of astrocytes GFAP in the medial prefrontal cortex of the experimental mice. In the control, saline, vitamin C and Diclofenac sodium + vitamin C treated groups, astrocyte processes (yellow arrows) are not overlapping, less expression of GFAP. In the Diclofenac sodium treated group, there are numerous reactive astrocytes/processes with thick fibrous astrocytes (red arrow head) (scale bar, 30 um)

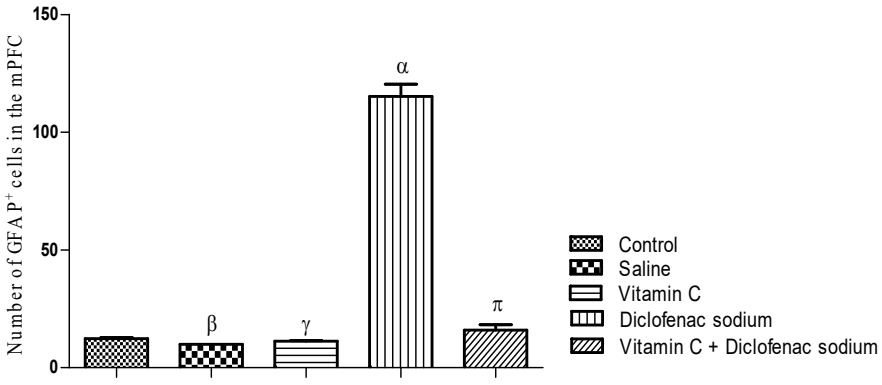

Fig. 5: GFAP immunoreactive cells in the mPFC of the mice. Data are presented as the mean ± SEM. Oneway ANOVA, post hoc Sidak's multiple comparison test. α, β, γ, and π values are significantly different at $p<0.05$

reactive astrocytic processes. In the micrograph, slight GFAP expression was observed at the perimeter of many vacuolated neurons.

The number of GFAP-positive cells in the mPFC of the mice is presented in Figure 5. There was no significant difference between the control, the saline, vitamin C and vitamin C + Diclofenac sodium co-treated groups, but significantly less ($p<0.001$) in the Diclofenac sodium treated group. Furthermore, there was no significant difference between the saline and the vitamin C and vitamin C + Diclofenac sodium co-treated groups, but significantly less ($p<0.001$) compared with the Diclofenac sodium treated group. The GFAP-positive cells was significantly less ($p<0.001$) in the vitamin C treated group compared with the Diclofenac sodium treated group, although there was no significant difference with the vitamin C + Diclofenac sodium co-treated groups. There was significantly more ($p<0.001$) GFAP-positive cells in the Diclofenac sodium treated group compared with the vitamin C + Diclofenac sodium co-treated group.

DISCUSSION

The neuroprotective effect of vitamin C on Diclofenac sodium-induced damage in the mPFC of experimental juvenile mice was studied. In a study by Rebec and Pierce (1994), it was observed that ascorbate was released from glutaminergic neurons as part of the glutamate reuptake process, leading to the postulation that ascorbate was capable of modulating the effects of dopamine in the mammalian brain. In many respects, Ascorbic acid appears to act as a dopamine receptor antagonist. Chattopadhyay et al. (2001) alluded to the fact that daily exposure to vitamin C in rats played a pivotal role in maintaining normal brain monoamines. Furthermore, Bornstein et al. (2003) reported that vitamin C acts as co-factor in catecholamine synthesis, which is involved in the conversion of dopamine to norepinephrine. Rebec and Pierce (1994), as well as Kocot et al. (2017) reported that vitamin C acts as a neuromodulator which positively enhances the release of neurotransmitters while inhibiting their binding to receptors.

The observed effect of vitamin C in the present study supports the assertion that ascorbate have important modulatory functions in the CNS. The effect of the treatment paradigm in the present study further attests to the role of vitamin C in modulating glutamatergic neurotransmission (Moretti et al. 2011). The increased glutamate level in the Diclofenac treated group may occur as a result of degenerative processes triggered by Diclofenac sodium administration, leading to excessive release, impaired uptake, or poor mop-up of glutamate. This observation is in tandem with previous reports (Coyle and Puttfarcken 1993; Domith et al. 1997). Studies by Rebec and Pierce (1994) and Domith et al. (2018) reported that ascorbate transport from astrocytes to the extracellular environment was linked to glutamate uptake, a process known as "ascorbate-glutamate heteroexchange", which reduces extracellular glutamate level and successively, decreases excitotoxicity and pro-oxidate damage (Oliveira et al. 2019; Kaźmierczak-Barańska et al. 2020). Vitamin C also decreases glutamate uptake, suggesting that the above-mentioned effects may have been triggered by the accumulation of extracellular glutamate. ‘

The inhibitory effect on the level of glutamate correlates with the reduced number of GFAP immunopositive cells and dead neurons, and the increased number of viable neurons promoted by vitamin C in the vitamin C only and vitamin C + Diclofenac sodium co-treated groups. It is significant to highlight that the increase in the level of glutamate seen in the Diclofenac sodium treated group is in consonant with the total number of dead neurons observed in the same treated group. This observation does not exclude the possibility that Diclofenac sodium is also controlling the transport mediated by some 'silent receptor' subtypes. The study by Agostinho et al. (1997) suggested that ascorbate in the presence of Fe^{2+} could inhibit glutamate transport via a mechanism related to the Fenton reaction. May (2012) also reported that 5,5'-dithio-bis-(2-nitrobenzoic acid), a thiol oxidant, decreases while dithiothreitol, an antioxidant increases glutamate uptake suggesting that the glutamate transporters may be regulated by a redox process. However, in our study, we observed that vitamin C inhibited the level of glutamate. Baek et al. (2016) reported that high concentrations of ascorbate increase protein kinase C activity and this kinase appears to regulate excitatory amino-acid transporters membrane levels, therefore, moderating glutamate uptake regulation. In this study, we cannot exclude the possibility of vitamin C regulation of protein kinase C activity, and consequently regulating the transport of glutamate in the model used in our study.

Nissl substance is a basophilic material mainly composed of ribonucleic acid (RNA) and proteins in aggregate with the rough endoplasmic reticulum. This substance is associated with protein synthesis and cellular metabolism. Nissl substance can vary in quantity, form, and distribution in different types of neurons (Garman 2011), and can be lost completely when a neuron is injured (Moon 2018). According to Nichols et al. (1993), morphological modifications affecting the availability, distribution, and functional integrity of Nissl substance is capable of inhibiting cell membrane, as well as protein synthesis, and could negatively affect the overall neurologic functions of the brain. In the present study, another fascinating morphological change was that of astrocyte proliferation in the studied brain regions. As the proliferation of astroglial cells usually is a reactive response to neurodegeneration or injury (Kettenmann and Verkhratsky 2011), it is possible to occur in

response to significant neuronal loss, as observed in the Diclofenac sodium treated group. This situation has the potential of further complicating impaired function arising as a result of axonal demyelination. However, the co-administration of vitamin C significantly preserved the Nissl substance.

In the present study, degeneration of neuronal cells in the mPFC of mice following exposure to Diclofenac sodium indicates deleterious effects in the juvenile period of brain development. This observation is in agreement with Yurt et al (2017) who reported that the processes involved in Diclofenac sodium toxicity are the same in different parts of the central nervous system. Statistically, significant cell losses have also been observed in the spinal cord of 20-week-old rats exposed to Diclofenac sodium in the prenatal phase of development compared to rats of the same age in the sham group. In a 2011 study, Özyurt et al. (2001) showed that exposure to Diclofenac sodium could trigger condensation of chromatin and shrinkage of cytoplasmic contents often resulting in cellular necrosis.

Additionally, a related study by Ragbetli et al. (2007), observed that Diclofenac sodium triggered a reduction in the number of Purkinje cells in the cerebellum of the experimental animals, and was consistent with other outcome (Gokcimen et al. 2007; Ragbetli et al 2007). Juvenile exposure to Diclofenac sodium triggered degeneration of neurons in the present study. This shows that this period of brain development and its associated topographies is very sensitive to Diclofenac sodium, as exposure can cause brain development alternations, and hence, increased degeneration of neuronal cells in the mPFC.

Over the past years, reports showed the antioxidant, enzyme co-factor, and brain neuromodulator roles of vitamin C (Adekeye et al. 2015; Meščić et al. 2019), with other reports on vitamin C inhibiting actions against deleterious effect of a neurotoxin, dexamethasone (Araque et al. 2014; Adekomi et al. 2019b). Yurt et al. (2017) further suggested that the deleterious effect of Diclofenac sodium could have been triggered following burst activities in the oxidative stress pathway within the central nervous system.

It was observed in the present study that Diclofenac sodium significantly reduced the number of neuronal cells in the mPFC of juvenile mice. This observation is similar to the study of Yurt et al. (2017), who reported significant cell loss in the hippocampus of rats exposed to Diclofenac sodium during prenatal life. A similar observation was also recorded by Gokcimen et al. (2007), Özyurt et al. (2011) and Ragbetli et al. (2007) Furthermore, the observed reduction in the number of neuronal cells in the mPFC may be linked to the ability of Diclofenac sodium to trigger the production of reactive oxygen species in the brain as reported by Chan et al. (2001), Gokcimen et al. (2007) and Yurt et al. (2017).

Previous laboratory-based studies have suggested that the mechanisms of Diclofenac sodium toxicity are a result of the production of oxidative stress, marked mitochondrial damage, and the interaction of its reactive metabolites with cellular macromolecules, resulting in a significant modification of cellular integrity of cellular proteins (Galati et al. 2002; Masubuchi et al. 2002; Owumi and Dim 2019).

Stereological, histological and immunohistochemical analyses showed that the Diclofenac-sodium group exhibited neuropathologic features in the mPFC neurons: Cell margins could not be easily differentiated. Vitamin C elicited neuroprotection against Diclofenac sodium in the vitamin C + Diclofenac sodium co-treated group. Exposure to Diclofenac sodium was associated with morphological modifications in astrocytes with increased population, which coincided with neuronal loss. In addition, the astrocytes possessed long thin processes, whereas, in the control, saline, vitamin C, and vitamin C + Diclofenac sodium treated groups there were relatively no glia to few glia cells with short processes. Most importantly, in the vitamin C + Diclofenac sodium treated group, there was relatively no immunopositive reaction with GFAP. Additionally, it could also be stated that the changes in the GFAP immuno-expression and astroglial morphology observed in the Diclofenac sodium treated group compared with the control, saline, vitamin C and the vitamin C + Diclofenac sodium treated groups respectively may be a manifestation of compensatory responses or survival modalities against neurodegeneration. As observed in this study, the significant increase in the GFAP immunopositive intensity may be a result of increased metabolic demand or owing to morphological alterations in the glia structure as a result of exposure to Diclofenac sodium (Yang and Wang 2015).

The observed alterations in GFAP-immunoreactive cells could have a profound impact on neural plasticity since astrocytes can actively modulate neuronal activity (Araque et al. 2014). The GFAP-immunoreactive cells in the mPFC following exposure to Diclofenac sodium is presumably the result of the chemotactic factors triggered by the degenerating neurons, and this further suggests that astrocytes can serve as facultative phagocytes in drug-related neurotoxicity (Adekomi et al. 2019a). In this context, Diclofenac sodium-induced neuronal degeneration model may provide a good system to study the neuron-glia interactions in response to neuronal injury. These observations are in consonant with Kudo et al. (2003). Exposure to Diclofenac sodium was accompanied by marked changes in the expression and significantly increased number of GFAP-labelled cells in the mPFC of the juvenile mice compared with the other experimental groups. In the non-human primate substantia nigra, the number of GFAP immunoreactive glia increased after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection

(Kanaan et al. 2008) suggesting that astrocytic activation may be vital in pathogenesis.

Astrocytes play intrinsic roles in brain defense mechanisms, and the control or regulation of homeostasis in the CNS (Omotoso et al. 2019). In the pathology of the CNS, astrocytes are stimulated and thereafter undergo marked changes in their histomorphology and molecular expressions (Hamby and Sofroniew 2010; Omotoso et al. 2019). The high expression of GFAP in the mPFC of Diclofenac sodium treated mice was evidence of ongoing reactive astrogliosis. Diclofenac sodium induced neuronal toxicity that potentiates astrogliosis, and is associated with inflammatory processes and neuronal degeneration. Although reactive astrogliosis is a non-specific response, it is highly characteristic and involves various morphological and molecular changes (Nash et al. 2011). A large number of astrocytes are post-mitotic in physiological state, and extremely active proliferation is usually connected with astrogliosis (Bardehle et al. 2013).

The administration of Diclofenac sodium triggered upregulation of the expression of GFAP and also altered the histomorphology of the neurons in the cytoarchitectural profile of mPFC of the present study. Furthermore, GFAP-immunoreactivity was sensitive in vitamin C intervention resulting in marked reduction of astrocytic count in the vitamin C and Diclofenac sodium co-treatment group.

In the mature astrocytes, GFAP is the primary filamentous protein, an indispensable constituent of the cytoskeleton involved in astrocyte formation (Middeldorp and Hol 2011). There are numerous populations of astrocytes in the CNS and are the foremost cells following many forms of injury to the CNS; therefore, GFAP has been employed as a neuropathological biomarker in various neurotoxicity studies (Lei et al. 2015; McMahon et al. 2015). The marked upregulation of GFAP immunoreactivities and the significantly increased number of dead neurons observed in the mPFC of the mice in the group treated with Diclofenac sodium alone is denotative of astrocytic response to the deleterious effect of Diclofenac sodium. The observed astrocytic reaction is suggestive of defense against the observed increased total number of dead neurons. Gylys et al. (2004) reported that astrocytosis with hypertrophied processes is a compensatory mechanism for neuronal and synaptic degeneration as represented by the observed neuronal cell degeneration in the mice exposed to Diclofenac sodium. Administration of vitamin C significantly reduced the number of dead neurons and astrocytic immunoreactivity, supporting its anti-inflammatory and neuroprotective properties.

Conclusion

The administration of Diclofenac sodium elicited astrogliosis and neuroinflammatory responses, with a significant increase in dead/dying neurons in the mPFC of juvenile mice, while co-administration of vitamin C protected against astrogliosis, neuroinflammation; significantly reduced neuronal loss, and improved the histoarchitectural profile of the mPFC.

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Conflict of Interest
None declared.

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Authors Contribution
DAA - conceptualized, designed the experiment, drafted the manuscript, and supervised the study; AOA, PBF, and OMD – carried out the study, handled the animals, and performed data collection and storage; OGO, OSF, and OSO – statistical analysis; SOA, ATH – carried out the editing and review of the manuscript; OOA, JOF – performed the biochemical analysis of glutamate

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