In *Invivo* model of Parkinson’s syndrome, concurrent administrations of anti-inflammatory drugs changed behavioral deficits

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In most of the laboratory and occupational studies, Parkinson’s syndrome (PS) is the major outcome of paraquat (PQ) toxicities. Nevertheless, the search for novel therapy against PS is still enormous. We therefore examined the effects of anti-inflammatory drugs against PS. Swiss mice (30-35g) were pretreated orally with saline, distilled water, acetylsalicylate acid (10mg/kg) or prednisolone (5mg/kg) for 7 days. They were concurrently treated for another 5 weeks against intraperitoneal injection of 0.01ml and 0.02ml of (10mg/kg) PQ which were given twice a week to induced PS. Subsequently, movement deficits in mice were closely examined by using the spontaneous motor activity test, the catalepsy, and hanging tests. Thereafter, the mice brains were dissected for the biochemical tests. The data were analyzed using ANOVA, and the values were statistically significant at (p<0.0001, p<0.05). In conclusion, aspirin and prednisolone produced strong antioxidant effects that may be associated with change in the behavioral deficits.

**Keywords:** behavioral; toxicity; antioxidant; anti-inflammatory


**Introduction**

Experimental model of Parkinson’s syndrome (PS) involves damaging the nigrostriatal dopaminergic neurons (NDN) to illustrate the neuropathology of the disease in human [¹].

Progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) leads to the reduction in the biosynthesis of dopamine in the brain [²], which eventually when dopamine shortage has been established in the brain, PS becomes a major health challenge. In such condition, the medications that are available such as levodopa and carbidopa, or bromocriptine have not been successful because they rather alleviate symptoms than to inhibit the neuronal degenerating mechanisms in PS brain. However, most of the victims do suffer from a serious symptomatic and majorly behavioral deficit which includes muscles rigidity, resting tremors, akinesia, mood changes, and postural instability with some other cognitive behaviors [²]. Indeed, if there is no proper treatment, PS could progress over 5-10 years into a severe condition whereby the victims become permanently disable and unable to care for themselves [², ³].

While the 6-OHDA and MPTP models are the most commonly applied in the preclinical studies, it is important to note that there are cogent caveats associated with these models. First of all, PS is generally described as a dopaminergic disorders, but recently it was extensively described that, the PS evolves from the previous changes in
the brain stem. Also, dopamine loss happens to be just one component in the pathogenetic cascade [2]. Thus, there are many other non-dopaminergic cells, such as serotonin cells from the raphe nuclei, noradrenergic cells of the locus coeruleus and the cholinergic cells of the septum which are also affected, all of which are expected to contribute to PS [2].

In some of the occupational and laboratory studies using PQ to induce PS, the pathophysiology of this disease have been unraveled [4]. For example, rotenone and PQ are in the same group of herbicide that causes mitochondrial dysfunction and massive oxidative stress [5-8], which for some reasons, PQ or rotenone has been considered to be greatly advantageous in animal models of PS, giving a likelihood of gradual loss of neurons that depict the disease. However, 6-OHDA and MPTP models are seemed to be very useful for targeting and for destroying the DA neurons rapidly, notwithstanding, this does not demonstrate how it usually take a long duration before developing PS in human [2-9].

Majorly, PQ is highly selective for the NDN and the cell loss is usually moderate, perhaps (20-30%), but it may be sufficient to trigger movement deficits and some other associated symptoms. Also, dose-dependent dopaminergic nerves damage do occur after multiple injections [10]. Again, PQ can cross blood brain barrier (BBB) through a neutral amino transporter, this process can also be well-facilitated through Na+ dependent reuptake mechanisms to allow continuous intoxication on the neuron’s mitochondria via redox cycling. Similarly, PQ can also bridge a contributing effort to inhibit complex I systems in order to triggers inflammatory responses [11]. Furthermore, well absorbed PQ can generate massive oxidative stress which may completely destroy the cell components. PQ will further act as a redox cycling compound via the formation of superoxide anions to impair the recycling of the oxidized glutathione into its reduced form, so as to result into destructions of the intracellular antioxidant systems efficiency [12].

Furthermore, in the intrinsic mitochondrial pathway pro-apoptotic activity was first revealed in PQ models of PD [13]. Similarly, vascular inflammation, leucocytes migrations and microglia activations are the major focus in the pathogenesis of PS [14]. Basically, only a few microglial cells are detectable at the vicinity of dopaminergic neurons, but when microglia systems are activated it may lead to neuronal damage [15]. These activated glial can act as defense mechanism to play a helpful role in the evacuating of the damaged neurons, but in this process some healthy neurons maybe affected [16]. Furthermore, another prominent factor seems to be the activation of complement system in the inflammatory processes [17].

Therefore, it maybe rightly expected that anti-inflammatory drugs can possible have the effects against the degenerative progress in PS. Hence, our study was aimed at investigating the effects of a non-steroidal and a steroidal anti-inflammatory drug on the behavioral and biochemical markers of PS.

Material and Methods

Experimental animals

Swiss mice (30-35 g, 10 weeks old) were obtained from central animal house of the University of Ibadan and they were put in plastic cages for one week at room temperature and at normal relative humidity. They were fed with commercial food pellets and water. The experimental procedures were carried out in compliance with National Institutes of Health Guide for Care and Use of Laboratory Animals.

Drugs and chemicals

Paraquat (PQ) herbicide 200mg/L (Made in UK), Aspirin (BDH), Prednisolone, Trichloroacetic acid-TCA (Burgoyne Burbidges & Co., Mumbai, India), thiobarbituric acid-TBA (Guangdong Guanghua Chemical Factory Co., Ltd), 5, 5’-dithio-bis (2-nitrobenzoic acid)-DTNB (Aldrich, Germany) and Tris (hydroxymethyl)-amino-methane (Tris-buffer) (Hopkin & Williams Company, USA), and Griess reagent.

Procedures

Mice were separated into four treatment groups (n=6) per group. Group 1, 2, 3 and 4 received normal saline (NS), distilled water (DW), acetysalicylate acid (ASA) 10mg/kg or prednisolone (PRED) 5mg/kg orally for 7 days. The mice were treated concurrently for another 5 weeks against an intraperitoneal injection of 0.01ml and 0.02ml of 10mg/kg PQ dissolved in phosphate buffer saline given twice a week in group 2, 3 and 4 to induced PS.

Behavioral assessments

All behavioral assessments were taken into considerations prior to commencing the treatments as part of the criteria to check for the gradual deteriorations of movements after multiple injections of PQ. Thereafter, the following tests were conducted again at end of the 1st-week and the 5th-week of the PQ challenged.

Spontaneous motor activity (SMA) test
SMA test was utilized to study the effects of ASA and PRED on mice spontaneous activity and novelty rearing [18]. Mice were placed separately inside electronic activity cage (Ugo Basile, Italy). Thereafter, the numbers of vertical and horizontal movements which were representing spontaneous movements and the rearing were measured for a period of 5 min.

Catalepsy

Catalepsy was investigated according to the method previously described by [19]. Mice forelimbs were gently placed on an inclined plane (H= 6 x W= 4 x L = 16cm) and the duration of akinesia (duration by which the animal remained in an abnormal position, before making any spontaneous movement) was scored in (sec) time.

Hanging Test

The hanging test was performed as previously described by [20]. Briefly, mice were placed on a horizontal grid and inverted upside down. The mice were allowed to hang by gripping the grid and the number of time it took for each mouse to drop off was scored.

Biochemical assays

A day after the behavioral tests, the mice were decapitated under ether anesthesia. Their brains were dissected and were kept in 10 % w/v sodium phosphate buffer (0.1 M, pH 7.4), placed in cold ice. Each brain was homogenized and it was centrifuged using cold centrifuge at 10000 rpm for 15 min. The clear supernatant was collected and the absorbance was then read at 412 nm against blank reagent at 532 nm using a UV spectrophotometer. The absorbance was calculated as μmol/g tissue and the data was expressed as percentage of control.

Estimation of reduced glutathione (GSH) concentration

GSH concentrations were determined using the method of [21]. The clear supernatant and 20% TCA (0.4 ml) was mixed together and it was centrifuged using a cold centrifuge at 10,000 rpm at 4°C for 20 min, to get 0.25ml of clear supernatant which was added to 2 ml of 0.6 mM DTNB and 0.75ml sodium phosphate buffer (0.2 M, pH 8.0). The absorbance was then read at 412 nm against blank reagent using a UV spectrophotometer. The concentrations of GSH in the brain tissues were calculated in μmol/g tissue and the data was expressed as percentage of control.

Estimation of brain level of malondialdehyde (MDA)

The brain level of MDA was estimated according to the method of [22]. An aliquot of 0.4 ml of the sample was mixed with 1.6ml of Tris-KCl buffer (pH 7.4). 0.5 ml of 30% TCA was also added. Then, 0.5 ml of 0.75% TBA was added and the reaction mixture was placed in a water bath for 45 min at 80°C. It was then cooled in ice and centrifuged at 3000 rpm for 15 min. The clear supernatant was collected and the absorbance was measured against blank at 532nm using a UV spectrophotometer. The absorbance was calculated as μmol/g tissue and the data was expressed as percentage of control.

Nitrite Estimation

Nitrite contents were estimated in the brain supernatant as previously described by [24]. The clear supernatant was incubated with ammonium chloride (0.7 mM) followed by addition of Griess reagent (0.1% N-naphthylethylenediamine and 1% sulfanilamide in 2.5% phosphoric acid). The reaction mixture was incubated for 30 minutes at 37°C and the absorbance was measured at 540 nm by UV spectrophotometer. The nitrite content was calculated using a standard curve and expressed in units of μmol/ml and the data were expressed as percentage of control.

Determination of Superoxide Dismutase (SOD) Activity

SOD activity was determined by using the method of [23]. 1ml of supernatant was diluted in 9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction was started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture. The increase in absorbance at 480nm was monitored every 60 secs for 180 secs. The valves were calculated using standard curve and the data expressed as percentage of control.

Statistical analysis

Data were expressed as the mean ± SEM. Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by bonferroni multiple comparison tests. Statistical significance was determined at (p < 0.05, and p<0.0001).

Results

Behavioral assessments

Effects of ASA and PRED on SMA

The effect of ASA (10mg/kg) and PRED (5mg/kg) on SMA was assessed by the number of vertical (rearing) and horizontal (motor) movement in the activity cage (Fig 1A&1B). One-way ANOVA revealed the number of SMA was enhanced when each of the values were compared with the initial SMA readings (Table 1).

Effects of ASA and PRED on catalepsy
Table 1. Data showing initial values for the vertical and horizontal movements in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1st week Vertical</th>
<th>1st week Horizontal</th>
<th>2nd week Vertical</th>
<th>2nd week Horizontal</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>34±0.02</td>
<td>787±0.05</td>
<td>24±0.06</td>
<td>759±1.01</td>
</tr>
<tr>
<td>DW</td>
<td>16±0.04</td>
<td>756±1.05</td>
<td>44±0.01</td>
<td>708±0.13</td>
</tr>
<tr>
<td>ASA</td>
<td>27±0.12</td>
<td>678±0.04</td>
<td>34±0.14</td>
<td>723±0.19</td>
</tr>
<tr>
<td>PRED</td>
<td>38±0.18</td>
<td>695±0.17</td>
<td>11±0.11</td>
<td>659±0.08</td>
</tr>
</tbody>
</table>

Data represent the Mean ± S.E.M (n=5) mice per group no analysis of significant or comparison tests between groups was required.

Similarly, the effect of ASA (10mg/kg) and PRED (5mg/kg) on duration of akinesia was assessed using catalepsy test. One-way ANOVA revealed duration of catalepsy was reduced significantly and movement deficits changed (Fig 2).

**Effects of ASA and PRED on Hanging test**

The effect of ASA (10mg/kg) and PRED (5mg/kg) on the duration of falling in the hanging test produced significant improvements over the duration of falling after mice were mounted on the grid and the grid was turned upside (Fig 3).

**Biochemical Results**

**Effects of ASA and PRED on MDA and GSH levels**
The effects of ASA and PRED on MDA and GSH concentrations, (Fig 4 and 5) One-way ANOVA revealed that there were significant differences between treatment groups, as there was increased in GSH level but decrease in MDA estimated by percent of control.

**Effects of ASA and PRED on Nitrite estimation and SOD**

The effects of ASA and PRED on Nitrite and SOD estimations, (Fig 6 and 7) One-way ANOVA shown that there were significant differences between treatment groups, as there was increased in SOD but decrease in nitrite estimated by percent of control.

**ASA and PRED increased Survival in treatment groups**

Fig 8 shows the number of mice that survival PQ challenged after the behavioral tests, the values represent the numbers of mice that were counted and expressed in percentage.

**Discussion**

Basically in this study, the various changes in the behavioral tests among the PS induced mice were observed adequately. We also evaluated changes in the biochemical components of the brain especially the reactive oxygen species (ROS), among those that are constantly involved in systemic inflammatory responses (SIRs). Furthermore, the analysis of data suggested that the anti-inflammatory drugs are useful medication in PS condition. First, the cataleptic test which was suitable for investigating duration of akinesia in PS mice was significant within the treatments group, and in the SMA test which was extremely useful for assessing movement disorders. In addition, the hanging test which was used in assessing paw deficits had provided insights for this study. Altogether, both drugs enhanced mice behavioral performances in SMA (Fig 1A and B), with the values been compared with the initial SMA readings (Table 1).
Generally, cataleptic mouse will remain immobile in an abnormal position for 40 sec to signify weakness or tiredness \[19\], but the duration of akinesia among the treatment groups was reduced (Fig 2). Similarly, the duration of falling after mice were been mounted on the grid and the grid was turned upside to assessed the level of paw’s rigidity was prolonged (Fig 3). Finally, the brain antioxidant effects were enhanced (Fig 4-7). In a way that the level of MDA (lipid peroxidation) was reduced with a simultaneous increase in GSH level. Furthermore, SOD level was elevated, but noxious nitric oxide in the brain was reduced. The lethality rate across the treatment groups was also reduced (Fig 8).

Thus, our findings maybe align with the followings. Firstly, in some of the occupational and laboratory studies using PQ to induce PS, the pathophysiology of this disease have been unraveled \[4\]. For example, rotenone and PQ are in the same group of herbicide that causes mitochondrial dysfunction and massive oxidative stress \[5-8\], which for some reasons, PQ or rotenone has been considered to be greatly advantageous in animal models of PS, giving a likelihood of gradual loss of neurons that depict the disease. However, 6-OHDA and MPTP models are seemed to be very useful for targeting and for destroying the DA neurons rapidly, notwithstanding, this does not demonstrate how it usually take a long duration before developing PS in human \[2, 9\].

Majorly, PQ is highly selective for the NDN and the cell loss is usually moderate, perhaps (20-30%), but it may be sufficient to trigger movement deficits and some other associated symptoms. Also, dose-dependent dopaminergic nerves damage do occur after multiple injections \[10\]. Again, PQ can cross blood brain barrier (BBB) through a neutral amino transporter, this process can also be well-facilitated through Na+ dependent reuptake mechanisms to allow continuous intoxication on the neuron’s mitochondria via redox cycling. Similarly, PQ can also bridge a contributing effort to inhibit complex I systems in order to triggers inflammatory responses \[11\]. Furthermore, well absorbed PQ can generate massive oxidative stress which may completely destroy the cell components. PQ will further act as a redox cycling compound via the formation of superoxide anions to impair the recycling of the oxidized glutathione into its reduced form, so as to result into destructions of the intracellular antioxidant systems efficiency \[12\].

Hence, massive neuronal damage, via oxidative stress, vascular inflammation and micro glial activations are mostly involve PS, and as a result behavioral deteriorations such as movement perplexity, sluggishness and muscle fatigue do occur later.

Perhaps, one may argue in this, if it is possible for anti-inflammatory drugs to have derived an ability to have inhibited PQ transportation into the brain. It is certainly doubtful. Because, as far as we know, anti-inflammatory drugs do not inhibit transport mechanisms in the brain, nor prevent uptake mechanisms at any point in the brain, but rather, this type of drugs inhibit markers of inflammation and oxidative stress.

However, our investigation is certainly in line with the growing evidences about the involvement of vascular
inflammation in the pathogenesis of PS. Furthermore, investigation in postmortem and in vivo studies shown some facts after some PS patients were been diagnosed of neuroinflammation [24, 25]. Also, it was revealed that neuroinflammation was majorly contributing to the pathological processes which preceded dopaminergic cell death in animal models of PS [25, 26]. Similarly, a genetic study has related risk of PS is to be associated with polymorphisms in inflammatory genes, including tumor necrosis factor, interleukin1, and high plasma concentration of interleukin 6 at the frontline. In that same study, it was shown that PS have common pathogenic mechanisms with Alzheimer’s disease, especially for its aggregation and deposition of misfolded proteins and chronic inflammation [27-29]. Thus, chronic inflammation, BBB dysfunction, and immune cell migrations do play a significant role in PS. For example, in another study, substantial infiltration of CD44+ T cells in the NDN, and in the striatum of PS mouse was detected [30], and with another report describing the significant increase in the number of leukocytes inside NDN and its dorsal extension in the capillaries of PS brain [31]. In another clinical study, high level of CD4+ and T cells were found to be expressing fast in the peripheral blood of some patients having PS [31,33]. Later, it was suggested that, the loss of DA neurons may be driven by neutrophil infiltration, reduced astrocyte density, and a consecutive increased in BBB permeability [33].

Nevertheless, largely supported information had ascertained that, the NSAIDs and some phytochemicals can inhibit cellular components of inflammation. They also have the ability to inhibit the release of chemoattractants which is contributing to the movements of the leukocyte to the site of injury [34, 35].

NSAIDs exhibited neuroprotective effects in animal models of PS [36], but, little information exists to explain for neuroprotective effect of SAIDs [25, 29], although, some theories had earlier postulated possible inhibition of COX-2, independent antioxidant effects, or modulation of the inflammatory responses through direct impact on gene expression or cytokine productions [24]. However, it is yet to differentiate any other contributing factor via another pathway to justify the neuroprotective effects of these medications.

Secondly, there was an increase in mRNA level of complement components which was found in a region of an affected PS brain [37]. The complement components and other constituents of membrane attack complex (MAC) were both identified in the intracellular lewy bodies and oligodendroglia in the SNpc of the brain [37]. Lewy bodies’ accumulation can apparently cause the activation of this complement, and it can as well cause the initiation of reactive changes in the microglia and in the release of some potential neurotoxic products such as the MAC, hydroxyl radicals and excess glutamate [38].

Nevertheless, among the toxic components which are released by the reactive glial, it is not clear which one of them is responsible for the neuronal death, maybe ROS, or hydroxyl radicals, nitric oxides (NO) and its peroxinitrite are the likely deleterious agents. Although, it seemed the inflammatory process and oxidative stress derived from DA metabolisms constitute to vicious cycle that may lead to the final demise of nigral DA cells [39].

Furthermore, the brain possesses COX-1 and COX-2 isoforms, but COX-2 up regulation during the stressful conditions such as cerebral ischemia, can trigger neuronal apoptosis and neurobehavioural deficit [37]. COX-2 appears to be expressed in dendrites and cell bodies of neurons in the several areas of the brain such as nigrostriatal pathways, hippocampus, and amygdale nucleus, where it can correspond to inflammatory and degenerative brain diseases [40]. Also, any increase in inflammatory process can generate different types of free radicals, capable of causing phospholipid peroxidation [39], the release of arachidonic acid can also inhibits glutamate uptake thereby contributing to the neurodegenerative processes observed in PS [41].

However, NSAIDs have the ability to ultimately inhibit COX isoforms irreversibly, and to dislodge both the release and the biosynthesis of inflammatory markers predominantly. On the other hand, SAIDs such as dexamethasone, or prednisolone can inhibit COX-2 gene expression, making glucocorticoids to possess widespread effects, including their dramatic effects over the manifestations of neuroinflammation, and the profound effects on the concentration and the distributions, as well the functions of peripheral leukocytes. They can also suppress the expressions of inflammatory cytokines such as TNF-alpha or interleukins-6 and also prevent the prostaglandin release [42]. Most especially, COX-2 selective inhibitors have changed the rigidity and movement impairments in a parkinsonian rat [43]. But, the histological study did not show any improvement among the already damaged SNC neurons [44].

Finally, the brain antioxidant effect was enhanced. ROS are involved in SIRs [45]. They play a crucial role while contributing to the onset and progress of inflammation in distant organs [45]. Free radicals exert their toxic effects at the site of inflammation by reacting with different cell components, as a result loss of function and cell death normally occur [46, 47]. Nevertheless, the deleterious effects of
ROS are usually been controlled by endogenous antioxidants such as superoxide dismutase (SOD), catalase, and glutathione which are synthesized from the cells [48].

Therefore, PQ remains one of the most widely used herbicides worldwide which have a trace to some diseases including PS [49], the potential for exposure to many of these pesticides, including rotenone and PQ, extends well beyond the occupational settings.

In conclusion, our findings can be summarized as follows, ASA and PRED are useful drugs to keep the neurons undeterred. Although, our study was based on the distributions of the behavior tests and the biochemical tests, further study is needed to further justify these effects.

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There is no conflict of interest

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