Protective effect of co-administration of vitamins C and E on reserpine-induced oxidative stress in mice

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ABSTRACT
Background: Several studies have shown potential benefits of antioxidants in the treatment of Parkinson’s disease (PD) but none have combined vitamins C and E targeting the oxidative stress (OS). Aim: To evaluate the neuroprotective effect of co-administration of vitamins C+E or single vitamin, on parameters of reserpine-induced OS in mice. Methods: Twenty-five mice were randomly assigned into 5 groups: Group I received only distilled water (control); other groups received reserpine 0.1 mg/kg intraperitoneally on alternate days. In addition, Group III received vitamin E 200 mg/kg/day orally; group IV, had vitamin C 250 mg/kg/day orally and group V, had both vitamins orally. All drugs were given concurrently for 28 days. The mice were humanely sacrificed and brain homogenate made to assess for biomarkers of OS. Data were expressed as mean ± SEM and values at p < 0.05 were considered significant. Results: The significant increase in malondialdehyde concentrations observed in the Res group (42.2±0.28 Umol/L) compared to control (37.5±1.27 Umol/L), was ameliorated in all the vitamin-treated groups with significance in the Res+Vit C group (35.0±1.69 Umol/L) compared to the Res group (p=0.002). Superoxide dismutase (SOD) activity increased significantly (p=0.003) across the vitamin-treated groups (24.9±2.11 Umol/mg, 24.0±1.78 Umol/mg and 22.4±1.50 Umol/mg in the Res+Vit E, Res+Vit C and co-administered groups respectively) compared to control (14.3±1.65 Umol/mg), with non-significant increase in the Res group (20.6±1.42 Umol/mg); catalase activity increased significantly in the Res+Vit C (28.0±3.70 Umol/mg) and co-administered (30.2±2.22 Umol/mg) groups compared to controls (14.3±1.65 Umol/mg) and Res (20.6±1.42 Umol/mg) groups (p=0.000), with non-significant increase in the Res+Vit E group (17.6±0.68 Umol/mg). The highest GSH level was seen in the Res group (45.2±2.65 Umol/mgpr) and the lowest level seen in the Res+Vit E group (38.5±1.78 Umol/mgpr) with no significant difference across all the groups (p=0.104). Conclusion: The co-administration of vitamins C and E fails to confer significant superior neuroprotection against reserpine-induced OS compared to single vitamin administration.

INTRODUCTION
Parkinson’s disease (PD), first described by James Parkinson (1817), is the most common movement disorder affecting about 1.5% of the world’s population over 65 years of age (~10million) (Blesa and Przedborski, 2014). This prevalence increases with advancing age to about 5% in those above 85 years (Farn, 2003) and the prevalence is projected to double by 2040 due to increasing life expectancy with higher aging population and increasing industrialization (Dorsey et al., 2018). It is the second most common neurodegenerative disease (NDD) (Tanner and Aston, 2000; Lee et al. 2009). Neurological disorders are now noted to be the leading cause of disability worldwide, among which, PD is the fastest growing (Dorsey et al., 2018).

Several conventional (Schapira, 2005; Clark, 2007; Davie, 2008; Weinreb et al., 2010; Thomas, 2017) and other treatment strategies such as nicotine (Barreto et al., 2015), non-steroidal anti-inflammatory drugs, NSAIDs (Wahner et al., 2007; Gagne et al., 2010), docosahexanoic acid (Ozsoy et al., 2011), caffeine

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(Joghataie et al., 2004; Saaksjarvi et al., 2008) etc. have been explored in the treatment of PD but some of these findings for example nicotine, are controversial (Ferrea and Winterer, 2009). Even the gold standard in PD treatment, l-dopa and carbidopa (a peripheral decarboxylase inhibitor), is associated with dyskinesia and end-of-dose “wearing-off” (Fahn, 1999) and it has been suggested that l-dopa might be neurotoxic, hastening the progression of PD via oxidative stress (Craciun et al., 2016). Although the motor symptoms initially respond well to pharmacologic therapies, none has been shown to slow down or halt the disease progression (Elmer and Hauser, 2013). Hence, the need to explore newer neuroprotective agents that can target the main pathophysiology of the disease with the aim of preventing further damage or neuronal loss, halting the progression, ameliorating symptoms and initiating repair or healing processes.

Given the well-established central role of oxidative stress (OS) in the pathogenesis of PD (Jenner, 2003), the focus in PD therapy is now shifting to antioxidants such as vitamins E and C, which might protect against oxidative damage by neutralising free radicals. Both vitamins C and E had been shown to have neuroprotective effects as individual antioxidants in some studies (Roghani and Behzadi, 2001; Miyake et al., 2011; Harrison, 2012) but the co-administration of both vitamins was shown to be of greater efficacy against oxidative stress and nephrotoxicity in rodents (Khadkhodaei et al., 2008), with more potent antidiabetic effects (Tanko et al., 2014), although, in another study, this was shown to be otherwise (Mohammed et al., 2015). The combined administration of the two vitamins has been shown to potentiate the effect of each other on reserpine-induced oral dyskinesia in rodents (Faria et al., 2005), also suggesting that the combination of both vitamins provide a complete antioxidant defence (Mahadik et al., 2001).

Vitamin C plays an important role in recycling of vitamin E (Kohen and Nyska, 2002; Harrison, 2012), a process that results in the formation of vitamin C (semial ascorbyl) radical (Powers and Jackson, 2008; Pillai and Yao, 2015). Therefore, a combination of the two vitamins, due to their synergistic interaction (Baptista-Ortega and Ruiz-Feria, 2010; Yarube and Ayo, 2011; Dawud et al., 2014; Bursac-Metrovic et al., 2016), may enhance their antioxidant benefit. The aim of this study is to evaluate the effects of co-administration of vitamin C (l-ascorbic acid) and vitamin E (α-tocopherol) on reserpine-induced oxidative stress and parkinsonism in mice.

METHODS

Vitamins C and E and reserpine were of analytical grade and purchased from MedChem Express, U.S.A. The reserpine was dissolved in 0.5% glacial acetic acid, vitamin E (alpha-tocopherol acetate) in dimethyl sulfoxide and vitamin C (l-ascorbic acid) in distilled water.

Ethical Consideration

Ethical clearance/approval was sought from the Ahmadu Bello University Ethical Committee on Research Animals Use and Care. The study was conducted in accordance with the guidelines of the A.B.U Animals Use and Care Policy.

Experimental Animals

A total of 25 mice, 8-12 weeks old and weighing 20-30g were housed in plastic cages under standard environmental conditions with free access to commercial grower mash feed and water.

Induction of Parkinsonism

Parkinsonism was induced by alternate day’s administration of 0.1mg/kg reserpine injection i.p over 4weeks (Sarmento-Silva et al., 2014).

Animal Grouping

The mice were randomly assigned into five (5) groups of 5 animals each:

Group I (n=5): were fed with normal diet ad libitum plus normal saline (1ml/kg) throughout the study period. This group served as normal control.

Group II (n=5): received reserpine (0.1mg/kg) i.p on alternate days (Sarmento-Silva et al., 2014) for 4 weeks. They served as positive control.

Group III (n=5): were pre-treated with vitamin C (250mg/kg) i.p daily plus reserpine (0.1mg/kg) i.p on alternate days for 4 weeks.

Group IV (n=5): were pre-treated with vitamin E (200mg/kg) i.p daily plus reserpine (0.1mg/kg) i.p on alternate days for 4 weeks.

Group V (n=5): were pre-treated with vitamin C (250mg/kg) i.p and vitamin E (200mg/kg) i.p daily plus reserpine (0.1mg/kg) i.p on alternate days for 4 weeks.

Homogenate Preparation

The mice were humanely sacrificed by anaesthetizing them with i.p ketamine (10 mg/kg) and i.p diazepam (2 mg/kg). As soon as the effect of anaesthesia is evident, the brain was carefully harvested and a homogenate was prepared as described by Freitas et al. (2005), using phosphate buffer solution. The homogenate was...
used for analysis of biomarkers for OS (according to the manufacturer’s manual/guidelines).

**Assay for Biomarkers of Oxidative Stress**

**Protocol for assay of superoxide dismutase (SOD) activity**

Activity of SOD was carried out according to the method described by Misra and Fridovich (1972). The assay is based on the principle of superoxide dismutase (SOD) inhibition of auto-oxidation of adrenaline at pH of 10.2. 0.2 ml of diluted homogenate (0.1 ml of homogenate plus 0.9 ml of distilled water i.e. dilution factor of 1:10) was mixed with 2.5 ml of 0.05 mM carbonate buffer (14.3 g of Na₂CO₃ and 4.2 g NaHCO₃ plus 1,000 ml distilled water at pH of 10.2) and 0.3 ml of 0.3 mM adrenaline (0.01 g of adrenaline plus 17 ml of distilled water). The absorbance was measured over 60s at 450 nm. The % inhibition was calculated as:

\[
\% \text{Inhibition of adrenaline oxidation} = \frac{\text{Increase in absorbance/min}}{\text{Increase in absorbance of blank/min}} \times 100
\]

Hence, the concentration of the SOD activity (in Umol/mg) is the amount of SOD required to elicit 50% inhibition of adrenaline auto-oxidation per minute.

**Protocol for assay of reduced glutathione (GSH) activity**

Reduced glutathione (GSH) activity was carried out according to the method described by Ellman (1959) as modified by Rajagopalan et al. (2004). It is based on the reaction of 5,5'-dithiobis nitro benzoic acid (DNTB) with GSH. To 150 μl of the homogenate 1.5 ml of 10% trichloroacetic acid (TCA) and 0.3 ml of 30 mM H₂O₂ (0.01 g of sodium) were added and centrifuged at 1500 g for 5 minutes. 1 ml of the supernatant was added to about 100 μl of the supernatant, forming a TBARS, an adduct that absorbs strongly at 532 nm. The supernatant was deproteinized by addition of the TCA and the mixture was heated in a water bath at 80°C for 30 minutes before cooling on ice then centrifuged at 2000 g for 10 minutes. The product of lipid peroxidation, MDA is expected to be released by the deproteinization and react with the TBA forming the coloured product, MDA-TBA (or TBARS), absorbance of which was measured at 532 nm with a UV spectrophotometer. The concentrations of TBARS were calculated as the absorbance/molar extinction coefficient of malondialdehyde: 1.56×10⁵ mol/L/cm. All TBARS concentrations are expressed in μmol/g tissue protein.

**Statistical Analysis**

Data were expressed as mean ± SEM and analysed using one-way analysis of variance (ANOVA), followed Tukey’s post-hoc test. Values at \( p < 0.05 \) were considered statistically significant using SPSS version 25.0.
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Level of SOD activity
There was a significant increase in the level of SOD activity across the vitamin-treated groups (24.0±1.78 Umol/mg by vitamin C, 24.9±2.11 Umol/mg by vitamin E and 22.4±1.5 Umol/mg by both vitamins). F = 5.660, p = 0.003.

Fig. 2. Level of SOD activity by group: Res= Reserpine, Vit= vitamin. *p < 0.05 indicates statistically significant difference compared to the control (One-way ANOVA followed by Tukey’s post-hoc test).

Level of CAT activity
There was significant increase in catalase activity in the Res+Vit C (28.0±3.70 Umol/mg) and co-administered (30.2±2.22 Umol/mg) groups compared to both control (14.3±1.65 Umol/mg) and Res (20.6±1.42 Umol/mg) groups, with non-significant increase in the Res+Vit E group (17.6±0.68 Umol/mg). F = 2.214, p = 0.000.

Fig. 3. Level of CAT activity by group: Res = Reserpine, Vit = vitamin. Superscripts *p < 0.05 indicates statistically significant difference compared to control and *p < 0.05 compared to Res group (One-way ANOVA followed by Tukey’s post-hoc test).

GSH level
There was no significant difference in the GSH level across the groups, the highest level seen in the Res group (45.18 ± 2.65 Umol/mg) and the lowest level seen in the Res + Vit E group (38.58 ± 1.78 Umol/mg). F = 2.214, p = 0.104.

Table 1: Reduced glutathione levels by groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Reduced Glutathione (GSH) (Umol/mgpr)</th>
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<tbody>
<tr>
<td>Control</td>
<td>39.72±1.45</td>
</tr>
<tr>
<td>Res only (0.1 mg/kg)</td>
<td>45.18±2.65</td>
</tr>
<tr>
<td>Res + Vit E (200 mg/kg)</td>
<td>38.58±1.78</td>
</tr>
<tr>
<td>Res + Vit C (250 mg/kg)</td>
<td>43.08±1.67</td>
</tr>
<tr>
<td>Res + Vit C + Vit E</td>
<td>41.82±0.74</td>
</tr>
</tbody>
</table>

Res= Reserpine, Vit= vitamin. No statistically significant difference (One-way ANOVA followed by Tukey’s post-hoc test).

DISCUSSION
Oxidative stress (OS) can be defined as an imbalance between the levels of ROS produced and the ability of the biological system to neutralize them, creating a state of possible cellular damage (Dias et al., 2013). It can also be defined as the presence of ROS in excess of the available antioxidant buffering capacity (Czerska et al., 2015). These ROS may damage DNA, proteins, lipids, proteins and carbohydrates changing the organism’s structure and functions (Czerska et al., 2015). There is normally a balance between production of ROS and the antioxidant defence against them and any disturbance of such balance is capable of causing OS. Within the cell where oxygen metabolism takes place, ROS are normally and constantly formed as part of normal physiological processes with the majority of endogenous ROS generated by the mETC during the production of ATP (Eckert et al., 2003). The antioxidant defence consist of enzymatic and non-enzymatic antioxidants.

The mechanism involved in toxin-induced PD in well-established animal models such as MPTP, 6-OHDA, rotenone and paraquat is associated with OS (Dias et al., 2013). Although, apart from PD, OS had also been linked to other NDD such as Alzheimer’s disease (AD), Huntington’s disease (HD) and amyotrophic lateral sclerosis (ALS) despite having distinct pathological and clinical features (Lin and Beal, 2006), supporting the role of oxidative stress in neurodegeneration (Anderson, 2004).

Markers of lipid peroxidation such as HNE and MDA are increased in the SN of PD patients, while PUFAs
are decreased (Montine et al., 2004). Our result showed a significant evidence of lipid peroxidation (figure 1), characterized by a significant increase in MDA concentration in the reserpine only treated group compared to control. This was however, significantly ameliorated by vitamins C and E as single administrations. This is in line with the findings of Nayak et al., (2018), which showed neuroprotective effect of vitamin E against lipid peroxidation at different regions of rat brain. Surprisingly, vitamin C conferred the greater significant protection against lipid peroxidation, not being a membrane lipid-soluble antioxidant itself. This finding on the co-administered group is unexpected, as vitamin C to regenerate vitamin E in a synergistic action to produce even much greater significant protection against lipid peroxidation. This might be due to pro-oxidant activity of vitamin C at certain dosages, depending on the presence of transition metal ions such as Fe$^{3+}$ (Paolini et al., 1999; Halliway et al., 1999).

The increase in SOD activity seen in the reserpine-only treated group, could be a compensatory response to the OS resulting from the excessive production of free oxygen radicals. This is a first line of antioxidant defence against OS, scavenging O$_2^-$ in both cytosol and mitochondrial inter-membrane space (Rodriguez et al., 2011). However, the activity of SOD in the vitamin groups was even significantly higher, probably due to augmentation of the endogenous antioxidant enzymes by the vitamins. Again, the co-administration did not show more efficacy probably due to the pro-oxidant effect of vitamin C. This finding is consistent with the work of Serra et al. (2001), but contrary to other more recent studies (Sunday et al., 2014; Cracium et al., 2016).

The significant increase in CAT activity across vitamin groups, with the greatest significance observed in the group given both vitamins is probably due to their synergistic effect against OS (Faria et al., 2005; Khadkhodaei et al., 2008; Baptista-Ortega and Ruiz-Feria, 2010; Yarube and Ayo, 2011; Dawud et al., 2014; Bursac-Metrovic et al., 2016). CAT is part of the second line antioxidant defence, scavenging for H$_2$O$_2$ generated by SOD activity intracellularly (Pillay and Yao, 2015). Hence, increase in SOD activity will lead to increase in H$_2$O$_2$ generation, leading to a compensatory increase in CAT activity.

The mild increase in GSH level, although not statistically significant, may be due to a compensatory response to increase generation of free radicals, which is contrary to the work of Sian et al. (1994), that showed decrease in GSH level in the SNc of PD brains. Although expected, there was no appreciable increase in the GSH level in the groups treated with vitamins. This may be due to an overwhelming level of OS and it’s in line with the findings of Nayak et al. (2018), who also found no significant alteration in GSH level in rat brain exposed to aluminium and ethanol toxicity, following vitamin E supplementation for 4 weeks. Since GPx catalyses a reaction in which GSH detoxifies H$_2$O$_2$ to water, the GSH level is expected to increase when there is decrease in the GPx activity, as seen in the present study. This is also in line with the findings of Sunday et al. (2014), who found decrease in GPx as a reciprocal to SOD activity, although other studies showed no significant alteration (Cracium et al., 2016) in the GPX activity.

All the above findings showed a significant evidence of OS induced by reserpine in agreement with several other studies (Sanghavi et al., 2010; Fernandez et al., 2012; Eftimov et al., 2014; Hsiang-Chien et al., 2015; Dwivedi and Tomar, 2016). However, the administration of the antioxidants (vitamins C and E) was significantly protective against the OS, with the co-administration of the two antioxidants more potent than single vitamin administration. These findings of the present study on biomarkers of OS is contrary to other studies who showed generalised decrease in the SOD, CAT, GPx activities and GSH level (Napolitano et al., 2011; Koppula et al., 2012).

The beneficial effect of antioxidants and antioxidant enzymes is seen in scavenging of free radicals generated during OS and neuroprotection. These antioxidant systems prevent the generation and actions of ROS and provide a potential mechanism for ameliorating OS. Vitamin E, being a chain-breaking antioxidant within the lipid membrane (Kamal-Eldin and Appleqvist, 1996), protected against lipid peroxidation to a very significant extent as seen in this study and, it was itself regenerated by the vitamin C (Ambali et al., 2010; Harrison, 2012), which is a powerful water-soluble antioxidant within the cytoplasm and outside the membrane, and may help to boost the endogenous antioxidant levels. The vitamin E in turn, probably played a role in the biosynthesis of more endogenous vitamin C (Machlin and Gabriel, 1980) to boost the antioxidant neuroprotective effect.

**CONCLUSION**

From this study, co-administration of vitamins C and E was only partially neuroprotective against reserpine-induced OS in mice. Although, no significant neuroprotection was observed over the single vitamin administration, this finding depicts a great potential in slowing down the progression of NDDs like PD. Introducing such neuroprotective therapy, which are readily available and affordable, into the standard
treatment of PD will be highly beneficial. More studies are however needed, to explore these vitamins C and E at different dosages.

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