ATTENUATION OF HESPERIDIN ON VANCOMYCIN-INDUCED NEUROTOXICITY IN RATS.

ABSTRACT

Vancomycin, a bactericidal antibiotic used for selective clinical infections confers its antibacterial activity via the inhibition of bacterial cell wall biosynthesis. This process thus results in oxidative stress. Hesperidin, a flavonoid found in citrus fruits has been reported to possess antioxidant activity. This study investigated the ameliorative effect of hesperidin on vancomycin-induced neurotoxicity. Male Wistar rats (n=24, 130-300 g) were divided into four groups of six rats each. Group 1 (Control) received distilled water, Group 2 received Hesperidin (50 mg/kg/day) orally, Group 3 received Vancomycin (10 mg/kg/day) intraperitoneally and Group 4 received Vancomycin (10mg/kg/day) and Hesperidin (50mg/kg/day). Administration was done for seven days. Data were analyzed using ANOVA at $P \leq 0.05$. Administration of Vancomycin significantly reduced the levels of Reduced Glutathione (GSH) and Ascorbic Acid (AA) in the brain sections of the rats relative to control. The activities of Glutathione Peroxidase (GPx), Catalase (CAT), Glutathione-S-Transferase (GST) and Superoxide Dismutase (SOD) were down-regulated relative to control. Furthermore, an increased level of Nitric Oxide (NO), Malondialdehyde (MDA), and activity of Myeloperoxidase (MPO) was observed relative to control ($P \leq 0.05$). However, co-treatment with Hesperidin significantly attenuated levels of GSH, AA, NO, MDA, and activities of MPO, GST, SOD, CAT, and GPx when compared with Vancomycin treated groups. Histopathological examination of the brain sections of Vancomycin-treated rats showed the presence of lesions. Administration of Hesperidin alongside Vancomycin reversed these lesions. Data obtained from this study showed that Hesperidin attenuated oxidative stress induced by Vancomycin in the brain of rats via antioxidant mechanisms.

Keywords: Vancomycin, Hesperidin, Neurotoxicity, antioxidants, Oxidative stress

INTRODUCTION

Several studies have identified free radicals playing a major role in the onset, progression and complication of almost all pathological disorders such as declined antioxidants in the central nervous system. Antibiotics are a group of medications used to treat infections caused by some bacteria and certain parasites. (Ebimieowei and Ibemologi, 2016). Vancomycin, a glycopeptide antibiotic with bactericidal activity has been used to treat infections caused by gram-positive bacteria including the methicillin-resistant Staphylococcus aureus. Its mechanism of action is via inhibition of bacterial cell wall biosynthesis or inhibition of peptidoglycan biosynthesis (Gupta et al., 2011). There have been several reported cases of vancomycin toxicity, of which are: ototoxicity (Gupta et al., 2011; Toxnet, 2013), alterations in free radical balance resulting in oxidative stress (Schoen, 2005; Costa et al., 2009), and nephrotoxicity (Dieterich et al., 2009; Shakan-khan et al., 2011).

Considering the fact that brain is predisposed to oxidative damage due to an abundance of redox-active transition metal ions, a relative dearth of an antioxidant defense system, high usage of inspired oxygen and a significant amount of easily oxidizable polyunsaturated fatty acid. Likewise, the brain possesses its endogenous antioxidant which limits free radicals damage in neuronal tissue (Vatassery, 1998). Oxidative stress is associated with cognitive declination in both animals and human models and it is also related to several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease among others (Giasson et al., 2002).

Furthermore, many plant natural products have shown antioxidant activity and have been employed since ancient times for their therapeutic properties. Biological activities, potential health

and therapeutic benefits of natural products, and their bioactive compounds have been intensively explored and investigated. These natural products have emerged as potential neuroprotective agents for the treatment of neurodegenerative diseases and some of these natural products that have been used over the years are Honey (Mohd sairazi et al., 2017), Propolis (Kwon et al., 2004; Swamy et al., 2014), Ginseng (Tan et al., 2015) and many others.

Hesperidin, a flavone glycoside occurs in the greatest concentration in green fruit such as citrus fruits (sweet oranges and lemon). Hesperidin possesses multiple beneficial effects such as antioxidant, anticarcinogenic, antihypertensive, antiviral, antidiabetic, hepatoprotective, and anti-inflammatory (Parhix et al., 2015). Hesperidin has also been demonstrated via in vivo studies of Parkinson's disease (Rasool et al., 2014; Antunes et al., 2015), Dementia and Alzheimer's disease (Badalzadeh et al., 2015) to possess antioxidant activity. In clinical studies, hesperidin has been established to be neuroprotective (Kean et al., 2015).

Despite the widely reported neuroprotective effect and antioxidant activity of hesperidin, there is a paucity of information on its role in vancomycin-induced neurotoxicity. Hence, this study aimed at underscoring the protective effect of hesperidin against vancomycin-induced neurotoxicity in rats.

MATERIAL AND METHODS

Chemicals and reagents

Hesperidin was purchased from AK Scientific, Inc. USA, Vancomycin was procured from CELON laboratories PVT LTD, India. Ellman's Reagent [5' -5'-dithiobis-(2-dinitrobenzoic acid], Sulphosalicyclic acid, Di-potassium orthophosphate, Potassium di-hydrogen orthophosphate, Bovin serum albumin (BSA), Thiobarbituric acid (TBA), Glutathione, 1-Chloro-2,4-

dinitrobenzene were procured from Sigma, Aldrich USA. All other reagents were obtained from the British Drug Houses (Poole, Dorset, UK) and are of good analytical grade.

Experimental animals

Twenty-four male Wistar rats weighing 130-200g obtained from Department of Physiology, university of Ibadan were kept in plastic cages on a 12-h light:12-h dark cycle, at room temperature of 22–24°C at the animal house, Ajayi Crowther University for the period of acclimatization and treatment. The rats were acclimatized for one week and were allowed unhindered access to food and water *ad libitum*. Handling of the experimental animals was consistent with international principles on care and use of experimental animals (National Research Council., 2011).

Animals were randomly placed into four groups of six rats each: Group I animals were the control that were administered distilled water only. While group II received Hesperidin (50 mg/Kg/day) orally, group III received Vancomycin (10 mg/Kg/day) intraperitoneally and group IV received Hesperidin (50 mg/Kg/day) and Vancomycin (10 mg/Kg/day). The doses for Hesperidin and Vancomycin were selected based on previous study.

Preparation of Tissue Homogenate

Animals were rapidly dissected to excise the brain sections (cerebellum, frontal cortex and hippocampus), rinsed in ice-cold 1.15% KCL before being blotted and weighed. Brain sections collected were homogenized in 10 volumes/weight of ice-cold 0.1M phosphate buffer pH 7.4. The homogenates was centrifuged at 10,000g for 15 minutes at 4ºC using eppendorf (UK) cold centrifuge and the supernatant were stored at -4˚C and used for subsequent biochemical assays. Brain sections were subsequently fixed in 4% phosphate-buffered formalin and further processed for histology.

Biomarkers of Oxidative Stress

Determination of Superoxide Dismutase Activity

The activity of superoxide dismutase in tissue homogenate was measured as described by Sun and Zigman, 1978. 20 µl aliquots of tissue homogenate mixed with 1.95 ml Tris-HCl buffer pH 7.2 and 30 µl of freshly prepared epinephrine (20 mM) was added and mixed by inversion to initiate the reaction. The blank cuvette contained all other components except the homogenates which were replaced with distilled water. Change in absorbance at 320nm was monitored every 30seconds for 150 seconds.

Determination of Catalase Activity

Catalase activity in tissue homogenate was assayed according to the method of Sinha, 1985. 1 ml of tissue homogenates was mixed with 19 ml distilled water to give a 1:20 dilution. 1 ml of the diluted sample was mixed rapidly with the reaction mixture (4 ml of hydrogen peroxide solution (800 µmoles), 5 ml phosphate buffer pH 7.0 by a slight swirling movement at room temperature. 1 ml of the reaction mixture was removed and blown in 2 ml of dichromatic acetic acid reagent at intervals of 60 seconds. The hydrogen peroxide contents of the withdrawn sample spectrometrically measured at 570nm as described by Sinha.

Estimation of Reduced Glutathione Level

Reduced glutathione level in the tissue homogenate was determined using method described by Jollow et al. (1974). 100 µl of the tissue homogenate was added to 900 µl distilled water and 1500 µl of 4% sulphosalicyclic acid. The reaction mixture was allowed to stand for 5 minutes and subjected to centrifugation at 4000rpm for 10 minutes. The clear supernatant was withdrawn. 400 µl of phosphate buffer pH 7.4 was added to supernatant withdrawn with 50 µl Ellman's reagent.

The absorbance was measured at 412nm. Reduced glutathione concentration was measured from the reduced glutathione standard curve.

Determination of Glutathione-S-Transferase Activity

The glutathione-S-Transferase activity was determined by the method of Habig et al. (1974). 6 µl of reduced glutathione, 30 µl 1-chloro-2, 4-dinitrobenzene (CDNB) and 564 µl phosphate buffer pH 6.5 were mixed. 6 µl of the tissue homogenate was added to the reaction mixture and mixed by inversion to initiate the reaction. Change in absorbance was read at 30 seconds for 150 seconds at 340nm against the reference blank.

Estimation of Ascorbic Acid Concentration

The tissue homogenate ascorbic acid concentration was estimated using the method described by Jagota and Dani, 1982. 125 µl of the tissue homogenate was added to 200 µl 10% trichloroacetic acid. The reaction mixture was mixed vigorously in a test tube, kept on an ice bath for 5 minutes and centrifuged for 3000rpm for 5 minutes. 125 µl of the clear supernatant was withdrawn, 375 µl distilled water and 50 µl Folin's reagent was added. Absorbance was read at 760nm against a reference blank. Ascorbic acid concentration of the tissue homogenate was extrapolated from the ascorbic acid standard curve.

Determination of Glutathione Peroxidase Activity

Glutathione peroxidase activity was determined according to the procedure of Rotruck et al. (1973) with some modifications. Two test tubes were selected for each sample. Each tube contained 250 µl phosphate buffer pH 7.4, 50 µl sodium azide, 100 µl reduced glutathione, 250 µl tissue homogenate and 100 µl hydrogen peroxide, the reaction mixture was maintained at 37°C. 250 µl of 10% trichloroacetic acid was added to the first tube after 1minute and the other tube after

3minute, each mixture was centrifuged at 3000rpm for 5minutes. 250 µl of the supernatant was dispensed from each tube respectively, added to each tube were 500 µl dipotassium hydrogen orthophosphate and 250 µl Ellman's reagent. The colour developed in the test tubes was spectrophotometrically read (420nm) as against the reagent blank.

Estimation of Lipid Peroxidation

Thiobarbituric acid reactive species (TBARS) amount in tissue homogenates were determined following the method of Varshney and Kale, 1990. Tissue homogenates (100 µl) was mixed with 400 µl of Tris-KCl buffer pH 7.4 and 125 µl of 30% trichloroacetic acid (TCA). 125 µl of 0.75% thiobarbituric acid (TBA) was added to the mixture and incubated for 1 hour at $90^{\circ C}$. The mixture was cooled on ice and centrifuged at 4000rpm for 10minutes. The clear supernatant obtained was carefully decanted and its absorbance read at 532nm against a reference blank.

Biomarkers of Inflammation

Determination of Nitric Oxide Concentration

Nitric oxide concentration was determined by estimating the level of NO as described by Green et al. (1982). The amount of nitrite was measured by incubating of the tissue homogenates (150 µl) with Griess reagent (150 µl) for 20minute at room temperature. The absorbance at 550nm was read spectrophotometrically. The concentration of nitrite was obtained by comparison with the OD 550 of standard solution of sodium nitrite concentration.

Myeloperoxidase Activity Determination

Activity of Myeloperoxidase was measured spectrophotometrically using O-dianisidine and hydrogen peroxide via the method described by Kim, 2012. 800 µl of O-dianisidine mixture (O-

dianisidine dihydrochloride, 3,3-dimethoxybenzidine in 100 ml phosphate buffer pH 6.0), with 50 µl of dilute hydrogen peroxide (4 µl 30% hydrogen peroxide in 96 µl distilled water) and 28 µl of tissue homogenates was added. The mixture was quickly mixed by inversion in the cuvette and three absorbance readings at 30 seconds intervals were taken at 460nm for 90 seconds.

Histopathology

Histopathological analysis on the brain sections were carried out using the method described by Fischer *et al*., 2008

STATISTICAL ANALYSIS

The results of the study were expressed as mean ± SD. Data were subsequently analyzed by subjecting data obtained to Tukey's test (ANOVA) using Graphpad Prism (V 6.0.1). (Graphpad Software, La Jolla, CA). P values < 0.05 were considered statistically significant.

RESULTS

From this study, it was observed that vancomycin caused perturbation in the antioxidant system of the brain resulting in oxidative stress which then progressed into inflammatory responses. Generation of free radicals and declined antioxidants play a major role in the onset, progression, and complication of cognitive declination. From this study, levels of non-enzymic antioxidants, Reduced Glutathione (GSH) and Ascorbic acid (AA) depleted in rats treated with Vancomycin. Administration of hesperidin increased the levels of GSH and AA (Figure i&ii).

Furthermore, activities of enzymic antioxidants; Superoxide Dismutase (SOD) and Catalase (CAT) were down-regulated in the brain sections (cerebellum, cerebrum and hippocampus).

The ameliorative effects of Hesperidin on Vancomycin-induced changes on the brain (cerebellum) SOD and catalase (CAT) activities in rats were presented in Table i. SOD and CAT activities significantly decreased in the Vancomycin-treated group by 45.8% and 44.8% respectively when compared with the control group ($P<0.05$). However, the combined administration of Hesperidin and Vancomycin significantly ameliorated against the reduction in SOD and CAT activities relative to the Vancomycin-treated group.

Table ii shows the ameliorative effects of Hesperidin on Vancomycin-induced changes on the brain (cerebrum) SOD and catalase (CAT) activities in rats. SOD and CAT activities were significantly decreased in the Vancomycin-treated group by 21.5% and 40% respectively when compared with the control group ($P<0.05$). However, the combined administration of Hesperidin and Vancomycin significantly attenuated the decrease in SOD and CAT activities relative to the Vancomycin-treated group.

Table iii presents the ameliorative effects of Hesperidin on Vancomycin-induced changes on the brain (hippocampus) SOD and catalase (CAT) activities in rats. SOD and CAT activities were significantly decreased in Vancomycin treated group by 23.7% and 44.8% respectively when compared with the control ($P<0.05$). However, the combined administration of Hesperidin and Vancomycin significantly protected against the decrease in SOD and CAT activities relative to the Vancomycin-treated group.

In addition, activities of enzymic antioxidants Glutathione Peroxidase (GPx) and Glutathione-S-Transferase (GST) were decreased significantly in Vancomycin-treated rats. This decreased activity observed was attenuated upon co-administration of hesperidin (Figure iii and iv).

Increased levels of Nitric Oxide (NO), Malondialdehyde (MDA) and increased activity of Myeloperoxidase (MPO) was observed in vancomycin-treated rats relative to control. These perturbations were reverted upon co-administration of hesperidin (Figure v, vi and vii). Histopathological examination of the brain sections of Vancomycin-treated rats showed the presence of lesions and administration of Hesperidin reversed these lesions (plate i).

DISCUSSION

The generation of free radicals and declined antioxidants play a major role in the onset, progression and complication of all pathological disorders. The brain is susceptible to oxidative damage as a result of an abundance of redox-active transition metal ions, high utilization of inspired oxygen, a large amount of easily oxidizable polyunsaturated fatty acids, and relative dearth of antioxidant defense system (Vatassery, 1998) and oxidative stress is associated with cognitive declination in both animals and human model (Giasson et al., 2002).

Vancomycin, a glycopeptide antibiotic with bactericidal activity has been used over time to treat several infections caused by the gram-positive bacteria, its mechanism of action is via inhibition of bacterial cell wall biosynthesis or inhibition of peptidoglycan biosynthesis (Gupta et al., 2011). There have been some reported cases of vancomycin alterations in free radical balance resulting in oxidative stress and oxidative stress is one factor involved in the pathogenesis of neurotoxicity (Schoen, 2005; Costa et al., 2009).

Hesperidin, a flavone glycoside present in green fruit such as citrus fruits (sweet oranges and lemon) possesses multiple beneficial effects such as antioxidant, hepato-protective and anti-inflammatory (Parhiz et al., 2015). In clinical studies, hesperidin has been established to be

neuroprotective (Kean et al., 2015). This study aimed at evaluating the attenuative effect of hesperidin against vancomycin-induced neurotoxicity in rats.

Data from this study demonstrated that vancomycin administration induced neurotoxicity as evident from the assays carried out and co-treatment with hesperidin ameliorated the effect seen. It was observed that the administration of vancomycin induced oxidative stress in the brain (cerebellum, cerebrum, and hippocampus) by causing a significant reduction in the activities of Superoxide dismutase (SOD), Catalase, Glutathione-S-Transferase (GST), Glutathione peroxidase (GPx), and the levels of Reduced Glutathione (GSH) activity and Ascorbic acid activity. Furthermore, an increase in the level of malondialdehyde was observed in vancomycin-treated rats which indicates some level of lipid peroxidation in the membranes of the brain sections, this was ameliorated in the group co-treated with hesperidin.

Superoxide dismutase and Catalase are the free radical scavenging enzymes that constitute a very important antioxidant defense against oxidative stress in the body (Landis and Tower, 2005). Superoxide dismutase catalyzes the dismutation of superoxide anion free radical (O^{2-}) into hydrogen peroxide (H_2O_2) and molecular oxygen, Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen (Casado et al., 2001; Valko et al., 2006) and SOD protects biological structures and membrane against oxygen free radicals by catalyzing the removal of superoxide radical (O^{2-}) (Yasui and Baba, 2006).

Decreased CAT activity (Table 1-3) is associated with oxidative stress in brain regions (Ankita et al., 2019). The decrease in activity of SOD in animals treated with vancomycin is associated with increased O^{2-}, which has been reported to inhibit CAT (Ghazaleh et al., 2017). However, in this study, the administration of hesperidin offered significant protection by up-regulating the activities of both SOD and CAT.

Furthermore, Glutathione peroxidase, an antioxidant enzyme in the brain protects cell membranes from lipid peroxidation by metabolizing peroxides such as H_2O_2 as hydrogen peroxide and removed through reduction to water, using the electron donor, glutathione (GSH). In this present study, the observed decrease in the activity of GPx in vancomycin-treated rats may be attributed to the reduction in the reduced glutathione level and an increase in the level of peroxides. Glutathione-S- transferase (GST) on the other hand is a family of enzymes that catalyze the addition of reduced glutathione to endogenous and xenobiotic substrates which invariably have electrophilic functional groups (Townsend and Tew, 2003). The glutathione-S-Transferase activity was also downregulated in brain sections of vancomycin-treated rats. Reduction in the activities of these enzymes in the brain sections thus result in oxidative stress, and this was attenuated by co-treatment with hesperidin.

Ascorbic acid is a non-enzymatic antioxidant and has been shown to protect various tissues against the damage caused by reactive oxygen species (Fadine, 2017). On the other hand, Reduced Glutathione is known for its effective antioxidant property by scavenging oxidative stress-inducing molecules such as hydroxyl radicals and singlet oxygen, detoxifying hydrogen peroxides and lipid peroxides (Katia et al., 2014). A depletion in the levels of Ascorbic acid and Reduced glutathione was observed in Vancomycin-treated rats. However, co-administration of Hesperidin was able to protect against oxidative stress-induced by Vancomycin as increased in the levels of ascorbic acid and GSH seen in rats co-treated with hesperidin.

In addition, the Malondialdehyde level is commonly known as a marker of oxidative stress. The elevation of malondialdehyde (MDA) level is an indication of lipid peroxidation which can be caused by reactive oxygen species. The increase observed in MDA level may be due to the effect of free radicals which can interact with polyunsaturated fatty acids in the phospholipids of the cell

membrane, inducing lipid peroxidation in brain tissues (Del et al., 2005). In this study, it was observed that MDA level was significantly increased in the Vancomycin-treated group, and thus implying that Vancomycin induced lipid peroxidation and therefore, oxidative stress in the brain. This increase seen was attenuated with treatment with Hesperidin, indicating that Hesperidin decreased lipid peroxidation and oxidative stress.

Nitric oxide (NO) is a non-enzymatic biomarker of inflammation (Sharma et al., 2007). An increase in the concentration of NO was observed in vancomycin-treated rats. Nitric oxide is a crucial molecule in acute and chronic inflammation and it is generated in high concentration in certain types of inflammation. However, co-treatment with hesperidin significantly reduced the increase in NO concentration observed in the vancomycin-treated group. Thus, hesperidin is effective against vancomycin-induced inflammation. In addition, Myeloperoxidase (MPO) is an enzymatic biomarker of inflammation that is released in response to inflammation. Myeloperoxidase produces hypochlorous acid (HOCL) from hydrogen peroxide (H_2O_2) and chlorine ion (CL-) (Ndrepepa, 2019). A significant increase in MPO activity was observed in the vancomycin-treated group as opposed to the decrease observed in rats co-treated with hesperidin.

Histopathological examination of the brain sections of Vancomycin-treated rats showed the presence of lesions and neuronal death which was reversed upon administration of Hesperidin.

CONCLUSION

From this study, Vancomycin was observed to induce oxidative stress in the selected brain sections (Cerebellum, Cerebrum, and Hippocampus) of experimental animals. However, Hesperidin, a potent antioxidant was able to ameliorate this effect and this may be due to its intrinsic antioxidant properties

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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