CELLGEVITY® AMELIORATES STZ-DIABETIC NEUROCHEMICAL CHANGES AND NEURONAL DAMAGES IN MALE RATS

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Abstract: Reactive-radicals' mediated neurochemical changes caused by diabetic hyperglycemia, participate in cognitive impairment and neurodegeneration. The present study investigated the effect of Cellgevity® (marketed glutathione enhancer) on cognitive function, inflammation, and oxidative stress in STZ-diabeticrats. Behavioural and cognitive parameters were assessed and the cerebral cortex region was assessed for acetylcholine esterase activity, oxidative stress markers, and inflammation. STZ-diabetes impaired rats' memory and it induced anxiety-like behaviour. It led to at least 2 fold increases in acetylcholinesterase activity, levels of TNF-α, nitrite, and lipid peroxidation respectively in the cerebral cortex. It also caused 3.25 fold and 1.62 fold decreases in the activities of glutathione peroxidase and superoxide dismutase respectively compared to non-diabetic control. Separate treatment of diabetic rats with Cellgevity® (25 or 40 mg/kgbw)for 28 days significantly attenuated cognitive deficit, increased acetylcholinesterase activity, decreased oxidative/nitrosative stress and inflammation. Our results show the involvement of oxidative/nitrosative and inflammatory events in diabetes-induced neurodegeneration in rats and it presents the possible therapeutic potentials of Cellgevity® in ameliorating these diabetic-associated neurochemical dysfunctions. Cellgevity® prevents the increase in acetylcholinesterase activity, showing that it can modulate cholinergic neurotransmission and consequently improve cognition. Taken together, our results confirm the capacity of Cellgevity® as an antioxidant, to neutralizeexcess free radicals generated by diabetes disease and consequently stalldiabetes-neurodegeneration.

Keywords: Diabetes, Cellgevity®, acetylcholine esterase, cholinergic dysfunction, oxidative stress

Introduction

Oxidative stress, defined as an imbalance between free radicals generation and innate antioxidant defence, plays important role in the pathogenesis of neurodegeneration (Brownlee, 2001; He and King, 2004; Romuk *et al*., 2017). The progression of diabetes encephalopathy is a multifactorial pathophysiological process that is dependent upon several events during which hyperglycemia-induced oxidative stressactivates glucose damages through auto-glycosylation, advanced glycation end products formation, and excessive ROS production (Bloomgarden, 2007; Gugliucci, 2000; Rajabally, 2011), it also alters the expression and activities of key enzymes (including Na-K-ATPase, catalase, 5′-nucleotidase, NTPDase, and acetylcholinesterase) necessary for neurological functions. It could also lead to endothelial and vascular damage, decreased vasodilation and nerve blood flow, endoneurial hypoxia, loss of neurons, disruption of calcium homeostasis, and deficit nerve conduction velocity(Brands *et al*., 2004; Singleton *et al*., 2003; Singleton and Smith, 2007). Excess cellular free radical pool (peroxides, superoxide, hydroxyl ions, and nitric oxide) damages lipid, DNA, and protein, by disrupting their structure and molecular functions thereby compromising cell integrity (Asmat *et al*., 2016). Diabetic brain mitochondria have high nitric oxide and nitric oxide synthase expression which may contribute towards neuronal damage and long-term neurodegeneration, excess superoxide radicals produced in the mitochondria electron transport chain during oxidative phosphorylation signals increased superoxide dismutase activity leading to hydrogen peroxide (H_2O_2) release into the cytosol, causing depletion of endogenous antioxidant enzymes and rendering the cells more vulnerable to oxidative damage (Brownlee, 2001; Fujita *et al*., 2009; Jakus, 2000). The brain cells are especially susceptible to oxidative injuries being rich in polyunsaturated fatty acids which are the targets for lipid peroxidation (Guan, 2008). Oxidative neuronal damage signals the influx of inflammatory cells into brain tissues; releasing pro-inflammatory cytokines, such as TNF-α and interleukin (IL)-1 which further increases free radicals production that could lead to aggravated cell damage and severe complications. Cholinergic dysfunction indicated by increased acetylcholinesterase activity is associated with cognitive and psychomotor impairments observed in diabetes mellitus; with the potentials to develop clinical dementia.

Cellgevity® is a popular glutathione supplement containing riboCeine (D-Ribose and L-Cysteine) as the active ingredient aside other components including; vitamin C, selenium, alpha-lipoic, broccolli seed extract, curcumin, resveratrol, grape seed extract, quercetin, milk thistle seed extract, cordyceps, black pepper, aloe leaf (Awodele *et al*., 2018a). Glutathione (a non-protein thiol tripeptide of cysteine, glutamic acid, and glycine) is a ubiquitous endogenously synthesized antioxidant that performs several physiological roles including; thiol-disulfides redox balance, antioxidant defence, drug detoxification, signal transduction, cysteine mobility, cell proliferation control, and apoptosis (Lutchmansingh *et al*., 2018; Meister and Anderson, 1983). Antioxidants therapies have been shown to protect against many neurodegenerative conditions by improving redox balance, energy metabolism, inflammation control, and ROS arrest and several effects of glutathione supplementations in ameliorating clinical syndromes have been well discussed (Lee *et al*., 2017; Lutchmansingh *et al*., 2018; Ueno *et al*., 2002).

Therefore, this study was designed to investigate the influence of Cellgevity® supplementation on cognition, brain oxidative parameters, TNF-α and nitrite levels (markers of inflammation), and acetylcholinesterase activity (a marker of cholinergic dysfunction) in STZ-diabetic rats.

Materials and Methods

Drugs and chemicals

Streptozotocin was purchased from Sigma Aldrich (USA), Accuchek glucose strip from Roche (Germany) and Cellgevity® was purchased from Max international (USA). Glutathione peroxidase assay kit and TNF-α ELISAkit were obtained from Abcam (UK). All other chemicals and reagents used were of analytical grade.

Experimental animals and design

The study was conducted in compliance with established protocol of biomedical research for the use of experimental animals as approved by the Animal and Human Health Ethics Committee of Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Ago-Iwoye, Nigeria in conformity with the guide for the care and use of laboratory animals - U. S. National Institutes of Health Publication (Research, 1996). Adult male Wistar rats (180–220 g), were properly maintained under standard laboratory conditions and provided fed and water ad libitum. All efforts were made to minimize animal suffering, to reduce the number of animals used.

Induction of Type 2 diabetes

Streptozotocin (STZ) is a cytotoxic compound, popularly used for diabetogenic toxicity in rodents (Konrad *et al*., 2001). Insulin resistance was first achieved in the animals (except those in the control group) by 3 weeks feeding with high sucrose diet (60% w/w) (Santuré *et al*., 2002). Afterward, intraperitoneally injection of streptozotocin (55 mg/kg) was given to induce diabetes. Seventy-two hours after STZ injection, hyperglycaemia was confirmed and diabetic rats (with fasting blood glucose > 250 mg/dl) were divided into three groups of 7 rats each: DM-untreated, DM-treated 1 (Cellgevity® 25 mg/kg), and DM-treated 2 (Cellgevity® 40 mg/kg); the dosage of Cellgevity® were selected based on the recommended human dosage (Awodele *et al*., 2018b). Treatments were administered as a single oral dose daily for 30 days.

Behavioural assessment

Open field (OF) test

The OF test was conducted on day 29 of treatment to evaluates the ambulatory activity, exploration, and anxiety (emotionality)behaviour in the rats (Arika *et al*., 2019). The open field is an opaque, Plexiglas (white)(50 X 50 cm) base and a 40 cm height unit placed in a quiet, well-lit room. Animals were acclimated to the test room overnight before testing. Each test rats is placed at the center of the unit and left for five minutes, while an independent observer recorded the number of entries into each zone and the time spent crossing. The floor of the unit is sectioned by 10 cm^2 gridlines into two major regions; central and peripheral region. The ambulatory distance and the time spent in each region were calculated; reflecting the locomotor activity and the anxiety behaviour.

Novel objects recognition (NOR) task

The NOR task was conducted following the OF test to evaluate recognition memory in the rats (Bădescu *et al*., 2016). The test is based on the natural tendency of mice to spend more time investigating an unfamiliar object than a familiar one. The preference to explore a novel object over a familiar one reflects the use of learning and recognition memory. The test animal is placed in an open field with two dissimilar objects having similar height and volume, but different shape and colour. The animals are allowed to habituate in an empty open field after which they are exposed to two identical objects. The next day, the mice are exposed to the open field in the presence of the familiar object and a novel object to test long-term recognition memory; the time spent exploring each object was recorded.

Necropsy

The rats were sacrificed under deep anaesthesia 24 h after the final test, blood was collected by cardiac puncture into plain pre-chilled bottles on ice, and serum was separated by centrifugation at 4000 rpm for 10 min andstored at -20ºC for subsequent biochemical analyses. The brain was rapidly removed and the cerebral cortex was separated, and homogenized in 5 volumes of chilled phosphate buffer (pH 7.4). The homogenate was centrifuged for 5 min at 4000 rpm to clear cell debris after which the supernatant was spurned at 15,000 rpm for 20 min at 4°C to obtain post mitochondrial supernatant (Kuhad and Chopra, 2007) which was stored at -20°C and used for biochemical analysis.

Biochemical assays

Measurement of fasting blood glucose (FBG)

The animal blood glucose was measured weekly after an overnight fasting period using AccuChek Active© glucometer and glucose strips with blood obtained by tail vein puncture. Measurements of the initial and final FBG were compared and presented.

Estimation of acetylcholinesterase activity

Cholinergic dysfunction was evaluated by measuring acetylcholinesterase activity in the cerebral cortex supernatant (Kuhad and Chopra, 2008). Acetyl-cholinesterase enzyme activity was determined based on the method of Ellman (Ellman *et al*., 1961) reported in (Alabi *et al*., 2019).0.1 ml tissue supernatant, 1.4 mL phosphate buffer saline (PBS), and 0.05 ml 0.01M DTNB (in 0.1M PBS)were incubated for 10 min at room temperature and the initial absorbance was read at 412 nm. The reaction substrate (0.05 mL 0.028M acetylthiocholineiodide in 0.1M PBS) was then added and the reaction allowed running for 2 min after which final absorbance was read at the same wavelength. The rate of acetyl-cholinesterase activity was expressed as µmol/min/g tissue

Estimation of tumour necrosis factor-alpha (TNF-α) level

The level of TNF-α (a marker of inflammation) was estimated in the cerebral cortex using a mouse TNF-α ELISAkit (Abcam, UK). The assay was conducted according to manufacturer's instruction; it is a solid-phase immunosorbent assay (ELISA) sandwich-enzyme-linked protocol using a 96 well micro-plate which is reader at 450 nm. The concentration of TNF-α was calculated from a standard curve and expressed as pg/mL.

Estimation of nitrite levels

Nitrite accumulation (an indicator of nitric oxide production) was estimated by a colorimetric assay method of Green (Green *et al*., 1982)as described in (Kuhad and Chopra, 2008). Equal volumes of sample and Griess reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% napthaylamine diaminedihydrochloric acid in water) were mixed and incubated for 10 min at room temperature in the dark; the absorbance at 540 nm was afterward determined. Nitrite concentration was calculated from a sodium nitrite standard plot.

Estimation of lipid peroxidation (MDA) level

Lipid peroxidation was assessed in the cerebral cortex supernatant by the TBARS assay. The extent of lipid peroxidation (LPO) was determined according to the method of Ohkawa (Ohkawa *et al*., 1979). The reaction forms a stable pink/reddish chromophore (malondialdehyde) which absorbs maximally at 532 nm. Lipid peroxidation was expressed as µmol of malondialdehyde (MDA) per mg protein.

Estimation of catalase (CAT) activity

Catalase activity was measured by a colorimetric assay method descried by Hadwan (Hadwan, 2018). Assay mixture contains 0.5 ml of the sample, 1ml 10 mM hydrogen peroxide (prepared by adding 0.1134 ml of 30% hydrogen peroxide to 100 ml phosphate buffer), and 1ml working solution (100 ml cobalt (II) solution, 100 ml Graham salt solution, and 1800 ml sodium bicarbonate solution. The mixture was vortexed for 5 s and kept at room temperature for 10 min in the dark. The changes in absorbance were recorded at 440 nm against the reagent blank. Catalase activity was calculated in terms of k min^{-1}

Estimation of superoxide dismutase (SOD) activity

The level of SOD activity is determined as previously reported (Misra and Fridovich, 1972). The principle is based

on the ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine. 0.2 ml of enzyme preparation is added to 2.5 ml of 0.05 M Carbonate buffer (pH 10.2) to which 0.3 ml of freshly prepared 0.3 mM epinephrine is added and the increase in absorbance at 480 nm is monitored over a time course against a reference. The unit of SOD activity is defined as the amount necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during an interval of one minute.

Estimation of glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity was measured using a commercial assay kit (Abcam, UK) according to the manufacturer's instructions. The assay is a coupled reaction involving the oxidation of glutathione (GSH) to GSSG as GPx reduces cumenehydroperoxide. Glutathione reductase (GR) then reduces the GSSG back to GSH, using NADPH. The decrease of NADPH is measured at OD=340 nm and is proportional to GPx activity.

Protein assay

Tissue protein concentration was estimated as previously described by Bradford (Bradford, 1976) using bovine serum albumin as the standard.

Statistical analysis

Results were expressed as mean ± standard deviation SEM. Data were subjected to one-way analysis of variance ANOVA. The groups were compared using Tukey's test. All statistics were carried out using GraphPad Prism 8.4.3 software (USA). Values of $p < 0.05$ were considered statistically significant.

Results and Discussion

Cellgevity® improved fasting blood glucose (FBG)

There was no change in fasting blood glucose levels of control animals but there was a markedly increased in the FBG of the DM-untreated rats (340%) compared to the control rats (Fig 1). Treatment of diabetic rats with Cellgevity® abrogated the diabetes-induced hyperglycemia.

Cellgevity® improved the long-term memory

Rats in the control group spent more time exploring the novel object than the familiar one (Fig. 2), but, DM-untreated rats did not discriminate between novel and familiar objects, and they spent similar time exploring both objects. The DM-treated 1&2 group that received Cellgevity® supplementation showed an object recognition pattern similar to the control.

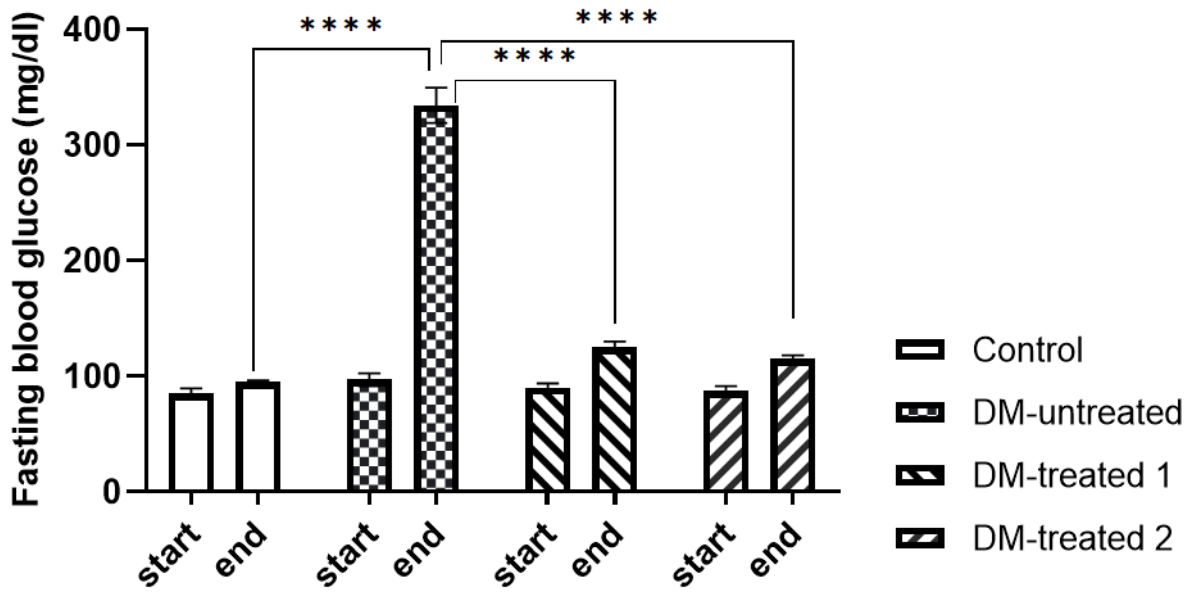

Fig. 1: Bar chart shows fasting blood glucose across the groups. The data are expressed as mean with error bar showing SEM. * represents a value of *P<0.001*. The comparison was with the corresponding value for control animals or diabetic untreated animals**

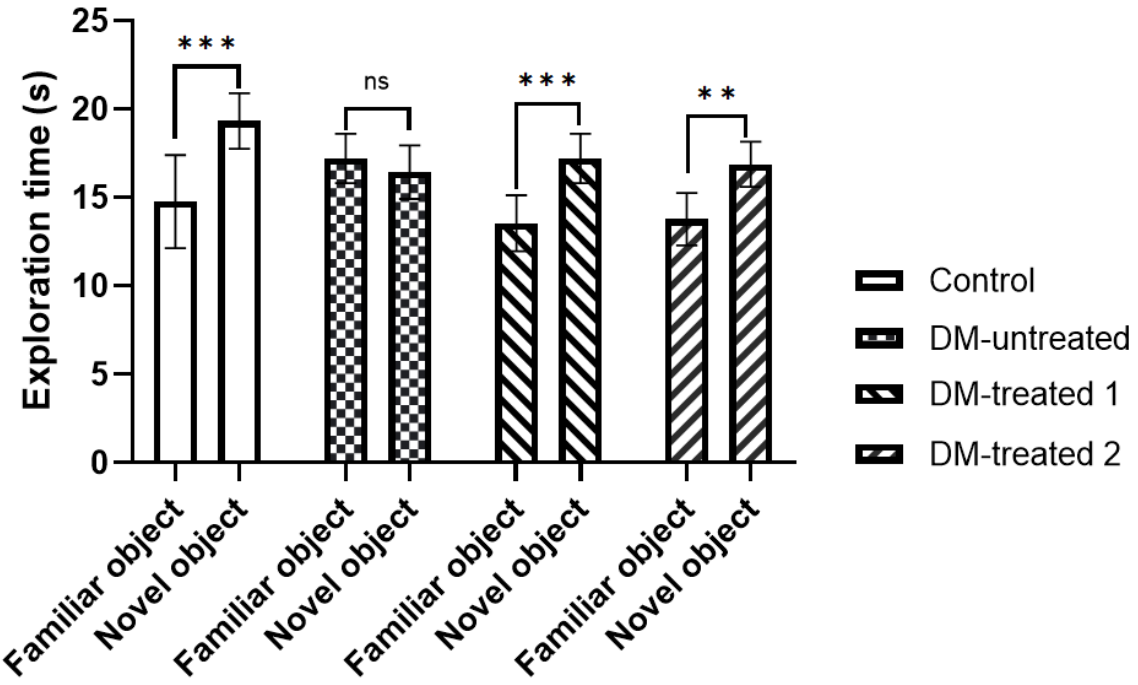

Fig. 2: Representative figure showing the exploration time spent on the familiar and novel object by each group. The data are expressed as mean with error bar showing SEM. * represents the value of *P<0.001* and ** shows a value of *P<0.01* and ns shows no significant difference. Comparisons were made between the familiar and novel object for each of the groups**

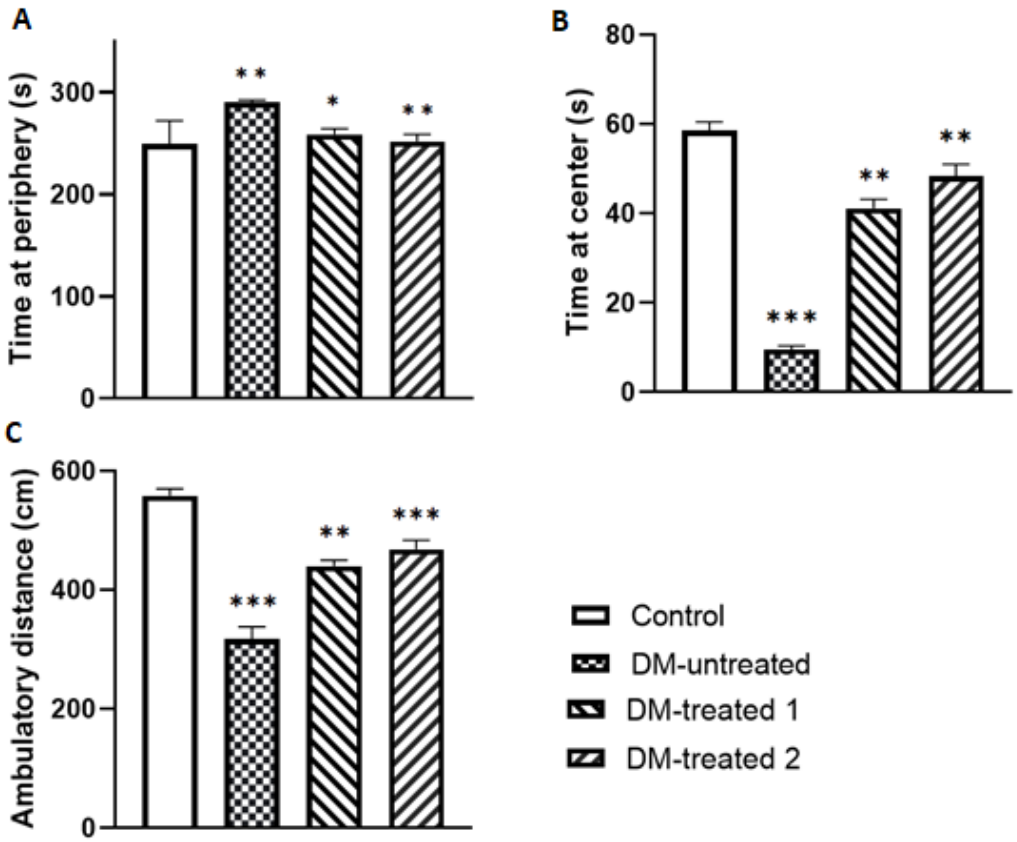

Fig. 3: Bar charts represent the result of the open field test: (3A) shows time spent at the periphery of the maze, (3B) shows time spent at the center, and (3C) shows the total distance recorded by animals within the open field. DM-untreated was compared with the corresponding value for control animals, while DM-treated 1 & 2 groups were each compared with DM-untreated, respectively. The data are expressed as mean with error bar showing SEM. * represents a value of *P<0.001* and ** represents a value of *P<0.01***

Cellgevity® reduced latency and anxiety

Control rats showed higher activity and exploration tendency (demonstrated by higher ambulatory distance travelled in the open field) compared with the untreated diabetic rats (Fig. 3C). The control rats also spent more time crossing and exploring the center of the open field (Fig. 3A and 3B). However, diabetic untreated rats showed increased anxiety-like behaviour by avoiding the center arena but staying more at the peripheral region of the open field and they also recorded shorter ambulatory distance compared to control animals (Fig. 3A, 3B and 3C). Cellgevity® treated diabetic rats had improved behaviour compared to the untreated rats but comparable to the control.

Cellgevity® improved cholinergic transmission

Results representing the activity of acetylcholinesterase in the cerebral cortex of the rats are presented in Fig. 4. Acetylcholinesterase activity was significantly (*P<0.001*) increased in the DM-untreated group compared to the control group. However, treatment with Cellgevity® significantly prevented cholinergic dysfunction in DM-treated 1 & 2 groups compared to theDM-untreated group.

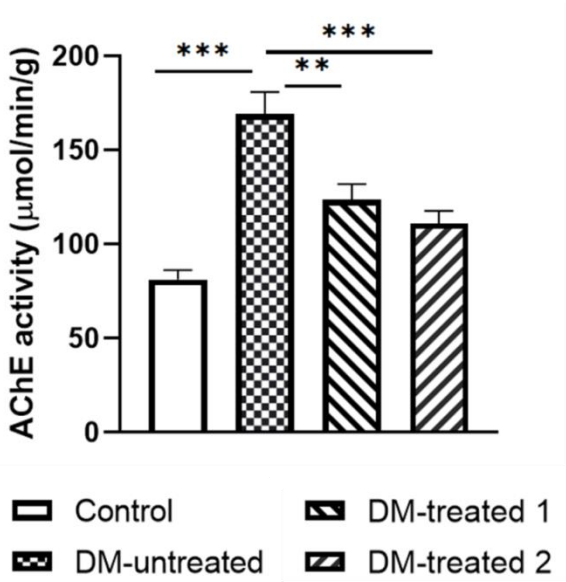

Fig. 4: Shows the level of acetylcholinesterase activity across the groups. The data are expressed as mean with error bar showing SEM. * represents the value of *P<0.001* and ** represents the value of *P<0.01*. The figure shows a comparison with the corresponding value for the control group or DM-untreated group**

Cellgevity® decreased inflammation and nitrosative stress

The level of tumour necrosis factor-alphain the cerebral cortex of diabetic rats was significantly increased compared to the control (Fig. 5A); however, Cellgevity® significantly prevented the diabetes-induced rise in TNF-α and inflammation as observed in the DM-treated groups compared to the DM-untreated group. Similarly, there was elevated nitrite concentration in DM-untreated group compared to the control but Cellgevity® also prevented this increase in the DM-treated rats compared to the DM-untreated (Fig. 5B).

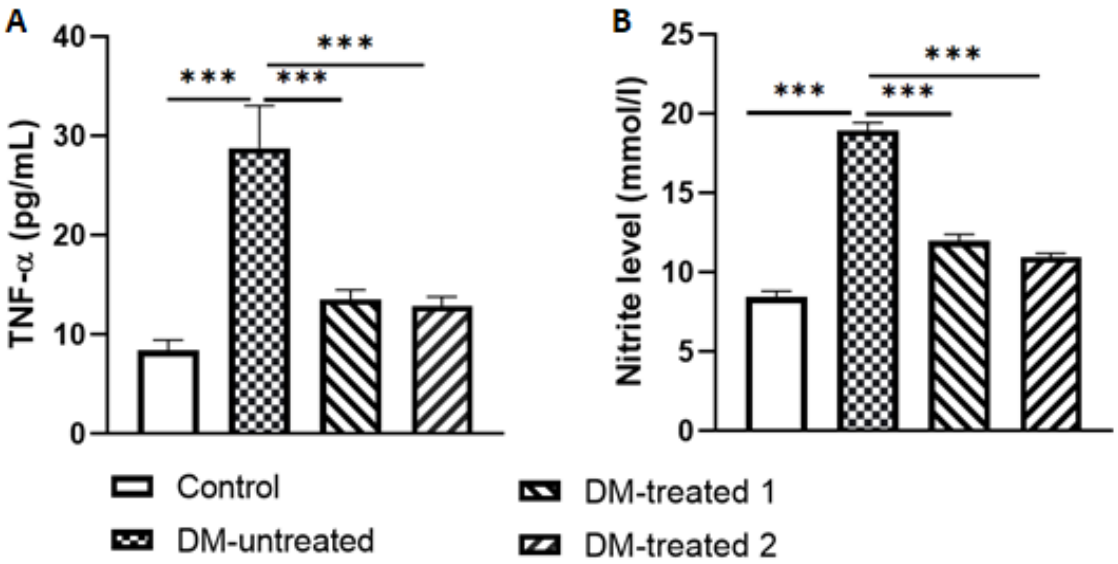

Fig. 5: Bar charts show levels of TNF-α (5A) and nitrite (5B) across the group. The data are expressed as mean with error bar showing SEM. * show a value of *P<0.001*. The figure shows a comparison with the corresponding value for the control group or DM-untreated group**

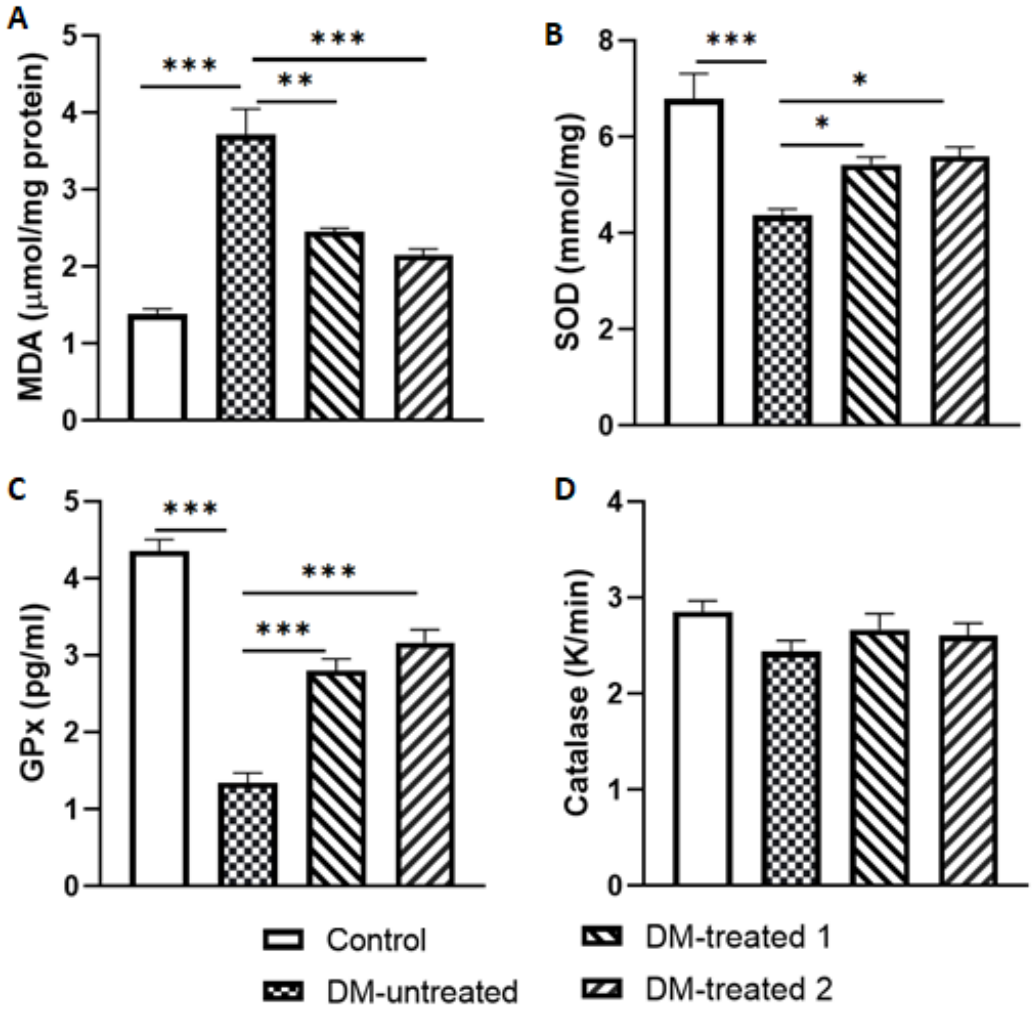

Fig. 6: Bar charts show the level of lipid peroxidation (6A) and the activities of antioxidant enzymes; SOD (6B), GPx (6C), and CAT (6D) in the cerebral cortex of experimental rats across the groups. The data are expressed as mean with error bar showing SEM. * represents value of *P < 0.001*, ** represents *P < 0.01* and * represents *P < 0.05* Comparison was with the corresponding value for control animals or diabetic untreated animals**

Cellgevity® reduced lipid peroxidation

The result showing the levels of lipid peroxidation in the cerebral cortex is presented in Fig. 6A. Diabetes caused a significant increase (*P<0.001*) in lipid peroxidation (malondialdehyde level)in the DM-untreated group compared to the control group. Supplementation with Cellgevity® significantly lowered the effect of diabetes-induced lipid peroxidation in the DM-treated 1 & 2 groups compared to the DM-untreated group by inhibiting lipid peroxidation.

Cellgevity® improved the activity of antioxidant enzymes

The result for antioxidant enzymes activity in the cerebral cortex is presented in Fig. 4. Activities of superoxide dismutase (SOD) (Fig.6B) and glutathione peroxidase (Fig. 6C), and were significantly (*P<0.001*) reduced in the DM-untreated group compared to the control group. Treatment with Cellgevity® significantly prevented diabetes-induced enzyme inhibition as shown in DM-treated 1 & 2 groups compared to the DM-untreated group. The activity of catalase (Fig. 4D) was similar across the groups with no significant difference.

Hyperglycemia induced mitochondria flux leads to excess production of reactive species which overwhelms the innate antioxidant defence of the body thereby facilitating oxidative-nitrosative stress. Glucose toxicity activates the major glucose-dependent ROS pathways (polyol pathway, advanced glycation end products formation, activation of protein kinase C, and the hexosamine pathway) and it amplifies neuroinflammation and neurodegeneration (Romuk *et al*., 2017; Sandireddy *et al*., 2014). The current report, represents a novel investigation on the impact of Cellgevity®, on STZ-diabetic neurodegeneration in malerats; its effects on behaviour and memory, acetylcholinesterase activity, TNF-α, and nitrite levels, the extent of lipid peroxidation and activities of SOD, CAT and GPx in the cerebral cortex of rat brain are presented. The results show that STZ-diabetes potentiates memory and learning impairment, lipid peroxidation, cholinergic dysfunction, inflammation and antioxidant deficiencies in the cerebral cortex of male rats similar to some other reports (Kuhad and Chopra, 2008; Schmatz *et al*., 2009). The result shows a significant increase in acetylcholinesterase activity in the diabetic untreated group; acetylcholinesterase has important roles in learning and memory and alterations in its activity and the level of acetylcholine are associated with cognitive deficiencies (Kuhad and Chopra, 2008; Silman and Sussman, 2005). Similarly, to our result, others have observed a significant increase in acetylcholinesterase activity in the brain of streptozotocin-induced diabetic rats (Brands *et al*., 2004; Kuhad and Chopra, 2008; Schmatz *et al*., 2009). The hyper-activation of acetylcholinesterase increases the degradation of acetylcholine and decreasing its concentration in synaptic cleft thereby reducing cholinergic neurotransmission efficiency

which could contribute towards progressive cognitive impairment and neurological dysfunctions (Bloomgarden, 2007; Downes and Granato, 2004; Guan, 2008; Soreq, 2001). Our results however show that treatment with Cellgevity® at the test doses (25 and 40mg/kg) ameliorated the diabetic-induced cholinergic dysfunction in the cerebral cortex of the diabetic treated rats; similarly to this result, treatment with some antioxidants have shown an inhibitory effect in the diabetes-induced rise in acetylcholinesterase activity (Kuhad and Chopra, 2008; Schmatz *et al*., 2009).

Furthermore, our result shows a significant increase in TNF-α, nitrite and lipid peroxidation levels, and reduction in the activities of glutathione peroxidase and superoxide dismutase but catalase activity was unchanged in the cerebral cortex of untreated STZ-diabetic rats; treatment with Cellgevity® normalized the levels of these biomarkers towards the control values. Hyperglycemia induced oxidative stress and inflammatory pathways interact via multiple cross talk resulting in several pathophysiological outcomes and influx of pro-inflammatory cytokines(Jin and Park, 2018; Navarro and Mora, 2005). Chronichyperglycemia executes the production of inflammatory cytokines such as TNF-*α*, IL-6, COX-2, and iNOS in microvascular and neural tissues; causing elevated microvascular permeability and hypercoagulability associated with direct neuroinflammation, peripheral nerve damage and neuropathies (Elmarakby and Sullivan, 2012; Jin and Park, 2018; Navarro and Mora, 2005; Sandireddy *et al*., 2014).High nitrite level parallels elevated nitric oxide (NO) and nitric oxide synthase (NOS) activity (Pereira-Maróstica *et al*., 2019; Vanhoutte *et al*., 2009). High NO may increase protein modification through nitrosylation and protein thioloxidation (Onozato *et al*., 2002), nitric oxide is also known to upregulate the expressions of glucose transporters (GLUT) in neurons (Lira *et al*., 2007);which under hyperglycemic condition could lead to high mitochondrial flux and mitochondrial membrane hyperpolarization resulting in elevated free-radical production (Friberg *et al*., 2002). High NO and NOS action could also result in increased production of peroxynitrite (formed by the reaction between superoxide and NO) production (Maddu, 2019; Pacher *et al*., 2012; Sandireddy *et al*., 2014); a potent oxidant that potentiates nitrosative stress (Facchinetti *et al*., 1998; MA, 2003).The decline in the antioxidant enzyme activities observed in the cerebral cortex of our STZ-diabetic rats could be oxidant-mediated; oxidative stress is known to also cause endoplasmic reticulum (ER) damage which leads to accumulation of misfolded proteins. ER participates in protein folding and processing, therefore, a dysfunctional ER would lead to production and accumulation of non-functional proteins (Lupachyk *et al*., 2013). Oxidative stress could also cause DNA and mRNA modification leading to diminished expression and activities of proteins (Guan, 2008). Finally, there was an increase in the level of MDA in the cerebral cortex of our diabetic untreated rats compared to the control; increased level of lipid peroxidation signals a state of oxidative-nitrosative stress which would lead high rate of cell membrane attacks by damaging agents (such as superoxides, peroxide radicals, hydroxyl radicals, and peroxynitrite) which would then culminate into cell-tissue damage and neuronal dysfunction (Asmat *et al*., 2016; Maddu, 2019). However, Cellgevity® prevented the rise in lipid peroxidation in the treated rats.

Overall, the supplementation of STZ-diabetic rats with Cellgevity® ameliorated the molecular symptoms associated with the progression of neuronal damage and neuropathic events. Cellgevity® contains several antioxidant ingredients (such as vitamin c, selenium, alpha-lipoic, and resveratrol) which could act in synergy to create a potent protective barrier against the plethora of excess reactive radicals generated in diabetes. Cellgevity® could also enhance the body's innate antioxidant defence thereby abrogating oxidative stress, tissue damages, and diabetic complications. These results taken together, present an appeal to the use of Cellgevity® and antioxidant supplements as adjuvant in diabetogenic management.

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Conflict of Interest

The authors declare that there is no conflict of interest related to this work.

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