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UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

ACTIVITY OF NATURAL COCOA ON MARKERS OF BRAIN HEALTH IN EXERCISE TRAINED RATS

BY

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DECLARATION BY CANDIDATE

I hereby declare that except for references to work of other researchers, which have been duly referenced, this project is the product of my own research carried out under supervision in accordance with regulations of the School of Research and Graduate Studies, University of Ghana. I further declare that this dissertation has neither in whole nor in part been presented for another degree elsewhere, and that I am solely responsible for any residual flaws in this work.

Signature……

Date…….……...

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DECLARATION BY SUPERVISORS

We declare that the practical work and presentation of this thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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Signature…… Date…….……

Dr. Kevin Adutwum-Ofosu

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DEDICATION

I dedicate this work to my Father, Mr Benonis Osae Baidoo, who has made sure that all my dreams come to life!

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LIST OF ABBREVIATIONS

AB	Amyloid beta
AD	Alzheimer's disease
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BDNF	Brain Derived Neurotrophic Factor
CNS	Central Nervous System
COX	Cyclo-oxygenase
DNA	Deoxyribonucleic acid
GSH	Reduced Glutathione Synthase
GPx	Glutathione peroxidase
IGF	Insulin growth factor
IL-1	Interleukins-1
IL-6	Interleukin-6
IL-8	Interleukins-8
LPS	Lipopolysaccharides
LOX	Liquid oxygen
LTP	long term potentiation
MCP	Monocyte chemoattractant protein
NCP	Natural cocoa powder
NGF	Nerve growth factor

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NO	Nitric Oxide
OS	Oxidative Stress
Pi	Pi
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
SOD	Superoxide dismutase
SD	Standard deviation
TNF-α	Tumor necrotic factor-α
VEGF	Vascu*lar endothelial growth factor
WHO	World Health Organization

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ABSTRACT

Exercise training enhances healthy brain function by regulating inflammation and oxidative stress and promotes the production of growth factors including brain-derived neurotrophic factor (BDNF). BDNF is essential for neuronal growth and survival in deleterious neurological conditions such as stroke, Parkinson's and Alzheimer's. Moreover, dietary nutrients in cocoa reduces oxidative stress and inflammation and may have a protective effect that affects the growth and survival of neurons.

Aim: To assess whether ingestion of natural cocoa additively affects structural and biochemical indices of brain health produced by regular exercise training in healthy rats.

Methodology: Five rats were randomly assigned in to four groups: Exercise + Natural cocoa (G1), Exercise only (G2), Cocoa only (G3), No exercise and No cocoa (G4). Exercised rats were subjected to running training for 6 weeks on a running wheel. Natural cocoa was administered as 2% (w/v) suspension which the rats drunk in place of water for 12 hours. Rats in G4 were given tap water and all rats were fed with standard chow. Rats in group 1 and 2 were subjected to a six-day pre-training period on the running wheel to help accustom them to the apparatus and reduce stress associated with novelty. Blood samples were collected at the beginning and end of the study via tail snipping and milking to assess the biochemical indices of oxidative stress (SOD), inflammation (IL-6) and neurotrophic growth factors (BDNF). The brains were harvested at the end of experiment and processed histologically for morphometric analysis by stereological methods.

Results: Stereological analysis showed that the volume density of unhealthy pyramidal neurons in G1($0.111\pm0.02\times10^3\mu m^3$), G2($0.085\pm0.01\times10^3\mu m^3$) and G3($0.046\pm0.01\times10^3\mu m^3$) was significantly lower (0.0002) when compared to G4($0.186\pm0.04\times10^3\mu m^3$). Activity of SOD was significantly increased in G2 (p value= 0.016) and G4 (p=0.0091) with mean SOD activity values of (0.449±0.016 U/ml) and (0.343±0.0091U/ml) respectively. GSH activity was found by paired t-test to be statistically significant in G3 (p value = 0.0474) and a mean activity value

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of 3.39 (SD1.27) mmol/g). ANOVA of post treatment levels of IL-6 showed significant difference ($p = 0.0484$) between the groups. G1 (33.9625, SD 11.9), G2 (38.6425, SD 10.8), G3 (37.9425, SD 8.6) showing higher levels of IL6 compared to G4 (19.565, SD 2.6). There was a significant difference in the post treatment levels of BDNF among the groups. However, G1 (1292.500, SD 149.30) recorded the highest mean BDNF when compared to G4 (760.333, SD 172.92).

Conclusion: Exercise training and cocoa individually as well as when combined exert potentiating benefits on selected markers of brain health by reducing oxidative stress, increasing anti-inflammatory markers as well as maintaining the structural integrity of the neurons.

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CHAPTER ONE

INTRODUCTION

1.1 Background to the study

The brain is one of the most complex structurers in the body and differs from other organs by its distinguished and organized topographic features (Eickhoff, Constable, & Yeo, 2018). Despite its complexity, the brain parenchyma is predominantly made up of two types of cells. The parenchymal cells of the brain are neurons and neuroglia (Brat, 2018). Besides these, there are meningothelial and mesenchymal cell types which occur in coverings of the brain. Both neurons and neuroglia are large families with many members that have highly specialized functions, yet the underlying structure and cell biology of each retain some central features (Brat, 2018).

The cells in the normal brain, respond adaptively to endogenous changes resulting from internal neuronal and glial activity. Similarly, the brain responds and adapts to exogenous changes, particularly, environmental stressors such as exercise and diet alterations (Camandola, 2017). According to the latter author, adaptive responses at the cellular level include strengthening of existing synapses and formation of new ones, as well as production of new neurons from stem cells. At the molecular level, normal endogenous challenges result in the activation of transcription factors that induce expression of proteins such as brain derived neurotrophic factor (BDNF) (Leal, Afonso, Salazar, & Duarte, 2015). The expression of these proteins bolster the resistance of neurons to the kinds of metabolic, oxidative, inflammatory stresses involved in the pathogenesis of brain disorders including stroke, Alzheimer's, and Parkinson's disease (Patterson, 2015). BDNF is the most widely distributed growth factor within the brain, and has profound influences on the survival and function of neurons and neuroglial cells (Park & Poo, 2012).

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Previous studies have shown that metabolic alterations such as inflammation and oxidative stress are strongly implicated in the instigation and progression of many brain pathologies and neurodegenerative disorders and an overall decline in brain health (Allen & Bayraktutan, 2009a; Amor et al., 2014; Verdile et al., 2015). Oxidative stress is a self-propagating phenomenon, which occurs when release of excessive reactive oxygen species (ROS) triggers cellular damage, causing the damaged cells to behave as or become ROS (Hulbert et al., 2007). The brain is an easy target for oxidative insult because of its high oxygen consumption, high energy demand, and relatively weak anti-oxidant capacity (Salim, 2017).

Under normal conditions, deleterious effects of ROS production during aerobic metabolism are neutralized by endogenous antioxidant system and in this manner the brain effectively regulates its oxygen consumption and redox generation capacity. When ROS production exceeds scavenging capacity of the innate antioxidant response system, extensive protein oxidation and lipid peroxidation occurs, causing oxidative damage, cellular degeneration, and even functional decline (Allen & Bayraktutan, 2009).

Inflammation is considered a natural defence mechanism against pathogens, and whereas it is associated with many pathogenic diseases such as microbial and viral infections; it is also a response to exposure to allergens, radiation and toxic chemicals, autoimmune and chronic diseases (Hussain et al., 2016). Inflammation in itself is not a disease condition but a relevant biological process (Sankowski, Mader, & Valdacs-Ferrer, 2015)

This complex network of reactions is initially designed to protect the host from injury and to heal damaged tissue. The activation and migration of leukocytes to the site of the lesion and the release of growth factors, cytokines, reactive oxygen species (ROS), and nitric oxide (NO) are known to play a crucial role in the inflammatory response. Constant overproduction of pro-

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inflammatory molecules leads to chronic inflammation (Perez-Cano, Massot-Cladera, Franch, Castellote & Castell, 2013).

Chronic inflammation however is implicated in many disease conditions, including those that affect the brain (Verdile et al., 2015).

Many experimental data show that in many chronic disease conditions, including certain neurodegenerative diseases, there is simultaneous existence of inflammation and oxidative stress (Biswas, 2016; Tucker, Scanlan, & Dalbo, 2015). Inflammatory cells liberate a number of reactive species at the site of inflammation leading to exaggerated oxidative stress. On the other hand, a number of reactive oxygen/nitrogen species can initiate intracellular signalling cascade that enhances inflammatory responses (Biswas, 2016).

Some studies have shown that oxidative stress plays a pathogenic role in chronic inflammatory diseases (Elwood, Lim, Naveed, & Galea, 2017; Sankowski et al., 2015; Tucker et al., 2015). Popa-wagner (2013) posited that damage such as oxidized proteins, glycated products, and lipid peroxidation that arise from the oxidative stress results in neuron degenerations mostly reported in brain disorders. Also, ROS generated in brain tissues can modulate synaptic and non-synaptic communication between neurons that result in neuroinflammation and cell death, and then in neurodegeneration and memory loss (Popa-wagner, 2013).

Other researchers have also alluded to the fact that neurons are mostly the primary targets of neurotoxic processes (Rissman et al., 2007; Yamin, 2009). Although the mechanisms remain unclear, symptoms of many brain pathologies are as a result of neuronal damage or apoptosis (McCoy & Tansey, 2008). Oxidative stress and inflammation play a major role in the expression of neurotrophins (Calabrese et al., 2014; Siamilis et al., 2009). Neurotrophins are a family of proteins that regulate neuronal survival, synaptic function, and neurotransmitter

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release, and elicit the plasticity and growth of axons within the adult central and peripheral nervous systems (Keefe, Sheikh, & Smith, 2017).

As indicated earlier, BDNF is the most abundant neurotrophic factor in the brain, and mainly exists in the hippocampus and frontal cortex. BDNF has a vital effect on the genesis, survival, growth and synaptic plasticity of neurons in mammalian brains, and it can improve learning and memory capacity (Gibon & Barker, 2017). So many evidences are converging on the concept that lifestyle factors such as exercise and diet can improve learning and memory, delay age-related cognitive decline, reduce risk of neurodegeneration, and play a part in maintaining and promoting brain health. For these reasons, there have been continuing efforts to find the agents that can protect against oxidative and inflammatory damage and increase the activity of neurotrophic growth factors and potentially treat or prevent neurodegenerative diseases.

Several studies (Marin et al., 2003; Speisman, Kumar, Rani, Foster, & Ormerod, 2013; Veerbeek, Koolstra, Ket, Van Wegen, & Kwakkel, 2011), have shown great significance of physical exercise in the treatment and rehabilitation of neuropathological conditions. In many human and animal investigations, it has been revealed that exercise targets many aspects of brain function and overall brain health (Chennaoui et al., 2015; Cotman, Berchtold, & Christie, 2007; Di Benedetto, Müller, Wenger, Düzel, & Pawelec, 2017). Physical exercise has been found to modulate oxidative stress by increasing the body's antioxidant defence system (Simioni et al., 2018). It also regulates the activities of inflammatory cytokines such as IL-6 (Svensson, Lexell, & Deierborg, 2015) and increases expression of growth factors such as BDNF in certain neuropathological conditions such as Alzheimer's (Mertikas & Kainulainen, 2010) and stroke (Ke, Yip, Li, Zheng, & Tong, 2011).

The beneficial effects of exercise have best been captured in learning and memory (Speisman et al., 2013), and protection from neurodegeneration by inducing structural and functional

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changes in various regions of the brain (Radak, Marton, Nagy, Koltai, & Goto, 2013). Exercise has been found to directly increase synaptic plasticity and strengthening of the underlying systems that support plasticity including neurogenesis, metabolism and vascular function (Gomez-Pinilla, Vaynman, & Ying, 2008; Kohman, DeYoung, Bhattacharya, Peterson, & Rhodes, 2012). Another important mechanism that mediates the broad effects of exercise is the induction of neuronal growth factors and growth factor cascades, leading to a downstream of structural and functional changes (Gibon & Barker, 2017).

According to some researchers, a common mechanism underlying the central and peripheral effects of exercise is related to mechanisms that interfere with growth factor signalling, specifically inflammation and oxidative stress (Chennaoui et al., 2015; Cotman et al., 2007; Radak et al., 2013). Hence, regulation of inflammation and oxidative stress by exercise is a common means by which exercise reduces peripheral risk factors for cognitive decline and neurodegeneration (Chennaoui et al., 2015; Simioni et al., 2018; Sleiman et al., 2016).

Additionally, exercise modulates peripheral risk factors such as diabetes, hypertension and certain cardiovascular diseases which usually lead to brain dysfunction and neurodegeneration (Cotman et al., 2007; Verdile et al., 2015).

The use of nutraceuticals in health promotion have in recent times been greatly considered. Researchers have considered the effects of many naturally occurring substances on various organs of the body including the brain (Grassi, Ferri, & Desideri, 2015; Simioni et al., 2018).

These nutraceuticals include grapes, green tea, curcumin, citrus fruits, and cocoa (Lien Ai Pham-Huy, Hua He, 2008; Simioni et al., 2018). Their benefits are often explored because of certain anti-oxidative and anti-inflammatory properties they possess (Hosseinzadeh, Roshan, & Mahjoub, 2013; Lee, Torosyan, & Silverman, 2017; Mancini et al., 2017). Currently, of peak interest is the use of cocoa in promoting and maintaining brain health. This is because, cocoa in its natural unsweetened state, is well known for its high anti-oxidant (Keen, Holt, Oteiza,

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Fraga, & Schmitz, 2005; Nehlig, 2013a) and anti-inflammatory capacity (Goya et al., 2016; Ishaq & Jafri, 2017). Cocoa contains numerous beneficial substances among which is found quite a large number of antioxidant molecules, mainly flavonoids. Demonstrated beneficial actions of flavonoids on the brain include:

1. Stimulation of brain perfusion and increased cerebral blood flow (Flanagan, Müller, Hornberger, & Vauzour, 2018).

2. Provocation of angiogenesis and neurogenesis (Magrone, Russo, & Jirillo, 2017).

3. Changes in neuron morphology (Vauzour, 2017).

These changes are considered to account for the numerous health benefits associated with cocoa.

Although many researchers have explored separately the benefits of physical exercise and cocoa consumption and their significance in the management of neuropathologies, as well as their promotion of brain health, apparently no studies have considered the additive and/or potentiating activity of combined physical exercise and consumption of cocoa on levels of markers of oxidation and inflammation as well as the production of the growth factor, BDNF.

1.2 Statement of the problem

The brain is susceptible to disorders that strike at every stage of life (Wekerle, 2002). Most brain disorders are age-related, and complicated disorders involve a multitude of determinants, including neuroinflammation (Amor et al., 2014), and oxidative damage (Chen, Zhang, & Huang, 2016) that consequently result in decreased neuronal growth factors such as BDNF (Gomez-Pinilla et al., 2008). These events can occur separately or together causing a decline in brain health (Uttara, Singh, Zamboni, & Mahajan, 2009).

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Currently, there is no singular effective therapy for these brain-related insults despite intensive research and on-going clinical trials. However, many promising studies have shown that determinants of brain health are to some extent modifiable. Thus, inflammation and oxidative stress, and regulation of neurotrophins may provide targets for intervention in the promotion of brain health (Rosano, Marsland, & Gianaros, 2012).

For a very long time, neurological disorders have been managed largely by the use of certain pharmacological agents, supplemented with rehabilitative strategies including regular exercises and dietary modifications. However, studies have shown that the chronic or sometimes terminal nature of these conditions may require long term intake of certain medicines which may have side effects on other organs such as the kidney (Winkelmayer, Waikar, Mogun, & Solomon, 2008); liver (Marcum & Hanlon, 2010); stomach (Sostres, Gargallo, Arroyo, & Lanas, 2010); and even the brain (Haag et al., 2008). Therefore, recent efforts in rehabilitation or treatment of neuropathological conditions rely mainly on lifestyle adjustments such as physical exercise (Barha, Galea, Nagamatsu, Erickson, & Liu-Ambrose, 2017) and dietary supplementation (Simioni et al., 2018).

Physical exercise has been reported by studies to promote release of BDNF(Cotman et al., 2007; Sleiman et al., 2016), as well as regulate oxidative stress (Simioni et al., 2018) and inflammation (Chieffi et al., 2017; Woods, Wilund, Martin, & Kistler, 2012). Similarly, many studies using certain natural substances such as grapes (Lee et al., 2017), citrus fruits (Ola, 2017), green tea (Mancini et al., 2017), curcumin (Wu et al., 2014) and cocoa (Ellinger & Stehle, 2016) have suggested that these possess anti-oxidative and anti-inflammatory properties. Cocoa has been reviewed for such properties in many studies (Ishaq & Jafri, 2017; Magrone et al., 2017; Nehlig, 2013b). It has high concentration of flavonoids which exert a multiplicity of neuroprotective actions, including the capacity to protect neurons (Nehlig, 2012).

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The current study explored whether combining consumption of cocoa and regular physical exercise will result in higher reduction of oxidative stress and inflammation, and increase levels of BDNF than they separately would, consequently affording better promotion of brain health.

1.3 Justification of the study

The need for treatment for brain disorders is urgent, since the World Health Organization (WHO) predicts that in 20 years, brain disorders that mainly affect motor functions will overtake cancer to become the second-most prevalent cause of death, after cardiovascular diseases (Gammon, 2014; Gitler, Dhillon, & Shorter, 2017).

Although the mechanism is not clear, maintaining brain health promotes successful ageing, minimizes occurrence of neurological disorders and facilitates recovery from neurodegenerative diseases (Rosano et al., 2012). Many promising studies have shown that determinants of brain health are to some extent modifiable. (Bayani Uttara et al., 2009; Verdile et al., 2015), making them good targets for intervention (Rosano et al., 2012). Physical exercise is the most noted intervention adopted in the promotion of brain health (Barha et al., 2017). It has been reported by studies to promote release of BDNF (Cotman et al., 2007; Sleiman et al., 2016), increase the endogenous antioxidant capacity (Simioni et al., 2018) and regulate the influx of inflammatory cytokines in the body (Chieffi et al., 2017; Woods et al., 2012). Fortunately, the use of animals especially rodents in the study of the effects of exercises have proven to be beneficial in many neurological studies (Chieffi et al., 2017; Svensson et al., 2015).

Nutraceuticals, which can simultaneously act on multiple targets to improve the overall neuronal health, in recent times appear to be an attractive option in brain health management. Cocoa, because of its overly stated nutraceutical properties comes across as an option worth

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studying. The present work extends previous demonstration of beneficial activity of ingested cocoa in this department (Aidoo et al., 2012, Sokpor et al., 2012). It is an additional advantage that Ghana is the second leading producer of cocoa in the world, and natural cocoa powder (NCP) is readily available and relatively affordable. Hence, its introduction into neurological studies in this part of the world may be easily considered and accepted.

1.4 Study hypothesis

Combination of natural cocoa consumption and exercise would have potentiating effects on selected markers of brain health.

1.5 Aim of the study

To determine whether combining natural cocoa ingestion and exercise would increase levels of selected biochemical markers of brain health in (normal) rat brain, and produce histomorphometric differences in selected parts of the brain.

1.6 Specific objectives

The specific objectives of the study are to determine the effect of cocoa ingestion and exercise training on:

i. serum concentration antioxidant markers, namely; superoxide dismutase (SOD) and reduced glutathione synthase (GSH).
ii. serum and homogenized brain tissue concentration of the most abundant neurotrophic factor, BDNF.
iii. serum concentration levels of interleukin-6 (IL-6) as an inflammatory marker.

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iv. The volume density of cortical neuronal cells as structural markers of brain tissue health.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Gross anatomy of the brain

The brain is an organ surrounded by meninges, enclosed in a bony cranium, and continuous with the spinal cord at the foramen magnum at the base of the skull (Desesso, 2014). The human brain usually weighs less than 400 g at birth, but the weight increases by the second year after birth to about 900g. The adult human brain weighs between 1,250 g and 1,450 g, with a gender differential by which brains of males generally weigh more than those of females. The adult human brain shows three broad regions; namely, the cerebrum, cerebellum, and the brainstem (Brat, 2018).

The rat brain is structurally similar to the human brain (Cappaert, Van Strien & Witter, 2015). The rat brain weighs about 1.5-2.2g. Like the human brain, the rat brain also has broadly, the cerebrum, the cerebellum and the brainstem. however, unlike the human brain where the cerebrum is the most prominent region, the rat brain shows almost equal size of the cerebrum and the cerebellum. This marked difference has been attributed to high balance and coordinative attributes of these rodents (Bear, Connor & Paradiso 2007). Also, the cortex of the human brain shows extensive convolutions of the cortex whereas the cortex of the rat is considerably smooth (Bear, 2007). Despite these differences, the rat brain is considered close in structure and function to the human brain (Palomero-Gallagher & Zilles, 2015).

2.1.1 The Cerebrum/cerebral hemispheres

The cerebral hemispheres are bilaterally symmetric large oval structures (Fig 1). Posteriorly, the cerebral hemispheres are narrower than anteriorly. At the posterior end is the occipital pole and at the anterior is frontal pole (Desesso, 2014).

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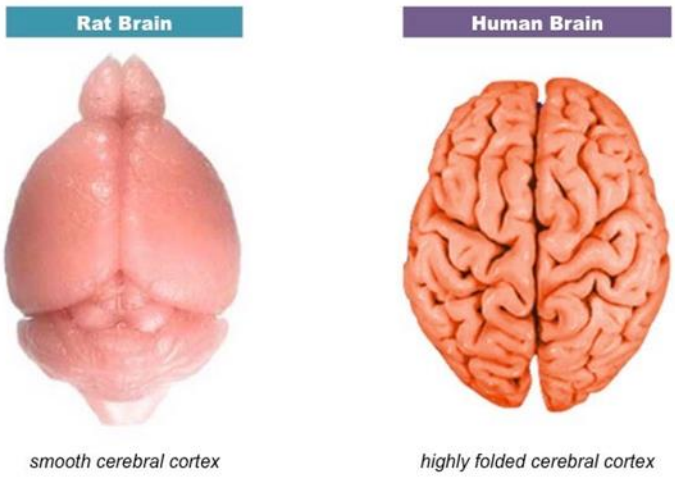

Figure 1: A diagram showing the cerebral hemispheres of the rat brain and human brain.

The cerebrum is divided incompletely into two halves by a midline longitudinal cerebral fissure. The floor of the cerebral fissure is formed by the corpus callosum, a large myelinated fibre tract that forms an anatomical and functional connection between the right and left hemispheres.

The cerebral hemisphere is composed of irregularly corrugated collection of grey matter, known as the cerebral cortex. The folding increases the surface area and presents elevations (gyri), and depressions (sulci). Deep to the cortex is a central core of white matter that forms the bulk of the cerebrum and represents fibre tracts, supported by neuroglia, ferrying information destined for the cortex and cortical responses to other regions of the central nervous system (CNS).

Within the mass of white matter are collections of neuronal cell bodies, some of which are lumped together under a rubric of basal ganglia, even though, technically, they are *nuclei*. Also in the cerebrum is a hollow structure and the cavities within the cerebral hemispheres are called the right and left lateral ventricles, which communicate with the third ventricle via the interventricular foramen (foramen of Monro).

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The two lateral ventricles are separated from one another by two closely adjoined non-nervous membranes, each known as a septum pellucidum. Ependymal cells line each lateral ventricle and protruding into each ventricle is a choroid plexus that functions in the secretion of cerebrospinal fluid.

Each cerebral hemisphere is subdivided into five lobes: the frontal, parietal, temporal, and occipital lobes, and the insula. Some researchers consider a sixth lobe which consists of cortical contents of the limbic system (Brat, 2018).

These lobes are separated from each other by various sulci. The sulci are generally smaller and shallower than the Fissures. The central sulcus (central sulcus of Rolando), separates the frontal lobe from the parietal lobe. The parieto-occipital sulcus, like the name suggests divides the parietal and occipital lobes (Desesso, 2014).

2.1.2 Histology of the cerebral cortex

The cerebral cortex is well endowed with neurons, neuroglia, nerve fibres, and a rich vascular supply. There are three types of neurons in the cerebral cortex, namely, pyramidal cells, granular neurons, and fusiform neurons. The arrangement of these three types of neurons that populate the cortex permit the classification of the cortex into three types: the archicortex (allocortex), mesocortex (juxtallocortex), and neocortex (isocortex) (Brat, 2018; Eickhoff, Constable, & Yeo, 2018).

The archicortex the oldest region, is composed of only three layers and is located in the limbic system. The mesocortex, phylogenetically younger, is composed of three to six layers, and is located predominantly in the insula and cingulate gyrus. The neocortex is the youngest region in the cerebral cortex. It is made up of six layers and forms the bulk of the cerebral cortex. Although the cerebral cortex is arranged in layers, superimposed upon this cytoarchitecture is a functional organization of cell columns (Eickhoff et al., 2018). Each cell column is less than

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0.1 mm in diameter, lies perpendicular to the superficial surface of the cortex, passes through each of the six cortical layers, and is composed of neurons with similar functions. All neurons of a single column respond to like stimuli from the same region of the body (Brat, 2018). The organization of the six layers of the neocortex is known as its cytoarchitecture, where each layer has a name and an associated Roman numeral (I – VI) as shown in Fig 2.

The six layers of the cerebral cortex from the surface to the white matter include molecular layer (I), outer granular layer (II), outer pyramidal layer (III), inner granular layer (IV), inner pyramidal layer (V) and polymorphic layer (VI).

Each of the layers is made up of cortical cells, for example, granular cells (▲) and pyramidal cells (↘).

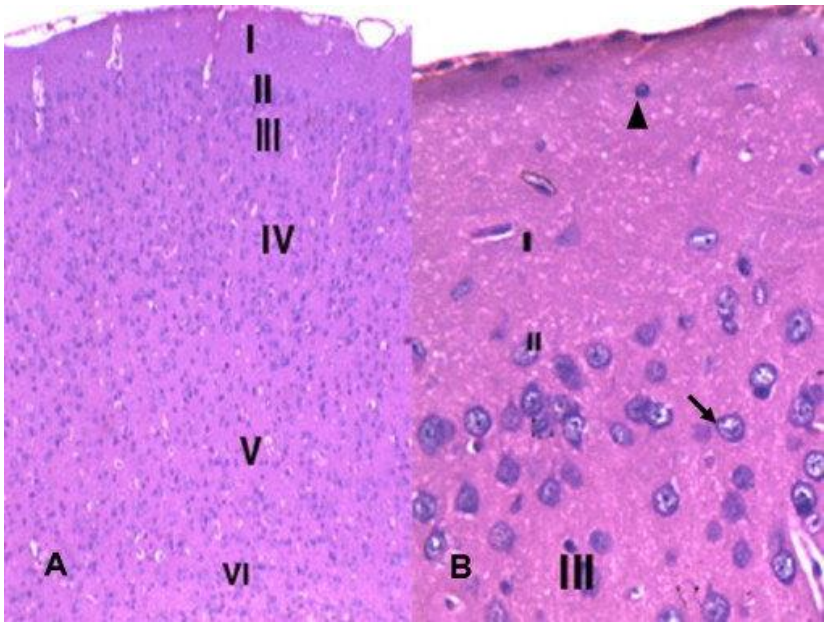

Figure 2: A diagram showing the histology of cerebral cortex showing layer I-VI (Adapted from El-Drieny et al., 2015)

2.1.3 Cellular organization in the cerebral cortex

The cerebral cortex is made up of two (2) general cell types; Neurons and Glia, which are both large families with highly specialized members.

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2.1.3.1 Neurons

They are specialized cells which are electrically excitable and responsible for sensing, integrating, and transmitting impulses. Neurons communicate with one another and with other cells via specialized connections known as synapses. Neurons vary in morphology and function however, each neuron is made up of a cell body or perikaryon, branching processes called dendrites and a longer process called the axon. The dendrites are responsible for integrating incoming signals, whereas the axon, with a terminal synapse for transmitting an electrical signal from one neuron to another or to a muscle cell through a neuromuscular junction (Desesso, 2014; Nervous, 2011).

Neurons include pyramidal cells which form a vast majority of the neurons in the CNS, especially in the cerebral cortex and in the subfields of the hippocampus. The cell bodies of the pyramidal neurons are large, triangular with a prominent apical dendrite which extends towards the surface of the brain and numerous finer branching dendrites. Another subtype of neurons is the cortical granular or stellate neurons. They are comparatively smaller than the pyramidal cells within the cortex. They possess shorter processes which remain within the confines of the cortex, enhancing their function as interneurons. Also found in the primary motor cortex are the largest neurons of the cerebral cortex known as the Betz cells. The Betz cells are the upper motor neurons (Brat, 2018).

The other large cellular family within the cerebral cortex is the Glia. The glia forms about 90% of the cells with the central nervous system. It provides structural and functional support for the neurons. Glia are divided into macroglia or true glia and microglia. The macroglia includes astrocytes, oligodendrocytes and ependyma. The astrocytes are multipolar and have a star-like appearance. Within the cerebral cortex is the protoplasmic astrocytes. The other type is the fibrillary astrocytes, which are found predominantly in white matter (Desesso, 2014; Nervous, 2011).

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The myelinating cells of the CNS are known as the oligodendrocytes. They are more predominant in white matter. In white matter, oligodendrocytes are disposed along the length of axonal processes, whereas in the cerebral cortex, they are scattered within the neuropil and concentrated immediately surrounding neuronal cell bodies (satellite cells).

The last of the macroglia is the ependyma. They are found in the ventricular system, where they serve as a single layered epithelium lining the ventricles. The microglia, are smaller and form about 20% of the cellular population of the CNS (Brat, 2018). They are phagocytic and responsible for natural debriding the CNS.

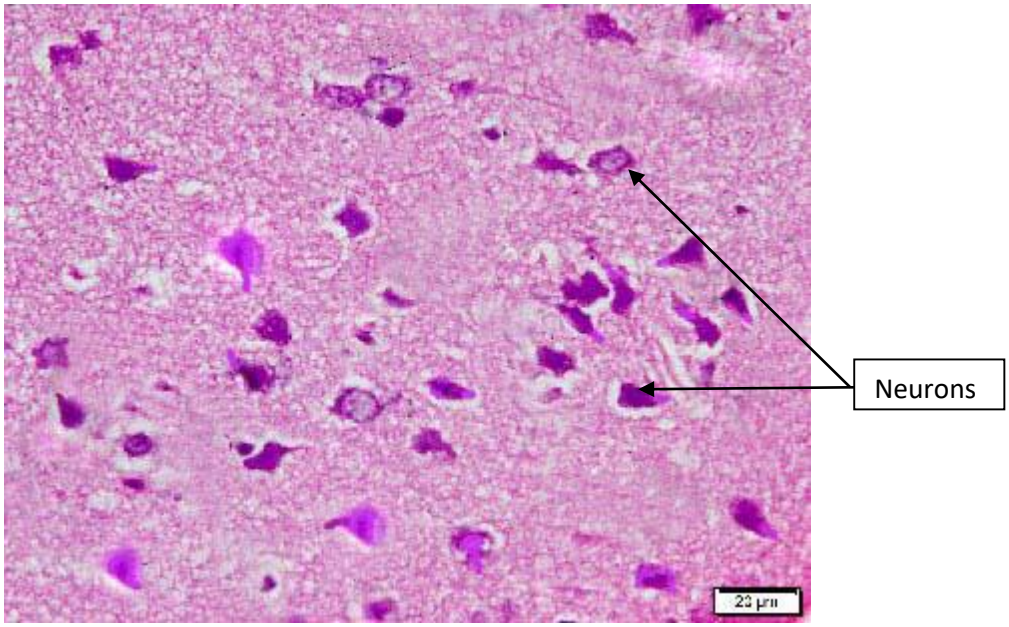

Figure 3: A diagram showing the neurons of the cerebral cortex (adapted from: Fattah et.al., 2016)

2.1.4 The use of rat models for brain studies

Animal models have contributed greatly to a better understanding of the complex physiologic and structural adaptations that occur in an organism in response to certain stimuli (Julho, Paulo, Rodrigues, & Lia, 2017). They have also been used elucidate the mechanisms processes of many pathologies as well as decipher treatment options, strategies and interventions (Jones et al., 2001).

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Animal models are warranted not only because human model use is restricted but also because animal offer a better contribution to assessing the cellular, molecular and genetic profiles in response to the stimuli under study (Julho et al., 2017).

Many studies have used rodent models including rats in well controlled experimental paradigms to measure changes in the CNS at levels of neurons, molecules and even signalling pathways involved in neurogenesis, synaptic plasticity, metabolism and even behaviour ((Feng et al., 2019; Kubera, Obuchowicz, Goehler, Brzeszcz, & Maes, 2011; Leal et al., 2015).

These rat models, of both normal (Franzoni, Federighi, Fusi, Agosta, & Cerri, 2017; Rossi et al., 2017) and pathological states (Kaur, Chauhan, & Sandhir, 2011; Praticò, 2008) have provided insight to the beneficial effects of many interventions across different populations.

Overall, there are many brain studies in humans which are consistent with rodent research suggesting that interventions many provide lasting benefits for brain structure and function (Voss, Vivar, Kramer, & Praag, 2013).

For instance, exercise has been shown to enhance hippocampus dependent spatial memory in rodent paradigms including Morris water maze, the Y maze and the radial arm maze (Clark & Hoffmann, 2013),. It has also been shown that running in rodents improves performances in tasks, new object recognition and passive avoidance learning. This form of learning in rodents may be similar to human learning during daily activities (Voss et al., 2013). Some human studies support the evidence that exercise training and fitness is beneficial to certain functions that depend on the functions of the hippocampus, which is responsible for learning (Feng et al., 2019).

2.2 Brain health

Throughout life, the brain has the responsibility of overseeing daily activities and operations of life. These include movement, cognition, learning, information management, logical

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reasoning among others (Jang et al., 2003). Brain health is therefore considered as the ability to perform these tasks, making the most of the brain and helping reduce risks to the brain as one ages or goes through different phases of life (Camandola, 2017). It also refers to protection of the brain from any form of neurodegeneration (Cotman et al., 2007) and alleviation of depression or any mental disorders (Kubera et al., 2011).

The only constant thing about the brain is that, it is constantly changing, hence change in brain function is expected as one ages or changes from one phase of life to the other. This process of change is known as brain plasticity or neuroplasticity (Combs, Jones, Kozlowski, & Adkins, 2016).

Neuroplasticity occurs as the brain practices habits, learns new information and adapts to certain lifestyle adjustments, causing increase in synaptic plasticity by directly affecting synaptic structure and potentiating synaptic strength within the brain (Arya, Pandian, Verma, & Garg, 2011). Brain health is also enhanced when the underlying systems that support plasticity including neurogenesis, metabolism and vascular function are also enhanced (Speisman et al., 2013).

Also, these changes that may occur in the brain is also attributed to the complex interplay of genetic (Praticò, 2008), environmental (Article, 2018) and lifestyle factors (Chieffi et al., 2017).

Furthermore, brain health is characterized by preservation of cognitive function during ageing while reducing the risks of neurodegenerative disorders (Gitler et al., 2017), reduced risk of dementia (Commenges et al., 2000), the ability to activate signalling pathways critical in controlling synaptic plasticity (Gibon & Barker, 2017). Also, the potential of the brain to induce vascular effects capable of causing new cell growth within certain areas of the brain is descriptive of brain health as well (Spencer, Vauzour, & Rendeiro, 2009; Vauzour, 2017).

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2.2.1 Selected markers of brain health

2.2.1.1 Oxidative stress

An essential element of survival in aerobic organisms is Oxygen. Oxygen has a high redox potential and serves as a terminal electron acceptor in the process of metabolic energy generation through a series of redox reactions (Valko et al., 2007). Unfortunately, because of this high redox potential, it is likely to cause damage to cells if not completely reduced. Partial reduction of molecular oxygen is an unintended occurrence during aerobic metabolism and may lead to the generation of highly reactive oxygen species (ROS). These include singlet oxygen, superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxyl radical; which can disrupt the redox balance inside cells if not properly neutralized (Wang & Michaelis, 2010).

In cells, over-production and/or under-detoxification of ROS, or even normal demands for these reactive species due to their beneficial effects, may cause oxidative stress(Allen & Bayraktutan, 2009; Salim, 2016).

In the brain, under normal circumstances, ROS are generated by certain reactions, either enzymatic or non- enzymatic, in the mitochondria and cytoplasm. In moderate amounts, these reactive oxygen species have beneficial effects on signalling, neurogenesis and in epigenetic regulation (Cobley, Fiorello, & Bailey, 2018). However, excessive production associated with pathological conditions leads to activation of certain deleterious enzymes including proteases, phospholipases, nucleases, and alterations of signalling pathways which subsequently lead to mitochondrial dysfunction, release of inflammatory factors and even apoptosis (Sun et al., 2008).

Many of the modifications of lipids, nucleic acids, and proteins result in structural changes in the respective macromolecules and lead to either dysfunction or loss of activity of these molecules. To protect themselves from the detrimental effects of these oxidative modifications,

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neurons employ a variety of defensive mechanisms that include lipid turnover, protein re-folding or degradation, and DNA base excision and repair. When these mechanisms are compromised, neuronal homeostasis is disturbed and oxidative stress (OS) ensues (Praticò, 2008). Among all organs in the body, the brain is particularly prone to OS-induced damage because of the high oxygen demand of this organ, the abundance of redox-active metals (iron and copper), high levels of oxidizable polyunsaturated fatty acids, and the fact that neurons are post-mitotic cells with relatively restricted replenishment by progenitor cells during the lifespan of an organism (Allen & Bayraktutan, 2009; Cobley et al., 2018).

Cells in the CNS are particularly vulnerable to OS (Praticò, 2008; Rossi et al., 2017; Siamilis et al., 2009). this is as a result of high demand of molecular oxygen, the enrichment of polyunsaturated fatty acids in membrane phospholipids and the relatively low antioxidant defence enzymes within the CNS (Pham-Huy, He, & Pham-Huy, 2008; Praticò, 2008; Valko et al., 2007).

Excess production of ROS in the brain has been implicated as a common underlying factor for the aetiology of a number of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and stroke (Radak et al., 2013); as well as certain age associated disorders (Vauzour, 2017; Verdile et al., 2015). Owing to its high reactivity and short lifespan, the direct detection of ROS is difficult and hence, the amount is often judged from the alteration of antioxidant status (Radak et al., 2013) or the accumulation of relatively stable products of lipids, protein and DNA (Allen & Bayraktutan, 2009b).

Induction of OS in neurodegenerative diseases may result from changes in the neuronal metabolism brought about by the accumulation of certain macromolecules. The latter includes oligomer form of amyloid peptides in neuronal cells, whose accumulation results in OS (Bayani Uttara et al., 2009). Studies have also shown that the redox potential of the different types of amyloid peptides matches the order of their toxicity to neurons (Praticò, 2008),

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suggesting OS plays a causative role in the development of Alzheimer's disease (Padurariu, Ciobica, Lefter, & Serban, 2013). In addition, neurons affected by Alzheimer's disease are characterized by decreased ATP production and diminished cytochrome c oxidase content, both of which are indices of mitochondrial dysfunction and, as dysfunctional mitochondria are a major source of ROS generation, increased OS (Zhu et al., 2004; Onyango & Khan, 2006).

Similarly, in Parkinson's disease, neurons in the substantia nigra pars compacta region are selectively lost (Kaur et al., 2011). The role of OS in this disease is supported by the observation of dramatically diminished levels of glutathione as one of the earliest detectable changes during the development of this disease in this region of the brain (Marosi et al., 2012). The iron levels in this brain region are high, as is the activity of monoamine oxidase, and both of these entities contribute to the generation of ROS in these neurons (Article, 2018)

Furthermore, some studies have shown that the levels of oxidative modification of lipids, proteins and DNA are generally used as markers of oxidative damage, which are increased with the neuropathology of ageing (Bayani Uttara et al., 2009; Valko et al., 2007). Some studies have explained that as ageing occurs, certain intrinsic activities cause an increase in the baseline oxidative stress. In these processes, the neurons become vulnerable to OS and lose their resilience in maintaining a healthy brain (Popa-wagner, 2013).

2.2.1.1.1 Superoxide dismutase (SOD).

Superoxide dismutase (SOD) is another endogenous enzyme that alternately catalyses the dismutation (or partitioning) of the superoxide (O^{2-}) radical into either ordinary molecular oxygen (O_2) or H_2O_2. Superoxide radical is produced as a by-product of oxygen metabolism /and causes many types of cell damages, including an acceleration of age-related muscle mass loss, cancer and a reduced lifespan (Marosi et al., 2012; Hamakawa et al., 2013). Finally,

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catalase is an endogenous enzymatic antioxidant that converts hydrogen peroxide into water and oxygen gas.

2.2.1.1.2 Glutathione (GSH)

Among molecules responsible for the protection of cells from OS, glutathione (GSH) serves as an effective oxygen radical scavenger. Hence a decrease in cellular GSH content increases oxidative stress (Allocati, Masulli, Ilio, & Federici, 2018). GSH, a tripeptide synthesized from glutamate, cysteine, and glycine, exerts protective function of cell survival against OS (Dasuri, Zhang & Keller 2013). In the brain, *in vivo* GSH is produced by the consecutive actions of two enzymes; γ dipeptide of γ-glutamylcysteine which is formed by -glutamylcysteine synthetase, using glutamate and cysteine as substrates. This dipeptide is further combined with glycine by the catalyzing action of glutathionine synthetase to synthesize GSH (Lee, Cho, Jantaratnotai, Wang, McGeer & McGeer 2010). GSH is involved in the following two types of reactions; Firstly, GSH, in its reduced form, is known to non-enzymatically react with ROS such as O^{2-} and $\cdot OH^{-}$ for the removal of ROS (Dasuri et al., 2013).

Secondly, GSH is the electron donor for the reduction of peroxides in the GP_X reaction (Dringen & Hirrlinger 2003). Reaction with ROS firstly oxidizes GSH, which generates glutathione disulfide, the final product of GP_X reactions. GSH can be regenerated from glutathione disulfide by the reaction with glutathione reductase that transfers electrons from NADPH to glutathione disulphide (Dringen 2000).

Several studies have reported that GSH is involved in inhibiting apoptotic cell death (Allocati et al., 2018) and DNA damage in cells following oxidative stress (Valko et al., 2007).

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2.2.1.2 Inflammation

Processes of neuroinflammation within the brain are believed to play a crucial role in the development of Alzheimer's disease (Amor et al., 2014), Parkinson's disease (Chen et al., 2016) as well as in injury associated with stroke (Amor et al., 2014), ageing (Rosano et al., 2012) and other brain related neuropathies (Patterson, 2015).

Inflammation is commonly considered as a complex reaction in the vascularized connective tissue in response to exogenous and endogenous stimuli (García-Bueno, Caso, & Leza, 2008; Yamada et al., 2011). The ultimate of this protective response is to rid the organism of both the initial cause of cell injury and the consequences of such injury (Ashworth, 2018).

However, exaggerated or unregulated prolonged inflammatory process can induce tissue damage and is the cause for many chronic diseases (Ashworth, 2018; Tucker et al., 2015). A critical component of inflammation is the infiltration of inflammatory cells, like neutrophils, monocytes, and lymphocytes, to the site of stimulus. The infiltration of leukocytes to the site of inflammation is a highly coordinated process involving margination, rolling, and adhesion of leukocytes to the vascular endothelium, transmigration across the endothelium, and migration toward a chemotactic stimulus (Biswas, 2016).

Brain inflammation is involved in neurological disorders such as stroke (Chen et al., 2016; Nabavi et al., 2015), Parkinson's disease (Amor et al., 2014), Alzheimer's disease and even epilepsy (Coupland et al., 2011) and can be both beneficial and detrimental to brain health (Xia et al., 2014). For instance, some studies have shown that brain inflammation alters the functional integration of new neurons into existing neural circuitries (Jakubs et al., 2008). In the intact brain, the maturation of new granule cells follows distinct morphological stages (Zhao et al., 2006), and they develop synaptic inputs closely resembling those of mature granule cells. Recent experimental evidence suggests that developing in an inflammatory

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environment may influence the synaptic connectivity of the new neurons (Jakubs et al., 2008). Additionally, other studies have shown that activated microglia secrete cytokines and growth factors, which can modulate synaptic transmission (Belarbi et al., 2012).

Cytokines are key regulatory mediators involved in the host response to immunological challenges, but also play a critical role in the communication between the immune and the central nervous systems (Calabrese et al., 2014). For this, their expression in both systems is under a tight regulatory control. However, some pathological conditions lead to an overproduction of pro-inflammatory cytokines that may have a detrimental impact on central nervous system (Belarbi et al., 2012). In particular, they may damage neuronal structure and function leading to deficits of neuroplasticity, the ability of nervous system to perceive, respond and adapt to external or internal stimuli (Di Benedetto et al., 2017; García-Bueno et al., 2008).

Interestingly, studies have shown that the same cytokines that in a physiological state are involved in the maintenance of neuronal integrity, may instead have detrimental effects under pathological conditions (Eyre & Baune, 2012). The increase of pro-inflammatory cytokines is not only due to a direct inflammatory stimulus (infection or trauma), but it could be caused by environmental stimuli such as stress (García-Bueno et al., 2008). The main consequence of a dysregulation of cytokine levels within the brain is the production of inflammatory, oxidative and nitrosative molecules that could affect the neural homeostasis and general brain health (Kubera et al., 2011; Stepanichev, Dygalo, Grigoryan, Shishkina, & Gulyaeva, 2014; Woods et al., 2012).

2.2.1.2.1 Interleukin 6 (IL-6)

IL-6 is a particularly interesting cytokine that is involved in a multitude of neuroprotective functions (Kubera et al., 2011). In physiological conditions IL-6 is able to activate pathways

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related to neural plasticity, neurogenesis, long term potentiation, and memory (Eyre & Baune, 2012). On the other hand, this cytokine is also responsible for showing some neurodegenerative properties, mediating synthesis of acute phase proteins, growth and differentiation of immune cells and regulation of pro-inflammatory factors (Audet & Anisman, 2013). Recent studies have confirmed IL-6 as a pleotropic cytokine, showing both pro inflammatory and anti-inflammatory characteristics (Hennigar, Mcclung, & Pasiakos, 2017). For instance, increased levels of IL-6 are associated with neuropsychiatric conditions such as depression (Stepanichev et al., 2014) and Alzheimer's disease (Wu, Hsu, & Wang, 2015). Furthermore, some studies have reported increased levels of serum IL-6 in brain atrophy during normal ageing (Al-hanbali et al., 2009; Briken, Cathérine, Keminer, Patra, & Ketels, 2016). Some researchers have however challenged the pro-inflammatory and neurodegenerative characteristics of IL-6 (Chennaoui et al., 2015; Scheller, Chalaris, Schmidt-arras, & Rose-john, 2011). These studies have provided results showing that this cytokine has several anti-inflammatory and immunosuppressive activities that may play a downregulating role in inflammatory conditions (Di Benedetto et al., 2017; Kubera et al., 2011).

In addition, IL-6 may play the role of developmental neurotrophic factor, because it has been shown to improve survival of neurons (Kubera et al., 2011), playing a protective role from excitotoxic and ischemic insults (Briken et al., 2016; Kubera et al., 2011).

Additional evidence gathered from some studies show that IL-6 plays a major role in promoting synaptic plasticity, long-term potentiation (LTP) and memory consolidation (Kubera et al., 2011). Taken together, findings from these different studies suggest that IL-6 has both neurodegenerative and neuroprotective biological functions.

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2.2.1.3 Neurotrophic growth factors/neurotrophins

Neurotrophic growth factors/Neurotrophins are a family of proteins that regulate neuronal survival, synaptic function, and neurotransmitter release, and elicit the plasticity and growth of axons within the adult central and peripheral nervous systems (Keefe et al., 2017). In the CNS of an adult, regeneration of axons after an injury is seemingly impossible (Gibon & Barker, 2017; Keefe et al., 2017). This is mainly as a result of decrease in the activation of intrinsic growth factors and to an extent certain extrinsic or environmental factors as well (Cotman et al., 2007; Vauzour, 2017).

In certain cases where axons are able to regenerate without external interference, they rarely target the correct post synaptic neurons or form synaptic connections that restore proper function (Keefe et al., 2017). Therefore, to facilitate proper regeneration of neurons, an optimal environment that would heighten the internal growth state and the presence of molecules such as the neurotrophic growth factors/neurotrophins that can overcome any inhibitory influences and induce maximal growth is required (Stepanichev et al., 2014).

The neurotrophins family comprises the Nerve growth factor (NGF) (Gibon & Barker, 2017; Gold et al., 2003; Keefe et al., 2017), the Brain derived neurotrophic factor (BDNF) (Keefe et al., 2017; Leal et al., 2015; Mattson, Maudsley, & Martin, 2004) as well as neurotrophins 3, 4 and 5 (Gibon & Barker, 2017). Some studies have also added to the neurotrophins family, bcl-2 (Kubera et al., 2011; Xia et al., 2014), and vascular endothelial growth factor (VEGF) (Speisman et al., 2013) as well as insulin growth factor (IGF)(Gomez-Pinilla et al., 2008; Kohman et al., 2012); all of which play a key role in neuronal survival and proliferation.

2.2.1.3.1 Brain Derived Neurotrophic Factor (BDNF)

BDNF stands out among all neurotrophins for its high and wide expression in the mammalian brain, in addition to its potent effects at many synapses (Keefe et al., 2017; Leal et al., 2015).

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It is a protein found predominantly in the CNS, primarily in hippocampus, cerebral cortex, hypothalamus and cerebellum (Briken et al., 2016). Furthermore, several aspects of the biology of this neurotrophin, including the release of BDNF, are regulated by neuronal activity. BDNF has emerged as a key molecule involved in the control of neuronal differentiation and survival, synapse formation, and in the regulation of activity-dependent changes in synapse structure and function (Park & Poo, 2012).

Central BDNF after being produced in the brain may be stored in other areas of the body, because of their ability to cross the blood brain barrier (Erikson et al., 2012). It can also be produced peripherally by the other tissues such as skeletal muscles (Murer et., 2001). This to an extent makes it difficult to detect whether changes in serum levels of BDNF are central or peripheral (Erikson et al., 2012).

2.3 Benefits of promoting brain health

2.3.1 Successful ageing

Maintaining brain health, promotes successful ageing. Ageing is a normal and inevitable process in life (Vauzour, 2017), however, its progression is dependent somewhat on certain lifestyle habits. In normal brain ageing, there is characteristic progressive decline in cognitive abilities in certain parts of the brain, such as the hippocampus, dentate gyrus and prefrontal cortex (Morrison & Baxter, 2012). Recent studies have also shown that the ageing process causes decline in both motor and cognitive functions even in the absence of neurodegenerative diseases in both animals and humans (Camandola, 2017; Rosano et al., 2012).

The main determinants of brain health including oxidative stress and inflammation as well as the preservation of cognitive function and remaining free from structural and metabolic abnormalities, including loss of neuronal synapses, atrophy, small vessel disease and focal

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amyloid deposits visible by neuroimaging change marginally with ageing (Rosano et al., 2012). Promising studies indicate that these determinants are to some extent modifiable, even among adults seventy years and older (Rosano et al., 2012).

As ageing occurs, there is a greater susceptibility of the brain to memory impairments following immune challenges that are usually characterized by increased and prolonged production of pro-inflammatory cytokines in the otherwise healthy aged brain (Barrientos et al., 2015).

Also, ageing is classically characterized by dysregulated interactions with synapses, resulting in neuronal loss, consequently representing the best pathological correlate of cognitive decline (Tay et al., 2016). Promoting brain health helps sensitize the aged brain to withstand these changes.

2.3.2 Improved cognitive function and memory

Promoting brain health preserves memory and improves cognitive function (Cotman et al., 2007; Speisman et al., 2013). this happens by the occurrence of the activation of various signalling pathways linked to the control of synaptic plasticity and memory (Spencer et al., 2009).

For optimal brain functioning, cerebral blood flow needs to be kept constant to allow regular oxygen and glucose supply to the neurons (Nehlig, 2013b), hence maintaining cerebral blood flow promotes brain health and promotes cognitive function and memory (Patterson, 2015).

2.3.3 Protection of brain from neurodegeneration

Neurodegeneration is a phenomenon that occurs in the CNS, generally characterized by loss of neuronal structure and function (Chen et al., 2016). It is often seen in the elderly and some of the conditions include Alzheimer's disease, multiple sclerosis, Parkinson's disease and

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amyotrophic lateral sclerosis that negatively affect mental and physical function (Camandola, 2017).

Promoting of brain health seeks to induce increases in neuronal spine density and synaptic plasticity (Jakubs et al., 2008). Such interactions may lead to improvements in memory through induction of synapse growth and connectivity, increases in dendritic spine density and the functional integration of old and new neurons(Park & Poo, 2012).

As mentioned above, inflammation and OS are implicated in the occurrence of many neurodegenerative conditions (Gitler et al., 2017; Uttara, Fau - Zamboni, Fau - Mahajan, & Mahajan, 2009). Many studies have shown inflammation and oxidative stress are determinants of brain health that are easily modifiable to promote maximal health and function (Verdile et al., 2015). In addition, neurodegeneration may be induced by viruses (Limongi & Baldelli, 2016), suggesting an important role of immune response in neurodegeneration involving healthy neuroglia cells (Kubera et al., 2011).

2.4 Strategies to improve brain health and consequences

In recent studies, a number of researches have focused attention on lifestyle patterns in reducing markers of oxidative stress (Nabavi et al., 2014; Rossi et al., 2017) and in altering expression of anti and pro-inflammatory markers (Hussain et al., 2016; Xia et al., 2014) within the brain considered to be specifically involved in the pathogenesis of many conditions such as Parkinson's and Alzheimer's disease (Gitler et al., 2017).

Similarly, these patterns have been channelled to focus on stimulating neurotrophic growth factors otherwise known as neurotrophins (Camandola, 2017; Gibon & Barker, 2017).

In keeping to this, particular relevance has been attributed to dietary flavonoids representing a diverse range of polyphenolic compounds naturally represented in certain fruits and vegetables (Lee et al., 2017; Simonyi et al., 2005; Vauzour, 2017) as well active engagement in certain

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physical exercises (Barha et al., 2017; Hamakawa et al., 2013; Ke, Yip, Li, Zheng, & Tong, 2011).

2.4.1 Diet as a lifestyle intervention for promoting brain health

Macronutrients such as lipids and carbohydrates are vital components of both neurons and glial cells and have been shown by many studies to play a huge role in brain function (Layer & Gómez-pinilla, 2008). The brain has a very high energy demand and as such utilizes in great proportions these dietary nutrients to function effectively (Spencer et al., 2009). Other studies have probed into other dietary-derived interventions such as foods and beverages derived from grapes (Lee et al., 2017), tea (Mancini et al., 2017), blueberry and cocoa (Ishaq & Jafri, 2017; Kim et al., 2014) in both animals and humans.

These foods and beverages have demonstrated beneficial effects in both vascular function and improving memory and learning (van Praag et al., 2007). These foods and beverages have in common a major dietary source of a group of phytochemicals called flavonoids (Kim et al., 2014).

In times past, the biological actions of flavonoids including those on the brain have been attributed to their ability to exert antioxidant activity (Keen et al., 2005; Shahidi & Ambigaipalan, 2015), through their ability to scavenge reactive oxygen species and or through their possible influence on the endogenous redox status (Sleiman et al., 2016). However, more recent studies have homed in on the direct protective effects of these flavonoids on the brain. Flavonoids have the ability to protect vulnerable neurons, enhance existing neuronal function, stimulate neuronal regeneration and induce neurogenesis (Goya et al., 2016). In long-term regard, flavonoids have been discovered to have neuroprotective action via their interactions with critical neuronal intracellular signalling pathways pivotal in controlling neuronal survival and differentiation (Nehlig, 2013).

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The underlying neurodegeneration seen in Alzheimer's and Parkinson's and other neurodegenerative diseases is triggered by a cascade of events. These include neuroinflammation, glutamatergic excitotoxicity, and depletion of endogenous antioxidants (Padurariu et al., 2013; Salim, 2016). More recent studies suggest that flavonoids and other polyphenols may be able to counteract these neuronal injuries, consequently delaying the progression of these diseases (Allen & Bayraktutan, 2009; Grassi et al., 2009).

For example, consumption of green tea, has been demonstrated to attenuate toxicity and protect against hippocampal injury during transient global ischaemia and to prevent nigral damage (Patterson, 2015).

2.4.1.1 Cocoa as a dietary intervention for promoting brain health

Cocoa and derivatives, especially chocolate and cocoa powder, are widely consumed worldwide, due to the highly attractive organoleptic characteristics (Goya et al., 2016) and its highly relatable health benefits (Andújar, Recio, Giner, & Ríos, 2012; Ishaq & Jafri, 2017; Keen et al., 2005).

Many epidemiological evidences buttress the concept that a diet rich in flavonoids from fruits and vegetables promotes health (Flanagan et al., 2018), attenuates or delays the onset of various diseases, like coronary heart diseases (Arranz et al., 2013; Keen et al., 2005), hypertension (Kim et al., 2014), cancer (Lambert & Elias, 2010) and age related degenerative disorders (Cimini et al., 2013).

Also, some experimental *in vitro* and in vivo evidence also support the potential beneficial effects of flavonoids on certain health risks through effects on atherogenesis (Blay et al., 2019), endothelial function (Katusic & Austin, 2016), inflammation (Andújar et al., 2012; Goya et al., 2016), platelet function and even glucose transport (Rios, Francini, & Schinella, 2015).

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Cocoa is well known for being a rich source of flavonoids, mainly flavan-3-ols (Goya et al., 2016; Grassi et al., 2015; Spencer et al., 2009). Cocoa is known to contain about 380 known chemicals (Andújar et al., 2012).

Cocoa beans in their natural state are inedible because of their high concentration of theobromine and polyphenols, which give them an extremely bitter taste (Andújar et al., 2012), however, in the final state product such as chocolate or sweetened beverages, polyphenol content might be decreased from 100% to about 10% (Magrone et al., 2017) throughout the different manufacturing processes. For this reason many studies purport the study of the beneficial effects of cocoa in their natural or close to natural state (Andújar et al., 2012). It is now known that natural (non-alkalized and unsweetened) cocoa powder has the highest flavanol content compared to other cocoa-derived foods (Sokpor et al., 2012 ; Aidoo et al., 2012).

These polyphenols which are found richly in cocoa (Andújar et al., 2012; Goya et al., 2016) have been proven to have many benefits on brain health (Grassi et al., 2015; Nehlig, 2013). They are capable of modulating intracellular signalling cascades (Flanagan et al., 2018; Spencer et al., 2009), gene expression and interactions with mitochondria (Hussain et al., 2016).

By affecting the above mentioned pathways, these polyphenol rich cocoa may be said to have the potential to induce new protein synthesis in neurons and thus an ability to induce morphological changes, which have a direct influence on memory acquisition, consolidation and storage (Nehlig, 2013). Alternatively, their well-established effects on the vascular system may also induce increases in cerebral blood flow capable of impacting on acute cognitive performance, or may lead to an increase in hippocampal vascularisation capable of inducing new neuronal growth (Grassi et al., 2015; Nabavi et al., 2015).

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2.4.1.1.1 Cocoa polyphenols and oxidative stress

The health benefit of cocoa is directly linked to its polyphenol antioxidant activity. It has the ability to directly scavenge free radicals and other nitrogen species *in vitro* (Vauzour, 2017). In lower amounts, like those found in diet, polyphenols have shown pharmacological activity within the cells with mechanisms that go beyond the classic antioxidant scavenging mechanisms (Flanagan et al., 2018; Vauzour, 2017).

The antioxidant activity depends on the structure of their functional groups. The number of hydroxyl groups greatly influences several mechanisms of antioxidant activity such as scavenging radicals and metal ion chelation ability (Goya et al., 2016; Pham-Huy et al., 2008). Indeed, the mechanisms involved in the antioxidant capacity of polyphenols include suppression of ROS formation by either inhibition of enzymes involved in their production, scavenging of ROS, or upregulation or protection of antioxidant defences (Arranz et al., 2013).

In addition, cocoa polyphenols may reduce the catalytic activity of enzymes involved in ROS generation (Andújar et al., 2012). ROS formation has been reported to enhance free metal ions by reduction of hydrogen peroxidase with generation of the highly reactive hydroxyl radical (Salvatore, Dennis, Achille, & Daniela, 2001; Siamilis et al., 2009). Some other researchers have shown that some polyphenols modulate the activity of arachidonic acid metabolizing enzymes such as cyclooxygenase (COX), liopogenase (LOX) as well as nitric oxide synthase (NOS) (Commenges et al., 2000; García-Bueno et al., 2008). They showed that when these enzymes are inhibited there is a reduction of arachidonic acid, prostaglandins and leukotrienes which are key mediators of inflammation.

2.4.1.1.2 Cocoa and inflammation

The anti-inflammatory properties of cocoa have been reported in some studies (Andújar et al., 2012; Ellinger & Stehle, 2016; Goya et al., 2016) involving acute and chronic inflammation.

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Goya and co-workers in their study considered dietary bioactive compounds with anti-inflammatory activities as an alternative source of prevention of inflammation-associated diseases (Goya et al., 2016). Apart from affirming the high potential of cocoa as an antioxidant, they also showed its role in modulating markers and processes of inflammation. Besides, one study showed that a cocoa flavonoid-enriched extract and the monomers epicatechin and isoquercitrin were able to decrease the production of inflammatory molecules such as tumour necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 by macrophages stimulated with lipopolysaccharide (LPS) (Guan & Fang, 2006).

Another study in a bid to add to the plethora of information about the health benefits of cocoa, showed that consumption of cocoa-rich food may reduce inflammation by lowering the activation of monocytes and neutrophils (Ellinger & Stehle, 2016). Furthermore, yet another study proposed that polyphenols many affect the enzymatic and signalling systems which are involved in the inflammatory processes, such as addition of tyrosine and serine threonine amino acid residues (Hussain et al., 2016). These enzymes are involved in cell activation processes such as T cell proliferation, B lymphocyte activation or cytokine production by stimulated monocytes (Hussain et al., 2016)

2.4.1.1.2.1 Cocoa and IL-6

Very few studies have specifically focussed on the effects of cocoa specific markers of inflammation such as interleukins6 (IL-6). One study among human participants to show the effects of flavanol rich dark chocolate consumption on the risks of cardiovascular mortality (Kuebler et al., 2016), found that flavanol-rich dark chocolate consumption relates to lower risk of cardiovascular mortality. However, some studies have shown inconclusive results in the anti-inflammatory potential of cocoa extracts or single flavonoids as monomers (epicatechin, catechin) or polymers (procyanidins).

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For instance, one study showed that epicatechin in stimulated whole blood cells culture suppressed the production of interleukin (IL)-6 and IL-8 (Al-hanbali et al., 2009). Whereas in another study, monomer to pentamer units and longer chain fractions of cocoa flavanols increased the secretion of TNF-α, IL-1, and IL-6 in LPS-stimulated peripheral blood mononuclear cell (Kenny et al., 2007; Wisman et al., 2008).

2.4.1.1.3 Cocoa and neurotrophic factors

Some studies have shown the neurotrophic action of certain polyphenols that lead to neuronal survival, growth, proliferation and differentiation (Cimini et al., 2013; Vauzour, 2017). In addition to the study of the antioxidant and anti-inflammatory properties of cocoa, some of these researchers have thrown light on the neurotrophic tendencies of cocoa as well (Cimini et al., 2013; Magrone et al., 2017; Nehlig, 2013).

In a study to analyse the neuroprotective activity of cocoa polyphenols in the progression Alzheimer's disease, the researchers showed that the cocoa polyphenolic extracts exerted neuroprotective effects. They attributed this characteristic to the activation of BDNF survival pathway leading to reduction of neurite dystrophy (Cimini et al., 2013). Another experimental study involving the administration of dark chocolate the Alzheimer's disease models showed a vast reduction of hyperglycemia and cholinesterase activity in hippocampal tissue homogenates as well as increase in cognitive ability. They also postulated that the increase in enzymatic activity is an indirect activity of the neurotrophic growth factors (Madhavas et al., 2016).

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2.4.1.1.3.1 Cocoa and BDNF

There are several lines of evidence pointing to the actions of polyphenols on the expression of a number of important genes including the BDNF gene, thus playing an important role in controlling neuronal survival, and synaptic function in the CNS (Cimini et al., 2013; Magrone et al., 2017; Simonyi et al., 2005; Vauzour, 2017). In one study involving human participants aged 65 and above, increases in global cognition after intake of cocoa flavanols was reported. Thus, the serum cocoa flavanol levels paralleled concurrent increases in serum BDNF levels (Neshatdoust, Saunders, Castle, Vauzour, & Williams, 2016).

Another study exploring the benefits of theobromine, the predominant methylxanthine found in cocoa beans revealed better working memory in group that consumed the theobromine as compared to the group that did not. They also found increases in cortical BDNF protein and mRNA levels in this same group of rats, suggesting that theobromine, which is found in naturally occurring cocoa beans upregulates the BDNF signalling pathway (Creb et al., 2019).

2.4.2 Exercise training

Exercise training is defined as any bodily movement produced by skeletal muscles that results in energy consumption. It may be structured or unstructured. It can be an everyday life activity, which includes prearranged, deliberate and repetitive activity, grassroots and competitive sports. Regular exercise training of moderate intensity such as walking, cycling or sports affords significant health benefits (Barha et al., 2017).

Regular participation in exercise is known to improve the physiological performance of skeletal muscle in the body. It has been shown by numerous studies to decrease the incidence of some diseases such as heart diseases, cancers and some other vascular diseases as well as type 2 diabetes (Radak et al., 2005). Many studies have implied the beneficial effects of exercise on brain function (Cotman et al., 2007; Di Benedetto et al., 2017; Shamsaei, Erfani, Fereidoni, &

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Shahbazi, 2017). Some of these studies have explored the preventive as well as therapeutic tendencies of exercise in brain neuropathologies such as Alzheimer's and Parkinson's diseases, as well as stroke (Katusic & Austin, 2016; Radak et al., 2013; Winstein et al., 2016).

2.4.2.1 Running wheel

The running wheel is a common method of exercise training in laboratory rodents (Novak et al., 2015). Most rodents ran readily and easily in exercise in running wheels. It is also an uncomplicated, easily quantifiable measure of physical activity (Holland, Mumford, Kavazis, & Roberts, 2018). Some studies employ the use of running wheels not only to assess the levels of general physical activity but also to model the effects of exercise to meet the demands of the study (Goh & Ladiges, 2013; Hayes et al., 2008; Holland et al., 2018).

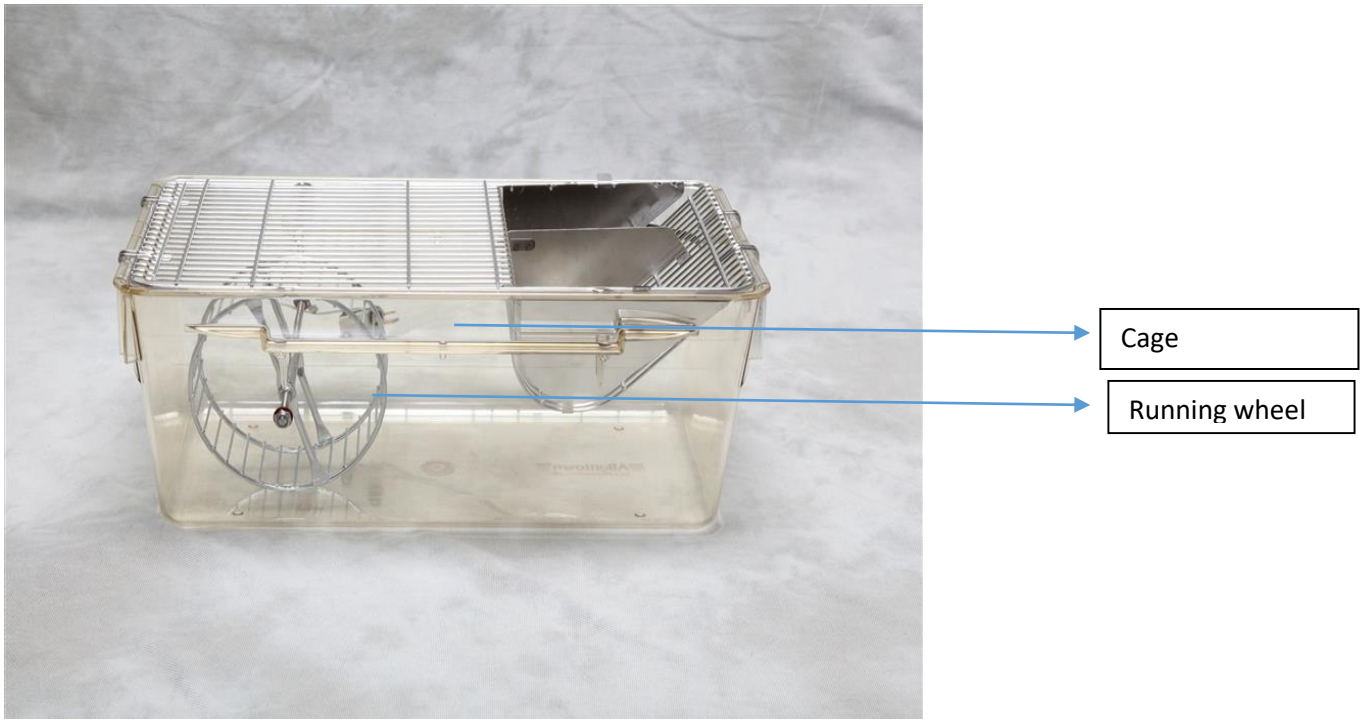

Figure 4: A photograph of an exercise training apparatus, consisting of a running wheel encased in a transparent plastic cage.

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2.4.2.2 Exercise and brain health

Certain lifestyle factors such as exercise has been greatly implicated in brain health (Barha et al., 2017; Cotman et al., 2007). Exercise can reduce the risk of age-related cognitive decline (Speisman et al., 2013; Woods et al., 2012) and neurodegeneration (Svensson et al., 2015). Exercise also generally protects the brain and stimulates proper function among all age groups (Barha et al., 2017; Cotman et al., 2007).

Also, many findings thus far suggest that physical exercise reduces the noxious effects of OS (Marosi et al., 2012; Radak et al., 2013), inflammation (Chennaoui et al., 2015; Simioni et al., 2018), promotes vascularization (Chieffi et al., 2017) and improves glucose metabolism (Vaynman, Ying, & Gomez-Pinilla, 2004).

Results from many animal studies demonstrates that exercise has been implicated for its benefits in promoting brain health. It has been demonstrated that moderate-to-vigorous exercises in animal models evokes a lot of activities including, increase in the production of antioxidant enzymes particularly superoxide dismutase (SOD), BDNF (Ke et al., 2011a), insulin-like growth factor and vascular endothelial growth factor, which are all responsible for neuronal protection (Vaynman et al., 2004).

In heathy adults, the benefits of exercise can be seen in some behavioural changes including significant increase in memory, attention, processing speed and executive functions (Smith et al., 2010). Also, in midlife, regular exercise is associated with decreased risk of dementia in later stages of life, indicating directly that exercise may have preventive effects on age –related cognitive decline (Hamer & Chida, 2009). Other studies have shown that chronic physical exercise reduces the production of free radical (ROS) in certain areas of the brain such as the cerebral and hippocampal regions associated with many neurodegenerative diseases (Cotman et al., 2007; Di Benedetto et al., 2017; Svensson et al., 2015).

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Furthermore, recent animal studies in varied contexts have been directed towards understanding the neurobiological basis for these benefits. These studies have reported many changes in the neuronal and glial structure, including neurogenesis and neuro plastic changes within the brain associated with exercise (Cho et al., 2013; Heo et al., 2014). For instance, a study carried out in Alzheimer's diseases (AD) mice models, involving running for 1 hour, 5 days in a week for 16 weeks showed an increase in the hippocampal volume as compared to the sedentary group of mice. In this study, the hippocampal neurogenesis was associated with synaptogenesis and improvements in learning capacity in the exercise trained rats compared with the sedentary control mice (Yeude et al., 2009).

Another study demonstrated a decrease in amyloid beta (AB) plaques, precursor signs in Alzheimer's disease mainly in frontal cortex on rat models of Alzheimer's disease with a consistent regimen of exercise (Radak et al., 2013). Exercise has also been highly implicated in the promotion of cognitive health during ageing as a non-pharmacological agent (Kempermann, Van Praag, & Gage, 2000) van Praag et al., 2005). In recent studies, there has been an increased interest in evaluating the effects of exercise training on various neurological factors that improve and maintain cognitive responses and processes as well as increase the resistance of the brain to injury (Hamakawa et al., 2013).

These studies have shown that voluntary exercise can increase levels of brain-derived neurotrophic factor (BDNF) and other growth factors, stimulate neurogenesis, increase resistance to brain insult and improve learning and mental performance providing a simple means to maintain brain function and promote brain plasticity (Chieffi et al., 2017; Ferris, Williams, & Shen, 2007; Gomez-Pinilla et al., 2008).

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2.4.2.3 Exercise training and oxidative stress

The generation of reactive oxygen species (ROS) is an inevitable and necessary consequence of aerobic metabolism (Ogonovszky et al., 2005). Usually, increased levels of ROS leading to oxidative damage have been associated with a single bout of exercise because of the limited adaptation of intensity and or duration (Ogonovszky et al., 2005).

Regular and consistent exercise is considered beneficial to health. However, acute and unaccustomed exercise may lead oxidative stress (He et al., 2016). The latter authors explained that repetitive muscle contraction involves accumulation of ROS (Zuo et al., 2015). Physiological levels of ROS play the role of signalling molecules, essential for normal cellular functions whereas an overproduction of ROS resulting from exhaustive exercise training can lead to oxidative stress (Radak et al., 2013).

Appropriate exercise training (moderate to high intensity) has been shown to induce adaptive responses and strengthen endogenous antioxidant defence systems to help resist excessive ROS, maintaining redox balance (He et al., 2016)

2.4.2.4 Exercise training and inflammation

2.4.2.4.1 Exercise and IL-6

Interleukin-6 (IL-6), that acts both as a pro-inflammatory and anti-inflammatory multifunctional cytokine, is produced locally by contracting skeletal muscles in response to exercise and after strenuous exercise sessions (Scheller et al., 2011; Vasconcelos & Fernanda, 2018). The high concentration of IL-6 during intensive exercise is due to the fact that this interleukin acts also as a myokine, because it increases exponentially but proportionally to the duration of the exercise and the amount of muscle mass involved in the exercise (Vasconcelos & Fernanda, 2018). During exercise IL-6 is thought to act in a hormone-like manner to mobilize

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extracellular substrates and/or increase the supply of nutrients to the muscle (Pattamaprapanont, Muanprasat, & Soodvilai, 2016).

2.4.2.5 Exercise training and neurotrophic factors

Exercise as proven by several studies modulates plasticity as well as the supporting systems that maintain brain function and health (Kohman et al., 2012; Vaynman et al., 2004).

The mechanisms mediate the effects of exercise on brain function and overall brain health are still extensively teased apart in various studies, however one striking concept is that exercise increases brain availability to several classes of growth factors that modulate brain function and health (Chieffi et al., 2017; Cotman et al., 2007; Gomez-Pinilla et al., 2008).

Presently these growth factors including BDNF, IGF-1 and VEGF are the common principal growth factors known to mediate the effects of exercise on brain plasticity, function and health (Keefe et al., 2017; Kohman et al., 2012; Speisman et al., 2013).

2.4.2.5.1 Exercise training and BDNF

Many evidences from animal and human research support the idea that BDNF is essential for brain function, synaptic plasticity, learning and modulation of certain brain related conditions. Some animal studies have shown that exercise increases BDNF in several regions of the brain including the cerebral cortex (Ding et al., 2004; Ke et al., 2011). One study showed that after several days of exercise, BDNF gene and protein production by neurons increased in all hippocampal subfields and levels continued to remain high for a week with sustained exercise. They suggested that the regulation of the hippocampal BDNF by exercise was mediated by neurotransmitter systems (Mertikas & Kainulainen, 2010).

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Another study involving animal models showed that exercise induced regulation of BDNF transcripts in the rat brain may help increase the brains resistance to damage and neurodegeneration that usually occurs with ageing (Strasser et al., 2006) and brain disorders (Briken et al., 2016).

In some human studies it has been shown that exercise increases BDNF concentrations in serum, suggesting that it plays a key role in enhancing neuronal volume as well as improving cognitive function (Mattson et al., 2004).

Of interest also was the reduced level of BDNF levels in patients with Alzheimer's disease and Parkinson's disease. These studies reported that with decline in the conditions of the patients, there was reportedly lower levels of BDNF as well (Svensson et al., 2015; Bayani Uttara et al., 2009).

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Materials procured for the experiment included: Rat running wheel (size: 28 cm diameter, Amazon, ASIN: B072QBF3BG), digital photo tachometer (Edhis Company, Amazon UK, ASIN: B076758QC6), Countdown timer (Model Number TR112N, China), Chemical weighing balance (Lab-Kits Analytical balance).

Natural cocoa powder (GoodFood Brand, Ghana, Batch Number KK1802A), Rat BDNF (Brain Derived neurotrophic factor) ELISA kit (Elabscience, USA, Catalog No: E-EL-R1235), Rat IL-6 (Interleukin-6) ELISA kit (Elabscience, USA, Catalog No: E-EL-R0015), Superoxide dismutase Assay kit (Cayman Chemical, USA, Item No: 706002), Glutathione Assay kit (Cayman Chemical, USA, Item No: 703002), Diethyl Ether (Auro Avenida PVT limited, India, Code: 16500) and Growers mash (GAFPA, Ghana).

3.2 Methods and Protocol for the study

3.2.1 Study design

The study design for this study was experimental. This was because the study involved randomization of the animals, manipulation of the treatments as well as the use of a control group.

3.2.2 Study site

The study was conducted at a designated space of the Animal Experimentation Unit of the School of Biomedical and Allied Health Sciences, University of Ghana, Korle-Bu. The designated area was well ventilated and pest free. The conditions of the designated area within the study site, including light, temperature and humidity were also adjusted to meet the requirements of the study.

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3.2.3 Animals

Twenty (n=20) male Sprague Dawley rats were purchased from the breeding division of the Animal Experimentation Unit and used for this study.

3.2.4 Inclusion and Exclusion criteria

Rats between ages of 8 -10 weeks were included.

Rats included weighed between 130g and 160g.

Rats that showed any form of physical deformity or ailment were excluded from the study.

3.2.5 Procedure

3.2.5.1 Acquisition and acclimatization of Rats

All experimental procedures for this study were approved by The ethical and protocol review committee of the College of Health Sciences under the protocol Identification number: CHS-Et/M.6 – 5.11/2018-2019). Experimental procedures followed the Institutional Animal Care and Use Committee (IACUC) guidelines for humane care and handling of laboratory animals.

As previously stated, twenty (n=20) male Sprague-Dawley rats with specified characteristics (aged range 8 – 10 weeks; weight range 100-160g) were acquired from the Animal Experimentation unit of School of Biomedical and Allied Health Sciences, University of Ghana. The rats were transferred from the Animal breeding facility to the designated experimental space using transport boxes with adequate ventilation holes. The rats were then housed in standard stainless-steel cages (28.7 cm x 20.3 cm x 17.3 cm). Each cage accommodated a maximum of 5 rats, and all were housed in a well-ventilated room within the unit, devoid of drought and ultrasonic and sudden noises, such as dripping tap water. The cages were placed on trays filled with wood shavings as bedding materials to mop up excreta and spilled fluids. The beddings were changed every 72 hours.

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The room was kept under regulated temperature of 28+/-2^{o} C and relative humidity of (70+/-4) % during the day and night. A fixed 12:12-h light/dark cycle (lights off from 7:00pm to 7:00am) was maintained in the designated room in the Animal Experimentation Unit.

The rats were provided with tap water *ad libitum* in graduated drinking bottles and fed commercially prepared chow (Growers mash) in the feeding troughs placed in each cage.

The rats were allowed to acclimatize to the new environment for a week. To facilitate acclimation to the human handling, the rats were weighed on three separate days during the week. The researcher handled all the rats with proper handling techniques. This was done to get the rats used to the researcher and to reduce the stress associated with the new handling techniques.

3.2.5.2 Allocation of Rats

The rats were randomly divided into four equal groups of five rats; three experimental groups and one control group. The groups were treated as follows; Group 1 received exercise training and natural cocoa powder (NCP), Group 2 had exercise training only, Group 3 received natural cocoa powder (NCP) only and Group 4 had neither training nor cocoa (control group) as shown in Fig. 5.

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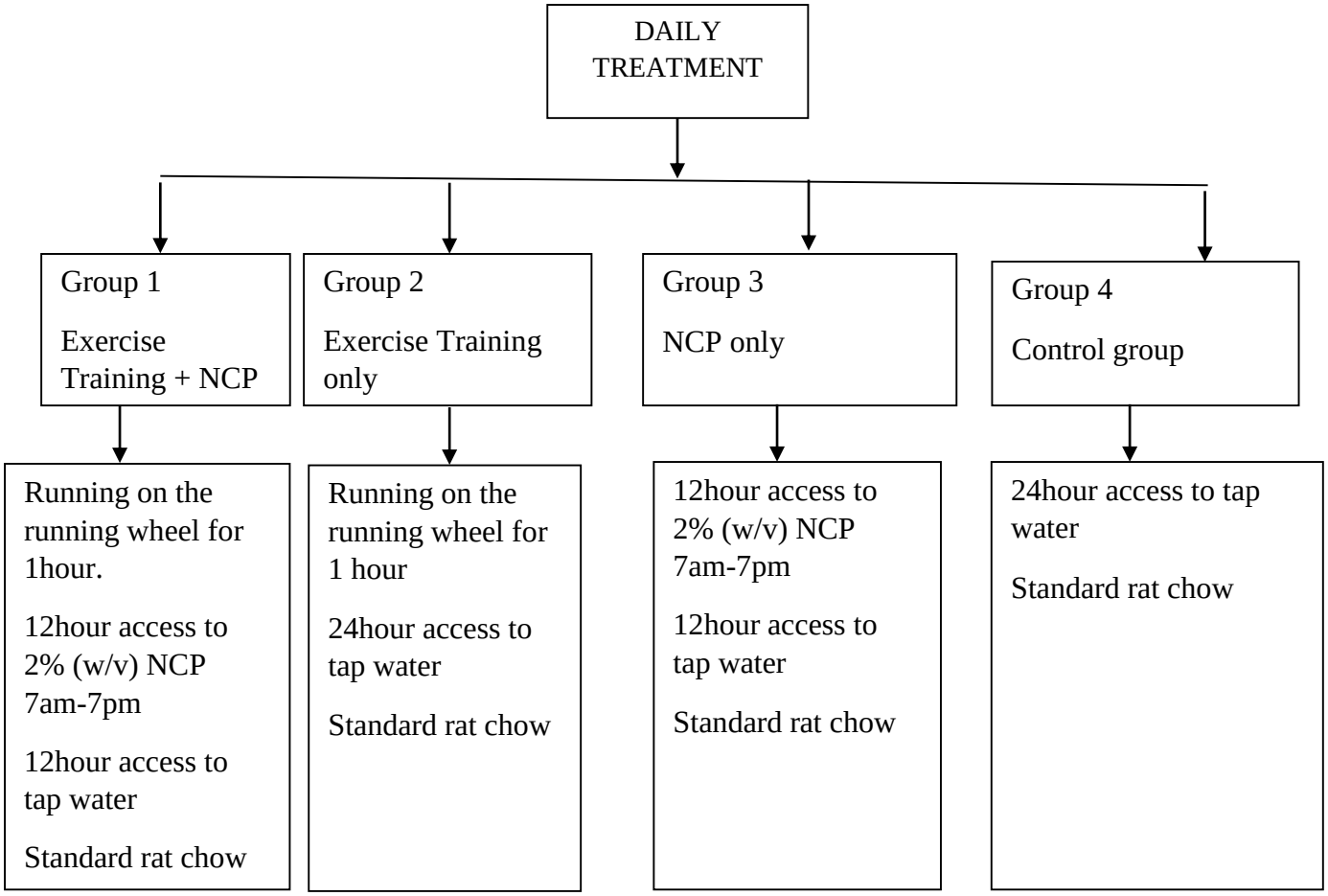

Figure 5: Flowchart summarizing the daily treatment of rats in this study.

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3.2.6 Exercise training apparatus and protocol

The rats were trained using recommended exercise training apparatus (Novak et al., 2015). The training apparatus consisted of the rat running wheel (diameter-28cm; width-8 cm) and the cage (30cm x 40cm x 20cm). The running wheel rotates on its shaft whenever the rat walks or runs in either direction in the wheel.

The number of revolutions made was recorded with the use of the digital photo tachometer. A reflective tape attached to the beam of the wheel provides signals picked by the digital photo tachometer and the number of revolutions, in units Revolutions per minute (RMP), is displayed and recorded on the display screen of the tachometer. The stop watch was used in recording the time spent on the wheel. The distance ran by each rat was calculated by using the formula below:

Diameter of the wheel x *pi* x number of revolutions x time = Distance travelled in Km.(Huber et al., 2017)

3.2.6.1 Pre training protocol

Prior to the main exercise training, ten (n=10) rats assigned to the experimental (training) groups (Groups 1 and 2) were exposed to the exercise apparatus to familiarise them with the exercise protocol (running wheel) for six days. During this period, each rat was allowed to run on the wheel. In order to discourage the rats from getting off the wheel and to maximize the time spent on the wheel, the wheel was elevated to 40cm above the floor of the cage.

In order to reduce stress and improve endurance, the running time on the wheel was increased progressively (Diederich, Bastl, Wersching, Teuber, & Strecker, 2017). In the first two days, the experimental training rats were allowed to run for 20 minutes. Subsequently, the running time was increased to 40 minutes for two more days and finally increased to 60 minutes for the

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remaining two days. The rats were then allowed to rest for one day prior to the commencement of the main training. The pre-training was done to reduce stress and behavioural changes associated with novelty to the running wheel (Clark & Hoffmann, 2013). Animals in Groups 3 and 4 did not receive any intervention at this time.

3.2.6.2 Exercise training protocol for Experimental period

After the pre-training period, the main exercise training was done for six weeks. Each rat was placed in the plastic cage of the running wheel and made to complete a 60-minute exercise session five days per week for six weeks. The exercise consisted of voluntary running. The exercise protocols were carried out during the day, between the hours of 7: 00 am and 12 noon each study day.

The time spent on the wheels as well as the number of revolutions made was recorded with a Countdown timer (Model Number TR112N, China) and tachometer (Edhis Company, Amazon UK, ASIN: B076758QC6), respectively for each training session (Goh & Ladiges, 2013). The running activity was voluntary, and each rat was rewarded with sugar free biscuit at the end of each training session.

Specifically, Group 1 rats were subjected to voluntary running and cocoa intake. Rats in this group were provided with 2% (w/v) NCP for 12 hours (7:00 am – 7: 00pm) and water for 12 hours (7: 00 pm – 7:00am). Group 2 rats were subjected to voluntary running only, and provided with water for 24 hours (7: 00 am -7:00 am). Group 3 rats were given 12-hour (7:00 am – 7: 00pm) access to 2% NCP suspension and 12-hours (7: 00 pm – 7: 00 am) access to water, but had no exercise training. Lastly, Group 4 rats (control group) were given standard rat chow and 24 hours (7: 00am -7:00pm) access to water. They were neither subjected to exercise nor provided with 2% NCP suspension.

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3.2.7 Preparation of Natural Cocoa powder (NCP) suspension

Natural cocoa powder (GoodFood Brand, Kakawa Enterprise Ltd. Accra, Ghana) was used to prepare the cocoa suspension in this study. A concentration of 2% weight per volume (w/v) was given as previously reported by Affram et al., (2008) and Sokpor et al., (2012).

The suspension of 2% w/v unsweetened cocoa was prepared daily. The suspension was prepared by dissolving in 330 ml of pre boiled water, 6.7 g of Natural Cocoa Powder. The volume of the pre boiled was obtained with the use of a measuring cylinder and the mass of the Natural cocoa powder was obtained with the chemical weighing balance.

The freshly prepared suspension was allowed to cool and then shaken thoroughly, before being dispensed to the rats in 500 ml graduated drinking bottles to be taken *ad libitum* as seen in Fig. 6. The suspension was shaken regularly within the day as the cocoa particles had the tendency to settle, disrupting free drinking by the rats.

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Figure 6: Photograph showing rat voluntarily consuming freshly prepared NCP suspension in a graduated feeding bottle.

3.3 Blood sampling for Biochemistry

Baseline blood samples were collected from each rat. This was done via tail snipping and milking. Prior to the blood collection, the animals were anaesthetised by inhalation in a glass chamber, containing cotton dubbed with adequate amount of diethyl ether. After the rat was properly anesthetized, the tip of the tail was snipped and allowed to hang to allow blood flow into the tubes. The bleeding was stopped immediately after collection by pressure application to the tip of the tail.

The blood was collected directly into the serum separating gel tube and covered immediately. The blood was allowed to clot at room temperature and then centrifuged at 3500 rpm for 7 minutes using the centrifuge (high speed refrigerated centrifuge TGL-16 M, Biochemistry Department, SBAHS, Korle-Bu).

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The serum was then transferred into 2 ml Eppendorf tube with appropriate labelling via a pipette and stored in a -20 freezer (Biochemistry department, SBAHS, CHS, Korle-Bu) for Glutathione reductase (GSH), Superoxide Dismutase (SOD), Brain Derived Neurotrophic Factor (BDNF) and Interleukins 6 (IL-6) analyses for assessing oxidative stress, inflammation and neurotrophic growth factors respectively. At the end of the experiment, blood samples were also collected using the same tail snipping and milking procedures employed at baseline.

3.4 Brain tissue homogenate preparation for biochemistry

The rats were transiently perfused transcardially with normal saline. The rats were immediately decapitated and the brain was carefully removed from the skull. The frontal lobes of the cerebrum of each rat were rapidly removed after decapitation and removal of the brain. The tissues were weighed in a 2 m L micro centrifuge tube and 500 µL of cell lysis buffer per 100 mg of tissue. The tubes were assembled in tissue lyser and homogenized in a homogenizer (Thermo Fisher scientific, USA) at 25 Hz for 3 minutes. The samples were then centrifuged at 16,000 × g for 10 minutes at 4°C. 7. The supernatant was then transferred to new Eppendorf tubes. Aliquots of the homogenates were immediately frozen and stored at – 80^{o}C.

3.5 Antioxidant assay

3.5.1 Glutathione assay

The Cayman's Glutathione assay kit (Cayman chemical company, USA, Item no. 703002) was used in the assessment of serum level of GSH. The serum samples were deproteinated to remove as much protein as possible from the serum samples to avoid interferences due to particulates and sulfhydryl groups on proteins in the assays. The deproteinated samples were then stored in -20^{o} C.

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An assay cocktail was prepared by strict adherence to the protocol outlined in the kit and used within 10 minutes of the preparation as recommended by the kit manufacturer.

The assay cocktail was then added to each well containing the standards and samples using the multichannel pipette. The plate containing the wells was then covered and incubated.

GSH concentrations were then determined by the End Point Method, which involved reading the plate at 405 nm after 25 minutes. The average absorbance values of each of the standards and samples were obtained and corrected as described by the manufacturers (Cayman Chemical, USA, Item No: 703002). The corrected absorbance values of the standards were plotted as a function of the concentrations of total concentrations provided by the kit. The concentrations of the GSH in the serum samples were calculated from the standard curve.

3.5.2 Superoxide Dismutase (SOD) assay

The SOD assay was prepared according the protocol stated in the Cayman's Superoxide dismutase assay kit (Cayman Chemical company, USA, item no. 706002). The serum samples were diluted 1:5 with the sample buffer before assaying for SOD activity. The Radial detector provided in the kit was added to each of the wells. The standards and samples were added as well to each of the wells.

The reactions were then initiated by adding diluted Xanthine Oxidase provided in the Kit. The plate was then thoroughly shaken and incubated for 30 minutes at room temperature. The absorbance values were then read at 430 nm using a plate reader.

The standard curve was plotted as described in the kit. The SOD activity of the serum samples was determined with the use of the standard curve.

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3.6 Assays for markers of inflammation

3.6.1 Interleukins-6 (IL-6) assay.

The Rat IL-6 ELISA kit (Elabscience, USA, Catalog No: E-EL-R0015), was used to determine the IL-6 concentrations in serum.

The standards and samples were added to the appropriate wells which had already been pre-coated with a specific antibody for IL-6. The Biotinylated detection antibodies provided in the kit together with the Avidin-Horseradish peroxide conjugates were added to each of the microplates containing the standards and samples and then incubated. The free components were then washed away. The Substrate reagent was then added to each microplate. The contents of each of the wells appeared blue. The Stop solution was then added and the contents changed to yellow colour.

The optical densities were measured at a wavelength of 450 nm. The concentrations of the samples were calculated by comparing the optical densities of the samples with the standard curve. The final concentrations obtained were analysed using the GraphPad Prism software version 5.0

3.7 Neurotrophic growth factor assays

3.7.1 Brain Derived Neurotrophic Factor (BDNF) assay.

The Rat BDNF ELISA kit (Elabscience, USA, Catalog No. E-EL-R1235) was used to determine the BDNF concentrations in serum and supernatants obtained from brain homogenates.

The standards and samples were added to the appropriate wells which had already been pre-coated with a specific antibody. The Biotinylated detection antibodies provided in the kit together with the Avidin-Horseradish peroxide conjugates were added to each of the

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microplates containing the standards and samples and then incubated. The free components were then washed away. The Substrate reagent was then added to each microplate. The contents of each of the wells appeared blue. The Stop solution was then added, and the contents changed to yellow colour.

The optical densities were measured at a wavelength of 450nm. The concentrations of the samples were calculated by comparing the optical densities of the samples with the standard curve.

3.8 Assessment of variables

Throughout the experiment, the individual body weights of the rats were taken weekly. The volume of fluids, which included NCP solution and water were measured daily. Also, the distance ran by each animal in the experimental training groups were recorded daily.

3.8.1 Weight

The weighing balance was used to weigh the animals on weekly basis. For accuracy, the scale was calibrated before each measurement was taken. The animals were allowed to settle properly on all four paws before readings were taken. Each animal was weighed three times and the average reading was recorded and used in the analysis. The weights were recorded on a weight chart created to study the weight change pattern of each animal.

3.8.2 Volume of fluids

The volume of NCP solution and water consumed by each animal was measured daily. The volume of the NCP solution consumed (Cc) in 12 hours was calculated by subtracting the volume left (Cf) in the graduated drinking bottles at the end of the 12 hours from the initial volume administered (Ci). The volume of water consumed (Wc) was also calculated by

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subtracting the volume of water remaining (Wf) in the bottles from the initial volume of water administered (Wi). A summary of the calculation is provided below:

Volume of NCP consumed

Cc (12hours) = Ci – Cf

Volume of Water consumed

Wc (12 hours) = Wi – Wf

The total volume of fluid consumed in a day was determined by adding the volume of NCP solution consumed to the volume of water consumed as;

Total fluid intake per day = Cc + Wc

The total fluid intake within 24 hours for each group was recorded and analysed.

3.8.3 Distance run on the wheel

The distance ran by each animal was obtained by using the formula below:

Diameter of running x *pi* x Revolutions (as taken by the tachometer in RMP) x time = Distance (Km).

3.9 Harvesting of tissues and histology

3.9.1 Harvesting of tissues

After 6 weeks of the experiment, the rats were conveyed from the Animal Experimentation Unit in well aerated transporter boxes to the Histology laboratory of the SBAHS for tissue harvesting and processing.

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A perfusion set-up comprised of normal saline provided in a sterile delivery kit. A glass gas chamber was provided, well cleaned and based with cotton ball of an appropriate size. The cotton ball was soaked with Diethyl Ether (Auro Avenida PVT limited, India) before each rat was placed in the chamber to be anaesthetised thoroughly before sacrifice. Inhalation of the diethyl ether in the chamber resulted in a full unconsciousness of the rat. Full unconsciousness of the rat was confirmed when there was not movement or twitch after a pin prick to the lateral aspect of the abdominal wall. The rat was then transiently perfused transcardially with normal saline. The rats were immediately decapitated and the brain was carefully removed from the skull. The brain of each rat was weighed twice on the chemical weighing balance (Lab-Kit Analytical Balance) and the average recorded. The brain was then post fixed in 10 % buffered formalin (p H 7.4) in sample collection bottles at 4^{o} C for 48 hours before processing.

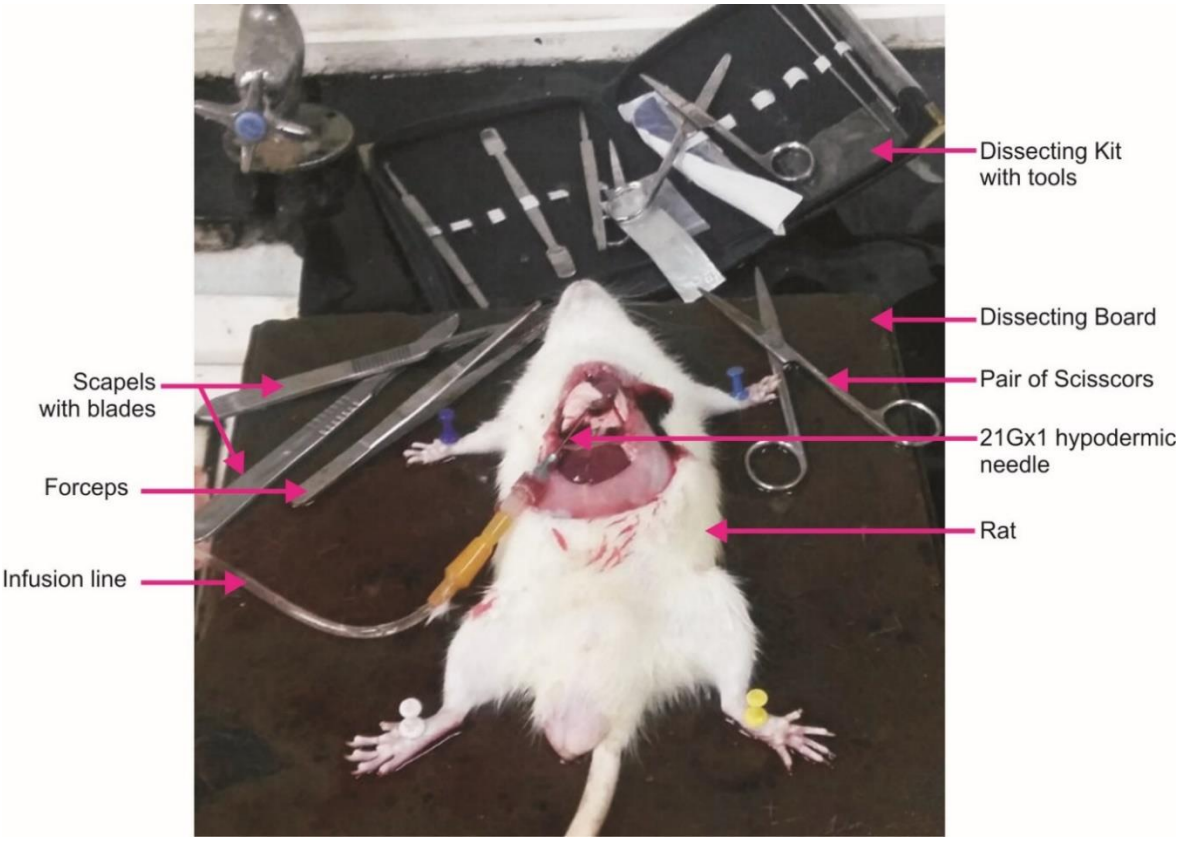

Figure 7: A photograph of rat undergoing perfusion

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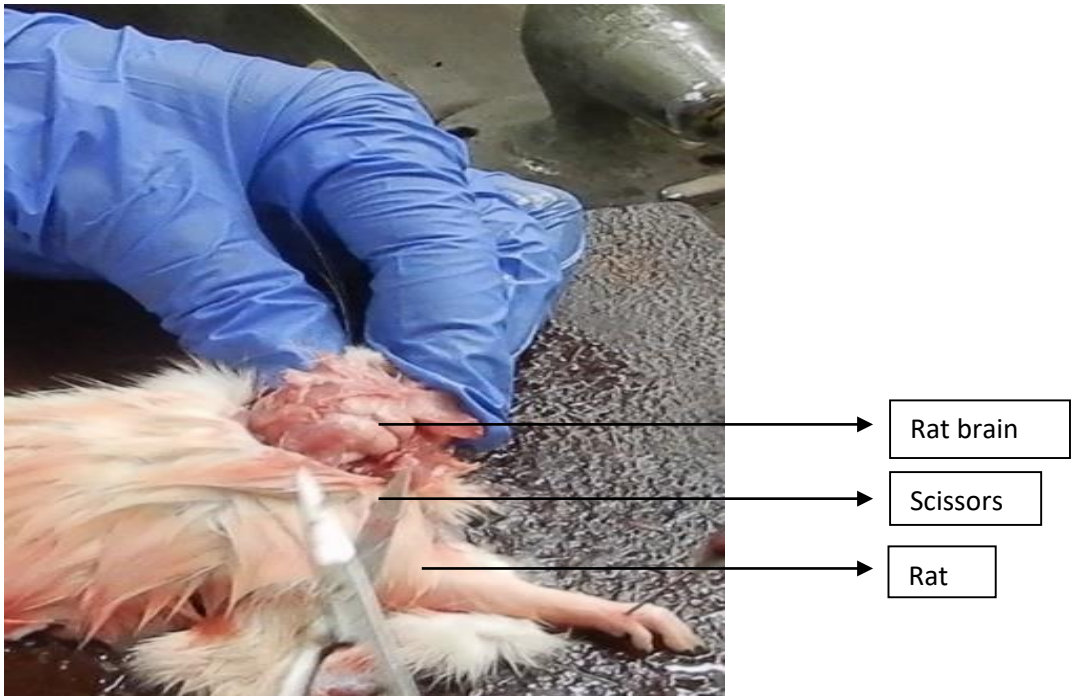

Figure 8: A photograph of rat brain being removed.

3.9.2 Tissue slicing and processing

The midbrain and the cerebellum of each brain were sliced off as well as the frontal lobes leaving intact, the rest of cerebrum. Each cerebrum was divided into two (2) coronal sections and place in well labelled cassettes. Tissue blocks were then processed from the coronal sections. The cerebral tissue slices were then fixed in 10 % buffered formalin (p H 7.4). In order to have a compact tissue, the tissue samples were treated with graded series of ethanol, 50% overnight, 70% for 2 hours and 95% for an hour. The tissues were then taken through three series of absolute ethanol for 2 hours. The tissues were then cleared in two subsequent steps of xylene, each step lasting for an hour. The cassetted cerebral tissues were then infiltrated with molten wax at a temperature of 60^{o}. The tissue was then embedded in a paraffin block.

3.9.3 Sectioning

Both coronal halves of the cerebrum per animal were embedded on one tissue block. The paraffin-embedded blocks formed were trimmed at a thickness of 10 microns with the use of

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the Leica rotatory microtome. This was done to obtain an optimal cutting surface that displayed the full profile of the brain tissue. After trimming appropriately, the rotatory microtome was set to create microtome sections of thickness of 5 microns.

Three sections were selected for each animal and this was done at the intervals of 5, 10 and 15.

The paraffin slices were then placed in 40^{o} C water in the flotation bath to allow expansion to take place and also remove wrinkles. The paraffin sections were fished out using the glass slide (25.4mmx76.2mm, thickness 1.0 mm-1.2mm) (AllPro, Middlesex, England) and a brush to position the section appropriately. The sections were then dried at 37^{o} C and mounted.

The tissues were then stained with Haematoxylin and Eosin (H&E) stain to assess the neuron morphometry.

3.10 Stereological analysis

3.10.1 Sampling of photomicrographs of brain sections

Photomicrographs of the stained sections on the slides were captured with the aid of an appropriate set up, comprising of the binocular bright-light microscope (Leica Galen III, Catalogue no: 317506, serial no: ZG6JA4), digital microscope eyepiece lens (Lenovo Q350 USB PC Camera) and a desktop computer (HP Compaq dx2300 Microtower).

The section was mounted under the optical light microscope and brought to focus with the x40 objective lens. The microscope field was then directed to one end of the slide. After a proper focus had been obtained, one eyepiece lens of the light microscope was replaced with a digital microscope eyepiece which captured the photomicrographs and displayed on the connected computer with the Ulead software.

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The tissue section on the mounted microscope slide was systematically sampled as follows. The stage was moved five microscope stage units on the x-axis and five microscope stage units on the y-axis and then photomicrograph taken. The stage was then moved horizontally to the left direction in the x-axis but in the same the direction on the y axis for the next micrograph to be taken. These movements were repeated till the whole section on a slide was sampled.

Out of the total number of micrographs obtained from all the 6 coronal cortical sections per animal, fifteen were selected by systematic random sampling from each coronal section. This was done by selecting the first micrograph as the starting point and selecting every third successive micrograph. Hence the selection involved the 1^{st}, 4^{th}, 7^{th} etc micrograph. A total of 90 photomicrographs per animal were pooled for further stereological assessment.

A final total of one thousand eight hundred (1,800) were selected systematically for all the animals in the four groups for point counting in the stereological grid.

Furthermore, the grid which was used for the point counting as well as determining the magnification of the micrographs was calibrated. This was done by taking snapshots of the microscope stage graticule (Leica Galen III, Catalogue no: 317506, serial no: ZG6JA4) at the same magnification,

The H&E slides were used to count the neurons in the cerebral cortex.

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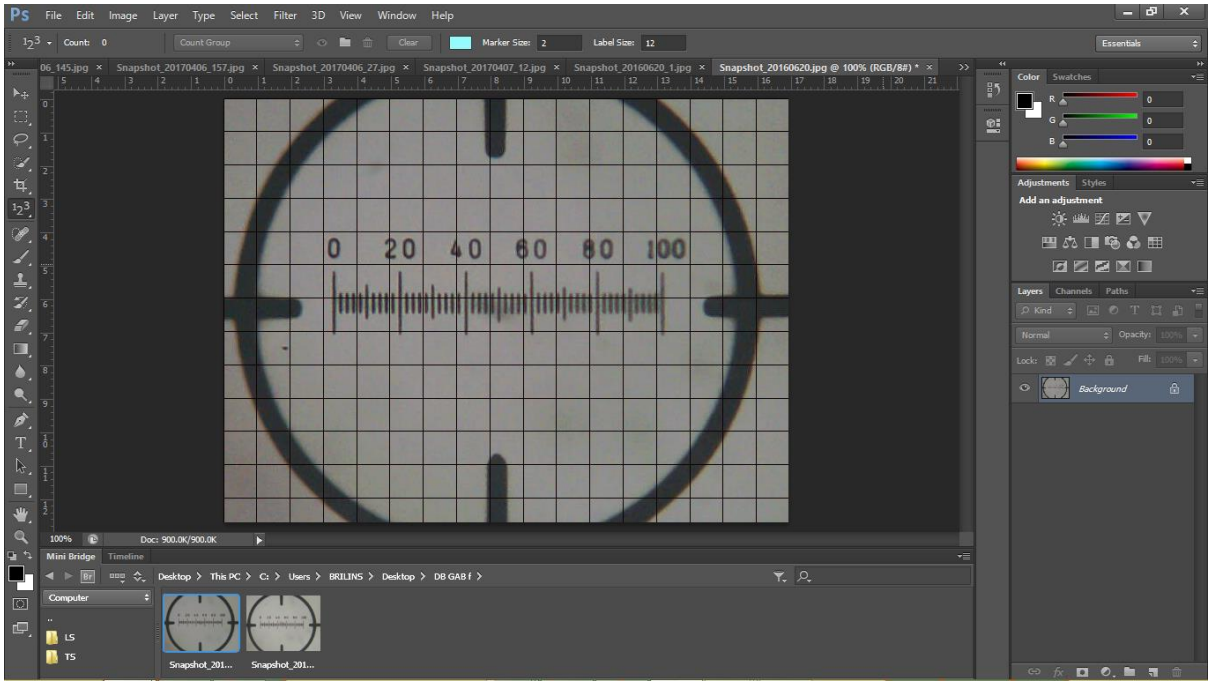

Figure 9: Photograph of a photomicrograph of microscope stage graticule superimposed on stereological lattice for calibration using Adobe photoshop. One graticule unit = 0.01mm

3.10.2 Stereological study of the brain tissue

A design-based stereological method was employed to measure volume density of cortical neurons in the cerebral cortex.

The Cavaliere's method with point counting (Dezfoolian et al., 2009) was employed to determine the Volume density of the neurons. A grid of dimension 1 cm × 1 cm in Adobe Photoshop CS6 (trial version 13.0.1) software was superimposed on each micrograph of the Cerebral cortex.

The volume density of cortical neurons was counted at the point of intersection of the grid lines.

The values from the point counting were entered into the formula (Cavalieri estimator of volume) below for calculation of volume density

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$$V = \frac{\Sigma P \times (\frac{a}{p}) \times t}{M^2}$$

Where V indicates volume of the cortical area in consideration, ΣP is the sum of all test points encountered, (a/p) is the area per point of the stereological grid, t is the thickness of the section and M is the linear magnification.

3.11 Statistical analysis

Statistical analysis was performed using Graphpad prism version 5.0. Descriptive data were summarised using means and standard deviations. Paired t-test was used to test for changes in outcome variables before and after the experiment for all groups. One-way analysis of variance (ANOVA) with Tukey's posthoc tests was used to compare the means of the outcome variables among the groups. Test for homogeneity of variance was done using Bartlett's test for equality of variance.

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CHAPTER FOUR

RESULTS

4.1 Experimental variables

4.1.1 Weight assessment

Table 1 shows the weight assessment of rats in the various groups in the experiment. At week 1, baseline of the experiment, there was significant difference ($p = 0.2713$) in the weights of the rats. The mean weights recorded were 140.0 (SD 27.4) g, 134.0 (SD 16.7) g, 162.0 (SD 21.6) g and 146.0 (23.0) g for G1, G2, G3 and G4 respectively.

At week 4, reduction of mean weight was seen in G2 and G4, but these reductions were statistically significant ($p<0.01$) in G2 only.

Also at week 5, the weights recorded for all the treatment groups, G1, G2, G3 were lower when compared to the control group, G4. However, only G2, the exercise only group showed a statistically significant difference ($p<0.01$). In the final week of the study, the trend from week 5 was maintained, with G2 showing statistical difference ($p < 0.05$).

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Table 1: Weekly weights of rats in grams during the 6 weeks of experiment. Values are means with standard deviation in bracket**.**

GROUPS → / WEEKS ↓	G1	G2	G3	G4	**P VALUE 1**
1	140.0 (27.4)	134.0 (16.7)	162.0 (21.6)	146.0 (23.0)	0.2713
2	157.1 (29.8)	145.7 (10.1)	164.7 (23.6)	159.0 (17.3)	0.5749
3	164.6 (30.6)	140.0 (7.0)	168.6 (22.8)	167.8 (17.5)	0.1495
4	194.4 (36.9)	121.8 (13.9)**	177.5 (25.2)	188.6 (16.9)[a]	0.0011
5	194.0 (31.8)	156.0 (19.4)**	214.0 (27.0)[a]	218.0 (21.6)[c]	0.0054
6	178.0 (33.4)	160.0 (20.0)*	226.0 (29.6)[b]	208.0 (21.6)[c]	0.039
P VALUE 2	0.0781	0.0064	0.0010	<0.0001	

Values are expressed as means (SD). P value 1 represent significance level for one-way ANOVA (followed by Tukey's post hoc) for between group comparison with *=P<0.05, **=P<0.01 and ***=P<0.001. P value 2 indicates significance level for one-way ANOVA (followed by Tukey's post hoc) for time course assessment within the groups with Alphabets indicating significant difference from week 1. c=P<0.001, b=P<0.01 and a=P<0.05. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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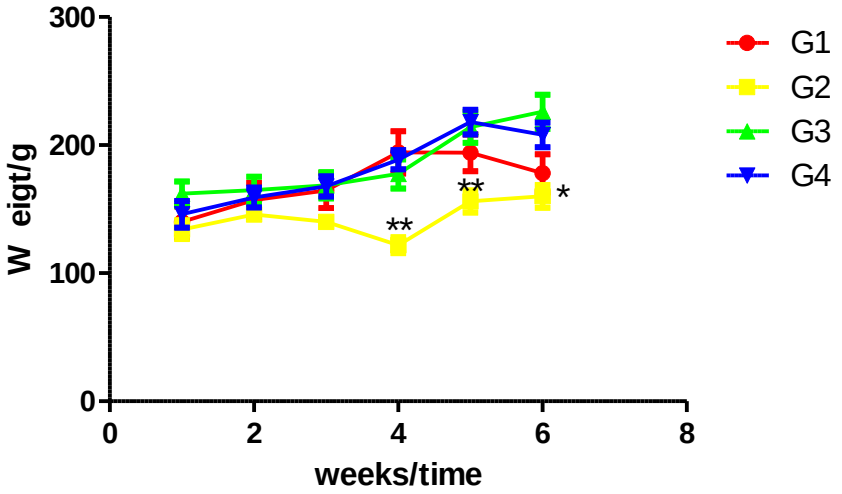

Figure 10: Line plot of weekly weight of rats during the experiment. Each point represents Mean weight of rats in the group for the specific week, error bars are standard deviation (SD). Probability values are for Tukey's post hoc comparisons with respective controls (G4). *P < 0.05, **P < 0.01, ***P < 0.001. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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4.1.2 Distance covered on the running wheel.

Exercise training done was recorded as distance ran by the rats on the running wheel in the course of the 6 weeks. Unpaired t-test revealed no significant difference between the two exercise groups G1 and G2. Time course assessment for intragroup analysis indicated no significant difference (p value 2 = 0.9300 and p value 2 = 0.9960 for G1 and G2 respectively) in the mean distance ran by rats.

It was however observed from the graph (Fig. 11), a steady increase in the distance covered by rats in G1, the exercise and Cocoa group. An alternate trend was observed in G2, as the mean distance covered by the rats reduced in week 2 of the study, increasing insignificantly from the 3^{rd} – 6^{th} week of the experimental period.

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Table 2: Average weekly distance ran on Running Wheel (Km) during the 6 weeks' experiment. Values are mean with standard deviation in bracket.

GROUPS →			**P VALUE 1**
WEEKS ↓	G1	G2	
1	1.0 (0.4)	1.2 (0.4)	0.2199
2	1.1 (0.4)	1.1 (0.5)	0.4487
3	1.1 (0.4)	1.1 (0.5)	0.4456
4	1.2 (0.4)	1.1 (0.4)	0.3774
5	1.3 (0.5)	1.1 (0.4)	0.3353
6	1.3 (0.5)	1.2 (0.4)	0.3513
P VALUE 2	0.9300	0.9960	

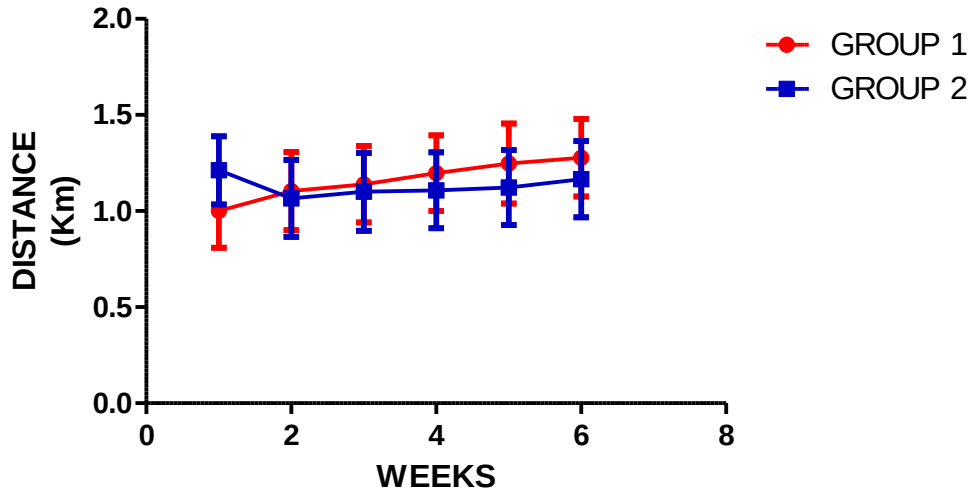

Figure 11: Line plot of weekly distance ran by rats during the experiment. Each point represents Mean distance ran by the rats in the group for the specific week. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group.

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4.1.3 Weight of Rat Brain

The Figure 12, reveals the analysis of mean weight of rat brains between the groups by one-way analysis of variance. The bar chart reveals no significant variations in the mean brain weights of the rats in the groups when compared to the control group (G4)

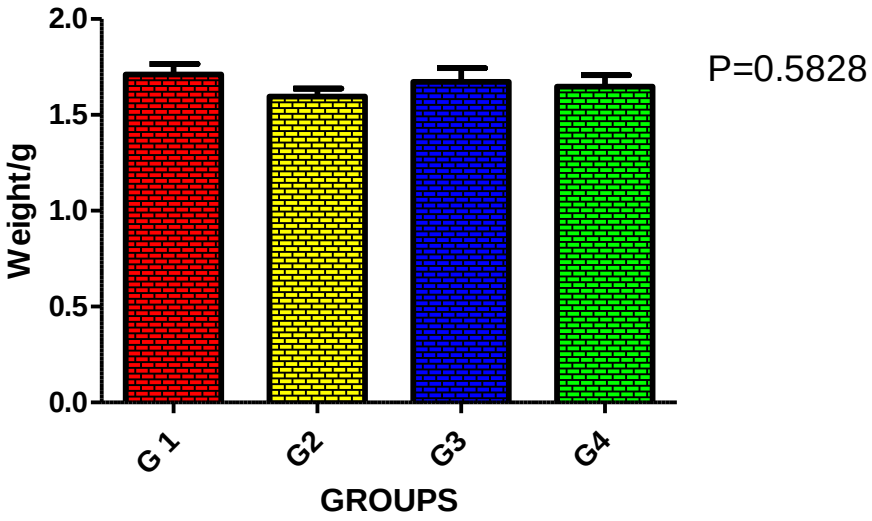

Figure 12: Bar chart of rat brain weights measured at the end of the experiment. Mean weight of rats brains in the groups, error bars are standard deviation (SD). G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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4.1.4 Fluid (Cocoa and Water) intake

One-way analysis of variance shown in Table 3 indicates a significant difference in the fluid consumption (cocoa and water) between the groups in the course of the six weeks' experimental period.

In comparison with G4 (control group), all the groups' intake of fluid differed significantly within each week. At the end of the week 1, the mean fluid intake recorded for the groups were 208.5ml, 187.7ml, 161.4ml and 210.0ml for G1, G2, G3 and G4 respectively. The fluid intake by G2, the Exercise only group, was lower when compared to the G4 group, nonetheless, significant reduction ($p = 0.001$) in fluid intake was only observed in the G3 group.

At week 2, there was significant increase ($p=0.05$) in the fluid intake in G1 when compared to G4 but significantly reduced ($p=0.01$) in G3 group. Significant increase ($p<0.001$) in the fluid intake was recorded in G1 and G2 groups at week 3. Also, the increase in the fluid intake in G3 was significant at $p<0.05$.

From week 4 to week 6, no significant increase in fluid intake was recorded for G1 and G3 when compared to G4 by one-way ANOVA. A significant increase in fluid intake by exercise working group (G2) was however statistically reduced ($p<0.001$ – $p<0.05$) from weeks four to six.

Time course analysis as revealed by the line graph in Figure 13, reveals a statistically significant increase in fluid consumption within the groups for the six weeks' duration. The p value recorded was less than 0.0001 for all groups.

From the graph Fig. 13, group 4 rats recorded significant increase in fluid consumption from weeks four to six with p values of $p<0.05$, <0.001 and <0.001 for weeks 4, 5 and 6 respectively when compared to week 1 by one-way analysis of variance. A similar trend was observed in the G3 however the values were only statistically significant at the end of week 5 and 6 at p

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values < 0.001. G1 and G2 recorded a significant increase in their fluid consumption from week 3 to 6 at $p<0.001$

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Table 3: Average weekly fluid intake in ml by rats during the 6 weeks' experiment. Values are recorded in means with standard deviations in bracket.

GROUPS ➡ WEEKS ⬇	G1	G2	G3	G4	P VALUE 1
1	208.5 (19.5)	185.7 (29.9)	161.4 (23.4)**	210.0 (11.5)	0.0010
2	242.8 (4.8)*	225.0 (17.5)[a]	188.5 (20.3)**	217.1 (11.1)	<0.0001
3	272.8 (22.1)***[c]	288.5 (8.9)***[c]	222.8 (16.0)*	227.1 (11.1)	<0.0001
4	347.1 (47.1)[c]	438.5 (26.7)***[c]	354.2 (75.2)	308.5 (57.2)[a]	0.0014
5	468.5 (8.9)[c]	498.5 (36.2)[c]	468.5 (33.8)[c]	457.1 (26.9)[c]	0.0683
6	490.0 (11.5)[c]	510.0 (11.5)*[c]	458.5 (40.1)[c]	410.0 (110.0)[c]	0.0231
P VALUE 2	<0.0001	<0.0001	<0.0001	<0.0001	

Values are represented as mean (SD). P-value (1) was obtained from one-way ANOVA test of mean fluid intake (ml) of groups G1, G2, G3 and G4 with * means p- value < 0.05; ** means p-value < 0.01. P-value (2) was from time course one-way ANOVA test of mean fluid intake (ml) within group. a=p<0.05, b =p<0.01 and c=p<0.001. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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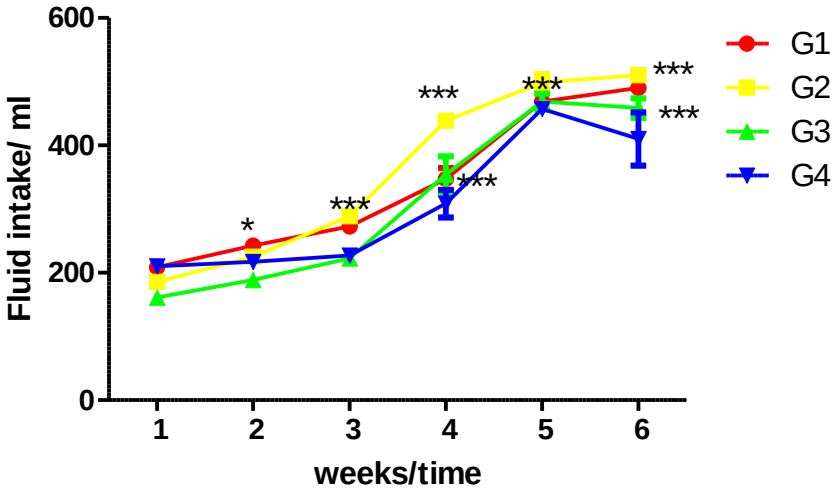

Figure 13: Line plot of weekly fluid intake by rats during the experiment. Each point represents Mean weight of rats in the group for the specific week, error bars are standard deviation (S.D). Probability values are for Tukey's post hoc comparisons with respective baseline (week 1). *P < 0.05, **P < 0.01, ***P < 0.001. G1 is Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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4.1.5 Antioxidant activity

4.1.5.1 Serum Superoxide dismutase (SOD) activity

Figure 14 shows an increase in serum SOD activity in all the groups at the end of the study. However, the change was statistically significant on in the G2 and G4 groups with p values 0.016 and 0.0091 respectively after paired T test.

Also, the one-way analysis of variance for between the groups assessment revealed no significant difference in the mean serum concentration of SOD for both pre and post treatment of SOD values between the groups. This is shown in Table 4.

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Table 4: Serum Superoxide dismutase (serum SOD) activity in U/ml. Means are recorded with standard deviation in brackets.

TIME	G1	G2	G3	G4	**P VALUE 1**
PRE TREATMENT	0.397 (0.3)	0.186 (0.0)	0.315 (0.0)	0.108 (0.0)	0.2080
POST TREATMENT	0.471 (0.1)	0.449 (0.0)*	0.402 (0.0)	0.343 (0.0)**	0.1672
P VALUE 2	0.7051	0.0160	0.1529	0.0091	

Values are expressed as mean (SD). P value 1 represent significance level for one-way ANOVA (followed by Tukey's post hoc) for between group comparison. P value 2 indicate significance level for Paired T-Test for time course assessment within the groups with alphabets indicate significant difference from week 0. ***=P<0.001, **=P<0.01 and *=P<0.05. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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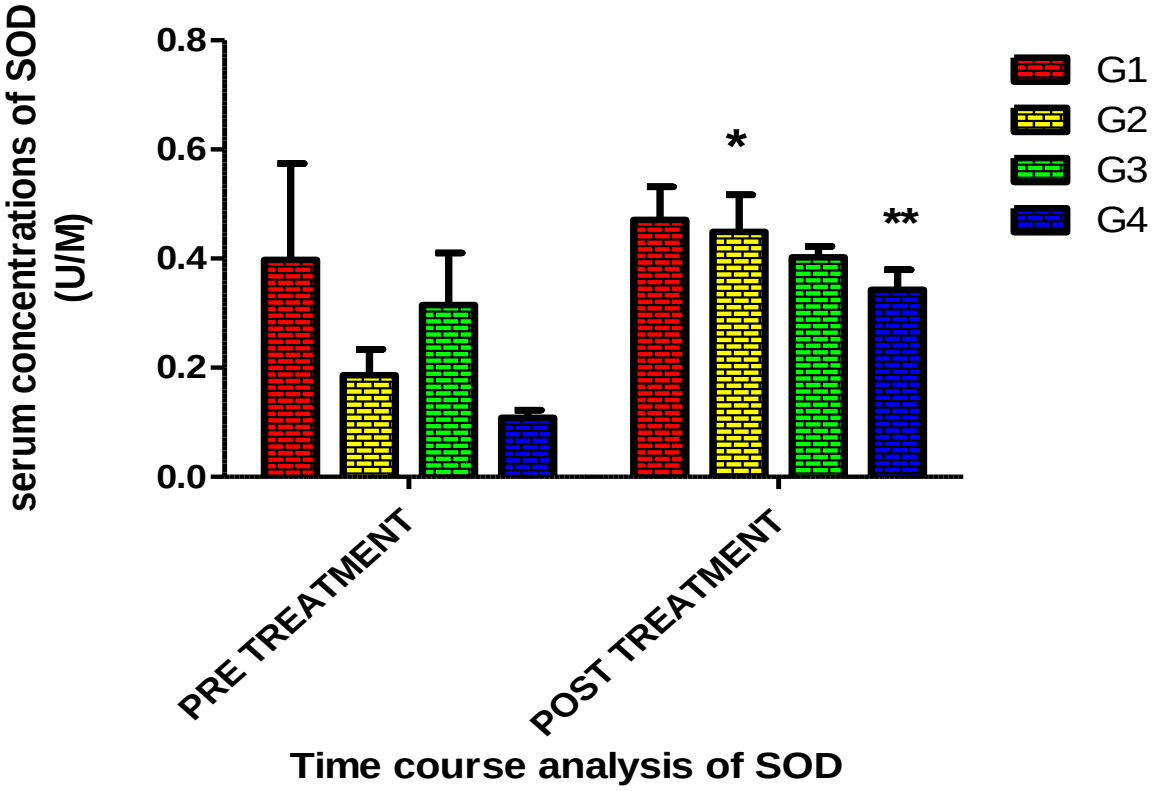

Figure 14: Bar chart of serum SOD before commencement and after the six weeks' experimentation. Each bar represents the mean value with S D as error bars. *P < 0.05, **P < 0.01, ***P < 0.001 compared to respective controls.

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4.1.5.2 Reduced glutathione (GSH)

Figure 15 showed that activity levels of GSH increased after 6 weeks' experimentation period in the various experimental groups, but one-way analysis of variance reported no significant difference among the groups.

However, paired t-test analysis revealed that post treatment value in G3, Cocoa only (3.39 (SD1.27) mmol/g) rats' serum concentration levels was significantly increased ($p = 0.0477$).

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Table 5: Serum levels of reduced glutathione (serum GSH) activity in mmol/g. Means are recorded with standard deviation in brackets.

TIME	G1	G2	G3	G4	P VALUE 1
PRE TREATMENT	2.01 (SD 1.71)	2.04 (SD 1.83)	2.41 (SD 0.77)	2.19 (SD 0.68)	0.9758
POST TREATMENT	3.82 (SD 1.15)	3.20 (SD 0.57)	3.39 (SD 1.27)*	2.80 (SD 0.67)	0.6416
P VALUE 2	0.3764	0.3978	0.0477	0.2111	

Values are expressed as mean (SD). P value 1 represent significance level for one-way ANOVA (followed by Tukey's post hoc) for between group comparison. P value 2 indicate significance level for Paired T-Test for time course assessment within the groups with alphabets indicate significant difference from week 0. ***=P<0.001, **=P<0.01 and *=P<0.05. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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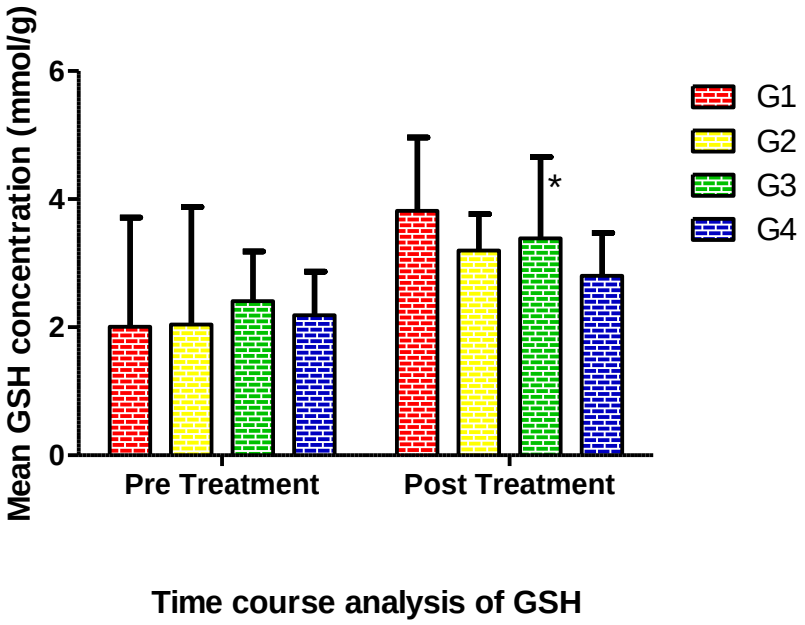

Figure 15: Bar chart of serum GSH before commencement and after the six weeks' experimentation in G1, 2 and 3. Each bar represents the mean value with S D as error bars. *P < 0.05, **P < 0.01, ***P < 0.001 compared to respective controls.

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4.1.6 Cerebral tissue homogenate content of BDNF

The mean BDNF concentrations obtained from cerebral tissue homogenates after the experiment were 106.3 (SD 13.77) pg/ml, 107.8 (SD 13.07) pg/ml, 99.7 (SD 12.28) pg/ml and 105.0 (SD 17.44) pg/ml respectively for G1, G2, G3 and G4. In comparison to the G4, the control group, BDNF values were found to be higher in G1 and G2 rats, although not statistically significant. The BDNF obtained from G3 rat cerebral tissue homogenate was lower, but not statistically significant when compared to the G4.

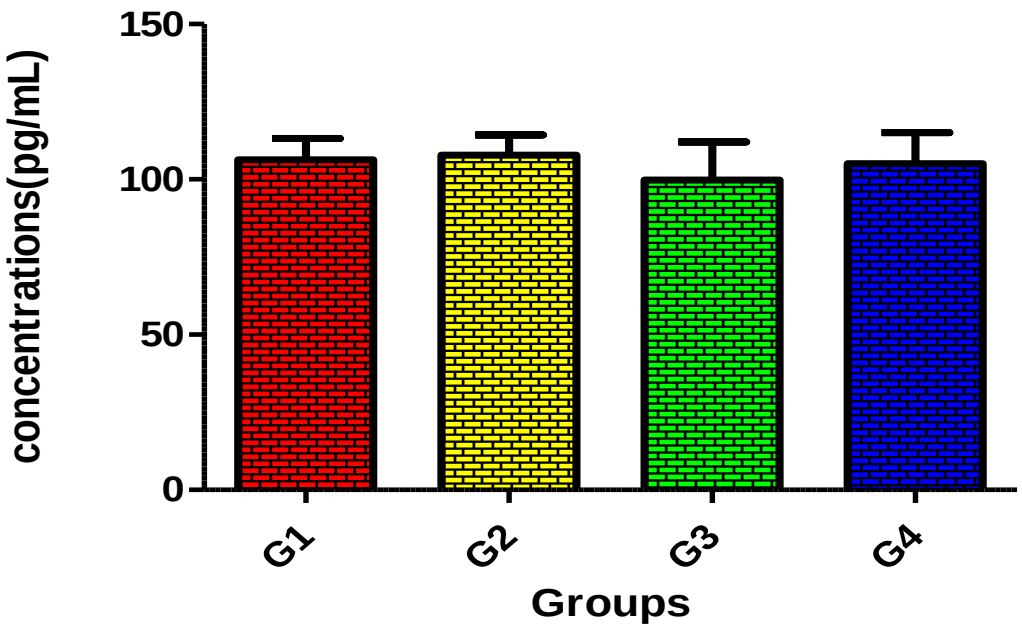

Figure 16: Bar chart of rat cerebral tissue homogenate content of BDNF measured at the end of the experiment. Each bar presents the mean concentration on BDNF in homogenate with errors bars as standard deviation. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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4.1.7 Concentration of BDNF in serum of experimental rats.

Table 6 shows statistical distribution and comparisons of BDNF concentration in serum of rats before commencement of various treatments and at the end of 6 weeks' experimentation period. One-way ANOVA of mean concentration for serum BDNF was not significantly different between the 4 rat groups ($p=0.9081$) at the beginning of the study. However, a significant difference ($p=0.0003$) was obtained for the mean serum BDNF between the groups such that, at 95% confidence level, there was significantly higher ($P<0.0001$) serum BDNF for rats in G1, G2 and G3 when compared to G4 at the end of six weeks' study.

Pre and post-treatment serum BDNF was not significant ($P>0.05$) in G4 rats, but significant increases were obtained for rats in G1, G2, and G3 by paired t-test.

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Table 6: Serum concentration in pg/ml of BDNF in rats pre and post treatment. Values are recorded in means with standard deviation in bracket.

GROUP	PRE-TREATMENT	POST-TREATMENT	P Value 2
G1	762.500 (149.30)	1292.500 (149.30)***	0.0006
G2	712.500 (82.61)	1272.500 (41.73)***	0.0004
G3	755.000 (147.08)	1262.250 (91.97)***	0.0124
G4	706.666 (127.41)	760.333 (172.92)	0.0965
P value 1	0.9081	0.0003	

Values are expressed as mean (SD). P value 1 represent significance level for one-way ANOVA (followed by Tukey's post hoc) for between group comparison. P value 2 indicate significance level for Paired T-Test for time course assessment within the groups with alphabets indicate significant difference from week 0. ***=P<0.001, **=P<0.01 and *=P<0.05. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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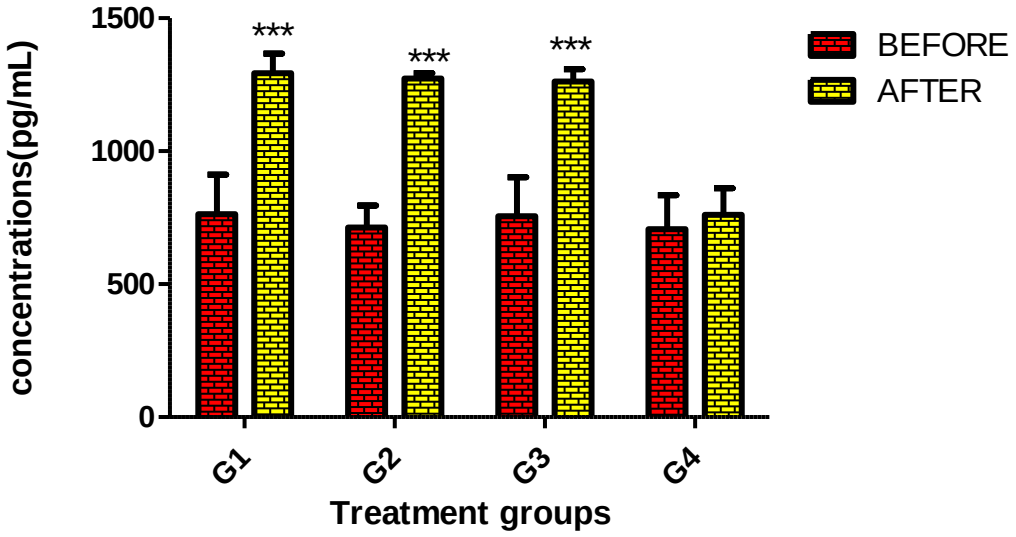

Figure 17: Bar chart of serum BDNF concentration in Rats before and after the 6-week experimentation period. Bars represent the mean values with SD as error bars *P < 0.05, **P < 0.01, ***P < 0.001 compared to respective controls.

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4.1.8 Serum concentrations of Interleukins-6 (IL-6)

Table 7 and Figure 18 show values and ANOVA statistics for interleukin 6 (IL6). One-way ANOVA between the groups revealed no significant difference (p=0.4764) for IL6 concentration before commencement of the experiment.

At the end of the study, a between the groups comparison showed a significant difference (P=0.0483) in the serum concentrations of IL-6 between the groups, G1, G2, G3 and G4.

Paired t-test indicated a significant increase in the IL6 in G1, G2, and G3 at p value of 0.0233, 0.010 and 0.010 respectively nonetheless no significant difference was observed in IL6 concentration after the experiment in G4 rats.

The quantum of change in the concentration of serum IL-6 of the treatment groups after the study when compared to the control was appreciably high. Serum IL-6 levels increased by 46%, 47% and 48% for G1, G2 and G3 respectively. However, a decrease by 5% was observed in the control group.

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Table 7: Serum IL-6 concentration values in pg/ml for rats studied, before commencement and after 6 weeks of treatment. Values are means with standard deviation in bracket.

GROUP	G1	G2	G3	G4	P value 1
PRE-TREATMENT	18.380 (1.5)	20.560 (1.8)	19.565 (2.6)	20.492 (2.4)	0.4764
POST-TREATMENT	33.963 (11.9)	38.643 (10.8)	37.943 (8.6)	19.570 (2.6)	0.0483
P Value 2	0.0233	0.010	0.010	0.428	

Values are expressed as mean (SD). P value 1 represent significance level for one-way ANOVA (followed by Tukey's post hoc) for between group comparison. P value 2 indicate significance level for Paired T-Test for time course assessment within the groups with alphabets indicate significant difference from week 1. ***=P<0.001, **=P<0.01 and *=P<0.05. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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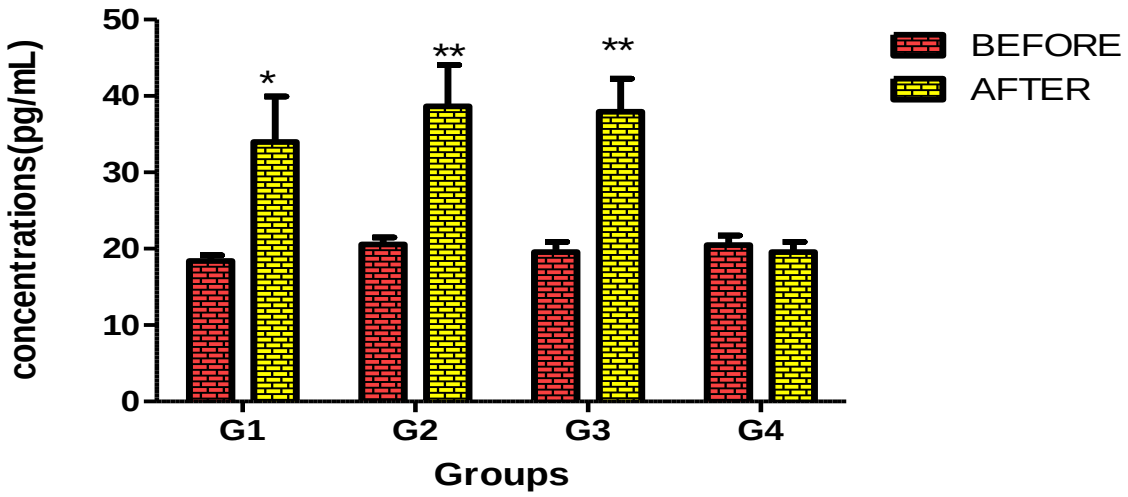

Figure 18: Bar chart of serum IL-6 activity in Rats. Each bar represents the mean with standard deviation (SD) as error bars. *P < 0.05, **P < 0.01, ***P < 0.001 compared to respective controls.

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4.1.9 Histomorphometric assessment of structural changes in neurons in the cerebral cortex

Table 8 represents a comparison analysis of the mean relative volume density (VD) of cortical cells between the groups by one-way analysis of variance.

Figure 20 shows there was a significantly lower ($p = 0.0002$) volume density of damaged pyramidal cells between the groups. The volume density of damaged pyramidal cells in G1, G2 and G3 when compared to G4 were significantly lower at P values of <0.05, < 0.01 and <0.001 respectively.

One-way ANOVA between the groups for volume density of undamaged pyramidal and granular (undamaged and damaged) recorded no significant difference between the rat groups (G1, G2 and G3) when compared to the control group (G4).

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Table 8: Morphometric values in Relative volume density ($\times 10^3 \mu m^3$) of cortical cells of the experimental groups. Values are expressed as mean with standard deviation in bracket.

Parameters (Neurons and Neuroglia)	G1 (Exercise training+ Cocoa)	G2 (Exercise Training only)	G3 (Cocoa only)	G4 (Control)	*p*-value
Undamaged pyramidal ($\times 10^3 \mu m^3$)	0.744 (SD 0.109)	0.675 (SD 0.131)	0.799 (SD 0.036)	0.625 (SD 0.034)	0.0738
damaged Pyramidal ($\times 10^3 \mu m^3$)	0.111 (SD 0.029)*	0.085 (SD 0.017)**	0.046 (SD 0.010)***	0.187 (SD 0.049)	0.0002
Undamaged granular ($\times 10^3 \mu m^3$)	0.854 (SD 0.080)	0.767 (SD 0.117)	0.9698 (SD 0.074)	0.929 (SD 0.2523)	0.2815
Damaged granular ($\times 10^3 \mu m^3$)	0.074 (SD 0.023)*	0.048 (SD 0.018)**	0.043 (SD 0.007)**	0.125 (SD 0.033)	0.0008

Values are expressed as means (SD). P values represent significant level of one-way ANOVA followed by Tukey's post hoc for between group comparison, with *=P<0.05, **=P<0.01 and ***=P<0.001. G1 is the Exercise and Cocoa intake group; G2 is the Exercise only group; G3 is the Cocoa intake only group and G4 is the control group (no exercise, no cocoa).

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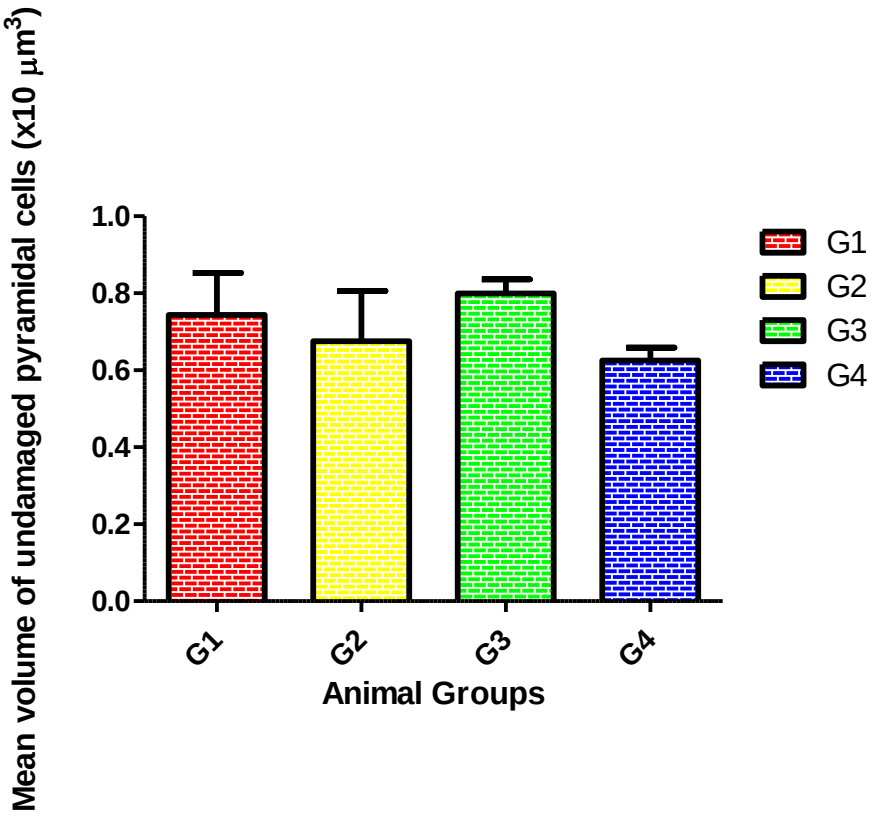

Figure 19: Bar chart of relative volume density of undamaged pyramidal neurons in μm^3 for between group comparisons. Error bars represents standard deviation (S.D). *P < 0.05, **P < 0.01, ***P < 0.001 compared to G4.

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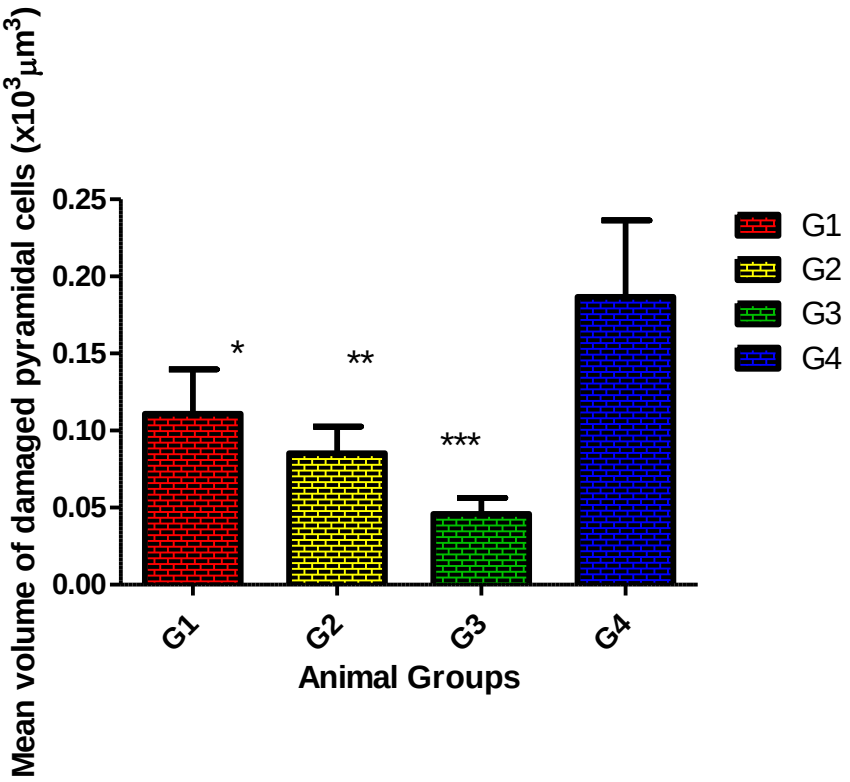

Figure 20: Bar chart of relative volume density of damaged pyramidal neurons in µm3 for between group comparisons. Error bars represents standard deviation (S.D). *P < 0.05, **P < 0.01, ***P < 0.001 compared to G4.

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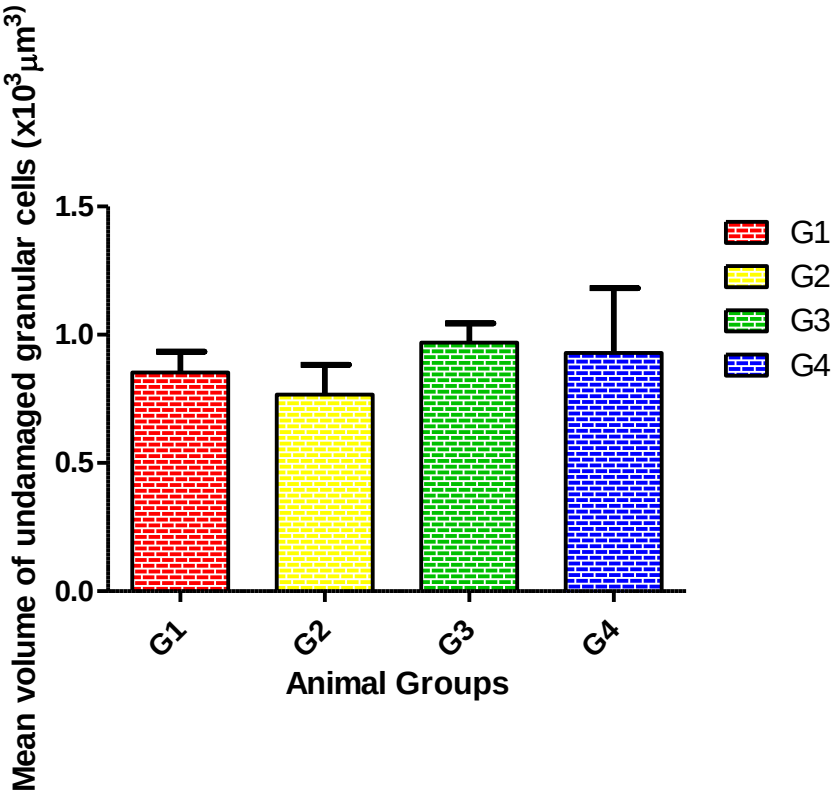

Figure 21: Bar chart of relative volume density of undamaged granular neurons in μm^3 for between group comparisons. Error bars represents standard deviation (S.D) *P < 0.05, **P < 0.01, ***P < 0.001 compared to G4.

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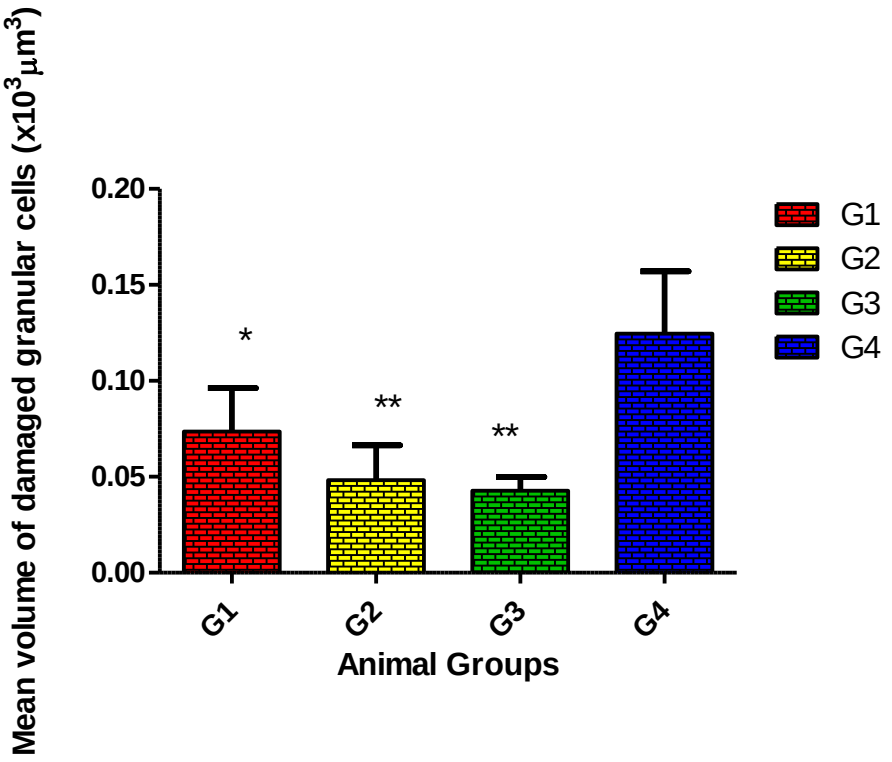

Figure 22: Bar chart of relative volume density of damaged granular neurons in μm^3 for between group comparisons. Error bars represents standard deviation (S.D). *P < 0.05, **P < 0.01, ***P < 0.001 compared to G4.

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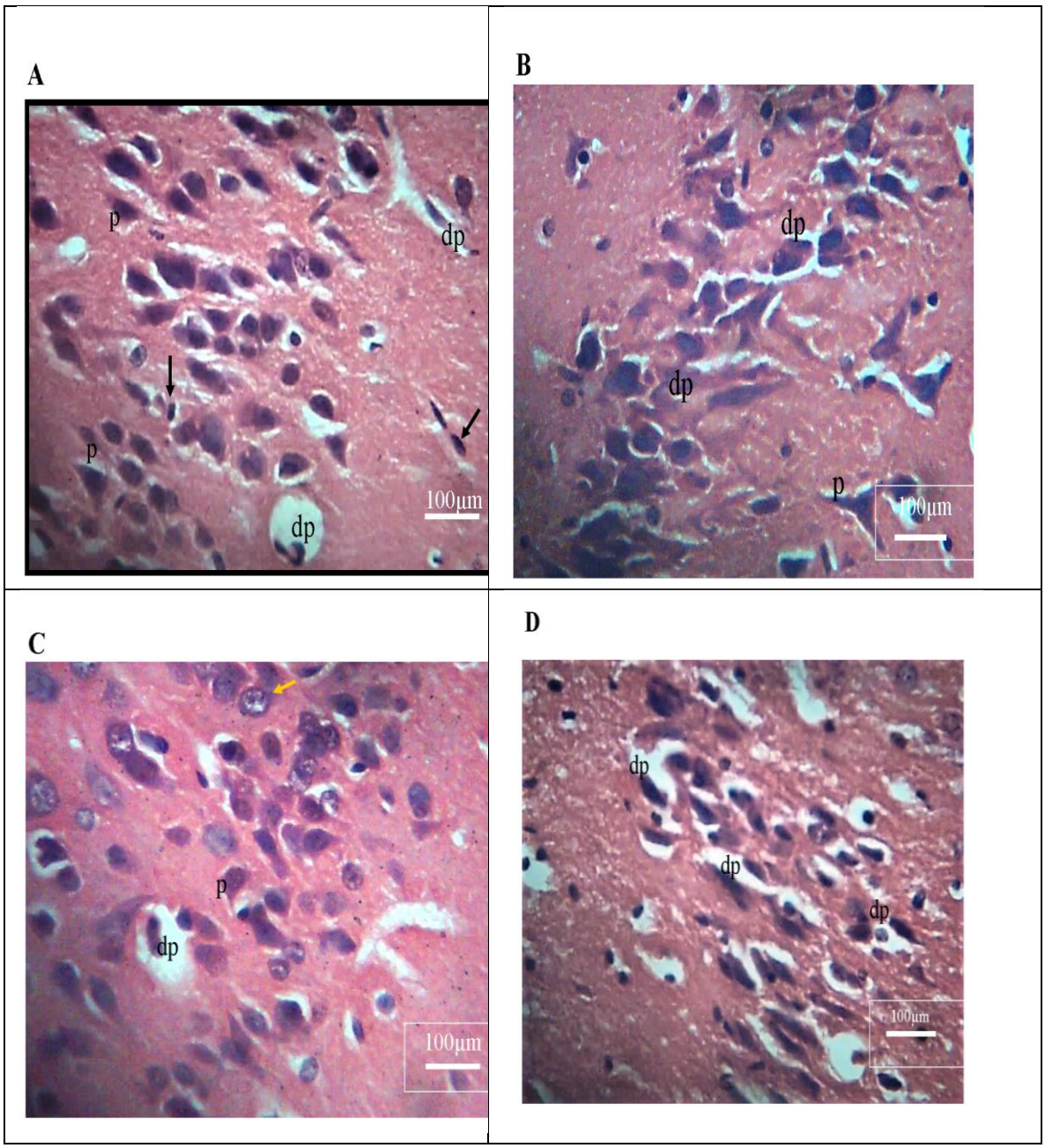

Figure 23: Photomicrographs of sections showing mostly pyramidal neurons in the cerebral cortex (H & E x 40). **(A)** of a rat in G1 (exercise + cocoa) group showing multiple pyramidal cells (p). Note few glial cells (black arrow) in neuropil. Few damage pyramidal (dp) characterized by acidophilic masses exhibited by dark nuclei surrounded by clear space, shrunken size, dense cytoplasm and dark pyknotic nuclei. **(B)** of a rat in G2 (exercise only) group showing multiple pyramidal cells (p). Note a number of damaged pyramidal cells (dp) characterized by shrunken size and pyknotic pyramidal neurons. **(C)** of a rat in G3 (cocoa only) group showing multiple undamaged pyramidal cells (p) and a few granular cells (yellow arrow). **(D)** of a rat in G4 (control) group, showing multiple damaged pyramidal cells (dp), exhibiting dark nuclei and surrounded by clear space.

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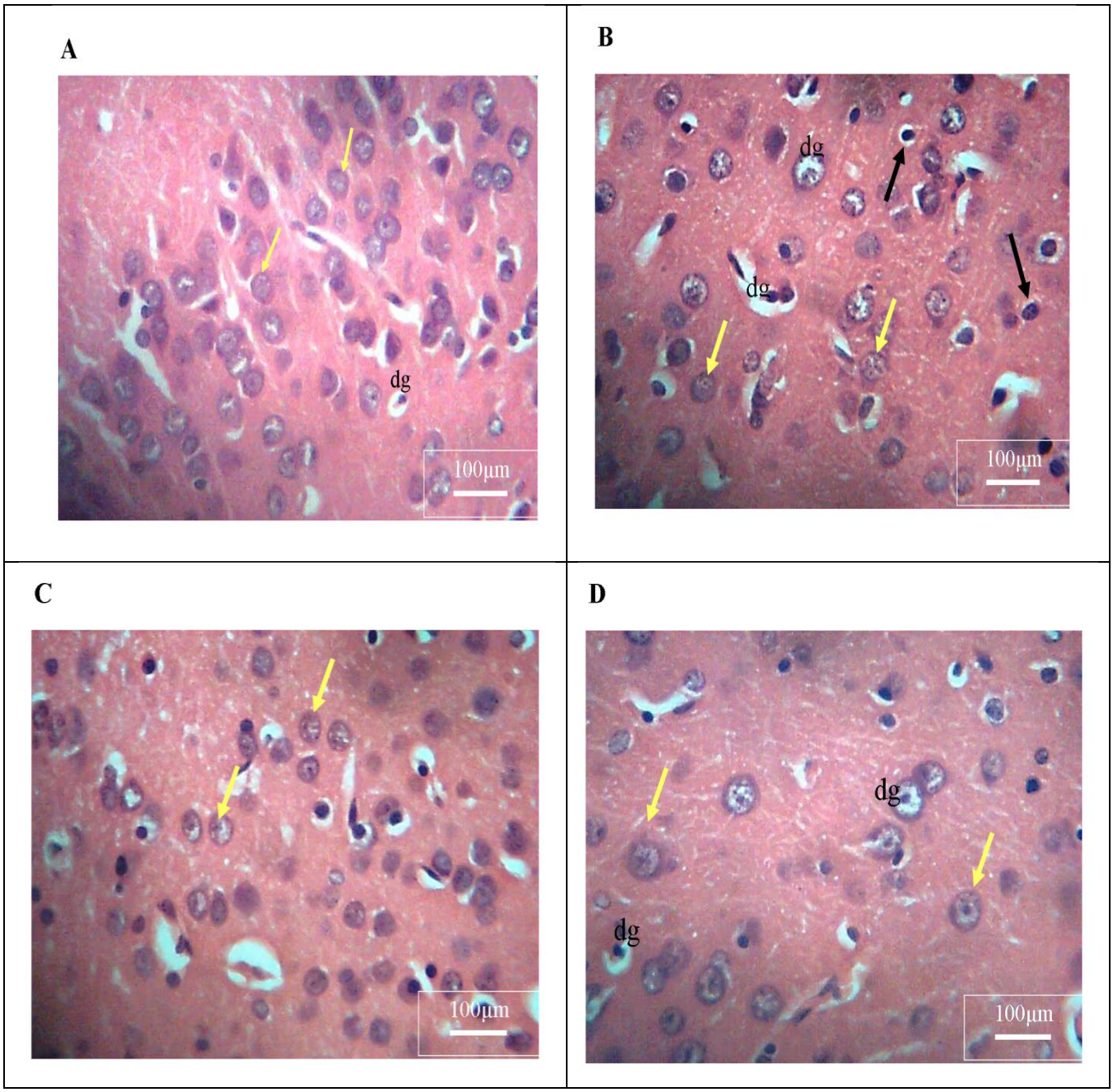

Figure 24: Photomicrographs of sections showing mostly granular neurons in the cerebral cortex. **(A)** of rats in G1 (exercise + cocoa) group showing multiple granular neurons (yellow arrow) and very few damaged granular neurons (dg) exhibiting dark nuclei surrounded by clear space. **(B)** of rats in G2 (exercise only) showing multiple granular neurons (yellow arrow) which are vacuolated. Note few glial cells (black arrow) within the neuropil. **(C)** of rats in G3 (cocoa only) group showing multiple undamaged granular neurons (yellow arrow). **(D)** of rats in G4 (control) group showing some undamaged granular neurons (yellow arrow) as well as damaged granular neurons exhibiting dark nuclei surrounded by clear space (dg)**.**

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CHAPTER FIVE

DISCUSSION

5.1 Introduction

The health benefits of Cocoa and exercise training have been investigated separately and independently by many studies. These benefits have generally been ascribed to their promotive activity on the neurotrophic factors responsible for promoting a healthy brain. However, the combined and possible potentiating activity of cocoa and exercise on key indicators of brain health has apparently never been investigated until the present study. This thesis reports the combined effects of six weeks' intake of natural cocoa as an aqueous drink and concurrent six weeks of regular exercise on some selected markers of brain health in rats. It was expected that combining cocoa intake and regular exercise in the form of wheel running would have potentiating benefits on the selected markers, as both cocoa and exercise have been implicated in promoting brain health (Camandola, 2017; Nehlig, 2013).

The primary and distinctive findings of this study are that Cocoa in combination with exercise training produces a profound potential effects on the selected markers of brain health under study.

5.2 Rat weight assessment

The impetus of this assessment was to study the weight change in the animals during the study. At the beginning of the study, week 1, the weights of the animals showed no differences, significant to affect the aim and objectives of the study.

As the study proceeded, in week 4, a marked decrease was observed in the weight of the animals in G2, the Exercise only group as well as G4, the control group. This decrease was however significant in the G2 group. In the last 2 weeks of the study, a general weight loss was

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observed in all the groups except G3, the Cocoa only group. This group showed a steady increase in weight throughout the study, suggesting that calorific property of cocoa.

A few studies have shown that regular levels of exercise training are consistently characterized by weight loss (El, Lac, Tabka, Gharbi, & El, 2011; Riyahi, 2019; Trottier et al., 2017).

The significant decrease in the body weight may be explained as a change in body composition by reducing fat mass (El et al., 2011), or alternatively, an increase in physical activity via exercise training participation causes an increase in energy expenditure which result in metabolically induced changes such as reduced adiposity and consequential weight loss (Garland et al., 2011).

Also, noteworthy was that, animals in G1 also participated in wheel running exercise training as G2, but had 12-hour access to NCP suspension during the period of the study. There was no significant change or decrease in their weight as seen in G2. This observation may suggest that even though exercise training is a high energy expenditure activity, cocoa has the ability to balance the calories lost thereby maintaining equilibrium in energy expenditure.

Cocoa, is also known to be well endowed with about 24%- 26% palmitic acid, a fatty acid component known to raise good blood cholesterol levels when consumed (Steinberg, Bearden & Keen, 2003).

5.3 Running distance

Running in a wheel has been used widely to study exercise behaviour of rats in several studies (Durrant et al., 2009; Ke et al., 2011; Naghshvarian, Zarrindast, & Sajjadi, 2017). In present study, there was no significant difference between the two groups, G1 (exercise + cocoa) group and G2 (exercise only) subjected to exercise training. A steady increase in the distance covered by rats in G1, the exercise and Cocoa group suggests in part that cocoa may have had a boosting effect on the exercise activity as a slightly reverse trend was observed in G2, the exercise only group.

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Even though running on the wheel seems to satisfy playing, escaping and certain metabolic related drives in rats (Manzanares & Gandra, 2019), and hence the possible adherence to the training protocol, the observed difference may be attributed to the cocoa intake by G1. This assertion is supported by human studies that found improved stamina in free-living footballers in Holland (Fraga et al., 2005). Relevant in this context is a study which showed that (−)-epicatechin, a flavonoid found in cocoa, was associated with increased capillary activity as well as mitochondrial biogenesis in the muscles of rats (Lee, Hüttemann, Kruger, Bollig-fischer, & Malek, 2015). The authors explained that cocoa independently increased the rate of mitochondrial respiration, thus boosting energy levels required for the activity. This could be a possible explanation for the activity spurt in the rats in the present study that had the exercise training and took cocoa as well.

5.4 Fluid consumption

Fluid consumption in all the groups increased significantly over the 6-week study period. This goes to point out that both exercise training and cocoa intake are directly or indirectly involved in increased fluid consumption.

The concept that thirst provides an adequate stimulus to maintain proper hydration during exercise training is a plausible explanation for increased fluid consumption in the exercise groups. Thirst is precisely regulated mechanism that protects plasma osmolality and circulating blood volume (Hoffman, 2013).

The increase in fluid consumption ran across all the groups including the control group, G4. This is to say normal growth over the period of the study increased the capacity of the rats to consume more fluids.

Another favourable explanation, may be angled from the chemical properties of cocoa and their notable physiological effects. Theobromine is an abundant methyl xanthine in cocoa well explored for its health promoting functions as a diuretic (Franco, Oñatibia-astibia, & Martínez-

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pinilla, 2013). When the body loses fluid via urine excretion, homeostasis is maintained by fervent replacement of the fluid, hence the increase in consumption.

5.5 Antioxidant activity

5.5.1 Superoxide dismutase activity (SOD) and Glutathione (GSH)

The results of this study provides an insight into the mechanisms that may contribute to the influence of exercise training in the form of running and cocoa intake on oxidative stress, a key determinant of brain health.

One possibility is that, exercise (Marosi et al., 2012) and cocoa (Ishaq & Jafri, 2017) increase the expression and the activity of SOD, an enzyme that reacts with and reduces the bioavailability of superoxide anion. In the present study, although there was an increase in the SOD activity in all the groups except control, affirming the antioxidant stimulation by exercise and cocoa, a combination of both exercise and cocoa did not have significant influence of SOD activity, even though their SOD activity was higher when compared to the control group. The increase of SOD activity was however significantly pronounced in the group that participated in only exercise (G2), suggesting the theory that exercise alone is a better stimulant of endogenous production of antioxidants (Franzoni et al., 2017; Marosi et al., 2012; Simioni et al., 2018). These findings were consistent with other findings in rodents (Durrant et al., 2009; Lawler, Kwak, Kim, & Suk, 2019) and some human studies (Barreiro, Rabinovich, Barbera, Gea, & Roca, 2008; Berzosa, Cebri, Piedrafita, & Mart, 2011).

A study by Durrant and others, implicated increases in SOD activity as a key mechanism by which exercise training, in this case, voluntary running regulates oxidative stress (Durrant et al., 2009). Another study with comparable findings, in healthy human subjects indicated that, exercise causes a significant augmentation of several antioxidant scavengers including SOD (Berzosa et al., 2011). It is possible that the lower serum antioxidant in exercise and cocoa

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group arose from excess oxidants arising from the trend of increasing distance run by rats in this group compared to decreasing distance in the exercise only group.

Glutathione is one of the primary components of the physiological antioxidant defence system and non-enzymatic antioxidant that reduces oxidative stress by scavenging oxygen radicals (Kaur et al., 2011).

The results of the present study revealed that exercise training and/or cocoa increases the levels of GSH but basal increases after the study was considered significant in G3, the group that consumed only cocoa. This appears to confirm the antioxidant activity of Cocoa.

Tomofuji et al (2009) in a study considering the effects of consuming rich diet on periodontitis-induced oxidative stress, found that cocoa-enriched diet increased the activity of reduced glutathione in the rat model. Contrary, another study considering another participant set, hypercholesterolemic rats, found out that consuming cocoa rich diet didn't have any apparent effect on the glutathione levels (Lecumberri et al., 2007). The latter work however measured glutathione levels in the liver of the rats and not serum, which may. account for their contrary finding to that in present study. In a more recent study involving healthy human subjects, daily consumption of cocoa for 4 weeks increased baseline measures of glutathione in serum (Davinelli et al., 2018). The authors of the study buttressed the claim of cocoa as an antioxidant, similar to the findings of this present study.

Taken together, these findings provide evidence that increases in SOD and GSH, determinants of brain health, may be a key mechanism by which habitual exercise training and regular consumption of cocoa may protect against oxidative stress and preserve the integrity of the brain.

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5.6 Anti-inflammatory activity

5.6.1 Interleukins-6 (IL-6)

After six weeks of either exercise training, or consuming cocoa or both, the levels of IL-6 increased as much as a two-fold from the baseline values. Several studies have exerted close relationship between exercise and circulating concentrations of this cytokine. In humans, some have shown that IL-6 increased in response to exposure to exercise (Pedersen, 2017; Petersen & Pedersen, 2019), revealing remarkable anti-inflammatory augmentation, which is in sync with the findings of the present study. However, some studies have reported contrary findings to that reported presently (Mohamadzadeh, Ali, Yusof, & Dehghan, 2016; Pablo & Trapero, 2019). It was noted that the training type and participants varied from our study.

Furthermore, the anti-inflammatory effects of IL-6 have been explained by its stimulation of other circulatory anti-inflammatory cytokines such as IL-1ra and IL-10, and consequent inhibition of the production of the proinflammatory cytokine TNF- α (Petersen & Pedersen, 2019).

This study therefore suggests that myokine (Pedersen et al., 2003), produced by contracting skeletal muscles may be involved in mediating the health beneficial effects of exercise and particularly involved in the protection of the body and brain thus promoting health.

The effects of cocoa on interleukins-6 (IL-6) is scanty in literature. Also whether cocoa invokes the proinflammatory or anti-inflammatory properties of IL-6 is still on the research debate table. In present study, the animals which consumed cocoa and did not exercise showed marked increase in the levels of IL-6, like the animals in the exercise groups. This probably suggests that cocoa has anti-inflammatory effects on IL-6.

A significant proportion of the flavanol content of fresh cocoa contains large molecular weight flavanol oligomers known as procyanidins. These exhibit biological actions in model systems including actions on increased cytokine production and metabolism, of which IL-6 forms part (Selmi, Mao, Keen, Schmitz, & Gershwin, 2006).

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The outcome of the combined effects of exercise training and cocoa consumption compared to the control group was not surprising as it showed similar anti-inflammatory trends.

These findings have important ramifications as they imply that exercise training and consumption of cocoa have anti-inflammatory effects by increasing levels of interleukin-6.

5.7 Brain neurotrophic factors

5.7.1 Brain derived neurotrophic factor (BDNF)

Several studies have reported exercise increases BDNF. This study sought to determine whether cocoa would potentiate the effect of exercise on the BDNF levels both in serum and the cerebral tissues.

Many clinical and experimental findings have shown that exercise enhances neurogenesis, modulates the release of neurotransmitters, reduces cell death as well as increasing brain plasticity. Most of these studies have postulated that elevated BDNF activity accounts for these changes (Ke et al., 2011; Maria, Marquez, Vanaudenaerde, Troosters, & Wenderoth, 2019; Sleiman et al., 2016).

In this study, comparatively higher BDNF levels in the cerebral tissues was detected in rats subjected to both exercise training and cocoa intake as well as the group that partook in the exercise only.

Based on the fact that BDNF has been confirmed by numerous studies to increase with intake of cocoa and its derivatives (Cimini et al., 2013; Grassi et al., 2015; Nehlig, 2013), it was expected that the cerebral content of BDNF in the groups that consumed the cocoa only would be higher than the controls. However, the results did not show this expected effect. It could be speculated that even though cocoa induced elevated levels of BDNF, the bioavailability and penetrations of the cocoa compounds into the brain was not enough to exert an elevated change.

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Serum concentration of BDNF found in this study was almost a 100 fold more when compared with the cortical tissue content of the BDNF in control rats. In this study, a marked significant increase was reported in all the treatment groups when compared to control group suggesting that exercise training and cocoa intake did promote brain health via the BDNF related mechanisms marking them as possible interventions for clinical application in neurologically related conditions.

It was observed in this work that a combination of exercise training and cocoa consumption provides greatest effects on serum BDNF production, suggesting that even though participating in exercise alone or consuming cocoa alone gives beneficial increase in BDNF, a combination would be of more interest.

Both exercise and cocoa are known to induce CREB activation in cortical neurons, a transcription factor that binds to several genes involved in synapse remodelling, synaptic plasticity as memory (Islam et al., 2019; Maria et al., 2019). BDNF acts as a receptor that regulates calcium/calmodulin-dependent protein kinase II (CaMKII) as well as extracellular signal-regulated kinase (ERK) systems (Spencer et al., 2009). Flavanols might activate similar signalling pathways, raising the possibility that the natural compounds in cocoa may have selective neuronal receptors as well (van Praag, 2009)

Also, increasing evidence from animal models show that flavonoids can promote cognitive benefits (Cimini et al., 2013; Magrone et al., 2017; Nehlig, 2013) through their ability to directly interact with the cellular and molecular architecture involved in memory and cognitive function, of which BDNF production forms a crucial part (Islam et al., 2019).

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5.8 Histomorphometric changes in structure of neurons of the cerebral cortex

Changes in the morphology of the brain seem to be responsible for certain characteristics of brain health such as learning and memory, reduced cognitive decline related to ageing and improvement in symptoms of neurodegenerative diseases (Camandola, 2017).

In the present study, the cellular anatomy of the cerebral cortex in the various treatment groups with exercise and/or cocoa were dominated by large neurons characterized by the distinct pyramidal or granular appearance of their soma. On histological examination with H&E staining, microarchitectural indicators of damage in the neurons of the cerebral cortex of the animals included previously described signs of early stages of apoptosis (Naqi, 2015), karyolysis (dissolution of the cell nucleus with loss of its affinity for basic stains sometimes occurring normally but usually in necrosis) and pyknosis (an irreversible condensation of chromatin and the nucleus, occurring in apoptotic and necrotic cell death). The injurious changes were also characterized by single cells or small clusters of cells around the degenerating neurons in the various treatment groups, making the cells appear round or oval mass, with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments as has been described by (Garman, 2011). Furthermore, cryptic changes seen in the soma of the neurons also suggested cell damage, in which cell shrinkage made the cells smaller in size, with dense cytoplasm with a number of vacuolated cells, dark pyknotic neurons (Julho et al., 2017).

The observation on the neuronal morphology in the cerebral cortex region in this study, suggests appropriate and normal functionality in these animals, since from the functional point of view, these neurons are the most important cells of the brain (Desesso, 2014). This observation could be attributed to the fact the all the rats recruited for this study were healthy. Also, the treatment interventions, exercise training and cocoa intake as discussed are both health promoting especially to the brain.

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Though not of statistically significant, the volume densities of the undamaged pyramidal neurons in G1 (Exercise Training-Cocoa) and G3 (Cocoa only) were higher when compared with G2 (Exercise only) and the control group. It may be hypothesized from this observation that cocoa in this group provided additional protective effect on these neurons.

Besides, as discussed above, cocoa stimulates the production of BDNF, a neurotrophin, well praised of its abilities to protect neurons in the brain and consequently promoting brain health. This speculation is backed by some studies with similar findings (Cimini et al., 2013; Nehlig, 2013a; Spencer et al., 2009).

Interestingly, significant difference was observed in the volume densities of damaged granular neurons. Animals in group 4 (Control) showed more damaged granular neurons expressed in volume densities than all the treatment groups. This finding supports the brain health promoting indications of exercise training and cocoa. Some studies have also deduced that an interplay of the factors of oxidative stress and inflammation stimulate an inherent protection of the brain, causing a release of enzymes and growth factors such as BDNF, VEGF (Speisman et al., 2013) and IGF(Gomez-Pinilla et al., 2008; Kohman et al., 2012), neurotrophins implicated in protecting neurons.

5.9 Summary of key findings

Additive effects of exercise training and cocoa was observed in the BDNF content in cerebral tissue. The serum levels of BDNF also displayed marked increase in all treatment groups, highlighting its possible mechanism for enhancing brain health. Six weeks of experimental protocol involving regular exercise and daily administration of cocoa caused microachitectural changes in the neurons of the cerebral cortex. Histologically, volume density of damaged neurons was higher in the control group (that did not exercise or drink cocoa). Other markers of health such as serum SOD and GSH activities showed significant changes in their basal levels after treatment (within group), even though no the significant differences were obtained

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between the treatment groups. Doubling of basal levels of IL-6 was observed with Exercise training and/or cocoa consumption, suggesting higher anti-inflammatory augmentation, by these treatments whether used individually or in combination.

5.10 Conclusion

The present study in part affirmed that Exercise training and cocoa intake additively have a positive effect on selected markers of brain health. This research reinforces the postulate that regular exercise in combination with naturally occurring polyphenols in the form of cocoa, enhances neuroprotection and promotes brain health.

5.11 Limitation of the study

1. The FITT (frequency, intensity, time and type) principle of exercise training could not be fully adhered to as the running wheel for the rat had no provision for the intensity of the exercise to be controlled.

5.12 Recommendation

1. More detailed studies of the neurons should be done, with better staining strategies and microscopic work.

2. A more modern stereological software should be considered for stereological analysis, to save time and improve reproducibility.

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APPENDICES

APPENDIX I

EXERCISE TRAINING PROTOCOL

Pre-training exercise protocol

1. 10 rats (G1 and G2) were exposed to the running wheel for 6 days.
2. In order to discourage the rats from getting off the wheel and to maximize the time spent on the wheel, the wheel was elevated to 40cm above the floor of the cage.
3. In order to reduce stress and improve endurance, the running time on the wheel was increased progressively over the 6-day period

   DAY 1-2 – rats given access to the wheel for 20 minutes

   DAY 3-4 – rats given access to the wheel for 40 minutes

   DAY 5-6 – rats given access to the wheel for 60 minutes
4. Rats were incentivized after each pre-training session

Exercise pre-training in session

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Exercise training protocol for experimental period

1. Carried out for 60 minutes, 5 days per week for 6 weeks.
2. Tachometer was used to record the number of revolutions made on the wheel.
3. Count down timer was used to record the time spent on the wheel.
4. Each rat was rewarded with sugar free biscuit at the end of each training session

Exercise training in session.

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APPENDIX II

Tail snipping and milking process

Materials

Rodent handling gloves, towel, cotton, collection tube and glass chamber, scalpel.

1. The animal was placed in a glass chamber with cotton dubbed with diethyl ether to make them unconscious
2. Local aesthetic was applied to the surface of the tail 30 minutes before the experiment
3. A cut of 0.5 cm was cut from the tip of the tail with a scalpel blade.
4. The tail was then allowed to hung and placed in collection tube to collect blood. The tail was hung to allow gravity facilitate blood flow.
5. After collection, pressure was applied to the tail to stop blood flow.

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APPENDIX III

Protocol for removal of rat brain

Method

1. Rat was deeply anaesthetized and perfusion done.
2. Cervical dislocation was done
3. A surgical scissor to remove the head with a cut posterior from the ears. Using the scissors, a midline incision in the skin was made.
4. The skin over the eyes was flipped to free the skull.
5. A small incision was made on the top of the skull starting from the caudal part of parietal bone, being careful not to cut through the brain.
6. A firm cut is then made through the most anterior part of the skull between the eyes.
7. With the use of the forceps, the parietal bone is broken off from both sides of the skull.
8. The brain was freed from meninges and the forceps were used to break the optic nerve and other cranial nerves, and the brain was gently lifted out of the skull.
9. These steps were performed within 2–3 minutes for each brain.

Rat brain removal process.

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APPENDIX IV

Protocol for tissue preparation

1. Place tissue in tissue cassettes and into 50% alcohol overnight
2. Remove tissue and place into 70% alcohol for 45 minutes
3. Transfer tissue into 90% alcohol for 30 minutes
4. Remove tissue and place in absolute alcohol (100%) I, II and III for 45 minutes each.
5. Transfer tissue into xylene I and II for 30 minutes each
6. Remove and place tissue into xylene III for 45 minutes.
7. Infiltration
8. Place tissue in tissue cassettes in molten wax I for 1 hour in an oven
9. Remove and place into molten wax II and III for 30minutes each in an oven
10. Tissues in cassettes are embedded in wax outside of oven and allowed to harden on ice.

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APPENDIX V

Protocol for Haematoxylin and Eosin staining (H&E)

Leica Auto Sectioner XL was used to section the embedded brain blocks at 5 micrometres prior to H & E staining for histomorphometry.

Staining Technique

1. De-wax sections in xylene for 1 minute.

2. Take sections to water (rehydrate) by passing them through graded series of alcohol in the order 100%, 95% and 70%

3. Stain in Haematoxylin (see below for preparation) for 15 minutes.

4. Wash in water for 2-3minutes

5. Differentiate in 1% hydrochloric acid in 70% alcohol for 1 minutes

6. Wash in water for 10 minutes

7. Stain in 1% aqueous eosin (see below for preparation) for 5 minutes

8. Rinse gently in a bowl under running tap water to wash off surplus stain.

9. Dehydrate in graded series of alcohol (75%, 95%, 100% and 100%) and keep in xylene for subsequent mounting with Dysterene Plasticised Xylene (DPX)

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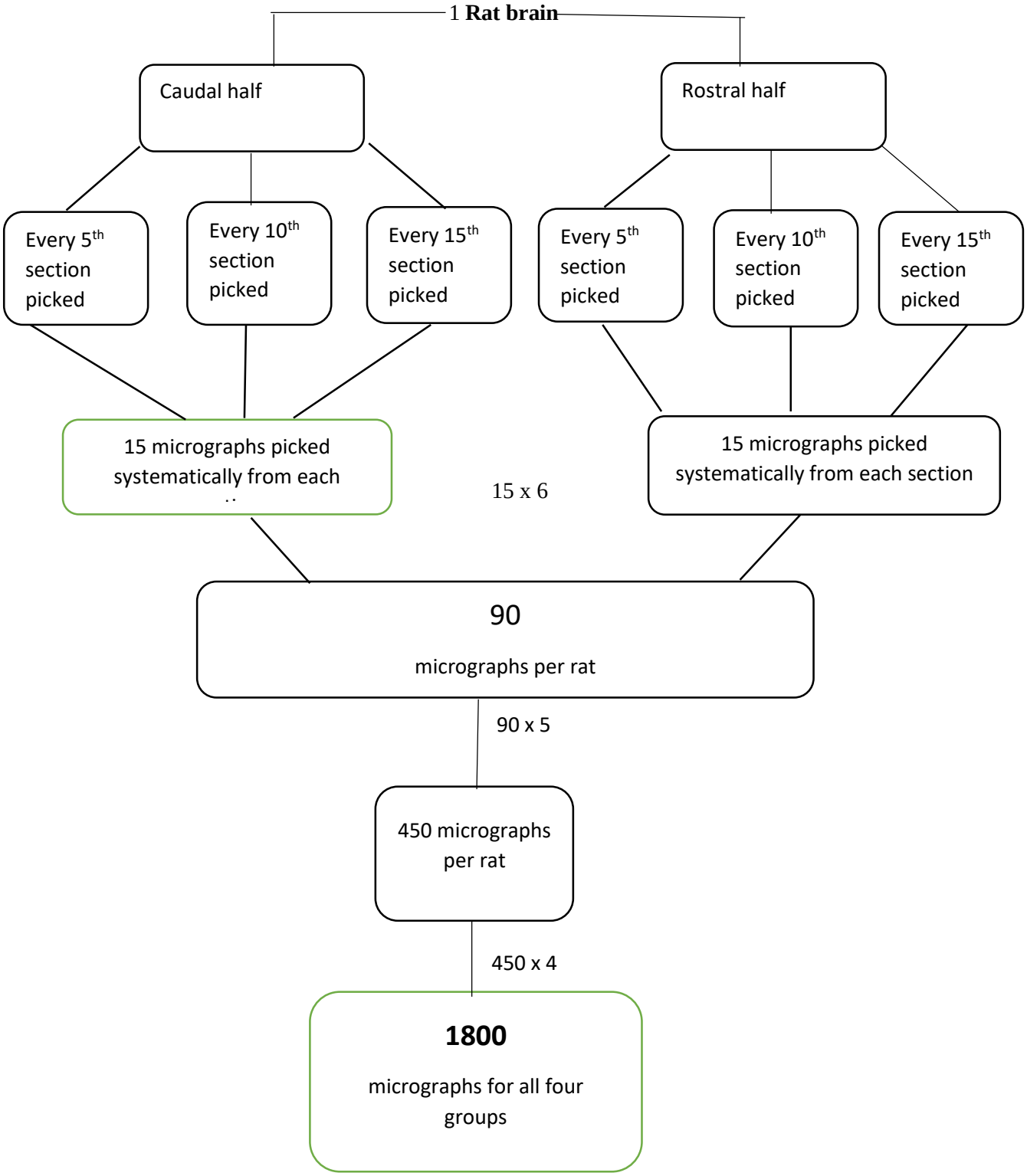
APPENDIX VI

FLOW CHART FOR STEREOLOGY



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